Interdisciplinary Biotechnological Advances

Daniele Ribeiro de Araujo
Marcela Sorelli Carneiro-Ramos *Editors*

Biotechnology Applied to Inflammatory Diseases

Cellular Mechanisms and Nanomedicine

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Preface

Inflammation is one of the most reported causes for several acute and chronic diseases, including obesity, cancer, dermatological, pulmonary, cardiac, vascular, bowel, and articular. For years, scientific research in this field have contributed to elucidate how cellular inflammatory pathways have influenced on diseases outbreaks, as well as stablishing relationships with infectious and oxidative processes, explaining how living organisms are able to sense and respond to the precise control between pathological and homeostasis conditions.

Biotechnology is currently defined as a broad area of Biological Sciences devoted to the extraction, purification, and/or the development of products applied to several fields of knowledge including pharmacotherapy and diagnosis of diseases. In fact, the recent advances of many biotechnological processes resulted from genomics, biochemical, biophysical recombinant gene, and applied immunology techniques, which significantly expanded their applications to new research fields such as Nanobiotechnology and Nanomedicine. In this sense, the innovative strategy of associating biotechnological advances and materials, at nanoscale level, opened perspectives to enhance pharmacological patterns of new and conventional drugs, as well as, to design new nanomaterials for tissue regeneration looking forward the treatment of inflammation in a variety of pathologies.

The purpose of the book *Biotechnology Applied to Inflammatory Diseases: Cellular Mechanisms and Nanomedicine* aims to integrate two challenges: the characterization of different inflammatory pathologies, in terms of cellular and molecular mechanisms, and to discuss the main biotechnological advances for understanding the molecular mechanisms involved in the progression of various types of inflammatory diseases, highlighting the contributions of nanomedicine to more efficient and biocompatible treatments with current precision medicine. Herein, the reader will find updated discussions and trends on cardiovascular, infectious, pulmonary, bowel, signaling molecules, and the intricate molecular mechanisms associated to mitochondrial activity and inflammatory-related oxidative process, as well as the design, the development and safety evaluation of

nanomedicine-based therapeutic and diagnosis strategies for both acute and chronic inflammatory diseases.

Finally, the editors would like to invite readers interested in science, and the world of opportunities it can provide us to appreciate this book. Especially, our sincere acknowledgments to all authors for taking part in this initiative by sharing their knowledge with us. We hope you enjoy it.

The Editors

Santo Andre, São Paulo, Brazil

Daniele Ribeiro de Araujo
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Chapter 1
Cardioimmunology: An Interdisciplinary Approach

Carolina Victória Cruz Junho, Ainhoa Rodríguez de Yurre, Emiliano Medei, and Marcela Sorelli Carneiro-Ramos

Abstract Although heart diseases continue to be the leading cause of death worldwide, advances performed in recent decades have facilitated a decrease in the mortality rate related to severe heart diseases. This is due to the recognition that has been acquiring the role of the immune system and its contribution to the progression of heart disease. Recent studies have shown that there is a close relationship between cardiac disturbances and inflammatory mediators produced by immune system cells since there is a close interaction between the innate and adaptive immune response in the pathophysiology of heart diseases. Regarding innate immune response, macrophages are the leading cells, which play a fundamental role in a wide variety of cardiac disorders. These cells produce a variety of cytokines that open up a wide range of therapeutic possibilities in the treatment of heart diseases. However, under certain circumstances, it is known that immune system cells can cause irreparable damage that contributes to heart failure. Therefore, it is essential to study the crosstalk between innate and adaptive response in order to better understand the mechanism of action of the different cardiac disturbances. In this sense, biotechnology emerges as a pioneering tool that allows on the one hand to effectively detect the various cardiovascular and inflammatory diseases, and on the other, to develop innovative therapies that result in effective treatments.

Keywords Heart · Inflammation · Cardiovascular diseases · Immune system · Cytokines · Biotechnology

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1.1 The Heart

The human heart is a void muscle, shaped as an inverted blunt cone, where the base forms a flat part, larger than its apex. The apex is the inferior end which tapers to a blunt, rounded point. It is positioned in the space between the lungs (mediastinum cavity), where the base lies posteriorly and superiorly, extending itself up to the level of the second rib. The apex lies anteriorly and inferiorly, resting on the central tendon of the diaphragm. Posteriorly, the heart rests against the vertebral bodies of the fifth to eighth thoracic vertebrae and is located behind the sternum (Durward 1950).

This important organ is composed of three layers of tissue, all involved by the pericardium (pericardial sac) which is a double-layered closed sac composed of a fibrous outer layer. Inside this sac, there is a viscous liquid known as pericardial fluid, which helps to lubricate the external surfaces involved in the heartbeat and to prevent friction between the fibrous and serous layers of the pericardium. From the outside in, the first layer is called epicardium. Some authors consider it an inner tier of the pericardium. It is composed of connective tissue fused with the muscular tissue on one side and the serous pericardium on the other. The second layer is the myocardium, composed of a muscle layer. It is considered the thickest layer and is where the contractions take place. This tissue is striated like skeletal muscle; however, it responds to involuntary stimulus of the autonomic nervous system. The third layer, and the most internal one, is the endocardium. It forms the layer which barks all cardiac chambers and is directly connected to all internal cardiac appendages (Durward 1950).

The main cell types that constitute the cardiac tissue are cardiomyocytes (or cardiac myocytes), cardiac fibroblasts, smooth muscle cells, and endothelial cells. Even though classically only those cell types were considered part of the heart, now we know that immune cells, mainly macrophages, also play a vital role in cardiac function (Zohman 1964). It can be seen in Fig. 1.1. Although cardiomyocytes are the ones which respond to electrical stimuli and correspond to approximately 75% of the total volume of the myocardium, cardiac fibroblasts are the predominant cells in this tissue, reaching about 2/3 of the total number of cells, having an essential role in the production of collagen I and III, the main constituents of the cardiac extracellular matrix (Bongartz et al. 2005).

The heart is divided into four chambers (two atria and two ventricles). The atriums are the upper ones, while the ventricles are the lower ones (both right and left). An important concept to consider is that the blood flow is unidirectional: the blood flows from the atrium to the ventricles → exits the ventricles out of the heart (lungs or body) → flows back to the atrium. This is only possible since the heart has two main kinds of valves: atrioventricular (AV) and semilunar. The AV valves (mitral—left ventricle, and tricuspid—right ventricle) separate the atriums from the ventricles. They are composed of thin wires of connective tissue (chordae tendineae) attached by papillary muscles to the heart wall. This tissue prevents the valves from opening upwards because of the high pressures achieved during some

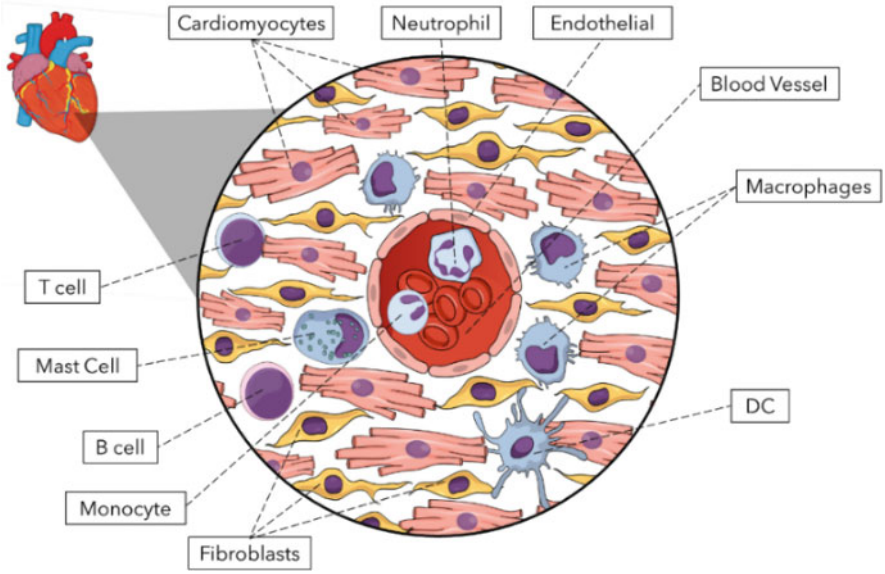

Fig. 1.1 The main cell types that constitute the cardiac tissue. They are cardiomyocytes (or cardiac myocytes), cardiac fibroblasts, and endothelial cells. There are also immune cells populating the cardiac tissue such as macrophages, dendritic cells (DC), B cells, T cells, mast cells, monocytes, and neutrophils

conditions. The semilunar valves (pulmonary and aortic) are located between the ventricles and the arteries. The pulmonary valve is located between the right ventricle and the pulmonary artery while the aortic valve is situated between the left ventricle and aorta (Tortora and Derrickson 2009). It can be better visualized in Fig. 1.2a.

As commented before, blood flow is unidirectional, and is divided into two types: systemic and pulmonary. Systemic circulation consists of oxygenated (arterial) blood being released from the left ventricle through the aorta to the body, where the cells will consume the oxygen in their processes. The blood returns to the heart. It is pumped from the ventricle to the lungs through the pulmonary artery. Gas exchange occurs in the millions of lungs' alveoli and capillary vessels that surround them, and it is called pulmonary hematosis. Re-oxygenated blood returns to the heart through the 4 pulmonary veins to the left atrium, and then it goes to the left ventricle passing through the left AV valve (mitral), restarting the route (Durward 1950).

This cardiac cycle happens because an electrical depolarizing and repolarizing cycle in the cardiomyocytes occurs. It is well described as a specialized electrical conduction system in the heart. The sinoatrial (SA) node is the heart's pacemaker and is located superior to the terminal groove of the right atrium, close to the opening of the superior vena cava. This group of special cardiac cells propagate electrical stimulus. From the SA the electrical stimulus goes to the atrioventricular (AV) node, which is also an area of specialized conduction tissue located between the atriums

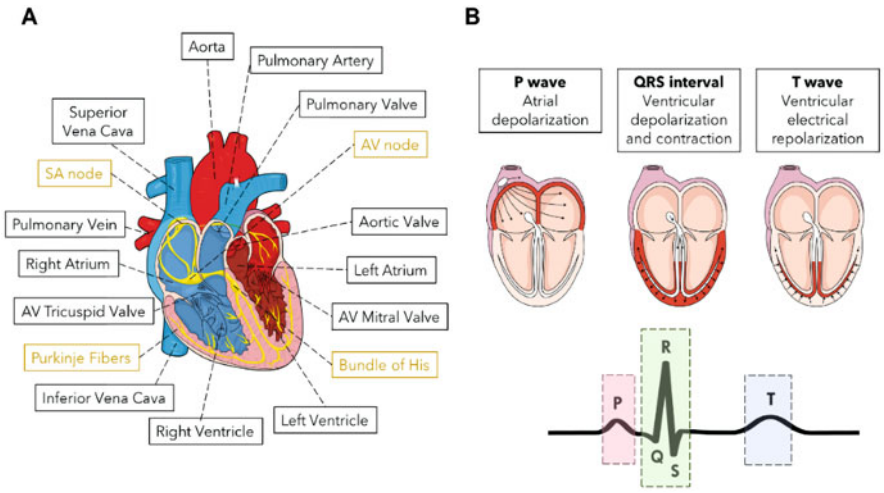

Fig. 1.2 The anatomophysiology of the heart. (**a**) Internal anatomy of the heart. (**b**) Physiological approach of the electrocardiogram (ECG)

and ventricles, which conducts electrical impulse from the atrium towards the ventricles. It can delay the passage of the electrical impulse, forcing the ventricles to contract later than the atriums.

The electrical impulse previously quoted is propagated through the membrane of each cardiomyocyte via action potentials (AP). The AP during the cardiac cycle includes two main steps: depolarization and repolarization of the cardiomyocyte. This process arises in T tubules, which are invaginations of the cardiomyocytes' plasmatic membrane containing voltage-sensitive receptors. Its depolarization is initiated after the stimulus from the sinus node is transmitted cell–cell by specific ion channel named connexin, mainly connexin-43. Afterward, some sodium (Na^+) channels open up and its ions move into the cell, resulting in the inside of the cell being positively charged. After the gradual depolarization of the cell, until the threshold is reached for triggering the next AP, the voltage-dependent L type Ca^{2+} channels open. This Ca^{2+} is sufficient to induce the opening of ryanodine receptors (RYRs) located in the sarcoplasmic reticulum (SR) membrane, the main calcium store in cardiac cells. Ca^{2+} ions diffuse out of the SR to interact with the contractile machinery. This process also is known as "calcium induce calcium release"—CICR. The contraction of cardiac myocytes is facilitated by myofilaments organized in sarcomeres, located along the long axis of the cell. The sarcomere is composed of actin and regulatory units: troponin (Tn) and tropomyosin (Tm). The binding of Ca^{2+} with TnC induces a conformational change allowing Tn/Tm to slide in the groove between the actin monomers, allowing the thick filament of the myosin to bind to the actin thus forming a cross-bridge. Through repeated and transient interactions of actin–myosin and, using energy from ATP hydrolysis, these two filaments slide in relation to each other, shortening the cell. The coordinated shortening of the entire

cardiomyocyte population by the spread of the AP leads to cardiac contraction. During systole, the main mechanism of Ca^{2+} efflux in cardiac myocytes is SERCA2A (sarcoplasmic reticulum Ca^{2+}-ATPase) which uses energy from ATP hydrolysis to pump Ca^{2+} back to SR; and the sodium-calcium exchanger (NCX), located in the cardiomyocyte membrane which uses the electrochemical gradient in the sarcolemma to translocate three Na^{+} ions into the cytosol and expel a Ca^{2+} ion (Bers 2002). The groups of these changes in cardiac AP and consequently membrane potential occurs in each cardiac beat, generating a cardiac cycle. The cardiac cycle consists of stages that occur in the interval of a heartbeat (called cardiac systole and diastole), which are the atrial systole, isovolumetric contraction, ventricular systole, isovolumetric relaxation, and ventricular filling (Patterson et al. 1914).

The SA node initiates an electrical impulse that flows over the right and left atriums causing their depolarization. Consequently, an atrial contraction happens (atrium systole). The blood will immediately be displaced into the ventricles through the opening of the AV valves, while the semilunar valves remain closed to avoid blood reflux from the great vessels. On an electrocardiogram (ECG), atrial depolarization is represented by the P wave. The AV and semilunar valves are closed. At that point, the ventricles begin to contract and, although the ventricular myocardial fibers shorten only a little, the intraventricular pressure increases rapidly. The electrical impulse spreads along the tissue reaching the AV node that conducts the stimulus through the bundle of the nerve fibers (His and Purkinje fibers) down the ventricle myocardium. Unlike atrial systole, during ventricular ejection the semilunar valves are opened while the AVs remain closed. This happens once the pressure in the ventricles exceeds the pressure in the arterial trunks and the valves are forced to open. It starts the ventricular systole, allowing blood to escape out of the heart. The left ventricle ejects blood to the body as well as right one ejects to lung circulation. During isovolumetric relaxation all cardiac valves close (Fig. 1.2b) (Tortora and Derrickson 2009; Farley et al. 2012).

All the phenomena described above work orchestrated to maintain the body function. It is known that a progressive decrease in cardiac function could be due to changes in the downgrading of sympathetic nervous system, in the calcium handling, in the reduction of myofilament function, in the heart anatomy, or as a result of a combination of these factors. Several pathologies may be considered when talking about abnormal calcium waves, among them are heart failure, myocardial infarction, ventricular tachycardia, and hypertrophy (González et al. 2015). It is known that inflammation is one of the most important point of convergence and can alter several cardiac conditions.

1.2 Immune System and Cardiovascular Diseases

Recent studies have explored the mechanisms involved in the progress of cardiovascular diseases from those related to the rupture of the atheromatous plaque to those observed in acute myocardial infarction, the process of self-repair, and the

development of heart failure. Results indicate that the occurrence of cardiovascular diseases depend largely on either inflammatory mediator produced by the cells of the immune system and by the immune system's cells themselves (Chiale et al. 2001; Thomas et al. 2017).

Immune system cells are numerous and diverse in form and function and are classified into: leukocytes of the innate immune system, responsible for the identification and removal of foreign substances present in organs, tissues, blood and lymph as neutrophils, monocytes, macrophages, dendritic cells, and NK cells; and adaptive immune system cells such as T and B lymphocytes, which eliminate or prevent pathogen threats.

The immune system plays a pivotal role in the heart's response to injury, but until recently, the amount of confusing data made it difficult to distinguish immune factors that promote recovery of the heart after a heart attack from those that lead to greater damage, for example.

1.2.1 Inflammation and Heart

There are several factors that affect the proper functioning of the heart leading to heart failure or even death. Myocardial infarction deprives a part of the heart of oxygen and can lead to ischemic injuries that can be fatal; myocarditis, that can occur due to a viral infection and generate autoimmune disease; endocarditis, mainly due to bacterial infections; and arrhythmia. Most of these conditions are associated with a complex immune response that can either spread or defend against the disease.

The immune system response is therefore divided functionally, into two types: innate and adaptive. The innate immune response acts as the first line of defense against infectious agents, and most pathogens can be controlled before they produce an infection. Besides, the adaptive system keeps memory of the infectious agent and can prevent it from causing later disease. Both responses play a fundamental role and, on several times, an interplay in the physiopathology of heart disease.

1.2.1.1 Innate Response

At the time it is well accepted that not only microorganisms are able to activate innate response thus also several diseases also can activate this kind of immune response, like lifestyle diseases, cancer, and heart disease (Swirski and Nahrendorf 2018).

One of the innate immune system's cellular populations that attract greater attention of researchers in the biomedical area are macrophages, which are responsible for phagocytosing damaged or infected cells or cell waste, as well as secreting various substances such as cytokines. Macrophages are involved in a wide variety of pathologies, such as heart failure, acute myocardial infarction, atherosclerosis, and obesity.

In the last decade, several studies have pointed out the macrophages as a key player in different cardiac diseases. In this regard, Epelman et al. demonstrated for the first time the presence of different resident macrophage populations in cardiac tissue (Epelman et al. 2014). After that, the group led by Dr. Medei was the first to demonstrate that cardiac macrophages can increase cardiac arrhythmic susceptibility (Monnerat et al. 2016). In the same line, the group led by Dr. Nahrendorf described a key role of cardiac macrophages consistently demonstrating that these cells also could be involved in the cardiac conduction system and that these cells actively participate in several cardiac mechanisms of repairs (Hulsmans et al. 2017).

The main action of macrophages is usually mediated by a high cytokine production such as IL-1β, TNF-α, and IL-6 (Duncan et al. 2010). One of the molecules that play a key role in the function of the innate immune system is interleukin 1β (IL-1β) (Cossio et al. 1974). The synthesis and maturation of IL-1β mainly depend on two signals: **signal 1**, classically mediated by the activation of Toll-like receptors (TLR's) and **signal 2** that depends on the activation/formation of inflammasome. In the cell membrane, the most important receptors involved in the synthesis of IL-1β in macrophages and monocytes are TLR's, which are pattern recognizers (Maenhaut and Van de Voorde 2011). In the last two decades, at least 13 molecules of the TLR's family have been described.

Signal 2 which results in the maturation of IL-1β involves a cytoplasmic protein molecular complex important in the inflammatory response, called inflammasome. Sensors that activate the inflammasome include the nucleotide-binding oligomerization (NOD) domains, receptors containing Leucine-rich repeats (NLRs), receptors such as those absent in melanoma-2 (ALRs), and proteins that contain a tripartite motif (TRIM) (Cossio et al. 1974; Malik and Kanneganti 2017).

It has been reported that in cardiomyocytes of patients with paroxysmal atrial fibrillation (AF) and with chronic AF, NLRP-3 inflammasome activity was increased (Yao et al. 2018). Moreover, patients with AF present increased circulating levels of inflammatory cytokines such as IL-1β, IL-18, and TNF-α. Inflammatory response mediators can also alter atrial electrophysiology and structural substrates, leading to increased vulnerability to AF (Hu et al. 2015). In this direction, it is currently known that NLRP-3 inflammasome plays a pivotal role in the development of cardiac disorders such as atherosclerosis (Grebe et al. 2018), coronary heart diseases (Libby et al. 2014), or cardiac arrhythmias related to renal ischemia-reperfusion (Alarcon et al. 2019). This points out, once again, the importance of NLRP-3 inflammasome as a target for the prevention or even the treatment of cardiovascular diseases.

1.2.1.2 Adaptive Response

The adaptive immune system develops as we are exposed to pathogens and other potentially harmful substances throughout our lives. This system comprises B and T lymphocytes and their products, including antibodies. In general terms, B lymphocytes are responsible for the antibody-mediated immune system, while T lymphocytes are responsible for the cell-mediated immune system.

Since 1976 it has been studied the role of G-Type immunoglobulins (Ig-G) from chronic chagasic patients, which are able to present agonist activity upon cardiac beta-adrenergic receptors (Cossio et al. 1974; Sterin-Borda et al. 1976). Neumann et al. described the presence of similar antibodies, but in patients with idiopathic dilated cardiomyopathy (Neumann et al. 1990). In this regard, our group has demonstrated that Ig-G from chronic chagasic patients is able to induce cardiac arrhythmias and AV conduction defects when perfused in isolated rabbit hearts (Farias de Oliveira et al. 1997). Besides, when studied in detail, it has been demonstrated that either Ig-G that activates beta-one adrenergic receptors or Ig-G that activates type-2 muscarinic receptors are involved in the mechanism of cardiac arrhythmias in chronic Chagas disease cardiomyopathy, modulating ventricular repolarization parameters (Medei et al. 2007). In 2006, Escobar et al. presented consistent data showing that Ig-G from chagasic patients was also able to activate beta-2 adrenergic receptors that induce cardiac conduction disturbances (Escobar et al. 2006). Several works were carried out to determine the clinical implication of the presence of these "functional autoantibodies" being Dr. Wallukat and his group in Germany the pioneer in this line. Wallukat's group began the challenge of the specific removal of beta1-adrenergic autoantibodies from patients with idiopathic dilated cardiomyopathy by extracorporeal immunoadsorption (Wallukat et al. 2002). Preliminary clinical results of this therapy demonstrated a long-term benefit; however, at the time, different practical and methodological limitations clearly limit the use of this method. In fact, strategies aimed at directly suppressing the generation of pathogenic autoantibodies and/or their activity in the patients' blood as intravenous Ig-G treatment (IVIG) or B cell depletion therapies could potentially be useful.

1.2.1.3 Adaptative-Innate Immune Response Crosstalk and Heart

In recent years, crosstalk between lymph and monocytes/macrophages emerged as a new mechanism to explain several diseases. Myocardial infarction (MI) is one of the most prevalent heart diseases in Western countries. MI occurs when blood flow decreases or stops in one part of the heart. Consequently, several cardiac cells die impairing the cardiac ventricular function. The left ventricular dysfunction is usually a consequence of the replacement of cardiac cells by fibroblast creating a "scar" area in the myocardium. In this sense, scar extension will determine cardiac function. Therefore, all clinical efforts are focused on preserving the ventricular mass by limiting the scar extension. In the last decade, several works consistently demonstrated the key role played by the immune system in the repair of cardiac mass after injury.

Immediately after the injury, takes place the clearance of cell debris and digestion of extracellular matrix, which is mediated by macrophages and neutrophils. It was demonstrated that this first instance, when exacerbated, could induce a higher scar region in the infarction zone. Conversely, some works remarked that the lack of these cells in the first moment of the MI could contribute to worse cardiac prognostic (Frantz et al. 2013) (Fig. 1.3).

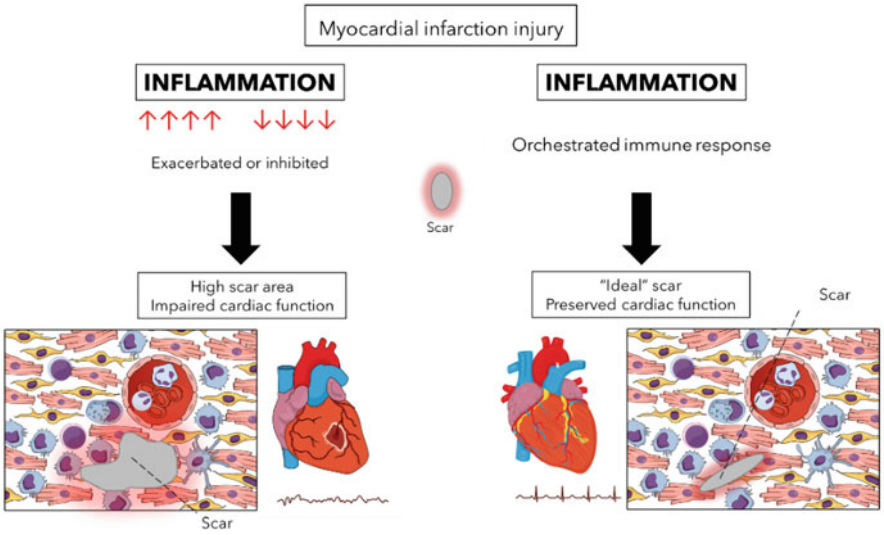

Fig. 1.3 After myocardial infarction (MI), an exacerbated or inhibited inflammation can lead to a bigger scar and impairment of cardiac function, while an equilibrated one leads to a smaller and "ideal" scar and better cardiac function

Chronologically, the first immune cells to act after a MI are the mast cells and 1 day later, monocytes and $Ly6G^+$ neutrophils play an important role. It has been observed that monocytes depicted a biphasic response after MI in mice. Thus, Ly-$6C^{high}$ and Ly-$6C^{low}$ monocytes peak is about 3 days and 5–7 days after MI, respectively (Swirski and Nahrendorf 2018). In humans however circulating inflammatory $CD14^+CD16^-$ monocytes expanded first (peak on day 2.6), followed by $CD14^+CD16^+$ monocytes (peak on day 4.8) (Tsujioka et al. 2009). Between days 4–7 post-MI, resident macrophages appear as the most important immune cells, and after 7 days of MI, B and T lymphocytes take a leading role. Thus, a higher concentration of these cells was documented in the mediastinal lymph node and also in the cardiac tissue. In addition, a shift from neutrophils to resident macrophages was observed in this phase. All these changes consistently help with cardiac healing. In this scenario, an elegant work from Stefan Frantz's Laboratory demonstrated the important interaction between $Foxp3^+CD4^+$ T cells/macrophage signaling crosstalk (Weirather et al. 2014). Thus, the authors demonstrated the important modulation of $Foxp3^+CD4^+$ T cells through IL-10, IL-13, and TGF-β1 on monocytes inducing their differentiation to macrophages. Additionally, TGF-β1 and IL-13 were shown to play a pivotal role on myofibroblast.

So, both extremes exist on the one hand the exacerbation and on the other the lack of immune response, which are deleterious for cardiac wound healing. Nowadays, the study of the equilibrium points of this orchestrated immune response, involving innate/adaptive crosstalk, is one of the most fascinating topics in the field of cardiology.

1.3 Biotechnological Tools Applied to the Treatment of Cardiovascular Diseases: New Insights

Biotechnology brings enormous benefits to many areas of knowledge, from agriculture to health sciences. It can increase productivity in crops and improve treatments of previously irremediable diseases. In the health area, biotechnology finds some of its most beneficial and comprehensive applications. When targeting cardiovascular and inflammatory diseases, biotechnology can be applied in two main ways: detection (through biomarkers and molecular diagnosis) and treatment. Innumerable techniques have been used in both scopes.

A biomarker is a characteristic molecule or substance that when measured and evaluated indicates physiological or pathological alterations. Classical biomarkers are measurable in plasma, serum, or urine. Specific ones leading to alterations of cell DNA or RNA, metabolite, and protein level are called molecular biomarkers. The biomarkers can track a disease progression over time or simply indicate a division or an endpoint in clinical studies. In other words, a biomarker reflects the state of some disease, being used for diagnosis or monitoring (Jain 2011a). Regarding cardiovascular diseases, many biomarkers have already been described (Table 1.1).

As heart cells die, their intercellular proteins are released out being exposed or degraded. Here lies the importance of the biotechnological techniques: capture this change or exposure of proteins that physiologically alter the cardiovascular system. Therefore, the most sensitive markers are those more abundant in the cell, whereas the ones involved in contraction are the most detectable in blood during heart diseases, as well as those markers involved in cytoskeleton (troponins, natriuretic peptide, ryanodine receptors, and others), enzymes responsible for cell energy (myoglobin and creatinine kinases), inflammatory cytokines, muscular tonus, cellular adhesion molecules, acute-phase reactants, rupture biomarker, and others. If such proteins have cardiac-specific forms, then specificity might be achievable as well as sensitivity (Lam et al. 2016).

In order to determine the number of biomarkers found in blood, many techniques were created. Basic technologies of molecular diagnostics are the Southern blot, DNA probes, pulsed-field gel electrophoresis, and polymerase chain reaction (PCR) (Jain 2011b). PCR has revolutionized the molecular diagnostics of heart diseases since it can be performed on even a few cells from body fluids or blood and is a key tool for genomics. Apart from that, we have proteomics, which has a fundamental role in the discovery of new and useful biomarkers of heart diseases. Proteomics comes to supplement the base already given by traditional genomics and traditional approaches. Proteomics investigates the protein alterations associated with the etiology of heart disease and its progression, outcome, and response to therapy. This is a systemic and sophisticated analysis of all protein profiles produced by a species in a determinate tissue. The term cardioproteomics is used for proteomic technologies applied to the cardiovascular system, providing a large-scale set of tools to study heart alterations, allowing the identification of pathological outcomes

Table 1.1 Principal biomarkers described in cardiovascular diseases

Disease	Biomarker	PMID
Myocyte Injury	NT-proBNP	24954516
	BNP	24954516
	MR-proANP	24756062
	FABP3	31733676
Myocyte Stretch	Troponin T	29278915
	Troponin I	29278915
	Creatine kinase	26623010
	Myoglobolin	30697054
	Fetuin-A	28213903
Oxidative Stress	Myeloperoxidase	30797769
	Oxi-LDL	25537066
	Serum amyloid A	26248570
Neurohormones	Renin	28985283
	Angiotensin II	28985283
	Aldosterone	28985283
	Chromogranin A	24325234
	Vasopressin	20653710
	Copectin	20653710
Inflammation	C-reactive protein	26973275
	TNF-α	26973275
	IL-1	25315037
	IL-6	31087601
	Galectin-3	28559694
	Fibrinogen	23034020
	Ischemia-modified albumin	24620936
Hypertrophy	MMP-2	28399617
	FGF23	30909513
	Colagens	24983265
	Myostratin	21467027
Calcium homeostasis	Secretoneurin	25634832
	Beta-2a protein	12042350

as well as new target treatments as in personalized medicine, the twenty-first century recent medicine goal (Jain 2011a).

Years of studies have led to many classic cardioprotective mechanisms, many of them pharmacological. There does not appear to be a repair of the heart tissue

itself but control of the risk factors in order to restore health. The most used therapeutic agents nowadays for the treatment of many heart disorders are the beta-blockers, the angiotensin-converting enzyme (ECA) inhibitors, and the calcium channels blockers, all particularly indicated as antiarrhythmic, antihypertensive, and cardioprotective after myocardial infarction. The beta blocker drugs reduce blood pressure, decrease heart rate, and soften irregular heartbeats. These drugs can also help relieve congestive heart failure and prevent secondary heart attacks and are atenolol, carvedilol, labetalol, metoprolol, and propranolol (among others). The ACE inhibitors work to lower high blood pressure by blocking ACE that creates a hormone that can cause blood vessels to compress (angiotensin II). When the vessels start to relax, blood pressure will drop. Some ACE inhibitors also help to relieve the symptoms of congestive heart failure as captopril, enalapril, lisinopril, quinapril, ramipril, benazepril, fosinopril, trandolapril or moexipril (Jain 2011a; Panico et al. 2019; Kumar et al. 2019).

The calcium channels blockers reduce blood pressure by opening blood vessels. They can also relieve angina by lowering the heart rate. They are amlodipine, diltiazem, nifedipine, nicardipine and verapamil. Many other types can be used in order to improve cardiovascular disorders as diuretics, nitrates, statins, and anti-inflammatories. Still in a pharmacological approach, some drugs have been used in a not so classic way. New strategies are being used to better understand the development of heart diseases. One of them is resveratrol. This molecule is considered an effective antioxidant by increasing the nitric oxide (NO) synthase, as well as the maintenance of intracellular redox. Its performance has already been shown in models of myocardial infarction, arrhythmias, hypertension, cardiomyopathies, fibrosis, atherosclerosis, thrombosis, and diabetes, proving to be very effective in reducing reactive oxygen species, improving vasorelaxation and angiogenesis, preventing inflammation and apoptosis, and delaying atherosclerosis as well as decreasing cardiovascular remodeling in those models (Dyck et al. 2019).

As mentioned above, NO plays an important role, mainly cardioprotective. This has been already discussed and it appears to be a common denominator among various causes of cardiovascular disease, serving as the main factor of the related treatments, pharmacological and nonpharmacological. Nitric oxide synthase (NOS) plays a very controversial role since some forms of iNOS seem to be deleterious and others as eNOS cardioprotective. Furthermore, NO interacts not only with nuclear factors but also with the electron transport chain in mitochondria, providing us with fundamental considerations about its molecular role during the mechanism of cardioprotection (Pieretti et al. 2020).

Besides that, new cell-based therapies are being developed for heart diseases. Cell therapy aims to treat some diseases by restoring or altering certain sets of cells, or by using the cells themselves to deliver treatment to the body. In cell therapy, cells are cultured or modified outside the body before being injected into the patient. Cells can come from the patient itself (autologous cells) or from a donor (allogeneic cells). The first type hinders the possibility of rejection since the patient's own cells are used. One technique well-used nowadays is the reprogramming of fibroblasts into pluripotent stem cells (iPSCs). This application allows cell reprograming using

transcriptional factors (Gata4, Mef2c, and Tbx5). Another option is to use the bone marrow (BM) cells or endothelial cells to induce reprograming to cardiomyocytes. These BM-derived progenitor cells have been observed to play an important role in vasculogenesis, already described to remodel this tissue. Despite these findings, critical points are being raised for the improvement of techniques. It is necessary to determine if the characteristics of cardiomyocyte will persist over time. It has been raised by investigators that the efficiency of the generation of functional and contractible cardiomyocytes through these techniques is about 1%. The outcomes of these trials have been ambiguous, and no robust result has been stipulated, mainly because of the variation in cell population and differentiation. Although cell therapy initiatives exist in the experimental context, there is no large-scale and productive clinical approach. The results in basic science are being now discussed and tested rigorously to stipulate the worthiest way to get closer to clinical research and medical industry (Jain 2011a; Ieda et al. 2010).

Not far from it, we can find some models of 3D contractile bioengineered heart muscle (BEHM) using cardiac myocytes from rats. Since about 2007, researchers are working on in order to develop potential 3D functional models that replace dead cardiac cells. It puts the researchers one step closer to the goal of growing a whole new heart for heart-injured rats. A 4-day experiment already showed the ability of myocytes from a heart tissue patch to contract with an active force of 800 mN. These myocytes spontaneously organize and begin to contract and appear to respond to external forces such as calcium. Experiments injuring this formed tissue already show us also the ability of regeneration and remodeling, and a significant finding of response to inflammation (Mohamed et al. 2017; Huang et al. 2007). Further studies will expose the BEHM tissue to more nutrients and other conditions that are present in the body. This methodology has several advantages when compared to the other approaches however still far from being the human failure treatment, accelerating the goal to the moment when we will be able to replace a whole heart constructed from the patient's own cells.

Another application of great importance for advances in the treatment of cardiovascular diseases is nanobiotechnology. Nanocardiology is the application of nanobiotechnology to cardiovascular diseases. Recent advances in nanotechnology and nanoscience offer a range of new opportunities for the diagnosis and therapy of cardiovascular and pulmonary diseases. Nanoparticles are an ample field and have strongly redefined molecular imaging for diagnosis and also the treatment of heart diseases. Magnetic nanoparticles are being used to target images of vascular inflammation, using conjugated nanoparticles with green fluorescent protein which reveals the inflammation location once it is injected in mice, as well as in different treatments as drug dealers. The dual role of these particles offers an individualized therapy since image-based treatments are selective and verify whenever the drug is reaching the target and point to the molecular effect that is occurring (Fan et al. 2020).

In general, nanobiotechnology facilitates the repairing and replacement of blood vessels, myocardium, and myocardial valves. It also can be used to stimulate regenerative processes such as therapeutic angiogenesis for ischemic heart disease. Within these technologies, we can name the nano-scaffolds and the nanofibers that

guide tissue repair and replacement of blood vessels and cardiac tissue. They are polymers that have drug-release properties. Using nanofibers, it is possible to produce biomimetic scaffolds that can mimic the extracellular matrix for tissue engineering, as nanofibers can guide cell growth along their direction. Combining factors like fiber diameter, alignment, and chemicals offers new ways to control tissue engineering. Scaffolds capable of mimicking cellular matrices should be able to stimulate the growth of new cardiac tissue and direct revascularization. New advances in electrospinning, especially in drug delivery, support the massive potential of these nanomaterials. Inside these materials, encapsulated, we can find cytokines, growth factors, or angiogenic factors, for example (Ashammakhi et al. 2009).

The nanoparticles offer many solutions for cardiac therapies such as the improvement of the solubilization of the drugs, the use of noninvasive administration routes, improvement of the instabilities of absorption of the compounds, improvement of the bioavailability and release rates, control of the particle size and surface's morphology, direct drug coupling to target restricted ligands, within others (Jain 2011a; Park et al. 2020). In addition to it, new strategies have been improved to better understand the role of many pathways including inflammation and immune system.

1.4 Final Considerations

The history of cardiology is marked by great names in science, art, and technology. Like other sciences, we are going through periods of progress and stagnation. However, it is inevitable to recognize that advances were greater and today, we can say that modern cardiology permeates areas that go beyond physiology. Interdisciplinarity has provided greater achievements since heart disease is the result of several other causes. The immune system has been shown to be a great ally in the understanding of cardiovascular diseases since it is the great systemic connector of the human organism. The breaking of barriers between the areas makes us think of medicine as being inter- and trans-disciplinary, turning technological studies to discover new treatments and disease prevention in a much more integrated way.

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Chapter 2
Vascular Inflammation: From Cellular Mechanisms to Biotechnology Advances

Fernanda Cardoso da Silva and Cristina Ribas Fürstenau

Abstract Blood vessels are an interconnected network of arteries, arterioles, capillaries, veins, and venules that carry blood to all body tissues. Blood vessels are formed by (1) a single layer of endothelial cells that regulates the transit of what needs to pass between the bloodstream and the surrounding tissue; (2) more or less prominent layers of smooth muscle fibers that control the vascular tone and resistance; and (3) an external layer of a fibrous and connective tissue that connects the layers of the vascular wall with the tissues. Inflammatory vascular pathology results from both the attack of the immune cells and the response of the vascular wall, providing both regeneration and maladaptive responses. Two main stages are recognized in the inflammatory process: (1) the acute phase, characterized by increased vascular permeability, intense blood flow, and accumulation of cytokines and immune cells; and (2) the chronic phase, which relies on the action of lymphocytes and macrophages, involving the stages of neoangiogenesis and fibroplasia. Altered or nonfunctional blood vessels are present in cardiovascular diseases (CVDs) which account for more than 30% of global deaths and are a major issue of public health worldwide. Vascular inflammation involves different mechanisms, such as the activation of the renin–angiotensin–aldosterone system; the excessive production of reactive oxygen (ROS) and nitrogen (RNS) species; the thrombi formation; among others. Thus, the processes that trigger vascular inflammation and its involvement with CVDs are relevant and can be clinically useful in the diagnosis and therapy of these diseases. This chapter presents biomarkers of vascular inflammation of molecular, cellular, and chemical nature, especially in the context of CVDs, and also brings the most recent research related to biotechnological advances in diagnosis and therapies for vascular inflammation.

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2.1 Introduction

The traditional separation between noninflammatory vascular diseases and inflammatory vasculopathies is incorrect. For example, it is well known that immune cells and inflammatory pathways participate in atherogenesis in atherosclerotic vascular disease, particularly in events that lead to plaque rupture and ischemia. In this sense, it is clear that inflammation is a critical component of atherosclerosis, which places it on the same level as classic vasculitis (Hoffman et al. 2012).

The inflammatory pathology results from the attack of the immune cells and the response of the vascular wall, making the vascular tissue an active participant in inflammatory diseases, being able to provide both regeneration and maladaptive responses (Hoffman et al. 2012). The correct functioning of blood vessels is essential for the maintenance of body homeostasis. Morpho-functional changes in blood vessels can lead to the development of several diseases, including cardiovascular diseases (CVDs) (e.g., coronary heart disease, cerebrovascular disease, rheumatic heart disease, and other conditions) that are the leading cause of morbidity and mortality worldwide (Aikawa et al. 2019). Vascular inflammation is one of the most aggravating factors of CVDs (Goyal et al. 2019) and involves different mechanisms, such as the activation of the renin–angiotensin–aldosterone system, the intensification of the production of reactive oxygen (ROS) and nitrogen (RNS) species, the activation of the innate and adaptive immune system, the thrombus formation, and the formation of new blood vessels (Kvietys and Granger 2012; Petrie et al. 2018).

In this chapter, the diversity of cellular and molecular markers involved in vascular inflammation is presented. Besides, the most recent research related to biotechnological advances directed both to the identification of new markers and to therapies for vascular inflammation are also discussed.

2.2 Blood Vessels and Vascular Inflammation

Blood vessels are an interconnected network of arteries, arterioles, capillaries, veins, and venules that carry blood to all body tissues. Blood vessels are formed by (1) the tunica intima, consisting of a single layer of endothelial cells and the subendothelial space; (2) the tunica media, which may be more or less prominent depending on its function, composed of smooth muscle fibers; and (3) tunica adventitia, formed by fibrous and connective tissue cells that externally cover the vessel (Bechara and Szabó 2006) as depicted in Fig. 2.1a.

Due to its location at the interface between the bloodstream and the surrounding tissue, the vascular endothelium forms a barrier that regulates the transit of what needs to pass between these two compartments (e.g., oxygen, nutrients, proteins,

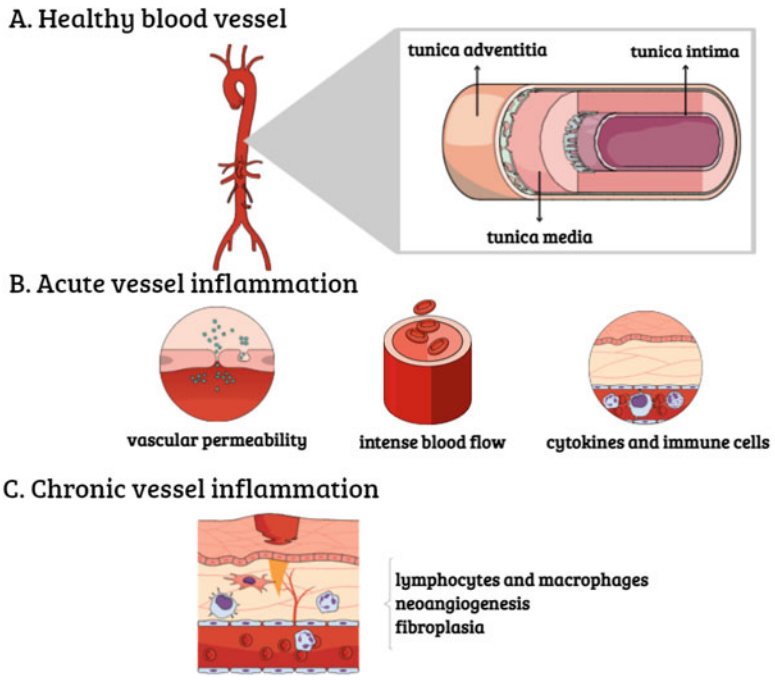

Fig. 2.1 Blood vessel structure and vascular inflammation. Panel (**a**) exhibits a healthy vascular wall consisting of the tunica intima, the tunica media, and the tunica adventitia. Panel (**b**) depicts the acute phase of vascular inflammation that is mainly characterized by increased vascular permeability, intense blood flow, and the accumulation of cytokines and immune cells. In (**c**), the chronic phase of vascular inflammation highlights the action of lymphocytes and macrophages, neoangiogenesis, and fibroplasia

molecules of different sizes, and immune cells). Among other functions, the endothelium stands out for its ability to alter its phenotype to control the inflammatory response, the anti or procoagulant function, and the vascular tone according to body needs (Geiger 2019).

Vascular smooth muscle cells determine the structure of blood vessels and their main function lies in the control of vascular tone and resistance and, consequently, in the modulation of blood pressure, through the mechanisms of vasodilation and vasoconstriction. The influx and removal of calcium from the cytosol of vascular smooth muscle cells are finely controlled by a range of biomolecules and intracellular pathways and damage to these signaling pathways can lead to vascular tone dysfunction with the consequent appearance of diseases such as hypertension and atherosclerosis (Geiger 2019).

The outermost layer of the vascular wall is formed by the tunica adventitia, which is composed of a rich-collagen layer that connects the layers of the vascular wall with the tissues. In addition to components of the extracellular matrix, adventitia also

contains fibroblasts that produce collagen in cases of injury or during healing processes (Geiger 2019).

Malfunctioning of blood vessels leads to the emergence of different pathologies, including CVDs. Some risk factors are characteristic of these diseases, such as endothelial dysfunction, inflammation and vascular remodeling, atherosclerosis, dyslipidemia, and even obesity (Petrie et al. 2018). In particular, vascular inflammation is one of the aggravating factors of these conditions and is characterized by a significant change in vascular dynamics, in addition to intensification in the process of chemotaxis and recruitment of cells from the immune system to the inflammatory site (Goyal et al. 2019).

In consensus, the researchers divide the inflammatory process into two main stages: (1) the acute phase and (2) the chronic phase. Acute phase is mainly characterized by increased vascular permeability, intensifying blood flow, and the accumulation of a variety of cytokines and immune cells at the site (Goyal et al. 2019) (Fig. 2.1b).

If the inflammation persists, it progresses to the chronic phase, which has been identified as the main cause of atherosclerosis in patients with CVDs (Goyal et al. 2019). This phase is more complex, of longer duration, and relies on the action of lymphocytes and macrophages, involving the stages of neoangiogenesis—the proliferation of new blood vessels—and fibroplasia—the proliferation of fibrous tissue (Bechara and Szabó 2006; Kreuger and Phillipson 2016) (Fig. 2.1c).

During the inflammatory process, different pathways are activated, resulting in the release of a variety of chemical and molecular mediators and cell activation (Kvietys and Granger 2012). Understanding the markers of the inflammatory process is essential in the search for new targets, both for the diagnosis and for the development of therapies for CVDs.

2.3 Cellular Markers of Vascular Inflammation

Vascular inflammation involves the activation and recruitment of *immune system cells*. Clinical studies have shown that patients affected by CVDs exhibit high counts of lymphocytes and neutrophils which are correlated with their high sensitivity to insulin that is released through inflammatory changes in adipose tissue (Moriya 2019). Leukocyte count is an easily determined marker in the blood, but the close relationship with cardiovascular risk factors and the non-specificity limits its use (Goyal et al. 2019).

In addition, it is widely accepted that the responses triggered by the innate and adaptive immune system are relevant to both the onset and progress of CVDs. In this process, besides lymphocytes and neutrophils, monocytes are also activated (Petrie et al. 2018). Neutrophils are the first cells of the immune system to migrate to the inflammatory site and promote subsequent events of inflammation through the recruitment of monocytes and the release of granules, constituting an important trap for pathogens and tissue debris. In turn, monocytes are activated and become

macrophages at the site of inflammation, especially in patients with atherosclerosis (Goyal et al. 2019).

Recent studies indicate that nonclassical monocytes act as prominent vascular rulers and are elevated in pathological states, such as chronic kidney disease, myocardial infarction, atherosclerosis, and vasculitis—a term that designates a set of rare diseases that lead to both inflammation of arteries and veins (Buscher et al. 2017). Furthermore, macrophages perform the elimination of apoptotic cells, a phenomenon called efferocytosis. In general, macrophages have regulated efferocytosis at the beginning of the inflammation, but with its intensification and other signaling pathways, they undergo cellular reprogramming, which results in unregulated action, causing post-apoptotic necrosis and chronic inflammation (Yurdagul Jr. et al. 2017).

The *erythrocyte sedimentation* is another marker of vascular inflammation, known for a long time and commonly used to verify this condition. The erythrocyte sedimentation rate (ESR) is an indication that erythrocyte aggregation occurred with the influence of blood proteins, such as fibrinogen and immunoglobulins (Bechara and Szabó 2006). This event occurs in inflammatory states and is an indicator of vascular inflammation due to CVDs (Kiyani et al. 2019). Methods for measuring ESR are fast, inexpensive, and easy to assess the inflammatory response and can contribute to determining the diagnosis and monitoring patient's condition (Tishkowski and Gupta 2020).

As previously mentioned, *endothelial cells* play an important role in vasculature. In addition to being a physical barrier that prevents the passage of pathogens from the blood to the tissues, they also produce biomolecules that modulate vascular tone, platelet aggregation, adhesion and leukocyte transmigration, and proliferation of vascular cells (Machado-Pereira et al. 2017). Therefore, changes in these cells can cause great damage and trigger vascular inflammation in a condition called endothelial dysfunction (Kvietys and Granger 2012).

Endothelial dysfunction is mainly characterized by the failure of mechanisms of endothelial repair (Yang et al. 2016) and by a reduction in nitric oxide (NO) bioavailability (Incalza et al. 2018). Consequently, there is an increase in the production of reactive oxygen species (ROS) and the onset of oxidative stress with a subsequent inflammatory process (Incalza et al. 2018).

Endothelial dysfunction is one of the causes of the main events that appear in CVDs: increased vasoconstriction, oxidative stress, alteration in the permeability of the plasma membrane, accumulation of immune system cells (Konukoglu and Uzun 2016), increased platelet aggregation, and proliferation of vascular smooth muscle cells (Yuyun et al. 2018). Thus, endothelial dysfunction can be monitored and used as a target for treatment approaches (Daiber et al. 2017).

Another important marker of vascular inflammation is the identification of many *endothelial cells in senescence*. This process is related to the development of heart failure and the progression of atherosclerotic plaques. Also, the senescence of endothelial cells is a factor inducing systemic glucose intolerance. However, since the aging process of these cells is extremely complex, there is still no known way to interfere with it (Katsuumi et al. 2018). Besides, cellular senescence concomitant

with dysregulation of innate immunity intensifies prolonged and persistent inflammation, even if the stimulus that caused the inflammation is removed or treated (Sanada et al. 2018). Cell senescence modifies the structural and functional properties of the vasculature (Maloberti et al. 2019), intensifying the inflammatory context.

In addition to playing a role in hemostasis, *platelets* are considered important agents of inflammation. For example, platelet activation leads to activation of the coagulation cascade via the intrinsic pathway, which causes thrombus formation and healing. Recent research indicates that platelets have receptors for complement system proteins and are closely related to inflammation (Mezger et al. 2019).

The increase in the volume of *perivascular adipose tissue* (PVAT) is also a marker of vascular inflammation. Due to this increase, PVAT becomes dysfunctional and starts to exhibit an inflammatory phenotype. PVAT thus produces pro-inflammatory cytokines, increases the production of oxidizing molecules, and decreases vasorelaxant and vasoprotective factors derived from adipocyte production. Together, these factors trigger subsequent inflammation processes (Nosalski and Guzik 2017).

2.4 Chemical Markers of Vascular Inflammation

Vasoactive amines (e.g., histamine and serotonin) play a role in the vascular inflammation process and are related to the acute stage of inflammation, increasing vascular permeability. These chemical markers are commonly stored in cytoplasmic granules of mast cells, basophils, and platelets and released by degranulation (Bechara and Szabó 2006). Vascular permeabilizing agents such as histamine and serotonin are involved in the angiogenesis process. Micromolar concentrations of these vasoactive amines can induce the proliferation of endothelial cells, migration, and formation of new blood vessels through the activation of TR3/Nur77 receptors (Qin et al. 2013).

Cytokines are molecules closely involved in vascular inflammation, both circulating and effector produced at the inflammation site. The central cytokines acting on the vasculature are TNF-α, interferon-γ, IL-1β, and IL-12. These cytokines contribute to vascular inflammation by influencing insulin sensitivity in peripheral tissues and modifying the release rate of this hormone (Petrie et al. 2018).

The IL-6/Th17/IL-17 activation pathway leads mainly to systemic inflammation related to cardiovascular diseases, and the IL-12/Th1/IFN-γ activation pathway is involved with inflammation of the vascular wall. This evidence is relevant to the development of new therapies (Keser et al. 2018).

Studies indicate the relevance of IL-1β in inflammation of the vasculature, making it a target for anti-inflammatory therapies. This interleukin is produced mainly by injured vascular cells and leukocytes (Libby 2017). Thus, the development of compounds such as the anti-inflammatory *Canakinumab*, a monoclonal antibody that neutralizes the action of IL-1β, is relevant because they have the

potential to significantly reduce the recurrent cardiovascular events of inflammation (Ridker et al. 2012).

C-reactive protein (CRP) is one of the most cited and researched chemical markers in the context of vascular inflammation. CRP is found mainly as a pentamer in circulation or insoluble monomers in tissues, performing different functions (Badimon et al. 2018). CRP participates in the innate humoral immune response contributing to the progression of CVDs by recognizing and binding multiple intrinsic ligands. This protein is considered a reagent in the acute phase of inflammation and is released by the liver into circulation. Some extrahepatic tissues (e.g., atherosclerotic plaques and vascular smooth muscle cells) can also synthesize CRP (Goyal et al. 2019). CRP inhibits nitric oxide production, increases the expression of cell adhesion molecules, and causes the recruitment of monocytes when binding to endothelial cells. Besides, it acts by modulating the innate immune response, activating the complement system, platelet aggregation, the coagulation cascade, tissue repair, and angiogenesis. Thus, CRP is involved in vascular inflammation through endothelial dysfunction, leukocyte recruitment, and thrombus formation (Badimon et al. 2018). Despite the recognized relevance of PCR for the diagnosis and prognosis of cardiovascular diseases, therapeutic options that target this protein are still little explored.

Another striking feature of vascular inflammation is oxidative stress that occurs due to the excessive production of *reactive oxygen* (*ROS*) and *nitrogen* (*RNS*) *species* and the reduction in the production of antioxidant molecules. Oxidative stress contributes to the activation of the five microvascular responses characteristic of inflammation: vasomotor dysfunction, recruitment of leukocytes to the site of inflammation, enhancement of vascular permeability, angiogenesis, and thrombosis (Kvietys and Granger 2012).

Both endothelial and immune cells like monocytes and macrophages can produce ROS. Particularly, the production of superoxide by NADPH oxidase 1 (NOX1) is central in the initial stages of many CVDs. Patients presenting a disease associated with the vasculature have elevated NOX1 expression compared to control patients (Gray et al. 2016). NADPH oxidase 4 (NOX4) is the most abundant oxidase, and patients with some vascular disease present a significant reduction in this enzyme. NOX4 is a generator of hydrogen peroxide (H_2O_2), which has a surprising protective effect, especially on atherosclerosis, since the deletion of the NOX4 gene in mice reduced the anti-inflammatory response and intensified the accumulation of vascular macrophages (Gray et al. 2016). NO is considered a vasoprotective biomolecule. However, when excessively generated by the activation of inducible nitric oxide synthase (iNOS) causes stress that can trigger endothelial dysfunction. This chemical molecule is one of the main RNS but the damage it can cause in the vasculature is still little known (Gliozzi et al. 2019).

Oxidized LDL (*oxLDL*) is also an important marker of inflammation in blood vessels that may also cause endothelial dysfunction. The mechanism by which oxLDL causes endothelial dysfunction is still poorly understood, but Gliozzi et al. (2019) suggested that oxLDLs negatively regulate nitric oxide endothelial synthase (eNOS) through the HMGB1-TLR4-Caveolin-1 pathway and also leads to the

activation of iNOS, causing oxidative stress in the endothelial cells (Gliozzi et al. 2019).

2.5 Molecular Markers of Vascular Inflammation

The *complement system* (*SC*) is a part of the immune system that "complements" the action of antibodies and phagocytic cells in organism defense. The *proteins and anaphylatoxins of the SC* also participate in the inflammatory process causing endothelial injury and the release of endothelial microvesicles. Anaphylatoxins C3a and C5a act by increasing vascular permeability via activation of histamine release (Fagerström et al. 2019). Anaphylatoxin C5a also acts by activating neutrophils and macrophages, causing the release of other chemical mediators (Bechara and Szabó 2006). Recent studies also point out that C3 proteins play functions in the intracellular and extracellular environments. Thus, the central roles of C3 are opsonization, the formation of the membrane attack complex, inflammation, and metabolic reprogramming (Elvington et al. 2016).

Proteins involved in the coagulation cascade contribute to the maintenance of hemostasis through the formation of clots, prevention of excessive blood loss, and initiation of the tissue repair process. The *proteins in the coagulation cascade* are involved in vascular inflammation. After cloning and knowing the coagulation factors (tissue factor, factors X, IX, V, XII, VII, VIII, and XI), it became evident that these proteins are involved both in thrombotic processes, in atherosclerotic processes, and in the response to ischemia–reperfusion injury. In these cases, as for the formation of atherosclerotic plaque, the activation of the coagulation cascade and the production of thrombin are initial events, since the coagulation factors can be produced by vascular smooth muscle cells and by cells of the immune system or recruited to the site of inflammation. Activated coagulation factors, acting as proteases, are related to a poor prognosis in patients with ischemia/reperfusion, so much so that anticoagulant drugs are used to improve patient survival. Other chemical mediators of the coagulation cascade are also involved in triggering and progressing vascular inflammation, like metabolites of arachidonic acid, lysosome granular compounds, and platelet-activating factors (Bechara and Szabó 2006).

A relevant role played by *hormones* is pointed out during the activation and resolution of inflammation at the systemic level. Thus, insulin is identified as a pro-inflammatory agent, while cortisol and glucagon have the opposite effect acting as anti-inflammatory agents (Bechara and Szabó 2006).

Fibrinogen is a protein involved in blood coagulation, being a determinant for platelet aggregation. In particular, it is worth highlighting the pro-inflammatory role of fibrinogen that has been reported in different types of inflammation, infections, cancers, and diseases that affect the cardiovascular system. According to Yakovlev and Medved (2018), products of fibrin and fibrinogen degradation present in the circulation or on the surface of endothelial cells promote leukocyte transmigration to the inflammation site, showing the signaling and chemotactic capacity of this

molecule (Yakovlev and Medved 2018). Deposits of fibrinogen are an almost universal characteristic of tissue injuries, making this molecule able to be used in early diagnosis methods and therapies (Luyendyk et al. 2019). Thus, circulating fibrinogen levels are a marker of vascular disease and there is a parallel effect of cytokines involved in the activation or inhibition of fibrinogen biosynthesis and vascular injury (Vasse et al. 1996).

The mechanism of action of the *renin–angiotensin–aldosterone system* in vascular inflammation is not yet fully understood. The type 1 (AT1R) receptor of angiotensin II (ANG II) is believed to perform most of the functions. Thus, AT1R triggers the activation of several signal transduction cascades capable of causing hypertension, vasculature remodeling, and significant damage to target organs. Among the activated signaling pathways, it is noteworthy that AT1R activation causes the regulation of vascular tone by stimulating vasoconstriction. This activation includes the release of ROS and other vasoconstrictor biomolecules and is a central event in CVDs (Forrester et al. 2018).

The *hyaluronan matrix* is altered during inflammation. Under homeostatic conditions, the vast majority of cells belonging to the immune system have a low affinity for this matrix. However, in inflamed tissue, this affinity increases significantly. During the inflammatory process, both the synthesis and the catabolism of the hyaluronan matrix are intensified and the degradation of the hyaluronan matrix generates fragments that act in chemotaxis and the spread of inflammation, recruiting mainly macrophages and dendritic cells (Grandoch et al. 2018).

Angiogenesis is the development of new blood vessels in response to hypoxia and/or ischemia, but it also contributes to the progression of CVDs and other inflammatory processes. The inflammatory environment increases the production of *pro-angiogenic factors* (bFGF, VEGF, PDGF, and TGF-β), which lead to the proliferation of endothelial cells, remodeling of the matrix, the release of growth factors, and budding of new vessels (Whiteford et al. 2016).

Non-coding RNAs, especially the long non-coding RNAs (*lncRNAs*), are emerging regulators of a variety of biological and pathophysiological processes, including CVDs. Several non-coding RNAs, for example, the CoroMarker lncRNAs (AC100865.1), MALAT1, lincRNA-Cox2, THRIL, and lincRNA-p21 present patterns of expression and involvement in regulatory pathways of inflammation and should be studied as possible targets (Haemmig et al. 2018).

Micro RNAs (*miRNAs*) are also involved in several pathological processes, including vascular complications that can be associated with other pathologies. The change in the expression of some miRNAs in patients who have diabetic cardiovascular complications is an example: miR-223, miR-320, miR-501, miR504, and miR1 have their expression increased while miR-16, miR-133, miR-492, and miR-373 have their expression reduced in these conditions, showing a relationship with vascular inflammation (Petrie et al. 2018).

Endothelial adenosine kinase (*ADK*) activity regulates intracellular endothelial adenosine levels. In the inflammatory process, ADK activity is increased, reducing adenosine levels and intensifying inflammation through signal replication in the intracellular medium, via the association of ADK with *S*-adenosylhomocysteine

(SAH) hydrolase (SAHH). Genetic knockdown of the ADK gene and the administration of exogenous adenosine attenuated the vascular inflammatory response (Xu et al. 2017).

The enzyme *α1AMPK* is also involved in vascular inflammation. α1AMPK-deleted mice have endothelial dysfunction caused by oxidative stress. Kröller-Schön et al. (2019) demonstrated that α1AMPK is responsible for reducing inflammation by limiting the recruitment of inflammatory cells and maintaining the antioxidant action of heme oxygenase 1 (Kröller-Schön et al. 2019).

Protein catabolism pathways, such as the ubiquitin-proteasome system, autophagy, and calpain, are altered during inflammation. The accumulation of certain defective proteins in blood vessel walls, which possibly triggers endothelial dysfunction, contributes to the pathogenesis of CVDs. Besides, the imbalance in protein catabolism leads to the release of pro-inflammatory and pro-angiogenic agents produced by dysfunctional endothelial cells (ROS and RNS) and the recruitment of phagocytic cells to the site, causing the progression of vascular inflammation (Miyazaki and Miyazaki 2017).

Table 2.1 Summarizes the major cellular, chemical, and molecular markers in vascular inflammation and their main features.

2.6 Biotechnology Advances in Diagnosis and Therapies for Vascular Inflammation

As seen in this chapter, the processes that trigger vascular inflammation and its involvement with CVDs are quite relevant and can be applied in the diagnosis and therapy of these diseases (Pechlivani and Ajjan 2018). Early diagnosis is a determinant for a better prognosis for patients with CVDs and consider that these new biomarkers of vascular inflammation can contribute to such progress. An example of this is the reduction of the expression of mitochondrial deacetylase Sirt3 as a marker of endothelial dysfunction since such enzyme is closely linked to the proper functioning of mitochondria (Dikalova et al. 2020).

Controlling inflammatory factors can represent a form of therapy and also of prevention of CVDs. Antioxidant therapies, for example, are used to reduce oxidative stress and inflammation. Some drugs with indirect antioxidant effects can be used for this purpose, such as angiotensin-converting enzyme (ACE) inhibitors, AT1R antagonists, and statins (Daiber et al. 2017).

Moreover, new biotechnological research and development expand the options for diagnosis and therapies (Raman et al. 2013). Monoclonal antibody therapies and gene regulation have been highlighted in the context of vascular inflammation (Guzik and Touyz 2017). For example, the neutralizing antibody to interleukin-1β, which reduces vascular inflammation and the chance of cardiovascular events (Aday and Ridker 2018). However, trials with this antibody have shown that it can cause an increase in fatal infections (Bäck et al. 2019).

Table 2.1 Cellular, chemical, and molecular markers involved in blood vessel inflammation

Markers	Characteristics/ Mechanisms
1. Cellular markers	
immune cells	high counts of lymphocytes and neutrophils that are correlated with their high sensitivity to insulin
sedimented erythrocytes	erythrocytes sedimentation rate (ESR) is an indication that erythrocyte aggregation occurred with the influence of fibrinogen and immunoglobulins
endothelial dysfunction	failure of mechanisms of endothelial repair, reduction in nitric oxide (NO) bioavailability, and increase in the production of reactive oxygen species (ROS)
vascular senescence	modification of the structural and functional properties of the vasculature, development of heart failure and the progression of atherosclerotic plaques, inductor of systemic glucose intolerance
platelets	activation of the coagulation cascade, which causes thrombus formation and healing
perivascular adipose tissue (PVAT)	increased volume of PVAT produces pro-inflammatory cytokines, increases the production of oxidizing molecules, and decreases vasorelaxant and vasoprotective factors derived from adipocytes
2. Chemical markers	
vasoactive amines (e.g., histamine, serotonin)	increase vascular permeability and are involved in angiogenesis
cytokines (TNF-α, interferon-γ, IL-1β, and IL-12)	influence insulin sensitivity in peripheral tissues
C-reactive protein (CRP)	endothelial dysfunction, leukocyte recruitment, and thrombus formation
reactive oxygen (ROS) and nitrogen (RNS) species	reduction in the production of antioxidant molecules
oxidized LDL	negatively regulates eNOS through the HMGB1-TLR4-Caveolin-1 pathway and also leads to the activation of iNOS
3. Molecular markers	
complement system proteins	increase vascular permeability, activate neutrophils and macrophages, opsonization, formation of the membrane attack complex, inflammation, and metabolic reprogramming
coagulation cascade proteins (plasma kinins)	act as proteases and are related to a poor prognosis in patients with ischemia/reperfusion
hormones	insulin is identified as a pro-inflammatory agent, while cortisol and glucagon act as anti-inflammatory agents
fibrinogen	promote leukocyte transmigration to the inflammation site
renin-angiotensin-aldosterone system	AT1R activation stimulates vasoconstriction, the release of ROS and other vasoconstrictor biomolecules
hyaluronan matrix	hyaluronan matrix fragments act in chemotaxis, recruiting mainly macrophages and dendritic cells
pro-angiogenic agents (bFGF, VEGF, PDGF and TGF-β)	proliferation of endothelial cells, remodeling of the matrix, the release of growth factors, and budding of new vessels
lncRNAs/ miRNAs	present patterns of expression and involvement in regulatory pathways of inflammation
endothelial adenosine kinase (ADK)	increased ADK activivy reduces adenosine levels and intensifies inflammation
endothelial α1AMPK	limits the recruitment of inflammatory cells and maintains the antioxidant action of heme oxygenase 1
defective protein catabolism	triggers endothelial dysfunction and recruits phagocytics cells

In addition, an anti-IL-6 receptor monoclonal antibody (MR16–1) has a protective effect against atherosclerotic lesions induced by dyslipidemia and/or inflammation (Akita et al. 2017). Other studies indicate that statins reduce the levels of CRP as well as the rates of occurrence of cardiovascular events (Aday and Ridker 2018). Similarly, studies point to a great of therapies to inhibit the action of type I interferons (IFNs), which are increased in CVDs (Chen et al. 2020).

Nanotechnology is an area of great prominence in biotechnology, making it possible to investigate the exclusive properties of nanomaterials. In this sense, there is a great potential for using nanomaterials to reduce the residual effect of certain drugs (Tu et al. 2015). For example, statins, antithrombotic, and thrombolytic agents are used for the treatment of cardiovascular diseases (Aday and Ridker 2018). In such a way, nanomaterial drug-delivery systems can be used for the treatment of vascular inflammatory diseases since vascular cells affected by inflammation have a greater tendency to incorporate nanometric materials.

The inhibition of the Toll-like receptor 9 (TLR9) is another therapeutic target for vascular inflammation, as it is known that the TLR9 activation impairs the recovery of blood flow in situations of ischemia (Nishimoto et al. 2018). Other molecular markers of vascular inflammation can be used as targets for the treatment of CVDs, as is the case for the proprotein convertase subtilisin/kexin 9 (PCSK9). Suppression of PCSK9 expression or its inhibition may be an effective way to decrease vascular inflammation in atherosclerosis (Tang et al. 2017).

Finally, current studies point to a greater need to invest in therapies aiming at the administration or activation of specialized pro-resolution mediators (SPMs), which include lipoxins, resolvins, protectins, and maresins. The administration of SPMs can take place via molecules specialized in the delivery of nanomaterials, causing a reduction in vascular inflammation, without, however, affecting the beneficial immune response (Bäck et al. 2019). Figure 2.2 illustrates the recent biotechnological advances in therapies for vascular inflammation.

2.7 Conclusion and Prospects

Cardiovascular diseases (CVDs) represent the largest cause of death worldwide. Inflammation of blood vessels is directly involved in the appearance and worsening of these pathologies. In this sense, all efforts are valid to recognize new diagnostic and therapeutic targets and curb the morbidity and mortality associated with CVDs.

The acute inflammatory process is established with increased vascular permeability, increased blood flow, and the accumulation of cytokines and immune system cells at the site of inflammation. If persistent, the inflammation reaches a chronic stage in which the action of lymphocytes and macrophages is observed, involving the formation of new blood vessels and fibroplasia. As mentioned, the identification of markers of vascular inflammation, both acute and chronic, is important in the search for new diagnostic and therapeutic targets. Thus, recent research has been

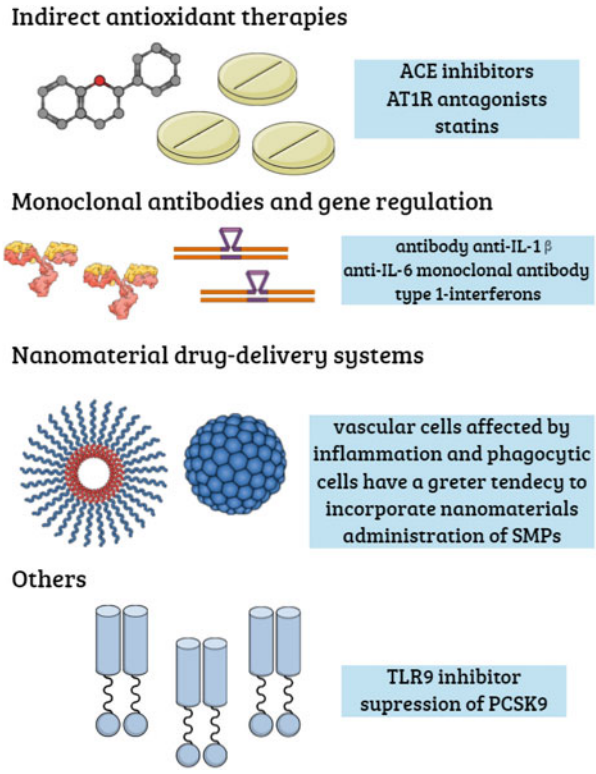

Fig. 2.2 Biotechnology advances in therapies for vascular inflammation. Indirect antioxidant therapies, antibody and gene regulation, nanomaterial drug-delivery systems, and other mechanisms (e.g., suppression or inhibition of specific enzymes and/or receptors) are effective to control the extent of blood vessels inflammation, helping to control the establishment and progression of CVDs

reporting (1) molecular; (2) cellular; and (3) chemical markers involved in the different phases of vascular inflammation.

Finally, biotechnology's contribution to the diagnosis of vascular inflammation and in the reduction of pathologies related to blood vessels is growing. Thus, therapies involving monoclonal antibodies and nanomaterial drug-delivery systems have gained prominence in this field and leveraged potentially useful targets for the clinical management of vasculature pathologies and CVDs.

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Chapter 3
Methods for the Analysis of Arachidonic Acid-Derived Metabolites in Platelets

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Abstract Arachidonic acid (AA) is the precursor of a series of bioactive lipids with relevant cell signaling and pathophysiological actions. Arachidonic acid signaling needs the first step of release from the membrane, being the released AA the substrate of four possible enzymatic pathways: prostaglandin endoperoxide H synthase (PGHS), lipoxygenase (LOX), cytochrome p450 (CYP 450), and anandamide pathways which lead to the formation of the bioactive 20-carbon oxygenated polyunsaturated fatty acids. The analysis of the different bioactive lipids formed in platelets, with AA as their precursor, is of relevance to the study of the mechanisms involved in platelet aggregation as well as for the development of novel antiplatelet and antithrombotic drugs. In this chapter, we will discuss the state of the art to detect and quantify different metabolites in resting and activated platelets.

Keywords Arachidonic acid · Platelets · HPLC · Mass spectrometry

3.1 Introduction

Arachidonic acid, all-*cis*-5,8,11,14-eicosatetraenoic acid (AA), is the precursor of a series of enzymatic and nonenzymatic oxidized-derived products with relevant cell signaling and pathophysiological actions (Brash 2001; Das 2018a, b; Hanna and Hafez 2018; Tsai et al. 2011). Its presence at the cell membrane not only is essential and necessary for membrane fluidity but also for membrane flexibility and function. At platelet membranes up to 25% of phospholipid fatty acids are AA, reaching levels near to 5 mM in resting platelets (Neufeld and Majerus 1983) and usually localized

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in the glycerol backbone sn-2 position (Brash 2001; Das 2018a, b; Hanna and Hafez 2018; Tsai et al. 2011). Human body requirements of AA are higher than the concentration found in the human diet. Thus, intake of the AA precursor linoleic acid (LA 18:2n-6) supports AA synthesis regulated by the activity of Δ6 and Δ5 desaturases which convert LA to gamma-linolenic acid (GLA, 18:3), dihomo-GLA (DGLA, 20:3), and AA (Das 2018a; Hanna and Hafez 2018).

The analysis of the different bioactive lipids formed in platelets, with AA as their precursor, is of relevance to the study of the mechanisms involved in platelet aggregation in physiology and pathophysiology but also the development of novel antiplatelet and antithrombotic drugs. In the current review, we will discuss the enzymatic oxidation of AA, which products are formed, and the state of the art to detect and quantify different metabolites in resting and activated platelets. We will discuss the benefits of different analytical methodologies as well as the pitfalls of their use, in addition to the description of recent methods to evaluate platelet metabolism.

3.2 AA Metabolism in Platelets: COX and LOX

Arachidonic acid signaling needs the first step of release from the membrane by phospholipase A_2 (PLA_2) which hydrolyze the AA present at the sn-2 position on the phospholipid backbone (Brash 2001; Davi and Patrono 2007; Holinstat et al. 2011; Maskrey et al. 2007; Thomas et al. 2010). The released AA is the substrate of four possible enzymatic pathways: prostaglandin endoperoxide H synthase (PGHS) or cyclooxygenase (COX), lipoxygenase (LOX), cytochrome p450 (CYP 450), and anandamide pathways which lead to the formation of the bioactive 20-carbon oxygenated polyunsaturated fatty acids called eicosanoids. Esterified AA can also be oxidized, i.e., by LOX with the bioactive products released after PLA_2 activity. In platelets, as well as in other tissues, the metabolic fate of AA depends on the pool of enzymes that catabolize the fatty acid-forming products with antagonistic function in different tissues, e.g., PGE_2 (Brash 2001; Hanna and Hafez 2018). Cyclooxygenase (COX) oxygenate AA forming the hydroperoxide prostaglandin G_2 (PGG_2), and then reduces it to prostaglandin H_2 (PGH_2) (Smith et al. 2000). PGH_2 is an intermediate hub that can be further metabolized by downstream enzymes to different eicosanoids in platelets as PGE_2, PGD_2, and $PGF_2\alpha$, or thromboxane A_2 (TXA_2). LOX pathway in platelets consists of AA oxidation mainly by the enzyme isoforms 12-LOX and 15-LOX, and then further transformed to leukotrienes (LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4) by other cell types (Brash 2001; Das 2018a, b; Hanna and Hafez 2018; Davi and Patrono 2007; Ikei et al. 2012; Marnett et al. 1999; Mollace et al. 2005; Murphy and Gijon 2007; Nascimento-Silva et al. 2005; O'Donnell et al. 2009) (Fig. 3.1).

Arachidonic acid metabolism is relevant for platelet function upon activation or returns the platelet bulk to the resting state. Platelet function is regulated by many agents with a central role being played by eicosanoids, i.e., TxA_2 (Jennings 2009).

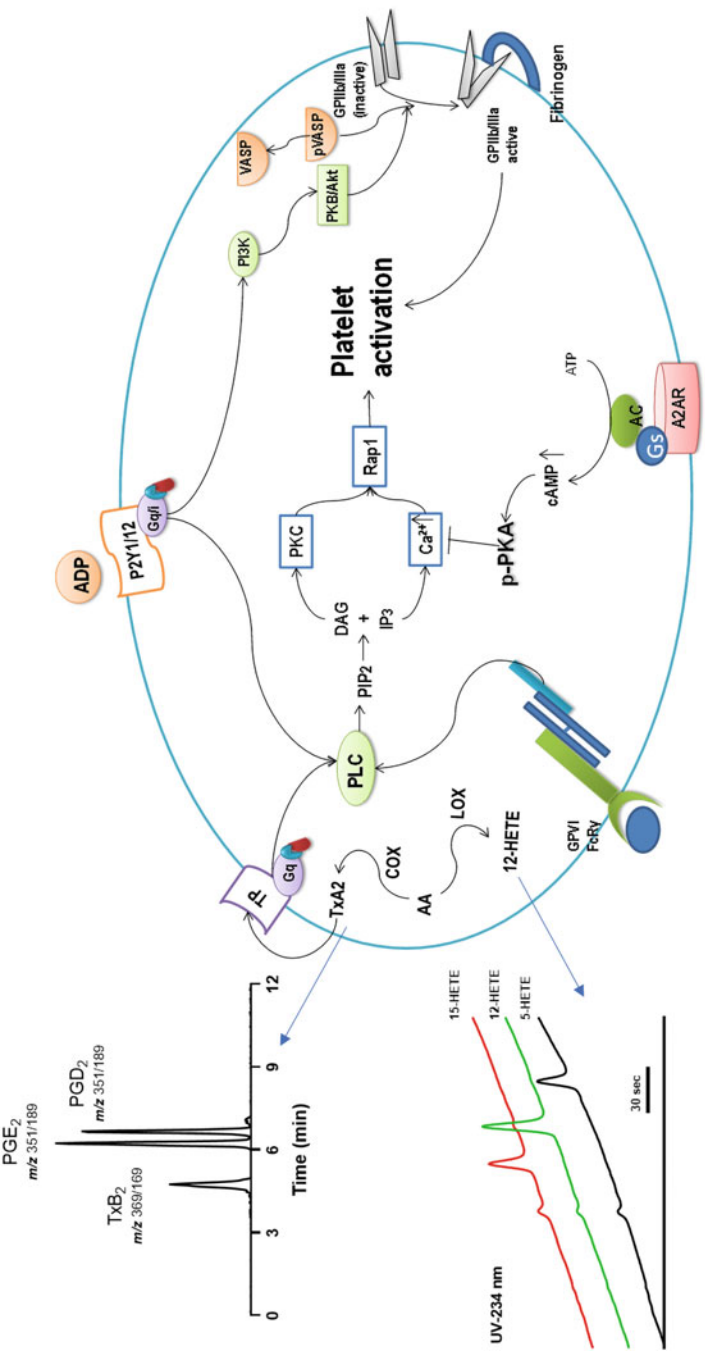

Fig. 3.1 Platelet aggregation can be activated by different agonists by interacting with receptors at the cell membrane. As a consequence, AA is released from the membrane being metabolized intracellularly, i.e., by COX and LOX. Lipid metabolites can be detected by HPLC-UV or HPLC-MS/MS studies as indicated. Figure modified from Exp Ther Med. 2012 Apr; 3(4): 577–584

The hemeprotein COX oxidizes AA to PGH_2 being COX-1 the major isoform present in platelets (Rouzer and Marnett 2003; Boutaud et al. 2001; Kurumbail et al. 2001). Traces of the COX-2 isoform is present in platelets as a result of the transcription of residual mRNA into protein or carried over from the platelet precursor cells (Maskrey et al. 2007; Marnett et al. 1999; Marnett 2002). In platelets, PGH_2 is further metabolized by TxA_2 synthase forming the pro-aggregant mediator TxA_2 which is released and autocatalytically induces platelet aggregation (Rouzer and Marnett 2003; Trostchansky et al. 2011). More recently, other relevant products from the COX-1 pathway in platelets have been described (Rauzi et al. 2016). In addition to the wide variety of eicosanoids formed by the COX pathway (Kirkby et al. 2015) (Fig. 3.1), other products such as 11-hydroxyeicosatetraenoic acid (11-HETE) and 15(*S*)-HETE are produced when AA is inserted at the active site of COX-1 in a different structural arrangement than the one necessary for PGH_2 synthesis (Rauzi et al. 2016). When high concentrations of AA are released from platelet membranes, e.g., at platelet hyperactivation both products can be formed at similar levels to TxA_2.

As shown in Fig. 3.1, AA can also be oxidized by the non-heme iron-containing enzymes LOXs. Hydroperoxy- (HpETE) and hydroxy- (HETE) eicosatetraenoic acids are the products formed by this enzymatic activity (Trostchansky et al. 2021; Wood et al. 2020). Different isoforms of LOXs are found depending on the carbon where the hydroperoxyl (–OOH) group is added. The main isoform present in platelets is 12-LOX, also known as p12-LOX, which oxidizes de fatty acid at C-12 forming the 12*S*-hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid [12(*S*) HpETE] (Trostchansky et al. 2021; Wood et al. 2020; Brash 1999), being reduced to its hydroxyl derivative 12-HETE due to the highly reducing environment (Wood et al. 2020; Brash 1999). The biological activity of 12-HETE in platelets in vivo remains under discussion with a suggested anti-aggregant and anti-inflammatory action, i.e., by lowering the release of AA from the membrane while others propose a prothrombotic activity (Kalgutkar et al. 1998a, b).

3.3 Analytical Techniques to Detect and Quantify Bioactive Lipids in Platelets

Due to their pivotal role, PG and Tx have been extensively studied in platelet function. The extremely low concentration of PG and Tx in biological fluids and the great variety and similarity between the different types of AA-derived molecules have been the main problems for their determination and identification and the need for the use of complex and sensitive techniques for its estimation. High-resolution gas chromatography (GC) in combination with tandem mass spectrometry (GC-MS/MS) has played a relevant role in the identification and quantification of bioactive lipids in biological samples (Kuksis 2000). However, in the last decade the simpler, rapid, and powerful high-performance liquid chromatography (HPLC)-MS/MS has

displaced GC-MS and GC-MS/MS in the area of instrumental analysis of physiological substances, drugs, and their metabolites (O'Donnell et al. 2014). Also, spectrophotometric and fluorimetric detection upon HPLC separation can be used in combination with LC-MS/MS for the analysis of PGs and Txs. Below we will describe different aspects and approximations for the detection and quantification of eicosanoids in platelets, discussing their benefits and pitfalls.

3.3.1 HPLC with Ultraviolet Detection

This method has been in use for several decades and has been refined ever since (Terragno et al. 1981). The standard ultraviolet (UV) detector HPLC samples, measures the absorbance of monochromatic light of fixed wavelength in the UV (190 nm) or visible wavelength range (400 nm in blue light) against a reference beam, relating the magnitude of the absorbance to the concentration of an analyte. This technique functions with molecules that contain unsaturated bonds, aromatic groups, or functional groups containing heteroatoms. In this sense, PGs and TXs, in contrast with leukotrienes, are particularly difficult to measure by UV. Spectral UV analysis of PGs reveals that they have a wavelength maximum of 192.5 nm (Puppolo et al. 2014). Now, UV detectors capable of working at low UV wavelengths are available, making it possible to detect nanogram quantities of PGs without the necessity to modify them chemically. However, chemical modification or "derivatization," is still in use in HPLC combined with fluorimetric methods (see below). Besides, PGs and TXs do not have strong chromophores and thus spectrophotometric detection after chromatography is difficult. Most PGs do not absorb UV light, so they have to be chemically converted before their determination, commonly in the carboxyl group to UV-absorbing phenacyl esters (Salari et al. 1987). The detection is then in order of nanograms to picograms.

Concerning the stationary phase, separation in reversed-phase (RP)-HPLC relies on the hydrophobic properties of the analytes and therefore remains the main method for the separation of the metabolites of AA (Puppolo et al. 2014). For Txs determination by HPLC-UV, chemical alterations are also commonly required, with panacyl bromide and methoxyamine to form methoxime-panacyl ester derivatives (Pullen et al. 1987). The normal plasma concentrations of thromboxane B_2 (TxB_2), the decay-stable TxA_2 end-product, are very low (10–370 pg/mL) and manipulation during the blood sampling alters the release from platelets (Nyyssönen et al. 1993).

3.3.2 HPLC with Fluorimetric Detection

This technique is based on the same principles of HPLC-UV, but with the use of a fluorescent detector after the HPLC separation. As mentioned above, PGs and TXs do not contain aromatic or natural fluorescent groups thus chemical derivatization of

these lipid molecules led to the formation of fluorescent complexes, before the HPLC separation, allowing their fluorescent detection. However, this makes the analysis more expensive and time-consuming (Liakh et al. 2020; Yue et al. 2004) but still less expensive than GC-MS. Some of the derivatizing agents used are *p*-(9-anthroyloxy)phenylacil bromide and anthryldiazomethane (Yamaki and Oh-Ishi 1986) for TxB_2 and bromomethyl 7-acetoxycoumarin for PGs (Tsuchiya et al. 1982).

3.3.3 MS Analysis of AA-Derived Metabolites

In the last decade, the improvement of sensitivity of mass liquid chromatography–MS (LC/MS) instruments, for example, electrospray ionization coupled to tandem (triple quadrupole or MS/MS) led to a better capacity to detect and quantify small amounts of lipids in diverse biological samples (O'Donnell et al. 2014). An advantage to GC-MS, LC/MS does not need sample derivatization before analysis with the increased use of LC/MS/MS methods in studies where the detection and quantitation of specific lipids are of interest for researchers (O'Donnell et al. 2014; Tacconelli et al. 2020; Tsikas and Zoerner 2014). Besides, LC-MS/MS methods show several-fold lower limits of quantitation values than reported GC-MS/MS methods (Tsikas and Zoerner 2014).

LC/MS/MS has been applied to the quantitation of eicosanoids and the identification of sphingolipids molecular species (O'Donnell et al. 2014; Tsikas and Zoerner 2014; Kornilov et al. 2019). When analyzing platelets' lipids, the use of prostacyclin or indomethacin should be avoided during cell purification procedures to avoid interference with lipid-sensitive signaling pathways (O'Donnell et al. 2014; Cebo et al. 2020a, b). Also, care has to be taken during the purification process to avoid undesired platelet activation (Trostchansky et al. 2011, 2019; O'Donnell et al. 2014). After cell purification, platelets' lipids can be isolated using several different methods. Among the most used are the Bligh and Dyer or the hexane/isopropanol-based solvent mixtures methods (Trostchansky et al. 2011, 2019; O'Donnell et al. 2014). The advantage of using the hexane method is that the organic phase is the upper one and less contamination with precipitated proteins is shown. Besides, a solid phase extract step, with C18 columns, can be included to obtain a cleaner lipid sample (Tsikas and Zoerner 2014; Cebo et al. 2020a). There exists a wide variety of labeled eicosanoids that are useful for identification and quantitation by LC-MS/MS (Trostchansky et al. 2011, 2018; O'Donnell et al. 2014; Kornilov et al. 2019). The standards have to be added before initiating the extraction procedure. Importantly, calibration curves have to be constructed in the same biological fluid that is analyzed to reduce the errors arising from ion suppression and ensuring accurate quantification (O'Donnell et al. 2014; Trostchansky et al. 2018).

Sample preparation is very important as artifactual formation of eicosanoid metabolites can be formed affecting lipid quantification. For example, ex vivo formation of TxA_2 by platelets can occur during blood sampling and processing

(Maskrey and O'Donnell 2008) leading to overestimation of platelet eicosanoids levels. Improvement of the sensitivity of LC-MS/MS methods would also be obtained by using larger sample volumes.

One of the most analyzed platelet-generated eicosanoids is the potent proaggregatory TxA_2. TxA_2 is extremely unstable and rapidly rearranges to TxB_2, which is released at ng amounts per 2×10^8 cells as measured by LC-MS/MS (Trostchansky et al. 2011, 2018; O'Donnell et al. 2014; Kornilov et al. 2019). We and others follow TxB_2 formation in the negative ion mode by following the *m/z* 369/169 transition (Fig. 3.1). When analyzing in vivo systemic changes in Tx formation, and due to TxA_2 short plasma half-life, generation of platelet Tx is best measured by quantifying the urinary metabolites, 11-dehydroTxB_2, or 2,3-dinor TxB_2 through LC-MS/MS (Trostchansky et al. 2011, 2018; O'Donnell et al. 2014; Kornilov et al. 2019). This is considered a reliable way for in vivo platelet reactivity measurement.

In contrast to TxA_2, which comes from the COX pathway, 12-HETE generated by 12-LOX is quantitatively more abundant (Fuentes et al. 2021; Mendez et al. 2020; Paes et al. 2019). However, the effects of 12-HETE remain in discussion. As discussed earlier, LOX products can be detected by UV absorbance (Bonilla et al. 2013; O'Donnell et al. 2000). To analyze by LC-MS/MS, lipids extracted from platelets are separated by HPLC employing a Spherisorb ODS2 column (5 μm, 150 × 4.6 mm; waters). HPLC settings at a flow rate of 0.5 mL/min are 50–90% in 40 min with mobile phase A = H_2O/acetonitrile/acetic acid (75:25:0.1 v/v), and B = methanol/acetonitrile/acetic acid (60:40:0.1 v/v) (Trostchansky et al. 2011, 2018; O'Donnell et al. 2000). These chromatographic conditions allow also us to identify and quantify other platelets' positional HETE isomers by following *m/z* 319/179 (12-HETE) and *m/z* 319/219 (15-HETE) (Murphy et al. 2005).

Small amounts of PGs, particularly PGE_2 and prostaglandin D_2 (PGD_2) are formed in platelets. Although both bioactive lipids present the same transitions *m/z* 351/189, both compounds present different retention times in the column allowing their identification and quantitation (Trostchansky et al. 2018).

3.4 Analysis of Arachidonic Acid-Derived Metabolites on Platelet Mitochondria

Arachidonic acid is an omega-6 polyunsaturated fatty acid (PUFA); it is a structural part of the cell membrane and is necessary for membrane fluidity, flexibility, and function in all cell types (Paes et al. 2019). Fatty acids fulfill structural, signaling, and energy storage functions; phospholipids are the main structural lipids of platelets and their metabolism produces very important secondary mediators for the regulation of platelet activation (Lepropre et al. 2018). AA is released from phospholipid membranes and acts as a precursor to eicosanoids (Olechowski et al. 2017). When platelets are activated, signal transduction generates the mobilization of intracellular

calcium, which increases and activates phospholipases, which catalyze the release of phospholipids, such as AA (Morel et al. 2016). As discussed, eicosanoids are produced in response to different cellular stimuli such as hormones, stress, and cytokines (Boer et al. 2018); with different effects such as pro- and anti-inflammatory (Trostchansky et al. 2019).

Current evidence suggests that LOX- and COX-generated AA metabolites can induce ROS generation by stimulating NAD(P)H oxidase (NOX) and that there is a potential signaling connection between LOX/COX and NOX metabolites (Cho et al. 2011). Platelet aggregation has been reported to exponentially increase reactive oxygen species (ROS), such as hydrogen peroxide, which acts as a second messenger and stimulates AA metabolism and the phospholipase C pathway (Trostchansky et al. 2019). Excess ROS of NOX and/or mitochondria are related to vascular dysfunction and hypertension (Martinez-Revelles et al. 2013). The COX pathway is important for platelet activation, specifically in prothrombotic activity and the production of pro-inflammatory mediators (Bijak and Saluk-Bijak 2017).

Among the main metabolites of AA, we find PGs, which are synthesized in response to various physiological stimuli (Fang et al. 2004). Among the evidenced effects of PGs on mitochondria, we found that the physiological increase of PGE_2 increased mitochondrial function and autophagy (Palla et al. 2021). In dendritic cells, PGE_2 has a protective effect on the mitochondrial membrane and it generates a decrease in the activity of caspase 3 and granzymes, regulating several pro-apoptotic molecules (Vassiliou et al. 2004). On the contrary, the authors point out that PGE_2 decreases the mitochondrial membrane potential in cells that carry out cellular respiration, associated with a reduction in oxidative phosphorylation, but does not show damage to the mitochondria (Sanin et al. 2018). In this case, prostaglandin E1 (PGE1) has been used as a pretreatment for ischemic reperfusion injury in various biological systems, mainly due to a protective effect on the mitochondria (Zhu et al. 2017).

In the case of HETE eicosanoids, it has been described that 12-HETE in isolated mitochondria increases the concentration of intramitochondrial ionized calcium, stimulates the activity of mitochondrial nitric oxide (NO) synthase (mtNOS), which causes mitochondrial dysfunction by decrease respiration and transmembrane potential, which ultimately induces the release of cytochrome c and stimulates the aggregation of mitochondria (Nazarewicz et al. 2007). About 15-HETE has been shown that increases the generation of mitochondrial ROS, especially in the electron transport chain (Li et al. 2016). 20-HETE is characterized by increasing the production of mitochondrial superoxide (Lakhkar et al. 2016); to the point of generating mitochondrial dysfunction and apoptosis in neurons (with traumatic brain injuries) (Cui et al. 2021). Besides, 20-HETE induces apoptosis of cardiomyocytes, since it induces a decrease in the mitochondrial membrane potential and stimulates the activity of caspase-3 (Bao et al. 2011).

In heart failure, HETEs open the mitochondrial permeability transition pore, increasing mitochondrial calcium that triggers mitochondrial inflammation and myocyte death. At the same time, in a healthy myocardial model, phospholipase A2 produces AA for the generation of protective epoxyeicosatrienoic acids (EETs)

(Wolf 2018). Studies have reported that platelets activated by endogenous agonists release AA metabolites, such as EET and 20-HETE (Jarrar et al. 2013).

ETTs are epoxygenase metabolites of AA by the activity of cytochrome P450 that are recognized for their cardioprotective role, specifically for the prevention of calcium overload and maintenance of mitochondrial function (Batchu et al. 2012). ETTs trigger a protective response that limits mitochondrial dysfunction and reduces cell death, by regulating the autophagic response, resulting in a healthier pool of mitochondria in starved heart cells (Samokhvalov et al. 2013). It has also been described that ETTs are involved in the maintenance of homeostasis and protection against cell injury, mainly by counteracting the loss of mitochondrial membrane potential (El-Sikhry et al. 2011). Specifically, 14,15-EET can promote cell survival during ischemia/reperfusion in neurons through a decrease in the mitochondrial apoptotic pathway (Geng et al. 2017) promoting mitochondrial biogenesis (Wang et al. 2014). In a model of mitochondrial damage in cardiomyocytes by dronedarone, it decreases the mitochondrial membrane potential, inhibits the mitochondrial complex I, and uncouples the electron transport chain; In this context, the exogenous pretreatment of H9c2 cells with 11,12-EET and 14,15-EET improved cytotoxicity, the decrease in ATP, and the alteration of the mitochondrial membrane potential (Karkhanis et al. 2018).

3.5 Concluding Remarks

The increased use of LC-MS/MS methodologies is having an impact on the study of platelet function and the development of antithrombotic drugs targeted to the AA-metabolizing enzymes. The techniques discussed in this chapter are faster, cheaper, and easiest to apply compared to GC-MS, increasing the number of laboratories capable of performing lipid analysis. Also, LC-MS/MS benefits allow the study of large cohort sample sets, and new data with the potential to obtain information to understand disease mechanisms. As data is being collected, continuous work is still required to improve separation and analytical conditions to analyze large data sets from clinical studies to increase the potential of these techniques in terms of understanding disease mechanisms.

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Chapter 4
Cancer Therapy-Induced Inflammation and Its Consequences

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Abstract The inflammatory process often modifies the natural history of cancers. There is broad evidence that chronic inflammatory responses, associated with, e.g., persistent viral or bacterial infections, promote carcinogenesis. Cancer treatment is also associated with an inflammatory process that may either induce an antitumor immune response or, conversely, favor tumor recurrence. Here, we will revise the major aspects of therapy-induced inflammation and its consequences for tumor recurrence or repopulation, emphasizing how the mode of tumor cell death elicits an antitumor response, the key elements associated with the clearance of dead cells within the tumor microenvironment and the unleashing of an innate tissue regenerative response, dependent on lipid mediators such as prostaglandin E2 and the platelet activation factor (PAF), that favor tumor regrowth. Therapy-induced inflammation may offer a window of opportunity for combination therapies that increase the effectiveness of conventional cancer treatment modalities. Nanobiotechnology offers versatile platforms for anti-inflammatory interventions. Here we also discuss RNA-based approaches in the nanoscale, which would allow targeted interventions of pro-tumoral inflammatory milieu assembled in the course of therapeutic regimens in order to avoid the emergence of treatment-resistant cancer cells that ultimately repopulate the tumor mass.

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4.1 How Cancer Therapy Induces Inflammation?

4.1.1 The Role of Cell Death

The main goal of most cancer therapies is to induce cancer cell death, in spite of the mechanisms of the distinct anticancer agents. Many cancer therapies are very effective in reducing the number of cancer cells by destroying these cells; however, the main challenge of these therapies is that they are not able to eliminate all cancer cells and residual resistant cells can proliferate and originate tumor reestablishment. Here, we will focus on the role of inflammation in cancer therapy resistance and we highlight the cell death process as a linker of these phenomena.

It is a consensus that the presence of microbes in injuries is a potent inducer of inflammation; however, sterile injury can also stimulate inflammation. Accumulated literature of more than 150 years after the first association between tissue injury and cancer made by Virchow reveals that inflammation is a crucial link between tissue injury and cancer. It is now well-accepted that tissue injury generates cell death that activates cytokine secretion by inflammatory cells to mediate wound healing. If inflammation gets chronic and is combined with carcinogen exposure, it can result in malignant transformation (Kuraishy et al. 2011; Fishbein et al. 2021). In this context, it is interesting to note that cancer therapies also result in cell death along with inflammation and can be viewed as a collateral effect stimulus for the survival and proliferation of residual cells. Thus, cell death-induced inflammation is linked to either tumorigenesis or cancer therapy resistance.

In 1994 Polly Matzinger introduced the "danger theory," which postulates that the immune system activity is not based on distinguishing self from non-self, but rather from dangerous or not stimulus (Matzinger 1994). Considering that cells often die as a consequence of an infection, dead cells can be recognized as danger signals by the immune system and trigger an inflammatory response in order to protect the host from a potential danger. However, not all dying cells induce inflammation, the way a cell dies dictates if an immune response will be initiated or not. According to Matzinger's argument, inflammation is induced by necrosis because this type of cell death is involved in cellular processes potentially dangerous to the host, such as infection, whereas apoptosis is associated with physiological processes. It has been assumed for a long time that the immune system triggers a strong inflammatory response upon cell membrane rupture during necrosis and in contrast, apoptosis was considered to be a silent cell death process. However, accumulating knowledge about cell death mechanisms revealed that there are programmed forms of necrosis (necroptosis, NETosis, and pyroptosis) that also induce inflammation. Moreover, it is now well-accepted that the concept of apoptosis as a noninflammatory process is an oversimplification.

Apoptosis is a silent process because at least initially, apoptotic cells maintain their plasma membrane integrity and are cleared by professional phagocytes [macrophages and dendritic cells (DC)]. Apoptosis can also be a tolerogenic process by preventing the release of anti-inflammatory cytokines [e.g., interleukin10 (IL-10) (Chung et al. 2006)] and transforming growth factor-β (TGF-β) (Huynh et al. 2002) by macrophages, suppressing DCs activation through decreasing IL-12 (Stuart et al. 2002) or attenuating type I interferon signaling by TAM receptor engagement (Lemke and Rothlin 2008). Interestingly, it has been demonstrated that a slow clearance of apoptotic bodies can lead to secondary necrosis, resulting in cell membrane permeabilization, release of pro-inflammatory contents, and stimulating the immune system (Majno and Joris 1995). Thus, the efficiency of apoptotic cell clearance is a key factor in determining between silent and inflammatory apoptosis. As mentioned above, the strategies to treat cancer are diverse and the same is valid for the cell death mechanisms elicited by them; however, all signaling pathways leading from cancer therapy-induced cell death converge to inflammation.

4.1.2 How Cell Death Signals in Inflammation and Immunity?

From a mechanistic view, how do dead cells induce inflammation? As aforementioned, how a cell dies matters to understand how they induce inflammation or immunity. The most predominant cell death process elicited by the majority of chemotherapeutic drugs and radiotherapy is apoptosis. When a cell dies through apoptosis, it immediately releases soluble signals, which are classified into (1) "find-me signals," which attract phagocytes, mainly macrophages; and (2) "eat-me signals," which promote their engulfment (efferocytosis). An effective clearance of dying cells is crucial to avoid the release of potential autoantigens; however, impaired clearance of apoptotic cells is often observed after anticancer treatment. One evidence of this is that neutropenia is a common consequence of cancer treatment, which limits the tolerable dose of chemotherapy (Crawford et al. 2004). Additionally, the efferocytosis activity of the remaining phagocytes can be inhibited by some FDA-approved chemotherapeutic agents, including tamoxifen, sorafenib, bevacizumab, vinblastine, and vincristine (Green et al. 2016). Additionally, it has been shown that upon epirubicin/docetaxel combination therapy, HMGB1 circulating levels are increased in breast cancer patients (Arnold et al. 2013). Considering that both drugs cause neutropenia and HMGB1 is released during secondary necrosis, this piece of evidence supports the notion that cytostatic therapies not only induce apoptosis but can also trigger secondary necrosis.

A major characteristic of secondary, primary, and regulated necrosis is plasma membrane rupture accompanied by the release of intracellular molecules that become damage-associated molecular pattern molecules (DAMPs). The number of intracellular compounds from dying cells that are able to trigger inflammation is

unknown and the list of DAMPs is still growing. The nature of DAMPs is diverse and they can be prevenient from almost any cellular compartment: cytosol (e.g., uric acid, heat shock proteins, ATP), mitochondria (e.g., mtDNA, formyl peptides, ATP), nucleus (e.g., HMGB1, histones, DNA), plasma membrane (e.g., syndecans, glypicans), and endoplasmic reticulum (e.g., calreticulin) (Bianchi 2007). Following radiotherapy or treatment with some chemotherapeutic agents, tumor cells can release DAMPs which bind to different receptors (TLR2, TLR4, TLR9, and RAGE) present on the membrane of innate immune cells. As a consequence of this recognition, DC is activated and triggers engulfment of dying tumor cells, followed by tumor antigen processing and presentation to T cells. Ultimately, CD4+ and CD8+ T cells and natural killer (NK) cells are recruited to execute their antitumoral response (Hernandez et al. 2016).

This beneficial antitumor role of DAMPs has been shown in experimental models. Apoptotic cancer cells generated by ex vivo exposure to certain anticancer agents (e.g., anthracyclines, oxaliplatin, and ionizing irradiation), mediate an "anticancer vaccine effect," in the absence of any adjuvants or immunostimulatory substances, when implanted subcutaneously into immunocompetent mice (Casares et al. 2005; Obeid et al. 2007). Interestingly, subcutaneous implantation of secondary necrotic cells, originated by doxorubicin treatment, into syngeneic immunocompetent mice induces an antitumoral response mediated by adaptive immune system. In contrast, primary necrotic cells did not induce a protective immune response (Casares et al. 2005). Thus, the final therapeutic outcome of antitumor therapies is cytotoxic effects with tumor burden reduction, but in parallel, they can subsequently prime the immune system and promote anti- or pro-tumoral responses.

It was long believed that DAMPs were exclusively released from necrotic cells; however, it is now well-accepted that specific forms of programmed cell death can also trigger DAMP release, leading to the process of "immunogenic cell death" (ICD) defined as a form of regulated cell death (RCD) that is sufficient to activate an adaptive immune response in immunocompetent syngeneic hosts (Galluzzi et al. 2020). ICD can be induced by different anticancer treatments such as chemotherapeutic drugs [including anthracyclines (doxorubicin and idarubicin), platinum-based compounds (oxaliplatin), cyclophosphamide, mitoxantrone, and dipeptides (bortezomib)], γ-irradiation and photodynamic therapy (PDT) (Krysko et al. 2012). It is worth noting that not all cytotoxic agents can drive ICD, despite their similar RCD-inducing capability. The reason for this divergence relies on the fact that ICD induction depends on specific intracellular responses driven by the initiating stressor such as reactive oxygen species (ROS)-based endoplasmic reticulum (ER) stress (Garg et al. 2012).

However, DAMPs may also have a key role in cancer progression and resistance to anticancer treatments. DAMPs mediate tumor progression via distinct mechanisms, for example, HMGB1 may contribute to immunosuppression, angiogenesis, tumor cell proliferation, and inflammation (Hernandez et al. 2016). Several studies have underlined the effect of DAMPs on the resistance of tumor cells to different anticancer treatments. Chemotherapy-induced release of HMGB1 results in docetaxel resistance in prostate cancer cells (Zhou et al. 2015) and favors the

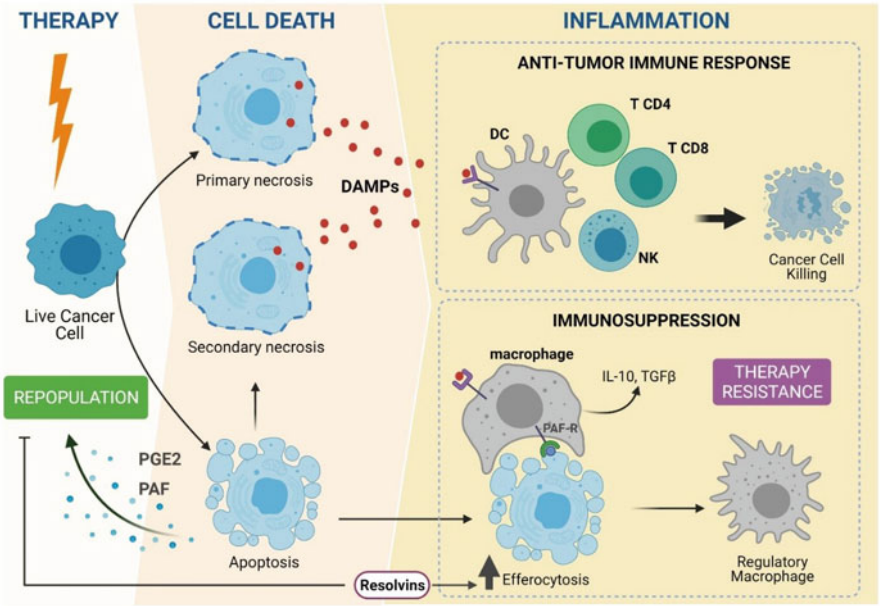

Fig. 4.1 Therapy-induced inflammation, friend or foe? Dying cells (necrosis) generated by anticancer therapy release damage-associated molecular patterns (DAMPs). DAMPs activate dendritic cells and increase tumor antigen presentation, resulting in an antitumor response that improves therapeutic outcome. Dead cells also recruit macrophages to execute their clearance (efferocytosis) and concomitantly it can polarize them toward a regulatory phenotype in a PAF-R-dependent manner, contributing to immunosuppression and rendering remnant cancer cells resistant to subsequent rounds of therapy. Dying cells also secrete lipid mediators, such as PGE2 and PAF, that can favor survival and proliferation of remnant cancer cells leading to tumor repopulation. Balance of this anti- and pro-tumoral consequences mediated by inflammation after therapy dictates the final therapeutic outcome (the figure was created using Biorender, biorender.com)

regrowth of remnant colon cancer cells after doxorubicin treatment (Luo et al. 2013). Additionally, released ATP can be hydrolyzed to adenosine, which has immunosuppressive activity and can promote a tumoral microenvironment that is associated with a reduction of antitumor immune responses efficacy (Ohta et al. 2006). Thus, while there is evidence that therapy-induced inflammation improves the therapeutic outcome by increasing tumor antigens presentation and consequent antitumor immune responses, there is also evidence that therapy-induced inflammation may promote tumor progression and favor therapy resistance. The ultimate response to anticancer therapy is dictated by the balance between anti- and pro-inflammatory mediators produced upon treatment, within a given dynamic immune landscape, which characterizes the tumor microenvironment (Fig. 4.1).

4.1.3 *Cytokines, Driving Mediators of Dying Cell-Induced Inflammatory Response*

Therapy-induced cell death stimuli trigger the release of DAMPs. DAMP-activated innate immune cells induce cytokines production, that are key mediators of inflammation (Fig. 4.2). The pivotal role of cytokines was initially considered as mediators of immune cell migration to the site of inflammation. Currently, we appreciate that these cytokines are also involved in tumor growth, progression, and therapy resistance (Chow and Luster 2014). Several studies have underlined the effect of cytokine production after anticancer treatments. Numerous in vitro and in vivo studies have shown evidence of changes in pro-inflammatory cytokine levels produced by a variety of cancer cells after administration of chemotherapy drugs (e.g., cisplatin, paclitaxel, 5-fluorouracil, and doxorubicin). We listed some of these findings in Table 4.1 to illustrate the diversity of pro-inflammatory cytokines generated by anticancer therapies. It has also been observed that inhibiting drug-induced cytokine signaling promotes the sensitivity of cancer cells to anticancer drugs. These studies suggest that the inflammatory cytokines released by tumor cells upon chemotherapy are implicated in mediating both resistance to cancer treatment. How do drug-induced cytokines alter tumor cell sensitivity to chemotherapy? One mechanism is altering pathways associated with apoptosis. Accordingly, the administration of recombinant human IL-8 to prostate cancer cells resulted in an increased expression of c-FLIP, an endogenous caspase-8 inhibitor (Wilson et al. 2008). Additionally, Sharma et al. (2013)) also demonstrated inhibition of spontaneous lung metastasis in

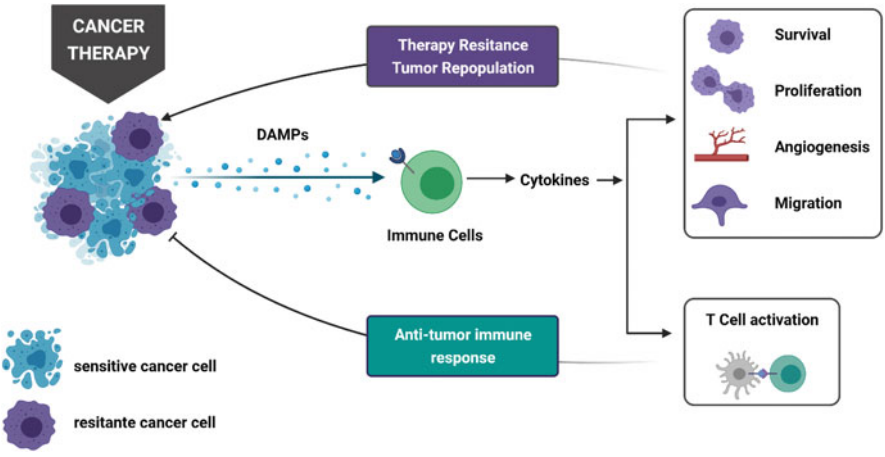

Fig. 4.2 Role of cytokines in therapy-induced inflammation. Following therapy, DAMPs generated by dying cancer cells activate cytokine-producing inflammatory cells. Cytokines bind to cognate receptors present in immune cells stimulating an adaptive antitumor immunity. Cytokines can also target residual cancer cells and induce pro-tumoral supportive phenotypes, such as cell survival, proliferation, angiogenesis, and migration, that promote therapy resistance and culminate in tumor repopulation (the figure was created using Biorender, biorender.com)

Table 4.1 Cytokine production upon anticancer therapy

Therapy	Cytokine	Cancer cell	References
Surgical resection	IL-1, TNF-α, IL-6	–	Desborough (2000)
5-Fluorouracil	IL-6, G-CSF, IL-1β	Head and neck squamous cell cancer	Reers et al. (2013)
Taxane	TNF-α	Breast and ovarian	Sprowl et al. (2012)
Cisplatin and paclitaxel	IL-8	Ovarian	Wang et al. (2011)
Oxaliplatin	CXCL8 and CXCL1	Prostate	Waugh and Wilson (2008)
Paclitaxel and doxorubicin	CXXL1	Breast	Sharma et al. (2013)
Irradiation	IL-6, -10, and TNFR1	Non-small cell lung cancer	Wang et al. (2010)
Irradiation	IL-6 and IL-8	Glioblastoma	Pasi et al. (2010)
Irradiation	IL-6 and IL-8	Human oral carcinoma cells	Tamatani et al. (2004)
Irradiation	IL-1, IL-6, and GM-CSF	Human lung cancer	Zhang et al. (1994)
Chemo-radiation	IL-6	Head and neck	Wang et al. (2010)

animals bearing CXCR2 knockdown tumors treated with paclitaxel. Chemokines can promote tumor cell migration as they act as attractant molecules, favoring the metastatic process. Indeed, several studies have underlined the effect of cytokines on promoting metastasis, as reviewed in Tanaka et al. (2005)). Considering that metastasis and chemoresistance in cancer are linked phenomena, cytokines production by tumor cells in response to chemotherapy are associated with the metastatic phenotype. Importantly, the role of cytokines in the tumor *milieu* goes much beyond their role as a chemoattractant, encompassing all tumor development steps, including tumor growth, angiogenesis, metastasis, and immune evasion, through immunoediting [reviewed in Raman et al. 2007 and Vyas et al. 2014].

Thus, tumor-promoting cytokines act in an autocrine or paracrine manner. Chemokines promote tumor growth by directly inducing cancer cell proliferation and migration, and indirectly by signaling to tumor stromal cells such as endothelial and immune cells favoring angiogenesis and immune evasion, respectively. CXCL8 is one of the cytokines produced by tumor cells that have both autocrine and paracrine pro-tumoral effects. This notion is supported by the evidence that IL-8-secreting prostate cancer cells were more resistant to docetaxel treatment and displayed increased vascular endothelial growth factor production along with increased microvessel density and abnormal tumor vasculature when compared with their vector-transfected control counterparts (Araki et al. 2007). In addition, IL-8 can signal for immune cell recruitment that contributes to cancer immune evasion. Tumor-derived IL-8 induces chemotactic recruitment of myeloid-derived suppressor cells (MDSC) (Alfaro et al. 2016) and DCs (Alfaro et al. 2011).

Interestingly, tumors producing IL-8 retain DCs and avoid their migration toward draining lymph nodes (Feijoó et al. 2005).

Not only tumor cells respond to chemotherapy-secreting cytokines, but also tumor stromal components, such as cancer-associated fibroblasts (CAFs) (Toste et al. 2016). It has been reported that gemcitabine treatment of CAFs induce upregulation of multiple inflammatory cytokines, including IL-8, that contribute to tumor-supportive phenotypes such as cell viability, migration, and invasion. Moreover, the inhibition of these cytokines attenuated these tumor-supportive functions. Considering all pro-tumoral roles of cytokines produced upon chemotherapy, they became a potential target for combined therapy. Indeed, in vivo studies have demonstrated that the specific inhibitor of CXCR4 receptor, AMD3100, sensitizes prostate cancer cells to docetaxel chemotherapy (Domanska et al. 2012). CXCR4 is the most common chemokine receptor expressed in most cancers and its ligand, CXCL12, is highly expressed on tumor stromal cells, mainly at the sites of tumor metastases and it is involved in homing of the tumors to different organs. Several in vitro and in vivo studies have demonstrated that the tumor-stroma interaction mediated by CXCR4/CXCL12 axis stimulates proliferation and migration of CXCR4-expressing cancer cells and is thought to protect them from cytotoxic chemotherapy [reviewed in Chow and Luster 2014]. Actually, the chemokine receptor inhibitor (CXCR4 antagonist AMD3100) is approved for the treatment of hematological malignancies (Mollica Poeta et al. 2019).

Not surprisingly, in vitro and in vivo studies have also demonstrated that radiotherapy induces an immediate inflammatory response with rapidly increased expression of many other inflammation-related cytokine genes (Hong et al. 1995; Schaue et al. 2012). Some examples of radiation-induced cytokine production are listed in Table 4.1. The general idea is that immediately after irradiation many cytokine cascades are activated sequentially, perpetuating an elevated cytokine production following irradiation. Fibrosis, a common late effect of radiotherapy, illustrates a consequence of this continuous cytokine response. Irradiation induced-cytokines unleash a persistent collagen production until apparent late effects of pathological fibrosis (Rubin et al. 1995). The cytokine cascade modifies the severity of the side effects observed post-irradiation. However, the biological implications of radiation-induced cytokine production go beyond its contribution to late radiation side effects, as cytokines can alter the primary tumor radiosensitivity. For example, IL-6 expression was positively linked with radiation resistance and IL-6 inhibition enhanced the radiation sensitivity of prostate cancer (Wu et al. 2013).

How irradiation-induced cytokines can modulate radiotherapy response? In mammalian cells, IR activates many pro-survival pathways that converge to transient activation of few transcription factors (TFs), including nuclear factor kappa B (NF-κB) and signal transducers and activators of transcription (STATs). IR induces a transient activation of NF-κB that is sufficient to produce multiple radioresistance signals, mainly by modulating anti-apoptotic pathways [reviewed in Magné et al. 2006]. The prevention of apoptosis, together with cell cycle arrest mediated by NF-κB activation after irradiation, favors a first moment DNA repair. However, sustained activation of NF-κB can allow the escape of radiation-induced DNA

damage cells from apoptosis (Jung et al. 1995). The central role of NF-κB regulation of radiation sensitivity and apoptosis after IR exposure was supported by the observation that cells from patients with ataxia–telangiectasia (AT) are hypersensitive to ionizing radiation but at the same time are defective in activating NF-κB and restoration of NF-kappa B regulation in these patients corrects the radiation sensitivity with a reduction of IR-induced apoptosis (Jung et al. 1995). In addition to apoptosis suppression, NF-κB activation regulates the transcription of a myriad of genes regulating immunity, proliferation, invasion, and angiogenesis, which favor radiotherapy resistance. Therefore, pharmacological inhibition of NF-κB would be a very interesting strategy to enhance tumor radiosensitivity. Indeed, compounds that suppress NF-κB activation, such as indomethacin and curcumin, enhanced radiation-induced apoptosis of Hela and prostate PC-3 cancer cells, respectively (Bradbury et al. 2001). Activation of the Jak-STAT pathway plays a significant role in radioresistance in different tumor models. Studies show that STAT3 mediates radioresistance of human squamous cell carcinoma (Bonner et al. 2009), prostate (Skvortsova et al. 2008), and breast cancer cells (Kim et al. 2006). Another member of the STAT family, STAT1, is also involved in renal cell carcinoma radioresistance (Hui et al. 2009). Targeting of STATs might also be a potential strategy to radiosensitize cancer cells; however, pharmacological inhibition of STAT for radiosensitization is not as far along in the drug development process, as compared to that of NF-κB inhibitors. Both transcription factors, NF-κB and STAT-3, regulate the expression of pro-inflammatory genes and cytokines that suppress apoptosis and induce invasion, metastasis, and angiogenesis processes, contributing to tumor cell radioresistance [reviewed in Di Maggio et al. 2015]. IR-induced IL-1β expression is one example of inflammatory IR response favoring tumor cell invasion and metastasis. Breast cancer patients have elevated IL-1β plasma levels persistent for a few weeks after radiotherapy (Sepah and Bower 2009) and in vitro studies demonstrate that IL-1β is involved in breast cancer cell invasion induced by IR (Paquette et al. 2013).

Irradiated tumor cells release several factors, including cytokines, involved in biological effects not only in irradiated cells but also in non-irradiated cells. There are three forms of non-target effects (NTEs) in radiotherapy, namely (1) bystander effect; (2) cohort effect; and, (3) abscopal effect (Wang et al. 2018). The bystander effect is defined as signals from irradiated tumor cells to neighboring non-irradiated cells. Cohort effects are responsible for the overall radiobiological response in irradiated cells that results from the direct energy deposition to target cells combined with indirect signals emitted from the neighboring irradiated cells. Abscopal effects are dependent on distant non-irradiated cells, which can also respond to irradiation consequences. These effects are mediated primarily by immune cells, such as T cells. In addition to nitric oxide and ROS, cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin 8 (IL8), and transforming growth factor beta (TGF-β), have been implicated as a source of NTEs (Iyer et al. 2000; Gandhi and Chandna 2017).

4.2 Beyond Cytokines. The Role of Lipid Mediators Produced by Cancer Therapy

4.2.1 Prostaglandin E2 (PGE2)

In addition to cytokines, other mediators of inflammation are secreted after anticancer treatments and contribute to pro-tumorigenic signaling pathways that are critical for tumor growth, immunosuppressive microenvironment, and therapy resistance. In this chapter, we emphasize the involvement of lipids as mediators of inflammation upon anticancer treatment. Huang-Li demonstrated that apoptotic tumor cells stimulate the proliferation of a small number of living tumor cells, resulting in an accelerated tumor repopulation. In this study, they demonstrated that ionizing radiation induces apoptosis by activating caspase-3, which is the master "executioner" of apoptotic cell death and in parallel generates PGE2, a potent growth-stimulating signal of surviving tumor cells. In accordance with these findings, (Kurtova et al. 2015) it has been shown that PGE2 secreted by chemotherapy-induced dying cells promotes neighboring cancer stem cell repopulation, contributing to chemoresistance and indicating a role for PGE2 in tumor repopulation.

PGE2 belongs to the prostanoid family of lipids and is enzymatically synthesized from membrane phospholipids oxidation by cytoplasmic phospholipase A2 (PLA2), releasing arachidonic acid (AA). Free AA is converted to prostaglandin G2 (PGG2), which is subsequently reduced to PGH2 by the cyclooxygenase (COX) enzyme. Finally, PGH2 is metabolized to PGE2 through one of three PG terminal synthases: [microsomal PGE synthase-1 (mPGES-1 and mPGES-2)] and cytosolic PGE synthase (cPGES). Upon its biosynthesis, PGE2 binds to their cognate cell-surface receptors, designated EP1–EP4, either in an autocrine or paracrine fashion (Sugimoto and Narumiya 2007).

Among prostanoids, PGE2 is the predominant member found in many cancers, including colon, lung, breast, and head and neck cancer, and predicts poor prognosis (McLemore et al. 1988; Rigas et al. 1993; Wang and Dubois 2004; Hambek et al. 2007). Several studies have demonstrated a key role of PGE2 in promoting tumor progression by inducing cellular proliferation and angiogenesis, enhancing invasiveness, making cells resistant to apoptosis, and modulating immunosuppression [reviewed in Wang and Dubois 2010 and Finetti et al. 2020].

Secreted PGE2, contributes to the inhibition of antitumor immune responses by mediating immune cells [myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), dendritic cells (DCs), natural killer (NK) T cells, and regulatory T cells (Tregs)] to establish a tumor immunosuppression microenvironment [reviewed in Finetti et al. 2020]. PGE2 controls MDSC differentiation, recruitment, retention, and activation (Yang et al. 2015; Porta et al. 2020). It has been extensively described that PGE2 regulates macrophage polarization toward an M2 polarization (Yin et al. 2020) and also controls the recruitment of these immune cells into the tumor (Oshima et al. 2011). Tumor-derived PGE2 plays a key role in controlling DC differentiation, inhibiting the antigen presentation ability of

BM-derived DCs and favoring DCs role of T cell tolerance instead of antitumor immunity. Notably, PGE2 secreted by tumor cells suppresses NK cell activity (Wang and DuBois 2018). In addition, PGE2 inhibits T cells proliferation, regulates CD4+ T cells toward Th2 development, and inhibits antitumor cytotoxic T lymphocyte (CTL) responses (Sharma et al. 2005; Shimabukuro-Vornhagen et al. 2013; Basingab et al. 2016).

PGE2 immunosuppression can contribute to immunotherapy resistance. Interestingly, exposure of PBMCs to PGE2 previous to stimulation results in a decrease of proliferating T cells and in parallel induces the expression of the co-inhibitory receptors, PD-1 and TIM3. Additionally, inhibiting PGE2 partially restores T cells proliferation (Gorchs et al. 2019). Another evidence that PGE2 is related to immunotherapy resistance is that PGE2 signaling through EP2 and EP4 receptors present in cytotoxic T lymphocytes (CTL) contributes to its suppressive function. Moreover, simultaneous blockage of PD-1 and PGE2 EP2 and EP4 receptors restore CTLs cytotoxic functions (Miao et al. 2017). In a murine model, it was observed that tumor cells induce PD-L1 expression in myeloid cells which exhibits upregulation of PGE2-forming enzymes COX2 and microsomal PGE2 synthase 1 (mPGES1). The pharmacologic inhibition of these two enzymes reduces tumor-induced PD-L1 expression in myeloid cells (Prima et al. 2017). Indeed, preclinical models shows that COX inhibitors synergize with anti-PD-1 mAb (Zelenay et al. 2015). Combination of celecoxib, a selective COX2 inhibitor, and anti-PD-L1 inhibit PD-L1 expression in myeloid cells together with a reduction in murine melanoma and breast cancer progression (Li et al. 2016). In accordance with these findings, COX2/PGE2 axis inhibition can render tumor cells susceptible to immune control and might contribute to unleashing anticancer immunity, emerging as an adjuvant strategy to PD-1 blockade immune-based therapies.

All these reports demonstrate the PGE2 role in promoting pro-tumoral characteristics and favoring an immunosuppressive tumoral niche which leads to tumor growth. Thus, interference in PGE2 production could be an alternative to prevent tumor progression and reprogram tumor immunity. PGE2 production can be reduced by non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit COX, the main enzyme involved in PGE2 production. Indeed, the contribution of the lipid mediator PGE2 to cancer development was evidenced by epidemiological observations showing that regular use of NSAID aspirin reduces mortality, metastasis, and incidence risk of various solid tumors (Veettil et al. 2017; Lin et al. 2018; Ma and Brusselaers 2018; Cho et al. 2020). However, the use of current targeting PGE2 therapies, NSAIDs, or COX-2 selective inhibitors (COXIBs) is limited due to their unacceptable cardiovascular and gastrointestinal side effects associated with their global proteinoid suppression. To avoid toxicity and achieve efficacy in reducing PGE2 levels, it is more clinically plausible blocking PGE2 biosynthesis by selectively targeting PGE2 EP receptors. Indeed, all PGE2 pro-tumorigenic roles are dependent on the activation of PGE2 EP receptors and they can be expressed on the surface of both tumor and tumor stromal cells. In this context, various small-molecule ligands targeting EP receptors have been identified, one example is the antagonist ONO-8711 specifically blocks EP1 receptors and exhibits chemopreventive activity

in several animal models of epithelial malignancy (Kawamori et al. 2001). However, EP antagonists have not been available in clinics up to now. Therefore, it is crucial to develop more effective and selective strategies to diminish PGE2 levels in cancer patients as an adjuvant strategy to conventional and immune-based cancer therapies.

4.2.2 Platelet Activating Factor (PAF)

Conventional chemotherapy and radiotherapy generate another lipid mediator of inflammation, platelet activating factor (PAF). Considering that PAF is synthesized in response to stress, including agents that induce DNA damage (Barber et al. 1998) and free radical formation (Lewis et al. 1988), it is intuitive to think that chemotherapy and radiotherapy may generate PAF. Indeed, a large number of studies have demonstrated that different anticancer therapy agents can induce overproduction of PAF agonists and increase the expression of its receptor, PAF-R, in diverse tumor cells. Chemotherapeutic agents (etoposide, dacarbazine, and cisplatin) and radiotherapy can generate native PAF and PAF agonists in melanoma tumors. Furthermore, PAF/PAF agonists generation by chemotherapy was partially blocked by antioxidants and PAF-R activation inhibits chemotherapy effectiveness by subversion of tumor-host immunity through regulation of Tregs in a COX-2-dependent process (Sahu et al. 2014, 2016). Additionally, chemotherapy induces PAF-R expression and PAF-R antagonist chemosensitizes melanoma cells in vitro and in vivo (Onuchic et al. 2012).

PAF is a potent pro-inflammatory lipid mediator which under physiological conditions is produced in small and continuous amounts by de novo synthesis and participates in membrane biogenesis. However, upon acute inflammation, such as that induced by radio and chemotherapy, large amounts of PAF are produced. Binding of PAF/PAF agonists molecules to its receptor activates many downstream survival pathways, including mitogen-activated protein kinase (MAPK) cascade and nuclear factor kappa-beta (NF-kB) (Ishii and Shimizu 2000). The role of PAF in tumorigenesis is complex, it can contribute to homeostasis by limiting cell proliferation and inducing apoptosis, and it can also promote tumorigenesis by stimulating cell growth, inhibiting DNA repair, inducing angiogenesis and metastasis (Tsoupras et al. 2009; Lordan et al. 2019). The balance between these opposing forces determines the final effect of PAF on tumorigenesis.

The role of PAF in inducing immunosuppression was well described in studies designed to define the molecular events involved in UV-induced immunosuppression. It has been shown that UVB-irradiated keratinocytes generate PAF/PAF agonists and administration of PAF-R antagonists in UV-irradiated mice inhibits UV-induced immune suppression. The general idea is that UVB irradiation generates PAF agonists which signal through PAF-R and activate downstream survival and immunosuppressive pathways, including the production of cytokines [e.g., TNF-α, IL-6, IL-10, COX-2, and PGE2 (revised in Ullrich 2005)]. It has been shown that this systemic immunosuppression contributes to the establishment of

murine melanoma tumors. Administration of cPAF enhances B16F10 tumor growth in vivo; however, this effect is not observed in immunodeficient NOD SCID mice, suggesting that it depends on targeting PAF-R on host immune cells (Sahu et al. 2012). This notion was also supported by animal models, whereby growth of two murine tumors, B16F10 melanoma and TC-1 carcinoma, was reduced in PAF-R KO, as compared to wild-type animals. Considering that TC-1 cells express PAF-R, whereas B16F10 do not this data reinforce the role of PAF-R signaling in immune cells. It also observed an increase in M2 macrophages frequency and intratumoral neutrophils, $CD4^+/CD8^+$ lymphocyte infiltration in PAF-R KO animals. These data suggest that tumor-derived PAF-R ligands regulate the recruitment and phenotype of immune cells, favoring tumor growth (da Silva et al. 2017). Accordingly, exogenous PAF was shown to potentiate the production of anti-inflammatory IL-10 by LPS-stimulated macrophages, driving them toward a regulatory phenotype (Ishizuka et al. 2016).

Similar PAF regulatory effects were also observed in LPS-stimulated murine DC. PAF-R is present on the DC membranes and its activation mediates DC phenotype and function. Koga et al. (2013) demonstrated that PAF-R activation during DC maturation resulted in a downregulation in antigen-presenting capacity of DC through the increased production of IL-10 and PGE-2 mediated by PAF-R. Moreover, in vitro treatment of DCs with PAF-R antagonists induce higher CD4+ T cell proliferation, indicating that the adaptive immune system is also involved in PAF-R-dependent tumor growth. This notion is supported by the evidence that exogenous cPAF does not affect tumor growth in immunodeficient NOD SCID mice, indicating the participation of Tregs in this pro-tumoral PAF-R response. Tumor growth mediated by PAF-R activation can be inhibited by depleting antibodies against Tregs and IL-10. Essentially, UVB-generated PAF agonists target host immune cells to orchestrate a systemic immunosuppression that favors murine melanoma tumor growth (Sahu et al. 2012). In accordance with these findings, it is appropriate to conclude that activation of PAF/PAF-R axis plays an important role in the regulation of inflammatory and immune responses.

Several chemotherapy regimens and mainly radiotherapy induce reactive oxygen species (ROS) production which can oxidize membrane phosphatidylcholine leading to PAF agonist production. Secreted PAF binds to PAF-R and in positive feedback, PAF-R activation promotes the synthesis of bona fide PAF. This amplified production of PAF results in an enhancement of PAF/PAF-R downstream biological processes discussed above. Briefly, PAF can signal in an autocrine way to tumor cells, stimulating proliferation and migration. Additionally, a paracrine signal of PAF to endothelial cells favors angiogenesis and to immune cells, mainly macrophages and T cells, promote immunosuppression by shifting these cells toward an immunoregulatory phenotype (Chammas et al. 2017).

Independently of ROS generation, all anticancer therapies result in cell death. As discussed above, when cells die they are engulfed by specialized phagocytes, the macrophages, through the exposure of several molecules on their surface which are recognized by macrophage receptors [reviewed in Gregory and Devitt 2004]. Importantly, macrophages do not simply engulf and digest apoptotic cells, they respond to

these cells by changing the profile of pro- and anti-inflammatory mediators that they release. Accordingly, it has been shown that the professional scavenger role of macrophages is dependent on PAF-R activation which reprograms these cells toward a regulatory phenotype. The phenomenon of efferocytosis of apoptotic and necrotic cells can be decreased by pretreating macrophages with PAF-R antagonists (de Oliveira et al. 2006). Another piece of evidence shows that efferocytosis of apoptotic cells requires the engagement of both CD36 and PAF-R (Rios et al. 2013). Coculture of mice bone marrow-derived macrophages with apoptotic thymocytes in the presence of PAF-R antagonists or specific antibodies against CD36 inhibited the phagocytosis of apoptotic cells by approximately 70–80%. Blocking PAF-R or CD36 also prevented efferocytosis-induced production of IL-10, inhibiting the regulatory cytokine profile IL-10 (high)/IL-12p40 (low) (Ferracini et al. 2013). All these reports indicate that the macrophage role of apoptotic cell clearance depends on PAF/PAF-R activation and is associated with a modulation of macrophage suppressor phenotype that contributes to tumor growth.

Additionally, apoptotic cell clearance results in immune implications dependent on the PAF/PAF-R axis that contribute to tumor repopulation. In animal models, coinjection of apoptotic cells promotes tumor growth from a sub tumorigenic dose of melanoma cells or Ehrlich ascites tumor. Moreover, results demonstrated that PAF-R antagonists significantly inhibited the tumor growth-promoting effect of apoptotic cells concomitant to the inhibition of early neutrophil and macrophage infiltration (de Oliveira et al. 2010; Bachi et al. 2012). Irradiated TC-1 cancer cells induce the proliferation of live TC-1 cells in vitro and in vivo in a PAF-R-dependent way. Tumor cell repopulation was correlated with increased infiltration of tumor-promoting macrophages (CD206+) (da Silva et al. 2017). It is worth noting that besides the development of PAF-R antagonists, none are in clinics due to toxicity issues and as far as we know there are no therapeutic strategies available to interfere in PAF synthesis. In this context, it would be of interest to study a putative beneficial effect of the combination of new strategies to inhibit PAF/PAF-R axis and radio or chemotherapy.

4.2.3 Resolvins

The notion that therapy-generated tumor cell death is a double-edged sword is now well accepted. Several manuscripts support this concept and show that tumor cell debris generated throughout chemotherapy, radiotherapy, or target therapy (Huang et al. 2011; da Silva et al. 2017; Sulciner et al. 2018) stimulate tumor growth. As previously discussed, PAF/PAF-R is involved in the dual effect of cytotoxic cancer treatments. Additionally, another lipid is recently reported to have a key role in tumor repopulation phenomenon. Proresolving lipid autacoids, specific RvD1, RvD2, or RvE1, stimulate the resolution of tumor-promoting inflammation (Sulciner et al. 2018). Interestingly, in this latter study, a critical role for phosphatidylserine in cell debris-stimulated tumors was described through the use of neutralizing anti-PS

antibodies. Anticancer therapies induce sterile inflammation by apoptotic cells release of inflammation "danger signals" that can either activate or suppress antitumor immunity. Stimulation of debris clearance process, in order to promote the termination of the inflammatory process, represents a new approach to inhibit tumor progression, growth, and recurrence (Serhan and Levy 2018). Thus, resolvins (i.e., RvE1, RvD1, and RvD2) can polarize the pro-tumorigenic and pro-inflammatory macrophages present in therapy-induced inflammatory microenvironment toward a pro-phagocytic state, inhibiting further pro-inflammatory cytokine secretion. Likewise, other lipid mediators derived from the activity of epoxide-hydrolases phenocopy the activities of resolvins (Zhang et al. 2014; Gartung et al. 2019; Fishbein et al. 2020). Findings provided by these reports further the interest in determining specific conditions in which therapy-generated cell debris activates or suppresses antitumor immunity to allow the design of new therapeutic approaches more efficiently in preventing tumor growth and recurrence.

4.3 Modulating Inflammation for Cancer Therapy by Nanobiotechnology

In cancer therapy, inflammation is an undesired but prevalent side effect that complicates treatment and, in some cases, can be a danger to the patient's health. Because of the possibility for severe adverse reactions, many developing treatments are delayed or stopped as they are deemed unsuitable for clinical use (Pecot et al. 2011). Furthermore, the induction of pro-inflammatory cytokines responsible for such inflammatory reactions also plays various roles throughout the hallmarks of cancer by promoting tumor growth and invasion (Dinarello 2006). Upstream of cytokine production in the cellular environment are pattern recognition receptors (PRRs) which activate a cascade of signals upon interactions with pathogen-associated molecular patterns (PAMPs) or in response to damage-associated molecular patterns (DAMPs) (Takeuchi and Akira 2010). These innate pathways are well-adapted to protect against pathogens, yet also can trigger the production of pro-inflammatory cytokines in response to cell death, even when favorable in the case of cancer therapies (Hernandez et al. 2016).

As a strategy for targeted and personalized medicine, nanotechnology offers a modular approach to overcoming unfavorable immune responses while maintaining the therapeutic effects of formulations. Established candidates for drug delivery can be selected based on their immunological profiles, even incorporating some known biological structures and PAMPs to fit the application as needed. For cancer therapies, the ideal candidates are those which can generate antitumor responses via inflammation without overstimulating a more chronic inflammatory response and ultimately aggravating cancer (Ilinskaya and Dobrovolskaia 2014; Barber 2015). Approaches may also focus on promoting anti-inflammatory activities or overall immunosuppression, which has its own consequences in the form of potential

myelosuppression, thymic suppression, and overall lowered immune function (Ilinskaya and Dobrovolskaia 2014). Nanobiotechnology can be used to deliver anti-inflammatory drugs to increase their overall solubility and bioavailability (Ilinskaya and Dobrovolskaia 2014). For example, dendrimers have been used as carriers for methotrexate and indomethacin in order to reduce inflammation, while the dendrimers themselves are anti-inflammatory, owing to their generation and surface group functionalization (Chandrasekar et al. 2007; Chauhan et al. 2009). Specific targeting of diseased cells only can be achieved using nanoplatforms in order to recruit particular cell populations to zones of inflammation. An example of this is the use of folic acid on chitosan nanoparticles to deliver siRNAs against COX-2 into activated macrophages (Yang et al. 2014). The highly customizable approach of nanotechnology allows for combinatorial strategies, such as for the codelivery of anti-inflammatory agents and targeting moieties.

Some of the foremost targets of PRRs are nucleic acids, owing to their roles in pathogenic invasion, but specifically for PAMPs which indicate the presence of non-self-genetic materials over self. As a result, nucleic acids offer a means to modulate therapy-induced inflammation and can be tailored, based on their sequences and resulting structures, to vary the resulting productions of pro-inflammatory to anti-inflammatory cytokines as desired. Agonists of the cGAS-cGAMP-STING pathway, for example, have been utilized for the development of vaccine adjuvants as well as cancer immunotherapies to activate antitumor T cell responses (Barber 2015). In addition to their immune recognition, nucleic acids retain their functional abilities to encode proteins, control posttranscriptional gene regulation, and interact with other classes of biomolecules, which then allows them to serve as vaccines encoding neoantigens, mRNAs for immunomodulation, viral mimics, and inducers of gene silencing in cancer therapy (Bisogno and Keene 2018; Lin et al. 2020). Nucleic acids have been demonstrated to silence the genes for immune checkpoints, play roles in cytokine regulation, and also act as vaccines (Lin et al. 2020). For example, siRNAs against TNF-α can be delivered to cells to reduce inflammation (Howard et al. 2009).

Individual strands of short synthetic nucleic acids can also be rationally designed to self-assemble into well-defined nucleic acid nanoparticles (NANPs), which are at the center of an emerging technology with the potential to manipulate and control biological processes at the molecular level (Dobrovolskaia 2019; Panigaj et al. 2019). NANPs may be composed of either DNA or RNA or their chemical analogs, all of which are programmed to interact via canonical Watson–Crick or non-canonical base pairing to result in the reproducible formation of specific nanostructures. NANP platforms have been developed to serve as biocompatible nanoscaffolds for the simultaneous codelivery of functional biomolecules (Afonin et al. 2014; Halman et al. 2017), therapeutic nucleic acids (Afonin et al. 2011), or fluorescent arrays (Yourston et al. 2020). However, in the biological environment outside of cells, NANPs are effectively invisible to the immune system. Due to their macromolecular structure, NANPs alone are too anionic to be efficiently taken up by cellular membranes or immune cells (Hong et al. 2018). The only way for a given NANP to enter a cell is thus via transfection using a delivery carrier, which is the only route to accessing the intracellular PRRs responsible for immunostimulation.

As was recently discovered, NANPs' interactions with the immune system can be controlled based on the structure, dimensionality, and composition of the NANP (Guo et al. 2017; Halman et al. 2017; Johnson et al. 2017, 2020; Rackley et al. 2018; Chandler and Afonin 2019; Hong et al. 2019; Ke et al. 2019; Dobrovolskaia and Afonin 2020), as well as the type of carrier used for NANPs' delivery (Dobrovolskaia and McNeil 2015; Halman et al. 2020; Avila et al. 2021).

Toll-like receptors (TLRs) are a particular class of PRRs which are produced and utilized by the immune system to interact with nucleic acids as a defense against foreign sequences. In humans, TLRs 3, 7, 8, and 9 are endosomal PRRs specific to nucleic acids and therefore are also involved in the recognition of NANPs composed of them. While these PRRs bind based on a variety of parameters, there are general structural trends to their activation. For instance, TLR3 binds to double-stranded RNA. This pathway leads to activating type I interferon production (Alexopoulou et al. 2001; Ranjith-Kumar et al. 2007; Leonard et al. 2008). TLR7 and TLR8 interact with single-stranded RNA (Heil 2004; Lund et al. 2004) and preferentially bind to uridine-rich sequences (Zhang et al. 2018). The MyD88 pathway is activated and results in the expression of type I interferons and pro-inflammatory cytokines (Heil 2004; De Marcken et al. 2019). TLR9 recognizes unmethylated CpG-rich DNA motifs and also induces the expression of type I interferons and pro-inflammatory cytokines (Latz et al. 2004). Besides endosomal PRRs, there are also several cytosolic sensors for non-self-nucleic acids. For example, the cGAS-cGAMP-STING pathway detects double-stranded DNA (Nakhaei et al. 2010; Motwani et al. 2019). RIG-I recognizes triphosphorylated RNAs (Hornung et al. 2006; Loo and Gale 2011) while longer double-stranded RNAs can be identified by MDA5 (Chandler et al. 2020). As a result of these trends in detection, the dimensionality, composition (DNA vs RNA), and functionalization with therapeutic nucleic acids on the NANP scaffold can determine the specific PRR and guide the resulting immune response (Chandler et al. 2020). Importantly, varying the sequence of a specific NANP does not seem to affect the immunostimulation so long as its structure is maintained (Chandler et al. 2019). The type of carrier also influences the route of delivery and thus the interactions which determine the response of the immune system (Halman et al. 2020; Avila et al. 2021).

The tailorability of nanotechnology is well-exemplified by NANPs, as variation in designs can be utilized to selectively aid in turning off or avoiding pro-inflammatory reactions or activating them as needed. For example, when delivered with a lipid-based carrier, three-dimensional RNA cubic NANPs have been consistently shown to interact with TLR7 for the downstream production of type I interferons, while three-dimensional DNA cubic NANPs are largely immune quiescent in human immune cells (Hong et al. 2018). Further investigations into the structure–activity relationship of NANPs to link a library of thoroughly physicochemically characterized NANPs with their associated panel of responses and their relative magnitudes hold much promise as an asset to effectively modulating the immune response. With the right combinations selected per patient, this technology could allow for more careful modulation of the inflammation associated with cancer therapy.

Much of the groundwork has been laid out by those that realized nucleic acids are much more functional beyond solely carrying information. As nucleic acid nanotechnology is further studied and fine-tuned, better treatments in favor of patient health grow closer to reality. Currently, there are several RNA-based therapies recently approved by the US Food and Drug Administration to treat a number of conditions, with many more in the pipeline (Afonin et al. 2020). While the systematic recognition by the innate immune system has previously challenged therapeutic nucleic acid development, the new outlook of harnessing these established routes for favorable modulation could allow for advanced applications in cancer therapy. This gap in knowledge requires further investigations of such therapies to produce safe and effective treatments (Afonin et al. 2020).

4.4 Conclusion and Prospects

Therapy induces the secretion of inflammatory mediators by dead or dying cells that recruit the immune system. In the first moment, these mediators orchestrate the clearance of dead cells and elicit an antitumoral immune response. However, in the long run, the pro-inflammatory mediators generated by dead cells induce the survival of the remnant tumor cells and promote a microenvironment that favors tumor recurrence. The challenge posed to the future is to identify clearly distinct phases in the post-therapy continuum in which inflammation could be either boosted or blocked. Understanding the different phases of therapy-induced inflammatory responses will allow further development of anticancer therapies that will likely exploit nanocarriers or nano-approaches that shape the immune/inflammatory landscape within tumors.

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Chapter 5
Advanced Therapies for Patients with COVID-19

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Abstract SARS-CoV-2 is a novel *Betacoronavirus* species that has caused the coronavirus disease 2019 (COVID-19) pandemic. At the moment, there is no definite or unified treatment protocol for patients with COVID-19; numerous therapies have been proposed and several are still under investigation, including mesenchymal stem cells (MSCs) and their bioactive products. Although still poorly addressed, MSC therapy has been evaluated for several viruses and proven safe. Allogeneic MSCs are especially attractive due to their potential to provide an immediately available therapy for patients with acute critical illness and multiple organ dysfunction, which makes them an interesting alternative as adjuvant therapy for SARS-CoV-2 infection. The immunoregulatory properties of MSCs have not entirely been elucidated, but it appears that cell–cell contact and trophic factors, ranging from cytokines to growth factors and extracellular vesicles, might exert key roles. Gene therapies have also emerged as a promising, though still experimental, option for treatment or prevention of a number of diseases. More recently, clustered

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regularly interspaced short palindromic repeats (CRISPR)-Cas systems and mRNA vaccines have been extensively investigated as therapeutic possibilities for patients with COVID-19. mRNA vaccines against SARS-CoV-2 demonstrated promising results in clinical studies, and have obtained both emergency approval and marketing authorization in several countries. Further validation studies are still needed, but they may become a feasible alternative to traditional vaccines. As COVID-19 has massively impacted worldwide public health since early 2020 and continues to carry a high fatality rate in 2021, the investigation of novel therapies, including advanced therapies, such as those described in this chapter, remains an urgent and pressing need.

Keywords SARS-CoV-2 · Cell therapy · Mesenchymal stromal cells · Gene therapy · mRNA-based vaccines

5.1 Introduction

The coronavirus disease 2019 (COVID-19), caused by a novel coronavirus—the severe acute respiratory disease coronavirus 2 (SARS-CoV-2, initially known as 2019-nCoV)—has a broad clinical spectrum ranging from mild respiratory infection, characterized mainly by fever and cough, to severe pneumonia and acute distress respiratory syndrome (ARDS), which is the major cause of COVID-19 death in intensive care units (ICUs) (Öztürk et al. 2020). In addition to acute respiratory failure, COVID-19 patients may also develop multiple organ dysfunction and long-term sequelae, including neurological and neuropsychiatric illness (Paterson et al. 2020). Currently, there is no specific treatment for SARS-CoV-2 infection; only supportive care is available, including noninvasive and invasive ventilatory support, management of organ injuries, symptomatic treatment, glucocorticoids, antivirals, and antishock therapy (Cancio et al. 2020). Accordingly, investigation into novel therapies for COVID-19 remains an urgent need. In this chapter, we will address three major groups of advanced therapies—mesenchymal stromal cells (MSC) and their derivatives, gene therapies, and mRNA vaccines—and the rationale for their use in this life-threatening condition.

5.1.1 Mesenchymal Stromal Cell Therapy for COVID-19

Stem cells are undifferentiated cells with the ability to self-replicate and differentiate into different cells in vivo (Weiss et al. 2008). In 1908, the term "stem cell" was first used by Alexander Maksimov. In the following decades, the existence of cells with the capacity for proliferation and formation of different tissues started to be extensively investigated, and, in 1963, the ability to form different cells in the blood tissue was attributed to the so-called hematopoietic stem cells (HSCs), the immature precursors of all blood cells (Becker et al. 1963).

Mesenchymal stromal cells (MSCs) were first discovered by Friedenstein et al. (1970), based on their observation that ectopic transplantation of bone marrow into the kidney capsule results not only in the proliferation of bone marrow cells but also the formation of bone, indicating the existence of a second stem cell population (in addition to hematopoietic cells) which gives rise to bone precursors. These bone marrow adherent fibroblast-like cells, when isolated, had the ability to differentiate in vitro into osteocytes, chondrocytes, and adipocytes, and to grow rapidly in vitro in the form of clonogenic colonies (Andrzejewska et al. 2019). In 1991, the term mesenchymal stem cell was first coined by Caplan (1991).

Since then, the use of MSCs in several diseases has been widely investigated due to their regenerative and therapeutic potential in animal models and human clinical trials and has grown exponentially over the past decade (Fig. 5.1). There has been growing research interest in MSCs derived from different tissue types, such as adipose tissue and perinatal tissues (umbilical cord, Wharton's jelly, and the placenta), as well as different cell types that MSC was reportedly able to differentiate into muscle, endothelium, neurons, heart, liver, and kidney cells generating controversies in the MSC field regarding methods for isolation, characterization, and nomenclature standardization. Consequently, in 2006, the International Society for Cellular Therapy (ISCT) recommended a new designation for the term MSC—multipotent mesenchymal stromal cells (Dominici et al. 2006). However, the definition of MSCs is still much debated in the literature, since MSCs derived from different tissues can differ drastically in their gene expression and in their ability to

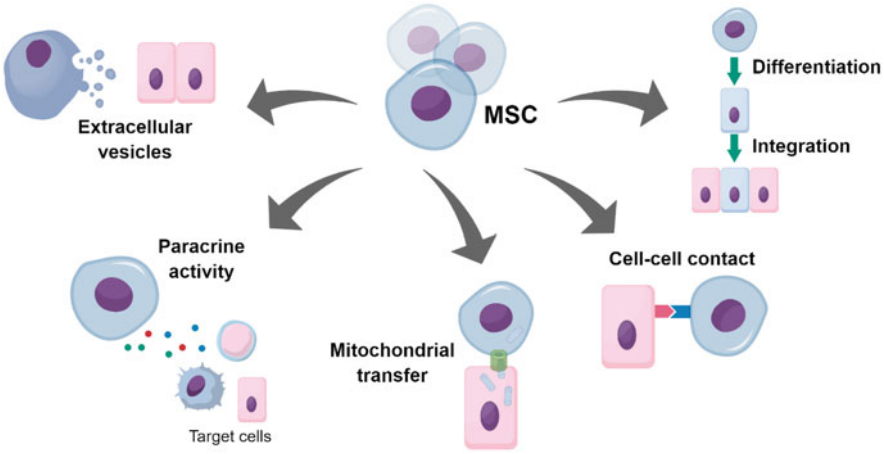

Fig. 5.1 Mechanisms of mesenchymal stromal cell (MSC) action. MSC rescue or repair of injured tissues can occur in different ways depending on the route of administration, quantity administered, and the injured tissue/organ: (1) through migration, cell differentiation, and integration into injured tissue, with immunomodulatory activities including (2) cell–cell contact, (3) mitochondrial transfer through tunneling nanotubes, (4) paracrine effects with secretion of cytokines, chemokines, growth factors, and (5) extracellular vesicles such as exosomes or microvesicles that can carry proteins and genetic material (e.g., different subtypes of RNA)

differentiate (Phinney and Sensebé 2013). Moreover, Caplan—who, as noted above, coined the term MSCs—called for another name change, asking investigators to adopt the term "medicinal signaling cells" to emphasize the mechanism of their therapeutic effects after transplantation (Caplan 2019).

5.1.1.1 Use of MSCs to Treat Other Viral Infections

Although cell-based therapies are among the most promising alternatives for several different disorders, their application in viral diseases is still poorly understood. MSC and HSC transplantations have proven safe (Peng et al. 2011; Lin et al. 2017; Xu et al. 2019; Li et al. 2016) and cell-based therapies have been evaluated for influenza virus (Chan et al. 2016; Chen et al. 2020), hepatitis B virus (HBV) (Peng et al. 2011; Lin et al. 2017; Xu et al. 2019; Li et al. 2016), and human immunodeficiency virus (HIV) (Zhang et al. 2013) infections. MSC administration improved mortality in H5N1- and H7N9-infected patients (Chan et al. 2016; Chen et al. 2020). HSC therapy, with donor cells not expressing the C–C chemokine receptor type 5 (CCR5) which is essential for the HIV entry process, has been used to achieve a functional cure of HIV (Hutter et al. 2009; Gupta et al. 2019).

Cell-free therapies using extracellular vesicles (EVs) have also been evaluated for influenza virus infection. Systemic administration of EVs from human umbilical cord-derived MSCs improved lung injury in H5N1-infected mice (Loy et al. 2019), and intratracheal administration of EVs from bone marrow-derived MSCs reduced the viral load in the nasal cavity, the replication of influenza virus in the lungs, and expression of cytokines and pro-inflammatory chemokines in a mixed viral infection (H9N5, H3N2, and H1N1) porcine model of lung injury (Khatri et al. 2018).

5.1.1.2 Rationale for Use of MSCs in COVID-19

The first reported study of MSCs in COVID-19 was performed by Leng et al. who observed the effects of MSC therapy in seven patients (Leng et al. 2020). Donor cells did not express ACE2, a key receptor used for SARS-CoV-2 to infect host cells. Furthermore, there were no adverse effects with cell transplantation, and lung function and symptoms were improved. Plasma levels pro- and anti-inflammatory mediators were also modulated after MSC therapy (Leng et al. 2020). Patients were followed only for 14 days, which may have been insufficient for proper evaluation, given that MSC therapy in HBV-infected patients did not improve clinical laboratory measurements in long-term follow-up (Peng et al. 2011), and raises concerns from our knowledge of septic and ARDS patients. In recent decades, a significant reduction in the mortality rates of critically ill patients has been observed in developed countries, reflecting an improvement in the general care of these patients. On the other hand, these advances have resulted in a growing population of survivors (Kaukonen et al. 2014), many of whom do not return to work, require prolonged hospitalization in step-down units, rehabilitation facilities, or at home, and suffer

from the long-term adverse effects of critical illness. As pointed out by Needham et al. (2012), new studies should evaluate not only short-term outcomes, such as in-hospital mortality, but also patient-centered outcomes such as quality of life after discharge and ability to return to the activities of daily living (Needham et al. 2012).

Neurological complications have also been observed in patients with SARS-CoV-2 infection, including encephalopathies with delirium or psychosis, inflammatory CNS syndromes including encephalitis, and acute disseminated encephalomyelitis, with the presence of hemorrhage, necrosis, and myelitis (Paterson et al. 2020). MSC therapy has great potential to modulate acute changes and improve neurologic conditions. Both experimental and clinical research has been conducted to evaluate the effects of cell therapies in neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and multiple sclerosis (Kwak et al. 2018; Suzuki et al. 2015; Yang et al. 2013; Gharibi et al. 2015); more recently, preclinical research demonstrated beneficial effects of MSCs as adjuvant therapy in infectious disease-associated encephalopathies (Silva et al. 2020; Lima et al. 2020).

Critically ill COVID-19 patients present increased levels of pro-inflammatory cytokines compared to patients not admitted to an ICU, as well as increased inflammation and extensive organ damage (Huang et al. 2020). Severe SARS-CoV-2 infection has been associated with increased blood levels of cytokines and chemokines, which have been proposed as potential predictors of disease severity (Huang et al. 2020). In this context, some groups aim for treatments to avoid the cytokine storm and thereby suppress the pro-inflammatory response (Leng et al. 2020; Conti et al. 2020). However, plasma levels of cytokines might not represent or predict lung inflammation, and targeting specific inflammatory mediator pathways at indiscriminate time points might not result in a good outcome (Sinha et al. 2020). For example, the use of tumor necrosis factor-α (TNF-α) inhibitors to mitigate inflammation failed to improve survival in clinical sepsis trials; a similar phenomenon might also occur in COVID-19. Another important observation is that cytokine storm theory originated from early reports from a small fraction of patients with elevated plasma cytokines. However, some of these markers, such as interleukin-6 (IL-6), appear to be lower than in previous cohorts of patients with ARDS, and the available data are insufficient to confirm even the presence, let alone a role, of cytokine storm in COVID-19 (Sinha et al. 2020). Nevertheless, like sepsis, COVID-19 is a highly complex disease that involves multiple immune pathways and different cell subsets. As such, cell-based therapies could be an interesting therapy, since MSCs are able to communicate with other cells and respond to the microenvironment, and MSCs are primarily entrapped in the lungs after intravenous delivery.

However, the amount of bone marrow cells trapped within the pulmonary vasculature after systemic administration is small (Araújo et al. 2010; Mei et al. 2010; Prota et al. 2010; Silva et al. 2011; Maron-Gutierrez et al. 2011), suggesting that cell migration and engraftment are not required for positive effects to occur. In this context, cell-free therapies with mediators released by MSCs, such as EVs, might be more effective in COVID-19. One of the main mechanisms of action of

MSCs is through their paracrine anti-inflammatory and immune-modulation effects to repair tissue damage. MSCs secrete bioactive mediators, such as cytokines, chemokines, and growth factors, as well as EVs (Jaimes et al. 2017; Spees et al. 2016). In general, EVs carry molecules such as lipids, proteins, and subtypes of RNAs (Raposo and Stoorvogel 2013). EV-based therapies have been shown to be safer than administration of their parent cells, with a lower risk of embolism. EVs are more stable and clinical outcomes achieved with their use could be potentially more reproducible than those of MSCs, as EVs are not influenced by the individual microenvironment and are not susceptible to viral infection. A prospective nonrandomized study in patients with COVID-19 evaluated the safety and efficacy of EVs derived from bone marrow MSCs; no adverse events were observed, and EV administration restored oxygenation and mitigated the inflammatory response (Sengupta et al. 2020).

Although cell-based and cell-free therapies show promising results in COVID-19, they raise some concerns, since these findings were obtained in pilot studies with a limited number of individuals; randomized multicenter trials should be carried out to confirm the positive effects observed (Öztürk et al. 2020). Both therapies present challenges, such as standardization of tissue origin and culture conditions, harvesting and purity of EVs, dosing, route and timing of administration, and outcomes (Khoury et al. 2020). The use of allogeneic cells and EVs is preferable to autologous cells and EVs, as the rapid deterioration seen in COVID-19 means there is not enough time for harvesting and culture (Yen et al. 2020). Another concern is that the stem cell industry is trying to sell unregulated MSC treatments for COVID-19, leading to public statements of concern from organizations such as the International Society for Extracellular Vesicles (ISEV) and the International Society for Cellular and Gene Therapies (ISCT) concerning their use without proper regulation (Borger et al. 2020).

Most clinical trials will deliver MSCs intravenously. When specified, umbilical cord is the most widely used source, followed by adipose tissue-derived MSCs and bone marrow-derived MSCs. However, adipose tissue-derived MSCs should be used with caution. Patients with COVID-19 often present thrombotic complications (Tang et al. 2020; Middeldorp et al. 2020) and adipose tissue-derived MSCs express more tissue factor than bone marrow-derived MSCs or those from other sources (George et al. 2018; Moll et al. 2012). Tissue factor is a transmembrane receptor that plays a key role in coagulation and thrombosis (Grover and Mackman 2018), and it has been demonstrated that platelet-monocyte interaction is determinant to tissue factor expression in the monocytes, which is associated with severity and mortality in critically ill COVID-19 patients (Hottz et al. 2020).

Even with the start of vaccination campaigns, the use of MSC therapy is still an interesting approach, since long-term consequences (e.g., neurological) have been shown in surviving individuals—even those with mild symptoms (Paterson et al. 2020; Battaglini et al. 2020)—and preclinical research has shown that adjuvant use of MSCs mitigates cognitive and behavioral damage in infectious disease-associated encephalopathies (Silva et al. 2020; Lima et al. 2020).

5.1.2 *Therapeutic Nucleic Acids/Gene Therapy*

Gene therapy emerged in the 1980s as a novel therapeutic approach for inherited diseases. However, the first breakthrough only came in 2012 with the approval of the European Medicines Agency of alipogene tiparvovec (Glybera®) for the treatment of patients with lipoprotein lipase deficiency (Carpentier et al. 2012). Although clinical advances have progressed slowly, research has moved steadily and widely towards successful applications. Currently, gene therapy is no longer restricted to inherited disorders but is also investigated for acquired diseases, with the concept of introducing genetic materials into host cells, either by using viral vectors or nanocomplexes, to promote inhibition or enhancement of the functional expression of target gene(s) (Arjmand et al. 2019). Along these lines, antisense oligonucleotides (ASOs), clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems, and mRNA-based vaccines are tools with promising therapeutic utility for SARS-CoV-2 infection.

5.1.2.1 CRISPR-Cas System

Mechanisms of modulation of gene expression and editing naturally occur in bacteria to defend themselves against invading bacteriophages and other external invaders, such as nucleic acids (Brouns et al. 2008; Jinek et al. 2012). CRISPR effectors may reprogram human cells to inhibit replication of double-stranded DNA or single-stranded RNA viruses, such as SARS-CoV-2, thus helping in the defense against these viruses (Ramanan et al. 2015; Wright et al. 2016; Yin et al. 2017; Ophinni et al. 2018). An increasing number of studies have been investigating orthologs of Cas13, as these have demonstrated stable and strong RNA knockdown and can be easily delivered into mammalian cells (Abudayyeh et al. 2017; Cox et al. 2017; Konermann et al. 2018) with minimal off-target effects on the host transcriptome (Bawage et al. 2018). The Cas13 protein interacts with the guide RNA through a short hairpin within the CRISPR-associated RNA (crRNA), and the specificity of the target is encoded by means of a ~30-nt spacer that is complementary to the target region.

Computational analysis has indicated hundreds of possible Cas13 crRNA target sites in mammalian cells (Freije et al. 2019). The Cas13a/b systems were found to inhibit the replication of single-stranded RNA viruses, such as influenza and SARS-CoV-2, by using the prophylactic antiviral CRISPR in human lung epithelial cells (Abbott et al. 2020). The Cas13d system also demonstrated the ability to target and cleave SARS-CoV-2 RNA sequences (Freije et al. 2019). Nguyen et al. were able to implement the CRISPR/Cas13d with over 10,000 potential guide RNAs to target ten coding regions of SARS-CoV-2, which inhibited SARS-CoV-2 genome replication by targeting ORF1ab and the spike transcript of the virus without the disrupting human transcriptome (Nguyen et al. 2020). In another study, Abbott et al. designed and screened several crRNAs targeting conserved viral genomic sequences to

identify those able to cleave SARS-CoV-2. Six crRNAs were able to target ~90% of the sequence of currently known coronaviruses, while a pool of 22 crRNAs was found to target all sequenced coronaviruses, which might prevent potential viral evasion (Abbott et al. 2020). Using a similar approach, a pool of crRNAs designed to target the influenza A virus demonstrated effective inhibitory effects (Abbott et al. 2020). Although such strategies still need to be further investigated and confirmed in animal models before testing in humans, they may represent promising alternatives to conventional vaccines, as the latter relies on the stimulation of the immune system by administering viral surface proteins that have a high rate of single mutations, and, therefore, a high chance of evading host immune responses (Pardi et al. 2018). Furthermore, targeting host factors using CRISPR-Cas can be an alternative to direct targeting of the viral genomic sequence, as it may create an unfavorable cellular environment for viral replication (Lu et al. 2008; Kumar et al. 2020).

5.1.2.2 Antisense Oligonucleotides

Antisense oligonucleotides (ASOs) are chemically synthesized, single-stranded nucleic acids ranging from 15 to 30 base pairs in length that are designed to bind RNA targets via complementary base pairing thus affecting translation of the target protein. The safety and efficacy of ASOs have been demonstrated in several deadly pathogenic viruses (Miller and Harris 2016), with ten ASO drugs currently approved for clinical use by the US FDA (Roberts et al. 2020). ASOs can bind to transcripts encoding proteins related to SARS-CoV-2 replication thus preventing the assembly and release of new viral particles.

A number of early studies demonstrated promising effects of ASOs to target SARS-CoV. Neuman et al. developed an ASO based on phosphorodiamidate morpholino-oligomers to target conserved RNA elements of SARS-CoV (Neuman et al. 2004). These ASOs had previously demonstrated enhanced cellular delivery (Moulton et al. 2003) and inhibited SARS-CoV replication by blocking translation of the ORF1ab (Neuman et al. 2005). However, SARS-CoV was able to develop resistance to this ASO and easily evade its protective effects (Neuman et al. 2005). Another study also demonstrated the ability of this ASO to reduce SARS-CoV titers in both in vitro and in vivo models (Burrer et al. 2007).

Although the viral genome of SARS-CoV-2 has been sequenced, the identification of conserved RNA sequences is still in progress. In an in silico study, Barrey et al. designed ASO candidates to target the genomic 5′-UTR, ORF1a, ORF1b, and nucleoprotein transcripts in SARS-CoV-2 (Barrey et al. 2020). These ASO candidates were predicted to have excellent properties to block SARS-CoV-2 replication, although experimental validation still needs to be performed to confirm such effectiveness (Barrey et al. 2020).

5.1.2.3 mRNA-Based Vaccines

mRNA-based vaccines are a novel type of vaccine that consists of an mRNA strand coding for a disease-specific antigen. Once the mRNA is delivered to host cells, it is translated into a specific antigen that is recognized and presented to immune cells. Experimental mRNA vaccines have been shown to elicit potent immune responses against several infectious pathogens in animal models (Pardi et al. 2018; Wadhwa et al. 2020). These vaccines also provide a number of advantages compared to traditional ones, including the ability to stimulate both cellular and humoral immunity, both prophylactic and therapeutic utility, and production which can be scaled up rapidly (Pardi et al. 2018; Zhang et al. 2019).

Several vaccines are under investigation for COVID-19, with eight mRNA-based vaccines demonstrating promising results in clinical studies and two already having received approval for emergency use or marketing authorization (Table 5.1)

Table 5.1 SARS-CoV-2 mRNA vaccine candidates in clinical trials

ClinicalTrials.gov ID (Phase)	mRNA vaccine	Target	Characteristics
NCT04523571 (I)	BNT162b1	Receptor binding domain of the spike protein	Lipid nanoparticle-nucleos modified mRNA
NCT**04649021 (II), NCT04733807 (IV), NCT04756813 (IV), NCT04760704 (IV), NCT04761822 (II), NCT04815031(IV)**	BNT162b2	Full-length, perfusion stabilized spike protein	Lipid nanoparticle-nucleos modified mRNA
NCT04283461 (I), NCT04405076 (II), NCT04470427 (III), NCT04649151 (II/III), NCT04748471 (II), **NCT04796896 (II/III)**	mRNA-1273	Full-length, perfusion stabilized spike protein	Lipid nanoparticle-encapsu mRNA
NCT04813796 (I)	mRNA-1283	Receptor binding domain and N-terminal domain of the spike protein	Lipid nanoparticle-encapsu mRNA
NCT04449276 (I), NCT04515147 (II), NCT04649021 (II), NCT04652102 (IIb), NCT04674189 (III)	CVnCov	Full-length spike protein	Lipid nanoparticle-encapsu mRNA
NCT04566276 (I)	ChulaCov19	Virus-specific antigen	Lipid nanoparticle-encapsu mRNA
NCT04758962 **(I)**	LNP-nCoVsaRNA	Perfusion stabilized spike protein	Lipid nanoparticle-encapsu self-amplifying mRNA
NCT04765436 (I)	PTX-COVID19-B	Spike protein	Lipid nanoparticle-encapsu mRNA

(Lopes-Pacheco et al. 2021). The mRNA-1273 vaccine (developed by Moderna) is a lipid nanoparticle-encapsulated, nucleoside-modified mRNA-based vaccine that encodes a full-length SARS-CoV-2 spike protein. In early-stage clinical studies, this vaccine was administered in a two-dose regimen and promoted anti-SARS-CoV-2 immune responses in all participants, with no severe safety concerns (Jackson et al. 2020; Corbett et al. 2020). The BNT162b2 vaccine, developed by BioNTech and Pfizer, is a lipid nanoparticle-formulated, nucleoside-modified mRNA-based vaccine that encodes the SARS-CoV-2 receptor-binding domain with insertion of two proline mutations to lock it in the prefusion conformation. In large-scale clinical studies, a two-dose regimen of BNT162b2 induced ~95% efficacy in preventing COVID-19 with no severe safety concerns (Polack et al. 2020; Walsh et al. 2020). Recently, an *Alphavirus*-derived replicon RNA vaccine candidate formulated with lipid inorganic nanoparticles that encode the SARS-CoV-2 spike protein was shown to promote immune responses and produce SARS-CoV-2 neutralizing antibodies in both murine and non-human primate models after a single intramuscular administration (Erasmus et al. 2020).

5.1.2.4 Challenges and Concerns in Therapeutic Nucleic Acids/Gene Therapy

Despite remarkable progress in developing therapeutic nucleic acids/gene therapies, there are still several challenges and concerns that need to be addressed in order for these strategies to be efficiently translated into clinical applications. These include poor cellular uptake, packaging for in vivo delivery, nuclease susceptibility, rapid clearance from circulation, and potential immunogenicity. For instance, ASOs can be less stable than small interfering RNA, and therefore the former should be modified (e.g., by 2′-*O*-methyl changes of the ribose sugar) to become a more stable product (Roberts et al. 2020). Naked mRNA vaccines are prone to nuclease degradation and exhibit poor intracellular uptake (Wadhwa et al. 2020). Efficient delivery is key to enhancing in vivo uptake. The delivery system is also important to minimize potential nonspecific targeting of nucleic acids and off-target effects by ASOs and CRISPR-Cas therapies (Wienert et al. 2019; Zuo et al. 2019). Furthermore, most Cas proteins have a high molecular weight, which poses difficulties to the use of certain viral vectors (such as recombinant adeno-associated virus) to carry Cas protein plus crRNA/guide RNA. Non-viral vectors (liposomes, inorganic particles, and others) have been investigated to circumvent this limitation (Wilbie et al. 2019).

5.2 Conclusion and Prospects

Cell-based therapies, CRISPR-Cas systems, and mRNA vaccines may become potential treatments for COVID-19 if efficiently translated into clinical applications and demonstrated to improve patient outcomes. We believe that research into such

advanced therapies should be considered essential and urgent to fighting the COVID-19 pandemic and its detrimental downstream effects.

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Chapter 6
Coupling Glucose Phosphorylation to Oxygen in Brain Mitochondria: Would It Be a Redox Set Point?

Antonio Galina

Abstract The brain is a crucial organ that integrates very rapidly several complex sensory functions contributing to the fitness of different mammal species to capture energy from its environment. To achieve this evolutionary success a high flow per specific brain mass of oxygen and glucose was adapted for obtaining ATP by oxidative phosphorylation. However, this high flow of glucose and oxygen inserts the brain at serious risk of oxidative stress, cell death, and inflammation that is present in degeneration process of inflammatory diseases and aging. Interestingly, during the development of the brain, several redox signals generated by reactive oxygen species (ROS) are necessary and are highly controlled in time and space by mitochondria. In central nervous system, the consumption of oxygen and the first reaction of glucose metabolism occurs together and mainly at the mitochondrial surface through the coupling of hexokinase in the outer mitochondrial membrane (OMM). The reasons for this strategic association go beyond the activation of glucose in cellular metabolism, because it involves a strong control of the release of mitochondrial ROS (mROS), mitochondrial uptake, management of calcium, and opening of the permeability transition pore. In this way, downstream redox signals can be indirectly regulated by the mitochondrial glucose phosphorylation by hexokinase and mROS release. The implication of this unique mitochondrial glucose phosphorylation extrapolates the central nervous system, contributing to the anti-aging and inflammation responses in different tissues and defense systems and species of mammals. Thus, hexokinase coupled to mitochondria may works as a glucose-OxPhos-redox transducer system, especially present in long-lived species tolerant to inflammation, viral infection, and pathogens.

Keywords Hexokinase · Mitochondria · ROS · Redox signaling · Inflammation · Aging · Glucose · Oxygen

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6.1 Introduction

Animal respiration was proved by Lavoisier and La Place 1780 to be in fact a combustion (oxidation) of organic matter (reduced carbon derivatives) by molecular oxygen forming essentially water (H_2O), carbonic gas (CO_2) and releasing heat:

$$\text{Organic matter (CH, CHO, or CHON)} + O_2 \rightarrow H_2O + CO_2 + \text{Heat}.$$

Despite the great difference in terms of kinetic rates of respiration (slow) and combustion (fast), the proportionality among heat/CO_2 was practically the same between burning of organic matter and respiration of organisms. These outstanding and elegant experiments wiped out the erroneous theory of phlogiston, accepted at that time (Woody AI et al. 2012; Keilin 1966; Lavoisier 1862).

Cellular respiration is vital for life and is a highly exergonic process in which redox reactions evolved to conserve part of the free energy transiently in molecules of phosphoanhydride and phosphoester bonds, such as ATP, PPi, phosphocreatine, and glucose-6-phosphate (G6P) (de Meis 1997, 2012); and also in carrier coenzymes of electrons and protons ($2e^- + 2H^+ \rightarrow H_{2(g)}$) such as NAD(P)H, $FADH_2$, or any other general $H_{2(g)}$ acceptors from nutrients avoiding $H_{2(g)}$ escape from biological systems to environmental atmosphere. Electrons and protons are ultimately attracted to molecular oxygen (O_2) by its great thermodynamic electron affinity expressed in a high standard reduction potential value ($E^{\circ\prime} = +816$ mV) forming H_2O (Skulachev et al. 2013).

The free energy released from the oxidations of NADH and flavoenzymes-$FADH_2$ are used to promote the charge separation through the vectorial transport of H^+ in the inner mitochondrial membrane (IMM), which was integrated quantitatively to the electron motive force (e.m.f.; $E^{\circ\prime}$) to protonmotive force (p.m.f.; Δp) by Peter Mitchell at the chemiosmotic theory (Mitchell 1961, 1976; Mitchell and Moyle 1969). In mitochondria, the electron transport system (ETS) is composed of iron/sulfur centers, cytochromes, and ubiquinones that give the electron physical conduits coupled to proton pumping to reach O_2-generating water. The redox energy released is converted into Δp. The H^+ downhill movements in favor of both the electrical polarization ($\Delta\Psi m$) (from positive to negative side) and H^+ gradient (ΔpH) (from high to low [H^+]) across $F_oF_1H^+$ATP-synthase complex drives ATP synthesis in oxidative phosphorylation (OxPhos) (Boyer et al. 1977). The Δp values are described in terms of electrical polarization ($\Delta\Psi m$) and the H^+ gradient (ΔpH) by the equation:

$$\Delta p = \Delta\Psi m - Z\Delta p\text{H}.$$

where Z is the conventional factor 2.303 RT/F, which is near 60 at 37 °C expressed in mV (Skulachev et al. 2013; Mitchell 1961). Where, Δp (~ −220 mV), $\Delta\Psi m$ (−180 mV), − Z (~60 mV), and ΔpH (0.5 pH unit). Thus, part of energy conservation can be written as:

$$(\mathrm{CHO, or\ CHON}) + \mathrm{O_2} \rightarrow \mathrm{H_2O} + \mathrm{CO_2} + \mathrm{Heat} + \Delta p.$$

Δp is the most negative H^+ attracting force inside the cell to mitochondrial matrix. Initially, it was thought that Δp was mainly involved in the ATP synthesis by driving the $P_i + ADP_{entry} \leftrightarrow ATP_{exit}$, and secondarily it was thought that organic anions, net Ca^{2+} uptakes, Ca/H^+, Ca/Na^+ were exchanged by anti and symporters in IMM (Lehninger et al. 1978). Thus, the ATP synthesized by OxPhos would attend instantaneous cellular works demand for high energy phosphates, but also provides secondary and integrative signals for energy homeostasis (Skulachev et al. 2013).

The coordination of energy demand contributes to the whole body in healthy state. More important is to find that these demands establish a synchronicity of signaling with Δp in mitochondria and cytosolic glucose metabolism. Reactive oxygen species (ROS) are by-products of mitochondrial O_2 metabolism, which accounts for about 2% of all oxygen consumed, and are involved in oxidative stress when their levels exceed steadily the antioxidant defense capacity of cell and/or mitochondria. This condition leads to oxidative stress in many diseases (Murphy 2009). The key independence discovered by Vladimir Skulachev, Boveris, Chance, and Kurshunov's group (Skulachev et al. 2013; Murphy 2009; Boveris and Chance 1973; Chance et al. 1979; Korshunov et al. 1997) was that high Δp value (Δp_{high}) drives the formation of ROS, and it was crucial to understand how the electron leakage rate from ETS impacts the entire cell. However, it is now well established that ROS are not only toxic but are also important integrating signals for many cell functions, such as differentiation, proliferation, apoptosis, inflammation, immunity, cell–pathogens interactions, and many other cellular works (Geary 2021; Figueira et al. 2013; Handy and Loscalzo 2012). How this ROS signaling synchronization is linked to mitochondrial and cell metabolism is just beginning to be revealed.

6.1.1 *Glucose and Oxygen Fluxes in Brain, ROS and Dependence to Mitochondrial* $\Delta \mathbf{p}$

Physiological variations in the cellular energy demands, such as electrical impulses in central nervous systems (CNS), promote the mitochondrial ADP/ATP exchange in IMM to attend to these demands in ATP. The OxPhos activation by ADP ↔ ATP exchange consumes a small portion of Δp (Δp_{low}) which alleviates, either thermodynamically and kinetically, the reductive state of centers in ETS (primarily complexes I and III, stabilized by Δp_{high}) avoiding monoelectronic reduction of the oxygen out of cytochrome c oxidase (complex IV) and forming superoxide anions ($O_2^{\cdot -}$), H_2O_2 and other ROS derivatives (Korshunov et al. 1997). These mitochondrial ROS escape may favor oxidation of lipids (peroxidation) and –SH, –OH groups in proteins that are redox sensitives. Thus, the OxPhos operation in phosphorylating state (ADP + P_i → ATP, net synthesis) circumvents a significant fraction of ROS

production by mitochondria by a mild depolarization of Δp (Δp_{low}) (Korshunov et al. 1997).

Notwithstanding, the CNS from a great variety of mammal species is extremely complex, organized, and regulated, but sustained almost exclusively by two primordial molecules that drive the evolution of sensorial systems—O_2 and glucose (Erecinska et al. 2004; Erecinska and Silver 2001; Vanderkooi et al. 1991). These molecules have a high probability to generates mtROS involved in damage or signals which may be critically driven by mild Δp transitions ($\Delta p_{low} \leftrightarrow \Delta p_{high}$) to less than 20% and involved in many functions, not only in CNS but in other cellular systems of the body (Korshunov et al. 1997; Geary 2021; Handy and Loscalzo 2012). Here, we will postulate a concept of redox sensor firstly described in rat brain by our group as an antioxidant defense (da-Silva et al. 2004) which integrates the rates of fluxes between O_2 to glucose in CNS making it tightly coupled to mild Δp fluctuations in energy demands and to mtROS formation (da-Silva et al. 2004; Meyer et al. 2006). This coupling is carried out by the reaction of glucose phosphorylation catalyzed by the enzyme hexokinase (mtHK) at the surface of mitochondria:

$$\text{Glucose} + \text{ATP} \rightarrow \text{glucose-6P} + \text{ADP}.$$

Consequently, it may be coupled to a redox adjustment of the mitochondrial Δp value by a slight depolarization induced by the ADP entry to the mitochondrial matrix for ATP synthesis, as follows:

$$\text{Glucose} + \text{ATP} + \Delta p_{high} + \text{ROS} \rightarrow \text{glucose-6P} + \text{ADP} + \Delta p_{low} + H_2O.$$

Therefore, this reaction may affect O_2, $O_2^{\cdot -}$, H_2O_2 levels, Ca^{2+} uptake, and IMM permeability—mPTPO (mitochondrial permeability transition pore opening) in mitochondria of brain and other tissues, and it is further implicated in other cellular systems in mammals (Figueira et al. 2013; Handy and Loscalzo 2012; Kowaltowski et al. 2001).

6.1.2 Controlling the Flow Mixture of Glucose and Oxygen in Brain. Do Mitochondria Play a Role?

The brain has a unique sophistication in organization and function; however, seems to have a fundamental strategy glitch: it consumes a large amount of energy but lacks a reservoir to store fuel for use when needed. Therefore, the brain receives energy substrates, primarily oxygen and glucose, "on the fly" through its constant blood supply (Iadecola 2017). Brain uses about 20% of all glucose and oxygen consumed daily despite the fact that it corresponds to only 2% of body mass, in humans (Erecinska and Silver 2001). Arteriovenous differences in the consumption rate of oxygen (CMR_{O2}) and the rate of glucose (CMR_{Glc}) across the brain stipulates the

ratio CMR_{O2}/CMR_{Glc}, the Oxygen/Glucose index (OGI) which in brain is closed to six (OGI ≅ 6) (Frackowiak et al. 1988; Hawkins et al. 1985; Nariai et al. 2001; Shishido et al. 1996). This value indicates a global preference for glucose oxidation with a high degree of coupling between oxidative phosphorylation (OxPhos) and glucose metabolism by the central nervous system (CNS).

This property of the brain in energy metabolism deserves attention because of its physiological and specific space-temporal activation in energy demands by high ATP turnover accomplished electrical impulse and by ionic fluxes activation involved in neurotransmission (Erecinska et al. 2004). This confirms that the brain is an interesting model to study the adaptations in glucose metabolism and its coupling to OxPhos (Erecinska et al. 2004; Frackowiak et al. 1988; Hawkins et al. 1985). In CNS the OGI is tightly coupled by the energy demands triggered by the electrical impulses in synapses. In fact, in patients with mitochondrial cytopathy and cerebral disease there is an OGI ≅ 3.8 indicating uncoupling of OGI of lactate and an imbalance of glucose and oxygen metabolism (Frackowiak et al. 1988; Hawkins et al. 1985; Nariai et al. 2001; Shishido et al. 1996), and thus the complete and rapid glucose oxidation to reach such coupling of OGI ≅ 6 is important for a healthy brain function and suggests that large deviation of this uncoupling, such as observed in stroke, ischemia, or hypoglycemia, may interfere with the signaling function, beyond cellular damage (Iadecola 2017; Frackowiak et al. 1988).

Looking in detail at the regulation of the delivery of O_2 and glucose to the brain's metabolism, this control is dependent on the neurovascular unit (NVU)—endothelium/vascular and muscle/astrocytes/perivascular macrophages, which makes the fine coupling between the cerebral blood flow (CBF) with the synaptic activity (Iadecola 2017). The development of positron emission tomography and MRI-based methods allowed investigators to monitor CBF in humans with great spatial resolution (Raichle and Mintun 2006). MRI-based functional brain imaging has also firmly established the concept that cerebrovascular function is intimately related to brain activity. In particular, the discovery of the blood oxygenation level-dependent (BOLD) effect, reflecting excess CBF delivery is related to local oxygen consumption, enabling the noninvasive detection of activity-dependent hemodynamic signals across the behaviour of the human brain (Raichle and Mintun 2006). As a consequence of these immediate adjustments in CBF, the basal oxygen levels vary widely in different brain regions (Lyons et al. 2016), and depending on local vascular topology and the intensity of the activating stimulus, regional hypoxia may develop, promoting vasodilatation of local vessels.

Thus, the physiological pO_2 ($[O_2]_{physiol}$) is a result of balance transfer-to-removal flows and it is not homogeneous for the whole brain. These observations show that the NVU plays a crucial role in the composition of the mixture of $[O_2]$ and [glucose] locally for the cells bathed and instantly receiving these "opened floodgates" of the blood vessel system (Iadecola 2017). The mechanisms involved in adjusting the energy demands of synaptic activity between oxygen and glucose and cerebral blood flow are assumed to be the result of blood flow activating signals (glutamate activation, Ca^{2+}, NO, etc.) and signals derived from synaptic energy metabolism (lactate, CO_2, adenosine, H_2O_2, etc.) (feed backward) that may support or inhibit this

flow (Iadecola 2017; Busija et al. 2016). In this way, the variable $[O_2]_{physiol}$ can activate mitochondrial H_2O_2 generation Δp_{high}-dependent (close to 100%) (Hoffman and Brookes 2009).

An approximate thermodynamic estimation of the variation of Gibbs free energy (ΔG) for mitochondrial generation of $O_2^{\cdot-}$ taking into account Δp_{high} ($\Delta G = -nF\Delta E + \Delta p_{high}$)(Quinlan et al. 2011) under these conditions of high $[O_2]_{physiol}$ (~50–100 μM), will favor the superoxide formation, by close to −40 kJ/mol* pushing the reaction (Nicholls and Ferguson 2013). Without the contribution of Δp, the free energy released would be approximately −14 kJ/mol (Murphy 2009; Nicholls and Ferguson 2013). In these estimations, the buildup of Δp would push the reaction to the direction of $O_2^{\cdot-}$ formation to almost threefold! However, at low $[O_2]_{physiol}$ (~5–10 μM) and with a Δp_{low} (close to 80%) ($\Delta G = -nF\Delta E + \Delta p_{low}$) it is around −15 kJ/mol*. It has observed a decrease of 50% in the driving force to form mitochondrial $O_2^{\cdot-}$. In this case, the Δp_{low} would not contribute to the reaction superoxide formation. The Δp_{low} level is reached during the activation of OxPhos by ADP availability to synthesize ATP for synaptic demands. In this context, it is important to note that the increase in CBF is not accompanied only by O_2, but also by an apport of glucose (Iadecola 2017; Raichle and Mintun 2006; Hoffman and Brookes 2009).

Thus, due to the presence of mtHK associated to VDAC at the outer mitochondrial membrane (OMM), and to cycling ADP_{entry}/ATP_{exit} exchange by the adenine nucleotide translocase (ANT) driven by mtHK catalyzed reaction, practically all electrons would flow to the cytochrome c oxidase (COX) forming H_2O, instead of $O_2^{\cdot-}$. This high rate of respiration (phosphorylating respiratory state) also decreases the $[O_2]_{physiol}$, which impairs, kinetically and thermodynamically, almost completely the formation of $O_2^{\cdot-}$ by blocking the electron leakage from the sites of CI and CIII (Murphy 2009; Korshunov et al. 1997).

However, an intriguing question arises: How the glucose's metabolism use would activate oxygen consumption speedily to counter the overfeeding of electrons to the ETS by high ratios of NADH/NAD+ and $FADH_2$/FAD+, formed by glycolysis and Krebs's cycle, avoiding an over formation of ROS by elevated rates of glucose oxidation (Murphy 2009; Adam-Vizi and Chinopoulos 2006; Brownlee 2003; Nishikawa et al. 2000; Votyakova and Reynolds 2001; Jezek et al. 2020)? The answer to this question started to be solved by the observation that in CNS close to 90% of the glucose phosphorylation, at the expense of the ATP formed by the OxPhos, occurs at the surface of the mitochondria by mtHK, high glucose affinity enzyme (Sui and Wilson 1997; Wilson 1997, 2003).

According to the traditional view, the G6P formed would be metabolized by the glycolytic pathway, producing pyruvate that enters the mitochondria and is oxidatively decarboxylated generating NADH and $FADH_2$. In this state, ETS would be flooded with electrons leading to a large electron leakage at specific sites for $O_2^{\cdot-}$ formation imposed by Δp_{high} (Murphy 2009; Adam-Vizi and Chinopoulos 2006; Brownlee 2003; Nishikawa et al. 2000; Votyakova and Reynolds 2001; Jezek et al. 2020). However, with the binding of HK to VDAC, its natural receptor, at OMM and the preferential use of ATP generated by OxPhos for the synthesis of G6P, it would

decrease Δp, accelerates oxygen consumption, and decreases the ratio NADH/NAD^+ and $FADH_2/FAD^+$. This also contributes to mitigate the generation of ROS by the excess of electrons delivered to ETS in the state of Δp_{high} (Sui and Wilson 1997; Wilson 1997, 2003). Therefore, we hypothesized that the association of HK with the mitochondria would immediately adjust the highest and lowest flow of oxygen and glucose consumption to CNS. In this context, an immediate redox adjustment to avoid reductive potential (excess glucose) and oxidative potential (excess oxygen) stresses would be achieved (da-Silva et al. 2004; Meyer et al. 2006). In addition, the cross talk with other sensitive redox downstream signaling pathways would be expected to occur.

6.1.3 *Glucose Phosphorylation at Mitochondria in Mammalian Cells by Hexokinase. Or Why Otto Meyerhof Failed to Activate Glucose Fermentation from Soluble Extracts from Vertebrate Muscle?*

Glucose phosphorylation and metabolism in eukaryotic cells have traditionally been seen major to occur in the cytosolic compartment of the cell (Heneberg 2019). This view is partly due to the pioneering and elegant experiments of Otto Meyerhof, discoverer of the hexokinase enzyme, the soluble glycolytic activator from yeast (Meyerhof 1930). By elucidating the fermentation of glucose in aqueous extracts of muscle, their deficiency in fermenting free hexoses was clear, but not glycogen. Meyerhof elegantly reconstituted the conversion of glucose to lactate by adding a soluble yeast factor plus coenzymes (hexokinase + ATP) responsible for the activation of glucose (formation of reactive glucose − G6P). These observations showed that in its biological preparations, muscle hexokinase was either lost or associated with some cellular structure.

This insightful observation that the muscle enzyme could be associated with some unknown cell structure can be seen in the following quote from Otto Meyerhof himself:

> *...These experiments tend to support the view that a hexokinase present in the tissues is involved here as well. Yet its separation from solid tissues components is undoubtedly dependent upon appropriate technical conditions, which are harder to achieve in mammalian tissue than in yeast.*

From "Conversion of fermentable hexoses with yeast catalyst (Hexokinase)" O. Meyerhof, "Die Chemischen Vorgänge im Muskel," J. Springer, Berlin (1930), pp. 149–155. [Cf. also, O. Meyerhof, *Biochem. Z.,* 183 (1926).]

Many years later, the work of Kennedy and Lenhinger on the liver demonstrated that the intracellular distribution of glycolytic activity and aldolase are concentrated in more than 80% in soluble fractions of the liver tissue by differential centrifugations (Kennedy and Lehninger 1949).

Nevertheless, from the seminal works by Utter, Otto Meyerhof itself, and Crane and Sols (Crane and Sols 1953; Meyerhof and Geliazkowa 1947; Utter et al. 1945) it became apparent that the particulate structure in which Meyerhof's hexokinase binds was in fact the mitochondria. Other workers confirmed and extended this view. As early as 1954, Samuel Bessman and coworkers suggested that hexokinases would be associated with mitochondria to prevent diffusion to the ATP cytosol formed by OxPhos (hexokinase theory as a phosphate acceptor effect) favoring G6P synthesis as a focal precursor for different pathways of glucose metabolism (Bessman and Gots 1975). This worker and colleagues (Bessman and Gots 1975; Gots and Bessman 1974; Gots et al. 1972; Srere and Mosbach 1974; Viitanen et al. 1984) presented evidence that this binding has functional significance in that ADP and P_i lead to higher rates of G6P formation than ATP for the mitochondrial-bound hexokinase. This would seem to indicate that the active site of hexokinase was oriented toward the mitochondrion to receive the ATP made within. This response would be associated with anabolic pathways. Further, Katzen et al. (1970), demonstrated that in different mammalian tissues exist four isozymes, named I–IV, but only HK I and II were mostly associated with mitochondria (Wilson 2003; Heneberg 2019; Colowick 1973). Subsequently, several studies confirmed the association of HK with mitochondria in carcinomas and the possible involvement in the proliferation and growth of tumors (Arora and Pedersen 1988; Shinohara et al. 1997).

Now it is recognized that, in mammals, exists, at least, five hexokinase isoforms, with intracellular localization with specific kinetics and regulatory properties, in addition to other unknown signaling functions that have been excellently revised (Heneberg 2019). In respect to these isoforms, only mtHK1 and mtHK2 have N-terminal domain of 10–15 amino acids that direct them to be associated with porin or VDAC, located in OMM (Sui and Wilson 1997; Azoulay-Zohar et al. 2004; Vyssokikh and Brdiczka 2003). Studies with diabetic rats have shown that the mtHK1/mtHK2 ratio is strongly altered in the skeletal and cardiac mitochondria with an almost complete disappearance of mtHK2 and maintenance of mtHK1 (Katzen et al. 1970). In a recent study (Amendola et al. 2019), the KRAS4A protein, a product of an important oncogene that drives metabolic reprogramming, directly regulates hexokinase 1. It demonstrated a direct and unique interaction between KRAS4A and hexokinase 1 (HK1) in cancer cells. They found that KRAS4A can directly bind to HK1 in mitochondria thereby blocking the allosteric inhibition of HK1 by G6P which could lead to enhanced aerobic glycolytic flux in cancer cells [Warburg effect, (Warburg 1956a, b)]. If this regulation exists in normal tissues and specifically in brain is yet a challenge and an opening question for the near future. This suggests that the G6P product of the different mtHK isoforms may be distinctly compromised with the metabolic pathways in different cellular specializations in healthy states (Calmettes et al. 2013; John et al. 2011). We will focus on these mtHK1 and 2 as players of glucose phosphorylation at mitochondrial surface.

In the brain of mammals and birds, mtHK type I has been associated with more than 80% indicating that even healthy tissues depend on this association of mitochondrial glucose phosphorylation for metabolic balance (Crane and Sols 1953; Katzen et al. 1970). The balance between the mtHK bound and unbound states to the

mitochondria may be correlated with the levels of G6P, glycemia, and sensitivity to back-inhibition by the product of the reaction, G6P, access to ATP generated by OxPhos and effects of insulinemia and other hormones (Wilson 2003; Bessman and Gots 1975; Knull et al. 1974). The bounded isoform of HK is more active and less sensitive to G6P feedback inhibition. Furthermore, high G6P levels can favor the dissociation of the mtHK from OMM. However, which isoform, type mtHK 1 or 2, would be more subjected to binding control by G6P is still unknown. There are some pieces of evidence suggesting that mtHK1 is more stable in OMM than mtHK2. MtHK 2 would have a more modulable location between mitochondria and cytoplasm in skeletal and cardiac muscle (Katzen et al. 1970). In the whole brain and in its sub-regions and different brain cell types, the evidence is still scarce (Calmettes et al. 2013; John et al. 2011; Knull et al. 1974; Leong 1991; Katzen 1966; Sun et al. 2008; Smilansky et al. 2015; Puthumana and Regenold 2019).

This set of data indicates that there is an authentic functional relationship between the preferential use of mitochondrial ATP by mtHK and G6P synthesis by mitochondria. However, the mechanisms of this fine regulation of the association of HK with mitochondria and how the metabolic distribution of G6P among different pathways is adjusted to specific demands are still poorly understood. Furthermore, it is unclear which signaling pathways and which factors are involved in these interactions. What would be the second messenger and how would it be the interconnection among different signals such as cellular nutrients and the major metabolic reprogramming, such as Crabtree, Pasteur and, Warburg Effects? In addition, how the signaling pathways of apoptosis, cell proliferation, and differentiation are integrated with the activity of glucose phosphorylation by mtHK remains a challenge to be solved.

6.1.4 Glucose Phosphorylation at Mitochondria in Mammalian Cells by Hexokinase. A Signal to Survive and Control of Apoptosis. Is This a Redox Role?

The link between events of apoptosis "programmed cell death or apoptosis" necrotic cell deaths were also to be associated with glucose's metabolism. At the beginning of the present century, important findings started the unmistakable demonstration that the activity and strategic location of mtHK 1 and 2 on the surface of the mitochondria linked to VDAC in OMM plays a crucial role in an anti-apoptotic's response and due to important consequences on cellular and outcomes in pathological or healthy maintenance states.

In 2001, the inhibition of the initial apoptotic events by Akt/PKB-mediated signaling was demonstrated, being dependent on mtHK-induced glycolysis (Gottlob et al. 2001). In the following years, it was demonstrated that mitochondrial binding of mtHK 2 to VDAC inhibits Bax-induced cytochrome c release and apoptosis (Pastorino et al. 2002) and that Akt-mediated mitochondria-HK interaction is

necessary to inhibit apoptosis regardless of the presence of Bax and Bak (Majewski et al. 2004).

Recently, it was shown that 4-phenyl butyric acid (4-PBA), a chemical chaperone that acts as an endoplasmic reticulum (ER) stress inhibitor in different cells types (Ozcan et al. 2004) and also to treat diabetic animal models produces beneficial effects on glucose metabolism and observed as a specific promoter of the binding of HK to mitochondria also modulates glucose metabolism in L6 myotubes cells and protects against ROS injury (da Silva et al. 2017).

Alterations in glucose metabolism have been shown for diverse disorders. HKI and II can bind to mitochondria through their N-terminal hydrophobic regions, and their overexpression in tissue culture protects against cell death. The truncated forms of the mtHK1 and 2 lacking the mitochondrial binding domains, and catalytically inactivated proteins in tissue culture were investigated (Sun et al. 2008). The overexpression of full-length proteins resulted in protection against cell death, decreased levels of reactive oxygen species, and inhibited mitochondrial permeability transition pore opening (mPTPO) in response to exogenous levels of H_2O_2. Remarkably, the truncated and mutant proteins without mtHK activity exerted only partial effects. The HK proteins also resulted in an increase in the phosphorylation of voltage-dependent anion channel (VDAC) through a protein kinase Cε (PKCε)-dependent pathway. These results suggest that both glucose phosphorylation and mitochondrial binding contribute to the protective effects of HKI and HKII, possibly through VDAC phosphorylation by PKCε (Sun et al. 2008).

Notably, the previous finding of the studies by da-Silva et al. suggested that mitochondrially bound HK plays a role in the rate of endogenous ROS generation, possibly through an ADP-recycling process (da-Silva et al. 2004). Thus, this study showed that the binding and activity of the mtHK1 and mtHK2 exert, even for exogenously added H_2O_2, is an important antioxidant defense and possibly a redox signaling control in different metabolic states in several tissues. In the next section, we will describe the new findings of the ROS modulation by mtHK in CNS and the possible involvement in other signaling redox responses in other cells and organisms.

6.1.5 Hexokinase as an Antioxidant Defense or Redox Signaling Modulator in Mitochondria?

The association of HK to the VDAC in OMM imposes a variable degree of coupling of its activity with Δp control, in addition to the escape of electrons from the ETS supercomplexes that are favorable to the formation of ROS (CI, CIII, and other electron leakage sites sensitive to Δp variations). And it can be summarized in the following equation as mentioned in Sect. 6.1.1:

$$\text{Glucose} + \text{ATP} + \Delta p_{\text{high}} + \text{ROS} \rightarrow \text{glucose-6P} + \text{ADP} + \Delta p_{\text{low}} + \text{H}_2\text{O},$$

where Δp_{high} and Δp_{low} are mild changes in their value (less than 10% of the maximum value) that can completely halt mitochondrial ROS. Glucose phosphorylation attaches to the surface of the cerebral mitochondria making mtHK a recycler of ADP/ATP for brain OxPhos in space and time. This coupling gives a strong control over the leakage of ROS (a decrease of 10% of the Δp can promote almost 100% decrease in the rate of formation of ROS) both intra- and extra mitochondrial. This demonstration was originally done for the first time by our group in 2004 (da-Silva et al. 2004) in isolated rat brain mitochondria. In the brain, mtHK activity corresponds to more than 80% of the total being mostly mtHK 1 (Wilson 2003). In this way, the brain presents itself as an excellent system for investigating how mitochondrial glucose phosphorylation is regulated and can affect the downstream redox homeostasis of the mitochondria and also of its different cell types.

An excellent summary of how this hypothesis was conjectured and applied to other tissues and cellular systems of different species and mammals, including long-lived mammals, was recently shown in a completed work of comparative bioenergetics by the group of Dr. Skulachev, Vyssokikh and collaborators ((Vyssokikh et al. 2020); see especially Fig. S1 of the Supplementary Material—"History of the Discovery of mild depolarization"). In this work, the Russian group confirmed our findings and extended the concept of redox regulator to different mammalian tissues and also confirms that mtHK activity is involved in an anti-aging program (see further description) (Vyssokikh et al. 2020).

We also investigated that, with the exception of hepatic mitochondria, the mitochondria obtained, from kidney and heart, present this mechanism for controlling the generation of ROS by mtHK isoforms coupled to mitochondria. In addition, there seems to be a negative correlation between mtHK activity and levels of mitochondrial peroxidases and catalase (Santiago et al. 2008). These data suggested that this new mechanism of redox control of glucose metabolism is present in different tissues. The presence of HK linked to mitochondria in several mammalian tissues had been investigated preliminarily in Katzen's studies (Katzen et al. 1970; Katzen 1966). These observations were also confirmed and extended to other tissues in the study by Vyssokikh et al. (2020). In tissues with high energy demand and ATP turnover, such as the brain, it has also detected a synergism of mtHK activity with the activity of mitochondrial creatine kinase (mtCK), a higher energy phosphate shuttle system, which can also be a mitochondrial ROS regulating redox system accessory to that of mitochondrial glucose phosphorylation by mtHK in situations of hyperglycemia (Meyer et al. 2006).

These observations led us to question whether in different brain regions the control could be exercised by mtHK in the same way. Hypothalamic control of hunger/satiety by glucose metabolism is exercised in neurons of the arcuate nucleus (AN), those of agouti-related protein (AgRP), neuropeptide Y, (NPY), and neurons pro-opio-melano-curtain (POMC) that respond to the increase in the generation of ROS initiating satiety (Horvath et al. 2009). In the presence of succinate (CII

substrate), the activation of mtHK promoted the clearance of ROS in hypothalamic and cortical synaptosomes. However, ROS clearance did not occur in hypothalamic synaptosomes when pyruvate, a substrate for complex I, mainly derived from glucose, was used (Cavalcanti-de-Albuquerque et al. 2018). These data suggest that mtHK regulation may be cell-specific for a given redox function consequent from the use of glucose.

These diverse responses of the mtHK system in brain-specific regions suggested that in models of chronic and proinflammatory diseases, such as in type I diabetes mellitus (DM1), there could be changes in the pattern of response to mitochondrial glucose phosphorylation by mtHK and also in different pathways signaling and transcription regulated by redox homeostasis. With this objective, Silva-Rodrigues et al. (2020) sought to evaluate the activity pattern of mtHK coupled with OxPhos, as well as possible changes in the OxPhos of mitochondria and target proteins of redox homeostasis. In fact, there was a decrease in C I activity in the mitochondria and a decrease in ROS generated from C I. There was also a preference by mtHK to control ROS-derived electrons entry coming from succinate. For the first time, it provided evidence that early progression of hyperglycemia, in brain tissue, changes the coupling of glucose phosphorylation at the level of mitochondria. In addition, DM1 increased the oxidation status of PTEN, as a downstream target of prooxidant action of released H_2O_2, which is a phosphatase regulated by H_2O_2, able to influence redox signaling and protein oxidation (Adam-Vizi and Chinopoulos 2006; Jezek et al. 2020; Hopkins et al. 2014) and decreased the activation of NF-kB, a transcription factor of genes related to the oxidative stress, in DM1. These results indicate that this reorganization of glucose–oxygen–ROS axis in mitochondria may impact turnover of glucose, brain amino acids, redox, and inflammatory signaling.

ROS are natural byproducts of oxidative metabolism that have an important role to modulate brain development (Kennedy et al. 2012; Wilson et al. 2018). Traditionally, ROS is correlated with oxidative damage and cell death in their physiology and in certain pathologies, such as cancer, neurodegenerative diseases, and psychiatric disorders. However, ROS and calcium handling are also recognized as important intracellular signalers that regulate various signaling pathways by modulating redox-sensitive proteins and molecules, such as transcription factors and cytoskeleton components (Borquez et al. 2016). They have been shown to be involved in the proliferation, migration, and differentiation of neural progenitor cells in physiological and pathological conditions (Haigh et al. 2016; Xie et al. 2015). These different possible contexts in the redox scenario led us to investigate then how the degree of coupling of mtHK in the rat brain mitochondria would evolve along the development of the CNS and how the redox and calcium handling would respond to the glucose metabolism coupled to Δp in OxPhos.

It was unequivocally demonstrated by de-Souza-Ferreira et al. (2019) that mtHK 1 plays a crucial role in the management of mROS and Ca^{2+} buffering by mitochondria throughout brain development in rats (de-Souza-Ferreira et al. 2019). Given the conditions of high demands for oxygen and glucose from the adult brain, the increased binding and activities of HK in the mitochondria confers to the isolated mitochondria the property of regulating glucose and oxygen consumption in a

singular way of control in Δp levels in OxPhos by its reaction G6P product. This distinctive relationship makes the CNS mitochondria themselves when equipped with this "glucose-OxPhos-redox transducer," a unit that controls tightly the flow of oxygen/glucose, that is, the brain mitochondria as a unit of OGI (de-Souza-Ferreira et al. 2019). But what would be the relevance of this intimate relationship? One proposal would be to meet the demands for redox and calcium signals crucial to neurodevelopment and synaptic neuroplasticity in the healthy brain, mainly governed by the supply and increasing demands for oxygen and glucose after weaning and switching from a high-fat to high-glucose diet.

Interestingly and counterintuitively, the main antioxidant enzyme defenses of brain mitochondria such as glutathione reductase and peroxidase do not accompany the mitochondrial mass gain in the brain, as in the case of thioredoxin reductase they decrease with development (de-Souza-Ferreira et al. 2019). In contrast, the mtHK 1 strongly correlates with the electron leakage capacity of the rat brain mitochondria (de-Souza-Ferreira et al. 2019). These data point to an increasing demand on brain development for mitochondrial released H_2O_2 molecules controlled by mtHK1. This delicate balance of redox signals would have an immediate connection to the availability of glucose phosphorylation mitochondrial coupled. Another new property described for glucose phosphorylation mitochondrial coupled relates to the ability of the highly activated mtHK activity to promote a substantial increase in the amount of Ca^{2+} stored when compared to its retro-inhibited state by G6P (de-Souza-Ferreira et al. 2019). Newly born animals are irresponsible to G6P and sequester 4 times less Ca^{2+} than 60-day-old animals. Adult animals have a greater capacity for Ca^{2+} mitochondrial sequestration and are clearly regulated by the product of the mtHK 1 reaction (de-Souza-Ferreira et al. 2019; Monteiro et al. 2020).

Evidence to support this view comes from different studies of mood disorders that demonstrate changes in mtHK levels in the postmortem brain of bipolar and/or schizophrenic patients (Regenold et al. 2012) and also from G6P levels and their correlation with pathway of pentose phosphate (Puthumana and Regenold 2019). Interestingly, it has also been observed that natural "de-novo" mutations of mtHK1 in humans are involved in different neurological developmental problems, such as blindness and cerebral palsy (Okur et al. 2019). Intriguingly, these mutants do not show significant changes in the kinetic activities of the HK1 isoform, but the mutations occur in the domain of the protein that may be involved in the way in which mtHK associates with VDAC (Okur et al. 2019). These mutations may be interfering in the manner of the mtHK 1 binding to VDAC/ANT and affecting the ADP/ATP cycling (Sui and Wilson 1997; Wilson 2003; Azoulay-Zohar et al. 2004; Vyssokikh and Brdiczka 2003; Okur et al. 2019).

This set of data points to a redox function of mtHK coupled to mitochondria beyond the classical glucose metabolism pathways. They also seem to be involved in the control, not only antioxidants, but for normal signaling between two extremes of the spectrum of the oxidation stress potential, as well as for reductive or electrophilic stress situations detected by mtHK and its delicate regulation of activity via Δp, ADP/ATP ROS (Figueira et al. 2013; da-Silva et al. 2004; Meyer et al. 2006; Busija et al. 2016; Heneberg 2019; Santiago et al. 2008; Cavalcanti-de-Albuquerque et al.

2018; Silva-Rodrigues et al. 2020; de-Souza-Ferreira et al. 2019; Monteiro et al. 2020; Saraiva et al. 2010). This "glucose-OxPhos-redox transducer" integrates a glucose sensor (mtHK) to its metabolism with redox molecules of signaling pathways involved in (a)—apoptosis, (b)—proliferation and differentiation, (c)—autophagy, innate, and adaptive immune responses in different CNS cells and NVU (see Sect. 6.1.2).

6.1.6 Going Beyond the Brains: Are Bats, Inflammation, and Mitochondrial Hexokinase Connected?

Finally, this integrative system of the use of glucose due to its phosphorylation coupled to the mitochondria by mtHK seems to be interconnected through ROS with longevity, inflammation, and tolerance to pathogens (Vyssokikh et al. 2020). This glucose-OxPhos-redox transducer (da-Silva et al. 2004; Meyer et al. 2006; Santiago et al. 2008; de-Souza-Ferreira et al. 2019) was confirmed by Skulachev's group, that mild depolarization of the internal mitochondrial membrane is a crucial component of an anti-aging program (Vyssokikh et al. 2020). In addition, the glucose-OxPhos redox transducer system appears to be involved in longevity and viral resistance against SarsCov-2 in long-lived species such as bats (*Carollia perspicillata*) and bare African moles (*Heterocephalus glaber*) possibly by strengthening the immune system and anti-inflammatory (Nunn et al. 2020; Irving et al. 2021; Gorbunova et al. 2020; Skulachev et al. 2020; Hayman 2019; Dammann 2017) (Fig. 6.1).

These redox and aspects deserve further investigation in the context of the recent scenario of the global pandemic of SARS-CoV-2. The SARS-CoV-2 presents with a mild to a very high inflammatory condition that can lead to death. Interestingly, patients with metabolic syndrome (overweight, diabetes, hypertension, and dyslipidemia) have a particularly bad result if infected with SARS-CoV-2 (Donath 2021; Yu et al. 2021). Yu et al. (2021) suggest that insulin therapy itself can promote fatality in patients with COVID-19 and diabetes. Hyper-inflammation can occur due to the conjunction of type 2 diabetes and COVID-19. The hypothetical mechanisms suggest that the cytokine storm in patients with COVID-19 and diabetes, glucose, lipids, and insulin may potentiate the activation of SARS-CoV-2 of the NLRP3 inflammasome by means of glucose metabolism in macrophages under insulin stimulation leading to insulin stimulation ROS production. This will lead to the splicing of pro-IL-1b into IL-1b with subsequent hyper-inflammation inducing respiratory and cardiac failure (Donath 2021; Yu et al. 2021).

It becomes opportune to further investigate how the glucose-OxPhos-redox transducer of the mtHK is involved in macrophages and immune cell responses (da-Silva et al. 2004; Meyer et al. 2006; de-Souza-Ferreira et al. 2019). And also, if there is some pharmacological intervention that could mitigate inflammatory responses to improve a better outcome for patients infected with COVID-19. Further

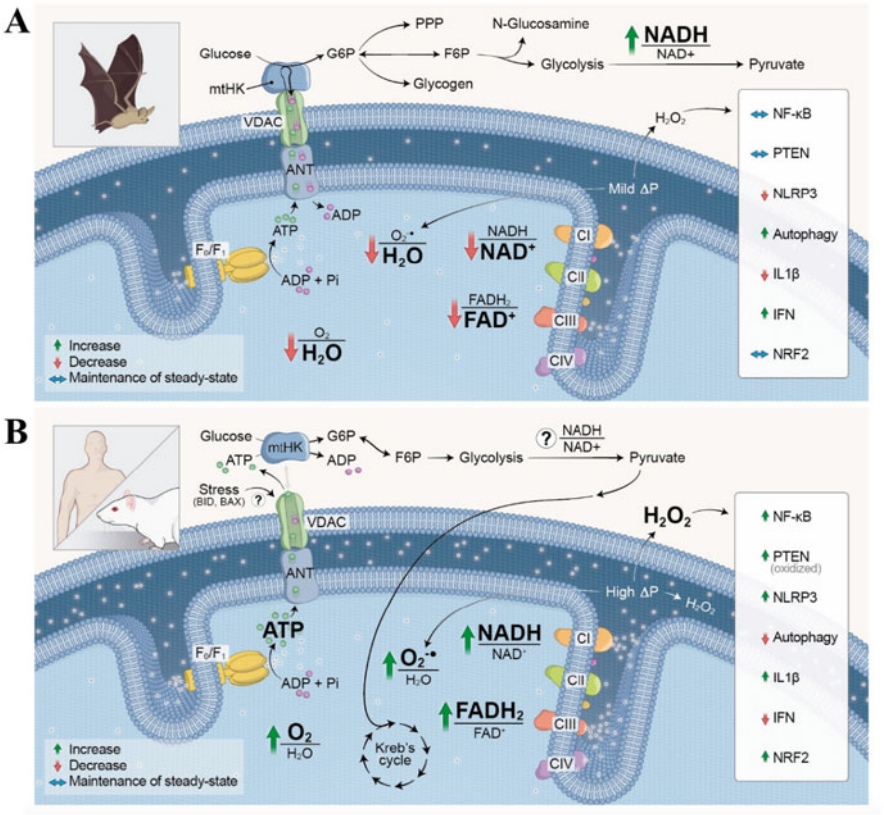

Fig. 6.1 Proposed glucose-OxPhos-redox transducer system coupling to mitochondria and downstream redox signaling responses. The association of HK with VDAC and ANT provides the immediate transmission of glucose phosphorylation from adenine nucleotide cycling (ADP_{entry}/ATP_{exit}) to ATP synthesis by F_oF_1ATP-synthase. The ATP synthesis by the OxPhos consumes the protonmotive force (Δp) avoiding the escape of electrons from the ETS and formation of the superoxide anion and its dismutation to H_2O_2. In cellular systems where HK is strongly linked to mitochondria or in long-lived species, such as bats and mole naked rats (**a**); this unique Glc-O_2-ROS balance can adjust secondary redox signals, such as NF-κB, PTEN, NRF2, suppressed inflammasome pathways—such as dampened NLRP3, autophagy, and downstream IL-1β. The constitutive expression of IFNs activates the immune response, downregulating inflammation and tolerating viruses. In cellular systems where HK is loosely bound or differently attached to mitochondria or in non-long-lived species, such as rats and man (**b**); the balance Glc-O_2-ROS is disrupted leading to a more susceptible interaction with apoptotic proteins Bax, Bid, interruption of ADP_{entry}/ATP_{exit} cycling, and high rates of H_2O_2 released from mitochondria (see Sect. 6.1.6 for explanations)

studies are promising to clarify the interplay of glucose metabolism in adaptive and innate immune responses.

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Chapter 7
Mitochondrial Dysfunction as a Trigger of Inflammation in Cardiomyopathies

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Abstract Mitochondrial dysfunction and bioenergetic failure are a hallmark of heart failure, diabetic cardiomyopathy, and myocardial infarction. An inadequate supply of oxygen and nutrients triggers a cascade of events in which mitochondria are a critical mediator, particularly mitochondrial calcium overload, permeability transition pore opening, oxidative stress, and the release of mitochondrial components that interact with immune cell residents in the heart. Depending on the degree of mitochondrial dysfunction, cardiac cells lead to the activation of the inflammasome and other inflammation pathways. On the other hand, the activation of immune cells depends on their mitochondrial metabolism, and they potentially contribute to cardiac diseases. This chapter reviews the main mitochondrial molecular mechanisms that compromise the heart's immune activation and their potential involvement in acute myocardial infarction, sepsis, and myocarditis.

Keywords Cardiomyopathy · Heart failure · Mitochondria · Inflammation · Immunometabolism

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7.1 Introduction

Cardiac inflammation is currently recognized as a condition state that perpetuates heart damage and leads to cardiomyopathies (Castillo et al. 2020). Inflammatory stimuli are caused by either direct injury to the heart (e.g., myocarditis) or systemic soluble mediators (e.g., diabetic cardiomyopathy), and they alter cardiomyocyte functioning, activate resident cardiac-immune system cells, and stimulate the recruitment of other immune cells (Tschöpe et al. 2020). Mitochondria are biosynthetic and bioenergetic organelles that dictate cell function, modulating metabolism and signaling pathways by calcium buffering and ROS production (Burgoyne et al. 2012; Dedkova and Blatter 2013). Thus, mitochondria act as a link between metabolism and immune system activation, facilitating adequate heart function. In this context, prior research has shown that two transcription factors, nuclear factor κB (NFκB) and peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α), negatively regulate each other (Alvarez-Guardia et al. 2010). The former is a key regulator of inflammation, whereas PGC-1α is an essential regulator in mitochondrial dynamics and processes, as well as in metabolism (Schilling et al. 2011). This chapter reviews the mitochondrial regulatory processes that facilitate efficient heart function and how the disruption of these processes contributes to immune system activation, including the role played by immune system cells in the development of cardiomyopathies.

7.1.1 Mitochondrial Structure and Function

The primary process associated with mitochondrial function is its capacity to regulate energy conversion by generating adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS). However, mitochondria also modulate cell signaling and cell death by buffering calcium and regulating the redox state of reactive nitrogen (RNS) and oxygen species (ROS) (Burgoyne et al. 2012; Dedkova and Blatter 2013). Therefore, the interconnection of mitochondria's fine-tuning mechanisms is essential to guarantee energy conversion (Gnaiger and Group MT 2020).

This organelle's unique feature is its two delimited inner and outer membranes, which are structurally and functionally distinct. While the outer mitochondrial membrane (OMM) allows the free transit of ions and small molecules, the inner mitochondrial membrane (IMM), consisting of cristae and inner boundary membranes, only enables their entry through specific transporters, and ion concentration gradients across the membrane create its membrane potential (Wolf et al. 2019). This characteristic allows physicochemical differences between the compartments associated with their particular functions in which the interaction among their components is crucial (van der Laan et al. 2016). The OXPHOS machinery (i.e., complexes of the respiratory chain and F1F0-ATP synthase) is anchored in the IMM cristae. The IMM surrounds the mitochondrial matrix, where mitochondrial DNA (mtDNA),

enzymes, and ions reside and the tricarboxylic acid cycle (TCA) occurs (Kühlbrandt 2015).

The space between the IMM and OMM contains various molecules, such as cytochrome c (Cyt c), which is essential for transporting electrons and, when released from mitochondria, acts as an apoptotic trigger (Zhao et al. 2019). Cyt c is released through the mitochondrial permeability transition pore (mPTP), which maintains proper membrane integrity, allowing only the passage of molecules <1.5 kDa (McCommis and Baines 2012). Although its structure is not yet well defined, the opening of the mPTP is triggered by calcium and regulated by adenine nucleotides and cyclophilin D (Cyp D) (Alves-Figueiredo et al. 2021).

7.1.2 Role of Mitochondria in the Heart

The heart is a demanding organ that needs large amounts of ATP to meet its bioenergetic requirements for rhythmic contractions and blood pumping. At high workloads, energy conversion can be completed in less than 10 s (Balaban 2009). Because mitochondria produce up to 90% of this ATP (Doenst et al. 2013), the mitochondrion comprises more than 30% of the volume of a cardiomyocyte, and various characteristics, such as its morphology, location, and interactions with other organelles, are crucial for proper functioning (Ventura-Clapier et al. 2011).

7.1.3 Metabolism

Although the heart can metabolize any substrate to produce energy, its primary fuel energy substrates are fatty acyl-CoA (fatty acids) and pyruvate (carbohydrates) (Kolwicz et al. 2013) to maintain a coordinated metabolic network and meet each heart contraction's energetic demands. Among them, fatty acids are the principal metabolic source (40–90%), and their utilization is controlled by the PPARα/PGC-1α/ERR (estrogen-related receptor) axis, from transport to oxidation (Duncan and Finck 2008). Fatty acid β-oxidation (FAO) provides higher energy yields than glycolysis, but more oxygen is required; thus, shifting mitochondrial oxidative metabolism from FAO to glucose oxidation increases energy efficiency. This shifting mechanism is employed by cardiomyocytes under certain pathological conditions, such as in hypertrophied hearts, to meet energetic needs (Sorokina et al. 2007). However, limiting fatty acid utilization results in detrimental heart function (Tuunanen et al. 2006). In contrast, if fatty acid utilization increases and surpasses the mitochondria's FAO capacity, lipids accumulate in the heart, which causes lipotoxicity (Nagoshi et al. 2011) and alterations in glucose transport, leading to mitochondrial dysfunction by impairing the redox state (Wright et al. 2009). Then, the increase in FAO and oxygen consumption increases the delivery of reducing equivalents to the electron transport chain (ETC), which decreases OXPHOS

capacity, ROS production, lipid peroxidation, and, in turn, cardiac energetic efficiency (Boudina et al. 2007). Thus, the exact coupling proportion of fatty acid and glucose oxidation is crucial to maintain the fine-tuning of energy versus ROS production for both ATP demands and signaling.

7.1.4 The Relevance of Mitochondrial Quality Control in Cardiomyocytes

As dynamic organelles, mitochondria continuously undergo morphological changes while forming continuous networks to adapt to environmental demands and achieve homeostasis. These morphological changes are called fusion and fission and are collectively known as mitochondrial dynamics. In conjunction with mitophagy and biogenesis, they maintain a healthy mitochondrial population (Fig. 7.1) by regulating mitochondrial quality control, and, therefore, cell survival (Ni et al. 2015). Additionally, a macrophage-dependent mechanism has recently been demonstrated, which involves the extrusion of autophagic vesicles with high mitochondrial content, also referred to as exophers, and incorporates the externalization of phosphatidylserine, which is an apoptotic cell recognition feature (Nicolás-Ávila et al. 2020) (Fig. 7.1). This process eliminates dysfunctional mitochondrial and avoids inflammation by preventing the activation of the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome by a process that we will discuss in Sect. 7.1.6 of this chapter.

7.1.4.1 Mitochondrial Dynamics

Mitochondrial dynamics are essential in situations involving increased energetic demands, mitochondrial damage during development, stressful conditions, and aging (Chen et al. 2012; Ong et al. 2010; Piquereau et al. 2012). Remodeling through fission and fusion is necessary to conserve mitochondrial function and protect the heart's cellular homeostasis (Ikeda et al. 2015).

Fusion is a GTPase-regulated mechanism by which damaged mitochondria join with intact ones and redistribute their soluble and membrane proteins, lipids, and mtDNA to maintain an adequate membrane potential (Chen et al. 2010). Thus, mitochondrial fusion helps mitochondria to avoid mitophagy, regulates their morphology, and facilitates positive regulation of cellular contractility and respiration (Givvimani et al. 2015). Fusion is mediated by the transmembrane GTPases mitofusin 1 (Mfn1) and Mfn2, which are located in the OMM, interact, and create homotypic or heterotypic units, culminating in OMM fusion (Franco et al. 2016). Moreover, Mfn2 is directly related to contractility by modulating Ca^{2+} and K^{+} ionic fluxes (Givvimani et al. 2015), and dynamic-like GTPase optic atrophy 1 (Opa1) in the IMM collaborates with cardiolipin and modulates IMM fusion (Ban et al. 2017).

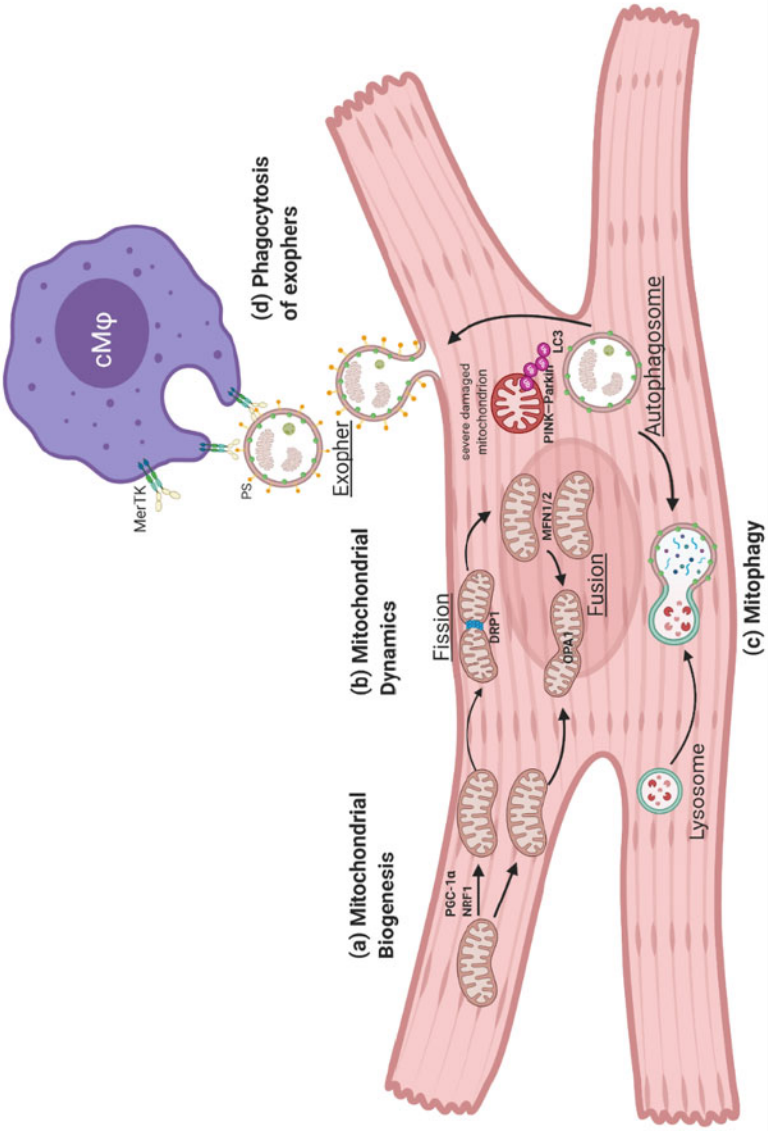

Fig. 7.1 Mechanisms of mitochondrial quality control in cardiomyocytes. (**a**) Mitochondrial biogenesis: a mitochondrion divides and gives two new renewal mitochondria. (**b**) Mitochondrial dynamics comprises fusion: lightly damaged mitochondria fuse with a healthy one to enhance mitochondrial function and fission: a senescent mitochondrion divides, giving up one healthy mitochondrion and other damage which is eliminated. (**c**) Mitophagy: allow the elimination of

Fig. 7.1 (continued) damaged mitochondria. (**d**) Phagocytosis of exophers: some autophagosomes are not eliminated within the cell and are extruded to the extracellular matrix to be engulfed. *cMφ* cardiac macrophages, *DRP1* dynamin-related protein 1, *LC3* microtubule-associated protein 1A/1B-light chain 3, *MerTK* Mer tyrosine kinase, *MFN1/2* mitofusin1/2, *NRF1* nuclear respiratory factor, *OPA1* dynamic-like GTPase optic atrophy, *Parkin* E3 ubiquitin ligase, *PGC-1α* peroxisome proliferator-activated receptor gamma coactivator 1-alpha, *PINK* PTEN-induced kinase 1, *PS* phosphatidylserine. (Created with BioRender.com)

In contrast, **fission** isolates nonfunctional mitochondrial fragments and is crucial in maintaining the quality of mitochondria (Youle and van der Bliek 2012). If the daughter mitochondrion has a normal membrane potential, it can undergo fusion. However, if it is impaired, it will be eliminated through mitophagy (Twig et al. 2008). On the other hand, increased fission impairs calcium handling in cardiomyocytes and Cyt c leakage (Givvimani et al. 2015). In conjunction with actin, the SR encircles the mitochondrion, marking the fission site and starting mitochondrial constriction (Korobova et al. 2013). Subsequently, cytosolic GTPase dynamin-related protein 1 (Drp1) migrates to the previously marked fission site. Its GTPase activity ends with OMM and IMM fission (Ji et al. 2015). In cardiomyocytes, the translocation of Drp1 to mitochondria depends on an increase in the cytosolic concentration of Ca^{2+} (Hom et al. 2010). Once Drp1 is translocated, it is recognized by the mitochondrial receptor fission 1 protein (Fis1), which is located in the OMM (Yoon et al. 2003).

7.1.4.2 Mitophagy

Mitophagy is an inherent process in quality control by which cardiomyocytes eliminate damaged mitochondria and retain functional ones, preventing heart failure and increasing survival (Kubli et al. 2013). As mentioned above, fission activation anticipates mitophagy (Twig et al. 2008). In the presence of low mitochondrial membrane potential, ROS increases production, or protein misfolding, and PTEN-induced kinase 1 (PINK1) accumulates in the OMM (Jin and Youle 2013). PINK phosphorylates Mfn2, blocking fusion, and activates Parkin; this cytosolic E3 ubiquitin ligase ubiquitinates the damaged mitochondria and associates with LC3, triggering the autophagic machinery (Kawajiri et al. 2010). Additionally, Parkin promotes mitochondrial biogenesis by the ubiquitination of PARIS (ZNF746), a Kruppel-associated box (KRAB), and zinc finger protein, which represses PGC-1α (Shin et al. 2011). As mitophagy requires mitochondrial depolarization, CypD, the mPTP regulator, acts as a crucial promoter (Carreira et al. 2010). Thus, assuring mitophagy prevents inflammation, as has been demonstrated that in the absence of Parkin-mediated mitophagy, NLRP-3 inflammasome is activated (He et al. 2019).

7.1.4.3 Mitochondrial Biogenesis

Considering that nuclear DNA encodes 99% of mitochondrial proteins, both nuclear DNA and mtDNA must be translated and transcribed to achieve mitochondrial biogenesis. Animal models have demonstrated that PGC-1α controls this process under stressful conditions but is nearly absent in steady-state cardiac conditions (Lehman et al. 2000). In adults, severe cardiac stress, such as starvation or ischemic conditions, causes mitochondria autophagy and, consequently, mitochondrial biogenesis (El-Sikhry et al. 2016; Huang et al. 2010). However, metabolic changes may also occur due to healthy lifestyle changes promoting metabolic flexibility, such as physical exercise (lactate) and fasting (ketone bodies), which confer cardioprotection (Kolwicz et al. 2013; Ismayil et al. 2005). Thus, mitochondrial turnover is associated with metabolic reprogramming, preconditioning, and cardioprotection (Gottlieb and Gustafsson 2011; McLeod et al. 2004).

Mechanistically, raising cytosolic calcium and the consequent Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activation and cAMP-response element binding (CREB) protein phosphorylation (Sun et al. 1994) promotes PGC-1α-mediated biogenesis while blocking the nuclear translocation of FoxO1 (Ozcan et al. 2012), preventing autophagy and oxidation by promoting FoxO3a (Olmos et al. 2013). PGC-1α is posttranscriptionally activated by sirtuin 1 (SIRT-1), an NAD^+-dependent protein deacetylase, allowing the transcription of nuclear respiratory factor 1 (NRF1) and NRF2. NRFs promote the expression of mitochondrial respiratory complexes, mtDNA transcription factors, and antioxidant defense genes (El-Sikhry et al. 2016; Olmos et al. 2013). They also regulate the transcription of SIRT-3 (Song et al. 2017), which controls the function of all these proteins within the mitochondria. In this context, SIRT-3 downregulation has been associated with mitochondrial and heart dysfunction related to metabolic changes in failing hearts (Castillo et al. 2019). Indeed, the concentration of the coenzyme NAD^+ is essential to prevent cell death (mPTP opening) (Castillo et al. 2019) and inflammation (NLRP3 activation) (Misawa et al. 2013); the latter in a SIRT-2 dependent manner (Misawa et al. 2013).

7.1.5 Calcium and ROS Regulation

Calcium is the second messenger responsible for many signal transductions in cells that respond to extracellular signals. In cardiac cells, such as cardiomyocytes, it controls contraction and cell death (Pérez-Treviño et al. 2020a). In immune cells, calcium determines the cells' activation and fate, including differentiation, replication, cytokine release, and cell death (Nunes and Demaurex 2010; Scharenberg et al. 2007). The regulation of Ca^{2+} within mitochondria facilitates the fine-tuning of calcium and ATP and ROS production (Görlach et al. 2015).

ATP synthesis is a calcium-dependent mechanism that results in ROS production, which is necessary to regulate cell signaling and perform the adequate contraction–

relaxation rhythm known as excitation–contraction coupling (ECC) (Burgoyne et al. 2012; Tarasov et al. 2012). During systole, the high influx of calcium, which facilitates contraction, depends on a mechanism of Ca^{2+}-induced Ca^{2+} release in which the L-type Ca^{2+} channel triggers Ca^{2+} release from the SR. This Ca^{2+} binds to troponin C, part of the contraction apparatus, and, consequently, heart contraction occurs (Altamirano and Bers 2007). Then, during the diastole or relaxation period, Ca^{2+} returns to the SR by sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), is exported from the cell through the Na^{+}/Ca^{2+} exchanger (Bers et al. 2006), and enters the mitochondria via the mitochondrial calcium uniporter (MCU). Ca^{2+} activates TCA cycle dehydrogenases in the mitochondria, ETC, and OXPHOS to produce ATP (Fernández-Sada et al. 2014).

As previously mentioned, OXPHOS takes place in the IMM, in which the ETC generates a proton gradient enabling the OXPHOS of ADP to ATP by F0/F1 ATP synthase. Due to FAO and glycolysis, Acetyl CoA enters the TCA cycle in the mitochondrial matrix. The resulting byproducts, nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$), are used in redox reactions by ETC complexes I and II. The generated electrons are then transferred through complexes III and IV, forming a proton gradient, which creates proton movement through F0/F1 ATP synthase, catalyzing the phosphorylation of ADP to ATP by restoring the electrochemical gradient. Consequently, the respiratory chain generates ROS (Hassanpour et al. 2018) (Fig. 7.2, Blue Box).

The process of fine-tuning regulation that allows ROS signaling includes crucial scavenging mechanisms. In the ETC, oxygen reduction generates superoxide anion radicals ($O_2^{\cdot-}$) in complexes I and III. The dismutation of these radicals to hydrogen peroxide (H_2O_2) is catalyzed primarily by manganese superoxide dismutase (MnSOD) (Holley et al. 2011). The final process that reduces H_2O_2 to water is performed by the enzymes catalase, glutathione peroxidase, and peroxiredoxin (Molavian et al. 2015). However, when ROS production overcomes the regulatory mechanisms, the frequency of oxidative posttranslational changes increases, limiting the reductive modifications' capacity. Consequently, important proteins that regulate calcium handling and ECC are compromised, which affects their function and leads to their constitutive activation, as is the case in calcium-calmodulin kinase II (CAMKII) and ryanodine receptor 2 (RyR2) (Burgoyne et al. 2012). As described previously, to prevent the entire cell from being compromised, highly damaged mitochondria are eliminated by mitophagy or even secreted into the extracellular space for elimination by macrophages (Nicolás-Ávila et al. 2020). However, if damage continues, ROS promotes several alterations within the cell, including the opening of mPTP, increasing ROS release, and, eventually, cell death and inflammation (Zorov et al. 2000).

In the heart, ATP cannot decrease, and ADP and Pi cannot increase during heavy workloads. Therefore, heart mitochondria balance the ATP production rate with the quality of utilization while maintaining ATP hydrolysis' energy-generating capacity, modulated by changes in the concentrations of mitochondrial Ca^{2+} in response to the increase in cytosolic Ca^{2+} (Yaniv et al. 2008). To achieve this balance, the MCU controls calcium buffering. However, under cardiac stress, such as severe metabolic

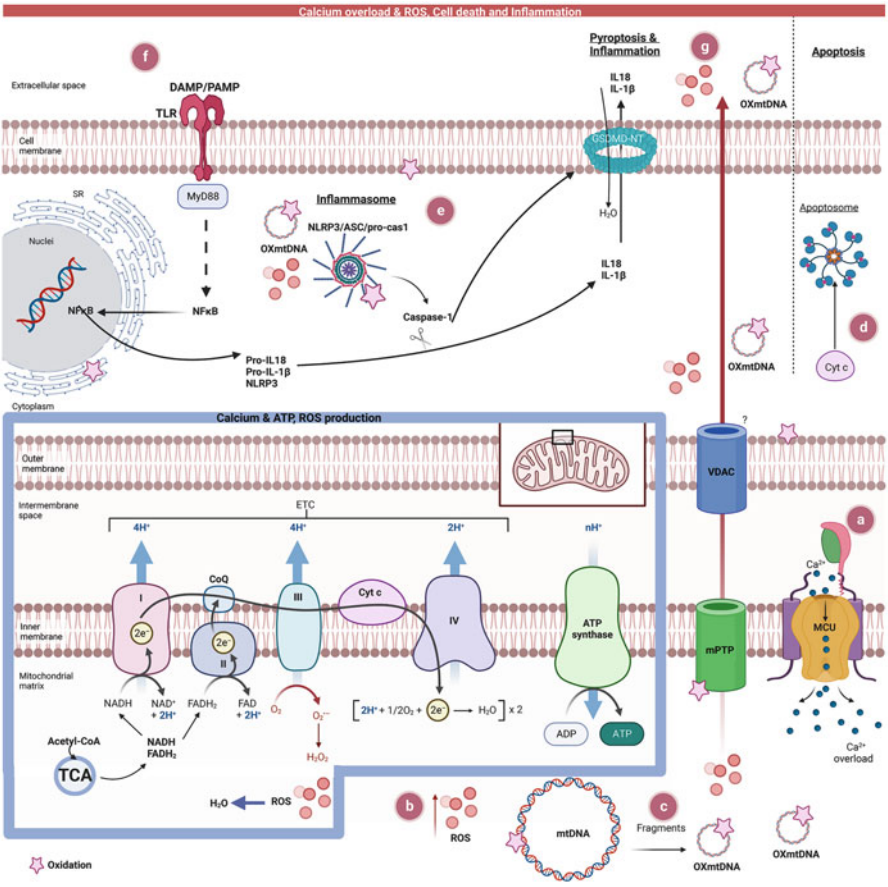

Fig. 7.2 Calcium and ROS regulation. Blue Box: oxidation of acetyl CoA produced NADH and $FADH_2$, which is further oxidated in the inner mitochondrial membrane by the ETC, generating a proton gradient and ROS. The proton gradient generates an electron force used by the ATP synthase to restore the electrochemical gradient and phosphorylation of ADP to ATP. (**a**) Under stress or pathological conditions, more Ca^{2+} enters the mitochondrial matrix by MCU transporter, promoting mitochondrial calcium overload and (**b**) ROS production increased. (**c**) Oxidative stress leads to mtDNA oxidation and fragmentation, and mPTP opening. (**d**) Cyt c is released into the cytoplasm, which interacts with other proteins to form the apoptosome and apoptosis cell death. (**e**) mtDNA and ROS activate NLP3 promoting caspase-1 activation (activation signal), which cleaves GSDMD producing NT-fragments and promotes maturation and release of IL-β and IL-18 through the GSDMD-NT pore leading to pyroptosis and cytokines released. (**f**) On the other hand, cardiomyocytes can also either be activated by extracellular DAMPs or PAMPS, which are recognized by TLRs and induce the synthesis of NLP3, pro-IL-1β, and pro-IL-18 (priming signal). These external activation signals by TLR also promote increased calcium and oxidative stress. (**g**) ROS and mtDNA are released from the cardiomyocyte and activate immune system cells such as macrophages, which recognized mtDNA as DAMPs. *ADP* adenosine diphosphate, *ASC* apoptosis-associated speck-like protein, *ATP* adenosine triphosphate, Ca^{2+} calcium, *Cyt c* cytochrome c, *DAMP* damage-associated molecular patterns, *ETC* electron transport chain, $FADH_2$ Flavin adenine dinucleotide, *GSDMD-NT* gasdermin D-N-terminal, H_2O_2 hydrogen peroxide, *IL* interleukin, *MCU* mitochondrial calcium uniporter, *mPTP* mitochondrial permeability transition pore, *NADH* nicotinamide adenine dinucleotide, *NFκB* nuclear factor κB, *NLRP3* NOD-like receptor pyrin domain-

changes (i.e., obesity) or ischemic conditions, mitochondrial overload occurs. Then, the balance between the production and detoxification of ROS is lost, leading to mPTP opening, the release of Cyt c, damage to mtDNA, cell death, and inflammation (Zhou and Tian 2018) (Fig. 7.2). It has been demonstrated that preventing mitochondrial calcium overload by targeting the MCU prevents all these mechanisms (de García-Rivas et al. 2006; Chapoy-Villanueva et al. 2019). Furthermore, it was recently documented that mitochondrial calcium overload could increase ROS production and cause membrane potential loss and energy decline. ROS can also affect critical proteins that control calcium handling, promoting unsolicited depolarization and pathological action potentials, which subsequently generate a suitable setting for arrhythmogenesis (Salazar-Ramírez et al. 2020).

7.1.6 *Mitochondrial Calcium Overload and ROS as Inflammation Triggers*

As previously mentioned, stressful conditions, such as ischemic and metabolic changes, promote calcium overload (Castillo et al. 2019; Oropeza-Almazán et al. 2017). Mitochondrial calcium overload or changes in the availability of adenine nucleotides or posttranslational modifications, such as oxidation, promotes the opening of mPTP and the release of Cyt c and other molecules, which initiate the formation of the apoptosome complex and results in apoptotic cell death (Carreira et al. 2010; Riojas-Hernández et al. 2015) (Fig. 7.2). Nonetheless, it has been suggested that mPTP opening may occur transiently during stress, such as in ischemic conditions, causing a mild depolarization of the IMM and resulting in a preconditioning mechanism; this mechanism prevents cell damage by favoring beneficial metabolic changes that increase mitochondria's capacity to respond to stress (Crescenzo et al. 2006).

However, when ischemic conditions promote excessive calcium overload and oxidative stress (Oropeza-Almazán et al. 2017), the increased mtROS induces mtDNA oxidation and specific fragments to be released through the mPTP (García et al. 2005; García and Chávez 2007). Unlike Cyt c, which promotes apoptotic cell death and prevents inflammation, oxidized mtDNA fragments lead to the activation of the NLRP3 inflammasome (Zhou et al. 2011) (Fig. 7.2). Upon activation, the NLRP3 forms a cytosolic complex with apoptosis-associated speck-like protein (ASC), which recruits the effector molecule pro-caspase-1 to form the NLRP3 inflammasome (NLRP3/ASC/pro-cas-1). NLRP3 belongs to the pattern recognition receptor (PRR) family, which recognizes a wide variety of specific motifs present on

Fig. 7.2 (continued) containing, O_2 oxygen, $O_2^{\cdot-}$ superoxide anion, *OxmtDNA* oxidized mitochondrial DNA, *PAMP* pathogen-associated molecular patterns, *Pro-cas1* procaspase 1, *ROS* reactive oxygen species, *TCA* tricarboxylic acid cycle, *TLR* Toll-like receptor, *VDAC* voltage-dependent anion channels. (Created with BioRender.com)

self and foreign antigens, triggering an innate immune system response. Both ROS and mtDNA are well-known NLRP3 activators that can activate the inflammasome within cardiomyocytes and then, when released, activate immune system cells (Pérez-Treviño et al. 2020b; Wu et al. 2019a).

It has recently been shown that ROS-associated mtDNA oxidation is partially mediated by proprotein convertase subtilisin/kexin type 9 (PCSK9) (Wang et al. 2020), which is secreted in response to inflammatory stimuli, such as LPS and ox-LDL, and increases in a pro-inflammatory milieu (Schlüter et al. 2017). On the cellular surface, both molecules are recognized by other PRRs, named toll-like receptors (TLRs), activating the NFκB transcription factor, which induces the synthesis of NLRP3, pro-IL-1β, and pro-IL-18, and providing the priming signal in NLRP3-mediated inflammation (Wu et al. 2019a) (Fig. 7.2). Moreover, TLR activation also contributes to increased Ca^{2+} and oxidative stress (Katare et al. 2017).

Once in the cytosol, damaged mtDNA activates the NLRP3 inflammasome, activating caspase-1 and promoting the maturation and release of pro-inflammatory cytokines IL-1β and IL-18 by a process called pyroptosis. Pyroptosis is a gasdermin (GSDMD)-mediated programmed necrosis that consists of the cleavage of GSDMD by caspase 1, generating N-terminal-GSDMD fragments that are oligomerized and form a pore in the cell membrane (Shi et al. 2017). mtDNA fragments and cytokines released by cardiomyocytes activate innate immune cells and induce immune cell recruitment, perpetuating cardiac inflammation. A recent study demonstrated that upon TLR activation, macrophages synthesize mtDNA for NLRP3 activation. Whereas pro-IL-1β and IL-18 synthesis is dependent on the NFκB pathway (Fig. 7.2), mtDNA is mediated by the IRF1 transcription factor (Zhong et al. 2018).

The mitochondrial-NLRP3-NFκB axis is a triggering factor for inflammation in many cardiomyopathies that lead to heart failure, whether due to sterile or pathogenic inflammation (Castillo et al. 2016). Moreover, its activation downregulates PGC-1α expression in both cardiomyocytes and macrophages (Kang et al. 2018; Palomer et al. 2009), which is involved in heart dysfunction via a direct association between the NFκB, p65 subunit and PGC-1α (Alvarez-Guardia et al. 2010). Therefore, as mitochondria modulate metabolic activity and immune function, mitochondrial dysfunction compromises immunometabolism and the cardiac-immune cells' relationship with heart function (Weinberg et al. 2015).

7.1.7 Role of Immune System Cells in Heart Function

Cardiac resident immune cells have been recognized as important regulators of heart function in steady-state conditions (Nicolás-Ávila et al. 2020; Adamo et al. 2020; Hulsmans et al. 2017). Cardiac-immune cells represent approximately 10% of all non-myocyte cardiac cells; Among them, macrophages are the largest immune population in the heart, comprising about 80%, followed by B cells, comprising 10% of all leukocytes (Pinto et al. 2016; Yu et al. 2016). In the atrioventricular node,

cardiac macrophages (cMφ) facilitate electrical conduction by direct contact with cardiomyocytes through connexin 43-gap junctions, which has been confirmed in patient biopsies (Hulsmans et al. 2017). Furthermore, connexin-43-gap junctions are also essential in the ventricle, where reduced levels are associated with fibrosis and ventricular dysfunction by aberrant mitophagy (Givvimani et al. 2014). Moreover, it was recently discovered that cMφ present in the ventricular myocardium are critical players in maintaining mitochondrial homeostasis, and, thus, healthy cardiac metabolism and function by phagocyting damaged mitochondria released by cardiomyocyte (Nicolás-Ávila et al. 2020). On the other hand, phagocytosis depends on bioenergetic production, which is finely tuned by the MCU. Recent data from a study of Mφ with MCU knock-down showed decreased pyruvate dehydrogenase activity, ROS production, and M2 polarization (Tedesco et al. 2019). These data reveal a new role for the MCU in alternative macrophage polarization and phagocytic activity. In this context, transgenic mice with dominant-negative MCU in macrophages showed a reduction in ROS and fatty acid oxidation and were protected from fibrosis. These findings suggest that macrophage MCU-mediated metabolic reprogramming is associated with fibrotic repair after lung injury (Gu et al. 2019). Notably, cardiac fibrosis and maladaptive remodeling in rodents are associated with MCU expression changes (Zaglia et al. 2017). However, the contribution of macrophage MCU in mediating fibrosis in the heart is not well understood.

Cardiac B cells (cB cells) are a B cell population that recirculates within the blood and spleen, delaying their heart transit. cB cells mostly remain intravascular and are located near the endothelium, which has been confirmed in patients with heart failure (Adamo et al. 2020). Although their exact function is not yet completely defined, the absence of cB cells also affects the recruitment of other immune cells and is associated with reduced cardiac mass and ventricular dysfunction. In this sense, cB cells are critical players in heart failure, several experimental therapeutics that target B cells are currently under examination (García-Rivas et al. 2020). Besides, immune synapse is crucial for B and T cell activation, and mitochondria have been shown a relevant role in this process by localizing nearly the immune synapse, regulating calcium signaling, and supplying energy locally. The interaction between B and T cells showed a significant mitochondrial depolarization after antigen exposure. Of note, antigen processing and antigen presentation were dependent on MCU activity (Bonifaz et al. 2015). Our group recently suggest that mitochondria participate during B cell activation through Ca^{2+} overload. Using primary murine B cells, we found that after BCR-independent stimulation, pretreatment with a mitochondrial antioxidant reduced B cell activation. It found that activated cells show higher mitochondrial calcium contents, suggesting that MCU participates in the B cell activation axis modulating mitochondrial ROS production (Torres-Quintanilla et al. 2017).

7.1.8 *Mitochondrial Dysfunction and Immune Cells Interplay in the Development of Cardiomyopathies*

As previously explained, mitochondria are exceptional organelles that regulate metabolism and immune function, and the interplay between cardiomyocytes and cardiac-immune cells is necessary to achieve ECC. In immune system cells, metabolic changes are indispensable to appropriate activation and polarization (Fracchia et al. 2013; Jang et al. 2015; Mills et al. 2016). During basal conditions, naïve/resting cells depend mainly on OXPHOS, but upon activation, their metabolism relies on aerobic glycolysis to directly produce ATP. This phenomenon is known as the Warburg effect (DeBerardinis and Chandel 2020) (Fig. 7.3). This shift increases

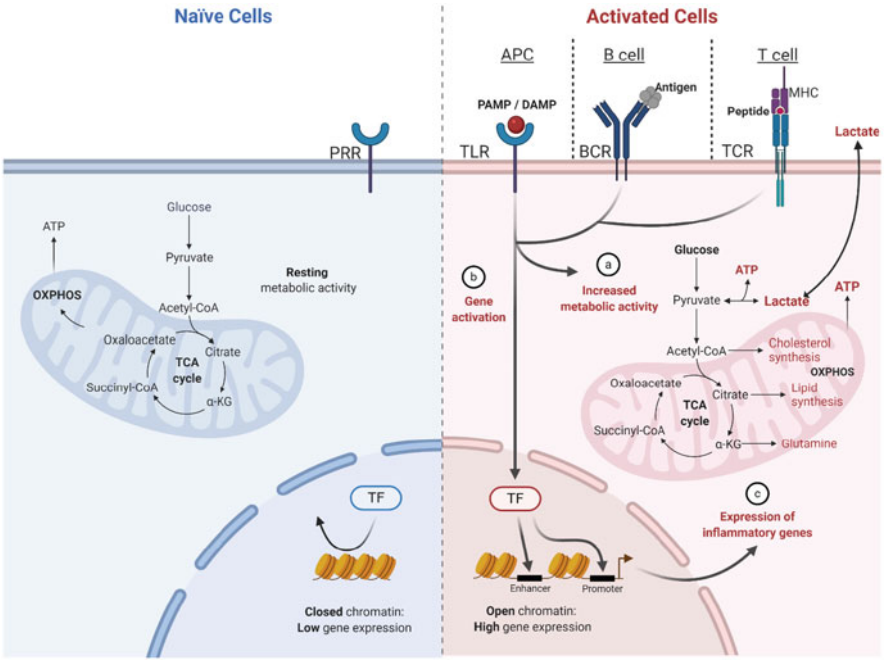

Fig. 7.3 Warburg effect: In the blue side, naïve or resting cells with resting metabolic activity producing ATP by OXPHOS after glycolysis fuels the TCA cycle. On the red side, immune cells become activated, APC, such as macrophages, by DAMPs or PAMPs recognition, B cell directly by the antigen and T cells by the antigenic peptide presented on the MHC-context by the APC. Either form of activation increases glucose utilization, and now cells also produce ATP directly by aerobic glycolysis, releasing lactate. *APC* antigen presenting cell, *ATP* adenosine triphosphate, *BCR* B cell receptor, *DAMP* damage-associated molecular patterns, *MHC* major histocompatibility complex, *OXPHOS* oxidative phosphorylation, *PAMP* pathogen-associated molecular patterns, *PPR* pattern recognition receptor, *TCA* tricarboxylic acid, *TCR* T cell receptor, *TF* transcription factor, *TLR* Toll-like receptor. (Created with BioRender.com)

the cell's bioenergetic capacity, allowing cytokines secretion, differentiation, and proliferation. However, the activation of polarization toward a regulatory cell, such as regulatory T cells or M2 macrophages, increases mitochondrial function by OXPHOS (Pålsson-McDermott and O'Neill 2020). Then, glucose metabolism in immune system cells changes over the duration of the immune response.

For instance, macrophages are the main population of immune system cells in the heart and are considered major players in cardiovascular homeostasis and disease (Lavine et al. 2018). In response to tissue injury, macrophages undergo M1 polarization. This pro-inflammatory phenotype produces TNF-α and relies on aerobic glycolysis to meet bioenergetic requirements, involving the downregulation of ETC genes. However, on subsequent days, a shift in tissue macrophages to the M2 phenotype promotes tissue reparation. This phenotype depends on OXPHOS by upregulating pyruvate metabolism and TCA cycle pathways (Rodríguez-Prados et al. 2010).

Cardiomyopathies can be triggered by either a direct insult to the heart, such as in acute myocardial infarction or myocarditis, or by indirect inflammatory processes that become chronic and systemic, such as in diabetic cardiomyopathy (Castillo et al. 2020). The trigger stimuli may also be sterile, associated with proper antigens, or pathogenic, associated with virus or bacteria. In this section, we will describe some of the previously described mechanisms in these types of cardiac disease.

7.1.9 Diabetic Cardiomyopathy

Diabetic cardiomyopathy is a sterile, chronic, and inflammatory process that becomes systemic and affects heart metabolism and function. Despite their hyperglycemic status, diabetes mellitus (DM) insulin-dependent patients exhibit decreased cardiac glucose uptake (Avogaro et al. 1990). The mechanism may be mediated by impaired GLUT4 signaling, as demonstrated in animal models (Camps et al. 1992), limiting the ability to produce energy by glucose oxidation and forcing cardiomyocytes to increasingly rely on FAO, disrupting the balance in the utilization of these metabolic pathways. Thus, mitochondrial dysfunction, elevated ROS production, and increased apoptosis are involved in diabetic cardiomyopathy. Under diabetic conditions, cardiomyocytes have a decreased capacity to store calcium in the SR due to decreased SERCA2a activity and increased membrane leakage (Zarain-Herzberg et al. 2014), promoting mitochondrial Ca^{2+} overload (Chaube and Werstuck 2016).

Furthermore, mitochondrial fragmentation mediated by fission proteins increases ROS and activates cell death (Yu et al. 2008). However, when hyperglycemia becomes chronic, associated fusion proteins and autophagy are also decreased (Makino et al. 2010). In response to all these changes, cardiac-immune cells become

activated, releasing cytokines and chemokines that promote the chemotaxis and activation of circulating immune system cells within the heart (Tan et al. 2019).

In addition to systemic metabolic changes, the chronic systemic inflammatory state also affects the heart. Systemic inflammation associated with DM patients makes circulating immune system cells more susceptible to activation (van Oostrom et al. 2004; Zhai et al. 2016). In this context, mtDNA, ROS, and cytokines released into the extracellular milieu become available to activate immune system cells and promote cardiomyocytes' detrimental pathways, even if they were not primarily produced in the heart. For instance, IL-1β increases the propensity for arrhythmia by increasing CaMKII oxidation phosphorylation and SR calcium leakage (Monnerat et al. 2016). Additionally, several studies of diabetic cardiomyopathy have described high levels of IL-1β are associated with cardiac mitochondrial dysfunction. These results indicate that mitochondrial failure observed in diabetic cardiomyopathy is due to lower mitochondrial content and decreased mitochondrial respiration complexes (Yurre et al. 2020). Based on this idea, a recent study of glucose-intolerant rats found mitochondrial dysfunction and proneness to mPTP opening. This susceptibility was mediated by the hyperacetylation of Cyp D and an increase in mitochondrial oxidative stress (Fernández-Sada et al. 2017). The same model identified a significant increase in serum pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6, and a nearly twofold incidence of ventricular fibrillation (Fernández-Sada et al. 2017).

7.1.10 Acute Myocardial Infarction

In contrast to diabetic cardiomyopathy, where the heart is exposed to a chronic stimulus, in acute myocardial infarction (AMI), cardiomyocytes are exposed to sudden and drastic metabolic changes, most notably a sharp decrease in the oxygen supply. Acute myocardial infarction is characterized by a strong inflammatory reaction in the necrotic myocardium, followed by tissue repair and fibrosis. Failure to keep inflammation in check results in inadequate tissue healing, adverse cardiac remodeling, and lower ejection fractions (Jia et al. 2019). Within the first day after AMI, significant macrophage recruitment occurs in the necrotic myocardium and non-infarcted myocardium as a remote ischemic event (Lee et al. 2012). Therefore, macrophages' metabolic activity may impact the healing of the necrotic myocardium and cardiac remodeling in the remaining viable myocardium. Local hypoxia promotes the expression of hypoxia-inducible factor 1 (HIF-1) in macrophages and a shift to glycolytic pro-inflammatory M1 macrophages (Mouton et al. 2018). When the oxygen supply is restored in a later phase, macrophages return to OXPHOS as their primary ATP source, changing their phenotype to M2 and thereby promoting inflammation resolution, fibrosis, and tissue healing (Mouton et al. 2018). However,

swelling, disruption of the cristae structure, and loss of density are observed in the cardiomyocyte's mitochondria, increasing fission (Tian et al. 2017). Fission augments mitochondrial permeability transition pore (mPTP) activity, which releases Cyt c and thereby promotes cell death (Ong et al. 2010).

7.1.11 Sepsis

Similarly, in sepsis, the classic activation of undifferentiated macrophages with LPS leads to M1 macrophage differentiation and is associated with metabolic reprogramming with lactate production (O'Neill and Pearce 2016). HIF-1 and mTORc1 mediate this metabolic shift (Sun et al. 2011). LPS-dependent HIF-1α activation is further stabilized by succinate accumulation (Tannahill et al. 2013) and pyruvate kinase M2 (PKM2), leading to increased glycolysis, inflammasome activation, and IL-1β expression (Palsson-McDermott et al. 2015). Both pharmacological inhibition and specific gene deletion in the macrophages of PKM2 reduce glycolysis and IL-1β production, contributing to decreased mortality in murine models of sepsis (Xie et al. 2016; Zhang et al. 2016).

The use of glycolysis as a direct source of ATP production allows the engagement of fatty acid synthesis, which may be used to synthesize pro-inflammatory mediators (O'Neill and Pearce 2016). The ETC however remains crucial during the early pro-inflammatory phase, as inhibition of complex II leads to reduced IL-1β serum levels, increased anti-inflammatory IL-10 levels, and a higher bacterial load (Garaude et al. 2016). Late sepsis is characterized by a shift towards M2 cells, which marks an immunosuppressed state (Watanabe et al. 2016). IL-10 promotes M2 differentiation by immunometabolic effects, suppressing glycolysis and stimulating OXPHOS. These actions are mediated by suppressing mTORc1, increasing mitophagy, and reducing ROS production (Ip et al. 2017). Instead of relying on glucose, M2 macrophages depend on FAO (Huang et al. 2014). The low glycolytic rate in M2 macrophages allows the glycolytic enzyme GAPDH to bind to TNF-α mRNA and suppress its translation by ribosomes (Millet et al. 2016). While M2 macrophages can oxidize glucose to drive fatty acid synthesis (Huang et al. 2016), M1 macrophages suffer from mitochondrial dysfunction. Therefore, it could be possible to reprogram M2 macrophages to an M1 phenotype, but the conversion from M1 to M2 would pose a more significant challenge (Van den Bossche et al. 2016).

7.1.12 Myocarditis

Myocarditis is characterized by a strong T CD4+ response that requires increased glycolysis. T cells from human patients with dilated cardiomyopathy (80% of whom had a previous diagnosis of myocarditis) were characterized by an increased

glycolytic rate, either during the basal condition or upon maximal metabolic stimulation, as assessed by the extracellular acidification rate (ECAR), without impairment in the oxygen consumption rate (OCR) (Wu et al. 2019b). The injection of exosomes from mice with experimental autoimmune myocarditis (EAM) into naïve mice induced cardiac damage associated with increased levels of Th1 and Th17 cells, as well as a reduction in T-reg cells. The predominance of Th1/Th17 cells was related to the increased glycolytic rate and lactate production. Further analysis identified the microRNA miR-142 as a key component in the ability of exosomes from EAM mice to induce disease. Inhibition of miR-142 blocked the increase in glycolysis and the proliferation of CD4+ T cells (Sun et al. 2020). Beyond the identification of miR-142 as a potential target in treating myocarditis, these findings highlight the relevance of glycolysis upregulation in T cell-mediated disease. During myocarditis, DRP1 is translated from the cytoplasm to mitochondria. Consequently, mitochondrial fission is stimulated (Lin et al. 2017) with the downregulation of OPA1-associated TNF-α and ROS in a TLR4-dependent activation, resulting in disorganized, fragmented mitochondria and damaged mitochondrial cristae in the cardiomyocytes of mice with dilated cardiomyopathy (Wu et al. 2018).

7.2 Conclusion and Prospects

As previously discussed, mitochondria are essential to maintaining cellular function by not only providing energy but also regulating the bioenergetic source, which dictates cellular fate. Thus, the regulation of mitochondrial biogenesis is critical for cell survival. In this chapter, we described how immune system cells contribute to this process by eliminating dysfunctional mitochondria released into the extracellular space, avoiding inflammation and allowing cardiomyocytes to meet their metabolic and mechanical demands (Nicolás-Ávila et al. 2020). Although we do not entirely understand the interplay between immune and cardiac cells, evidence has shown that imbalance among these cells has a detrimental effect on cardiac function and even proper heart development (Adamo et al. 2020; Hulsmans et al. 2017). However, when inflammation occurs, whether sterile or pathogenic, chronic or acute, immune system cells release soluble mediators that alter mitochondrial function in cardiomyocytes by altering calcium handling and favoring glycolysis (Palomer et al. 2009; Palsson-McDermott et al. 2015). This change in the bioenergetic source of cardiomyocytes promotes mitochondria fragmentation, as well as increases ROS production, protein oxidation, mPTP opening, and cell death (Boudina et al. 2007). However, the activation of cardiac-immune cells also promotes glycolysis, which facilitates the proliferation and production of cytokines (Tedesco et al. 2019), compromising the relationship between cardiac-immune cells and cardiomyocytes that exist in a steady state (Adamo et al. 2020; Hulsmans et al.

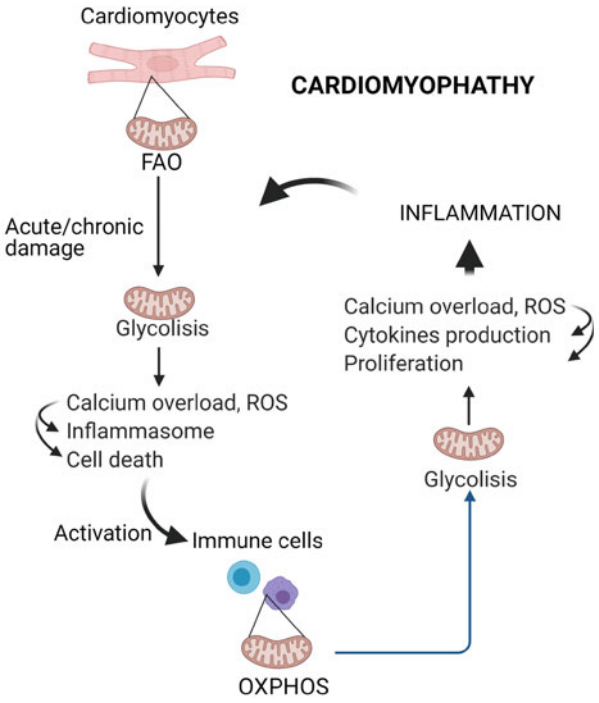

Fig. 7.4 Mitochondrial dysfunction as a trigger of inflammation in cardiomyopathies. When alterations in mitochondria promote bioenergetic changes that relay in glycolysis, it increases ROS production leading to inflammasome activation, cell death, and immune cell activation. To become activated, immune systems cells must change their bioenergetic source from oxidative to glycolytic, increasing the inflammatory milieu, and perpetuating cardiomyocyte dysfunction that leads to cardiomyopathies. (Created with BioRender.com)

2017). Therefore, mitochondrial dysfunction compromises immunometabolism and the cardiac-immune cell relationship, leading to cardiomyopathies (Fig. 7.4).

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Chapter 8
Cross-Talk Between Gut Microbiota and Immune Cells and Its Impact on Inflammatory Diseases

Eloisa Martins da Silva, Renan Willian Alves, Lorena Doretto-Silva, and Vinicius Andrade-Oliveira

Abstract The collection of microorganisms that inhabits the human gastrointestinal tract is usually called the gut microbiota. The gut microbiota is an important component for the development and function of the immune system, operating as a complex of microorganisms that produce substances that interact with the immune cells and respond to internal and external stimuli in the body. The gut microbiota has a different composition in healthy individuals and those who have a disease suggesting that it can be a disease marker. It is also suggested to educate the host immune response and keep homeostasis through sophisticated microbial cross-talk with the mucosal immune system that includes huge integrated signaling pathways and gene regulatory circuits. The imbalance of these delicate interactions between microbiota and immune cells is associated with the development not only of inflammatory diseases but of also several diseases such as neurological, autoimmune disease, and metabolic syndrome. Therefore, a better understanding becomes vital for comprehending the factors linked with the development and/or occurrence of these disorders. This chapter focuses on the current findings of the role of gut microbiota in the activation and function of immune cells and how this relation modulates homeostasis and health disorders associated with microbiome dysbiosis. Moreover, we point up new nanotechnology therapies concerning manipulating the microbiome for the management of microbiota alterations-related human disease, giving and discussing future challenges and the perspective for this emerging area.

Keywords Gut microbiota · Immune cell · Innate immune system · Adaptive immune system · Inflammatory disease

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8.1 Introduction

Our body is endowed with the ability to respond to external environmental signals while maintaining host's homeostasis. This task is particularly relevant in sites with closer contact with the external world. The gut microbiome is a reunion of all microorganisms inhabiting the intestine, such as fungi, viruses, and bacteria. The gut microbiota, in this case, is related to all the different bacteria that are somehow living in the intestine. It is appreciated now that gut microbiota composition as well as its product produced by different pathways are important to maintaining tissue homeostasis. Any perturbation either in its composition or in the substances released by the microbiota directly impacts gut-residing immune cells, triggers inflammation, and, thus, these microbiota factors have been associated with different inflammatory diseases, such as metabolic syndrome, autoimmune diseases, infectious diseases, and cancer. In this chapter, we summarize the findings reporting the cross-talk between gut microbiota and the activation and stimulation of immune cells in the gut and the potential association with inflammatory diseases. Moreover, we highlight the recent nanotechnology approaches focused on gut microbiota manipulation to treat intra- and extraintestinal diseases.

8.2 Gut Microbiome

The human gut microbiome, or gut microbiota, refers to the assembly of microorganisms including, archaea, fungi, bacteria, and even viruses that coexist in the digestive tract (Arumugam et al. 2011). A growing number of studies demonstrates that the microbiota is an important manager of physiological, metabolic, and protective functions (Schluter et al. 2020).

In healthy conditions, the gut microbiota composition is predominantly composed of six main phyla; Firmicutes (*Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus,* and *Ruminicoccus* genera) and Bacteroidetes (*Bacteroides* and *Prevotella* genera) making up about 90% of gut microbiota and the remainders are constituted by Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (Krajmalnik-Brown et al. 2012). Diversity in the microbiota composition seems to be important for health since recent studies have shown that a high diversity rate and high microbial gene richness indicate a healthy gut microbiota (Lloyd-Price et al. 2016). Moreover, by responding to several environmental stimuli such as diet, lifestyle, and medication, the gut microbiota composition is constantly challenged and susceptible to rapid change in its composition (Fig. 8.1) (David et al. 2014; Radjabzadeh et al. 2020) which may persist and impact over generations (Sonnenburg et al. 2016).

The type and quantities of food consumed affect the composition and function of gut microbiota, indicating that a diverse diet modulates the composition and functions of bacteria in the intestine (Maurice et al. 2013). For instance, recent studies

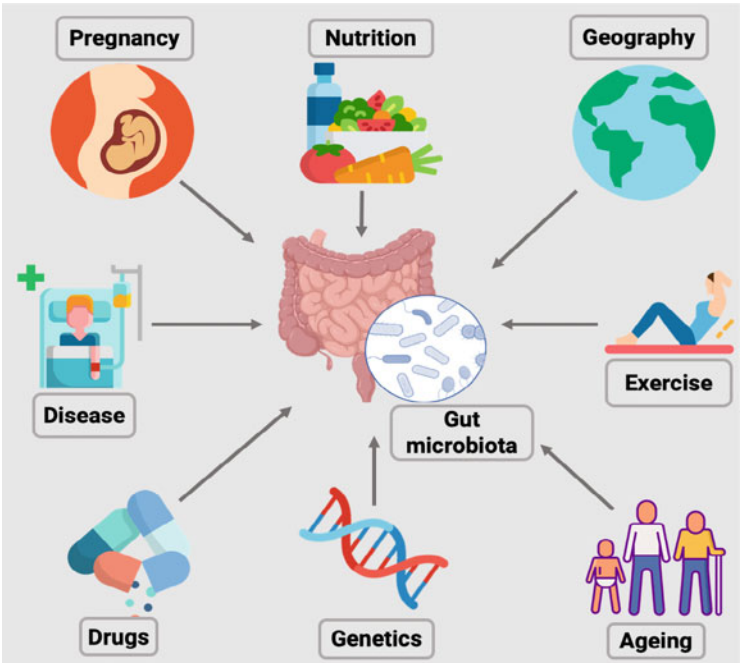

Fig. 8.1 Factors that influence the development, diversity, and composition of gut microbiota. The influences of exogenous (exercise, nutrition, geography, drugs therapy, disease) and endogenous (aging, genetics, pregnancy) factors upon gut microbiota. Arrows indicate interactions that might occur between the gut microbiota and a particular factor

have shown that a diet high in fat and sugar causes shifts in gut microbiota that may be correlated to the increase in diseases such as type 2 diabetes, obesity, and inflammatory bowel diseases (IBD) (Charbonneau et al. 2016; Zhu and Goodarzi 2020). As well with the increase in Western diet consumption, modern dietary pattern characterized by high intakes of ultra-processed foods, for instance pre-packaged food, can trigger major distresses of the gut microbiota, inducing an abnormal gut microbiota profile with consequences for well-being that are not always well comprehended (Wu et al. 2017). Therefore, the obvious interrelationship between the diet and how it modulates microbiota and affects the host still demand studies that in the future may lead to dietary therapeutic applications.

Besides, drug therapy such as antibiotics, painkillers, and diabetes medication also impacts gut microbiota. It is well established now that antibiotics use act not only on pathogenic bacteria that cause infections but also affect the resident commensals, diminishing levels of bacterial diversity and changing relative abundances, and in some cases, leading to gut microbiota dysbiosis-associated diseases (Elvers et al. 2020). For instance, metformin changes microbiota composition both in vitro and in vivo, increasing short-chain fatty acid (SCFA)-producing bacteria (Uranga et al. 2016). As well, proton pump inhibitor drugs increased the number of bacteria

typically found in the oral tract in the gut (Imhann et al. 2017). Besides that, the indiscriminate use of antibiotics can lead to antibiotic-resistant bacteria present in the intestinal lumen, and, thus, can enter into the bloodstream of vulnerable patients, resulting in disseminated infection. Antibiotic therapy results in changes in intestinal microbiota composition that diminishes the resistance to colonization by antibiotic-resistant bacteria in the intestinal lumen (Keith and Pamer 2019).

8.2.1 *Dialog Between Gut Microbiota and the Immune System in Homeostasis*

One of the known benefits of the gut microbiome to the host is the contribution to the development of the immune system (Fig. 8.2). This concept comes from germ-free (GF) studies that revealed that these animals do not present a fully functional immune system (Round and Mazmanian 2009). By being generated in a sterile environment, and, thus, having never ever been exposed to any bug, GF mice are a valuable tool to study the impact of gut microbiota in different host conditions. The immune system is also involved in shaping and preserving the microbiota community (Round and Mazmanian 2009). Antibiotic-treated mice that eliminate bacteria in

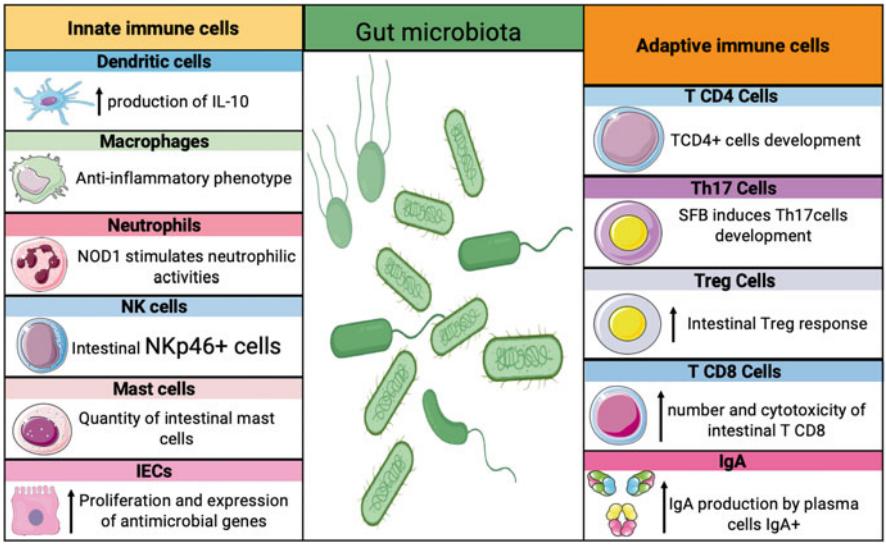

Fig. 8.2 Gut microbiota shape innate and adaptive immune cells. Some of the ways that the intestinal microbiome modulates host immunity are illustrated, including effects on innate and adaptive immune cells. *IECS* intestinal epithelial cells, *IgA* immunoglobulin A, *IL-10* interleukin 10, *NK cells* natural killer cells, *NKp46* natural cytotoxicity receptor, *NOD1* nucleotide-binding oligomerization domain containing 1, *SFB* segmented filamentous bacteria, *T reg cells* regulatory T cells

the gut exhibited an impaired immune response (Lazar et al. 2018). Importantly, dysbiosis in gut microbiota may trigger an exacerbated immune response, culminating with the development of allergies, inflammatory, or autoimmune diseases (Lazar et al. 2018).

8.2.1.1 Interactions Between the Innate Immune System and the Microbiota

Numerous reports provide direct evidence of relevant protagonist for the gut microbiota in regulating the development of macrophages, neutrophils, conventional natural killers (NK) cells (Khosravi et al. 2014; Luo et al. 2015; Smythies et al. 2005) and hematopoiesis (Shi et al. 2011). For instance, antibiotic therapy decreases bone marrow granulocyte-macrophage colony formation in animal models, and also GF animals present a deficiency in innate immune cells (Goris et al. 1985; Maslowski et al. 2009). Thus, the gut microbiota has been shown to modulate the innate immune response.

A critical feature for innate immune cells in the intestine, mainly antigen-presenting cells (APCs), is their capacity to protect the body against possible infections and, at the same time, to maintain a tolerogenic state to the normal gut microbiota (Imaoka et al. 1996). Indeed, the gut microbiota modulates the development of APCs. In GF animals were observed a reduction in the number of intestinal but not in the systemic DCs. Notably, monocolonization of GF mice with *Escherichia coli* led to the recruitment of DC to the intestine (Iwasaki and Kelsall 1999; Smythies et al. 2005). Furthermore, peritoneal macrophages of GF mice present an impairment of chemotaxis, phagocytosis, and microbial activities (Mikkelsen et al. 2004) besides displaying a lack of activation markers, such as MHC II (Wu and Wu 2012), once again demonstrating the impact of the gut microbiota on immune cell function at distant organs. Interestingly, CX_3CR1^{hi} mononuclear phagocytes, an intestinal cell population, are capable of trafficking antigen bacteria from the intestinal lumen to mesenteric lymph nodes modulating gut barrier repair and immune response, such as T cell responses and IgA production. Inhibition of this capture pathway through a MyD88-dependent mechanism restricts immune priming against intestinal antigens and can be a mechanism by which pathogens bacteria evade the immune response (Kim et al. 2018).

The gut microbiota also influences the regulation of neutrophils function. For instance, a gut microbiota bacteria cell wall component, peptidoglycan, is recognized by the cytosolic receptor-nucleotide oligomerization domain 1 (NOD1), and this interaction intensifies the killing capacity of bone marrow neutrophils (Clarke et al. 2010). Conversely, GF rats are neutropenic and have diminished nitric oxide and superoxide anion generation and decreased phagocytosis in blood neutrophils (Ohkubo et al. 1990). When those GF rats were transferred to a conventional environment it has not observed a recovery in superoxide anion production, indicating an impairment of neutrophils functions perhaps due to a lack of bacteria antigenic stimulation by gut microbiota.

NK cells, which are innate lymphoid subsets responsible for antitumor and antiviral responses, are also found in the gut mucosa. Recent studies identified two different subsets of intestinal NK cells that expressed the natural cytotoxicity receptor NKp46. One subset of gut NKp46+ cells is very similar to conventional NK cells while the other subset diverges from classical NK which displays restricted IFN-γ translation and lacks perforin production (Satoh-Takayama et al. 2008). In contrast, these NKp46+ cells expressed the nuclear hormone receptor retinoic acid receptor-related orphan receptor gamma t (RORγt) and interleukin-22 (IL-22) in the presence of *Citrobacter rodentium*. In GF mice there is an absence of IL-22-producing NKp46+ cells indicating that signals from the gut microbiota contribute to the differentiation of IL-22-producing NKp46+ cells (Sanos et al. 2009).

Also, mast cells in the lamina propria (LP) represents 2–3% of cells in the GI tract (Boeckxstaens 2018) and in the GF mice there is a reduction in the proportion of the intestinal mast cell and an increase in mast cells circulating in the blood compared to normal mice (Kunii et al. 2011). These results suggest that intestinal bacteria may regulate the migration of blood mast cells to the intestine. Mechanistically, this migration is promoted by the releasing of the CXCR2 ligands from intestinal epithelial cells (IECs) in a TLR-dependent fashion and MyD88$^{-/-}$ mice had lower densities of intestinal mast cells than raised mice (Kunii et al. 2011).

8.2.1.2 Interactions Between the Adaptive Immune System and the Microbiota

Gut microbiota also has an essential role in B cells. Generally, B cells are found in the gut-associated lymphoid tissues, like Peyer's patches and mesenteric lymph nodes, differentiated in immunoglobulin (Ig)A-secreting plasma cells (Hapfelmeier et al. 2010). IgA is an important form of secretory antibody found in gut mucosa composing a physical barrier and regulating the expression of genes by microbes in the intestine thus maintaining gut homeostasis (Peterson et al. 2007). Mechanistically, secretory IgA binds and obstructs the uptake of microbial antigens in the lumen, leads to bacterial disturbance and agglutination, and besides neutralizes pathogenic bacterial toxins (Cong et al. 2009). In addition, secretory IgA modulates and downregulates the expression of pro-inflammatory surface epitopes in the commensal bacterium *Bacteroides thetaiotaomicron* (Lindner et al. 2012). The absence of intestinal microbiota leads to a reduction in the number of plasma cells IgA+ in the gut, mostly in PP and in the LP, and a lower level of IgA (Wei et al. 2011). Likewise, deficient mice for Toll-like receptor 5 (TLR5), known to recognize bacterial flagellin, exhibit reduced levels of IgA leading to an abnormal expression of genes related to flagellum structure commensal bacteria (Cullender et al. 2013). In addition, people with IgA deficiency present a higher rate of bacteria with potentially inflammatory properties (Friman et al. 2002). Although gut IgA diversity is individual-specific, specific pathogen-free (SPF) mice have a much greater richness of gut IgA repertoires when compared to mono-colonized mice with bacteria or GF mice (Hapfelmeier et al. 2010). During mice aging, the IgA repertoire gets more

complex while new B cell clones are persistently generated against new possible microbiota antigens. Interestingly, B cell clones acquired during the beginning of life are also kept, revealing a long-lasting memory B cell response (Lindner et al. 2015).

T cells are a key component of the adaptive immune system involved in killing infected host cells, activation of other immune cells, production of cytokines, and regulation of the immune response. T cells that express CD4 can be found in every organ of the body, and include a high amount of the T cells of the LP of the intestine (Lindner et al. 2012). Once activated, naive T CD4+ cells can differentiate in four subsets: T helper 1 (Th1), Th2, Th17, or regulatory T cell (Treg), which differ by distinct expression of various transcription factors and cytokines. The correct adjustment and balance of T cell subtypes is an essential element in shaping homeostasis state. Unrestrained Th responses are related to pathological disorders, for example, the Th1 and Th17 responses have been related to autoimmune diseases whereas the Th2 response has been related to allergic response (Geuking et al. 2011).

Likewise, gut microbiota modulates TCD4+ cell development, inside the LP and in other tissues. Thereby, GF mice exhibit a noticeable reduction in the number of T CD4+ cells in LP (Macpherson et al. 2002). In addition, observed defects in spleens and mesenteric lymph nodes of GF animals, with the absence of lymphocyte zones in these animals (Mazmanian et al. 2005). Also, GF animals have been shown a Th1/Th2 imbalance, their immune response going toward a Th2 response (Mazmanian et al. 2005). Some recent findings revealed that some specific bacterial species play a key role in the development of distinct T cell subtypes. For example, *Bacteroides fragilis*, through its polysaccharide A (PSA), induces the proliferation of CD4+ T cells into Foxp3+ Treg cells directing to a proper Th1/Th2 balance in the host (Round and Mazmanian 2010).

In addition, gut microbiota induces the development of Th17 cells. For instance, segmented filamentous bacteria (SFB) were revealed to be strong inducers of Th17 in the LP. Contrary to that, in GF mice the number of Th17 cells is reduced in the LP in their gut (Ivanov et al. 2009). Recent reports have shown that the physical interaction of SFB with intestinal epithelial cells (IECs) and Th17 that express T cell receptors (TCRs) specific for adhesive forms of these bacteria is essential for Th17 differentiation (Atarashi et al. 2015), postulating that SFB colonization must trigger distinctive signaling pathways in the intestine to induce Th17 response.

Intestinal Tregs are essential for the preservation of the immune tolerance to dietary antigens and the gut microbiota and the suppression of tissue damage suffered by an immune response against pathogenic bacteria. It was demonstrated that the number of peripheral Treg is diminished in GF mice (Bilate and Lafaille 2012). Some endogenous bacteria, such as Clostridium (cluster IV, XIVa, and XVIII), and bacterial products (PSA of *B. fragilis*) or SCFA can induce functional colonic Treg in LP (Atarashi et al. 2011) and modulate the pathogenesis of inflammatory diseases (Atarashi et al. 2013). Indeed, PSA of *B. fragilis* can interact with TLR2 on Treg cells and consequently suppress Th17 response (Round et al. 2011). In addition, DNA from gut microbiota activates TLR9 signaling and maintains immunity by controlling Treg cell conversion in the LP (Hall et al. 2008). Furthermore, colonic Tregs induced by microbiota colonization express low levels of

Helios, a key transcription related to thymus-derived Treg (Yang et al. 2016) indicating that these cells are a consequence of induction of peripheral Treg not thymic Treg cells.

CD8+ T cells are cytotoxic cells able to kill infected cells, as well as cancer cells (van der Leun et al. 2020) and intestinal T CD8+ are found, mainly, in the intraepithelial layer of the gut (Imaoka et al. 1996). GF mice exhibit a reduction in the number and cytotoxicity of intestinal CD8+ T cells, suggesting that microbiota provide essential signals required for the maintenance of CD8+ T cell population (Wu and Wu 2012). The gut microbiota takes part in shaping CD8+ T cells perhaps due to the modulation of other peripheral immune cells, such as invariant natural killer T cells, plasmacytoid DCs, and marginal zone B cells (Wei et al. 2010). Interestingly, during the dysregulation of the gut barrier type I IFN signaling is needed leading to CD8 T cell accumulation and effector functions within the small intestine. In fact, blocking type I IFN receptor signaling or depleting CD8 T cell prevented barrier leakage caused by a viral infection, indicating that CD8 T cells response can be a crucial factor of intestinal leakage in the pathogenesis of chronic infections (Labarta-Bajo et al. 2020). Besides, recent findings indicate a novel role for butyrate on CD8+ T cells modulating the gene expression of effector molecules in CD8+ T lymphocytes (Luu et al. 2018).

8.3 Dysregulation of Gut Microbiota and the Association with Inflammation-Mediated Disease

The microbiota can modulate several cells of the immune system, through signaling molecules, as well as by its metabolites, such as SCFAs. Immune cells are responsible for the secretion of cytokines, which are signaling molecules that can, for example, recruit other cell types for the inflammatory site (O'Shea and Murray 2008). One of the ways in which the cross-talk between microbiota and the immune system occurs is through cytokines, such as IL-22 which is produced mainly by CD4+ Th17 T cells (Leung et al. 2014) and innate lymphoid cells (ILCs) (Zeng et al. 2019) and in GF mice, the production of IL-22 is impaired (Sanos et al. 2009; Satoh-Takayama et al. 2008). A recent study demonstrated that the microbiota regulates the production of IL-22 through SCFAs in T cells and ILCs inhibiting the inhibition of histone deacetylase (HDAC) and GPR41 and promoting the expression of aryl hydrocarbon receptor (AhR) and hypoxia-inducible factor (HIF-1α) (Yang et al. 2020). In addition, SCFAs propionate and butyrate facilitate the generation of Foxp3+ Tregs (Arpaia et al. 2013; Smith et al. 2013).

Thus, demonstrating that an intestinal dysbiosis influences the population of immune cells, being able to trigger inflammatory, infectious diseases, dysplasia, and others related to dysregulation of the immune system (Fig. 8.3).

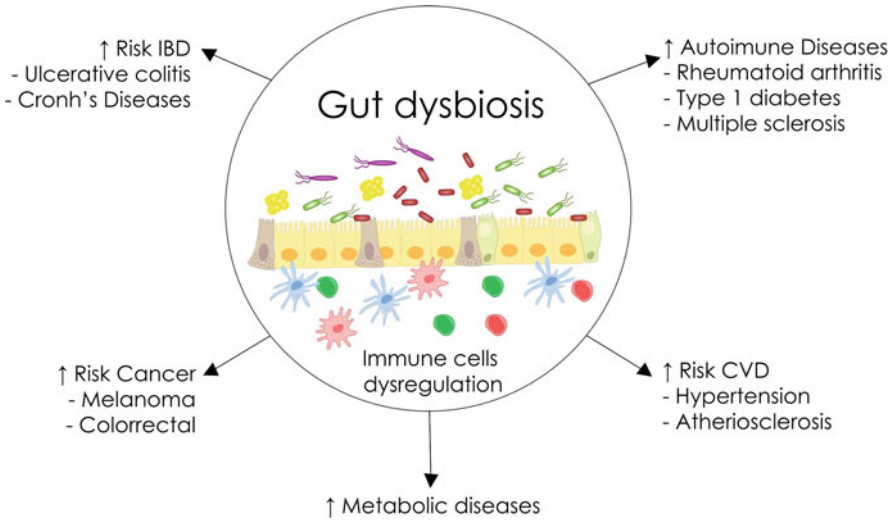

Fig. 8.3 Gut dysbiosis and diseases. Intestinal dysbiosis changes the immune cells and increases the risk of some diseases, such as rheumatoid arthritis, type 1 diabetes, multiple sclerosis, metabolic diseases, hypertension, atherosclerosis, melanoma, colorectal cancer, ulcerative colitis, and Crohn's disease. *CVD* cardiovascular diseases, *IBD* inflammatory bowel diseases

8.3.1 Inflammatory Bowel Disease (IBD)

IBD comprises a range of diseases including Crohn's disease (CD) and ulcerative colitis (UC) affecting the GI tract (Khor et al. 2011). Several strong evidence reveals that alterations in gut microbiota influence the pathogenesis of IBD (Comito et al. 2014). Indeed, most IBD patients show significant changes in the gut microbiota when compared with normal adults (Manichanh et al. 2006). Antibiotic therapy ameliorates the clinical condition both in patients with IBD and animal IBD models (Khan et al. 2011). Using IBD animal models it was demonstrated that GF rederivation leads to a milder form of the disease in the IL-2 knockout (KO) IBD model or protects against disease (in the T cell receptor α/β KO or IL-10 KO IBD models), suggesting that habitual gut microbiota modulates the inflammatory state of IBD (Schultz et al. 1999; Sellon et al. 1998). Lately, it has been observed a reduction in the relative abundance of Firmicutes and Bacteroides species and an accentuated growth of proteobacteria in feces/mucosa-associated microbiota of IBD patients (Frank et al. 2007).

CD patients present discontinuous lesions that affect any parts of the GI and include chronic and relapsing transmural inflammation resulting in severe abdominal pain, diarrhea, obstruction, and/or perianal lesion. Currently, CD pathogenesis involves the complex balance between genetic, microbiological, immunological, and environmental factors (Neuman and Nanau 2012). In fact, there is evidence that changes in the microbiome are involved with the inflammatory response of the disease (Baker et al. 2009). The intestinal commensal populations are distinctive in

CD patients compared to healthy individuals. Especially, some protective microbes and normal anaerobic bacteria, such as *Bacteroides* sp., *Eubacterium* sp., and *Lactobacillus* sp., are remarkably reduced in active CD patients (Alhagamhmad et al. 2016). Also, adherent–invasive *E. coli* has been related to a higher prevalence of CD, due to over colonization of epithelial cells, mostly in ileal regions (Palmela et al. 2018). Interestingly, a study that compared the cytokines production by intestinal intraepithelial lymphocytes (IELs) in health and CD patients, found that IELs from subjects with CD secreted significantly larger amounts of TNF-α, IFN-γ, and IL-17A (Regner et al. 2018).

UC is triggered by chronic inflammation in the colon, which has been increasing in the number of patients in recent years. A variety of environmental and genetic factors are associated with the development of UC (Feuerstein et al. 2019). Also, the intestinal microbiota in balance with the immune system is essential for maintaining the epithelial barrier and homeostasis. Therefore, an imbalance between the microbiota and the immune system can trigger excessive intestinal inflammation, consequently leading to the development of a UC (Shen et al. 2018). In patients with UC, the diversity and quality of the microbial population are altered, with an increase in *Proteobacteria*, mainly *E. coli*, and variable changes in *Bacteroidete*s (Hansen et al. 2010). In experimental models, these changes vary according to the model used, in general, the variations are similar to the human (Lupp et al. 2007). Another interesting factor that is changed in UC is the immune cell profile, a study was done on CD45+ blood cells of patients with UC showed differences in the expression of Treg, T cell, and CD8+ tissue-resident memory cells (Boland et al. 2020).

The modulation of the microbiota through antibiotic treatment for UC alters the microbial composition in different forms varying according to the experimental model, being an alternative therapy that needs to be better explored (Rooks et al. 2014). Genetic ablation of genes responsible for an encoded protein that recognizes bacteria components is also important to maintain microbiota composition. Animals with innate immunodeficiency in the TLR5 that promotes intestinal inflammation are likely to develop colitis (Vijay-Kumar et al. 2007). Likewise, NLRP6 inflammasome KO mice are more susceptible to develop severe colitis (Elinav et al. 2011). Thus, the gut microbiota seems to be a determinant for this development, in which there is an increase in *Proteobacteria* and consequently a greater susceptibility to infections by *E. coli* (Carvalho et al. 2012). Another experimental model of UC used is through IL-10 deficient mice that develop inflammation in the colon spontaneously, with a CD4+ Th1 cell response and excessive production of pro-inflammatory cytokines IL-12, IL-17, and IFN-γ (Keubler et al. 2015). According to Maharshak et al., the IL-10−/− germ-free animal colonized with microbiota from SPF loses its wealth 4 weeks after colonization with an increase in *Proteobacteria* and *Tenericutes* (Maharshak et al. 2013), strengthening the idea of the role of immune cells in controlling gut microbiota composition.

The IL-22 signaling molecule plays an important role in maintaining intestinal homeostasis (Shohan et al. 2020) and can be regulated by microbiota through metabolites derived from microbial tryptophan such as kynurenine (Kyn) and indole-3-acetic acid (IAA) that are Ahr ligands (Zelante et al. 2013). The signaling

of IL-22 and Ahr ligands has been associated with the expression of the CARD9 gene (Lamas et al. 2016) which is related to susceptibility to IBD (Lanternier et al. 2015). CARD9 deficient mice are more susceptible to colitis, by decreasing antimicrobial peptides REGIII-γ and REGIII-β, change in fungal and bacterial microbiota (Lamas et al. 2016). Fecal microbial transfer experiments from CARD9−/− mice into GF are sufficient to increase susceptibility to DSS-induced colitis, potentially by a deficiency in the induction of IL-22 by T cells and ILCs and decreasing AhR ligands (Lamas et al. 2016). AhR signaling is an essential component of intestinal immune response and the microbiota is one of the main ones responsible to produce Ahr ligands (Lamas et al. 2018). Thus, AhR signaling is one of the pathways whereby the gut microbiota and immune cells communicate (Modoux et al. 2021). Animals treated with indole-3-aldehyde (IAId), a tryptophan metabolite synthesized primarily by *Lactobacilli*, promote IL-22 production and reduce DSS-induced colitis (Zelante et al. 2013). In addition, according to Qiu et al. Rorc GFP+ AhR−/−, SPF and aged mice have increased Th17 cells producing IFN-γ and IL-17 and the development of chronic spontaneous colitis (Qiu et al. 2013). In Rag−/−AhR−/− animals the inflammation worsened, and it was later reversed with a decrease in the inflammatory infiltrate in the colon, through treatment with antibiotics (Qiu et al. 2013). Also, animals treated with indole-3-carbinol (I3C) AhR ligand of plant origin have their microbiota altered, and in the colitis model have an attenuation of inflammation with lower intestinal permeability, increasing the production of IL-22 by ILC3, with an increase in bacteria producing butyrate and consequently an increase in regulatory T cells (Busbee et al. 2020). Innate immunity cells participate in the colitis development process (Geremia et al. 2014); however, studies focusing on microbial AhR ligands and their association with innate immune system cells in UC models are scarce, and it is important to explore the role of Ahr in innate cells in colitis. In fact, in vitro experiments with LPS-stimulated macrophages showed the potential anti-inflammatory function of indole-3-acetate (I3A), by decreasing cytokines at mRNA levels of the IL-1β, MCP-1 e TNF-α (Krishnan et al. 2018). In an in vivo UC model, treatment with the microbial metabolite ligand of AhR, Indole-3-pyruvic acid (IPA), had an anti-inflammatory effect with increased DCs CD103+ CD11b− (Aoki et al. 2018). Together these findings indicate an essential function of the microbiota in the development of UC through AhR ligands signaling in AhR-expressing immune cells.

Another group of microbial metabolites important in the modulation of immune cells and inflammatory diseases is SCFA. The three most studied SCFA are the butyrate, produced mainly by phylum *Firmicutes*, and the acetate and the propionate, produced by the phylum Bacteroidetes (Bilotta and Cong 2019). SCFA receptors are G-protein coupled receptors or GPCR or GPR expressed in several immune and intestinal epithelial cells and the stimulation of GPRs by SCFA in these cells contributes to the maintenance of gut homeostasis (Bilotta and Cong 2019). The SCFAs themselves induce Treg in the colon GPR43-dependent manner (Smith et al. 2013), and butyrate and propionate, but not acetate, are involved in the increase in peripheral Treg, through the inhibition of HDAC (Arpaia et al. 2013; Furusawa et al. 2013). Corroborating with data showing that in an IL-10−/− colitis model, mice

treated with antibiotics have a reduction in CD4+ Treg and Th1 cells, a decrease in bacteria belonging to the Firmicutes and Bacteroidetes groups, and a reduction in the total levels of SCFA (Shen et al. 2019), known Treg cell regulators (Arpaia et al. 2013). In addition, animals deficient in HIF-1α in epithelial cells with DSS colitis induction reduce the amount of butyrate-producing bacteria and after treatment with sodium butyrate there was a decrease in F4/80+ cells, cytokines IL-6, TNF-α, and IL-1β (Zhou et al. 2020). Also, acetate is another SCFA with anti-inflammatory potential, reducing migration (Kamp et al. 2016) and infiltration/activation of neutrophils in UC, GPR43-dependent (Maslowski et al. 2009), indicating that bacterial metabolites SCFAs have an anti-inflammatory effect.

Intestinal dysbiosis and alteration of the immune system are present in patients and experimental models of UC, requiring a balance between these factors for a better prognosis of the disease. Indicating an immunomodulatory capacity of microbiota and its metabolites, which are possible therapeutic targets for UC.

8.3.2 Cancer

One of the triggers to the development of cancers is due to an accumulation of genetic changes within the cell, which can be a consequence of genetic predisposition, environmental factors, and individual habits such as smoking and food, among others (Lewandowska et al. 2019). There is a necessary immune balance for cell types to behave in an antitumor manner, as the abundance of both Treg cells and effector T cells (CD8+) can cause pro- and antitumor effects, respectively (Farhood et al. 2019).

The intestinal microbiota is an important factor when we talk about the development of cancer, in which the change in its composition can impact patient prognosis (Gopalakrishnan et al. 2018a). This is exemplified by a study demonstrating that GF animals with colorectal cancer (CRC) induction by azoxymethane (AOM), when conventionalized with feces from patients with CRC, has an increase in Th17 and Th1 cells, directly involved in CRC development process, in addition to positive regulation of proliferation, metastasis and angiogenesis genes (Wong et al. 2017). Consequently, these animals develop high-grade dysplasia when compared to controls (Wong et al. 2017). In a CRC model using AOM+ sodium dextran sulfate (DSS), the bacterial composition proved to be essential, in which the colonization of GF animals with *Bacteroides fragilis* decreased the infiltration of granulocytes and the formation of tumors (Lee et al. 2019), showing the potential of modulation of the microbiota in the development of the CRC.

The impact of the microbiota during treatment with checkpoint inhibitors, which target immunomodulatory T cell molecules, has been gaining emphasis in recent years. Oral administration of the bacterial genus *Bifidobacterium* improved antitumor immunity, with the recruitment of CD8+ T cells for TME and the combination with anti-PDL1 checkpoint inhibitor led to tumor elimination in an experimental model of melanoma (Sivan et al. 2015). According to Routy et al.

animals and patients treated with antibiotics had a response to anti-PDL1 therapy compromised (Routy et al. 2018). In addition, patients who received anti-PDL1 therapy and with a high abundance of *Faecalibacterium*, *Clostridiales*, and *Ruminococcaceae* had high frequencies of CD8+ and TCD4+ T cells as well as a better response to therapy, while the abundance of Bacteroidales is related to increased Tregs and a lower response to therapy (Chaput et al. 2017; Gopalakrishnan et al. 2018a). Another checkpoint inhibitor used in cancer therapies is anti-CTLA-4, and it has been shown that microbiota modulation is essential for its efficiency and affects Th1-type responses in melanoma and CRC models (Vetizou et al. 2015). Taken together, the efficacy of checkpoint blockade treatment should consider gut microbiota composition to increase the extraordinary potential of this therapy to treat cancer patients.

8.3.3 Hypertension and Cardiovascular Diseases

In addition to influencing diseases related to the gastrointestinal system, a change in the microbiota can favor cardiovascular diseases (CVD), through the modulation of the immune system by the intestinal microbiota causing systemic immune effects (Kitai and Tang 2018). Among the CVD that are altered or alter the intestinal microbiota are hypertension, heart failure, and atherosclerosis (Roth et al. 2017), and research with a focus on alternative therapies to combat these diseases is extremely necessary.

Blood pressure (BP) is regulated by several factors, such as genes, environment, hormones, and it is currently suggested that the intestinal microbiota is also a regulatory factor (Kitai and Tang 2018). It is observed in animals with hypertension, a reduction in the production of SCFAs and changes in the composition of the microbiota, with an increase in *Firmicutes* and a decrease in *Bacteroidetes*, as well as in patients who have a lower microbiome richness and diversity (Yang et al. 2015). In addition, SCFAs receptors that are present in various cardiac tissues can modulate blood pressure through SCFAs signaling (Pluznick 2014). Propionate produced by the intestinal microbiota, the intravenous infusion of propionate resulted in a drop in BP; however, GPR41-deficient mice this effect is attenuated, suggesting that Gpr41 mediates the hypotensive effects of propionate (Pluznick et al. 2013). Also, to the direct influence of the microbiota on hypertension, it can act by modulating the immune system, Toral et al. performed fecal transplantation of hypertensive in normotensive animals and observed an increase in BP and inflammatory markers in the aortic infiltrate, TNF-α, IFN-γ, Rorγ, and IL-17 as well as a decrease in FoxP3 and IL-10, after administration of neutralizing IL-17 antibody there was a decrease in BP and pro-inflammatory markers (Toral et al. 2019).

Arteriosclerosis is considered another chronic inflammatory disease, involving the entire immune system modulating the onset and progression of lesions, being characterized mainly by the accumulation of fat in the arterial walls (Gui et al. 2012). Intestinal dysbiosis can also contribute to the development of atherosclerosis through

systemic inflammation (Duttaroy 2021) indicating an immunomodulatory role of the microbiota through its bacterial products. A study using GF animals showed a decrease in arteriosclerosis, as well as a decrease in plasma levels of LPS and inflammatory markers, IL-6, IL-1β, and TNF-α (Kasahara et al. 2017). In addition, trimethylamine and trimethylamine N-oxide (TMAO) are metabolites derived from the intestinal microbiota and oral supplementation with TMAO has a pro-atherogenic effect linked to cardiovascular risks, dependent on the microbiota (Koeth et al. 2013) with enrichment of specific microbial group, increase in *Coriobacteriaceae*, *Erysipelotrichaceae,* and *Allabaculum,* and a decrease in *Candidatus arthromitus*, *Lachnospiraceae*, *Oscillospira,* and *Ruminococcus* (Zhu et al. 2016). The effects of TMAO in atherosclerosis have recently been associated, in endothelial cells, with increased oxidative stress, the activation of NLRP3 with the release of inflammatory cytokines, IL1β and IL18, an increase in protein kinase C (PKC) activation and Nuclear factor κB (NF-κB) phosphorylation, consequently inducing positive regulation of Vascular cell adhesion protein 1 (VCAM-1) and an increase in monocyte adhesion (Ma et al. 2017; Sun et al. 2016).

The balance between the immune system and microbiota is altered in experimental models and patients with CVD and its modulation has been shown to be effective in reducing pathology, highlighting the therapeutic potential targeting the immunity-microbiota axis in CVD (Adnan et al. 2017).

8.3.4 Autoimmune Diseases

Changes in gut microbial populations have been connected to autoimmune diseases modulating the immune sensing that recognizes between self and nonself, impacting autoimmune diseases (Leipe et al. 2010).

Rheumatoid arthritis (RA) is also an inflammatory and systemic disease that causes the destruction of bone and cartilage and in consequence, evolving into functional disability. Recent studies demonstrated that RA is related to the Th1- and Th17-mediated inflammatory response and it seems that the disproportion between Th17 and Tregs has been linked to the etiology and progression of RA (Xu et al. 2019). Using experimental collagen-induced arthritis (CIA) animals Mui et al. identified the influence of gut microbiome on arthritis susceptibility (Wu et al. 2010). There were observed changes in the gut microbiota composition between CIA-susceptible and CIA-resistant or healthy mice. During RA, in CIA-susceptible, the relative abundance of families Bacteroidaceae, Lachnospiraceae overlap significantly the *Lactobacillus* genus found before arthritis onset (Wu et al. 2010). Using the model of RA study (IL-1 receptor antagonist deficient (IL-1Rn−/−) it was observed that the gut microbiota was involved in RA development in mice while GF IL-1Rn−/− mice did not exhibit the disease (Abdollahi-Roodsaz et al. 2008). Furthermore, monocolonizated with *Lactobacillus bifidus* of the GF IL-1Rn−/− mice restored the disease. Besides, the decrease of Th17 and Tregs cells observed in the lymph nodes and spleens were correlated with disease intensification in non-GF

TLR2−/− IL-1Rn−/− mice and disease improvement in non-GF TLR4−/− IL-1Rn−/− mice (Abdollahi-Roodsaz et al. 2008). In the K/BxN mouse, a reduction in RA symptoms in GF-K/Bx was observed (Wu et al. 2010). Mechanistically, the authors demonstrated that the gut microbiota-induced LP small intestine Th17 cell migrated into the peripheral lymphoid tissue, then, stimulated B cells differentiation and autoantibody production in an IL-17-dependent fashion that can lead to the progression of the disease (Wu et al. 2010).

In humans, RA patients show less diverse gut microbiota composition compared with controls, indicating a relationship between the RA duration and autoantibody levels in these patients (Picchianti-Diamanti et al. 2018). A taxon-level analysis revealed that control samples have higher *Actinobacteria* levels when compared to RA patients (Chen et al. 2016). A study using forest algorithms indicated that *Collinsella*, *Eggerthella*, and *Faecalibacterium* are correlated to RA. The abundance of *Collinsella* was related to increased levels of alpha-aminoadipic acid and asparagine as long as the production of IL-17A, and *Collinsella* is involved in the process of disrupting gut permeability and RA severity in the experimental arthritis model (Wang and Xu 2019).

Type 1 diabetes (T1D) is one more disorder framed in autoimmune disease in which β cells are abolished by T cell-mediated response and in consequence very little or no insulin is produced by the islets of Langerhans in the pancreas (Katsarou et al. 2017). The observation that intestinal Tregs were reduced in T1D patients, indicated the likely implication of the gut microbiota in T1D pathogenesis (Badami et al. 2011). Using non-obese diabetic (NOD) mice have been observed that diabetic incidence is markedly higher in GF NOD mice when compared with their SPF controls (Alam et al. 2011). In harmony with these findings, SPF MyD88−/− NOD, lacking MyD88 protein, did not develop T1D, whereas GF MyD88−/− NOD mice readily developed TD1. Interestingly, colonization of GF MyD88-negative NOD mice with a microbial consortium, likely to the phyla normally present in the human gut attenuates T1D, the same result was observed when GF NOD mice were exposed to the microbiota of SPF MyD88-negative NOD donors ameliorating T1D in the GF mice (Wen et al. 2008).

Multiple sclerosis (MS) is an autoimmune disease featured by an abnormal immune system response directed against the central nervous system, leading to demyelination of this system (Oh et al. 2018). Since there is no specific murine model for human MS, the researchers have focused on the use of experimental autoimmune encephalomyelitis (EAE), the most common and accepted experimental model for the human inflammatory demyelinating disease (Constantinescu et al. 2011). Using GF induced for EAE models, Lee et al. noted an attenuation of disease phenotype in these mice, associated with reduced production of pro-inflammatory cytokines, such as IL-17 (Lee et al. 2011). In addition, intestinal colonization with SFB, a known stimulator of IL-17 production in the gut, induces IL-17A-producing CD4(+) T cells (Th17) in the CNS and restoring the phenotype of EAE and worsening the progression of the disease (Lee et al. 2011), indicating a role for SFB in EAE pathogenesis. On the other hand, some commensals may be beneficial in lessening EAE development. The colonization with human commensal *B. fragilis*

can attenuate disease, due to the expression of PSA enhancing the number of Treg cells and CD5+ B cells in the animals treated with *B. fragilis* (Ochoa-Reparaz et al. 2010). Interestingly, the treatment with Lactobacillus strains (*L. paracasei* DSM 13434, *L. plantarum* DSM 15312, and DSM 15313) lead to suppression and reversion suppressed of the clinical symptoms of EAE, and these therapeutic effect was due to IL-10-producing Tregs stimulated by Lactobacillus presence (Lavasani et al. 2010).

8.4 The Gut Microbiota Manipulation as a Treatment in Diseases

As mentioned on the topics above, the gut microbiota composition and/or molecules produced by the microbiota are important to prevent or attenuated intestinal and extraintestinal diseases as well as to maintain the health states of the body thus demonstrating that regulation of the microbiota composition and microbiota-producing product are extremely regulated where different pathways, cell, and molecules take place. In this sense, targeting the microbiome as a strategy to prevent or treat disease may be an interesting approach. A review discusses the importance of alteration in the gut microbiota in pancreatic ductal adenocarcinoma patients, point out that these patients had a decreased microbial diversity, decreased beneficial bacteria, and abundance of potential pathogens (Yu et al. 2021).

There is a strategy already approved by the Food and Drug Administration (FDA) in the USA as a treatment for severe, recurrent *Clostridium difficile* infections (Napolitano and Covasa 2020) called fecal microbiota transplantation (FMT). This technique appears to be a potential therapy that can modify the human gut microbiome, once the transfer of living microorganisms from a donor to an afflicted person, presents an improvement of the response of the body related to not only *Clostridium difficile* infections (Napolitano and Covasa 2020) but also obesity and metabolic syndrome (Marotz and Zarrinpar 2016) cancer (Gopalakrishnan et al. 2018b) and liver disease as hepatic encephalopathy (Hassouneh and Bajaj 2021).

In the field of innovation, nanotechnology brings nanoparticles (NPs) and their application in drugs, and medication delivery (Wang et al. 2021) facilitating the treatment of several diseases.

One of the options for the treatment of IBD is Cyclosporine A (CYA) which some patients present resistance to the steroid and adverse effects like toxicity and infections after the treatment (Kornbluth 1999). Targeted delivery of CYA by polymeric nanoparticles shows to improve the therapy of IBD on DSS-induced acute colitis in mice model and shows that the effect of the drug is focused on the intestinal mucosa and not in the systemic absorption by oral administration (Melero et al. 2017). This work also demonstrated that nanoparticles and microparticles develop the same effect on IBD.

Not only CYA polymeric nanoparticles for IBD but a lot of other options of nanoparticle-based drugs have been studied as treatment strategies. Deliverable targets include intestinal epithelium, mucus, immune cells, LP, and the extracellular matrix are targets for NP and routes of NP administration such as oral, injection, and rectal administration (Yang and Merlin 2019). The oral administration of platinum nanoparticles (PtNPs) shows to improve and attenuate colonic and systemic inflammation in DSS-induced colitis mice model, protect their gut barrier from acute colitis, and in macrophage RAW264.7 cell murine culture, PtNPs attenuate inflammation from LPS, suppression Toll-like receptor 4/Nf-κB signaling, although this administration results in dysbiosis (Zhu et al. 2019). Gold nanoparticles (AuNPs) display a potential therapeutic action, and like PtNPs, AuNPs protect against colitis, suppressing TLR4, impacting negatively in mice microbiota, and inducing gut dysbiosis (Zhu et al. 2018).

Another option is the hyaluronic acid-bilirubin nanomedicine which accumulates in the colonic epithelium, restoring the epithelium barriers in an experimental model of acute colitis, which can modulate gut microbiota, associate with pro-inflammatory macrophages, regulating innate immune response (Lee et al. 2020).

Nanobiotechnology proved to be a promising action in the treatment of diseases, mainly for IBD. For a more efficient action of these NPs, it is necessary to interact with the immune system so that both help each other and manage to fight the disease.

8.5 Conclusion and Prospects

As discussed in this chapter, the gut microbiota is capable of modulating, directly or indirectly, the aspects and functions of innate and adaptive immunity directly impacting inflammatory, autoimmune, metabolic, and neurologic diseases. Although promising, there is still a lot to understand about how gut bacteria mechanistically affect local and systemic immunity. Beyond the genetic and immunological factors, environmental factors are a fundamental key to shape the gut microbiota. These features should be considered with precaution as inapt practices such as indiscriminate use of antibiotics is related to higher risks of inflammatory diseases mediated by the microbiota immunomodulation. The impact of intestinal commensals on health state and disease due to the regulation of immune system function has become a new field of science with potential clinical importance for disease therapy. For instance, nanotechnology approaches have emerged as a powerful strategy for manipulating gut microbiota to prevent intestinal and extraintestinal diseases, such as IBD, obesity, and metabolic syndromes. Such therapies when considered for human application must take into consideration the wide variation in gut microbiome diversity and innate immune responses that occur between each individual.

A better understanding of the mutual interactions of the microbiota and host immune system, as well as gut microbiota role, modulate the immune system will contribute to many strategies for manipulating the intestinal microbiome for therapeutic benefit, especially in inflammatory and autoimmune diseases.

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Chapter 9
In Vitro Models and Molecular Markers for Assessing Nano-Based Systems Inflammatory Potential

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Abstract When nanotechnology proved to be a promising science with applications in several areas, there was a need for studies regarding the toxicity of nanomaterials. In vitro evaluation is a tool of potential interest among different study models since it can provide early signals of the possible behavior of the nanomaterial quickly and often accurately. In vitro studies allow the evaluation of both toxicological potential and nanomaterial activity. For confidence in these tests to reduce experiments using animal models, evaluative markers began to be studied and refined, along with different cell culture models, to ensure compatibility with in vivo exposure. Thus, two strands should be developed and used together for the application of in vitro models. One of them regards cell seeding and exposure techniques, and the other is the study of valuable markers to detect possible cellular alterations and their consequences. Although there are well-established techniques to evaluate cell viability and genotoxicity, these are not always appropriate for assessing cells exposed to nanocomposites due to the unique characteristics of these new materials. In this way, it is still necessary to verify the actual efficiency of the existing techniques when evaluating nanomaterials and envision possible changes and adjustments.

Keywords Cell culture · Nanoparticles · Organ-on-a-chip

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9.1 Introduction

Almost a century ago, most of the innovations and technologies that emerged in the research fields were directly or indirectly related to cell culture. Most cancer-related studies are directly related to Henrietta Lacks (HeLa) cells.

Since the discovery and culture of HeLa cells in 1952, innovations such as the polio vaccine in 1954 and sequencing of the human genome in 2000 have significant social and economic relevance showing that new technologies and discoveries play an essential role in the future of science (Freshney 2016; Masters 2002; Skloot 2010).

Over the years, it is possible to observe an increasing interest in developing protocols for in vitro cell culture. In 1885, when the embryologist Wilhelm Roux succeeded in conserving an embryonic tissue of chicken in a warm saline solution, a constant search for better culture conditions has begun. Harrison (1907) demonstrated the development of frog nerve fibers in a coagulated blood suspension, Carrel (1912) observed the importance of the nutrient content in the culture media, and in 1952, George Gey propagated the HeLa cell line from a cervical tumor tissue which has been used to this day (Freshney 2016; Gruber and Jayme 1994; Verma et al. 2020).

The emergence of nanomaterials triggered the need for new study models, mainly regarding in vitro evaluation since the assessment of newly developed nanomaterials aims to establish rules for their application and manipulation. As these are materials with totally differentiated characteristics, a new view concerning the applied tests must be considered, since the biological impacts on health and the environment are the primary concern of researchers to avoid future risks (Srivastava et al. 2015; Savage et al. 2019).

One of the possibilities presented using cell cultures is the rapid screening of these materials. However, the use of in vitro tests for the evaluation of nanomaterials goes far beyond screening. Nanomaterials act at the molecular level so that in vivo investigation studies will not keep up with the advances in nanotechnology, requiring a connection between in vivo and in vitro studies (Romeo et al. 2020). Therefore, tests should not be replaced but used in combination. The association between in vitro and in vivo tests can give accurate answers about nanomaterials, reduce animal experimentation, and introduce optimized tests. In vivo tests can track the routes of biodistribution and bioaccumulation of nanomaterials. However, previous evaluations using in vitro tests can identify highly dangerous nanocomposites (Hartung 2009, 2010; Berg et al. 2011).

Most studies involving the assessment of nanomaterials follow a sequence of in vitro tests with subsequent in vivo evaluations. However, it is predicted that this context will change within a few years. Both types of tests will be performed in an interconnected way, mainly regarding the evaluation of nanomaterials. Different cell culture models and markers are being developed, which nowadays enhance the assessments and will have even more impact in the future (Fig. 9.1).

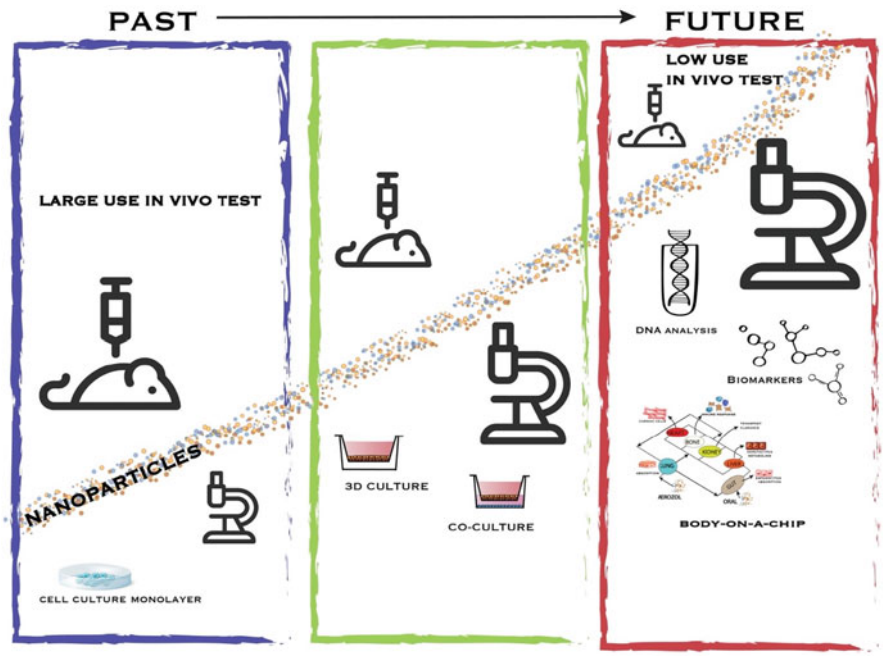

Fig. 9.1 Trends in nanomaterial research over time

Studies for improving some aspects of in vitro evaluation enable the development of models that employ different structures and materials and simulate a living organism (body-on-a-chip). It means that in the future, more elaborated in vitro evaluations could have a more significant contribution than the simple determination of risk potential, toxic dose, or as precursors to in vivo tests. In addition, it will be possible to reduce the use of animal tests, which will be performed just when the material is entirely safe (Frey et al. 2014; Romeo et al. 2020; Chen et al. 2021).

9.2 Evolution of Cell Culture Models

9.2.1 Cells

Multicellular organisms exist because cells can adhere to each other. This adhesion occurs through physical and biochemical mechanisms that happen in the extracellular matrix. In addition to enabling cell–cell adhesion, the extracellular matrix also promotes cell–substrate adhesion. The loss of cell adhesion can occur due to genetic mutations that cause alterations in the extracellular matrix proteins and, consequently, destabilize the tissue and alter the transduction of signals from the external

environment. Thus, it is a dynamic process that continuously moves and responds to changes in the microenvironment (Armingol et al. 2020; Windisch et al. 2019).

When using cell cultures as a strategy for performing in vitro tests, there is a need to provide a microenvironment-like in vivo systems, guaranteeing its homeostasis. One of the main limiting factors for this strategy is cell adhesion and cell–matrix–cell interaction (Oliveira et al. 2019; Zhou et al. 2018; Bich et al. 2019), which often are not maintained. The extracellular matrix comprises metabolites, receptors, ions, and multifunctional proteins such as growth factors, hormones, cytokines, chemokines, and neurotransmitters (Armingol et al. 2020). Cell adhesion molecules (CAMs) play a fundamental role, both physical and regarding cell signaling, influencing cell migration, mesenchymal remodeling, and contributing to critical processes such as embryogenesis, organ development, and wound healing (Canel et al. 2013; Windisch et al. 2019; Thiery et al. 2009; Epifano and Perez-Moreno 2012).

Therefore, over time and with the evolution of cell models, much has come to be questioned regarding the maintenance of the cell culture microenvironment, such as, for example, interactions mediated by cell adhesion molecules (Daley et al. 2008). These structures are widely distributed in the plasma membranes or clusters near the cellular junctions, which are responsible for maintaining the rigidity and strength of the tissues and epithelial barrier, transmission of information between intracellular and extracellular compartments, and the movement of molecules and ions from the cytoplasm of a cell into the cytoplasm of the adjacent cell (Saraiva et al. 2016; Nzou et al. 2019; Bergmann et al. 2018; Gloushankova et al. 2017). Cadherins, integrins, selectins, and immunoglobulins are examples of CAMs (Honig and Shapiro 2020; Mui et al. 2016; Aplin 2003; Juliano 2002).

9.2.2 Cell Cultures

Cell cultures have been used for material evaluation since the late nineteenth century when cells began to be isolated and cultured in the laboratory (Curtis et al. 1983; White 1946; Eagle 1955). An overview from 1907 to the present day (Fig. 9.2) shows that the techniques have evolved a lot concerning the employed technology, with several problems being solved over time, from cell adhesion in culture plates (1980) to the solution of issues related to the current three-dimensional cultures (Pardo et al. 2005; Sharrer 2006; Andrysiak et al. 2021; Hennies and Poumay 2021).

Depending on the type of culture, cells show different morphological properties and changes in gene expression, proliferation potential, cell interaction, and signal transduction (Fang and Eglen 2017; Riedl et al. 2017). An example was the work carried out by Ma et al. (2018), who compared the genome of glioblastoma multiforme cells in 3D cultures (polylactic acid scaffolds) and 2D cultures and found that cells cultured in a 3D system showed positive regulation of 8117 genes and negative regulation of 3060 genes in comparison with 2D cultures.

In vivo, the cellular response to external factors depends on the adhesion between cells and proteins of the extracellular matrix, mediated by the transmembrane

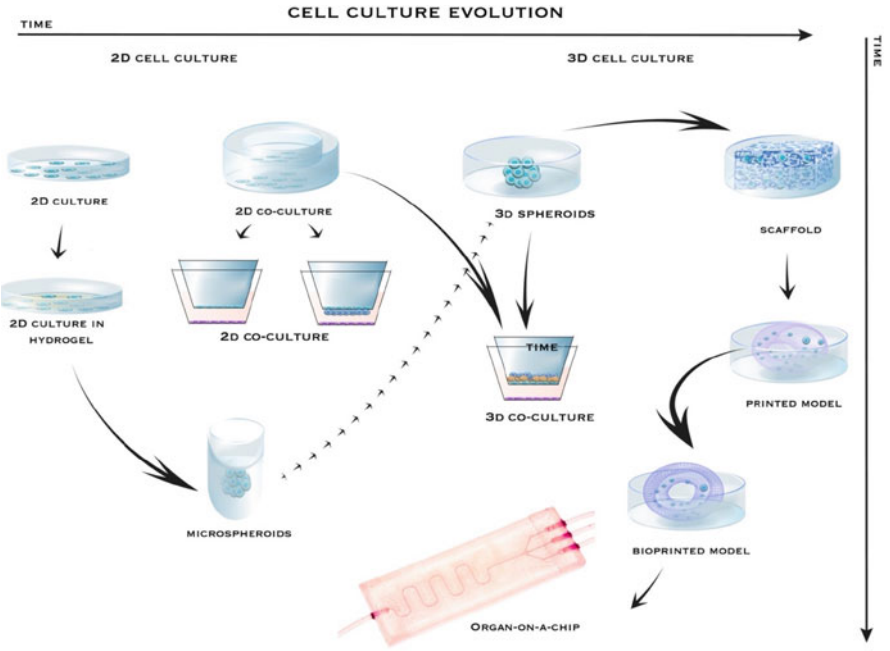

Fig. 9.2 Cell culture models over time. Cells grown in monolayers, in general, have a flat shape, not corresponding to the actual morphology. Coculture systems can mimic cellular interaction. 3D cultures are inserted in microenvironments like in vivo, being more representative

receptor system (Bachmann et al. 2019). In 2D culture, cell surface receptors have a structure and spatial arrangement different from in vivo organization. This change influences the way drugs and other substances bind to the cell, triggering varied responses (Edmondson et al. 2014). An example is a study by Loessner et al. (2010) in which ovarian cancer cells showed a viability decrease between 40% and 60% in 3D culture and 80% in 2D culture when exposed to paclitaxel. In this study, an increase in the expression of surface receptor integrins a3/a5/b1 and MMP9 protease was observed in 3D culture compared to the 2D culture model.

Analyzing the metabolic profile, Russell et al. (2017) found that in 2D culture, due to the monolayer arrangement, all the cells die when exposed to a cytotoxic drug, while in the 3D model, the cells form a protective barrier so that only those in the edge die. Soares et al. (2012) compared cardiac cells in 2D and 3D cultures and observed several differences, among them structural differences. The 3D culture showed a higher number of intercellular junctions, organized myofibrils, and preserved mitochondria and desmosomes, making the connection of neighboring cells and more significant deposition of extracellular matrix. A higher frequency of spontaneous contractions and an increase in the expression of the cardiac differentiation markers cadherin, sarcomeric alpha-actin, and desmin were also observed in the cells of the 3D model.

Liu et al. (2021) investigated the genomic architecture of mouse hepatocytes (AML12) in 2D and 3D cultures and observed differences in cellular organization, cell shape, and nucleus shape. They also observed differences in genomic interactions and a higher expression of genes involved in physiological processes in 3D culture. Chen et al. (2017) observed differences in the face of genomic regions related to structural changes in human fibroblasts grown in 3D and 2D models. More than 3000 genes showed altered expression.

Cells in 2D culture grow in monolayers attached to a plastic surface. Due to this arrangement, they present different morphology, physiology, interaction, and communication than the cells that compose living organisms (Edmondson et al. 2014). Thus, cells in 2D culture may be more sensitive when exposed to some substances (Chen et al. 2017; Lv et al. 2017); moreover, in this arrangement, all the cells receive the same amount of nutrients and growth factors, different from cells in natural conditions (Huang et al. 2013). In general, even though it is a low-cost and widely used practice compared to in vivo tests, 2D cultures present some limitations, mainly due to the impossibility of mimicking tissue architecture and the cellular microenvironment (Hartung 2013; Kieninger et al. 2018).

Since the presumption that the culture of monolayer cells limited cell–cell interaction and altered cell signaling, consequently causing discrepancies in the results of tests with cell cultures and organisms, new models of cell culture began to be studied and evaluated (Langhans 2018; Sieber et al. 2018; Chou et al. 2020; Turnbull et al. 2018). Then, the absence of a third dimension and a concentration gradient in the cell population in 2D models and the demand for more accurate models have triggered further studies.

Although there are gaps between the different types of cell models, the use of cell culture for the evaluation of new materials has been established, with varying attempts at combinations to obtain tremendous success (Fig. 9.2). With this exhaustive search for better in vitro evaluation parameters, it is possible to observe an increase in the number of studies that bring more effective and differentiated tests (Langhans 2018). All the advancement in this technology aims the search for study environments that resemble the in vivo cellular environment since many clinical trials fail in phase II and III due to safety and efficacy problems (Arrowsmith and Miller 2013).

3D cell culture models have advantages underrepresented in 2D cultures since they provide a complex cellular microenvironment closer to the in vivo environment, composed of proteins and extracellular matrix glycoproteins. Moreover, depending on the cellular composition, it is possible to simulate the signaling from other tissues (Vinci et al. 2012; Jedrzejczak-Silicka 2017; Chaicharoenaudomrung et al. 2019). In summary, the critical characteristic of 3D culture is the maintenance of the natural shape of the cell, which allows heterogeneous exposure to the medium, cellular communication, and better development (Chen et al. 2017; Lv et al. 2017). This system can be obtained using structures produced with biocompatible material denominated scaffolds or through the development of spheroids (Maia-Pinto et al. 2021; Saydé et al. 2021; Wang et al. 2020; Sokolova et al. 2020). In addition, it is

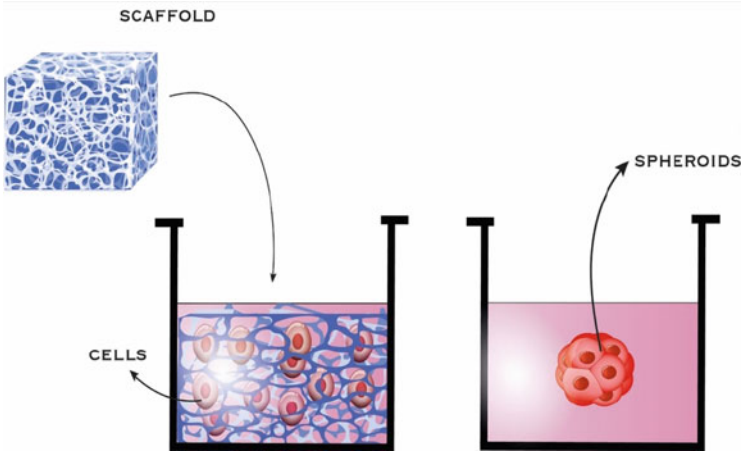

Fig. 9.3 Schematic representation of the two most used 3D cell culture models. The material used for the production of the scaffold may vary according to the needs of the study

worth mentioning that 3D models enable better exploration of space dimensions, providing greater cell–cell and cell–environment interactions (Fig. 9.3).

In the 3D cell culture model, the cellular organization is heterogeneous; that is, each cell is at a stage, with proliferating cells in the edges and cells in necrosis or quiescent within the system (Langhans 2018; Bonnans et al. 2014). Due to the cell–cell and cell–extracellular matrix interactions similar to in vivo experiments, the 3D cell culture model has become one of the most used methods for studying drugs and new materials (Jensen and Teng 2020).

Studies of scaffolds were introduced in the last decades, and, initially, these structures were composed of animal biomaterials such as collagen, gelatin, and chitosan. However, new biomaterials based on plants started to be studied and applied over time, including pectin and cellulose derivatives. Some studies showed that these scaffolds have favorable characteristics for developing cell cultures and contribute to the control of contamination and the improvement of cell–matrix interaction (Ravi et al. 2015; Campuzano and Pelling 2019; Mizoguchi et al. 2017).

Cellular interactions and communication play an essential role in several cellular functions, such as differentiation and proliferation, vitality, gene expression, response to stimuli, and metabolism, and are greatly influenced by the cell culture model (Kapałczynska et al. 2018). In addition to affecting cell–cell communication, the culture model also influences the extracellular matrix organization and its interaction with cells (Jensen and Teng 2020). The extracellular matrix biomolecules such as proteins, glycoproteins, and growth factors regulate cell proliferation, migration, differentiation, adhesion, and survival (Bonnans et al. 2014; Langhans 2018). Alterations in this organization, common in 2D cell cultures, give rise to inaccurate evaluations (Jensen and Teng 2020).

9.2.3 Coculture Models

Coculture models enable the study of two or more cell populations of different lines, the interactions between cell populations, exchange of substances, cell signaling or prediction of some events, as well as the development of methods for the creation of artificial tissues (Moraes et al. 2012; Costa and Ahluwalia 2019).

Another promising application of the coculture model, especially for nanotechnology, is tracking the transport of nanoparticles and other substances. These models enable the evaluation of materials permeation through biological membranes (Costa and Ahluwalia 2019). It is possible to assemble different models, from confluent monolayers and bilayers to 3D cell cultures, simulating different pathways such as pulmonary, cutaneous, and digestive, and then evaluate the permeability, translocation, and toxicity of substances and nanomaterials (Fig. 9.4).

These new evaluation methods employing cell cultures are more realistic. They provide essential strategies for the advancement of tissue engineering studies, discovering new drugs, organogenesis studies, and the modeling of diseases. In addition, they follow the 3Rs principle (reduction, replacement, and refinement), which boosts activities related to in vitro evaluation (Ravi et al. 2015; Jaroch et al. 2018). Another critical factor is that the development of 3D printing using biomaterials enabled new study models such as tridimensional organs (Ma et al. 2021).

One of the models of great importance for the evaluation of compounds using cell culture is the 3D coculture for the ocular surface. It is composed of rabbit conjunctival epithelium and lacrimal gland spheroid cells. According to the cell organization, Lu et al. (2017) tested different models for optical surface studies, which they named top, bottom, and membrane. The results proved that coculture introduced a beneficial effect on secretory function, mimicking the healthy ocular surface. This study provided a new platform for pathophysiological studies of the ocular surface.

Nanomaterials require molecular evaluation due to their unique characteristics, and the use of cellular cultures comes out as a great combination. Numerous studies involving different cell culture models have been developed for this purpose, which is increasingly well-elaborated, aiming not only for the previous assessment of nanomaterials but also for more robust analyses (Table 9.1).

Even in the face of different strategies of cocultures and 3D cultures, to obtain even more effective models, it is necessary to integrate different areas such as materials, molecular biology, and computational modeling, among others (Kamm et al. 2018). Moreover, although already used for a long time, in vitro analyses still need improvements mainly concerning current tests involving human biometric pathophysiology, which have a gap (Franzen et al. 2019; Ma et al. 2021). In the future, this gap may be completed using organ-on-a-chip, which enables the reproduction of organs or tissues in vitro, mimicking the architecture and functionality of in vivo systems, as an attempt to replace in vivo tests.

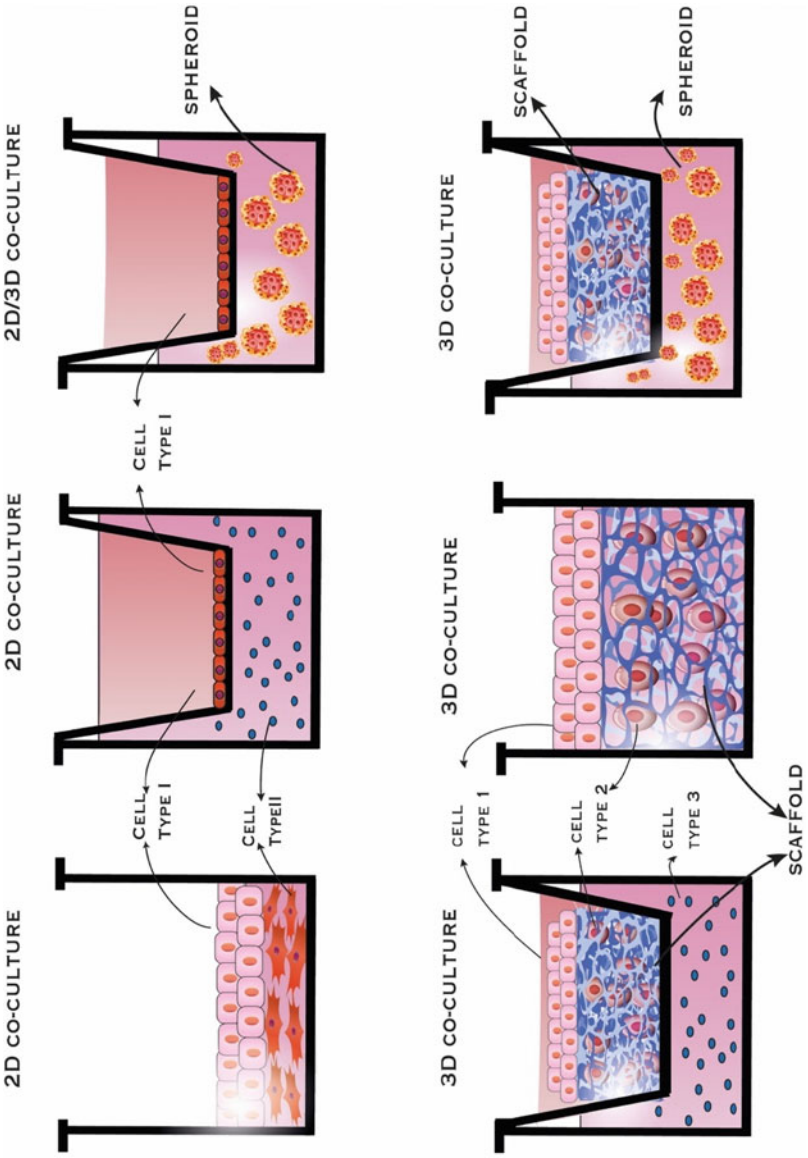

Fig. 9.4 Schematic representation of some coculture models

Table 9.1 Studies using cell culture for the evaluation of nanoparticles

Nanoparticle	Objective	Culture/cell type	In vitro tests	References
Magnetic nanoparticle-loaded human adipose-derived mesenchymal cells spheroids	Levitation of cell culture, maintenance of properties, and improvement of performance	Primary 2D and 3D cultures Human primary adipose-derived mesenchymal cells	• Cell viability: MTT • Cell migration • Assessment of osteogenesis, chondrogenesis, and adipogenesis	Labusca et al. (2021)
Silver nanoparticles synthesized with flavonoids from *Reinwardtia indica* leaves	Antioxidant, antimicrobial, and cytotoxic potential	Monolayer (2D) Human cervical tumor cells (SiHa)	• Cell viability: MTT • Enzymatic assays	Upadhyay et al. (2019)
Zinc oxide nanoparticles	Non-apoptotic cell death	Monolayer (2D) Murine microglial cells (BV-2)	• Cell viability: MTT • Trypan blue	Sruthi et al. (2020)
Cellulose nanofibers/hydroxyapatite/silver nanoparticles	Efficiency as a scaffold for tissue engineering applications	Monolayer (2D) Chicken embryo fibroblasts (CEFs)	• Cell viability: MTT • Cell fixation/microscopy	Sofi et al. (2021)
Silver and titanium dioxide nanoparticles	Toxicity	Monolayer (2D) Porcine ovarian granulosa cells	• RT-PCR • ELISA test	Sirotkin et al. (2021)
Silver nanoparticles with different surface modifications	Digestion on gastrointestinal fate and uptake	Monolayer (2D) and coculture Human colorectal adenocarcinoma cells (Caco-2) Human mucus-secreting adenocarcinoma cells (HT29-MTX)	• Cell viability: WST-1 • Integrity assessment: transepithelial electrical resistance • Cellular uptake/association and transport	Abdelkhaliq et al. (2020)
Natural organic matter coated silver nanoparticle	Kinetics of transport across the cell membrane	Monolayer (2D) Human lung carcinoma epithelial cells (A549)	• Cell viability • Uptake kinetics • Relationship intracellular/suspension nanoparticles • Cellular uptake pathway • ROS level	Zhong et al. (2021)

ROS-scavenger nanoceria encapsulated within mesoporous silica nanoparticles (Ce@MSNs)	Osteoporosis treatment/in stressed and normal conditions	Monolayer (2D) coculture Murine primary macrophage (RAW264.7) Pre-osteoblast cells (MC3T3-E1)	• Cell viability: MTT • Uptake • Antioxidant activity • Osteoblast differentiation • Therapeutic activity	Pinna et al. (2021)
Cytarabine-loaded poly(ε-caprolactone) nanoparticles	Sustained-release/anticancer activity	Monolayer (2D) Acute myeloid leukemia cells (KG-1) Breast cancer cells (MCF-7)	• Cell viability: MTT • Apoptosis quantification (flow cytometry) • Western blot	Jan et al. (2021)
Polyvinylpyrrolidone (PVP)-coated silver nanoparticles	Investigation of molecular mechanisms underlying AgNP induced lung cellular senescence	Monolayer (2D) Human fetal lung fibroblast (MRC5)	• SA-β-galactosidase staining • Western blotting • RNA-sequencing and data analysis • Immunofluorescence • PGE2 immunoassay • Apoptosis and senescence • Cell cycle	Chen et al. (2020)
Chitosan nanoparticles	Test and overcome the unfavorable influences of aggregated chitosan nanoparticles	Monolayer (2D) Human pancreatic adenocarcinoma (CFPAC-1)	• Cell viability: MTT • Uptake	Ozturk et al. (2020)
Cancer-specific prodrug nanoparticle (doxorubicin) with Bcl-2 anti-apoptotic inhibitor (Navitoclax)	Overcome acquired drug resistance during chemotherapy using nanoparticles	Monolayer (2D) Human breast adenocarcinoma (MDA-MB231) Human dermal fibroblast (HDF) Rat BDIX heart myoblast (H9C2)	• Uptake • Analysis of Bcl-2 expression (western blot) • Cytotoxicity assays (flow cytometry)	Kim et al. (2021)
Engineered silica nanoparticles	Investigate if the nanoparticles are biologically safe to deliver drugs or genes to liver cells	Monolayer (2D) Epithelial-like human hepatoblastoma cell (HuH-7) Liver sinusoidal endothelial cells (SK-HEP-1)	• Cell viability: MTT and Sulforhodamine B • Colocalization analysis with lysosomes and mitochondria • Flow cytometry • Genotoxicity/micronuclei • Hemolysis assay • Clonogenic assay	Tüncel et al. (2021)

(continued)

Table 9.1 (continued)

Nanoparticle	Objective	Culture/cell type	In vitro tests	References
Calcitonin-loaded octamaleimic acid-silsesquioxane nanoparticles in a hydrogel scaffold	Enrich the hydrogel scaffold with hydroxyapatite and platelet-rich plasma for bone tissue engineering	Monolayer (2D) Human osteosarcoma cells (MG-63)	• Cell viability: MTT and trypan blue • Alizarin red staining • Enzymatic assays • Osteogenic gene expression (qRT-PCR)	Ahmadipour et al. (2021)
Hyperthermic Ag and Au Fe_3O_4 nanoparticles	Anticancer activity	Monolayer (2D) Human embryonic kidney cells (HEK293) Human colorectal carcinoma cell (HCT116) Mouse mammary carcinoma cells (4T1) Epithelial-like human hepatoblastoma cell (HUH7)	• Cell viability: MTT • Hyperthermia • qRT-PCR	Katifelis et al. (2020)
Polydopamine (PDA)-coated magnetite nanoparticles (NPs) and spheres (sMAG) with PAMAM dendrimers and functionalized with NHS–PEG–Mal (N-hydroxysuccinimide–polyethylene glycol–maleimide) linker	Chemo- and photothermal therapy Generation of reactive oxygen species	Monolayer (2D) Hepatocellular carcinoma cells (HepG2) Human liver epithelial cells (THLE-2)	• Viability: WST-1 • Oxidative stress quantification assay	Jędrzak et al. (2020)
Cadmium oxide nanoparticles	Cytotoxicity and genotoxicity	Monolayer (2D) Human lymphoblastoid cells (TK6) Hepatocellular carcinoma cells (HepG2) Mouse lymphoma cell (L5178Y/Tk+/−3.7.2C)	• Cellular uptake (transmission electron microscopy) • Viability: MTS and ATP • Lactate dehydrogenase (LDH) activity assay • Micronucleus (flow cytometry) • Comet assay • Mouse lymphoma thymidine kinase assay (MLA)	Demir et al. (2020)

Multifunctional gelatin nanoparticles modified by NIR-emitting gold/silver alloy nanoclusters and loaded with ovalbumin (OVA) as a model antigen	Functionalized multifunctional nanovaccine for targeting dendritic cells and modulation of immune response	Monolayer (2D) and coculture murine bone marrow-derived dendritic cells (BMDCs) T cells derived from OT-I and OT-II mice spleens	• Viability, uptake, and cell activation • Analysis of chemokines• And cytokines	El-Sayed et al. (2021)
Copper oxide nanoparticles	Toxicity on intestinal barrier	Monolayer and 3D Human colorectal adenocarcinoma cells (Caco-2)	• Barrier integrity assay (transepithelial electrical resistance evaluation) • ELISA test	Bertero et al. (2020)

9.2.4 Organ-on-a-Chip

From the year 2010, with the construction of lung-on-a-chip (Huh et al. 2010), organ-on-a-chip systems started to be recognized with the development of several studies involving different tissues over the years (Si et al. 2020; Ma et al. 2016; Musah et al. 2017; Glieberman et al. 2019; Ugolini et al. 2018; Poceviciute and Ismagilov 2019; Koo et al. 2018; Bein et al. 2018). The use of chip systems for multi-cultures enables the control of interconnected independent cell cultures arranged to simulate tissue and organ physiology, which cannot be accomplished using only 2D or 3D cell cultures. This system also enables evaluating incompatible cultures in the same model simulating a specific microenvironment, leading to the discovery of new signaling mechanisms. The application of organ-on-a-chip models allows molecular and immunological analyses to promise future in vitro analyses (Ma et al. 2021; Chen et al. 2021).

Organ-on-a-chip systems enable the investigation of the toxicity of nanomaterials and other substances intermediating preclinical models such as 2D culture and animal models and population studies (Lu and Radisic 2021). A 3D culture system that involves fluid flow technology simulates living organisms' conditions with the continuous nutrient exchange, oxygenation, gas exchange, removal of residues and metabolites, shear stress, and other characteristics of in vivo systems. Among the advantages of organ-on-a-chip compared with static cultures such as 2D is that this system simulates cellular metabolism. An example is a study by Trapecar et al. (2020) in which the metabolism and inflammatory responses of CD4 T effector cells were observed in a multi-organ-on-a-chip model created with human hepatocytes and Kupfer cells, mimicking the liver, and ulcerative colitis epithelium, dendritic cells, and macrophages mimicking the gut.

Specifically, regarding the evaluation of the toxicity of nanomaterials, it is known that the dynamism of tissues has a significant influence on their behavior (Lu et al. 2020; Lu and Radisic 2021). In this way, different organ-on-a-chip systems are being developed, aiming at the investigation of nanomaterials effects. Huh et al. (2010) developed a biomimetic microsystem mimicking the alveolar-capillary interface of the human lung with human alveolar epithelial cells and microvascular endothelial cells to investigate the toxicity of silica nanoparticles. They observed high levels of intercellular adhesion molecule-1 (ICAM-1) expression in the underlying endothelium in the microvascular channel and an increase in reactive oxygen species production, which were intensified by mechanical stretching, suggesting that the toxic effects of nanomaterials may be induced by physiological breathing. Zhang et al. (2018) also developed a lung-on-a-chip system to investigate the effects of TiO_2 and ZnO nanoparticles. The system consisted of three parallel channels, with the culture of primary human lung epithelial cells (HPAEpiCs) on one side, a layer of 3D matrigel membrane with fluid flow in the center, simulating the human lung alveolar-capillary barrier, and vascular endothelial cells (HUVEC) on the opposite side. An increase in the system's permeability and the production of reactive oxygen

species were observed, especially in epithelial cells directly exposed to the nanoparticles, and apoptosis, with more significant effects of ZnO nanoparticles. Still focusing on the respiratory system, Chen et al. (2016) developed a human lung microtissue array using bronchial epithelial cells BEAS-2B to investigate the fibrogenic potential of multi-wall carbon nanotubes. After 72 h of exposure to carbon nanotubes, an increase in the microtissue contraction force and the fibrogenic marker miR-21 expression was observed, indicating the fibrogenic potential of the nanomaterial.

Directing the organ-on-a-chip model to investigate possible impacts of nanomaterials on the cardiovascular system, Ahn et al. (2018) evaluated the effects of TiO_2 and silver nanoparticles on the cardiac contraction tissue using a 3D mussel-inspired microphysiological model. The system consisted of bioadhesive polydopamine (PDA)/polycaprolactone (PCL) nanofibers introduced with neonatal rat ventricular myocytes, which developed into mature and functional cardiac tissue. The nanoparticles caused structural damage to the tissue architecture with disruption of the sarcomeric alignment and calcium signaling, decreasing the contractile function of the microphysiological system. Lu et al. (2020) also used a heart-on-a-chip system to evaluate the toxicity of air pollution CuO and SiO_2 nanoparticles. They developed a 3D vascularized microfluidic system that simulates cardiac tissue with cardiomyocytes derived from human pluripotent stem cells and human umbilical vein endothelial cells (HUVEC) into a bioscaffold. CuO nanoparticles showed high toxicity translocating from endothelium to cardiac tissue and causing electrical and contractile dysfunction, whereas SiO_2 nanoparticles did not translocate but induced the release of inflammatory cytokines.

Advancing even further, the inclusion of different organs in the organ-on-a-chip model to assess the effects of nanomaterials may present different results. Esch et al. (2014) developed a microfluidic body-on-a-chip system to evaluate the impacts of carboxylated polystyrene nanoparticles, combining in vitro models of the human intestinal epithelium with the coculture of enterocytes (Caco-2) and mucin-producing cells (HT29-MTX), and liver, with HepG2/C3A cells. When comparing the system which combined the intestinal tract and liver to a system that simulated a unique organ, the first one showed more significant toxic effects of the nanoparticles. Because of this, the authors suggest the greater effectiveness of multi-organ in vitro models for nanomaterials toxicity assessment.

Another essential point to be evaluated for the specific study of nanomaterials are the biomarkers, which can be safely employed to investigate how inert or potentially toxic a nanomaterial is. According to Salieri et al. (2020), there is a tendency to conduct more in vitro evaluations to replace in vivo tests in the future. However, new study strategies are necessary to use better data provided by in vitro analyses.

9.3 Inflammatory Effect Biomarkers of Exposure to Nanoparticles

Inflammatory effects occasioned by the exposure of cell cultures to nanomaterials are generally assessed by analyzing the release of soluble factors such as cytokines, chemokines, and growth factors by enzyme-linked immunosorbent assay (ELISA), with detection through flow cytometry or microplate reader (Drasler et al. 2017). Some studies have evaluated inflammatory responses using the ELISA assay with inflammatory markers, such as that performed by Huk et al. (2014), who investigated the inflammatory effects of silver nanoparticles (50, 80, and 200 nm) coated by polyvinylpyrrolidone (PVP) through the analysis of IL-8 and MCP-1 biomarkers in human lung carcinoma epithelial cells (A549). Greulich et al. (2011) quantified the release of the pro-inflammatory cytokines IL-6, IL-8, and TNF-α, the anti-inflammatory IL-1ra, and the IL-2 and IL-4 cytokines derived from T cells exposed to silver nanoparticles. Hackenberg et al. (2011) also quantified the release of the inflammatory cytokines IL-6 and IL-8 and the vascular endothelial growth factor (VEGF) in human mesenchymal stem cells silver nanoparticles exposed.

The evaluation of inflammatory proteins is widely used in studies of nanomaterials; however, it is still subject to interference from the evaluated nanomaterial, as it can interact with the culture medium or with the marker proteins. In addition, it is crucial to work with concentrations below the limit of cytotoxicity since a cytotoxic nanomaterial reduces cell viability and consequently reduces the release of cytokines, causing false-negative results (Drasler et al. 2017).

Due to the previously addressed problem, some authors prefer to use gene expression analyses, such as those performed by Shannahan et al. (2015), who evaluated the expression of the inflammatory marker TNF-α in mouse macrophages (RAW264.7) exposed to silver nanoparticles with and without protein corona. The assay consisted of the exposure of macrophages to the nanoparticles for 6 h, followed by the extraction of total RNA, reverse transcription for cDNA, and real-time PCR to quantify TNF-α. Cheng et al. (2020) evaluated the expression of the pro-inflammatory cytokine genes IL-1β and IL-6 and the chemokines CXCL1, CXCL2, CXCL3, CCL20, and CXCL8 in keratinocytes differentiated from embryonic stem cells exposed to ultrafine carbon nanopowder.

The pro-inflammatory potential of Al_2O_3, SiO_2, and CeO_2 nanoparticles was evaluated using a mouse alveolar macrophage cell model. The evaluation of the pro-inflammatory markers TNF-α, IL-1β, and IL-6 expression was performed, as well as the quantification of IFN-γ, IL-12p70, IL-1β, IL-6, IL-10, TNF-α, and mouse keratinocyte chemoattractant (KC) in the cell culture supernatant, using the Mouse ProInflammatory 7-Plex Ultra-Sensitive kit (Flaherty et al. 2015). The quantification of TNF-α, IL-1β, IL-8, and IL-6 markers in a 3D reconstruct of human bronchial tissue was performed by Di Cristo et al. (2020) after repetitive exposures to graphene oxide nanomaterial. The model simulated prolonged and repetitive human occupational exposure to the nanomaterial by nebulization using an air–liquid interface culture for 30 days. In this way, biomarkers are widely used for the evaluation of

nanoparticles. Some examples of studies that used biomarkers are shown in Table 9.2.

9.4 Evaluation of Genic Mutations for Exposure to Nanoparticles—Genetic Markers

In addition to the detection of biological markers that indicate inflammation triggered by cell exposure to nanomaterials, it is also possible to identify mutations through genetic features. Genes such as Tk (thymidine kinase) and Hprt (hypoxanthine guanine phosphoribosyltransferase) may be used to evaluate genetic mutations occasioned by nanomaterials (Kazimirova et al. 2020; Du et al. 2019; Doak et al. 2012).

Mouse lymphoma cell line L5178Y/Tk+/− (MLA) is employed to evaluate mutagenicity using the TK gene. At a specific time after exposure, trifluorothymidine (TFT), an analog of thymidine, is added to the cell culture, and then, only the cells that have undergone TK mutation in the presence of the nanocomposite can form colonies (Chen et al. 2014a; Demir and Castranova 2016; Du et al. 2019).

The test for mutation evaluation with the Hprt gene is performed according to the standardization proposed by the OECD Guidelines for the Testing of Chemicals 476 (OECD 2016) using 6-thioguanine (6-TG), a toxic guanine analog (Huk et al. 2014). Kazimirova et al. (2020) investigated the mutagenic effects of titanium dioxide anatase/rutile nanoparticles on different dispersions of V79–4 cell lines through the mammalian heart gene mutation test. Huk et al. (2014) used the same technique to evaluate the effects of polyvinylpyrrolidone (PVP) coated silver nanoparticles with different sizes (50, 80, and 200 nm) on the V79–4 cell line. Table 9.3 shows some studies that used the genes hprt, tk, and other genetic markers to detect the mutagenicity of different nanomaterials on in vitro cell cultures.

9.5 Conclusion

The emergence of nanotechnology has led to greater attention to new in vitro culture techniques. In addition to studies focused on the impact of the environment and health, it has also been necessary to improve molecular studies for more excellent knowledge of this new material. Therefore, different in vitro assays have developed an increasingly more effective approach to in vivo systems, which has led to a reduction in animal experimentation.

Coculture, 2D, 3D models, and new organ-on-a-chip models, together with a greater understanding of biomarkers, place in vitro analysis as one of the main tests that can safely assess the effects of nanomaterials, as well as collaborate to evaluate their inflammatory potential.

Table 9.2 Inflammatory and oxidative stress biomarkers for evaluation of nanomaterials effects on cell cultures

Nanomaterial	Cell line	Inflammatory or oxidative stress biomarkers	References
PVP-coated silver nanoparticles	Human lung carcinoma epithelial cells (A549)	IL-8 and MCP-1	Huk et al. (2014)
Silver nanoparticles	Human peripheral blood mononuclear cells (monocytes and lymphocytes)	IL-6, IL-8, TNF-α, IL-1ra, IL-2, IL-4 Reactive oxygen species (ROS)	Greulich et al. (2011)
Silver nanoparticles	Human mesenchymal stem cells	IL-6 and IL-8, and VEGF	Hackenberg et al. (2011)
Silver nanoparticles with and without protein corona	Mouse macrophages (RAW264.7)	TNF-α	Shannahan et al. (2015)
Ultrafine carbon nanopowder	Human embryonic stem cell (hESC)-based differentiation system towards keratinocytes	IL-1β and IL-6, CXCL1, CXCL2, CXCL3, CCL20, and CXCL8	Cheng et al. (2020)
Al_2O_3, SiO_2, and CeO_2 nanoparticles	Mouse alveolar macrophages (ATCC® CRL-2019)	TNF-α, IL-1β, IL-6 IFN-γ, IL-12p70, IL-1β, IL-6, IL-10, and TNF-α ROS	Flaherty et al. (2015)
Graphene oxide nanoparticles	EpiAirway™ tissues (AIR-100, PE6–5), 3D reconstruct of human bronchial tissue	TNF-α, IL-1β, IL-8 and IL-6	Di Cristo et al. (2020)
Silver nanoparticles	HeLa cells in infection with *Toxoplasma gondii*	IL-1β, TNF-α, IL-12p70, IL-8, IL-6, and IL-10 Nitric oxide, ROS	Machado et al. (2020)
Zinc oxide nanoparticles	Isolated human eosinophils	IL-1β and IL-8, ROS	Silva and Girard (2016)
Titanium dioxide and silica nanoparticles	Rat alveolar macrophages (NR8383)	84 rat chemokines and cytokines	Schremmer et al. (2016)
Multi-walled carbon nanotubes	A549 cells and normal human bronchial epithelial cells (HBEpC)	8-nitroG formation (iNOS expression, endocytosis, HMGB1, RAGE, TLR-2, and TLR-4), nitric oxide, GSH	Hiraku et al. (2016)
Zinc oxide nanoparticles	Transfected cells derived from A549 with reporter genes for IL-8 (Luc, RFP, and GFP)	IL-8	Stoehr et al. (2015)

Zinc oxide nanoparticles	A549 cells	NF-B-mediated NLRP3 inflammasome activation, IL-1β, and IL-18, ROS	Liang et al. (2017)
Silver nanoparticles	Human liver-derived hepatoma cells (HepG2)	NLRP3 inflammasome activation, IL-1β	Mishra et al. (2016)
Silver nanospheres and wires coated with PVP	A549 reporter cells possessing luciferase reporter genes	IL-6, IL-8, TNF-α, and NF-kB	Stoehr et al. (2011)
Spherical and wire-shaped aluminum oxide nanoparticles	Primary mice splenocytes	IL-1β and IL-18 NLRP3 inflammasome activation	Manshian et al. (2018)
Silver and silver-lipoencapsulated nanoparticles	THP1 monocytes and THP1 differentiated macrophages (TDM)	IL-1 β, IL-6, IL-8, and TNF-α	Yusuf and Casey (2019)
Silver nanoparticles	Caco-2/THP-1 coculture mimicking the intestine in a healthy or inflamed state	IL-1β, IL-8, TNF-α MCP-1, MIP-1α, IFN-γ, IL-4, and IL-6	Kämpfer et al. (2020)
Silver and metal oxide nanoparticles (CuO, Fe_3O_4, ZnO, TiO_2, NiO, and CeO_2)	GFP-tagged mouse embryonic stem (mES) cells	Bscl2-GFP, Srxn1-GFP, and Btg2-GFP	Karlsson et al. (2014)
Vegetable carbon (E153) and TiO_2 (E171) nanoparticles	GFP-tagged mouse embryonic stem (mES) cells	Bscl2-GFP, Rtkn-GFP, Btg2-GFP, Srxn1-GFP, BlvrbGFP, and Ddit3-GFP	Brown et al. (2019)
Silver nanoparticles	Human embryonic stem cell (hESC)-derived neural stem/progenitor cells (NPCs)	ROS	Oh et al. (2016)

Table 9.3 Genic mutation biomarkers for evaluation of nanomaterials effects on in vitro cell cultures

Nanomaterial	Cell line	Genic mutation biomarker	References
TiO_2 nanoparticles	Human lymphoblastoid cells (WIL2-NS)	Hypoxanthine guanine phosphoribosyltransferase (*hprt*)	Wang et al. (2007)
Ni and NiO nanoparticles	Human bronchial epithelial cells (HBEC3-kt)	*Hprt*	Åkerlund et al. (2018)
Cd/Se semiconductor quantum dots	Human lymphoblastoid-B cells (TK6)	*Hprt*	Manshian et al. (2016)
TiO_2 nanoparticles	Chinese hamster lung fibroblasts (V79)	*Hprt*	Chen et al. (2014b)
TiO_2 nanoparticles	V79	*Hprt*	Kazimirova et al. (2020)
Multi-wall carbon nanotubes (NM401)	V79	*Hprt*	Rubio et al. (2016)
Ag nanoparticles coated with PVP	V79	*Hprt*	Huk et al. (2014)
TiO_2 nanoparticles	Chinese hamster ovary cells (CHO-K1)	*Hprt*	Wang et al. (2011)
TiO_2 nanoparticles	V79	*Hprt*	Jain et al. (2017)
Multi-wall carbon nanotubes	Chinese hamster lung cells (CHL/IU)	*Hgprt*	Asakura et al. (2010)
TiO_2 nanoparticles	Mouse lymphoma cells (L5178Y)	Thymidine kinase (*tk*)	Du et al. (2019)
Ag nanoparticles	L5178Y	*Tk*	Mei et al. (2012)
Ag nanoparticles	L5178Y	*Tk*	Kim et al. (2010)
Poly(anhydride) nanoparticles	L5178Y	*Tk*	Iglesias et al. (2017)
Tungsten carbide–cobalt (WC–Co) nanoparticles	L5178Y	*Tk*	Moche et al. (2014)
Multi-wall carbon nanotubes	Mouse embryonic stem cells (ES)	Adenine phosphoribosyltransferase (*aprt*)	Zhu et al. (2007)
Zinc oxide nanoparticles	Human-hamster hybrid cells (AL)	*CD59*	Wang et al. (2015)

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Chapter 10
Macrophage-Targeted Nanomedicines

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Abstract Macrophages are versatile cells of the innate immune system responsible for the control and progressions of a variety of autoimmune inflammatory, infectious, and metabolic diseases and cancer. Macrophage polarization (pro-inflammatory and tissue injury M1 or anti-inflammatory, tissue repair, and proangiogenic M2) occurs in health tissues and in diseases, being a vital element of disease development or reversion. Macrophages play important roles in diverse diseases that affect millions of people and have significant health and economic costs since generally they are chronic, relapsing, and disabling. Therapies focus on elimination, repolarization, reduction of pro-inflammatory mediators, activation of antimicrobial activity, or induction of immune response by macrophages is being considered of increasing interest. However, issues associated with inappropriate pharmacokinetics, lack of tissue selectivity, and poor intracellular delivery make such pharmacological approaches poorly efficient and/or toxic. Macrophage-targeted nanomedicines may increase intracellular drug concentration on activated macrophages, reduce toxicity, and improve activity. In this chapter, we will describe strategies for macrophage targeting employing nanoparticles for treatment of inflammatory diseases such as cardiovascular diseases, lung inflammatory diseases, inflammatory bowel diseases and rheumatoid arthritis, and infectious diseases such as leishmaniasis, tuberculosis, and nontuberculous mycobacterial disease developed in the last 5 years.

Keywords Inflammatory diseases · Infectious diseases · Liposomes · Nanoparticles · Active targeting

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10.1 Introduction

Macrophages (Mϕ) are cells of the innate immune system with diverse functions. Mϕ sense their environment, kill pathogens, take up apoptotic and necrotic cells, heal tissue damage, present antigens to T cells and produce cytokines and chemokines that serve many important roles in innate and adaptive immune responses (Hume et al. 2019). Mϕ are found as resident self-maintaining populations distributed as sinus-lining and interstitial resident Mϕ in lymphohematopoietic and other tissues, such as Langerhans cells of the skin, alveolar Mϕ (AMϕ), Kupffer cells (KC) of the liver, intestinal Mϕ, microglia cells, and osteoclasts, with specialized functions and phenotypes (Ginhoux and Guilliams 2016). Bone marrow-derived blood monocytes restock resident Mϕ with high turnover and are recruited to places of injury, infection, sterile inflammation, and in response to metabolic, atherogenic, and neoplastic stimuli, generating infiltrating activated tissue Mϕ.

Mϕ are dynamic plastic cells, they can alter their functional phenotype depending on the microenvironment. Mϕ sense multiple signals from pathogens (though pathogen-associated molecular patterns, PAMPs, such as toll-like receptors-TLRs), from damage tissue (though damage-associated molecular patterns, DAMPs), and from normal tissue environment (lineage-determining growth factors and cytokines) (Shapouri-Moghaddam et al. 2018). According to the combination of these stimuli, Mϕ polarized into two subsets, classically activated (M1) or alternatively activated (M2) (Murray 2017). Briefly, interferon-gamma (INF-γ) produced by Th1 lymphocyte and PAMPs or DAMPs induce the M1 phenotype with antimicrobial, inflammatory, and antigen-presenting activities, whereas cytokines produced mainly by Th2 lymphocytes promote the M2 phenotype characterized by anti-inflammatory actions and antiparasitic actions (Fig. 10.1).

Generally, M1 Mϕ are involved in microorganism and cell matrix debris phagocytosis, in the early phase of tissue healing and antigen presentation. M1 Mϕ produced pro-inflammatory cytokines (TNF, IL-1β, IL-6, IL-12, IL-23), low levels of anti-inflammatory cytokines (IL-10), and several chemokines. M1 Mϕ highly express cyclo-oxygenase 2 (COX 2) and inducible nitric oxide synthase (iNOS) for nitric oxide (NO) synthesis, secrete high levels of reactive nitrogen intermediates (RNIs), reactive oxygen species (ROS), and produce collagenase, that leads tissue damage.

M2 Mϕ, on the other hand, are associated with allergy, extracellular parasitic infection, tissue remodeling and healing, acetogenesis, and tumor progression (Mantovani et al. 2013). M2 Mϕ are highly endocytic and partially phagocytic, secrete high levels of IL-10 and low levels of IL-12 and IL-23 (Arora et al. 2018), produce angiogenesis mediators such as transforming growth factor (TGF-β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and express scavenger, mannose, and galactose receptors and COX 1. M2 Mϕ can be further divided into M2a, M2b, M2c, and M2d subsets depending on the activation stimulus (Martinez and Gordon 2014). M2a and M2c Mϕ express high levels of Arg-1 that plays a role in catalysis polyamines, which is necessary for collagen synthesis,

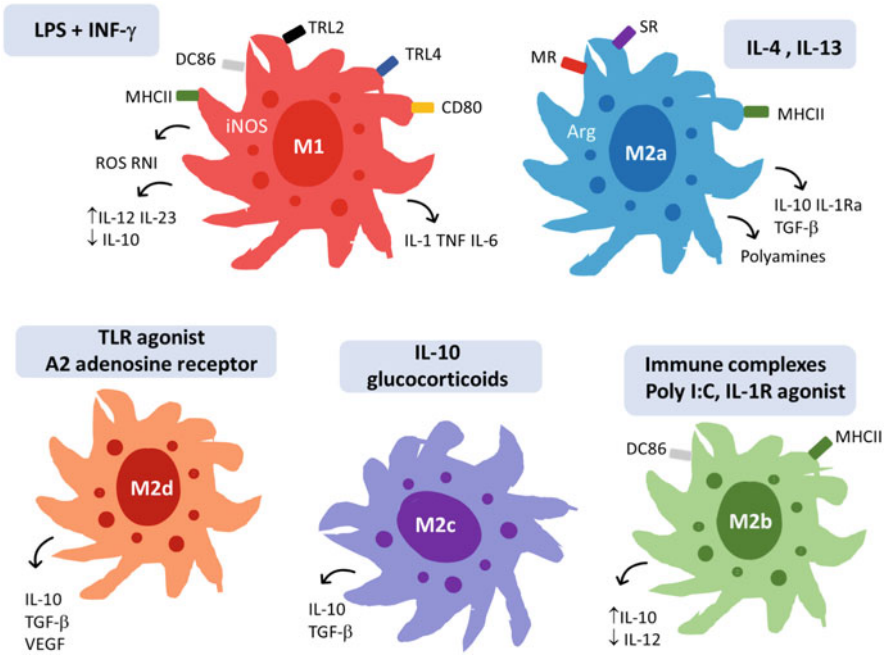

Fig. 10.1 M1 Mϕ are induced when naïve or M0 Mϕ are exposed to bacterial moieties including LPS and Th1 cytokines including IFN-γ, IL-2, IL-12, IL-18, and TNF-β [lymphotoxin β (LT-β)]. M1 Mϕ express pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and type I IFN, and promote cytotoxic adaptive immunity by upregulating MHC class II molecules in conjunction with co-stimulatory molecules CD40, CD80, CD86, TLR2, and TLR4. Also, M1 macrophages express Th1- and Th17-polarizing cytokines IL-12, IL-23, IL-27, and Th1-recruiting chemokines CXCL9, CXCL10, CXCL11. M1 Mϕ are finally characterized by microorganism and matrix debris phagocytosis in the early phases of healing and high antigen presentation capacity

fibroblast proliferation, fibrosis, and other tissue remodeling functions. Healthy tissues associated with immune-suppressed states such as placenta and lung are rich in M2 Mϕ. In this chapter, we will generally refer to M2a as M2 Mϕ.

Classification into two subsets, M1 and M2, is a simplified description of Mϕ heterogeneity and plasticity, being a continuum of functional states more realistic. Repolarization or switching Mϕ phenotype in response to new environmental influences is possible (Martinez and Gordon 2014).

M2 Mϕ are developed upon exposure to Th2 cytokines including IL-4, IL-5, IL-6, and IL-10. The M2 Mϕ can further be divided into M2a, M2b, M2c, and M2d depending on their stimulus for the activation.

Upon stimulation with IL-4 or IL-13, M2a (a stands for alternative) Mϕ express high levels of MR (CD206), IL-1 receptor (IL-1R) and CCL17, and secrete profibrotic factors, such as TGF-β, IGF, and fibronectin, essential for tissue repair. M2 phenotype expressed MMP2, MMP7, MMP9, and arginase receptors.

M2b Mϕ or "regulatory" Mϕ are induced by poly I:C or TLR or IL-1R agonists, which leads to activation of multiple transcription factors such as NF-κB, MAPK, and interferon regulatory factor 3, as well as PI3K–AKT signaling. Besides producing several pro-inflammatory cytokines (IL-1β, IL-6, TNF, CCL1, and TNF SF14), M2b cells also secrete anti-inflammatory cytokines such as IL-10 and low levels of IL-12 thereby opposing M1 Mϕ.

M2c or "deactivation" Mϕ are induced by IL-10 and glucocorticoids, which leads to high levels of IL-10 and TGF-β. This anti-inflammatory phenotype of M2c Mϕ is further driven by their efficient capability to phagocytose apoptotic cells by high expression of Mer receptor tyrosine kinase.

M2d or tumor-associated Mϕ are induced by TLR agonists through the adenosine receptor, which leads to production of high levels of IL-10, TGF-β, and VEGF and low levels of IL-12, TNF, and IL-1β thereby providing proangiogenic properties with the features of tumor-associated Mϕ (TAMs).

Mϕ polarization occurs both in physiological and pathological conditions and is a key element of disease development and progression or resolution (Sica et al. 2015). For instance, Mϕ with sustained M1 phenotype are implicated in the development and maintenance of diseases-sepsis, infections, chronic inflammatory diseases [atherosclerosis, chronic obstructive pulmonary disease (COPD), asthma, rheumatoid arthritis (RA), inflammatory bowel disease (IBD), diabetes, lupus, muscle injury, psoriasis, etc.], and neurodegenerative disease. M1 Mϕ release pro-inflammatory cytokines, chemokines, extracellular matrix (ECM) digestive enzymes, prostaglandins, and ROS that aggravate and accelerate damage to the tissues during diseases. On the other hand, M2 Mϕ contributes to lung remodeling and fibrosis leading to lung dysfunction in asthma and COPD (Arora et al. 2018). M2 Mϕ also suppress anti-tumour T cell responses and stimulate tumor angiogenesis (Lee et al. 2019). Additionally, Mϕ are host cells of numerous intracellular pathogens such as *Mycobacterium tuberculosis and Mycobacterium avian complex, Leishmania* parasites, *Salmonella enterica*, *Legionella pneumophila, and Listeria monocytogenes*. These microorganisms have developed different strategies to survive inside Mϕ including modulation of Mϕ phenotypes and reduction of their immune response.

Mϕ targeting aiming at their elimination, reduction of pro-inflammatory cytokines production, phenotype switching or repolarization, enhancement of antimicrobial activity and immune response could improve the current therapy of diverse diseases. However, issues associated with pharmacokinetics (PK, plasma transient peaks), biodistribution (BD, lack of tissue selectivity), and pharmacodynamics (PD, poor intracellular delivery) make such pharmacological approaches poorly efficient and/or toxic. Mϕ targeting nanomedicines may help to solve these limitations suffered by free drug-based therapies.

Therapeutic nanomedicines are composed of nano-object [generally nanoparticles (Nps), nano-objects with three dimensions in the nanoscale, such as liposomes, micelles, lipidic, and polymeric Nps] associated with an active pharmaceutical ingredient (API, low-molecular-weight molecules, or macromolecules such as nucleic acids, polysaccharides, or proteins). Due to their small size and high surface area, nanomedicines (1–1000 nm) exhibit related dimension-dependent

properties or phenomena, that are different from bulk materials, such as endocytic uptake by cells. However, the most important aspect of therapeutic nanomedicines is that upon incorporation into its structure, the API's PK, BD and intracellular traffic no longer depend on the chemical structure of the API but on the structural features of the Nps, such as size, shape, charge, and surface properties. Hence, incorporation into Nps could avoid unspecific distribution in healthy tissues and reduce the adverse effects of API's. Besides, increased solubility and protection from degradation could be achieved by API's incorporation into Nps.

Plain, pegylated, or surface ligand-modified Nps are used for passive and active Mϕ-targeting. The use of plain Nps is based on the natural capacity of Mϕ to engulf micro and nano materials that are anatomically accessible. For instance, KC in the hepatic sinusoids, spleen, and bone-marrow Mϕ (Mϕ of the mononuclear phagocyte system, MPS) rapidly eliminate intravenously (iv) administered plain Np, while AMϕ eliminate inhaled plain Np. On the other hand, pegylated Np [Nps covered by poly(ethylene glycol) (Peg) shield] should be used to access Mϕ others than those of the MPS. Pegylated Np could partially, evade or delay such uptake, circulate for a longer time, and extravasate by convection in zones where vascular endothelium barrier dysfunction and increased permeability occurs for example in tumors or inflamed tissues [enhanced permeation and retention (EPR) effect]. Though the lymph drainage in inflamed zones is not impaired, as in tumors, accumulation in inflamed zones of pegylated Np is anticipated (Maeda 2012; Chen et al. 2017). After site-specific accumulation, either API release or phagocytosis of Nps by accessible Mϕ may occur. However, this last option is less possible since pegylation strongly inhibits cellular uptake (Hatakeyama et al. 2011). Then, active targeting, where ligands (i.e., antibodies, peptides, proteins, sugars) of endocytic receptors are superficially exposed, could increase selective intracellular delivery of nanomedicines to Mϕ. Mannose receptors (MR), scavenger receptors (SR), macrophage galactose lectin (MGL), folate receptor (FR-β), and CD44 are endocytic receptors overexpress on inflammatory Mϕ generally used for active Mϕ targeting nanomedicines (Table 10.1).

Once a nanomedicine is up taken by Mϕ it follows, such as a pathogen, the endo-lysosomal pathway where Np and the loaded API are degraded. Thus, unless the molecular target is localized in the endo-lysosomal system, the loaded API should scape such system to access the cytoplasm and other organelles like the nucleus, mostly relevant for the delivery of DNA, RNA, and proteins. Numerous mechanisms including membrane fusion, membrane destabilization, particle swelling, and osmotic rupture could be used as strategies for Nps and API endosomal escape (Smith et al. 2018).

In this chapter, we will describe the strategies for Mϕ targeting nanomedicine for inflammatory and infectious diseases developed and tested in in vivo models in the last 5 years.

Table 10.1 Surface phagocytic receptors used for active macrophage targeting nanomedicines

Name	Type	Ligands	Cell expression	Overexpression
Mannose receptor (CD206) (MR)	Type I membrane glycoprotein composed of short cytoplasmatic domain, a transmembrane domain, and an extracellular region comprising eight C-type lectin-like domains, a fibronectin type II domain, and an N-terminal CRD	Mannose, fucose, sulfated sugars (sLex), collagen, CD45, tumoral mucins, and neutrophil-derived myeloperoxidases	MØ, endothelial cells: hepatic sinusoidal endothelial cells, dermal endothelial cells lymphatic endothelial cells	Inflammatory conditions M2 MØ Upregulated by IL-4, IL-13, IL-10 Downregulated by IFN-γ
MGL (CD301)	Type II C-type lectin composed of N-terminal cytoplasmic domains, transmembrane domains, extracellular stalk domains, and C-terminal CRD	Galactose and *N*-acetylgalactosamine		Inflammatory conditions M2 MØ
Folate receptor (FR-β)	Glycosylphosphatidylinositol-linked protein receptor	Folic acid	Rapid dividing cells	Inflammatory conditions (IBD, RA, atherosclerotic lesions, TAM) Cannot be detected on resting MØ or any other normal cells M1 and M2 MØ
Scavenger receptors SR-A1 (CD240)	Type II membrane protein, composed of short N-cytoplasmic tail, a transmembrane domain, a spacer region, an α-helical coiled-coil domain, a collagen-like domain, and a C-terminal scavenger receptor CRD	Polyionic ligands including gram-positive LTA, gram-negative bacteria lipid-A moiety of LPS, AcLDL, OxLDL, malondialdehyde-LDL, maleylated-LDL	MØ monocytes	M2 MØ
CD44	Type I transmembrane protein	Hyaluronic acid Chondroitin sulfate	Present in almost all cells: endothelial cells, epithelial cells, fibroblasts, keratinocytes, and leukocytes	Cancer cells, inflammatory epithelial cells, and MØ

AcLDL acetylated LDL, *CRD* cysteine-rich domain, *CD* cluster of differentiation, *LDL* low-density lipoprotein, *LPS* lipopolysaccharide, *LTA* lipoteichoic acid, *MGL* macrophage galactose lectin, *OxLDL* oxidized LDL, *RA* rheumatoid arthritis, *TAM* tumor-associated macrophages

10.2 Macrophage-Targeted Nanomedicines for Inflammatory Diseases

10.2.1 Cardiovascular Diseases and the Role of Macrophages in Atherosclerosis

Cardiovascular diseases (CVD) are the foremost cause of morbidity and mortality worldwide, representing 31% of all global deaths in 2016 (World Health Organization 2017). Atherosclerosis is the main underlying factor of the most common forms of life-threatening CVD such as coronary artery disease and cerebrovascular disease (Frostegård 2013). Atherosclerosis is a chronic arterial disease of inflammatory and metabolic origin. It takes place upon binding of aberrantly elevated levels of low-density lipoproteins (LDL), to the rich proteoglycans extracellular matrix of the endothelium. In there, LDL are chemically modified, mostly oxidated, and also suffering aggregation (Hansson and Hermansson 2011). Oxidized LDL are anomalously deposited in the intima, which inhibit the production of the anti-atherosclerotic labile liposoluble radical NO. NO prevents smooth muscle cell proliferation, leukocyte adhesion, and regulates the vascular tone. Decreased levels of NO prompt coronary vasospasm and cardiac ischemia producing the characteristic anginal pain (Steinberg 2002; Witztum and Steinberg 2001). Oxidized LDL are potent chemoattractant provoking the secretion of monocyte-chemotactic protein 1 (MCP-1) by the inflamed vascular endothelium, which also express cell adhesion molecules, including selectins, cell adhesion protein 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) that attach and collect circulatory monocytes (Libby 2012). The inflammatory process initiated in the endothelium thus is magnified by the arrival of attracted circulatory monocytes that accumulate in the intima and differentiate into tissue Mϕ. Such Mϕ display multiple phenotypes, being the prevailing M1 phenotype releasing pro-inflammatory cytokines and chemokines such as MCP-1, that recruit C–C chemokine receptor-2 (CCR2)-expressing monocytes and promotes the transfer of vascular smooth muscle cell (VSMC) from the media to the intimal layers (Adamson and Leitinger 2011).

The asymptomatic thickening of the intima could start as early as the first months of life. Later, the continuous cell collection and production of inflammatory mediators may induce progressive vascular alterations. A protagonist role in this process is played by Mϕ that express receptors relevant to perpetuate the local inflammation. TLRs for instance, recognize molecular patterns foreign to the body like bacterial pathogens (Krieger 1997), mostly TLR2 and 4, and produce cytokines such as TNF-α that augment local inflammation and VSMC proliferation (Ionita et al. 2010). Mϕ also express SR such as CD36 and SR-AI/II, which account for 75–90% of oxidized or acetylated LDL uptake and degradation; SR-B1 and lectin-like oxidized LDL receptor-1 (LOX-1) participate also in the uptake of oxidized LDL. The phagocytosis of oxidized aggregated and ECM-bound LDL causes intracellular cholesteryl ester accumulation in cytoplasmic lipid droplets. The resulting lipid-laden Mϕ or foam cells are the typical cells present in atherosclerotic

plaque. These foam cells result from cholesterol (chol) imbalance caused by increased chol influx or its esterification [regulated by Acyl coenzyme A: chol acyltransferase-1 (ACAT1) and neutral cholesteryl ester hydrolase (nCEH)] or decreased chol efflux [mediated by ATP-binding cassette transporters A1 (ABCA1), ABCG1 and SR-B1] (Chistiakov et al. 2017; Kzhyshkowska et al. 2012; Li and Glass 2002). The pathological thickening of the intima progress through the accumulation of VSMC, Mϕ, and foam cells, and end up in an anomalous structure known as fibroatheroma, which occludes in variable degree the vascular light. Common features of important lesions such as late core fibroatheromas are the acellular necrotic, hypoxic, acidic cores (made of chol released from senescent cells), absence of ECM (containing hyaluronan, proteoglycans, collagen) immersed in a fibrous cap produced by VSMC, irrigated by variable neovascularization. VSMC and endothelia also capture oxidized LDL employing SR-B1, contributing to the fibroatheroma structure.

Fibroatheromas can be structurally stable for a long time, or the fibrous cap may tend to erode or experience sudden rupture. Stable plaques are clinically silent while unstable plaques can rupture and produce vessel-occluding thrombosis and end-organ damage. Stable plaques have a thick protective fibrous cap, which largely consists of VSMCs that express CCR2, synthesize fibrin and collagen (Sakakura et al. 2013), and produce mostly MMP-2 (Sluijter et al. 2006). Different from stable fibroatheromas, thin (below 65 μm thick) cap fibroatheromas (TCFA) are characterized by immune cell infiltration, inflammatory cytokines production, decreased apoptosis of Mϕ, and necrotic processes. TCFA contain few VSMC, and are rich in inflammatory Mϕ producing proteases that degrade ECM, such as MMP-1, MMP-8 and 9, gelatinases, and stromelysin, which break down collagen and lead to fibrous cap thinning, plaque destabilization, and rupture (Libby 2013). Overall, the presence of senescent cells and prolonged inflammation promotes plaque instability, including elastic fiber degradation and fibrous cap thinning (Goetzl et al. 1996; Childs et al. 2016).

Importantly, plaque Mϕ show *reduced* migratory properties, which hinder the inflammation resolution, attract lymphocytes, and stimulate the progression of lesions into TCFA. The persistent inflammation induces Mϕ apoptosis, which in the absence of efficient phagocytic clearance of apoptotic cells (efferocytosis) and accumulation of debris, enables the plaque necrotic core development (Tabas 2000, 2010; Bäck et al. 2019). The rupture or erosion of TCFA exposes necrotic core contents to the circulating blood and induces platelet activation, which generates rapid thrombotic vascular occlusion and can induce myocardial infarction, stroke, acute limb ischemia, and cardiovascular death (Lusis 2000). Mϕ and smooth muscle cells within atherosclerotic plaques also overexpress the CD40 ligand (CD154), a potent procoagulant tissue factor. Inflammation and thrombotic complications of atherosclerosis are linked since both integrity of the protective fibrous cap and the thrombogenicity of the plaque are controlled by inflammation.

10.2.2 Current Therapeutics for Atherosclerosis

Most of the currently available therapies for atherosclerosis do not focus on the disease-causing pathways active in the vessel walls, but target risk factors such as hypertension and hyperlipidaemia. Current pharmacological treatments aimed to diminish the chol/LDL levels by inhibiting chol synthesis employing statins (atorvastatin, simvastatin, rosuvastatin, and pravastatin). Statins decrease plasma levels of LDL chol and triglycerides and increase plasma levels of high-density lipoprotein (HDL) chol reducing the risk of atherosclerotic CDV and suppressing the progression of atherosclerosis. However, statins induce myopathy, hepatotoxicity, and increase the risk of diabetes mellitus. Other drugs are also used to decrease morbidity and mortality of CVD such as antiplatelet agents, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, beta-adrenergic antagonists, calcium channel blockers, and diuretics (Arnett et al. 2019). Advanced cases require stent-assisted therapies, associated with complications, such as restenosis, inflammation, and thrombosis or coronary artery bypass surgery, the last needing of a longer time for recovery (d'Souza et al. 2017).

Remarkably however even if chol levels are strongly reduced, only a limited regression of the fibroatheroma is achieved (Nicholls et al. 2016) and is not sufficient for many patients to avoid major adverse cardiac event (Sabatine et al. 2017). The underlying reason is that atherosclerotic CVD is the consequence of the unresolve inflammatory response (Moore et al. 2013; Ross 1999; Witztum and Lichtman 2014). Besides the poor outcome of pharmacological treatments, current analytical techniques do not allow satisfactorily detect dangerous TCFA. Altogether these facts underscore the urgent need for better therapeutic and diagnosis strategies to treat and detect TCFA.

Vascular inflammation enhances the risk of recurrent atherothrombotic events, while Mϕ contribute to inflammatory risk (Barrett 2020). Therefore, therapies aimed to induce Mϕ efferocytosis, Mϕ emigration, or Mϕ polarization to a pro-resolving phenotype, should have clinical benefits. Counting on agents capable of selectively localizing within TCFA and performing specific targeting on enriched Mϕ and foam cells, would be key tools to meet such aims.

10.2.3 Macrophage-Targeted Nanomedicines for Atherosclerosis

Nanomedicines may provide the tools to overcome the challenges posed by the treatment and diagnostic of TCFA. Nanomedicines-mediated TCFA treatments and diagnostics should pursue two goals: the first is deceptively simple and consists of accumulating nanomedicines into the plaque; the second is detecting features of plaque lability, such as inflammatory Mϕ or any inflammatory Mϕ related activity. Intravenously injected nanomedicines are optimal to accomplish the first objective,

since different from conventional low-molecular-weight drugs, Nps below 300–400 nm tend to extravasate by convection at inflamed sites. Plaques progress at the walls of the vasculature of blood flow disturbance and low shear stresses. Upon convective extravasation, nanomedicines may passively accumulate at places of high vascular permeability present at inflamed vessels from fibroatheromas by passive targeting (Hu et al. 2018). The extent of nanomedicines accumulation into the plaque is dependent on their size, shape, and surface characteristics (tailored to minimize their liver uptake), and also on the site permeability. Plaque-specific extravasation brings nanomedicines close to plaque Mϕ, but alone is insufficient: to specifically deliver carried therapeutics or execute diagnosis, nanomedicines must be captured by inflammatory Mϕ. To do so, nanomedicines must display ligands that will be recognized and internalized by plaque Mϕ overexpressing specific receptors. Many of these candidate receptors for specific delivery however are ubiquitous. Such is the case of SR-B1 (also expressed in tumors); p-selectin, endothelial cell junctional molecules, such as PECAM1, expressed on most leukocyte sub-types, platelets, and at junctions between endothelial cells (overexpressed not only in infarcted myocardium, but also in arthritis, renal and hepatic diseases, acute lung injury, and graft rejection). Most of the preclinical active targeted nanomedicines for atherosclerosis rely on targeting either the transferrin receptor 1 (TfR1) (overexpressed not only in foam cells, Mϕ, and VSMC, but also on tumor cells) or the FR-β (overexpressed on activated, but not on resting Mϕ, and implicated in a diversity of inflammatory and autoimmune diseases) (Chen et al. 2020). The use of targeted delivery to a population with prevalent comorbidities thus may hamper its effective translation (Wang et al. 2021a). Nonetheless, even the newest strategies target Mϕ receptors which are not selectively expressed on plaque Mϕ, such as MR CD-206 (expressed only on immature monocyte-derived DCs); CD9 (ubiquitously expressed tetraspanin protein); hyaluronan acid receptors; CD36 SR (upregulated during monocyte-to-Mϕ differentiation and stimulated by hypertension, high glucose and oxidative stress, also expressed on DC, erythrocytes, adipocytes, and platelets), SR-AI/II (not expressed on monocytes, upregulated during monocyte-to-Mϕ differentiation), and chemokine receptor CCR2-binding motif of MCP-1.

Active targeting strategies for plaque Mϕ may be aimed at diagnostic or therapeutic purposes. The current diagnostic methods cannot detect nascent lesions. Targeting specific molecules or cells such as inflammatory Mϕ as a sign of plaque vulnerability are an emerging field for molecular imaging. Therapy instead pursues to reduce the inflammation within the plaque, on the basis of an array of strategies, such as (1) shifting Mϕ phenotype from M1 to M2 (to reduce the inflammatory component), (2) minimizing Mϕ chol content, by reducing its uptake and/or increasing its efflux (to reduce the formation of foam cells), (3) avoiding foam cells necrosis by delivering anti-senescent agents (to reduce their input to inflammation), and (4) increasing their ability to make efferocytosis (to avoid the senescence of foam cells).

In the last 5 years, a growing number of approaches showing the response of ex vivo and in vivo fibroatheroma/TCFA models to pharmacological treatments, as well as improvements in image diagnosis techniques performed with

macrophage-targeted nanomaterials, has been published. A detailed description of such voluminous information is out of the scope of this section, but the emerging landscape shows that most of the preclinical approaches developed between 2017 and 2020 employed polymeric [mostly *poly*(*lactic-co-glycolic acid*), PLGA, and hyaluronic acid, HA, based] and lipid-based (liposomes, HDL, other) nanomedicines, followed by micelles, dendrimers, cyclodextrins, and carbon-based, inorganic, biomimetic NPs. Most therapeutic nanomedicines combined macrophage targeting plus increased cholesterol efflux. Along 19 years (2001–2020), 27 clinical trials assessed the performance of mainly lipid-based therapeutics nanomedicines (Chen et al. 2021).

A selection of relevant strategies illustrating the latest experimental approaches will be discussed in the following sections. Several targeted nanomedicines do not show therapeutic improvements but are proof of concepts for effective macrophage targeting. Included are examples of macrophage targeting achieved only by passive targeting to the plaque, by tailoring nanomedicines size, shape, and surface features to control PK and extent of extravasation.

10.2.3.1 mAbCD9-Targeted Nanomedicines for Anti-senescence Drug Delivery

Statins are used to reduce blood chol level by inhibiting HMG-CoA reductase and are also being experimentally used as anti-senescent agents, because of their telomerase shortening inhibition, anti-inflammatory, and antioxidant activities (Boccardi and Paolisso 2014; Sørensen et al. 2019). Since dying Mϕ and foam cells are deleterious for fibroatheroma stability, a recent work has developed Mϕ-targeted NPs for delivery of statins as anti-senescent agents (Pham et al. 2021). To that aim, NPs were designed that combined monoclonal antibody (mAb) anti-CD9-mediated senescent Mϕ targeting, with delivery of rosuvastatin (RSV). CD-9 is a cell surface glycoprotein highly expressed in some smooth muscle cells and in Mϕ-rich plaques in atherosclerotic lesions that controls cell migration, proliferation, and adhesion (Nishida et al. 2000). CD9 expression is considered a marker of inflammatory cells; it induces cellular senescence through the phosphatidylinositide 3 kinase-AKT-mTOR-p53 signal pathway and aggravates atherosclerotic plaque formation in apolipoprotein E knockout apo E (−/−) mice (Cho et al. 2020). RSV was loaded in mesoporous silica Nps (MSN) (137 nm, $-\zeta$ potential 16.3 mV, 2.8 nm pore size, 0.1517 cm^3/g pore density) and covered by 3 layers of polymers: poly(ethylene glycol)-*block*-poly-glutamic acid) [PGA], poly-lysine (PLL), and hyaluronic acid (PHA). Then Nps were surface decorated with ~55 anti-CD9 mAbs (CD9-HMSN@RSV) per Np. The inner PGA and PLL layers avoided fast RSV leakage, inhibited plasma protein opsonization, decreased MPS uptake, and prolonged MSN circulation. In an in vivo model of atherosclerosis, the anti-CD9 mAb was observed to efficiently target the MSN, delivering RSV to inflammatory Mϕ; an additional release of free anti-CD9 mAb, that restrained the progression of

cell senescence, occurred upon dissociation of the external PHA layer by plaque hyaluronidase.

10.2.3.2 MCP-1-Targeted Nanomedicines for Competitive Inhibition of MMP1

To recognized and treat rupture-prone plaques possessing thin fibrous caps monocyte-binding, collagenase-inhibiting, and gadolinium-containing peptide amphiphile micelles (MCG PAMs) were recently developed (Chin et al. 2020). The micellar structure was made of three components: MCG (a peptide binding motif of MCP-1 to target monocytes and VSMC); Col-1 peptide (peptides having collagen cleavage recognition site sequence, [VPMS-MRGG] which are recognized by MMP-1 undergoing rapid degradation) to inhibit MMP collagenases and preserve the integrity of plaques; and diethylenetriamine pentaacetic acid (DTPA)-chelated Gd^{3+} to allow simultaneous magnetic resonance imaging of plaques. These micelles bonded to monocytes and Mϕ and were small enough ~15 nm since leaky endothelial tight junctions are 20–1330 nm range (Chin et al. 2019) to extravasate and labeled atherosclerotic aortas in apo E (−/−) mice in proportion to the severity of the lesions. Furthermore, micelles successfully detected plaques in diseased mice and acted as contrast agents for molecular imaging. Micelles competed with collagenases, treated mice showed 61% and 113% increase in fibrous cap thickness compared to non-targeting micelle- and PBS-treated mice, respectively. Overall, this multimodal Nps offers new opportunities for noninvasive diagnosis and treatment of atherosclerotic plaques.

The efficacy of the (a) and (b) approaches may be counterbalanced however by their huge structural complexity, which would make difficult its industrial scaling up.

On the other side, the following approaches employed nanomedicines of higher structural simplicity:

10.2.3.3 Hyaluronan-Targeted Nanomedicines

Hyaluronan (HA), a key component of the extracellular matrix, is a linear polymer of *N*-acetylglucosamine and a β-glucuronic acid. HA regulates cell adhesion, migration, and proliferation. The HA lining on vascular endothelium mediates immune cell rolling and extravasation during inflammation. Mϕ express several HA-binding receptors, including CD44, ICAM-1, LYVE-1, RHAMM, and TLR-4. The biological activity of HA depends on its degree of polymerization: low-molecular-weight (MW) HA stimulate inflammation and angiogenesis, whereas high MW (megadalton) HA inhibit these processes. The lack of immunogenicity and the low cost of HA have driven its application in biomedicine; however, its systemic administration is hampered by its rapid blood clearance and susceptibility to hydrolysis. Nanoparticulate HA can be prepared by deposition on the surface of either

lipid or polymeric Nps, or through transformation of its polymeric backbone in Nps by chemical modification of their carboxyl groups. A recent report showed that HA-Nps (90 nm, ζ potential −31.3 mV) are structurally stable under hydrolysis and efficiently target fibroatheroma-associated pro-inflammatory Mϕ in an apo E (−/−) atherosclerotic mice (Beldman et al. 2017).

10.2.3.4 Oxidized Phosphatidylcholines-Targeted Nanomedicines

Since oxidized phosphatidylcholines (oxPCs) of oxidized LDL bind to the CD36 receptor of intimal Mϕ in atherosclerotic lesions, Mϕ-targeted liposomes were designed by including a type of oxPCs (1-palmitoyl-2-(4-keto-dodec-3-enedioyl) PC) in the liposomal bilayer (Dhanasekara et al. 2021). Targeted liposomes (90 nm) co-localize with intimal Mϕ and CD36 receptors and show 1.4-fold higher accumulation in aortic lesion areas than non-targeted liposomes. This strategy could be useful to detect early stages of lesions and identify Mϕ amounts and distribution in the lesion, providing evidence of lesion vulnerability.

Biomimetic nano-carrier platforms are nanomedicines inspired by the way cells (erythrocytes, leukocytes, thrombocytes) or lipoproteins that interact with the vascular system behave and represent an alternative to drug synthetic Nps because of their longer circulation times, MPS evasion, and favorable interactions with target cells (Zinger et al. 2021). The following examples illustrate recent biomimetic approaches used to target plaque Mϕ.

10.2.3.5 Synthetic HDL Biomimetic-Based Passively Targeted Nanomedicines for LXR Delivery

Synthetic HDL (sHDL) Nps mimic the structure and function of pre-β HDL a specific small fraction (2–5%) of endogenous HDL (Fielding and Fielding 1995). sHDL are nanodisc structures of 8–12 nm of lipid bilayers wrapped around apolipoprotein A-I (apoA-I) or apoA-I synthetic peptide (ETC-642) at 1:2 w/w peptide to lipid ratio. Pre-β HDL accumulate in the atheroma area where they efflux the excess of chol from foam cells and deliver it to the liver for elimination (Kingwell et al. 2014). Acute treatment with sHDL, 4–6 times a week, reduces plaque burden in coronary artery disease patients (Tardif et al. 2007, 2014).

Liver X nuclear receptor (LXR) agonists induce the expression of chol transporters (ATP-binding cassette transporters ABCA1 and ABCG1), which stimulates excess chol efflux from plaque Mϕ to endogenous HDL acceptors. LXR agonists reduced plaque burden in apo E (−/−) murine models of atherosclerosis (Kratzer et al. 2009). However, LXR agonist induce liver toxicity. LXR agonists activate lipogenesis in liver, which induce hepatic steatosis and excretion of excess triglycerides into systemic circulation, increasing the levels of very low-density lipoproteins (VLDL) and pro-atherogenic LDL and intermediate-density lipoprotein. Besides, the high doses of LXR agonists needed to get anti-atherosclerotic effects,

the poor aqueous solubility, and the low levels of endogenous HDL acceptors in patients, limit the clinical translation of LXR agonists.

sHDL has been used as a carrier for the LXR agonist T0901317 (T1317), where sHDL acted also as an acceptor of chol efflux from Mϕ. The formulation induces atheroma regression in a severe model of atherosclerosis (Guo et al. 2018; Aye et al. 2010; Costet et al. 2000). Then HDL delivers efflux chol to the liver resulting in reduction of fibroatheromas (Kingwell et al. 2014).

In a recent work, the original formulation of sHDL-LXR agonist was adjusted to maximize encapsulation efficiency, drug retention, Np purity, upregulation of ABCA1/ABCG1 gene expression, and chol efflux. The improved formulation was observed to halt the development of atherosclerotic lesions on an early onset plaque formation apo E −/− murine atherogenesis model, with an average atheroma area of 4.9% compared to 12.2% and 10.8% after treatment with T1317 or sHDL alone, respectively (Yuan et al. 2021). sHDL-mediated delivery of LXR agonists would avoid hepatic toxicity by achieving effects with doses between 15 and 78-fold lower than those administered by oral route. In this approach however the sHDL were administered by the intraperitoneal (ip) route, which is not suitable for clinical use. Besides, despite two decades of preclinical studies, the industrial production of HDL proteins is still dealing with unsolved challenges (Brusinia et al. 2020). An interesting alternative to the industrial synthesis of lipoproteins is the therapeutic platform based on squalene (SQ), a cholesterol precursor, that can be bonded to drugs and upon iv injection, it is spontaneously inserted within lipoproteins, such as LDL, to be used for targeted delivery. SQ-based Nps (SQ Nps) were recently shown to target atherosclerotic plaque (Sobot et al. 2017). Indeed, significant accumulation of SQ Nps in both early and advanced atherosclerotic plaque, in apo E (−/−) mice, and interaction with plaque resident Mϕ was found (Brusinia et al. 2020).

10.2.3.6 Plaque Targeting Via Biomimetic Liposomes

Platelets, that interact with multiple substrates and release active factors, are fundamentals for atherosclerosis initiation and progression (Gawaz et al. 2008; Wu et al. 2017; Huo et al. 2003). In the early-stage platelets adhere to the injured endothelium, then platelets induce release of chemo-attractants, upregulation of endothelial adhesion molecules, and secretion of MMPs (Langer and Gawaz 2008). Activated platelets attract leukocytes, promote smooth muscle cell and fibroblast proliferation, and stimulate collagen synthesis, contributing to atherosclerotic lesion progression and maturation (Ross 1985). P-Selectins expressed on platelets directly interact with inflammatory cells (Totani and Evangelista 2010).

Another example of biomimetic nanomedicine made of liposomes covered with platelet proteins for targeted delivery of rapamycin (RAP) to the inflamed endothelium was recently published (Song et al. 2021). To that aim, hybrid vesicles made of platelet membranes and artificial lipid membranes (P-lipo) of (90 nm, ζ potential −20 mV) were prepared. RAP P-lipo reduced the average plaque area from ~53 to 14% and stabilized the atherosclerotic plaques in an apo E (−/−) mice. P-Lipo

showed a 5.91-fold increase in accumulation into the atherosclerotic lesion compared to conventional liposomes.

The last is an example of preclinical succeeding nanomedicines that because of their chemical nature and poorly explored biocompatibility and biodistribution, regulatory organisms may find difficult to accept.

10.2.3.7 Increased Efferocytosis with Passively Targeted Nanomedicines to Monocytes

Accumulation of apoptotic cells in the necrotic core is characteristic of atherosclerotic plaque. These cells are removed by efferocytosis (Latin: "to take to the grave"), a highly conserved process triggered by "eat me" ligands that signal phagocytes to induce uptake (Arandjelovic and Ravichandran 2015; Yurdagul Jr. et al. 2017). Conversely, cells may overexpress "don't eat me" ligands to avoid removal. Such is the case of the CD47 molecule, a major mechanism by which red blood cells enable immune evasion, and cancers establish and propagate disease. The anti-phagocytic CD47 signaling has a critical role in atherosclerosis, being upregulated in the atherosclerotic plaque (Kojima et al. 2014, 2016). CD47 binds with the signal regulatory protein-α (SIRPα), a transmembrane protein expressed in Mϕ and activates the SH2 domain-containing phosphatase-1 (SHP-1) initializing the intracellular signaling that inhibits phagocytosis. In this way, disease vascular cells resist to be removed and plaque expansion is promoted. Antibody-mediated blockade of CD47 accelerates the off-target removal of healthy tissue, such as the elimination of red blood cells in the spleen (Kojima et al. 2016), causing anemia and reduced oxygen-carrying capacity, which limits the translational potential of systemic pro-efferocytic therapies.

In a recent approach, nanomedicines to efficiently reduced plaque inflammation by interrupting CD47-SIRPα signaling in monocytes and Mϕ were developed (Flores et al. 2020). The system involves Peg-functionalized single-walled carbon nanotubes (SWNTs) loaded with a small-molecule inhibitor of SHP-1. Peg-SWNTs showed ability to accumulate within Ly-6Chi inflammatory monocytes (Smith et al. 2014), the primary circulating cells recruited to the diseased artery, where they differentiate into lesion Mϕ (Swirski et al. 2007). Peg-SWNTs were shown to accumulate within the atherosclerotic plaque, reactivate phagocytosis, and reduce plaque burden in atheroprone apo E (−/−) mice without compromising safety.

10.2.4 Inflammatory Lung Diseases and the Role of Macrophages

Pulmonary inflammation is generated by the innate immune system in response to harmful foreign stimuli such as invading pathogens, allergens, air pollutants, toxic chemicals, cigarette smoke, or by endogenous signals such as damaged cells.

Inflammation is an underlying pathology of several common respiratory diseases, such as COPD, asthma, acute lung injury (ALI), pulmonary fibrosis, and infectious diseases such as bacterial pneumonia or respiratory viruses. Although each disease expresses a unique inflammatory response, there are some shared characteristics: persistent inflammation, impaired repair process, and lung remodeling. The environmental adaptation of pulmonary macrophages plays a central role in pulmonary immunity response and is a determining factor in the establishment of chronic inflammatory pulmonary pathologies (Ogger and Byrne 2021). There are two distinct populations of pulmonary Mϕ: AMϕ, which are in the lung lumen in contact with the alveolar epithelial cells; and interstitial Mϕ, which reside in the parenchyma between the respiratory epithelium and the blood vessels. In healthy conditions, the main function of AM is to clear apoptotic cells and cell debris, express CD206, CD169, CD11c, CD163, and MARCO. In an inflammatory context, monocytes are recruited in the lung where they differentiate and add to the pool of AMϕ. M1 activation occurs by the classical stimulus or by losing their exposure to regulatory ligands like IL-10 or CD200 after epithelial cell injury during inflammation. The outcome of AMϕ activation is determined by pathogen-specific properties and by the host immune response to them. For example, during COVID-19-associated pneumonia Mϕ can produce a hyper-inflammation known as Mϕ activation syndrome or cytokine storm which is associated with constant production of pro-inflammatory cytokines (e.g., IL-6, IL-8, TNF-α, IL-1β) leading to acute respiratory distress syndrome (ARDS) (Ogger and Byrne 2021). Resolution of inflammation is achieved after clearance of foreign agents, elimination of recruited immune cells such as neutrophils by efferocytosis and IL-4/IL-13-mediated M2 Mϕ switching to initiate lung tissue repair (Schett and Neurath 2018). The switch to M2 phenotype has a central role in the resolution of pulmonary inflammatory conditions but could also contribute to fibrotic pathology through increased production of TGF-β as in idiopathic pulmonary fibrosis, or when leading to allergic airway chronicity as in asthma (Hussell and Bell 2014; Schett and Neurath 2018). An excess of Mϕ MMPs release produces structural changes in the lungs that can lead to pulmonary emphysema. In addition, during lung emphysema, AMϕ upregulates TLR2 and TLR4 expression and increases inflammation in response to infections. Conversely, cigarette smoke and COPD lead to decreased TLR2 and their phagocytic capacity, impaired bacterial killing, and neutrophil efferocytosis, but increase pro-inflammatory cytokines, chemokines, MMPs, and ROS production.

10.2.5 Macrophages-Targeted Nanomedicines for Pulmonary Inflammatory Diseases

Mϕ targeting nanomedicines for lung inflammatory diseases could decrease production of pro-inflammatory cytokines, induce M2 phenotype polarization, or reduce profibrotic activity of M2 Mϕ (Table 10.2). Local administration of Nps via intratracheal instillation or inhalation is a direct and more straightforward alternative

Table 10.2 Summary of macrophage-targeted nanomedicines for the treatment of inflammatory lung diseases

Disease	Receptor	Ligand	Nanomedicine	Drug	Aim	References
ALI	ud	Hexapeptide CLPFFD	Au Nps	–	M1–M2 switching	Wang et al. (2020, 2021a)
Lung fibrosis	CD44	Anti-CD44 antibody	Au Nps	Imatinib	Reduced M2 macrophages	Codullo et al. (2019)
COPD/interstitial lung disease	ud	PEI	Calcium phosphate PLGA Np	CCL-2, IP-10, and IFN-γ siRNA	Anti-inflammatory	Frede et al. (2017)
Staphylococcal pneumonia	ud	Peptide CRVLRSGSC	Porous silicon Nps coated with fusogenic liposomes	IRF5 siRNA	M1–M2 switching	Kim et al. (2018)
VILI	–	–	Lipid Np	pre-miR-146a	Mitigation lung injury	Bobba et al. (2021)
Pulmonary fibrosis	–	–	Cationic liposomes	Mdb2 siRNA	M1–M2 switching	Wang et al. (2021c)
Toxic industrial chemicals	CD44	HA	Multilamellar liposomes	DEX and *N*-acetyl cysteine	Anti-inflammatory	Rivkin et al. (2017)
ALI	–	–	Liposomes	DEX and TPGS	Anti-inflammatory	Shah and Banerjee (2019)
Asthma	SR-A1	PGP-Me	pH-sensitive archaeosomes	DEX-P	Anti-inflammatory	Altube et al. (2016, 2017)

ALI acute lung injury, *AM* alveolar macrophages, *DEX* dexamethasone, *DEX-P* dexamethasone phosphate, *PEI* polyethylenimine, *PLGA* poly lactide-*co*-glycolic acid, *TPGS* tocopheryl polyethylene glycol succinate, *ud* undetermined

than iv administration (that leads to MPS accumulation) for AMϕ targeting. Local pulmonary administration avoids the first-pass metabolism, has low enzymatic activity (compared to the gastrointestinal tract, GIT), and allows the use of lower doses than those necessary in a systemic administration, avoiding unwanted systemic effects (Loira-Pastoriza et al. 2014). For instance, intratracheal administration of AuNps coated with the hexapeptide CLPFFD for macrophage targeting (~13 nm, ζ potential −36 mV), in contrast to iv and intraperitoneal (ip) administration, led to more accumulation of AuNps in the lungs but less in the liver and other organs, in lipopolysaccharide (LPS)-induced ALI mouse model (Wang et al. 2021b). After being uptaken, CLPFFD-AuNp blocked the acidification process of the endosomes, inhibiting TLR4 signaling and the activation of NF-κB and IRF3 and the further pro-inflammatory response (Yang et al. 2016). Treatment with CLPFFD-coated AuNps increased granulocyte colony-stimulating factor (G-CSF) levels in the lung, IL-4 and IL-13 in serum, and M2 Mϕ in lungs (Wang et al. 2020).

Pulmonary fibrosis is a pathological consequence resulting from altered wound healing in response to persistent lung injury. CD44 is overexpressed by lung fibroblasts and Mϕ isolated from bronchoalveolar lavage fluid (BALF) of systemic sclerosis patients with interstitial lung disease. Anti-CD44-AuNps loaded with Imatinib (Imb), a tyrosine kinase inhibitor able to interfere with the downstream activation of profibrotic pathways (21 nm, ζ potential −46.3 mV) reduced the percentage of M2 Mϕ and IL-8 release in BALF of interstitial lung disease patients. Intratracheal administration of anti-CD44-AuNps-Imb resulted in AMϕ accumulation and reduction of pathological changes (collagen deposition and fibrotic tissue) as effective as ip administration of Imb however avoiding Imb systemic side effects, on bleomycin lung fibrosis murine model (Codullo et al. 2019). Possible translocation of AuNps to blood and local and systemic toxicity should be studied. Although intratracheal instillation is a useful tool to test nanomedicines in preclinical settings because it deposits a large number of Nps in the lungs, this administration route is not currently used in clinics.

In spite that large amount of dose could be ingested, a major part of intranasal administration of polyethyleneimine (PEI) coated calcium phosphate/PLGA Nps loaded with a mixture of pro-inflammatory cytokines siRNAs (~145 nm, ζ potential +23 mV) was observed in lung Mϕ and DC 1 h after administration to a sterile inflammation mice model. The expression of CCL-2, INF-γ inducible protein-10 (IP-10), and IFN-γ were downregulated in lung, and less loss of weight and a reduced number of cells within the BALF were observed, indicating a less severe inflammation (Frede et al. 2017). However, cationic Nps are known to be more toxic than negative or neutral counterparts and could lead to inflammation.

Silencing the Irf5 gene, that upregulates TNF, IL-1, IL-6, IL-15, IL-18, IL-23, and downregulates IL-10, induce repolarization of Mϕ toward the M2 phenotype. An interesting approach to decrease acute lung inflammation, promote bacterial phagocytosis and tissue repair during *Staphylococcal* pneumonia used porous silicon Nps with a fusogenic pegylated liposomal coating to Mϕ targeting and intracytoplasmic release Irf5 siRNA after iv administration. Nps (~190 nm, ζ potential −10 mV) targeted monocyte-derived Mϕ recruited to infected lungs and

significantly lowered expression of Irf5 in Mϕ collected from BALF, but not in pulmonary homogenates (composed of epithelial, endothelial, and interstitial cells) from *S. aureus* infected mice. This strategy allowed all the mice to be rescued from a lethal dose of *S. aureus* (Kim et al. 2018).

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly transmissible and pathogenic coronavirus that emerged in late 2019. SARS-CoV-2 infection in humans manifests as mild symptoms to severe life-threatening pneumonia. SARS-CoV-2 begins replicating in the epithelial cells of the respiratory tract, then migrates down into the airways and enters alveolar epithelial cells in the lungs (Hu et al. 2020). This rapid replication and the destruction of lung cells triggers a local immune response, recruiting Mϕ and monocytes, release cytokines, and activate T and B cell immune responses, which in some cases, may lead to a dysfunctional strong immune response (Tay et al. 2020). This is evidenced in patients with severe COVID-19 with high plasma levels of Mϕ inflammatory protein 1α (MIP1α), G-CSF, IP-10, MCP-1, TNF-α, IL-1β, IL-2, IL-7, and IL-10 (Huang et al. 2020). Also, IL-6 plasma levels increase over time and are more elevated in non-survivors than survivors (Guirao et al. 2020). Despite that the mechanisms of lung injury and organ failure are still under investigation, these elevated cytokine levels plus Mϕ activation syndrome, elevated C-reactive protein, *d*-dimer levels, and renal dysfunction, suggest that cytokine storm may contribute to the pathogenesis of COVID-19 (Fajgenbaum and June 2020). In the context of Mϕ targeting, some works have recently shown that nanomedicines could mitigate lung injury and reduce lung fibrosis by Mϕ repolarization.

Mechanical ventilation is the standard of care for patients with ARDS including COVID-19 patients. However, the physical forces produced by mechanical ventilation aggravate lung dysfunction through a phenomenon known as ventilator-induced lung injury (VILI) (Slutsky and Ranieri 2013). To mitigate lung injury caused by VILI, AMϕ modestly increase expression of microRNA (miR-146a), (small non-coding RNA that acts as negative posttranscriptional regulators) associated with innate immunity and inflammation. A pre-miR-146a-PEI polyplexes loaded in lipid Np containing the antioxidant tocopheryl polyethylene glycol succinate (TPGS) (160 nm, ζ potential −0.6 mV) increased miR-146a expression in vitro, preferentially accumulated into AMϕ and mitigated lung injury during mechanical ventilation after intratracheal administration to mice (Bobba et al. 2021).

Current clinical evidence supports the possibility that pulmonary fibrosis may be one of the major complications in severe COVID-19 survivors after recovery (Han et al. 2021; Zou et al. 2021). M2 Mϕ polarization contributes to the pathogenesis of pulmonary fibrosis producing TGF-β1 and activating fibroblasts to myofibroblasts. A promising therapeutic target to reduce M2 polarization is the methyl-CpG-binding domain protein 2 (*Mbd2*) that mediates transcriptional repression in methylated DNA regions. Fibrotic lungs of patients with severe COVID-19 exhibited significant M2 Mϕ infiltration and *Mbd2* overexpression (Wang et al. 2021c). Intratracheal administration of *Mbd2* siRNA encapsulated in cationic liposomes (~100 nm, ζ potential +3.2 mV) protected mice from bleomycin-induced lung injury and fibrosis

by a significant reduction in the expression of fibrotic markers (fibronectin, collagen I, and α-SMA) and the M2 Mϕ marker Arg 1 (Wang et al. 2021c).

Systemic (iv hydrocortisone or oral DEX) corticosteroid therapy for 7–10 days in patients with severe COVID-19 is recommend by WHO to reduce mortality. Inhaled corticosteroids however could lower levels of inflammatory markers and improve lung physiology during ARDS, and potentially could inhibit coronavirus replication in host cells (Nicolau and Bafadhel 2020; Yamaya et al. 2020). Nowadays, several clinical trials are ongoing for the use of inhaled corticosteroids to treat or prevent COVID-19. Pulmonary administration of DEX-loaded Np however could specifically target AMϕ that initiate and spread inflammation in the lung, blood, and myeloid and lymphoid tissues, being a strategy to intervene in the sub-acute phase of COVID-19. This would better control Mϕ activation syndrome and cytokine storm, that would help patients recover faster and more efficiently than with free DEX treatment (Lammers et al. 2020).

Most clinical studies for pulmonary administration of Nps use nebulizers to generate aerosols (da Rocha Sandro et al. 2019). The use of nebulizers is the most direct, quick, and easiest way to aerosolize an aqueous suspension, also allowing a quick translation from animals to clinical tests with the same formulation and the same administration device previously evaluated preclinically (Cipolla et al. 2013). Furthermore, liposomes are almost the only Nps found in clinics (liposomal amikacin Arikayce) and in advanced clinical trials and this is mainly due to their high lung biocompatibility. Nebulization is not a gentle process and liposomes may lose their structure (modify vesicle size, lamellarity, membrane fluidity, and the amount of encapsulated drug) due to exposure to shear forces and the air–liquid interface (Carvalho and McConville 2016). However, liposomes with lipid bilayers of transition temperature above the nebulization temperature, such as those containing saturated phospholipids, are more robust to the nebulization process. For instance, Arikayce are unilamellar liposomes of 300 nm composed of dipalmitoylphosphatidylcholine (DPPC) and chol at 2:1 weight ratio.

There are few works studying inhalation delivery of liposomal DEX. In one of the pioneer work, it was shown that intratracheal administration of plain liposomes loaded with DEX increase the retention time of DEX in the lung as well as improve prophylactic efficacy in counteracting LPS-induced lung injury compared to free DEX (Suntres and Shek 2000). An active targeting strategy with DEX loaded in mannosylated liposomes was able to further decrease the production of TNF-α when administered in a model of inflammation induced by LPS in rats (Wijagkanalan et al. 2008). Recently, it was shown that co-delivery of DEX and antioxidants into liposomes effectively reduce lung inflammation. For instance, DEX and *N*-acetyl-cysteine (antioxidant) co-loaded in hyaluronan covalently linked multilamellar liposomes (HA-MLV, soybean phosphatidylcholine (SPC): dipalmitoylphosphatietanolamine (DPPE):chol 75:5:20 molar ratio) reverted animal weight loss to a level similar to that of control mice in a model of pulmonary inflammation and edema caused by exposition to toxic industrial chemicals (Rivkin et al. 2017). On the other hand, DEX disodium phosphate (DEX-P) loaded into plain liposomes (DPPC/1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol ammonium

salt, POPG) containing TPGS (~270 nm, ζ potential −15 mV) significantly lowered oxidative stress, IL-1β, IL-6, and TNF-α levels in BALF in a mouse model of acid-ALI (Shah and Banerjee 2019).

None of the above strategies however consider the cytoplasmatic delivery of DEX where its cytoplasmic receptor is located. Novel pH-sensitive nanovesicles (ApH) that incorporated the natural SR-A1 ligand, the archaeolipid PGP-Me (2,3-di-*O*-phytanyl-sn glycero-1-phospho-(3′-sn-glycerol-1′-methylphosphate), were designed to increase the delivery of DEX-P to the cytoplasm of AMϕ. PGP-Me, an archaeolipid extracted from halophilic archaebacteria, can be incorporated into lipidic nanostructures such as vesicles or lipid Nps without any chemical synthesis. Besides, the chemical structure of PGP-Me (sn1,2 glycerol ether having fully saturated polyisoprenoid chains) make archaeolipids resistant to lipolytic enzymes, hydrolytic or oxidative attacks, and too harsh conditions such as nebulization. ApH (~150 nm, ζ potential −40 mV) showed increased cellular internalization than conventional liposomes in AM NR8383 and in J774A.1 cells. Due to their great cellular internalization, ApH were able to improve the anti-inflammatory and antioxidant activity of DEX-P in LPS-activated Mϕ. In addition, ApH were efficiently aerosolized with a vibrating mesh nebulizer, proving to be more stable than high stable [hydrogenated SPC (HSPC): chol 3:1 weight ratio] liposomes to the nebulization process (Altube et al. 2016). In addition, after nebulization, ApH could overcome a pulmonary surfactant barrier and deliver its hydrophilic cargo into J774A.1 cells to a greater extent than conventional liposomes (Altube et al. 2017). These strategies could be further investigated to evaluate their anti-inflammatory capacity in the context of COVID-19.

Overall, local administration via inhalation with stable and biocompatible Np has shown to be the best option for Mϕ targeting to reduce lung inflammation or fibrosis, protecting extremely sensitive siRNA or microRNA, and reducing access to corticosteroids such as DEX to health tissues.

10.2.6 Inflammatory Bowel Diseases and the Role of Macrophages

Inflammatory bowel diseases (IBD) such as Crohn's disease (CD, transmural inflammation in the complete intestine) and ulcerous colitis (UC, diffuse superficial mucosal inflammation in colon) are chronic, relapsing, progressive disabling disorders of the gastrointestinal tract (GIT), that affect millions of persons in the world (Palmela et al. 2015). Intestinal inflammation and epithelial damage induced by the uncontrolled activation of the immune system characterized these diseases.

Mϕ in the lamina *propria* of the intestine are vital for keeping homeostasis and the balance between the commensal microbiota and the host (Bain and Mowat 2014). These Mϕs are TLR-hyporesponsiveness acting as noninflammatory scavengers of microbes, express high levels of IL-10, contribute to the maintenance of

regulatory T (Treg) cells, and stimulate epithelial cell renewal (Bain and Mowat 2014). During intestinal inflammation, neutrophils and monocytes are sequentially recruited to mount a suitable immune response. These cells, following activation by PAMPs, produce IL-12, IL-23, and IL-1β that promote Th1 and Th17 cell responses toward invading microorganisms and produced epithelial damage. In a healthy person, efferocytosis of apoptotic neutrophils induces suppression of pro-inflammatory cytokines production and enhanced IL-10 and TGF-β production switching of M1–M2 Mϕ and starts the resolution phase. M2 Mϕ reduce the Th1 and Th17 responses and are indispensable to regenerate the epithelial barrier. In IBD patients however this process is dysregulated, conducting to accumulation of M1 Mϕ in the inflamed colon, which induces Th17 cells with increased expression of pro-inflammatory markers (IL-23, TNF-α, IL-1b, IL-6, and iNOS), contributing directly to the defective intestinal barrier function (Na et al. 2019). Besides, a defective signaling through TGF-β, which impairs M2 Mϕ recutting and deficiencies in efferocytosis is also involved in the pathogenesis of IBD.

10.2.7 Current Therapeutics for IBD

Treatment of IBD is symptomatic and depends on the stage of the disease. The classic oral drugs include 5-aminosalicylic acid, corticosteroids, and immunosuppressive drugs (azathioprine and methotrexate, MTX). Intravenous anti-TNF-α Mab infliximab and adalimumab, or the subcutaneous certolizumab pegol are used when conventional drugs fail. However, all treatments have limited benefits because of their systemic adverse effects displayed during long-term use. Therapy with Mab is expensive and could lead to serious adverse reactions, such as infection, anaphylaxis, and myelosuppression (Abraham et al. 2017).

10.2.8 Macrophages-Targeted Nanomedicines for IBD

Mϕ-targeted nanomedicines for IBD could reduce or block pro-inflammatory cytokines production, switch M1–M2 Mϕ phenotype, and promote wound healing (Table 10.3). Oral is the most ideal administration route, as it presents great safety, patient compliance, and is cost-effective for production. Oral administration also allows direct access to the intestinal colonic mucosa. However, the success of oral-targeted Nps depends on their ability to remain structurally stable along the GI transit, and on the possibility of accessing Mϕ. If well, the GIT is the most hostile environment in the organism, the differences between the inflamed mucosa of IBD patients and the normal gut can be exploited for Nps-mediated targeted delivery. IBD patients show loss of the inner adherent and the outer mobile mucus layer; infiltration of immune cells such as neutrophils, Mϕ, lymphocytes, and DC (Antoni et al. 2014); accumulation of positively charged proteins such as transferrin (Tirosh

Table 10.3 Summary of macrophage-targeted nanomedicines for the treatment of IBD and RA

Disease	Receptor	Ligand	Nanomedicine	Drug	Aim	References
IBD	MR	Mannose	Polymeric Np (carboxymethyl inulin)	Apremilast	M1–M2 switching	Sun et al. (2018)
		Mannose	Polymeric Np (chitosan)	miR-146b	M1–M2 switching, wound healing	Deng et al. (2019)
	CD44	HA	Polymeric Np (PLGA-chitosan covered)	CD98 siRNA KPV	Anti-TNF-α	Xiao et al. (2016, 2017)
		HA	HA-bilirubin conjugate	Bilirubin	Antioxidant	Lee et al. (2020)
		CS	pH-sensitive natural silk fibroin	Curcumin	Antiinflammatory	Gou et al. (2019)
	MGL	Galactose	Polymeric Np (PLGA-chitosan covered)	TNF-α siRNA	Anti-TNF-α	Huang et al. (2018) and Xiao et al. (2018)
	FR	FA	Pegylated polymeric Np (PLGA-Peg-FA)	6-Shogaol	Anti-inflammatory	Zhang et al. (2018b)
	PepT1	KPV	Polymeric Np (PLGA)	CyA	Anti-inflammatory	Wu et al. (2019)
	SR-A1	PGP-Me	Archaeosomes	DEX and BR	Anti-inflammatory Anti-oxidant	Higa et al. (2017, 2020)
RA	FR	FA	Stimulus-sensitive pegylated polymeric Np (PLGA-Peg-FA)	Mcl-1 siRNA	Macrophages apoptosis	Sun et al. (2019)
		FA	Redox sensitive pegylated AgNp	Ag+	M1–M2 switching	Yang et al. (2021)
	SR	DS	DS-5β-cholanic acid conjugate	MTX	Anti-inflammatory	Heo et al. (2017)
		DS	DS-MTX conjugate	MTX	Anti-inflammatory	Yang et al. (2017)
	CD44	HA	pH-sensitive pegylated HA-5β-cholanic acid	MTX	Anti-inflammatory	Alam et al. (2017)

AO antisense oligonucleotide, *BR* bacterioruberin, *CS* chondroitin sulfate, *CyA* cyclosporine A, *DEX* dexamethasone, *DS* dextran sulfate, *FA* folic acid, *HA* hyaluronic acid, *IBD* inflammatory bowel diseases, *KPV* lysine–proline–valine, *LRP-1* low-density lipoprotein receptor-related protein, *MTX* methotrexate, *PLGA* poly lactide-*co*-glycolic acid, *RA* rheumatoid arthritis, *SR* scavenger receptor

et al. 2009), bactericidal/permeability increasing protein, and antimicrobial proteins (Canny et al. 2002; Ramasundara et al. 2009); and disruption of the epithelial barrier function with the concomitant increase of permeability (Goggins et al. 2013). In this pathological context, macrophages can be found at the luminal side of the inflamed mucosa, favoring its accessibility from the oral route. On the other hand, the local pH is decreased [from 6.8 to 7.2 in normal mucosa to 5.5 to 2.3 in IBD patients' mucosa (Fallingborg et al. 1993)] and the GI transit is enhanced, with frequent diarrhea. Overall, to reach mucosal macrophages in an intact form, oral Nps must overcome low pH, the activity of degradative enzymes, and avoid being trapped within the mucus layer during the transit across the healthy portions of the gut. Negatively charged and small-sized Nps have been reported to accumulate to a high extent into the inflamed mucosa (Lamprecht et al. 2001). Nps can passively accumulate in the inflamed colon sites based on the epithelial enhanced permeability and retention (eEPR) effect (Watanabe et al. 2016; Lamprecht 2010). Np could then release their content; however, only if Np is taken up, loaded drug could be intracellularly released and hence therapeutic efficacy could be enhanced.

Mannose is widely used as a superficial ligand due to its high binding affinity for MR, its simple structure, non-immunogenicity, and for being inexpensive. Mannose-modified chitosan Nps was used to encapsulate microRNA mimic (miR-146b mimic) complexed with PEI to inhibit M1 Mϕ activation and promote wound healing. Oral administration of Nps (213 nm, ζ potential +28.3 mV) switched M1–M2 phenotype by regulating TLR4 signaling pathway resulting in the repression of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and promotion of intestinal epithelial cells regeneration by regulating STAT3-dependent IL-10 production (Deng et al. 2019). Initial burst release in the upper GIT from polymeric Nps and hydrolysis of the ligand-Nps linkage could be reduced by incorporation into enteric-coated capsules or hydrogels. For example, mannosylated decamethylenediamine-grafted-carboxymethyl inulin Nps loaded with apremilast, a phosphodiesterase 4 inhibitor that switches M1- into M2-phenotype, were freeze-dried and encapsulated into enteric-coated capsules for oral administration. The synthesis of the self-assemble mannosylated amphiphile took several steps: carboxymethylation of inulin, introduction of hydrophobic segments of decamethylenediamine through an acid amide bond, and mannose linkage through a Schiff's base. The mannosylated-Nps (323 nm, ζ potential −11.7 mV, 5% mannose graft) showed great uptake in inflamed Mϕ and large accumulation in inflamed colon of DSS-model (60%); however, in vivo activity was not reported (Sun et al. 2018).

HA and chondroitin sulfate (CS) were also used for superficial Nps modification to target CD44 which is overexpressed on colonic epithelial cells and Mϕ in UC tissues. HA is biocompatible, biodegradable, and has several modification sites; however, HA is degraded by GI hyaluronidases. Nps can be embedded into chitosan/alginate hydrogels that protect Nps along the passage through the upper GIT, and then disassemble in the colon, releasing the loaded Nps. HA-modified PLGA Nps were prepared to simultaneously deliver CD98 siRNA (transmembrane protein complex related to mucosal damage and inflammation) and the antioxidant and anti-inflammatory curcumin. Nps (~246 nm, ζ potential −14 mV) were prepared

by a complex with several steps, double emulsion-solvent evaporation method. Briefly, first CD98 siRNA-spermidine complex was loaded into PLGA-polyvinyl alcohol (PVA) containing curcumin Nps, then chitosan was superficially adsorbed, and finally Nps were functionalized with HA via ester bond formation. Nps embedded in a chitosan/alginate hydrogel prevented mucosal damage and reduced inflammation, inhibiting the DSS-induced overexpression of CD98 and TNF-α in the colon (Xiao et al. 2016). In further work, same Nps were loaded with the naturally occurring anti-inflammatory tripeptide lysine-proline-valine (KPV). Oral administration of HA-Nps-KPV (270 nm, ζ potential −5.3 mV) exhibited a much stronger capacity to prevent mucosa damage and downregulation of TNF-α compared with non-targeted Np (Xiao et al. 2017).

A different strategy based on the self-assembling of an amphiphilic conjugate of hydrophilic HA and the hydrophobic endogenous antioxidant bilirubin that confers hyaluronidase resistance has been recently developed. The conjugate synthesis takes several steps starting from an acid form of HA and an aminoethylene–bilirubin conjugate and the latter conjugation through amine linkage in a ~4 molecules of bilirubin per each 100 kDa HA molecule ratio. The HA-bilirubin Nps (~400 nm, ζ potential −46 mV) accumulated in inflamed colonic epithelium and restored the epithelium barriers in DSS-induced colitis model (Lee et al. 2020). Other self-assembled Nps based on CS-modified natural silk fibroin were used for curcumin delivery by oral and iv routes (Gou et al. 2019). Hydrophobic domains of silk enable the self-assembly of Nps where curcumin is kept trapped using a mild desolvation method, followed by CS superficial conjugation via amide bonds. CS-silk Nps (175 nm, ζ potential −35.5 mV) embedded in chitosan/alginate hydrogel accumulated in colitis tissues after oral administration and undergo internalization by macrophages. Remarkably, due to the pH-sensibility of silk fibroin, curcumin could be released into the cytoplasm upon endocytic uptake. Interestingly, intravenous administration resulted in higher colonic Nps accumulation than oral administration.

Lactobionic acid (LA) was used for modification of chitosan through amidation reaction to target MGL. Galactosylated-chitosan coated PLGA Nps loaded with TNF-α siRNA were prepared by laborious double emulsion-solvent evaporation method. Galactosylated-Nps (~300 nm, ζ potential +12.2 mV) resisted the harsh conditions of the GIT and displayed superior efficacy in TNF-α gene silencing than galactose-negative Np in colon of DSS-mice (Huang et al. 2018). Co-embedded galactosylated-Nps (260 nm, ζ potential −8 mV, galactose content 0.23 mg/g Np) with recombinant IL-22 (inductor of epithelial regeneration) in a chitosan/alginate hydrogel significantly inhibited TNF-α, infiltration of mucosal neutrophils, and promoted the colon epithelia regeneration (Xiao et al. 2018). Chitosan Np could release the siRNA into the cytoplasm since it is known to overcome lysosomal sequestration by membrane destabilization or through a proton sponge effect (Coya et al. 2019).

FA-pegylated PLGA Nps loaded with the ginger active compound 6-shogaol to target both colon epithelial cells and Mϕ were prepared using commercial PLA-PEG-FA block copolymer added to a PLGA polymer/6-shogaol emulsion.

FA-PLGA Nps (250 nm, ζ potential −24 mV) embedded in a chitosan/alginate hydrogel relieved colitis signs and enhanced wound repair in DSS-mice through downregulation pro-inflammatory molecules (TNF-α, IL-6, IL-1β, and iNOS) and upregulation anti-inflammatory (Nrf-2 and HO-1) players (Zhang et al. 2018a).

The tripeptide KPV, additionally being an anti-inflammatory agent, is a ligand of peptide transporter 1 (PepT1) that is highly expressed in inflammatory colon epithelial cells and Mϕ (Wang et al. 2018). Complex method with several steps was used to prepare cyclosporine A (CyA) loaded KPV-PLGA (covalent linked) Nps were further coated with montmorillonite/chitosan for reduced CyA leakage in the upper GIT and mucus adhesion, respectively. Oral administration of Nps (185 nm, ζ potential +30 mV) decreased the levels of inflammatory cytokines and relieved colitis symptoms (Wu et al. 2019).

SR-A1 is involved in the innate immune response in intestinal inflammation (Komai et al. 2017). SR-A1 negative regulates NF-κB signaling and stimulates production of reparative cytokines, shifting Mϕ phenotype (Zong et al. 2018). However, SR-A1 has been sparsely explored as a receptor for Mϕ targeting. Solid lipid nanoparticles (SLN) containing the natural SR-A1 ligand PGP-Me has shown to deliver DEX to Mϕ of inflamed mucosa with enhanced anti-inflammatory activity and reconstitution of the epithelial barrier. Ultrasmall SLN containing PGP-Me (∼67 nm, ζ potential −41 mV) were highly uptaken and significantly reduced the levels of TNF-α, IL-6, and IL-12, compared to SLN without PGP-Me, by macrophages stimulated with LPS (Higa et al. 2017). Besides SLN showed enhanced mucus penetration. Further, incorporation of the high antioxidant C50 carotenoid bacterioruberin into SLN-containing PGP-Me displayed high anti-inflammatory and antioxidant activities on a gut inflammation model made of Caco-2 cells and LPS stimulated THP-1 derived Mϕ reducing TNF-α and IL-8 release and ROS production. Nps also reversed the morphological changes induced by inflammation (normal microvilli, well-defined tight junctions, desmosomes, interdigitations, and F-actin filaments) and increased the transepithelial electrical resistance, partly reconstituting the barrier function (Higa et al. 2020). One of the most important aspect of this work is that after in vitro digestion, the anti-inflammatory activity of Nps was retained, indicating the high structural resistance of Nps prepared with lipids extracted from halophilic archaebacteria.

Overall, oral administration with highly stable or embedded into protective capsules, for GI degradation and drug leakage reduction, active targeted Nps have shown to be a good option for Mϕ targeting to reduce intestinal inflammation, oxidative stress, and promoted wound healing in IBD. Some receptors used for targeting however, such as CD44 and PepT1, are not only expressed on inflammatory Mϕ but also on epithelial cells. Additionally, mucus penetration and retention of Nps in inflamed intestine were scarcely studied.

10.2.9 Rheumatoid Arthritis and the Role of Macrophages

Rheumatoid arthritis (RA) is a systemic, chronic, autoimmune disease with a high world prevalence (0.5–1%) that causes long-term disability and low quality of life (Davis and Matteson 2012). RA cause progressive destruction of joints and vascular, metabolic, osseous, and psychiatric comorbidities. Multiple causes are associated with RA pathogenesis, but all generate inflammatory cell infiltration, synovial hyperplasia, autoantibodies production, and excess of synovial fluid. These in turn cause joint swelling, pain, progressive stiffness, joint destruction, and bone erosion. During this process, principally Mϕ produce pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), which stimulate synovial fibroblast (synoviocytes) to produce MMP that degrade the joint and activate osteoclast which produce bone erosion (McInnes and Schett 2017). Besides the inflammatory mediators, ROS and RNS produced by Mϕ play a key role in RA pathogenesis. Due to the hypoxia-inducible factor (HIF-1α) expression and the high ROS level, in arthritic joints Mϕs are M1 subtype (Peiser and Gordon 2001).

10.2.10 Current Therapeutics for RA

Current therapeutic agents are divided into four categories: disease-modifying anti-rheumatic drugs (DMARD, such as MTX, hydroxychloroquine, and sulfadiazine), glucocorticoids (DEX, hydrocortisone, prednisone), nonsteroidal anti-inflammatory drugs, and biological agents (anti-TNF-α Mabs such as adalimumab, certolizumab and infliximab, the anti-IL-6 Mab sarilumab and the anti-IL-17 Mab ixekizumab). Even though these treatments are effective up to a certain level, there are still numerous limitations. For example, many patients do not respond to DMARD and all drugs showed numerous adverse effects. Mabs to some degree reverse the progression of RA however ~50% of patients who respond at the beginning, stop responding after 1 year (Singh et al. 2011). Additionally, these patients are prone to acquire local or systemic infections, such as tuberculosis or tumors, and the cost of treatments with Mabs are extremely high.

10.2.11 Macrophages-Targeted Nanomedicines for RA

M1 Mϕ should be eliminated/switched to M2 phenotype to alleviate synovial inflammation. Vascular permeability in RA sites is high, which allows passive accumulation of pegylated Nps through so-called ELVIS effect (Extravasation through Leaky Vasculature and subsequent Inflammatory cell-mediated Sequestration) (Wang and Goldring 2011) like the EPR effect observed in solid tumors. For instance, DEX loaded into pegylated micelles or pegylated liposomes showed

accumulation into inflamed joints and reduced inflammation. DEX loaded into polymerized stealth liposomes suppressed pro-inflammatory cytokines (TNF-α and IL-1β) in joint tissues, decreasing the swelling of inflamed joints in the adjuvant-induced arthritis rat (AIA) model (Fang et al. 2020). Since pegylation reduces Mϕ uptake of Np, accumulated Nps in the inflamed joints act as depots where DEX should be released, active targeting to Mϕ could enhance the intracellular accumulation of the loaded drug and improve therapeutic efficacy.

FA, dextran sulfate, and HA were used as ligands of Nps for targeting FR-β, SR-A, and CD44 receptors on Mϕ, respectively, to treat CIA mice or AIA rat by the iv route (Table 10.3).

Myeloid cell leukemia-1 (Mcl-1) is an anti-apoptotic signal overexpressed in Mϕ from RA joints. Polymeric Nps composed of a commercial FA-Peg-PLGA as targeting ligand, a novel polyketal (PK3) as a pH-sensitive polymer, and a Mcl-1 siRNA/DOTAP (dioleoyloxy)propyl-trimethylammonium methyl-sulfate) lipoplexes core (143 nm, ζ potential 3.6 mV) was shown to be taken up by Mϕ, while the siRNA was released into the cytosol. Nps showed to be accumulated in inflammation zones and high efficacy in the AIA rat model (Sun et al. 2019).

In a different strategy, glutathione-sensitive FA modified silver Np (FA-AgNps) was used to induce M1 Mϕ apoptosis and M2 switching. FA-AgNps were obtained in several steps. Briefly, heterofunctional lipoyl-FA-Peg (LA-Peg-FA) was synthesized through DCC/NHS coupling chemistry (~45% of LA-Peg modification) and then LA-Peg-FA was attached through Ag-sulfide bond on the AgNps surface. After entering cells, FA-AgNps (30 nm, ζ potential −6 mV) released Ag^+ in response to intracellular glutathione (~1000-fold higher than in extracellular fluids), and induced M1 Mϕ apoptosis and scavenged ROS causing M2 polarization. FA-AgNps displayed long-circulation life and showed biodegradability. FA-AgNps accumulated in inflamed joints decreased clinical score and showed better end outcomes, than MTX and MTX-AgNps in CIA mouse. FA-AgNps reduced TNF-α, IL-1β, and IL-6 almost to a normal level, reduced M1, and increase M2 Mϕ-specific biomarkers in inflamed joints (Yang et al. 2021).

Mϕ and synoviocytes of patients with RA overexpressed the SR-A that is in part responsible for pro-inflammatory cytokines and MMP generation. Dextran sulfate (DS) is a hydrophilic biocompatible and biodegradable polysaccharide ligand of Mϕ (SR-A). Self-assembled Nps of DS-5β cholanic acid and MTX-DS showed to be accumulated in inflamed joints and reduced the pro-inflammatory status. An amphiphilic DS derivative was synthesized by a simple two-step procedure where 9 hydrophobic 5β-cholanic acid were conjugated to 100 sugar residues of DS to obtain self-assembled Nps (220 nm) where MTX was loaded by dialysis. Fluorescently labeled Nps showed accumulation in inflamed knee and ankle of CIA mice; however, substantial fluorescence was found in liver and kidney. Despite rapid MTX released from Nps in the initial 3 h (~50%), MTX-Nps showed higher efficacy against CIA mice compared to free MTX alone (Heo et al. 2017). To reduce the MTX leakage, MTX was covalently linked with DS through condensation reaction. The amphiphilic DS-MTX conjugate obtained self-assembled into ~100 micelles and accumulated at inflamed site after iv administration; however, organ distribution

was not shown and 60% of MTX was released in the first hours. DS-MTX micelles mitigated synovitis and protected articular cartilage (Yang et al. 2017).

CD44 is overexpressed in the early stages of inflammation to recruit immune cells. Self-assembled pegylated HA-5β-cholanic acid was used for MTX delivery to Mϕ in CIA mice. Peg was linked to amphiphilic HA-5β-cholanic acid conjugate (11 5β-cholanic acid moieties per 100 sugar residues of HA). Pegylated-HA acts as the hydrophilic shell and 5β-cholanic acid as the hydrophobic core, where MTX was encapsulated. Calcium phosphate was further loaded into HA to selectively release MTX in acidic media (50% at pH 5). Nps (~220 nm) showed accumulation in arthritic paws and reduction of inflammation safety with a high dose of MTX, in CIA mice (Alam et al. 2017). However, the nonspecific uptake by hepatic sinusoidal endothelial cells and the low stability in physiological conditions of HA-based Nps should be considered (Choi et al. 2012).

Overall, iv administration of active targeted Nps has shown to reduce joint inflammation in RA. However, a great accumulation of Nps into Mϕ of the MPS and the use of receptors that are also expressed in other tissues such as MR or CD44, or receptor that could be overexpressed by comorbidities could reduce the effectivity and increase the potential off-target toxicity of these strategies.

10.3 Macrophages-Targeted Nanomedicines for Infectious Diseases

Mϕ-targeted nanomedicines have been used to intracellular co-localize antimicrobial drugs with infections agents like leishmanial parasites, tuberculous, and nontuberculous mycobacterium.

10.3.1 Leishmaniasis and the Role of Macrophages

Leishmaniasis, caused by the protozoa parasite *Leishmania*, affects around one million persons annually and causes 20,000–30,000 deaths. Most of the leishmaniasis cases occur in Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan.

There are three clinical manifestations of leishmaniasis: cutaneous (CL), mucocutaneous (MCL), and visceral (VL). Leishmania promastigotes enter the skin by the bite of sandflies and invade local phagocytic cells. Promastigotes transform into amastigotes and survive into the phagolysosomes, where they multiply. After being released, promastigotes are distributed to local or distant phagocytes. VL is characterized by parasites colonization of liver, spleen, and bone marrow Mϕ, skin Mϕ. CL is characterized by Langerhans cells and DC colonization, while in MCL, lymph nodes and mucosal cells are also colonized.

10.3.2 Current Therapeutics for Leishmaniasis

Pentavalent antimonials (Sb^v) were the first antileishmanial agents used, but given their toxicity, treatment evolved depending on the clinical manifestation to parenteral liposomal amphotericin B (AmB) (AmBisome) and paromomycin, and oral miltefosine. The iv infusion of AmBisome (plain unilamellar liposomes of 80 nm) is a standard treatment for the lethal VL and clinical efficacy in CL patients was shown (Wijnant et al. 2018; Wortmann et al. 2010). However, all treatments show disadvantages such as variable cure rates, toxicity, high costs, and emerging resistance.

10.3.3 Macrophages-Targeted Nanomedicines for Leishmaniasis Treatment

Since liver and spleen Mϕ are targets of both leishmania parasites and iv administered plain Nps, in a seminal work it was shown that Sb^v-liposomes eliminate 99.8% of the parasites in vivo but with a 100-fold lower dose than free Sb^v (Alving et al. 1978). Almost 20 years later, FDA approved AmBisome for the treatment of VL. AmBisome alters PK, BD, and PD properties of AmB resulting in improved efficacy, tolerability, and reduction of the nephrotoxicity associated with conventional AmB deoxycholate administration (Fungizone). The main limitation of AmBisome however is its high cost, even a single dose (5 mg/kg 97.5% cure rate in VL patients in India) is costly. Besides, AmBisome is unstable above 25 °C (Croft and Olliaro 2011), increased size and decreased AmB content have been reported after 72 h storage at room temperature (Zia et al. 2017). Even slight changes in the AmB to phospholipids molar ratio or modifications in the manufacturing procedure affect the efficacy and toxicity of liposomal AmB (Olson et al. 2008).

Several different types of Nps (liposomes, polymeric Nps, SLN) encapsulating diverse clinically approved or preclinical antileishmanial drugs have shown promising results in experimental models of VL and CL as recently been reviewed (Singh et al. 2019, 2020; Nafari et al. 2020; Sousa-Batista and Rossi-Bergmann 2018; Espuelas et al. 2016). However, it should be relevant to find only one or a few doses of treatment that could be economically affordable. Here, only the more recent Mϕ targeting strategies that employed approved drugs and with the best chances of translation will be described.

Immunomodulation could be a way to improve the current therapy by enhancing efficacy and/or reducing drug intake. The *Leishmania* parasite has developed several strategies, which can inhibit Th1 response by diverting DC to a state that induced parasite-infected Mϕ toward anti-inflammatory Th2 response. These lead to reduced production of pro-inflammatory cytokines (TNF-α, FN-γ), ROS, and RNS. Additionally, these early controls in Th1 response may help in the early control of innate immunity that eventually leads to compromised adaptive immunity characterized by decreased proliferation of CD4+ and CD8+ T cells and enhanced Th2 response by

means of anti-inflammatory cytokines (IL-4, IL-10, and TGF-β). Co-loading of an immunomodulatory drug with a leishmanicidal drug into Mϕ targeting nanomedicines could reverse the immune bias from Th2 to Th1 response. However, excessive inflammatory response may worsen the prognosis of CL, whereas restoration of Th1 response is necessary to cure VL patients (Murray et al. 2000).

MR, MGL, SIGN-R1, DCSIGN, and other cell surface Mϕ receptors that recognize polysaccharide residues on parasite could lead to immune stimulation. For instance, AmB loaded into mannan-PLGA nanospheres and mannosylated-chitosan Nps have shown immunomodulation in the treatment of VL. High expression of MHCII and co-stimulatory molecules (CD40, CD80, and CD86), induction of a pro-inflammatory response (IL-6, IL12p40, and TNF-α) on Mϕ and higher in vivo efficacy was shown by AmB-loaded mannan-PLGA nanospheres against *L. infantum* infection in comparison with Fungizone (Barros et al. 2015). Interestingly both empty and AmB-loaded mannosylated-chitosan Nps (~200 nm, ζ potential +31.7 mV) induced high expression of pro-inflammatory mediators (IFN-γ, IL-12, and TNF-α), suppress levels of immunosuppressive cytokines and increased iNOS production in *L. donovani* infected hamsters. However, AmB-loaded mannosylate-Nps significantly reduced splenic parasite burden (~90%) compared with non-targeted Np (Asthana et al. 2015a). AmB was also loaded into lactoferrin-coated PLGA Np (~200 nm, ζ potential +21.7 mV). Lactoferrin binds to multifunctional glycolytic protein (GAPDH) but also to MR and DCSIGN. Infection results in overexpression of GAPDH to accomplish iron requirement of parasite. Lactoferrin-Nps has shown to increase production of pro-inflammatory mediators while downregulated disease-stimulating cytokines in *L. donovani*-infected hamsters, resulting in higher reduction of splenic parasite burden (~88%) compared with AmBisome (~68.8%) and fungizone (~55.6%) (Asthana et al. 2015b). More recently, galactofuranoside containing liposomes showed to produce a mixed polarization profile into Mϕ (induction of genes encoding M1 pro-inflammatory cytokines (IL-12, IL-1β, and TNF-α and iNOS) and the M2 cytokine IL-10). Galactofuranoside-liposomes enhanced Th1 immune response showing induction of crucial pro-inflammatory cytokines and iNOS in target organs, but reduced serum inflammatory cytokines in mice. Treatments however only modestly reduce parasite loads in liver and spleen, compared with AmBisome, against *L. donovani* infection (Guegan et al. 2019).

The oral route is suggested for CL and VL however because of their poor aqueous solubility and bioavailability, most drugs, except miltefosine, are administered by parenteral routes. New approaches intended to change the administration route have recently been described. For instance, to enhance AmB oral absorption and minimize its side effects, three formulations (cochleates, chitosan Nps, and self-emulsifying drug delivery systems, SEDDS) are in clinical trials (Serrano and Lalatsa 2017). While cochleates and SEDDS are based on conventional pharmaceutical technology AmB-chitosan Np allows oral targeting to Mϕ of lung, liver, and spleen, but avoids delivery to kidneys. AmB was encapsulated in core-shell Nps (200 nm) made of palmitoyl-methyl dimethyl-trimethyl-6-*O*-glycol chitosan (GCPQ). The palmitoyl chains of GCPQ form a nanocomplex with AmB in the core while the hydrophilic

quaternary ammonium groups interact with carboxylate group of AmB and form the particle shell. The AmB-GCPQ Nps obtained are very stable and can be reconstituted from a dry powder. AmB-GCPQ Nps are taken up by enterocytes and Peyer's patches that enable the translocation of AmB-GCPQ Np. High levels of AmB were found in liver, lung, spleen, and bone marrow, as a result of Mϕ phagocytosis of Nps. Lower AmB concentrations were found in target organs after oral administration of AmB-GCPQ Nps compared to iv AmBisome; however, there were no differences in the efficacy of both formulations in *L. infantum*-infected Balb/c mice (Serrano et al. 2015).

Topical treatment of CL should be advantageous compared to parenteral treatments since it could eliminate the local parasites preventing the risk of dissemination, reduce scar formation and disfigurement, reduce the toxicity of parenteral drugs, improve patients' compliance, and reduce treatment costs. However, the location of infected Mϕ in the border of the lesions with epidermal thickening difficult drug penetration. Nps could enhance drug permeation and target drug intracellularly, besides Nps could have immunomodulatory or wound-healing properties.

Several works show the effectivity of Sb^V or miltefosine-loaded liposomes for the topical treatment of CL. For example, deformable liposomes loaded with Sb^V (195 nm, ζ potential +32.8 mV) showed tenfold higher skin retention in the deeper skin layers than free drug, without the use of classical permeation enhancers, and reduced parasite burden in *L. tropica* infected Balb/c mice (Dar et al. 2018). Recently, deformable liposomes co-loaded with miltefosine and the polyphenol apigenin (120 nm) showed 3.2-fold higher skin permeation compared with free drug and a 9.5-fold reduced parasitic burden in *L. mexicana*-infected Balb/c mice (Dar et al. 2020). Stearylamine (with per se antileishmanial activity)-bearing liposomes loaded with Sb^V improved the Sb^V permeation compared with Sb^V cream and reduced lesions size in *L. major* infected Balb/c mice (Moosavian et al. 2019).

The use of AmB-loaded Nps for topical treatment of CL however is more complex. On one hand, the structural properties of drugs (MW and hydrophobic/hydrophilic balance) directly impact skin penetration and their leishmanial activity. On the other hand, susceptibility differences of leishmania species and their lymphatic nodule dissemination could contribute to this problem. The low permeation of AmB (high MW and insolubility in water) through uninfected and infected skin explained the unsuccessful topical AmB treatment on *L. major* infected mice (El-On et al. 1984). Liposomal AmB with increased skin permeation has shown different efficacy depending on the leishmanial strain. SinaAmpholeish 0.4% gel is a semisolid formulation of liposomal AmB (80 nm) produced by Exir Nano Sina (Tehran, Iran) for topical treatment of CL. It is claimed that sinaAmpholeish permeates *stratum corneum* and reaches the dermal and epidermal macrophages. SinaAmpholeish was effective against *L. major* using a Balb/c back rump infection model (US Patent US 20150147382A1). Clinical studies show that SinaAmpholeish has a 95% effectiveness for rural leishmaniasis with *L. major* and 30% for urban leishmaniasis with *L. tropica*. However, topical SinaAmpholeish was unable to cure a murine *L. mexicana* infection model that closely resembles clinical disease

(Varikuti et al. 2017). The virulence of this leishmania strain might require a higher dose, an earlier or a more prolonged treatment.

Interestingly, recently it was shown that iv administration of AmB-loaded chitosan-TPP Nps (70 nm, ζ potential +25.5 mV) reduced lesion size and parasite load in *L. major* infected Balb/c mice, with more efficacy than AmBisome. However, poor AmB permeation into and through mouse skin showed that AmB-loaded chitosan Nps are not appropriate candidates for topical treatment of CL (Riezk et al. 2020). These Nps release the AmB on the skin, which then should permeate into and through the skin in the free form. In the same sense, topical chitosan-coated poly (isobutyl cyanoacrylate) Nps (187 nm, 53.8 mV), gelled with pluronic F127 resulted in partial and incomplete healing lesions in *L. major* infected Balb/c mice (Malli et al. 2019).

Overall, if well for more than 20 years AmBisome has shown to be effective for leishmaniasis treatment, we are still searching for inexpensive treatments that could be orally or topically applied.

10.3.4 Tuberculosis and the Role of Macrophages

Tuberculosis (TB) is a life-threatening disease caused by *Mycobacterium tuberculosis* (*Mtb*). TB is one of the top 9 causes of death worldwide. Mycobacteria, transmitted by the air, enter the respiratory tract, and are phagocytized by AMϕ, where they spread across the lungs. Weeks later, mycobacteria could distribute to liver and kidneys. Inside Mϕ, mycobacteria multiply within phagosome arresting their fusion with lysosomes. In this way, Mtb subvert host immune responses and utilize the lung Mϕ as a niche for growth and proliferation.

10.3.5 Current Therapeutics for TB

Although the BCG (Bacille Calmette–Guérin) vaccine prevents childhood TB, it fails to protect adults already infected or sensitized to mycobacteria. Six-month treatment with 4 drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) is the standard chemotherapy for drug-susceptible TB. Prolonged treatments (up to 24 months) with pyrazinamide combined with second-line drugs (e.g., fluoroquinolones, ethionamide, cycloserine, capreomycin, or prothionamide) are required for multidrug-resistant strains or low proliferating phases. Limited bioavailability and poor absorption; rapid degradation or excretion; systemic distribution; toxicity and high costs of these drugs, as well as low patient compliance to the long-lasting treatments, are drawbacks of the current therapies.

10.3.6 Macrophages-Targeted Nanomedicines for TB

Passive and active targeting is possible for Np delivery in TB by the iv route. Pegylated Nps could be accumulated in granulomas by the recruitment of Mϕ that phagocytosed the Nps (Trousil et al. 2019) or via a process that resembles EPR effect (Fenaroli et al. 2018). Several studies demonstrating in vivo achievements using passively targeted Nps against TB have recently been reviewed (Hussain et al. 2019; Donnellan and Giardiello 2019). Although great number of works, from more than 10 years, have focused on active targeting Mϕ demonstrating enhanced uptake of the targeted Nps relative to non-targeted formulations, only a small portion of works shows in vivo results (Baranyai et al. 2021). Besides, biodistribution studies corroborated that KC of the liver is the cell population that concentrates the highest proportion of the injected Nps dose. Additionally, a change of the currently used oral administration route by iv administration should ensure the complete elimination of Mtb with few doses.

The inhalation route appears the most promising drug entry to achieve high local concentrations of the drug in the infected Mϕ, since ~75–80% of infections persist localized in the lungs. Besides, local administration could reduce dose level and prevent adverse reactions, avoiding GI degradation and first-pass metabolism. Despite this, very few studies have focused on Mϕ targeting nanomedicines for inhalation therapy against TB. Spray drying of Nps using suitable carriers can preserve Nps during dehydration and can provide microparticles (MP) with aerodynamic diameter (daer) optimal for alveolar deposition (1–5 μm). Upon contact with pulmonary fluid, these MP could dissociate and release the Nps for further Mϕ uptake. For example, inhaled mannitol MP encapsulating SLN loaded with rifabutin with daer of 4–5 μm delivered higher amounts of rifabutin in lungs compared with free drug in mannitol MP, additionally relevant quantities of drug were also detected in liver and spleen in mice. This system efficiently reduced the bacterial burden in lung, spleen, and liver of Mtb-infected mice (Gaspar et al. 2017). Mannosylated-SLN and liposomes were also designed for powder inhalation. Mannosylated-SLN [containing hexadecanoic acid (aminoethyl α-D-mannopyranoside)amide] achieved a respirable particle fraction of 30–50%, showed high Mϕ uptake even in the presence of a commercial replacement of natural pulmonary surfactant and showed high rifampicin retention in lungs after intratracheal powder aerosolization in mice (Maretti et al. 2019a, b; Truzzi et al. 2020). Mannosylated-liposomes loaded with moxifloxacin co-spray drying with dextran improved liposomal physical stability, achieved a respirable particle fraction of more than 75%, and showed deep lung deposition after intrapulmonary administration using dry powder inhaler in rats (Hamed et al. 2019). The efficacy of these two approaches on TB models remains to be tested.

Self-assembling hydrophobized HA-nanogels (500 nm, 2.4 mV) loaded with an antimicrobial peptide (LLKKK18) were nebulized to *M. avium* or *Mtb*-infected mice. HA-nanogels were highly internalized by Mϕ and reduced the intracellular levels *M. avium* and *Mtb* in vitro, together with reducing pro-inflammatory cytokine

levels (IL-6 and TNF-α). Intratracheal administration using a MicroSprayer® aerosolizer of peptide-HA-nanogels significantly reduced bacterial levels in the lungs (Silva et al. 2016).

If well only recently has been addressed, local administration via inhalation with passive or active targeted Np could be a good option for Mϕ targeting to increase antimicrobial activity, while reducing access of antibiotics to healthy tissues.

10.3.7 Nontuberculous Mycobacterial Disease

Pulmonary nontuberculous mycobacterial (*Mycobacterium avium* complex [MAC]) disease is a chronic, frequently progressive infection characterized by necrotizing inflammation, bronchiectasis, associated irreversible lung damage, and increased mortality. In 2018, the FDA approved liposomal amikacin for inhalation (LAI; Arikayce) to treat refractory MAC lung disease, becoming the first liposomal formulation specifically approved to be administered by the inhalation route. Arikayce are neutrally charged liposomes (~300 nm) administered via a PARI eFlow vibrating mesh nebulizer. Arikayce improved the efficacy of conventional treatment (macrolide, ethambutol, and rifamycin) for MAC lung disease in terms of microbiological results, although a clinical benefit has not yet been established (Olivier et al. 2017; Shirley 2019). Treatment of nontuberculous mycobacteria infections can be challenging because they can persist in biofilms or as intracellular infections within Mϕ. One of the main advantages of inhaled LAI treatment versus conventional amikacin-free intravenous administration is its ability to target pulmonary Mϕ. In vitro, LAI can improve amikacin uptake by ~fourfold into THP-1 Mϕ compared with free amikacin. In rats, nebulized LAI increased amikacin concentrations in pulmonary Mϕ by eightfold at 24 h post-dose relative to free amikacin. Furthermore, compared to iv-free amikacin, LAI increased 274-fold the mean AUC-time curve in Mϕ (Zhang et al. 2018b). Consequently, LAI can improve lung retention time while minimizing systemic exposure, compared to iv administration of free drug.

10.4 Conclusions and Prospects

AmBisome, one of the first FDA-approved nanomedicine in the 90s, and the recently approved Arikayce, are based on plain liposomes that are naturally highly uptaken by MPS Mϕ and AMϕ to deliver massively and specifically the loaded amphotericin B and amikacin by the iv or inhalatory routes, respectively, to the infected macrophages. AmBisome and Arikayce significantly reduced the toxicities of free drugs (nephrotoxicity of AmB and renal and auditory toxicities of amikacin) and improved the efficacy of conventional treatments. As we have described throughout this chapter, the active Mϕ targeting nanomedicines to selectively

eliminate them, switching their phenotype, decreasing production of pro-inflammatory mediators, increasing antimicrobial activity, and modulating their immune response could improve the treatment of several diseases. However, currently, no translation studies have been done. Some crucial challenges should be addressed to accelerate clinical studies.

The first issues are related to the selection of the administration route that is fundamental to get access to macrophages and imposed the structural design of nanomedicines. For instance, direct access of nanomedicines to intestinal Mϕ or AMϕ could be achieved by oral or pulmonary routes, respectively. However, not only Np should be structurally resistant, but ligands and linkages should not be degraded during GI transit or the stress of aerosolization. In addition, for iv-administered Np, stability during blood circulation is fundamental to have the chance for reaching the inflammation sites. Colloidal instability, aggregation, and opsonization produce drug leakage, uptake by Mϕ of the MPS, and reduce targeting opportunities.

The second issues are related to the selection of the ligand and its binding method to Nps that are key to minimized off-target effects and for translation of results. On one hand, the selected receptor should be overexpressed on activated Mϕ and no in other cells or rest Mϕ, to reduce the off-target effects. For example, although CD44 is typically overexpressed by inflammatory Mϕ HA-targeted Np in principle can also accumulate where HA catabolism takes place such as in the skin and liver, and this has a clearly negative effect on therapeutic efficacy. Besides, receptor expression in comorbidities may hamper its effective translation. On the other hand, ideally, ligands should be highly specific, with a simple structure, readily available, economic, and stable. Usually, highly specific ligands, such as antibodies, are structurally complex and highly instable. The methods for binding ligands (pre- or post-preparation Nps) usually include several steps or are performed in organic solvents and under harsh reaction conditions, resulting in potential toxicity and high cost. Besides, it should be considered that structural heterogeneities in Np population obtained can reduce batch-to-batch consistency, and the accuracy of its chemical characterization, which leads to unwanted unpredicted BD and activities after in vivo administration.

Surface functionalization and excessive structural complexity are two factors that will delay future market implementation. In this sense, the use of natural ligands that could be incorporated as one component of Nps, that could not require chemical synthesis and that could be easily quantified, could be advantageous. In addition, sufficient data on the best density of ligands to insurance effective targeting is lacking, and examples show that excessive ligand density could be conduced to off-targeting.

The third issues are related to the in vivo models used to preclinical test Mϕ targeted nanomedicines. Animal models of inflammation are easy to use but are not particularly reflective of the mechanisms of action in human disease since they do not recapitulate complete human diseases. For example, DSS-colitis models are characterized by acute instead of chronic inflammation probably with a higher

eEPR effect than that present in patients, and hence site-specific accumulation of nanomedicines could be artefactually magnified (Danese et al. 2016). Besides, in recent years, it has been noticed that in cancer the EPR effect is highly heterogeneous, changing during tumor growth, changing among tumor types of the same origin, and between tumors and metastases in the same patient, and varying between mouse models and patients (Tanaka et al. 2017; Golombek et al. 2018). However, eEPR effect was not fully characterized in patients with inflammatory diseases.

The fourth issues are related to potential nanotoxicity of iv-administered nanomedicines. It is well known that because of their size and high specific surface, Nps may be recognized as foreign bodies by the immune system, by macrophage uptake and/or complement activation. Besides, nanomedicines can induce the production of binding and/or neutralizing antibodies that hamper or suppress pharmacological activity such as the accelerated blood clearance effect (Moghimi 2018; Szebeni 2018). Besides, the iv infusion of certain nanomedicines, for instance, AmBisome and Doxil (liposomal doxorubicin) are known to induce idiopathic hypersensitivity (HSR) (Jiskoot et al. 2009). Additionally, data on how Nps modulate the innate immune response is mostly unknown. For example, plain and mannosylated-chitosan Np control the expression of cell-cycle-related genes, inflammation, and upregulate stress response genes on Mϕ (Coya et al. 2019). Besides, the effects of Mϕ repolarization on autoimmune or inflammatory diseases promotion have been not addressed at all (Ardura et al. 2019).

Finally, counting with a production method that could be industrially scalable and cost-effective is fundamental for translation. For instance, there are no available industrial methods for the solvent precipitation method predominantly used to prepare polymeric Nps at the laboratory scale (Khayata et al. 2012). It is important to underly that, because of their non-biological complex drug (NBCD) nature, switching from lab scale to industrial production methods is expected to alter the structural features of the final product, modifying its therapeutic performance and toxicity (Smith et al. 2016; Coty and Vauthier 2018). The scalable, controlled, and reproducible production of nanomedicines under good manufacturing practice (GMP) conditions still present unique challenges (Paliwal et al. 2014; Agrahari and Agrahari 2018). The operative challenges involved in their industrial production have been already solved for liposomes. However, industrial manufacture of Np exhibiting major structural complexity, such as displaying surface protein ligands, remains troublesome (Paliwal et al. 2014). This is reflected in the pipeline of products in clinical trials and in the market; most of them are plain or sterically stabilized liposomes, while targeted nanomedicines constitute a minor fraction of the total.

Overall, Mϕ targeting, different from cancer targeting nanomedicines, has been only recently address, but it could be a straight way to modulate the behavior of these versatile cells involved in a considerable number of diseases. Plain and Mϕ targeted liposomes have more promising translational opportunities compared with other Nps.

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Chapter 11
Nanomedicine Applied to Inflammatory and Infectious Pulmonary Diseases

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Abstract Chronic pulmonary diseases often include inflammation in the upper or lower respiratory tract causing severe/extreme discomfort. To subside inflammation, currently, small molecules are administered to patients. Nano-based therapies have the potential to replace drugs by significantly increasing efficacy and decreasing dosage for long-term relief. In this chapter, we discuss the various nanotherapeutics being employed in preclinical and clinical settings to ameliorate inflammation. We also describe different nano-drug delivery systems used for diverse modes of treatment. To specifically target pulmonary inflammation, nanoparticles developed

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and tested via inhalation are also discussed. Recent developments summarizing the last 20 years for a variety of pulmonary diseases are explored. Although these nano-based studies are promising, long-term toxicity and clearance strategies are still debated and must be investigated.

Keywords Inflammation · Nanoparticles · Respiratory · Drug delivery · Nano-based therapy · Airway disease

11.1 Introduction to Inflammation in the Respiratory System

It is hard to imagine life without respiration. Yet, millions of lives are lost to respiratory ailments each year. Science has progressed enough to identify the causes and symptoms of respiratory diseases. The real challenge is to eliminate these pathological conditions and alleviate the suffering of a patient. Understanding respiratory diseases in a better way take us to history; many pieces of evidence point to the existence of these illnesses as far back as 10,000 years ago (Comas et al. 2013). The long history of humanity has allowed pathogens to coexist in our immediate community. However, infection or affliction by disease does not always explain discomfort during respiration. The human body's evolved response against a pathological disturbance may create emergent symptoms in an individual. The host immune response against a pathological disorder within the pulmonary system has been well characterized over several decades of medical history. Inflammation is one of the many articulated and cascaded pathways among these. Still, due to pathogen-induced subversion of inflammation, prior genetic conditions, smoking, hypersensitivity, and coexisting infections, it is often dysregulated in individuals, leading to more severe disorders accompanying the original (Herbst et al. 2008). The presence of lifestyle diseases such as obesity, hypertension, and type 2 diabetes mellitus in modern times exacerbates inflammation in the human body, leading to extended periods of discomfort and multiple disease complications (Sharma et al. 2019; Rodríguez-Hernández et al. 2013).

Inflammation is primarily mediated by an imbalance in the Th1 or Th2 immune response against a pathological condition, elevated levels of pro-inflammatory cytokines such as TNFα and IL-1β result in increased susceptibility to diseases such as tuberculosis (Piergallini and Turner 2018). To combat inflammation in the lungs arising due to a pathological condition, drugs must be delivered in a manner that does not affect homeostasis and causes fewer or no side effects. Nanotechnology has previously been employed for purposes of better deliverability of drugs to the target site, sustained release, better solubility of hydrophilic drugs, and many others (Alam et al. 2014; Azarmi et al. 2008). The variety and easy manipulation offered by nanotechnology in therapeutics make it an application-based science, which is essential for therapeutics in today's world. In this chapter, we discuss and elaborate

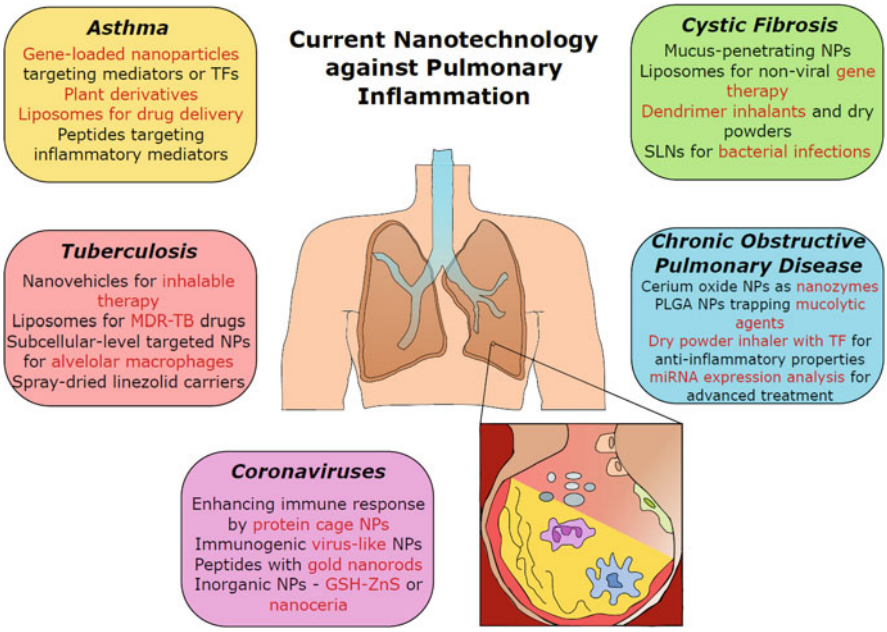

Fig. 11.1 (Introduction): An overview of recent developments in nanotechnology for treatment of inflammation in the respiratory system due to various pathologies. The inset schematic represents an inflamed alveolus

on the inflammatory mechanisms in the lung correlated to some of the most common afflictions in humans, along with a detailed description of nanotechnology developments to tackle unwanted inflammation and the pathological condition itself (Fig. 11.1). Further, we address knowledge gaps and important discoveries that can aid nanoscience in the construction of newer, multipurpose devices that help in the elimination of disease and reduce lung inflammation.

11.2 Asthma: Improving Nanotherapeutics for Longer Relief

Asthma is a chronic, non-communicable respiratory disease originating from the downstream effects of several genetic polymorphisms interacting with a reaction-inducing environment (Wang et al. 2019). A comprehensive study conducted from 1990 to 2010 noted that asthma is prevalent in over 330 million people worldwide (Vos et al. 2012). Smoking, increase in air pollution, and frequent occurrence of smog-like weather with increased particulate matter in the lower strata of the atmosphere are important risk factors for asthma after infancy, especially in genetically susceptible individuals (Ober and Vercelli 2011). The term “asthma” is used

for a set of clinically diagnosable characteristics, which includes inflammation of the bronchial passages (Wenzel 2012). Conventionally, early-onset asthmatic individuals are given symptomatic relief by inhalable vaporized β_2-adrenoreceptor agonists using a metered-dose inhaler. Newer drugs targeting inflammatory symptoms via systemic or inhalation routes have also been developed (Keil et al. 2020).

Nanotherapeutics in the context of asthma is an oxymoron. There are many pieces of evidence of inhaled environmental and occupational nanoparticles creating a chronic inflammatory response within the pulmonary system (Lu et al. 2014; Ferreira et al. 2013; Qiao et al. 2015). In 2005, PM10 was closely correlated with daily hospital admissions for asthma, acute or chronic bronchiolitis, and lower respiratory tract infections. However, in the same study, it was also noted that nanoparticles (NPs) have a larger surface area than bigger particles and penetrate deep into the respiratory tract (Inoue et al. 2005). Therefore, studies aim to target inflammation using micro- and nano-scale particles, devices, and medicine. Initially, experiments focused on micro-particle delivery to the pulmonary system. Shifting from metered-dose inhalers to dry powder inhalers provides benefits of ease-of-use, multidose capability, and greater chemical stability of the drug (Porta et al. 2005). These rapidly gave way to the development of new nano-based therapeutic technologies against pulmonary inflammation in asthma.

11.2.1 Nucleic Acid Supplementation

Recent advances in nanotechnology have made direct pulmonary delivery possible. Nucleic acid supplementation is an important and upcoming field in this respect (Kaczmarek et al. 2017). Gene therapy technology is known for its longer effectiveness. To develop NPs for alleviating asthmatic inflammation, cationic lipid NPs were conjugated with locked nucleic acid oligonucleotides to target miR-154, a sequence identified in regulating asthma of mouse models. Conclusively, the study correlates miR-154 nanoparticle treatment of asthmatic mice with the downregulation of multiple cytokine and chemokine genes associated with inflammation (Ramelli et al. 2020). Targeting transcription factors provides yet another route. Keil et al. attempted downregulation of GATA3, which is essential for the control of inflammatory processes. To stably transport a negatively charged GATA3-silencing siRNA to activated T cells, polyethyleneimine (PEI), a positively charged polymer, was conjugated with transferrin (Tf) for uptake to the T cells. However, due to the inability of PEI to cross the mucus barrier, these nano-in-micro particles are being tested in vivo with oligospermine instead for biological compatibility, as well as spray-drying characteristics to develop a bench-to-bedside formulation (Keil et al. 2020). da Silva and colleagues developed murine models to test the efficiency of thymulin gene-loaded biodegradable NPs for therapeutic relief from asthma. Mice treated with the NPs indicated a reversal of inflammatory processes and a significant reduction of eosinophil counts, CXCL1, CCL11 (BALF chemokines), and M2 macrophage counts. However, discontinuous mouse exposure to the allergen as

compared to human asthma may play a critical role in demonstrating effectiveness in humans (da Silva et al. 2020).

11.2.2 Plant-Derived Nanotherapeutics

An alternative approach to the development of nanotherapeutics for asthma is the introduction of plant products. Quercitin and celastrol are examples of plant-derived hydrophobic molecules introduced into liquid crystalline NPs and their in vitro characteristics have been studied. Effects on cytokine suppression were observed to conclude that these NPs could be a potential nano-based drug therapy to reduce inflammation in asthma (Cherk Yong et al. 2019; Chan et al. 2020). Other plant-derived products such as the extract of *Eriobotrya japonica* leaves and *Hyssopus cuspidatus* Boriss can be employed in the NPs to alleviate inflammation (Kim et al. 2020; Yuan et al. 2019).

11.2.3 Drug Delivery Systems

NPs alone may not be the solution. Deposition of particles into the deep lungs requires a diameter of 1–5 μm, which can be achieved by encapsulating NPs in a microgel easily dissolvable in the lower lung environment, targeting cells by introduction of enzyme-responsive crosslinkers on their surface. These nano-in-microgel particles were designed as a drug delivery system and had a retention time of several hours followed by clearance in a murine model (Mejías and Roy 2019). NPs as drug delivery systems were previously coupled with salbutamol sulfate, the current mainstay for asthmatic relief. Newer technologies include liposomal delivery vehicles, which are biodegradable, safe, and sustainable as inhalable technology. Synthetic liposomal carriers show enhanced persistence in the lungs of mice (up to 18 h), whereas free salbutamol sulfate was retained for about 8 h (Yang et al. 2012). Liposome bilayers shield less soluble molecules such as curcumin from the pulmonary environment and provide a mechanism for sustained release in vitro (Ng et al. 2018). Curcumin was also part of hydrogel microspheres encapsulating PLGA NPs to be used as a delivery system to the lungs. El-Sherbiny and Smyth concluded that these particles could evade the rapid phagocytosis by macrophages, although in vivo studies were not carried out (El-Sherbiny and Smyth 2012).

As an example of a prescription drug going back to the bench, montelukast is a leukotriene receptor antagonist that binds to the CysLT1 receptor. Nanostructured-lipid carriers encapsulating montelukast can be an alternative to conventional montelukast tablets for temporary relief. These nanostructures were tested to be safe in lung epithelial cell line A549; increased bioavailability, higher lung deposition, greater residence time, and slow release of the drug by the nanocarrier were reported (Patil-Gadhe et al. 2014). Another drug delivery system utilized the

tea-based compounds theophylline or budesonide for long-term relief from asthma. A potent bronchodilator, theophylline, is commonly recommended to asthma patients for late-stage management; however, it has a narrow therapeutic range. Buhecha et al. studied the loading efficiency of budesonide and theophylline into mono-encapsulated and co-encapsulated PLA NPs as well as their sustained release to lung cell lines (Buhecha et al. 2019). Prior studies also evaluated the role of cyclosporine A as an option for inhalation therapy (Sato et al. 2016).

11.2.4 Peptide Nanoparticles

Peptides and other biologics are increasingly employed as first-line drugs. Similarly, nanotechnology is increasingly employing peptides and/or whole proteins. For example, the overactive Th2 response in asthma induces a higher concentration of IL-4 and IL-13. Both IL-4 and IL-13 share the IL-4α subunit in their structures which presents itself as a target for nanotherapeutic development. Halwani et al. synthesized an anti-IL-4α NP formulated using superparamagnetic iron-oxide NPs that could control inflammation in ovalbumin-sensitized mice (Halwani et al. 2016). Athari et al. developed a PLGA nano delivery system for the anti-inflammatory vasoactive intestinal peptide. Their study was limited to in vitro observations and in vivo characteristics of the particles remain to be tested (Athari et al. 2016). However, peptide mimetics can be associated with adverse effects on high doses of administration. Gene therapy of specific peptides might be useful in this respect. To combat Th2-mediated airway inflammation, a chitosan NP incorporating the IFN-g gene into plasmid DNA was administered intranasally to mice models. By promoting a Th1-type response and balancing the immune reaction, airway inflammation was successfully reduced in mice via a STAT4 signaling pathway (Kumar et al. 2003). The challenges accompanying asthma treatments are slowly being overcome by nanotechnological advances. The development of newer systems to counteract inflammatory reactions in asthma patients due to an imbalanced Th2 response to allergens is encouraging. A greater number of microbubble- and hydrogel-based technologies incorporating natural products also provide a positive outlook (Corthésy and Bioley 2017; Secret et al. 2014).

11.3 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD), a combination of chronic bronchitis and emphysema, is a chronic inflammatory lung disease that results in obstructed airflow to and from the lungs and difficulty in breathing. According to the Global Burden of Disease Study, 251 million cases of COPD were reported globally in 2016, and around 3.17 million fatalities were estimated in 2015 (Soriano et al. 2020). Characterized by an abnormal inflammatory response, COPD patients suffer from

chronic inflammation of the airways and damaged alveoli of the lungs (Hogg et al. 2004). Being a progressive disease that worsens over time, COPD is among the leading causes of death due to respiratory illnesses. Cigarette smoking (CS) is known to be the primary risk factor for COPD, and the immune response is mainly driven by inflammatory cells such as macrophages, neutrophils, and T cells (Zuo et al. 2014). Smoking elevates lavage iron and ferritin levels in the lungs which produce oxidative stress leading to inflammation (Ghio et al. 2008). Other secondary risk factors, including long-term exposure to inhaled noxious particles, chemicals, or gases, also contribute to COPD pathogenesis (Boschetto et al. 2006).

11.3.1 No Smoke without Fire: Inflammatory Aspect

COPD is associated with inflammatory mucus accumulation, disruption of the epithelial barrier, widespread damage to the bronchial epithelium, and lung parenchymal tissue destruction. The small airway morphology in a COPD patient shows thickened airway wall, narrowed lumen along with mucus, and cellular debris (Baraldo et al. 2012). This obstruction occurs due to the surge of tissue volume of the bronchial walls, which occurs due to the infiltration of macrophages, neutrophils, CD4, CD8, and B lymphocytes. Alveolar macrophages show impaired phagocytosis of non-typeable *Haemophilus influenzae*, which increases the severity of the disease by complementing bacterial colonization (Berenson et al. 2013). A complex network of inflammatory cytokines, reactive oxygen species (ROS), and proteases are produced by phagocytes and epithelial cells damaging lung tissues. Cigarette smoking elevates the expression of TNFα, IL-1, IL-6, and reduces the expression of the anti-inflammatory cytokine IL-10 (King 2015) (Fig. 11.2).

Recent research has shown that autoimmunity linked to emphysema is also among the most commonly associated factors with inflammation in COPD. Deficiency of alpha 1-antitrypsin (A1AD) due to a mutation in the SERPINA1 gene resulted in increased release of proteases like neutrophil elastase and decreased production of inhibitors (alpha 1-antitrypsin), leading to disruption of lung tissues (Alvarado 2018). Current therapeutic options with long-term adherence can control the symptoms but do not cure the underlying disease. The most commonly followed treatment includes antioxidant therapeutic agents like *N*-acetyl-L-cysteine (NAC), and Nrf2 activators to reduce oxidative stress in the body during COPD. Other treatment strategies include corticosteroids, bronchodilator inhalers, anticholinergics, mucolytics, bronchodilator tablets, and even gene therapy (Vogelmeier et al. 2017). Nano-based therapeutics such as drug delivery systems overcome major challenges like low diffusion rate, mucociliary clearance, acute inflammation, and blocked airways which are generally encountered by conventional drug delivery treatments (da Silva et al. 2017).

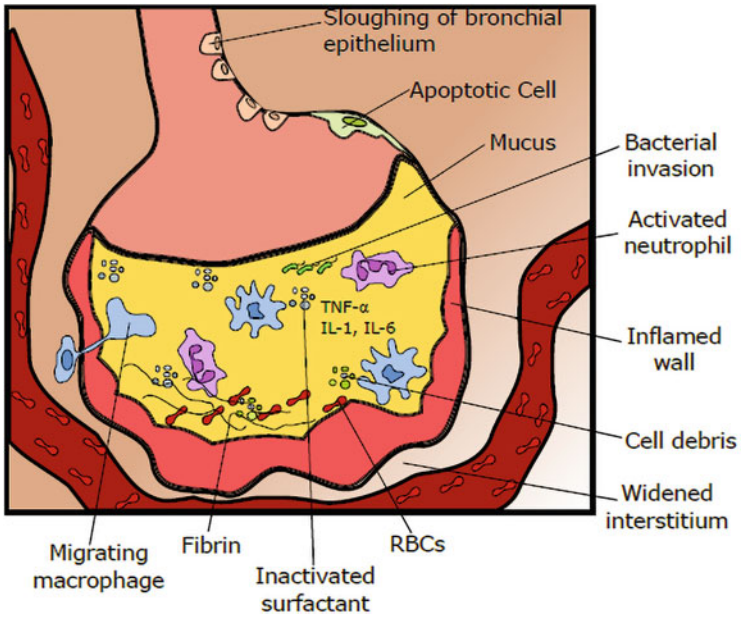

Fig. 11.2 Schematic representation of a diseased COPD alveolus. The bronchial epithelium begins to slough off, while the cavity of each alveolus reduces due to wall thickening. The alveolus itself becomes a host for pro-inflammatory processes resulting in bacterial infections and severe blockage to gaseous exchange

11.3.2 Optimal Nanoparticle Characteristics

Nanocarriers for efficient drug release need to be optimized, like conjugation with specific ligands, to enhance the targeted delivery and therapeutic effect of nanomedicine. AuNPs have shown enhanced epithelial targeting in mice with COPD/emphysema and can be utilized for targeting alveolar epithelial cells and macrophages (Geiser et al. 2013). Similar to cystic fibrosis, nanoparticles to be used as nanocarriers should be small-sized with a negative surface charge along with surface modifications that facilitate easy penetration through a highly thick and viscoelastic mucus layer. Mucus also acts as a barrier to inhaled gene therapy for respiratory illnesses like cystic fibrosis and COPD which can be overcome with the involvement of nanoparticles (Duncan et al. 2016). Li et al. used black phosphorus quantum dots (BPQDs) along with PEGylated chitosan nanospheres which facilitate the delivery of antibiotic amikacin through the mucus layer. BPQDs, being biocompatible and biodegradable, play a significant role in enhancing amikacin delivery to the lungs (Li et al. 2020). Cerium oxide nanoparticles mimic the activity of superoxide dismutase (SOD) and catalase due to their tendency to coexist either in a reduced or oxidized state and act as a potential nanozyme to treat oxidative stress in COPD (Passi et al. 2020). Bhushan et al. synthesized biocompatible cerium oxide

nanoparticles encapsulated in albumin nanoparticles that subsided intracellular ROS (Bhushan and Gopinath 2015).

In an ex vivo model of COPD, the antioxidant and anti-inflammatory activity of *N*-acetylcysteine (NAC) was demonstrated (Cazzola et al. 2017). NAC acts as a mucolytic agent by loosening thick mucus in airways in patients with COPD or cystic fibrosis, but it displays low bioavailability (6–10%), limiting its therapeutic potential. To refine its therapeutic effect, nanoparticles that can stabilize it inside the body, increasing its bioavailability are being developed. Lancheros et al. accomplished the NAC-loaded PLGA nanoparticles by nanoprecipitation method, which is simple, economical, and results in the increased load capacity and efficient entrapment of the compound (Lancheros et al. 2018). Muralidharan et al. reported for the first time the use of therapeutic dry powder inhalers carrying micro or nanoparticulate powders of dimethyl fumarate (DMF) that can be administered using a DPI device. DMF is a nuclear transcription factor, Nrf2 activator, and has shown both antioxidant and anti-inflammatory properties targeting the lung Nrf2/Keap1 pathway to treat pulmonary inflammation. Nrf2 (Transcription factor nuclear factor erythroid 2-related factor) triggers cellular protection against inflammatory or oxidative stress in lung cells. Their administration via DPIs has shown excellent aerosol dispersion performance and enhanced penetration to lower airways (Muralidharan et al. 2016). NPs have also been employed for early and better diagnosis of COPD. In a study by Faraj et al., MR imaging coupled with antibody-conjugated superparamagnetic nanoparticles was developed for targeting a specific macrophage subpopulation which offers an attractive approach for timely diagnosis (Al Faraj et al. 2014). The narrow safety range of theophylline can cause adverse systemic side effects if administered in high doses hence nano-based sensors are also developed for detection in drug analysis. One such electrochemical sensor was developed by the fabrication of poly-sulfosalicylic acid on carbon fibers to detect theophylline level (Duan et al. 2021).

11.3.3 Inhalation Therapy

Administration of pharmaceuticals for the treatment of lung disease by inhalation show certain advantages over orally given drugs in terms of targeted delivery, decreased side effects, and higher retention and their incorporation with nanotechnology can further enhance the efficacy of treatment (Kuzmov and Minko 2015). Novel nano-based theranostics that can offer a real-time diagnosis of COPD along with drug delivery can be an enticing invaluable approach for tackling COPD (Vij 2011). Furthermore, nanoparticles can also be incorporated into dry powder microparticles (NCMPs) to facilitate their deposition in the lungs. NCMPs have been developed for carrying miR-146a with PFA-*co*-PDL nanoparticles. Micro RNAs are short, regulatory, non-coding RNAs involved in the pathogenesis of COPD (Ebrahimi and Sadroddiny 2015). A study indicated the role of miR-146a in the severity of disease and differences observed in miRNA expression in COPD patients

versus healthy individuals (Pottelberge et al. 2011). The expression level of miRNA was found to be 2.5-fold lower in patients, which results in overexpression of the cyclooxygenase 2 gene and in turn, prostaglandin E2 production in fibroblasts contributing to chronic inflammation of the pulmonary system (Sato et al. 2010). Hence, the administration of miR-146a by spray drying of NCMPs has emerged as an attractive therapy in the management of COPD (Mohamed et al. 2019). Differential expression analysis of other miRNAs such as miR-223/1274a, miR-1, miR-150, and let-7c have also been tested, but a more vigorous understanding of disease complexity and relevance of these miRNAs is required (Ezzie et al. 2012; Fujita et al. 2013). In a recent finding, it was found that miR-155 expression is increased in lung tissues and alveolar macrophages in CS-induced inflammation and COPD, which can also serve as a new therapeutic tool for COPD treatment (De Smet et al. 2020).

Recent studies by Beyeler et al. have revealed that nanomaterials like multi-walled carbon nanotubes are responsible for the polarization of alveolar macrophages toward pro-inflammatory M1 phenotype and affect the pulmonary mucosal immune cells in mouse model studies (Beyeler et al. 2020). This can cause increased susceptibility to adverse side effects in COPD patients and therefore marks the importance of clinical assessment for the toxicity of nanomaterials. COPD is estimated to be the world's third leading cause of death; hence, it should be among the prime focuses of the scientific community (Soriano et al. 2020). Efficient drugs with high bioavailability, increased stability, and strongly targeted to the site of action need to be developed to combat this complication. Nanotechnology is a potent tool to accomplish the shortcomings faced by conventional drugs. More than a billion people smoke globally on a daily basis and cigarette smoking is the primary risk factor that poses a significant risk for airway blockage and the development of COPD.

11.4 Crossing Mucus in Cystic Fibrosis

Cystic fibrosis (CF) is a chronic genetic disease affecting nearly 70,000 patients worldwide (Cystic Fibrosis Foundation 2018; Velino et al. 2019). Characterized by the build-up of thick, sticky mucus that can damage many of the body's organs, CF is an autosomal recessive disease caused by defects in the CFTR gene, encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Turcios 2005). A membrane protein, CFTR forms a chloride channel for regulating transport across the membrane of epithelial cells in pulmonary, gastrointestinal, renal, and male reproductive tissues (Vankeerberghen et al. 2002). It regulates the secretion of chloride ions out of cells producing mucus, sweat, tears, saliva, and digestive enzymes thereby controlling fluid flow across epithelial cell membranes (Rey et al. 2019). More than 1900 mutations are described since the discovery of CFTR in 1989 (Kerem 1989; Rowe and Verkman 2013). Among all the mutations, the most common is a 3-bp deletion, F508del, preventing protein movement to the cell surface

(Kälin et al. 1999). 84.7% of individuals in the CF Foundation Patients Registry have at least one copy of this mutation (Cystic Fibrosis Foundation 2018).

Mutations of the CFTR gene lead to impaired Cl^- ions secretion and overabsorption of Na^+ ions, resulting in an imbalance of ion concentration across the cell membrane. As a result, cells lining lung passageways produce mucus that is usually thick, sticky, and difficult to clear, and in the end, ducts become plugged and atrophic. Chronic airway infection, progressing to bronchiectasis, persistent high-intensity inflammation in lung epithelium, gas trapping, hypoxemia, and hypercarbia, ultimately damage leading to respiratory failure are the hallmarks of CF lung disease. Neutrophils are the first cells migrating into the pulmonary compartment, releasing oxidants and proteases like elastase, which eventually overwhelm the antiprotease capacity of the lungs, leading to bronchiectasis (Cantin et al. 2015). IL-10, anti-inflammatory cytokine production by bronchial epithelium cells, is downregulated in CF airways, which may contribute to enhancing local inflammation and tissue damage (Bonfield et al. 1995). On cell death, DNA released by neutrophils also increases mucus viscosity leading to airway obstruction (Elizur et al. 2008). CF causes breathing difficulties, recurrent lung infections, persistent bacterial infections (particularly *Pseudomonas aeruginosa*), edema, progressive impairment of lung airways, malnutrition, chronic endobronchial inflammation, pancreatitis, and death (O'Sullivan and Freedman 2009).

11.4.1 Nano-Based Therapies

Presently, treatment strategies used include CFTR modulators (potentiators, correctors, and amplifiers) that restore CFTR functions, mucociliary clearance, antibiotics, anti-inflammatory, gene therapy, etc. (Edmondson and Davies 2016). The disadvantage of current treatments is their inability to reach the site of action due to thick mucus (Velino et al. 2019). The use of NPs for CF creates new perspectives to counter mucus formed within the alveolus and eliminate resulting bacterial infections. The mucus layer is a mesh structure composed of highly cross-linked mucin-fibers, cytoskeletal fragments of actin, and DNA along with other macromolecules creating hydrophobic and electrostatic barriers, reducing drug delivery efficacy (Duncan et al. 2016). Viscoelasticity is increased dramatically due to changes in mucus structure and composition in CF patients with mesh size reduced to 100–400 nm in size as compared to >500 nm in healthy individuals (Yaakov et al. 2007). Small NPs bypass steric hindrance effects and diffuse through the mucus network. Electrostatic interactions can be countered by coating NPs with electrostatically neutral molecules or muco-inert polymers such as polyethylene glycol (PEG) or by using mucolytic agents like *N*-acetylcysteine (NAC) (Ong et al. 2019). Suk et al. illustrated that PEGylation for 200 nm particles increased penetration into the mucosal barrier in CF sputum pretreated with NAC and also increased NP mobility in *Burkholderia multivorans* and *Pseudomonas aeruginosa* biofilms (Suk et al. 2011).

Mucus penetrating particles (MPPs), used as vehicles for drug delivery, possess nonadhesive coatings to rapidly penetrate mucus through pores in the mesh at rates similar to pure water. Highly compacted DNA NPs with block copolymers of poly-L-Lysine and PEG have been shown to mediate effective gene transfer (Ensign et al. 2012). The molecular weight of PEG should be sufficiently less to avoid adhesive interactions with mucins, and PEG coating density should be appropriate to effectively shield or protect the hydrophobic NP core. The conformation should also be taken into account; brush-like PEG facilitates penetration while mushroom conformation increases the time of an adhered fraction of particles on the mucus layer (citation). Another approach is the formation of nano-embedded microparticles (NEMs). In an interesting article, Porsio et al. produced PEGylated, and Transactivating transcriptional activator peptide (Tat)-decorated FNPs linked to PHEA-PLA to deliver Ivacaftor to the pulmonary epithelial cells across the mucus barrier and promote lung cellular uptake of the drug. FNPs showed proper nanometric sizes (~70 nm), slightly negative ζ potential (~ −12 mV), and high cytocompatibility. Tat, a cell-penetrating peptide (CPP), strongly enhanced the uptake of FNPs by lung epithelial cells. Moreover, nano into micro strategy was applied by encapsulating NPs into Matryoshka microparticles, inhalable by dry powder inhalers (DPI) devices to achieve an inhalator therapy (Porsio et al. 2018).

The rapid progress of nanomedicine enhances the efficacy of inhalation treatment for CF. Alton et al. conducted a randomized, placebo-controlled Phase2b trial (clinicaltrials.gov ID NCT01621867) for the application of pGM169/GL67A gene therapy formulations to CF patients. The GL67A, a cationic lipid mixed with equal amounts of pGM169 drug, was given to patients once a month for a year with a nebulizer. The administration of non-viral CFTR gene therapy gave statistically significant results with improvements in Forced expiratory volume (FEV1), forced vital capacity, and gas trapping (Alton et al. 2015). Over the last decade, different types of NPs like liposomes, polymeric NPs, dendrimers, solid and lipid NPs have been designed as nanocarriers for drug and gene delivery in CF treatment (Table 11.1) (Upadhyay and Ganguly 2015; Mansour et al. 2009).

LIPOSOMES are the most widely used and best characterized lipid-based drug delivery systems, especially for pulmonary applications, as it is prepared primarily from phospholipids, which are inherent in the lungs. Liposomes can entrap both lipophilic and hydrophobic drugs due to their amphiphilic nature. Studies have shown that drugs encapsulated within cationic liposomes exhibit greater antibacterial efficacy since they target bacterial biofilms via electrostatic interactions thus allowing drug release close to the pathogen (Messiaen et al. 2013). But they were found to be toxic to human lung cells and can introduce genetic aberrations (Shah et al. 2013). Liposomes have been used to develop antibiotics. Inhalation formulation of Amikacin liposome consisting of neutral liposomes (DPPC:Chol) completed Phase III of clinical trials and was approved by FDA in 2018 (ClinicalTrials.gov ID NCT03905642) (Paranjpe and Müller-Goymann 2014).

SOLID LIPID NANOPARTICLES (SLNs) are lipophylic particulate colloidal drug delivery systems comprised of solid lipids with mean diameters ranging in size between 50 and 1000 nm. Various drugs for CF have been encapsulated within SLNs

Table 11.1 Different types of nanocarrier approaches were tested for the treatment of cystic fibrosis in vitro and in vivo

Nanocarrier	Properties	Composition	Drug	Key finding	References
Liposomes	Prepared from compounds endogenous to the lungs, such as components of lung surfactant. Delivered in liquid and dry powder form	DPPC/Chol	Amikacin Gentamicin Tobramycin	Improved penetration within PA biofilm Refine drug efficacy when encapsulating in cationic liposomes	Meers et al. (2008), Mugabe et al. (2006) and Okusanya et al. (2009)
		DPPC, DOPC, DPPG	Tobramycin	Encapsulation in cationic liposomes improves drug penetration and efficacy	Messiaen et al. (2013)
		DOTMA/ DOPE	siRNA	Effective rectification of mucociliary defects and airway clearance	Tagalakis et al. (2018)
Polymeric	Composed of biodegradable or biocompatible materials such as PLA, PCL, PLGA, alginic acid, gelatin, chitosan	PLGA	Curcumin	Enhanced efficacy and drug bioavailability	Cartiera et al. (2010)
		PLGA	Ciprofloxacin	Drug antimicrobial activity and improved penetration within mucus	Günday Türeli et al. (2017)
		PLGA/ Chitosan	cmRNA	Chloride secretion is lowered and lung functions restored	Haque et al. (2018)
Solid lipid NPs	Composed of solid lipids, surfactants, and water	SA/PC	Myriocin	Lung inflammation significantly reduced	Caretti et al. (2014)
		DMA/ DSPC/Chol/ DMG	cmCFTR	Positive CFTR restoration	Robinson et al. (2018)
Dendrimers	Polymers with hyperbranched structures and layered architectures for gene transfers	PAMAM G4	siRNA	Enhanced cellular uptake and gene silencing	Bielski et al. (2017) and Agnoletti et al. (2017)
		PAMAM-DEN	Cysteamine	Reduced *Pseudomonas aeruginosa* infection and rescue of CFTR protein	Brockman et al. (2017)

DPPC dipalmitoyl-phosphatidylcholine, *Chol* cholesterol, *DOPC* dioleoylphosphatidylcholine, *DPPG* dipalmitoylphosphatidylglycerol, *DOTMA* dioleyloxypropyl-trimethylammonium chloride, *DOPE* dioleoylphosphatidylethanolamine, *PLGA* poly(lactic-*co*-glycolic acid), *SA* stearylamine, *PC* phosphatidylcholine, *DMA* dimethylaminobutyrate, *DSPC* distearoylphosphatidylcholine

as it provides physical stability and low cytotoxicity. Amikacin-loaded SLNs were developed previously (Varshosaz et al. 2013), and the results showed sustained release and increased efficacy of the drug with respect to free drugs. Another interesting approach for the treatment of *Pseudomonas aeruginosa* infection in CF utilizes SLN loaded with quorum sensing inhibitor. The SLN penetrated artificial sputum and also demonstrated a sevenfold higher anti-virulent effect in comparison to free compounds (Nafee et al. 2014).

DENDRIMERS are ordered, highly branched structures synthesized and studied for biomedical applications. Dendrimers for pulmonary therapies are developed as both inhalable suspensions and dry powders. Considering gene therapy, Agnoletti et al. prepared dendrimer-siRNA nanocomplexes, processed into microparticle-based dry powders for inhalation, which showed enhanced cellular uptake and gene silencing efficiency (Agnoletti et al. 2017). Brockman et al. developed a polyamidoamine (PAMAM) dendrimer with terminal groups modified to obtain Cysteamine-like structure (Cysteamine is an FDA-approved drug with antioxidant, anti-biofilm, and mucolytic properties) to reduce *Pseudomonas aeruginosa* infection in addition to preventing delF508-CFTR sequestration to aggresome bodies (Brockman et al. 2017).

POLYMERIC NANOPARTICLES, a major class of nanotherapeutics widely used as drug delivery systems, are highly versatile, having the ability to a prolonged and controlled drug release, stabilize encapsulated drugs and promote cellular uptake (Kuzmov and Minko 2015). PLA and PLGA are widely used because of their biocompatibility and biodegradability. Developing efficient non-viral delivery systems for gene therapy had been a major challenge for CF. Guan et al. developed synthetic peptides able to self-assemble to poloxamines and nucleic acids to form compact and monodisperse NPs. This led to increased expression of both mRNA and plasmid DNA expression in the lungs of CF mice with negligible toxicity thus providing a new strategy for the development of non-viral gene delivery (Guan et al. 2019).

11.5 Tuberculosis: A New Approach

Tuberculosis (TB) is an ancient illness associated with humans (Comas et al. 2013), leading to its description as a "heritage disease" (Cambier et al. 2014a). The causative agent, *Mycobacterium tuberculosis* (Mtb), has developed strategies to avoid the hosts' immune responses by removing the need for colonization before infection. Instead, *M. tuberculosis* manipulates the hosts' immune system deeper inside the respiratory tract (Cambier et al. 2014b). Although a significant fraction of the population is infected with pulmonary tuberculosis, most of the population is asymptomatic, harboring a very low bacterial load and resulting in a latent disease period. Poor health triggers active infection in 5–10% of the infected individuals, allowing transmission of disease (Piergallini and Turner 2018). The active infection

however is often fatal, with an estimated 1.7 million deaths from TB in 2019 (World Health Organization 2019).

11.5.1 Subverted Inflammation in Tuberculosis

Initial latent infection of the Mtb bacteria occurs ca. 80% in the lungs, serving as the entry and exit points of transmission of the disease (Kaufmann and Dorhoi 2013). On contact with the immune system, controlled inflammation is the first line of response. Macrophages are the primary target of Mtb, where it lives in a modified membrane-bound vacuole—altered Rab GTPase composition, increased pH, and presence of the protein TACO are primary characteristics (Glickman and Jacobs 2001). Current knowledge indicates that Mtb can modulate processes such as the production of the pro-inflammatory cytokines IL-1, TNFα, and interferons. IL-1 production requires caspase-1 and inflammasome complex activity, while also regulating TNFα synthesis and TNFR expression. TNFα, on the other hand, can be modulated directly or indirectly via eicosanoids by Mtb. A positive feedback loop between TNFα and IL-1 works to the benefit of the bacterium, creating an intensely pro-inflammatory environment (Kaufmann and Dorhoi 2013). Further, IFNγ forces T cells into apoptosis while lymphocyte activation is downregulated (Cooper et al. 2002). Inflammatory granuloma formation in tuberculosis is a disease hallmark, with the observation of the granuloma replacing functional tissues, while also being necrotic and damaging surrounding cells (Cooper et al. 2002). It is evident that inflammatory responses in tuberculosis should be controlled to surmount a significant response.

11.5.2 Emerging Nanotherapeutics

Although antitubercular drugs have been available via prescription for the last two decades, patient noncompliance due to extended treatment is a common cause of treatment failure. Biocompatible NPs encapsulating current drugs showed promise with improved bioavailability. Other advances include the altered route of administration, compatibility with hydrophilic and hydrophobic drugs, increased dosage capacity, and better stability (Gelperina et al. 2005). As an example, Pandey et al. described a poly-(DL-lactide-*co*-glycolide) (PLG) nanoparticle system containing rifampicin, isoniazid, or pyrazinamide for inhalable therapy against tuberculosis (Pandey 2003; Azarmi et al. 2008). Sustained release is also one of the main goals for scientists currently developing better treatments (Gelperina et al. 2005). Considering this, the study detected the presence of rifampicin in the circulation for 4 days and isoniazid and pyrazinamide for 9 days. They also reported a reduction in the treatment period (Pandey 2003). In a similar light, wheat germ agglutinin lectin coated or conjugated onto PLG NPs was used to deliver isoniazid, pyrazinamide,

and rifampicin to Mtb-infected guinea pigs. The study noted severe necrosis in the lungs of untreated guinea pigs, while the treated animals had no necrotic regions and no observable hepatotoxicity (Sharma et al. 2004).

11.5.3 Tuberculosis and Drug Resistance

Multidrug treatment is usually prescribed to patients with tuberculosis infections for the prevention of drug resistance. However, long periods of intensive care (6–-9 months) and high doses of drugs resulting in health and economic issues often cause patients to opt out of the treatment course. An increase in drop-out rates and drug resistance eventually led to the rise of multidrug-resistant tuberculosis (MDR-TB) (Blasi et al. 2009). A separate category of drugs developed to treat MDR-TB suffers from low effectiveness and higher toxicity. Other problems that mark the treatment of patients include higher doses (125–200 up to 1000 mg/day), even longer administration periods (up to 2 years), low cure rates (60%), and daily intramuscular injections (Blasi et al. 2009). A compilation of several newer formulations against tuberculosis is presented previously (Hussain et al. 2019).

Liposomal carriers easily take up hydrophobic drugs and exhibit low toxicity. In a study, Le Conte et al. described capreomycin liposomes as being effective against *Mycobacterium avium* infection (Le Conte et al. 1994). A study to increase the content of capreomycin within liposomes was also reported (Ricci et al. 2006). Another report by Adams et al. used clofazimine in liposomes for the treatment of acute and chronic tuberculosis in mice. Their experiments conclusively indicated that treatment with liposome-encapsulated clofazimine reduced mononuclear cell infiltration significantly by localizing the granuloma formation (Adams et al. 1999). Rifabutin is a broad-spectrum anti-mycobacterial agent, particularly in use for its low resistance development against Mtb. To increase the bioavailability of the drug to the lungs, liver, and spleen on intravenous administration, multilamellar liposomal vehicles were developed by Gaspar et al. In vivo studies in BALB/c mice infected with Mtb indicated an increase in drug efficiency and a significant reduction in the lung inflammatory response. Although the liposomes were not effective in reducing bacterial load within the lungs, the liver and spleen sections indicated improvement in colony-forming units as compared to free rifabutin (Gaspar et al. 2008).

11.5.4 Alveolar Macrophages: Aiming for the Heart

The symptoms and duration of active disease widely vary person-to-person in tuberculosis. The activation of inflammatory responses depends on bacterial ligand expression and host cell type. Primarily mediated via MyD88 and TLR2 as observed in murine tuberculosis, the production of pro-inflammatory cytokines is beneficial to

the bacterium by inhibition of the expression of major histocompatibility complex class II on the surface of antigen-presenting cells (Sasindran and Torrelles 2011). To this effect, alveolar macrophages should be targeted; they are most modulated by Mtb, making them a critical component of infection. A step in this direction was the treatment of macrophages with PLGA NPs containing rifampicin to treat BCG infection. Although the loading of rifampicin was insufficient, appropriate loading enabled the clearing of infection from the cells (Kalluru et al. 2013).

Targeting lung macrophages has also been achieved by the development of Stealth® liposomes coated using O-stearyl amylopectin, the liposomes increase their affinity toward mice lung tissue (Deol and Khuller 1997). The development of other nanocarriers for sustained drug release by targeting macrophages may be of interest in newer studies. For example, *O*-palmitoyl mannan and *O*-palmitoyl pullulan can be coated on the drug vehicle to assist in eradicating the bacterium from its host (Vyas et al. 2005). Depending on the inherent design of NPs, such as the charge, size, composition, and coating, they can be directed to not just the correct cell type, but also the right cellular sub-compartment. This would increase the possibility of the drug delivery system ending up together with the bacteria during infection and aid in the targeted killing of the bacteria (Lawlor et al. 2011).

In this context, nonstructured lipid carriers containing linezolid were developed and studied for their characteristics in vitro as well as in vivo. These drug carriers exhibited phagocytosis by macrophages and were able to cross the mucus barrier in the lungs. The particles showed effectivity as aerosols for inhalation therapy by spray drying. This study could be used as a framework for the development of more patient-friendly tuberculosis treatments using nanomedicine (Makled et al. 2020). In summary, TB remains a major respiratory disease, affecting millions each year. Although the prescribed treatments exhibit efficiency in pathogen clearance, severe inflammatory response generation, and side effects often make patients discontinue their treatment. To combat the growing drug resistance of Mtb, it is imperative to develop newer nanomedicines that directly target macrophages, if not the site of the residence of latent bacteria in order to eradicate the disease.

11.6 A Balancing Act: Battling Coronaviruses

COVID-19 or the Coronavirus Disease 2019 began with a coronavirus (SARS-CoV-2) infecting several visitors in a seafood market in Wuhan, China. The virus binds to the host cell's ACE2 (angiotensin-converting enzyme 2) receptors present on the epithelial cells of alveoli, trachea, bronchi, and serous bronchial glands of the lower respiratory tract, rapidly producing new viral particles inside the host cell and infecting more cells as the disease progresses (Shereen et al. 2020). Transmission occurs by droplets of viral particles released on coughing, sneezing, or respiratory distress (Rothan and Byrareddy 2020). Clinical features indicated by chest imaging are pneumonia, RNAemia, acute respiratory distress syndrome, and incidence of ground-glass opacities that led to death (Zhu et al. 2020).

11.6.1 Cytokine Storms: Battling Inflammation

Similar behavior was previously observed in the SARS virus, an epidemic that affected the human population in 2003 (Nile et al. 2020). Pathogenetic similarities do not end there. The host response against SARS-CoV-2 includes aggressive inflammation, which is conducive to causing increased damage to the airways (Tay et al. 2020). Inflammation is typically caused by pro-inflammatory cytokine activation in epithelial cells, endothelial cells, and alveolar macrophages induced by the identification of damage-associated molecular patterns, including ATP and nucleic acids released by pyroptotic host cells (Tay et al. 2020). Primary mediators of cytokine-related inflammation include IL-6, IL-10, IP-10 (Laing et al. 2020), macrophage inflammatory protein 1α (MIP-1α), MIP1β, and MCP1. These proteins establish a chemoattractive gradient and a positive feedback loop of inflammation is generated by the incoming monocytes, macrophages, and T cells at the site of infection (Tay et al. 2020).

11.6.2 Potential Nanotherapy: Lessons from SARS and MERS

Previously encountered coronaviruses SARS and MERS (Middle East Respiratory Syndrome) exhibited quite a similar pathophysiology (Channappanavar et al. 2016). Therefore, strategic targeting to cure infections can be derived from studies that report effectiveness against SARS or MERS, albeit with some differences. By modulating the immune response, Wiley et al. induced the formation of bronchus-associated lymphoid tissue (iBALT) by the use of protein cage NPs in the lung parenchyma. In subsequent experiments, they indicated how iBALT in the lungs could help prevent the alleviation of SARS infection. Although it is unknown how the NPs enhanced the host immune response, where B cell and/or CD4+ T cell-dependent mechanisms are involved (Wiley et al. 2009).

In an attempt to synthesize an immunogen, a study in 2009 carried out a step-wise assembly of SARS-CoV subunit virus-like NPs exhibiting properties of easy protein expression, purification, and high stability. These NPs were conformation-specific, had neutralization activity toward the virus, and did not infect any host cells (Pimentel et al. 2009). A different approach to synthesizing vaccines could be the DNA route, as in a report using polyethyleneimine NPs coated with spike protein-encoding DNA to intranasally immunize mice from SARS (Shim et al. 2010). Similarly, nucleocapsid protein-encoding DNA of SARS was loaded into biotinylated chitosan NPs and intranasally administered to mice to target mucosal dendritic cells. The NPs elicited a humoral immune response, exhibiting elevated levels of IgG in mice (Raghuwanshi et al. 2012). The development of spike protein NPs can be of interest to target multiple coronaviruses, as predicted in Coleman et al. They synthesized spike protein NPs for SARS and MERS and depicted that these

particles could generate an immune response in mice. However, none of these NPs provided specificity to both diseases, leaving a potential gap for multiple-coronavirus targeting NPs (Coleman et al. 2014). Peptide mimics that interact with the viral particles in place of cellular receptors could be therapeutic in nature. Huang et al. developed a peptide mimic conjugated with gold nanorods against MERS, increasing its inhibitory effects by ten times and preventing membrane fusion of the virus both in vitro and in vivo (Huang et al. 2019).

11.6.3 Current Drug Repurposing and Nanotherapeutics

In severe cases of COVID-19, different drug cocktails are being used such as chloroquine (antimalarial drug), lopinavir/ritonavir (from AIDS treatments), favipiravir (against influenza), and ribavirin (from Hepatitis C treatments) (Bhavana et al. 2020). Better clinical outcomes by prevention of viral replication within the host are required, either by aiding the immune response in capturing viral particles or preventing their attachment to the ACE2 protein (Alphandéry 2020). Alphandery et al. review several options available for modification and specific targeting of NPs to SARS-CoV-2 (Alphandéry 2020). The majority of the antiviral nanotherapies involved silver NPs; this may be due to their previously established role in ameliorating other viral infections (Bhavana et al. 2020).

Additionally, Zhou et al. developed a GSH-ZnS NP that could effectively target multiple viruses, including both RNA and DNA viruses. The observed action of NPs against the porcine epidemic diarrhea virus belonging to the *Coronaviridae* family implied their probable use against SARS-CoV, SARS-CoV-2, and MERS (Zhou et al. 2020). Interferons, critical components of the antiviral immune response, could be induced to reduce the viral load and enable the strengthening of defenses. Therefore, nanoceria or cerium oxide NPs are hypothesized to be a possible agent in curbing inflammation—they attenuate cytokine signaling by modulation and inhibition of MAP kinase/NF-κB, p65-NF-κB, and Nrf2/NF-κB pathways, significantly disrupting and halting disease progression (Allawadhi et al. 2020). Targeting viral proteins such as proteases (3CLpro and PLpro), RNA polymerase (RdRp), and the spike protein involved in cellular uptake is critical to the development of any therapeutic against coronaviruses. Although these proteins do not directly undertake immunomodulation, their role in the viral life cycle is indispensable, and inhibition will substantially prevent inflammation. To this end, neutralizing antibodies coated on the surface of NPs might be an effective nanotherapeutic approach available (Chauhan et al. 2020).

11.7 Conclusion and Future Prospects

Chronic lung diseases are often driven by the inflammatory state due to the complex microenvironment involving close communication between the respiratory and immune systems. Any dysregulation of the immune half results in inflammatory processes sabotaging the exchange of gases. Action against inflammation is now being explored through the use of nanotechnology, which promises better drug availability, safer delivery, and efficacy. Although many preclinical studies have been conducted for the diseases mentioned, clinical studies using nanotechnological advances to improve patient rehabilitation are still far from reality. This may be due to the unknown impact of nanodevices or vehicles as a systemic supplement. To avoid this, several dry powder inhaler technologies have been developed to specifically target the lung and avoid contact with other organs for maximal retention and safety. Newer developments in drug technology, among other investigations, revealed that drug delivery via oral or inhalation or IV route by nanovehicles is a feasible strategy for application in pulmonary therapy. Further, liposomal and dendrimer carriers are noted as drug vehicles gaining importance to improve efficacy, targeting, and safety. To explore the full potential of nano-based drug carriers and delivery systems, further studies and clinical trials are vital to test the toxicity and long-term effects. Several natural derivatives can be employed as anti-inflammatory agents in disease and the potential for combination with existing treatments is noted as well.

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Chapter 12
Nano-Based Therapies for Acute and Chronic Lung Diseases

Mohammad Doroudian, Michelle E. Armstrong, and Seamas C. Donnelly

Abstract *Nanotechnology in medicine*—known as nanomedicine—has opened a new era for accurate and precise diagnosis and treatment of pulmonary inflammatory diseases. This novel approach has developed into a broad range of preclinical and clinical applications. Nanoparticles can be designed and employed as drug delivery systems to defeat the limitations of current medical treatments and cross biological barriers, such as mucosa, microenvironmental, and cellular levels. Nano-drug delivery systems are able to improve drug stability, enhance drug solubility, minimize drug first-pass metabolism, and facilitation of controlled release of payload. The applications of *nanotechnology in* pulmonary inflammatory diseases provide numerous benefits compared to the traditional way of therapeutic agent administration. *This novel strategy c*an be engineered for targeting specific site to deliver therapeutic agents to the desired tissues and cells in a more effective manner including higher level of bioavailability, less toxic side effects, and drug dose reduction. Another attractive application of nanotechnology in the diagnosis of pulmonary inflammatory diseases is nano-based sensors, known as nano-biosensors, which are extensively used for the molecular detection of biomarkers associated with the diagnosis and detection of pulmonary diseases. The surface area to volume ratio which is a unique feature of nanomaterials provides higher sensitivity and shorter response times compared to traditional method for detection. Nano-biosensors, also, offer attractive features such as specificity, ease of diagnostic procedures, and multiplexed measurement ability with high diagnostic accuracy ensuring accuracy of outcomes in

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high-throughput experiments. Therefore, it has been used for the early pulmonary inflammatory diseases' diagnosis even before symptoms' presentation. Unique properties of nanomaterials may help overcome traditional and current challenges for the treatment of lung inflammatory diseases.

Keywords Nanomedicine · Nanotechnology · Lung diseases · Nano-drug delivery systems · Nano-biosensors · Pulmonary inflammatory diseases

12.1 Introduction

Pulmonary inflammatory diseases, such as Chronic Obstructive Pulmonary Disease (COPD), Acute Respiratory Distress Syndrome (ARDS), Idiopathic Pulmonary Fibrosis (IPF), and Asthma are the leading causes of severe illness globally (Soriano et al. 2020; Li et al. 2020). Despite recent advances in therapeutic strategies, for many patients, these diseases are progressive with associated enhanced morbidity and mortality (Barnes et al. 2021; Soriano et al. 2020). In fact, the number of deaths from chronic inflammatory lung diseases has increased by 20% over recent years (Li et al. 2020). Hence, there is an urgent requirement to develop innovative approaches to diagnose and treat pulmonary inflammatory diseases. To this end, numerous novel treatment strategies and diagnostic methods are required. Nanotechnology in medicine—*Nanomedicine*—offers the promise of delivering such novel innovative improvements in therapeutic strategies going forward. Nanomedicine is defined as the manipulation of materials range of 1–1000 nm for specific medical applications (Kim et al. 2010). This involves the application of nano-carriers and nano-biosensors for the prevention, diagnosis and treatment of lung inflammatory diseases (Doroudian et al. 2019). Nano-drug delivery systems are an active area of research that offers numerous opportunities to deliver both hydrophilic and hydrophobic small molecule drugs or bio-macromolecular therapeutic agents (i.e., recombinant proteins and nucleotides) (Doroudian et al. 2021). Various types of nanomaterials such as polymeric, liposomal, dendrimer, micellar, metallic, nanogel, and carbon-based nanomaterial (i.e., nanotube, graphene) have been employed for the treatment and detection of pulmonary inflammatory disease (Fig. 12.1).

Nano-drug delivery systems have unique physicochemical properties (Table 12.1), that provide optimum biocompatibility, biodegradability, and thus facilitate the intracellular delivery of various therapeutic agents to lung tissue (Xu et al. 2020). Encapsulation, conjugation, or trapping of drugs in nano-drug delivery systems improves the solubility of poorly water-soluble therapeutic agents which has been a significant historical rate-limitation in the development of many candidate small molecule weight candidate drugs by the pharmaceutical industry (Patra et al. 2018; Doroudian et al. 2021). The nano-carriers packaged with a therapeutic payload have been shown to:

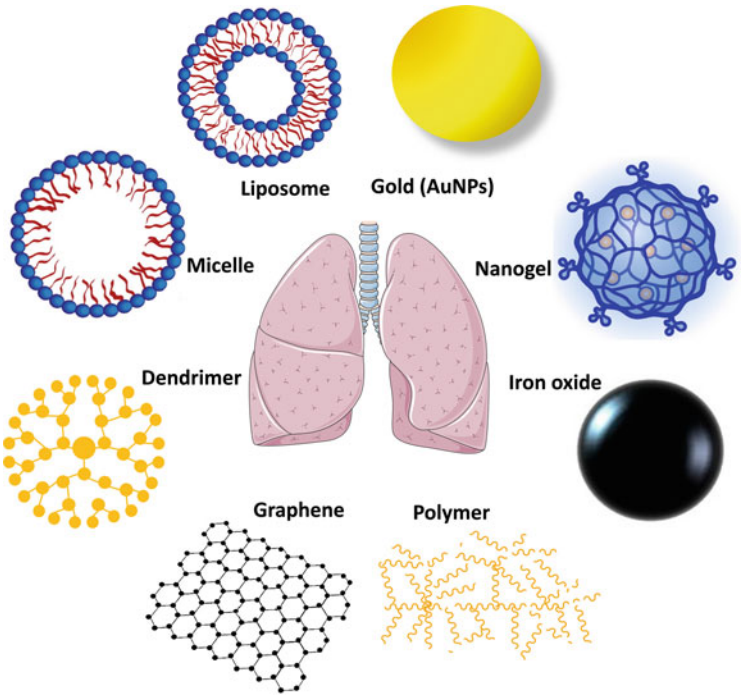

Fig. 12.1 Schematic diagram representing types of nanomaterials frequently used as a drug delivery system or nano-sensors for the treatment of pulmonary diseases

Table 12.1 Advantages and disadvantages of different nanomaterials for treatment of pulmonary inflammatory diseases

Nanomaterials	Advantages	Disadvantages
Lipid-based	• Non-immunogenic • High biocompatible • High bioavailability • Formulation simplicity	• Fast degradation by phagocyte system • Low encapsulation efficiency
Polymeric	• Biodegradable • Payload flexibility • High stability in circulation	• Difficulty for their scale-up • Possibility for aggregation and toxicity
Dendrimers	• Increasing solubility of extremely lipophilic drugs	• Unsuitable candidate for hydrophilic drugs
Nanogel	• Well-suited for stimuli nondrug delivery system strategy • Avoid quick phagocytic clearance	• Expensive procedure to remove solvents and surfactants
Metal-based	• Feasible for industrial applications • Well-suited for imaging and theranostic applications	• Toxicity concern • Solubility limitations
Carbon-based	• Extremely small and lightweight • Economic synthesis procedure • Mass production	• Toxicity concern • Not easy to handle

- improve the stability of the drug (Woods et al. 2020),
- enhance the circulating half-life (Hoshyar et al. 2016),
- reduce dosing frequency (Durham et al. 2016),
- improve cellular internalization (Doroudian et al. 2020),
- *decrease enzyme degradation* and *immunogenicity of the drugs* (Yu et al. 2016).

*Moreover, e*mploying nanoparticles to deliver therapeutic agents can overcome the natural structural and biological barriers found in pulmonary inflammatory diseases (Kaneko et al. 2020). Drug-loaded nanoparticles can be administered via different routes of administration, such as intravenous injection, oral, and via nebulized/inhalation route (Muralidharan et al. 2015; Mitchell et al. 2020). In the context of lung disease, the direct delivery to the target organ via the nebulized/inhaled route offers significant advantages which would include:

- noninvasive delivery,
- high bioavailability,
- rapid onset of action,
- enhanced drug accumulation in the target organ, the lung,
- avoiding gastrointestinal upset,
- escaping first-pass liver metabolism,
- fast drug absorption,
- ease and convenience for patients, leading to improved patient compliance (Anderson et al. 2020; Doroudian et al. 2020).

Historically, it has been well described that inhaled nano-drug delivery has the capacity for deep lung deposition of one selected drug. More recently, a more nuanced approach is now possible where controlled drug release within the lung can be regulated via specific internal and external stimuli such as temperature, pH, and externally via ultrasound frequency or exposure to magnetic fields. These are termed "smart nano-drug delivery systems" and allow lower therapeutic payloads to be administered to give an equivalent therapeutic response hence reducing the risk of dose-related adverse drug reactions and improving patient compliance (Doroudian et al. 2021). In this chapter, we focus on the current trends and developments in the nanomedicine field relating to the lung, and specifically for pulmonary inflammatory diseases, such as IPF, COPD, ARDS, and Asthma.

12.1.1 Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive fibrosing interstitial pneumonia of unknown etiology which is associated with fibroproliferation, myofibroblast differentiation, excessive collagen, and extracellular matrix deposition leading to impairment of gas exchange and respiratory failure (Fischer and Donnelly 2017; Wongkarnjana et al. 2019).

Pirfenidone is an anti-inflammatory and anti-fibrotic oral medication which has been approved by regulatory authorities as a treatment for patients with IPF (Kim and Keating 2015). This medication has both a high elimination half-life (~150 min) and low clearance saturation after multiple doses which reduces the drug accumulation in the body. Consequently, high daily doses are required (>2 g) to be administered, leaving individual patients susceptible to enhanced risk of systemic side effects (Raghu and Selman 2015; Trivedi et al. 2012). The use of nanotechnology offers the potential to solve these issues by optimizing the drugs pharmacokinetic and improving patient compliance. In vivo studies in an IPF murine model study, using nano-encapsulated pirfenidone, resulted in enhanced drug accumulation in the lung with evidence of enhanced anti-fibrotic activity as manifested by a significant reduction of inflammatory cells and extracellular matrix found in the lungs (Trivedi et al. 2012). Another attractive feature of nanotechnology delivery systems is its ability to adapt to targeting specific organs of interest. For example in excessive scar tissue in the skin—employing a transdermal delivery of pirfenidone via a nanogel-based carrier has been shown in in vivo models to deliver enhanced drug concentrations compared to the historical oral route (Abnoos et al. 2018).

Prostaglandin E_2 (PGE_2) is a well-described inflammatory mediator that has anti-fibrotic activity and augmentation of this mediator's activity in targeted organs represents a valid therapeutic strategy for a variety of inflammatory diseases (Vancheri et al. 2004). In order to effectively deliver PGE_2 into the lung for local therapy, PGE2 was encapsulated in a nano liposome and examined in an animal model of bleomycin-induced IPF. This investigation showed that 80% of the pulmonary administrated nano-liposomal PGE2 can accumulate in the lungs compared to 6% when compared to the intravenous route. Liposomal PGE2 was found to significantly inhibit the overexpression of matrix metalloproteinase-3 (MMP3), CCL12 chemokine (C–C motif) ligand 12 (CCL12), and *hypoxia-inducible factor 1-alpha* (HIF1A) which have been implicated in the pathogenesis of pulmonary fibrosis (Ivanova et al. 2013).

There has been recent interest in combination drug therapies administered via nano-carriers with the aim of enhancing treatment efficacy combined with limiting both the development of drug resistance and side effects (Doroudian et al. 2021). In this regard, an investigation was carried out where PGE2 along with three types of siRNA (MMP3, CCL12, and HIF1A mRNAs) were packaged in nanostructured lipid carriers (NLC-PGE2 + NLC siRNAs). The aim of this study was to decrease the adverse effects of the therapeutic agents while increasing the efficiency, stability, and solubility of active components by development of an inhalation nondrug delivery system. This combined treatment was administered in a murine IPF model and a significant reduction of fibrotic injury in the lung was shown in Fig. 12.2 (Garbuzenko et al. 2017). Chang et al. conducted another combination study in which they encapsulated two drugs, astaxanthin (AST) and trametinib (TRA), simultaneously into a poly(lactide-*co*-glycolide) or PLGA-based nano-carrier in order to enhance synergetic effect of the drugs to inhibit myofibroblast activation and to stimulate damaged lung regeneration. This approach showed significant therapeutic effects and exceptional anti-fibrotic efficacy compared to

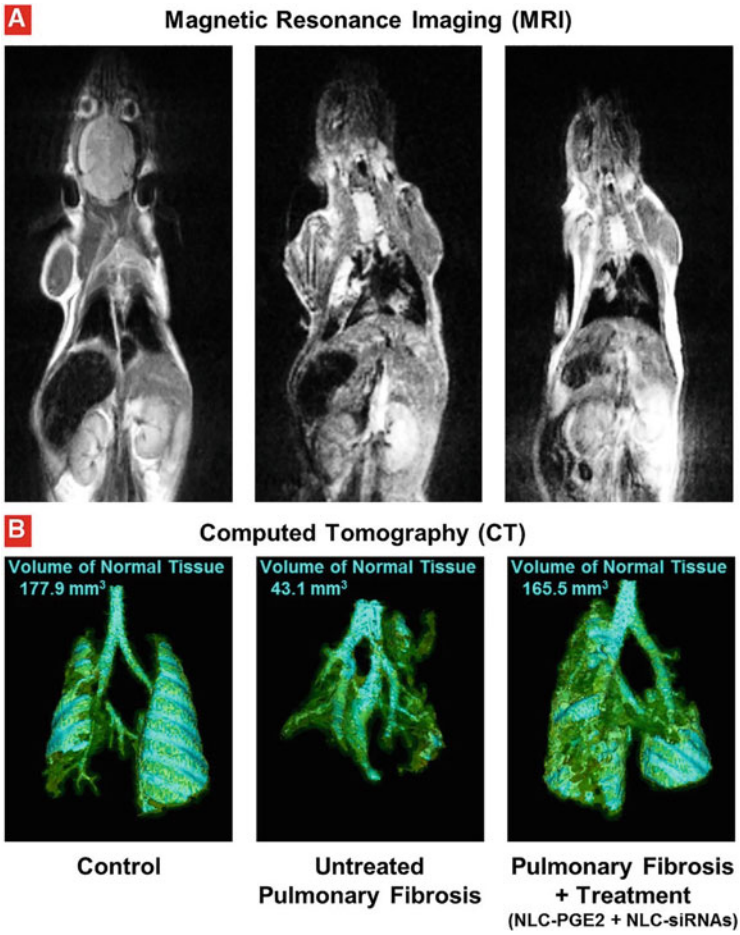

Fig. 12.2 Impact of drug-loaded nanoparticles on a bleomycin-induced IPF mouse model after 3 weeks treatment (**a**) illustrative magnetic resonance from healthy control, untreated, and treated mice (**b**) computed tomography. Cyan-colored areas show healthy lung tissue while green color represents fibrotic tissue areas of the lung (Garbuzenko et al. 2017) (Open access Journal)

traditional treatment, indicating that nano-drug carriers can be considered as a promising approach for deep lung deposition and maximum drug accumulation in local delivery for the treatment of IPF (Chang et al. 2020).

12.1.2 Chronic Obstructive Pulmonary Disease (COPD)

Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of death globally (World Health Organization 2018). It is characterized within the airways by

chronic inflammation, goblet cell hyperplasia leading to excessive mucus production which contributes to progressive irreversible airway obstruction. Earlier diagnosis offers the opportunity to limit disease progression. Currently, by the time the patients present with symptoms the disease has progressed significantly within their lungs (Andreeva et al. 2017). Thus, a global clinical unmet need would be the development of a noninvasive rapid screening test that would enable large-scale population screening to facilitate early disease diagnosis. Electronic nose (E-nose) is one of the extensively evaluated nanotechnology-based sensors capable of detecting and distinguishing specific volatile organic compounds (VOCs) in exhaled gas (Ratiu et al. 2021). This novel method offers attractive advantages such as fast data processing (<ms), sensitive to very low concentrations, simple and easy-to-use interface which facilitates high-throughput screening (Dragonieri et al. 2017). E-nose method has been evaluated from a clinical research perspective and has the discriminatory ability to differentiate COPD from Asthma (Fens et al. 2009), with an accuracy of 96%. It has also shown that E-nose method can discriminate eosinophilic- and neutrophilic-driven inflammation and their activation status in mild and moderate COPD, indicating the promises of exhaled breath analysis for specific VOC expression readouts in the evaluation and monitoring of airway inflammation in COPD (Fens et al. 2011). Bacterial and viral infections are major causes of COPD exacerbation (D'Anna et al. 2021). Recent investigations show that E-nose technology has the capacity to identify the presence of airway bacterial infection and also distinguish patients with COPD exacerbation from stable COPD (Shafiek et al. 2015). The results of a clinical trial (NTR4601), conducted to assess the ability of this method to distinguish infections in COPD patients suffering from exacerbation, achieved to detect both viral and bacterial infections delivered an area under the curve (AUC) of 0.74 and 0.72, respectively (Van Geffen and Kerstjens 2016). In another clinical study, Vries et al. integrated nano-sensor-based E-nose method with spirometry which resulted in an acceptable discrimination with high accuracy (almost 87%) to distinguish asthma, COPD, and lung cancer patients (De Vries et al. 2015). Recently, a clinical trial (NCT01976117) has been designed and carried out to compare E-nose method with protected specimen brush (PSB), which is a gold standard, but invasive, method for the diagnosis of lower respiratory tract infections. The use of E-nose technique shows a high accuracy (88%) to discriminate the colonized and non-colonized patients with COPD; (Sibila et al. 2014). These studies highlight the potential of E-nose nanotechnology as a noninvasive, practical, and reliable technique in clinical practice for both the clinical diagnosis of disease and clinical exacerbations in COPD.

COPD results in enhanced secretion of a viscous mucous layer, which forms a "barrier" that decreases substantially efficacious drug delivery (Murgia et al. 2018; Ramos et al. 2014). This also provides an optimum environment for opportunistic infections to colonize and subsequently form a thick bacterial biofilm further limiting drug delivery and activity (Zhang et al. 2017; Dua et al. 2018). Aminoglycosides are frequently used antibiotic medications for the treatment of hospital-acquired infection in COPD (Quon et al. 2014). Previous investigation had shown that a strategy to package amikacin, an aminoglycoside antibiotic, into nanoparticles

Table 12.2 Clinical trials involving LAI as a liposomal nano-delivery system against bacterial infections in chronic pulmonary diseases

Clinical trial registration no.	Indication	References
NCT03905642	*Pseudomonas aeruginosa* infections	Clancy et al. (2013)
NCT00777296	*Pseudomonas aeruginosa* infection	Clancy et al. (2013)
NCT00558844	Bacterial infections	Clancy et al. (2013)
NCT00558844	*Pseudomonas aeruginosa* infections	Clancy et al. (2013) and Okusanya et al. (2014)
NCT01315236	*Mycobacteria* infection	Olivier et al. (2017)
NCT02081963	Acute exacerbation of bronchiectasis	Ailiyaer et al. (2018)
NCT02344004	*Mycobacterium avium complex* (MAC) lung infection	Griffith et al. (2018)
NCT03038178	*Mycobacterium abscessus* lung infection	Siegel et al. (2018)
NCT01316276	*Pseudomonas aeruginosa* infections	Doroudian et al. (2019)
NCT00775138	Lung infection in bronchiectasis patients	Doroudian et al. (2019)
NCT01315678	Chronic *Pseudomonas aeruginosa* infections	Bilton et al. (2020)
NCT02628600	*Mycobacterium avium* complex (MAC) infections	Winthrop et al. (2020)

improves the stability of the drug with a sustained-release profile (Ghaffari et al. 2011), enhances the drug concentration and cellular uptake in respiratory track which facilitates targeted and localized drug delivery to desired site while minimizing drug systemic side effects (Varshosaz et al. 2013). These earlier advances paved the way for the development of an encapsulated amikacin in liposomal nanoparticles that was adapted for aerosolized administration, and hence its name liposomal amikacin for inhalation (LAI), also known as Arikayce. In several clinical studies LAI has been found to be an effective and well-tolerated treatment therapeutic formulation in lung infections as a consequence of pulmonary inflammatory diseases (Griffith et al. 2018; Olivier et al. 2017; Siegel et al. 2018) (Table 12.2). In 2018, Arikayce was approved by US Food and Drug Administration (FDA) for the treatment of bacterial infections. Also, Arikayce is the first FDA-approved nanoformulation in the United States for the treatment of refractory *Mycobacterium avium* complex (MAC) (Shirley 2019; Eleraky et al. 2020).

Oxidative stress has been implicated as a key injurious drive of disease pathogenesis in COPD (Barnes et al. 2015, 2021). Consequently, there has been a recent focus within academia and industry to develop anti-inflammatory therapies, and in particular antioxidant strategies in COPD. Oral administration of antioxidants presents significant challenges such as low diffusion/absorption rate, sub-optimal drug pharmacokinetics, and a short half-life of antioxidants. Therefore, there is a pressing need to develop novel specific drug carriers to deliver antioxidant and anti-inflammatory drugs to the target organ, namely the lung in COPD (Xu et al.

2020). Intranasal administration of encapsulated non-steroidal anti-inflammatory, ibuprofen, in PEGylated PLGA nanoparticles, which was also conjugated with an anti-neutrophil antibody (NIMP-R14) to target neutrophils, was recently investigated in a murine model of COPD. Intranasal administration of this nano-formulation in two in vivo models, a *Pseudomonas aeruginosa* lipopolysaccharide (LPS)-induced model and a chronic cigarette smoke-induced inflammatory lung disease model, led to a significant reduction in the NF-κB activation and a consequent dramatic decline in the number of infiltrating neutrophils within the lung (Vij et al. 2016).

12.2 Acute Respiratory Distress Syndrome and Acute Lung Injury

Acute Respiratory Distress Syndrome (ARDS) represents a fulminant systemic injurious inflammatory response that takes out the lung as the favored target organ, with the breakdown of alveolar capillary integrity with consequent leakage of an inflammatory exudate into the airspaces with resultant progressive respiratory failure, requiring intensive care support (Williams et al. 2021). In the context of therapeutic strategies in ARDS, nanoparticles as drug carriers can be employed to enhance the bioavailability, to improve biodistribution, and to provide prolonged-release drug by which the pharmacokinetics and pharmacodynamics of current and future therapeutic agents can be improved (Sadikot et al. 2017). A growing recent body of evidence has highlighted the potential of novel delivery systems of specific anti-inflammatory and antioxidative therapeutics as front-line treatment of ARDS (Shurbaji et al. 2021; Yu et al. 2020; Wang et al. 2020; Bobba et al. 2021; Zhang et al. 2019; Matthay et al. 2017). Previously published work in acute lung injury (ALI) murine models showed that *N*-acetylcysteine-loaded nano liposomes decrease lung permeability index by circa 60%, with a consequent significant reduction in lung pro-inflammatory cytokines [i.e., CINC-1, IL-1β, and TNF-α (Hoesel et al. 2008)]. Oleic acid has been shown to possess anti-inflammatory properties. Fang and colleagues encapsulated oleic acid in a lipid-based nano-carrier to increase the bioavailability and improve its pulmonary biodistribution. They found that these drug-loaded nanoparticles significantly decreased neutrophilic infiltration and inhibited both superoxide and elastase production in this murine ALI-model (Yu et al. 2020). Nitric oxide is well recognized to have anti-inflammatory and vasodilator activity but possesses a relatively short half-life which, to date, has limited its potential as a therapy in ARDS. The application of a nanogel carrier loaded with nitric oxide has been shown in an in vitro model of murine lung airways to lead to the slow release and a relatively prolonged anti-inflammatory effect inflammatory effect in this model (Shurbaji et al. 2021).

Stimuli responsive and targeted nanoparticles are characterized as smart nano-drug carriers which offer numerous advantages over standard nano-carriers and have

shown promising results in recent ARDS and ALI studies to prolong the therapeutic effects, reduce required drug dose, and improve the pharmacokinetic properties of the drugs in in vivo models (Zhang et al. 2019; Patil et al. 2018; Brenner et al. 2018).

12.3 Asthma

Asthma is characterized by repetitive insults leading to airway hyper-responsiveness, airway inflammation, and subsequent airway obstruction (Holgate et al. 2015; Chung 2013). The classical delivery modalities for asthma treatments are via the oral or inhaled routes. The ability to directly target the lung via the inhaled route offers distinct advantages but it also has significant challenges such as even lung deposition—centrally and peripherally, drug absorption kinetics, stable tissue retention, and the systemic spill-over and the risk of systemic side effects. This has led to extensive investigation into effective nondrug carriers in an attempt to overcome many of these challenges (Kan et al. 2020).

Extracellular vesicles (EVs) are a natural, lipid-based, and cell-derived nano-drug carrier which has several unique properties which include their ability to move through the natural barriers with extended in vivo circulation time, maximum biocompatibility, minimum toxicity, and immunogenicity (Vader et al. 2016). Previously published work has shown that packed-immune-modulators in EVs can inhibit allergic sensitization and lung inflammation by stimulation of immunosuppression in allergic asthma animal models (Almqvist et al. 2008; De Castro et al. 2017; Du et al. 2018). These encouraging results led to a clinical trial study (NCT03059017) to examine the therapeutic efficacy of EV-based carrier packaged with salbutamol sulfate, a bronchodilator used in asthma. This study revealed a controlled release profile and rapid onset of efficacy with satisfactory safety profile for the treatment of acute asthma (Doroudian et al. 2019). While standard EVs as a nano-carrier offers significant improvement, particularly in terms of delivery efficiency in asthma, they suffer from sub-optimum drug loading efficiency (Mitchell et al. 2020). To address this issue, polymeric nanoparticles can be designed to provide high loading efficiency as well as slow and sustained drug release (Mukherjee et al. 2019; Doroudian 2019). Polymeric nanoparticles have also been shown to persist for longer in the respiratory tract, and enhance the solubility of anti-inflammatory agents in murine allergic asthma models (Roy et al. 2020; Wang et al. 2018). An additional strategy to limit drug degradation and thus prolong drug activity within the airways has focused on the additional packaging of peptide drugs (Athari et al. 2016), antibodies (Rincon et al. 2017), or oligonucleotides (Givens et al. 2018; Ramelli et al. 2020; Da Silva et al. 2020) within the polymeric nanoparticles, all of which has shown safe and effective strategies to decrease the inflammatory factors level. These studies have shown in vivo animal models of asthma to significantly reduce airway hyper-responsiveness (AHR). These in vivo results show promise and add to the expanding scientific literature to support future

investment in human clinical trials aimed at the optimum management of clinical disease in Asthma.

12.4 Conclusion

Pulmonary inflammatory diseases represent a major global clinical burden on healthcare providers, and on patients and their families. Over recent years, we have witnessed exponential growth in scientific published work in the nanomedicine field in the developments and applications of nanotechnology for the diagnosis and treatment of pulmonary inflammatory diseases. It is now well recognized that nanotechnology-based approaches can overcome many of the limitations of conventional therapies not only by developing targeted nano-drug carriers with selected properties that deliver therapeutic agents to the lung but also therapies with significantly improved pharmacokinetics and efficacy. It is imperative as we welcome a new dawn and the future potential of a suite of nanomedicine-based clinical offerings, that we proceed with due rigor to ensure that full characterization is undertaken of the proposed clinical application that maximizes treatment efficacy and minimizes nano-specific toxicity.

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Chapter 13
Nanomedicine Applied to Inflammatory Bowel Diseases

Cintia M. S. Cereda and Giovana R. Tofoli

Abstract Inflammatory bowel disease (IBD) includes Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis. IBD is a diverse disorder with contributions from genetic background, microbiota, host-related factors, and environmental factors. It is not curable, and treatment aims to increase quality of life and diminish evolution and complications of disease. Conventional treatment for IBD includes aminosalicylates, systemic corticosteroids, topical corticosteroids, antibiotics, immunomodulators, and biologic therapies. Usually, these treatments strategies rest on a considerable dosage which are often associated serious potential negative consequences. Another important issue is the requirement to obtain the delivery of therapy directly in the intestinal inflamed tissue. Thus, the incorporation of nanomedicine delivery systems that will improve therapeutic efficacy and decline systemic side effects seems to be a useful and beneficial option for IBD. Nanomedicine has the capacity of modernized IBD treatment and it might improve compliance and quality of life. Thus, in this chapter we will review nanomedicine systems with the usual drugs used for the treatment of IBD and the benefits of such approach. We will describe how to target the release of 5-ASA to colon using enteric polymer coating or nanoparticles. Corticosteroids formulations for oral administration with a pH- and time-dependent release and topical formulations will also be described. Finally, strategies to improve direct delivery of biological agents such as siRNAs to the gastrointestinal tissues are also covered in this chapter.

Keywords Inflammatory bowel diseases · Nanomedicine · Drug delivery · Crohn's disease · Ulcerative colitis

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Abbreviations

5-ASA-SiO_2 NPs	5-ASA-loaded silicon dioxide nanoparticles
ASA	Aminosalicylic acid
CD	Crohn's disease
cKGM	Cationic konjac glucomannan
CS	Chitosan
DSS	Dextran sulfate sodium
Gal-siTNF-NPs	siTNF into galactosylated polymeric nanoparticles
HPMC	Hydroxypropyl methylcellulose
HP-β-CD	Hydroxypropyl-β-cyclodextrin
HSA	Human serum albumin
IBD	Inflammatory bowel disease
IFN-g	Interferon gamma
IL	Interleukin
MPO	Myeloperoxidase
NO	Nitric oxide
NPs	Nanoparticles
PEI	Polyethyleneimine
PG	Prostaglandin
PLGA	Poly(*d*,*l*-lactide-*co*-glycolide acid)
PS-ATNF-α	Phosphorothioated antisense oligodeoxyribonucleotide of TNF-α
siTNF	TNF-α gene silencing
Th	T-helper
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumor necrosis factor alpha
UC	Ulcerative colitis

13.1 Introduction

Inflammatory bowel disease (IBD) is the range of disorders that include Crohn's disease (CD), ulcerative colitis (UC), and indeterminate colitis (Kelsen and Sullivan 2017; Flynn and Eisenstein 2019). There are clinical differences among Crohn disease and ulcerative colitis. While inflammation in UC is limited to the colon and categorized as a neutrophilic inflammation, CD usually presents transmural pleomorphic inflammation that might comprise all gastrointestinal tract. In this case, patients may develop fistulas, granulomas, and abscesses (Kelsen et al. 2019). Common symptoms comprise diarrhea, bloody stools, chronic pain in the lower abdomen, abnormal defecation patterns, weight loss, general discomfort, malnutrition, anemia, delayed growth and sexual maturation in children, arthralgias, arthritis, mucocutaneous lesions, ophthalmologic complications, bone

abnormalities, hepatobiliary and renal diseases, increased colorectal cancer risk, high morbidity, and loss of quality of life (Sairenji et al. 2017; Yang and Merlin 2019; Lázaro et al. 2020). Commonly, due to disease swings, patients will possibly experience periods of disease exacerbation alternating with clinical remission (Shivashankar and Lichtenstein 2018).

The cause of IBD is incompletely understood and it is a diverse disorder with contributions from genetic background, microbiota, host-related factors, and environmental factors (Kelsen and Sullivan 2017; Flynn and Eisenstein 2019). IBD patients may have genetic polymorphisms, with 30 specific loci for UC and 41 for CD. But it is important to note that 137 loci are associated with both CD and UC, indicating that these diseases might show common inflammatory pathways (Ramos and Papadakis 2019). These genetic changes cause an enlargement in T cell function in IBD. In CD, inflammation is augmented by T-helper (Th) 1 and Th17 responses that causes secretion of interleukin (IL)-17, interferon gamma (IFN-g), and tumor necrosis factor alpha (TNF-a) causing an uninterrupted inflammation cycle. In UC, the response is Th2 intermediated, which causes more activation of B cells and natural killer T cells (Flynn and Eisenstein 2019).

Environment factors, diet, drugs, geography, social stress, and psychological element are also involved in the development of IBD (Zhang and Li 2014). Diet composition is also an important factor in IBD development. Eating fat and processed meat in excess are linked with IBD, while high-fiber diets are related to a decrease in the risk of CD. Diversity of the host microbiome is also an important factor for the development of IBD. In this sense, any factor that disturb the host microbiome such as antibiotics, non-steroidal anti-inflammatories, contraceptives, and statins increase the risk of development of IBD (Flynn and Eisenstein 2019).

IBD is not curable and main objective of treatment is to improve quality of life and minimalize progression and complications of disease (Sairenji et al. 2017). Medical treatment for IBD will depend on factors such as type, localization, and severity of the disease and treatments of CD and UC differ significantly and need to be individualized (Leitner and Vogelsang 2016; Sairenji et al. 2017). Conventional treatment for IBD includes aminosalicylates, systemic corticosteroids, topical corticosteroids, antibiotics, immunomodulators, and biologic therapies (Jacob et al. 2020).

For UC treatment, severity of disease and the degree of colonic involvement are key factors for drug choice and delivery method. Patients with mild to moderate disease may benefit from oral or topical aminosalicylates alone or in combination with topical steroids. For severe UC, the treatment might include an anti-tumor necrosis factor, immunosuppressive, and anti-inflammatory. For CD treatment, topical medications are less effective due to its diffuse nature. Therapy usually starts with less potent drugs, often with fewer side effects. Then, the therapy might be "stepped up" for more potent drugs such as immunomodulators or biological agents. The decision to change therapy includes the patient's goals, risk tolerance, side effects, cost, and patient compliance (Sairenji et al. 2017).

In the 1990s, management of IBD was largely performed with steroids and 5-aminosalicylic acid drugs. In 2000s, the emergence of biologic treatments, such

as anti-TNF-α antibody, changed disease concept and therapeutic objectives. However, the best protocol and the long-term safety of each drug have not yet been clarified. Also, the traditional strategies depend on repeated administration of high dosages of drugs, such as antibiotics, non-steroidal anti-inflammatories, biologics, and immunomodulators. Some of these medications are effective in relieving inflammation, but their long-term effectiveness is affected by its common adverse effects. Immunosuppressive and anti-inflammatory present serious potential negative consequences that must be considered in treatment decisions (Yang and Merlin 2019; Nakase 2020; Ahmad et al. 2021).

Considering the wide spectrum of adverse effects evoked by drugs used in IBD treatment and the need to obtain the delivery of therapy directly in the intestinal inflamed tissue, the incorporation of nanomedicine delivery systems in the treatment of IBD seems to be a useful and beneficial option. The use of nano-drug delivery systems could help to sustain remission and relapse of IBDs, it might also increase therapeutic efficacies and decrease systemic side effects (Jacob et al. 2020). Thus, in this chapter we will review nanomedicine systems with the usual drugs used for the treatment of IBD and the benefits of such approach.

13.1.1 Nanomedicine in Inflammatory Bowel Disease

Nanomedicine is the application of materials in the scale range from 1 to 1000 nm to health and medicine. Nanoparticles composed of different materials, shapes, sizes, and diverse physicochemical properties are able to offer unique interactions between biomolecules and cell surface or interior, which can improve the effectiveness or reduce the profile of adverse effects (Laroui et al. 2013).

Although considered a scientific area still in its initial stages, nanomedicine research has resulted in significant impact through a range of applications (Nance 2019; Jacob et al. 2020). The use of nanomedicine delivery systems for the treatment of IBD is also in its beginning, nevertheless recent evidences have made it look promising (Jacob et al. 2020). New studies have suggested that nanosized molecules can penetrate the epithelium and interfere with the inflammatory process (Takedatsu et al. 2015).

Nano-drug delivery's type, compositions, structure and morphology can be designed for various purposes. It can achieve increased bioavailability and minimize adverse effects, it is possible to improve transport of molecules to the inflamed tissue through epithelial enhanced permeability and retention, and it is also possible to occur selective uptake of nanoparticles by the immune cells at the inflamed site (Jacob et al. 2020). Several strategies and nanotechnology have been described to enhance success of IBD. These approaches include a variety of size, charge, pH response, degradation, ligand–receptor, and microbiome-influenced drug delivery systems (Laroui et al. 2013; Yang and Merlin 2019). Nowadays it is possible to obtain biocompatible nanomedicines with a highly controlled physical–chemical properties (shape, size, and surface charge, for example) that can avoid some of

the limitations of traditional therapy (Brusini et al. 2020). Figure 13.1 displays some of developed nanocarriers systems.

Nevertheless, all therapies have one mutual objective: increase the local drug concentration at the site of inflammation in order to take full advantage of the anti-inflammatory effect of the loaded/encapsulated drug and minimize systemic adverse effects. In this chapter, we will discuss the possibilities of change proposed in traditional treatments at a preclinical stage of 5-aminosalicylic acid, corticosteroids and biological agents such as TNF-antibodies. Table 13.1 shows some of the preclinical studies with nano-drug delivery systems for aminosalicylates (5-ASA), corticosteroids, and biologic therapies.

13.1.2 Nanomedicines with Mesalazine (5-ASA)

5-ASA is widely used for the treatment of IBD, being considered safe and effective. It can be used with oral, rectal, or combination dosage. The action mechanism of 5-ASA includes suppression of nuclear factor-kappa B pathway, protection of epithelial barrier function against peroxynitrite, scavenging free radicals, synthesis inhibition of pro-inflammatory cytokine, leukotriene, and prostaglandin. These actions provide immunosuppressive, anti-inflammatory, and antioxidant properties of 5-ASA (Sardo et al. 2019).

After oral administration of 5-ASA, a rapid and extensive absorption in the upper intestinal region occurs, which leads to low efficacy and increase rate of adverse effects, such as nephrotic syndrome, myopericarditis, and fever. Also, this rapid absorption occurs before the drug reaches the inflamed tissue and it could produce a small drug concentration in the colonic region (Yuri et al. 2020). For that reason, targeting the release of 5-ASA to colon might reduce these problems. For IBD treatment, an ideal 5-ASA delivery system should maximize delivery to the colon and minimize systemic absorption in the upper parts of the gastrointestinal tract (Ahmad et al. 2021). There are few 5-ASA delivery systems, orally or rectally, that are commercially available. On the other hand, there are a huge number of studies that describes research and development of delivery systems for 5-ASA (Sardo et al. 2019).

To release 5-ASA in the colon after oral administration, commonly the nanomedicine carrier is pH-dependent (Yuri et al. 2020). One approach to achieve release in the ileum is to coat the 5-ASA with pH-dependent polymers, such as Eudragit (Sardou et al. 2019). This substance is used as an enteric coating polymer and it can avoid the gastric acidic surroundings to successfully achieve colon epithelium (Duan et al. 2017; Ahmad et al. 2021). This enteric polymer coating can be used in association with various kinds of polysaccharides, cellulose derivatives, and with nanocarriers (El-Bary et al. 2012; Tang et al. 2017; Sardou et al. 2019; Foppoli et al. 2019; Ahmad et al. 2021). In general, the results showed that the enteric polymer alone or in combination with other technologies were effective

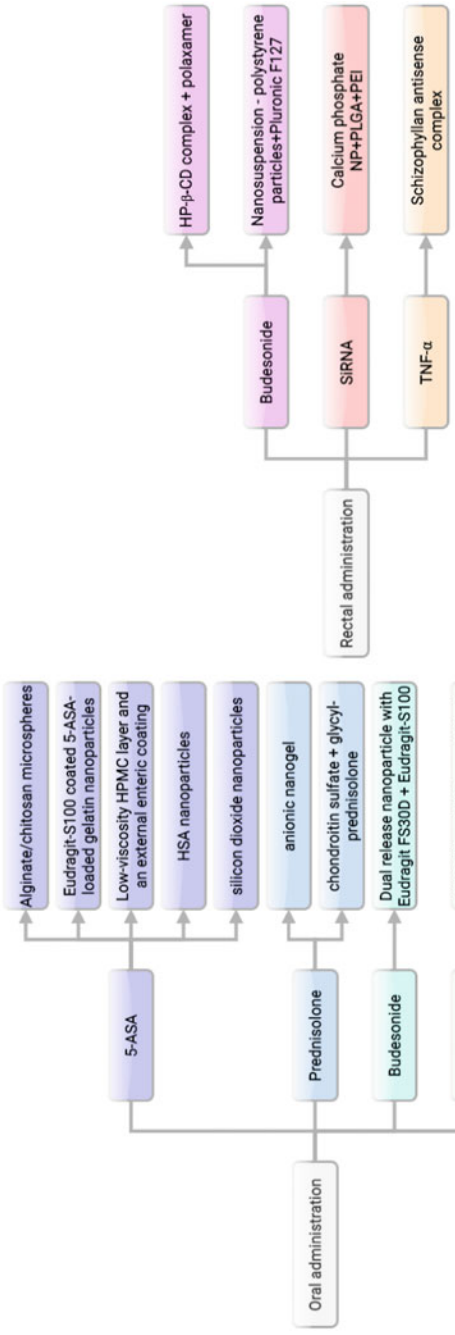

Fig. 13.1 Nanomedicines systems for inflammatory bowel diseases in preclinical development for oral and rectal administration

Table 13.1 Preclinical studies with mesalazine (5-ASA), corticosteroids, and biological agents with nano-drug delivery systems

Nanocarrier	Inflammatory induction and animal model	In vivo experiments	Main results	References
Co-delivery of zinc and 5-aminosalicylic acid from alginate/*N*-succinyl-chitosan blend microspheres	TNBS-induced colitis in male Wistar rats	In vivo therapeutic efficacy was assessed by visible colonic damage and clinical scoring system. Main blood parameters were determined	Microspheres system relieved colonic inflammation and enhanced the efficacy of 5-ASA in healing of colitis in rats. There was no toxicity after oral administration of the new formulation	Duan et al. (2017)
5-ASA-loaded silicon dioxide nanoparticles	DSS-induced colitis in male BALB-c mice	The therapeutic effect was assessed based on the disease activity index, colon histopathology, myeloperoxidase (MPO), and levels of tumor necrosis factor-α and interleukin-6	5-ASA-SiO NPs were able to release the drug into the inflamed tissue and highly increases therapeutic efficacy. The 5-ASA-SiO_2 NPs achieved similar effects to higher dosages of 5-ASA	Tang et al. (2017)
Nanoparticles prepared with human serum albumin conjugated with 5-cid (5-ASA-HSA NP)	DSS-induced colitis in mice	The clinical activity of colitis was evaluated by disease activity index and samples colon were observed by histopathology and immunohistochemistry analysis	The results indicate that colon inflammation could be inhibited by the 5-ASA-HSA NP formulation. The colon remains structurally intact after the treatment	Iwao et al. (2018)
The system consists of a tablet core containing 5-ASA, covered by a low-viscosity HPMC layer and an external enteric coating	NA	A pharmacoscintigraphic investigation was undertaken in an oral 5-ASA delivery system in 6 healthy male volunteers	The imaging study showed that disintegration of the administered units was in no cases observed prior to colon arrival. Fragmentation mainly occurred in the caecum, ascending or transverse colon. Markedly, the small intestinal transit time was proved fairly reproducible	Foppoli et al. (2019)
Gelatin nanoparticles loaded with 5-ASA, with an outer coating composed of Eudragit-S100 polymer	DSS-induced colitis in Swiss albine mice	Abundance of crypts foci in colon of mice, among different treatment groups was studied and better	Treatment with 5-ASA NPs resulted in significant attenuation in mast cell infiltration	Ahmad et al. (2021)

(continued)

Table 13.1 (continued)

Nanocarrier	Inflammatory induction and animal model	In vivo experiments	Main results	References
		therapeutic efficacy of 5-ASA NPs over free 5-ASA was further assessed by hematoxylin and eosin-stained histological sections of colon		
Eudragit-S100 coated 5-ASA-loaded gelatins NPs	Acetic acid-induced colitis in white albino rats	To assess the degradation of the polymeric coat of the microspheres by the colonic microflora, the microspheres were instilled by performing cecal ligation	In vivo study in rats performed by colonic inflammatory lesions demonstrated a remarkable reduction in ulcer index treated with microspheres. Histopathological study confirmed no signs of ulceration or bleeding	Patole and Pandit (2017)
Nanoparticles loaded with budesonide with dual release pH (Eudragit FS30D) and time (Eudragit-S100)-dependent mechanism for oral administration	Dextran sulfate sodium (DSS)-induced colitis in mice	To assess the efficacy of budesonide in the dual system (pH/time-dependent NPs), pH NPs or time NPs the authors observed colitis severity by disease activity index, micro and macroscopic assessment of the disease	Dual system pH-/time-dependent NPs-treatment showed increased body weight and decreased in disease activity index, histological damage and inflammatory cell infiltration in colon tissue. The dual pH/time-dependent NPs were effective to promote oral colon-targeted delivery of budesonide	Naeem et al. (2015)
Budesonide complex with HP-β-CD in a poloxamer-based thermoreversible hydrogel for rectal administration	Rat model of colitis induced by trinitrobenzene sulfonic acid (TNBS)	Efficacy of the new formulations was assessed via MPO activity, macroscopic, and microscopic damage score in colon tissues. TNF-α, IL-1β, IL-10, and endogenous glucocorticoids levels	MPO activity after treatment with $BUD_{HP\text{-}\beta\text{-}CD}$ poloxamers formulations was similar with rats without colitis. All tested new formulation decreases TNF-α and IL-1β levels. $BUD_{HP\text{-}\beta\text{-}CD}$ + PL 407 (20%) and $BUD_{HP\text{-}\beta\text{-}CD}$ + PL 407 (18%) + PL	Lázaro et al. (2020)

			403 (2%) induced lower levels of systemic glucocorticoids when compared to plain BUD	
Budesonide nanosuspension (NS) composed of polystyrene particles with 200 nm with a muco-inert Pluronic F127 for rectal administration	Mice model of colitis induced by trinitrobenzene sulfonic acid (TNBS)	Efficacy of a new budesonide NS enema using an acute TNBS mouse model of IBD. Micro and macroscopic damage were assed as well as number of inflammatory macrophages and IL-β producing $CD11b^+$ cells in colon tissue in colonic lamina propria cells with flow cytometry	Daily budesonide NS enema reduced the macroscopic and microscopic symptoms of IBD compared to untreated controls. Also, the new enema significantly reduced number of inflammatory macrophages and IL-β producing $CD11b^+$ cells in colon tissue in colonic lamina propria cells	Date et al. (2018)
Prednisolone in an anionic nanogel system (NG) for oral administration	Rat model of colitis induced by trinitrobenzene sulfonic acid (TNBS)	Gastrointestinal distribution and plasma concentrations of prednisolone were investigated after the intragastric administration of this new system	After 6 and 12 h of the administration, prednisolone was distributed in the lower parts of the gastrointestinal. The systemic absorption of prednisolone with NG(S) was very low. These results showed that prednisolone was slowly released and this promoted accumulation of the drugs in the colonic parts	Zhou et al. (2020)
Chondroitin sulfate and glycyl-prednisolone were conjugated in a nanogel for oral administration	Rat model of colitis induced by trinitrobenzene sulfonic acid (TNBS)	Efficacy of the new formulation was assessed by the observation of the severity of colitis, stool consistency, and colonic damage. Also, the authors determined the drug biodisposition in the gastrointestinal tract	The presence of the nanogel with chondroitin sulfate and glycyl-prednisolone improved the therapeutic efficacy of prednilosone. The system enhanced the drug delivery to the lower intestines	Onishi et al. (2019)
Phosphorothioated antisense oligodeoxyribonucleotide of TNF-α (PS-ATNF-α) in a chitosan alginate hydrogel	Dextran sulfate sodium (DSS)-induced colitis in mic	Efficacy of the new formulation was evaluated after oral administration with MPO, TNF-α, and malondialdehyde levels	The new formulation reduces TNF-α production and MPO activity	Duan et al. (2019)

(continued)

Table 13.1 (continued)

Nanocarrier	Inflammatory induction and animal model	In vivo experiments	Main results	References
siRNA-loaded nanoparticles of a calcium phosphate core encapsulated into poly(*d*,*l*-lactide-*co*-glycolide acid) (PLGA), and coated with a final outer layer of polyethyleneimine for rectal administration	Dextran sulfate sodium (DSS)-induced colitis in mice	Evaluated the in vivo efficacy with disease activity index (loss of body weight, rectal bleeding, stool consistency) and severity of colitis was assessed by measuring hematocrit levels, colon length, and histological analysis Mice were treated with the new formulation for 5 days administered intrarectally	TNF-α, IP-10, and KC siRNA-loaded nanoparticles covered with polyethyleneimine effectively deliver siRNA into the cytoplasm of epithelial cells and immune cells in vivo and thus induce active gene silencing via RNA interference. The siRNA-loaded nanoparticles promoted specific knockdown of target genes at the site of inflammation and ameliorate intestinal inflammation	Frede et al. (2016)
TNF-α siRNA packed in PLGA into nanoparticles with galactosylated chitosan	Dextran sulfate sodium (DSS)-induced colitis in mice	Evaluated the in vivo efficacy with weight loss, myeloperoxidase activity, colon length, and TNF-α in blood and colon tissues	The nanoparticles efficiently delivered TNF-α siRNA as demonstrated by the improvement of colitis parameters such as weight loss and MPO activity	Huang et al. (2018)
Combination of TNF-α siRNA (siTNF) and IL-22. siTNF in galactosylated polymeric nanoparticles plus IL-22 embedded in a hydrogel (chitosan/alginate) for oral administration	Dextran sulfate sodium (DSS)-induced colitis in mice	Spleen weight and colon length were measured. Colon samples were used for histopathological analysis ND to MPO and RNA analysis	The new formulation showed robust capacity to downregulate the expression of pro-inflammatory factors and promote mucosal healing. This formulation also improved colon mucosa healing	Xiao et al. (2018)
Macromolecular complex with schizophyllan-antisense TNF-α complex for intrarectal administration	Dextran sulfate sodium (DSS)-induced colitis in mice	Evaluated the expressions of TNF-α, IL-1β, and IL-6, in the mucosa of DSS-treated mice were examined via real-time PCR. Colon length and histological analysis were also performed	TNF-α production was significantly inhibited by SPG-antisense TNF-α. The topical therapy by SPG-antisense TNF-α ameliorated intestinal inflammation	Sakisaka et al. (2020)

strategies to promote optimization pH-dependent, colon-targeted, and/or sustained-release 5-ASA.

The mesalamine-loaded alginate microspheres filled in enteric-coated hydroxypropyl methylcellulose (HPMC) capsules were tested for local treatment of UC by Patole and Pandit (2017). The authors observed both in vivo and in vitro experiments, that the combination of HPMC with Eudragit FS30D provided a high degree of protection from premature drug release in the stomach and small intestine and was found to release the drug in colon (Patole and Pandit 2017).

In 2017, Tang and colleagues developed 5-ASA-loaded silicon dioxide nanoparticles (5-ASA-SiO_2 NPs) and assessed its therapeutic effect using a colitis model in mice. The authors observed that 5-ASA-SiO_2 NPs were able to target the inflamed colon, and the analysis of disease activity index, colonic histopathology, myeloperoxidase (MPO), serum IL-6, and TNF-α levels demonstrated a significant improvement in comparison to control group (Tang et al. 2017).

More recently, Tang et al. (2018) evaluated a hydroxypropyl-β-cyclodextrin (HP-β-CD) inclusion complex for a sustained-release system for mesalazine, carried by chitosan (CS) nanoparticles (NPs). The results showed that the presence of the inclusion complex with NPs inhibited the production of NO, PGE_2, and IL-8, demonstrating that this new formulation had better anti-inflammatory effects compared with free mesalazine in colon cancer cells.

Iwao et al. (2018) developed a new strategy site-specific drug delivery of 5-ASA in the colon for UC treatment. The authors prepared human serum albumin nanoparticles (HSA NPs) conjugated with 5-aminosalicylic acid (5-ASA) that potentialize the interaction between myeloperoxidase (MPO) and human serum albumin (HSA). The results achieved after UC induction in mice demonstrated that HSA NP formulation was able to deliver 5-ASA to the inflamed area with high expression of MPO.

Besides these preclinical data, there are some formulations that are already approved by FDA for IBD treatment with 5-ASA, such as ASACOL HD®, PENTASA®, APRISO®, CODES®, DELZICOL®, LIALDA®, and MEZACANT®. These formulations use nanotechnology to release 5-ASA with a pH and/or time-dependent mechanism (Yang and Merlin 2019).

13.1.3 Nanomedicines for Glucocorticosteroids

Corticosteroids are systemic anti-inflammatory drugs commonly used to treat autoimmune conditions such as IBDs. It can be used during the active phase of the disease or after the resolution of disease, in maintenance therapy. Both oral and topical modalities can be effective depending on extent and severity of disease, and in some cases, it is necessary to use intravenous application. Hydrocortisone (topical form), prednisone (oral route), methylprednisolone (intravenous route), and budesonide (oral or topical form) are the most common glucocorticoids used in IBD treatment (Kelsen et al. 2019; Sairenji et al. 2017; Yang and Merlin 2019; Lamb

et al. 2019). However, long-term use of conventional formulations of corticosteroids can lead to several adverse effects, such as diabetes, venous thromboembolism, poor wound healing, Cushing's syndrome, osteoporosis, infections related to *Candida* spp., acne, weight gain, mood swings, moon face, and hair loss (Waljee et al. 2018; Jacob et al. 2020).

Budesonide is a topical anti-inflammatory synthetic steroid that can be used either after oral or topical administration. Due to its low systemic absorption, budesonide has a minor rate of systemic adverse effects when compared to conventional corticosteroids (33% vs 55%), and it is not associated with adrenal suppression or important reduction in bone mineral density (Abdalla and Herfarth 2016; Lamb et al. 2019; Brusini et al. 2020).

New formulations of budesonide associated with nanocarriers have been developed to improve its efficacy. A series of strategies have been used to minimize early drug release in the stomach and small intestine since the release and permanence of budesonide must be in the colon (Brusini et al. 2020). Formulations with polymer nanoparticles composed of Eudragit FS30D lead to a pH- and time-dependent release to improve the efficacy of budesonide in a mouse model of colitis (Naeem et al. 2015).

In fact, there are three formulations approved by the FDA for oral application of budesonide that were designed to boost uptake into the colon such as Entocort EC®, Ulceris®, and Targit®. Entocort EC® uses gelatin capsules with enteric-coated granules as a dual pH- and time-dependent release mechanism. Ulceris® also uses dual pH- and time-dependent release strategy but it is also associated with an extended release obtained with a multi-matrix system with hydrophobic and hydrophilic coating. Targit® is an oral form that has a pH-sensitive coating onto injection-molded starch that relies on pH and bacteria degradation to release the drug in the colon (Yang and Merlin 2019).

Recently, Gareb et al. (2019) developed an ileo-colonic-targeted zero-order sustained-release tablet containing budesonide. The authors use a ColoPulse coating technology that consists of a suspension with Eudragit S100/PEG 6000/CS/talc in a solvent mixture of acetone/water. In this in vitro evaluation, the authors observed that the release profiles of the novel formulations were compared with Entocort®, Budenofalk®, and Cortiment® (budesonide MMX) (Gareb et al. 2019).

Budesonide is also considered the model of the topically acting corticosteroid form in IBD treatment. It is recommended as first-line therapy for induction of remission in mild to moderate CD disease with the distribution involving the distal ileum and/or right colon. In patients with UC, it is recommended for proctitis and left-sided colitis (Abdalla and Herfarth 2016). Budesonide rectal topical formulations are available as an enema (Entocort®) or foam (Budenofalk® and Uceris®).

Topical therapy can be extremely valuable for the treatment or maintenance of IBD, molecules that present low bioavailability and act directly on inflamed mucosa without reaching significant concentrations in systemic circulation are very interesting and useful for this end. In this sense, budesonide can be considered an extremely beneficial option especially associated with nanomedicines and drug delivery systems, that may boost the exposure of it to the diseased sites (Pastorelli et al. 2020).

Despite these advantages, only one in four patients receives topical therapy (Lázaro et al. 2020). Several factors, such as retention problems, unpleasant feelings, rectal/abdominal pain, and flatulence, may limit the use of rectal therapies (Pastorelli et al. 2020). Thus, effective and more comfortable and acceptable topical formulations might rise treatment adherence by decreasing dosage, administration frequency, and adverse effects (Lázaro et al. 2020).

Lázaro et al. (2020) evaluated drug delivery systems composed of budesonide in HP-β-CD and its incorporation into a poloxamer-based thermoreversible hydrogel in a rat model of colitis induced by trinitrobenzene sulfonic acid (TNBS). These novel budesonide inclusion complex formulations improved microscopic damage and reduced colonic MPO activity and TNF-α levels.

Date et al. (2018) described the development of a budesonide nanosuspension coated with muco-inert Pluronic F127 that augments penetration in mucus and ulcerated colorectal tissues. The authors observed in a mouse model of acute IBD induced by TNBS, significant reduction in macroscopic and microscopic symptoms of IBD as well as a significant reduction of number of inflammatory macrophages and IL-β. This new formulation improved local delivery of budesonide with a significant influence on local colorectal tissue inflammation.

Another important tool in IBD treatment is the use of oral prednisolone. Kumari et al. (2018) used prednisolone loaded in microsponges and coated with Eudragit S100 for targeting the drug to the colon. In vitro drug release studies revealed that the drug starts releasing after 5 h, which is approximately the time that the drug will enter the proximal colon. Therefore, it is expected that a maximum amount of drug could be released in the colon which may result in reduced dose and frequency as well as side effects.

A conjugate between chondroitin sulfate and glycyl-prednisolone was incorporated in a nanogel for oral administration of prednisolone to rats with TNBS-induced colitis. This new formulation enhanced drug delivery to the lower intestines and it can be considered as a potential new way to deliver prednisolone for the treatment of ulcerative colitis (Onishi et al. 2019). Zhou et al. (2020) evaluated a novel anionic nanogel system to deliver prednisolone in the colon. This anionic nanogel is composed of succinylated Glycol Chitosan-Succinyl Prednisolone Conjugate, modified into a negatively charged conjugate by succinic anhydride. The authors evaluated this new formulation in a TNBS-induced IBD rat model and observed that anionic nanoparticles have shown to be accumulated in the colon due to epithelial enhanced permeability and retention effect. The use of this anionic nanogel system showed a favorable accumulation in the colonic parts and promoted the prolonged release of the drug.

13.1.4 Nanomedicines with Biological Agents

Patients who are steroid-dependent or refractory to the traditional agents used in IBD can be treated with biological therapy. These agents can be used in

corticosteroid-sparing maintenance therapy with increases in the disease outcomes and adverse effects profile of prolonged steroid use. Certainly, early use of corticosteroid-sparing medications is seen as a significant measure to improve the quality of IBD care (Damião et al. 2019). IBD can be treated with biological agents using gene silencing via RNA interference (RNAi) that accurately regulates genes to decline the expression of pro-inflammatory cytokines related to IBD (Guo et al. 2016). Biological treatment strategies for IBD involve the neutralization of pro-inflammatory cytokines, the use of anti-inflammatory cytokines, and the inhibition of neutrophil adhesion or T cell signaling (Takedatsu et al. 2015).

Biological agents relieve IBD progression and stimulate intestinal mucosa recovery. Anti-TNF-α antibodies, such as infliximab, adalimumab, certolizumab, and golimumab, have established success against IBD. Infliximab was the first monoclonal antibody to be approved for the treatment of pediatric and adult patients with moderately to severely active Crohn's disease (CD) and ulcerative colitis (UC) (Hemperly and Vande Casteele 2018). However, the low penetration of small interfering RNAs (siRNAs) across cell membranes requires quite high doses of these therapies which increases the risk of adverse effects, such as lymphoma, infections (especially tuberculosis reactivation), and lupus-like syndrome (Takedatsu et al. 2015).

Strategies that improve the direct delivery of siRNAs to the gastrointestinal tissues or decline the incidence of adverse effects include chemical modifications to the RNA molecule and the use of nanotechnology to target the disease areas (Chevalier 2019).

In 2015, Huang and colleagues report an orally administrated microspheric vehicle loaded with an antisense oligonucleotide against TNF-α. This new formulation designed with cationic konjac glucomannan (cKGM), phytagel, and an antisense oligonucleotide against TNF-α acts in the colonic macrophages and decreases the local levels of TNF-α. The results showed that oral administration of the treatment alleviated the symptoms of colitis induced by DDS in mice (Huang et al. 2015).

A new strategy for the delivery of phosphorothioated antisense oligodeoxyribonucleotide of TNF-α (PS-ATNF-α) was evaluated by Duan et al. (2019). The aim of this research is to target intestinal inflammation with a colon-specific degradation of the chitosan alginate hydrogel. After an evaluation in a dextran sulfate sodium (DSS) model of colitis in mice, the new formulation reduces TNF-α production and MPO activity (Duan et al. 2019).

Frede et al. (2016) evaluated the local interference of cytokine signaling mediated by siRNA-loaded nanoparticles. These NPs present a calcium phosphate core encapsulated into poly(*d*,*l*-lactide-*co*-glycolide acid) (PLGA) and coated with polyethyleneimine (PEI). After DSS-induced colonic inflammation in mice, intrarectal application of these nanoparticles improve the intestinal inflammation demonstrated by a significant reduction of the target genes in colonic biopsies and mesenteric lymph nodes (Frede et al. 2016).

PLGA was used to pack TNF-α siRNA into nanoparticles grafted with galactosylated chitosan for oral administration in mice with DSS-induced colitis

(Huang et al. 2018). The use of this new strategy for delivering TNF-α siRNA are efficient as demonstrated by the improvement of colitis parameters such as weight loss and MPO activity.

A synergistic combination therapy of ulcerative colitis was tested by Xiao et al. (2018) using the TNF-α gene silencing (siTNF) mediated by orally targeted nanoparticles combined with interleukin-22. The oral administration of this formulation showed an excellent capacity to downregulate the expression of pro-inflammatory factors and promote mucosal healing (Xiao et al. 2018).

Sakisaka et al. (2020) evaluated a therapeutic efficacy of a macromolecular complex with schizophyllan (SPG) and antisense oligonucleotides using a dextran sodium sulfate (DSS)-induced colitis model, topically administrated. They observed that TNF-α production both in vitro and in vivo was significantly inhibited by SPG-antisense TNF-α, demonstrating the possibility of new topical therapeutic approach against the inflammatory bowel disease Sakisaka et al. (2020).

13.2 Conclusion and Prospects

Nanomedicine technology is still evolving to promote better results in IBD therapy. However, nowadays it is possible to observe robust results in IBD therapy aiming at selective targeting and local drug delivery with reduced systemic side effects and toxicity. Nano-drug delivery systems are optimized in recent research and results showed efficient drug delivery to the inflammation site along with a decrease in the amount of drug administered and/or reduction of side effects.

Despite the fact that some commercially available products already use this kind of technology, a lot of ground has to be covered to make these new strategies part of the clinical routine during IBD treatment. Focus should be given to the conversion of nanocarrier systems used in preclinical into clinical trials. One important factor is the requirement of a well-established colitis model to validate the benefits (or harmful events) of the new formulations. However, as with any animal model available, the present models do not fully relate to the complex nature of IBD in humans. Some other factors may be overcome in order for these new approaches to become clinically available, such as safety profile, premature release or enzymatic degradation in the gastrointestinal tract, stability, costs, and large-scale production. Thus, these issues must be addressed to allow that new nanomedicine drug form to become a reality to increase the quality of life of patients with IBD.

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Chapter 14
Micro and Nanostructured Drug Release Systems for Skin Cancer Treatment

Cristina Wöhlke Vendruscolo, Beatriz Ewert de Oliveira, and Gislaine Ricci Leonardi

Abstract Skin cancer is the most common type of malignancy world wide. The use of topical medications is considered the safest and most effective route of administration for cutaneous diseases. However, in skin cancer, the available drug options have poor skin permeation and retention, resulting in a high incidence of side effects and disease recurrence after treatment interruption. Therefore, topical treatment for premalignant and malignant skin lesions is just recommended when surgical excision is not possible. In this matter, nanocarrier systems are to promote targetable delivery of drugs, enhancing the efficiency and reducing side effects, showing great potential for skin cancer application. This chapter describes the main types of skin cancer and the commercially available topical treatment options. Then, nanocarrier-based delivery systems are pointed out, indicating the main uses and applications in topical cancer treatment. For instance, lipid-based vesicles, such as solid lipid nanoparticles, liposomes, and ultra-deformable liposomes, have a great affinity to skin tissue, being applied to enhance skin permeation and retention of drugs. Polymeric, carbon-based and metal-based nanoparticles have been used for delivering high amounts of drugs with diverse polarity and size, being able to modulate drug release. Finally, immuno stimulating complexes (ISCOMs) are antigen carrying molecules which can provide specific drug delivery. Although many researches show promising improvement in skin cancer treatment, most studies are in preclinical stages.

Keywords Nanostructures · Nanocarriers · Drug delivery systems · Skin cancer

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14.1 Introduction

It is known that the efficiency and tolerability of topical treatments are largely influenced by the properties of the vehicle used. Patients' adherence to treatment also suffers direct interference from the pharmacotechnical and sensory characteristics of the pharmaceutical form, especially in cases where treatment is long (Schirm et al. 2003; Shirata and Campos 2016). When referring to the treatment of skin cancer, it is very important to have strategies for drug release as specific and oriented as possible to the target tissue. Standard chemotherapy treatments are often nonspecific, attaining healthy tissue as well as cancer cells (Stone and DeAngelis 2016). Another obstacle faced in the treatment is the development of drug resistance by tumor cells (Mansoori et al. 2017). The use of nanotechnology may be a strategy to overcome these obstacles and deliver a more targeted, effective treatment with less side effects.

In this chapter, the main premalignant dermatoses and malignant tumors will be approached just to contextualize the possible treatments that use the various forms of nanotechnology, which are the focus of this chapter.

14.1.1 Premalignant Dermatoses

In the concept introduced by Sittart and Pires (2007) premalignant dermatoses would be skin diseases with a potential to become malignant within a certain period, which can be counted in months or years. These dermatoses can be classified according to oncogenic risk (high or low). The relevance of this type of disease has been gaining prominence as it has a direct impact on the patient's life expectancy.

Actinic keratosis (AK), also known as solar keratosis or senile keratosis, is one of the main high-risk premalignant dermatoses. Actinic keratosis lesions mainly appear in areas exposed to the sun, such as face, forearms, hands, and ears. Treating actinic keratosis is very important, mainly because lesions could possibly evolve into squamous carcinomas, which is a very common type of skin cancer (Ceilley and Jorizzo 2013).

The use of several topical therapies to treat actinic keratosis lesions has been documented. Among the treatment options for actinic keratosis, the most commonly used include 5-fluorouracil (5-FU), cryotherapy, diclofenac, photodynamic therapy (PDT), imiquimod (IQ), retinoids, and ingenol mebutate (IM). Recently, advances in treatment options have been reported, including the emerging use of innovative active ingredients, such as resiquimod, betulinic acid, piroxicam, and dobesilate. Moreover, therapies combination has shown relevant results due to the reduction and duration of therapy and side effects (de Oliveira et al. 2018).

14.1.2 Malignant Tumors

Skin malignant tumors, also called "skin cancer", are the most common types of malignancies worldwide. According to the classification described by Sittart and Pires (2007), skin cancers include tumors derived from keratinocyte cells: basal cell carcinomas (BCC) and squamous cell carcinoma (SCC) and tumors derived from melanocytes: melanoma (Culen et al. 2020; Estimativa 2020).

CBC is the most prevalent type, corresponding to 80% of non-melanoma skin cancer found clinically. BCC begins in the basal cells, which are found in the deepest layer of the epidermis, there is a low lethality rate and it usually affects the face and back of the hands of middle-aged or elderly patients. Like most skin cancers, exposure to ultraviolet radiation is a risk factor with a significant impact on the appearance of BCC (Culen et al. 2020). For BCC patients with low-risk superficial tumors or nodular tumors, treatment with topical 5% imiquimod has been recommended. 5-FU treatment has also been cited as an option (Culen et al. 2020; Vílchez-Márquez et al. 2020).

SCC is the second most prevalent type of skin cancer derived from keratinocytes, affecting about 20% of patients. It is known that SCC usually derives from AK. Like CBC, the appearance of AK and SCC lesions is associated with exposure to ultraviolet light, and most of the lesions appear in areas of the body with greater sun exposure, such as the face, head, ears, and neck (Chetty et al. 2015; Craythorne and Al-Niami 2017; Alam et al. 2018; Culen et al. 2020). The use of topical medications such as 5-FU 0.5–5%, Imiquimod 5%, and Diclofenac 3% gel have been recommended for the topical treatment of AK (Chetty et al. 2015).

Although melanoma is the least common type of carcinoma, affecting about 2% of patients, this is the type of lesion with the worst prognosis and the highest lethality rate. The pre-carcinogenic stage of the disease is called dysplastic nevi and early carcinoma is known as melanoma in situ (MIS). In situ melanoma can be subdivided into two types: malignant lentigo (SCI) and malignant melanoma lentigo (LMM) (Alam et al. 2018; Culen et al. 2020).

It is known that MIS has a good prognosis and low metastatic potential, notwithstanding, topical treatment is indicated only for precancerous or transitional lesions. For SCI, topical treatment with Imiquimod 5% can be recommended as primary therapy in cases where surgery is contraindicated or as secondary treatment after surgical removal of the lesion (Sober et al. 2001; Alam et al. 2018; Culen et al. 2020).

In general, physical removal by surgery or cryoscopy is still the most suitable treatment for pre-carcinogenic and carcinogenic skin lesions as those are the options with the greatest scientific evidence of effectiveness (Chetty et al. 2015; Craythorne and Al-Niami 2017; Alam et al. 2018; Culen et al. 2020). However, there are cases in which surgical excision is contraindicated, due to the patient's comorbidities, extent, number, and location of lesions. An alternative widely used to replace surgical excisions is the use of topical medications (Florin et al. 2012; Jansen et al. 2017; Williams et al. 2017; Tio et al. 2019; Borgheti-Cardoso et al. 2020).

14.2 Topical Treatment

The use of topical medications is considered the safest and most effective route of administration for dermatological treatments because it has fewer systemic side effects when compared to other drug administration routes (Gilman et al. 1987; Ceilley 2012).

However, the use of topical medications for skin cancers is still limited due to the difficulty of conventional medications in reaching the deeper layers of the skin combined with the high incidence of local irritating effects (Tambunlertchai et al. 2021).

Several drugs have been used clinically for the treatment of skin cancers, Culen et al. (2020) describe the main topical drugs clinically available, the date is summarized in Table 14.1. In addition to conventional drugs, the efficiency and safety of new compounds have also been evaluated, for instance, the drugs BIL-010t and Patidegib are in the clinical studies I and II phase, respectively, showing promising results. Moreover, some studies deal with topical methotrexate for the treatment of skin cancer; however, it is found in the pharmaceutical form of a tablet or solution.

14.3 Release Systems

14.3.1 Emulsions

Emulsions are conventional pharmaceutical drug delivery systems widely used in the pharmaceutical industry. Emulsions consist of a dispersed phase (internal or discontinued phase), a dispersing medium (external or continuous phase), and generally a third component known as an emulsifier (Leonardi 2004; Müller-Goymann 2004; Djekic and Primorac 2008). The preparation of emulsions using high mechanical energy emulsification methods promotes the formation of emulsions with reduced size of dispersed phase droplets, increasing the capacity to dissolve large amounts of drugs and the total interfacial area of the dispersed phase, which results in increased drug bioavailability, stability, and protection against degradation (Mohamadi Saani et al. 2019). Emulsions with a decreased dispersed phase droplet can also promote greater skin hydration and increased efficiency of skin release and retention of the drug, reducing systemic adverse reactions (Ahmad et al. 2017). Many formulations used for skin cancer topical treatment are conventional emulsions, which usually require a higher concentration of the drug to achieve the desired effect thus causing skin irritation. One way to mitigate unwanted effects and increase the effectiveness of drugs is to use micro and nanostructured drug delivery systems. Nanostructured compounds with anticancer activity against melanoma have been evaluated with promising results (Pinho et al. 2019; Beiu et al. 2020).

Table 14.1 Clinically available topical treatments for skin cancer

Active ingredient	Commercial name	Pharmaceutical form and dosage	Posology	Active ingredient	Composition/other ingredients
5-Fluorouracil	Efurix®	Cream (emulsion) 5% (50 mg/g)	Application twice a day in quantities to cover the lesions (3–6 weeks)	50 mg of fluorouracil in 1 g cream (5%)	Stearyl alcohol, white petrolatum, polysorbate 60, propylene glycol, methylparaben, propylparaben, and purified water
5-Fluorouracil	Carac® (fluorouracil cream) cream	5-FU 0.5% in Microsponge® and creamy base system	Application once a day for not more than 4 weeks	5 mg of fluorouracil in 1 g of cream (0.5%)	Microsponge® (methyl polymer methacrylate/glycol dimethacrylate and dimethicone), carbomer 940, glycerin, methyl glucet-20, methyl paraben, hydroxy octyl stearate, polyethylene glycol 400, polysorbate 80, propylene glycol, propylparaben, purified water, stearic acid, and trolamine
Imiquimod	Aldara®, Ixium®, Modik®, and Imoxy®	Cream (emulsion) 5%	For basal cell carcinoma, the application should be 5× per week, for 6 weeks; a thin layer should be applied for 6–10 h	12.5 mg of imiquimod in 250 mg of cream (5%)	Iso-stearic acid, benzyl alcohol, cetyl alcohol, stearyl alcohol, white petroleum jelly, polysorbate 60, sorbitan stearate, glycerol, methyl parahydroxybenzoate (E218), propyl parahydroxybenzoate (E216), xanthan gum, purified water
5-Aminolevulinic acid	Levulan®, Kerastick®, e Metvix®	Cream (emulsion) 160 mg/g	Application of a 5–10 mm layer and cover with an occlusive dressing (3 h),	Methyl aminolevulinate hydrochloride	Self-emulsifiable glyceryl Monostearate, cetostearyl alcohol, PEG-40 stearate,

(continued)

Table 14.1 (continued)

Active ingredient	Commercial name	Pharmaceutical form and dosage	Posology	Active ingredient	Composition/other ingredients
			remove the adhesive, and expose the lesion to red light		methylparaben, disodium edetate, glycerol, white petrolatum, cholesterol, isopropyl myristate, arachis oil, refined almond oil, oleyl alcohol, and purified water
Ingenol mebutate	Picato®	Gel 150 mcg/g or 500 mcg/g	The drug should be applied 1× daily for 2 or 3 consecutive days	70 mcg or 235 mcg of ingenol mebutate	Isopropyl alcohol, hyethellosis, citric acid monohydrate, sodium citrate monohydrate, sodium citrate dihydrate, benzyl alcohol, and purified water
Retinoids (tretinoin, isotretinoin, adapalene and tazarolene)	Vitanol A®(tretinoin)	Cream 0.25 mg/g (0.025%) or 0.50 mg/g (0.05%) or 1.00 mg/g (0.1%)	Apply a thin layer at night and remove after waking up for 24 weeks	0.25 mg/g (0.025%) or 0.50 mg/g (0.05%) or 1.00 mg/g (0.1%) of tretinoin	Cetyl alcohol, butyl hydroxyanisole, butyl hydroxytoluene, decamethylcyclopentasiloxane, and trimetilsiloxissilicato, glycerol, methyl paraben, propyl paraben, triglycerides of capric acid/caprylic, pentylene, octyldodecanol, decamethylcyclopentasiloxane and cross polymer dimethicone, cetostearyl and glucopyranoside cetostearyl alcohol, ectoin, hydroxyethylacrylate/ acryloyldimethyltaurate sodium and polysorbate 60 and purified water

Sinecatechin—dry extract of unfermented leaves of *Camellia sinensis*	Veregen®	Ointment 100 mg/g	Application of aa 0.5 cm layer 3× a day, the treatment should not exceed 16 weeks	1 g of ointment contains 100 mg of extract (in the form of dry, refined extract) of *Camellia sinensis* (L.) O. Kuntze, folium (green tea leaf) (24–56:1), equivalent to 55–72 mg (−) epigallocatechin gallon	First extraction solvent: water White Vaseline (contains all rac—alpha—tocopherol), white beeswax, isopropyl myristate, oleyl alcohol, propylene glycol monopalmito stearate
Tacrolimus monohydrate	Tarfic®	Ointment at 0.03% or 0.1%	Apply twice a day	0.03% equivalent to 0.300 mg of tacrolimus 0.1% equivalent to 1.0 mg of tacrolimus	Yellow petrolatum, liquid petrolatum, propylene carbonate, white beeswax, and white paraffin

14.3.2 Nanostructured Systems

The cutaneous route is attractive for the administration of drugs in the treatment of skin cancer. Although, the stratum corneum (SC) is a barrier that hinders drug skin penetration. Within this context, liposomes, and other micro or nanostructured structures appear as potential carriers to improve topical distribution of therapeutic agents (Carita et al. 2018).

Nanostructured systems are based on nanoparticles, which can be defined as particles with dimensions between 1 and 100 nm. According to Vogt et al. (2005) and Antonio et al. (2014), these particles can be organic and inorganic, being classified according to the following parameters: shape, size, surface, physicochemical properties, and also for their malleability. The malleable particles are described as those that change their shape if subjected to some type of external pressure and are composed of organic materials (lipids and proteins). The rigid particles are composed of inorganic materials (metals or ceramics), encapsulating the drug to be later released, inside the organism. Several nanostructured systems have been evaluated for topical administration of chemotherapy drugs for the treatment of skin cancer, as summarized in the topics below. Figure 14.1 schematizes the structure of some described systems.

14.3.2.1 Solid Lipid Nanoparticles (SLN)

Solid lipid nanoparticles (SLN) are defined as colloidal carriers with particle size between 50 to 1000 nm made by physiological lipids, dispersed in an aqueous surfactant solution. Generally, SLNs contain a solid hydrophobic core with a phospholipid coating. They can carry lipophilic or hydrophilic drugs. The advantages of SLNs can include ease of production compared to biopolymeric nanoparticles, controlled release, and large-scale production potential. In addition to better control of the kinetics of the encapsulated compound, specific delivery and greater penetration of the drug into the skin through topical application (Lalotra et al. 2020).

For instance, Khallaf et al. (2016) developed lecithin and Poloxamer 188-based SLN loaded with 5-FU. Then, the 5-FU SLN was incorporated into a hydrogel matrix and topically applied, twice a week, to mice with Ehrlich Ascites Carcinoma. Histological results demonstrated that the 5-FU SLN showed greater penetration capacity and decreased the hemorrhage and inflation in the tumor area when compared with the negative control and the free drug. Although the results are promising, the systemic effects of the drug were not evaluated in the study.

A new generation of solid lipid particles which have been developed to overcome the SLN limitations. According to the review published by Lalotra et al. (2020), NLC are more than solid lipid matrices because they can be associated with liquid lipid particles, resulting in a less ordered system that is able to increase transported drug concentration and prevent its degradation during storage. The system was used

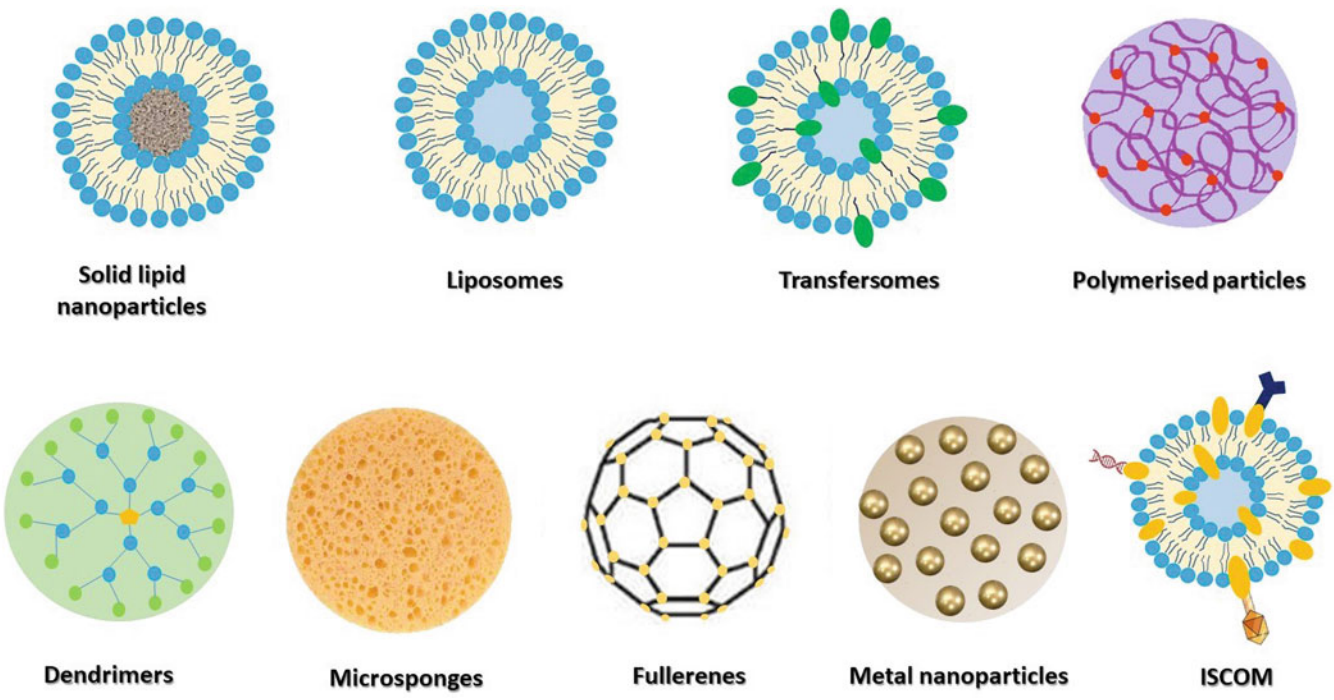

Fig. 14.1 Solid lipid nanoparticles are formed by a solid hydrophobic core with a phospholipid coating. Liposomes and transferosomes are double layer vesicles formed by phospholipids and phospholipids/non-ionic co-surfactant mixture, respectively. Polymerized particles are made of synthetic polymers. Dendrimers are composed of poly(amidoamine) in a branched structure. Microsponges are polymeric microspheres with a large porous surface area. Furullenes are nanostructures composed of carbon atoms. Metal nanoparticles are particles of metal atoms such as zinc oxide, silver, and gold. Immuno-stimulating complexes (ISCOM) are antigen carrier matrixes

with Silymarin, an anti-inflammatory, antioxidant, anti-proliferative, and antitumor activity. The silymarin-NLC system showed better results when compared to the conventional silymarin gel (Lalotra et al. 2020).

14.3.2.2 Liposomes

Liposome spherical and circular microscopic vesicles composed of phospholipid layers (the main component of the cell membrane), which gives liposomes a great affinity to biological membranes, including the skin (Krishnan and Mitragotri 2020; Tambunlertchai et al. 2021). Liposomes can consist of just one layer: (Large Unilamellar Vesicle—LUV and Small Unilamellar Vesicle—SUV) or multiple, concentric layers separated by layers of the aqueous phase. The vesicle diameter range from 30 nm to hundreds of micrometers (Raminelli et al. 2018). The topical application of liposomes was first described in 1987 by Mezei and Gulasekharam demonstrating increased absorption and cutaneous retention of corticosteroids, with a significant reduction in drug systemic concentration.

Topically applied liposomes have the ability to adsorb to the skin, fusing with the stratum corneum lipids and releasing the drug of interest directly into the tissue (El Maghraby et al. 2008). In addition to their great affinity for biological membranes, liposomes have amphiphilic characteristics, which can solubilize a range of polar and nonpolar drugs (Bozzuto and Molinari 2015; Singh et al. 2017; Borgheti-Cardoso et al. 2020). The use of liposomes generally improves the pharmacokinetics of drugs, increasing efficacy, providing dose reduction, and, consequently, reducing side effects.

It is also possible to associate recognition molecules with the liposome membrane, increasing selectivity for specific cell groups in order to intensify the efficiency of the drug (Ewert de Oliveira et al. 2020).

The efficiency of a liposomal vehicle in delivering T4 endonuclease V enzyme for topical application in patients with xeroderma pigmentosum was evaluated in a clinical trial. Xeroderma pigmentosum genetic disease that increases the skin's sensibility to UV light and predisposes patients to skin cancers. The T4 endonuclease V loaded liposomes efficiently delivered the enzyme through the stratum corneum lowering the rate of development of AK and SCC lesions (Yarosh et al. 2001).

The high resemblance with the skin and the possibility to be associated with other molecules, make liposomes a great option as a topical skin cancer drug delivery system. For instance, Petrili et al. developed cetuximabe-immunoliposomes loaded with 5-FU for the topical treatment of SCC. Cetuximab (Erbitux™) is an IgG1 monoclonal antibody that binds to the extracellular EGFR domain with high affinity therefore preventing its activation by endogenous ligands. It is known that overexpression of epidermal growth factor (EGFR) receptors is not only a recurrent feature in SCC but is also associated with poor prognosis. The immunoliposome developed by Petrili et al. was proven to have predilection and selective toxicity for EGFR-positive cancer cells in vitro (Petrilli et al. 2017, 2018).

14.3.2.3 Transferosomes

Transferosomes is also called ultra-deformable liposomes; their main characteristics include the ability to squeeze through stratum corneum pores driven by the transdermal hydration gradient without coalescence or aggregation. Transferosomes are composed of phospholipids and non-ionic co-surfactants, soy lecithin, and acrylate in different concentrations. When applied in non-occlusive conditions, transferosomes from 200 to 300 nm can penetrate intact skin (Cevc and Gebauer 2003; Benson 2006; Werner et al. 2015).

For instance, sodium diclofenac-loaded transferosomes significantly increase trans epidermal flux and prolong the release of the drug in comparison to free sodium diclofenac (El Zaafarany et al. 2010).

Calienni et al. (2019) incorporated the drug Vismodegib (Erivedge®, Genentech) into ultra-deformable liposomes of soy phosphatidylcholine and sodium cholate in order to develop a topical delivery system. Vismodegib is a first-class inhibitor of the hedgehog signaling pathway used now in the treatment of BCC. Vismodegib Ultradefromable Liposomes (UDL-Vis) presented size, deformability, and encapsulation efficiency compatible with topical use. In a topical penetration study using human skin, it was observed that UDL-Vis presented cutaneous penetration 7 times greater when compared to the drug solubilized in DMSO after 1 h of testing. Moreover, UDL-Vis presented a good distribution, being found in the stratum corneum, in viable epidermis tissues, and in the dermis while Vismodegib in DMSO was found only on the surface of the stratum corneum. Finally, considering the daily dose of 150 mg of oral Vismodegib, the bioavailability of the drug and the minimum volume of distribution, the authors calculated that after 8 h of oral intake the theoretical amount of Vismodegib in the skin is around 3 μg/mL while the concentration of UDL-Vis ap After 8 h was 8.4 μg/mL in the viable epidermis and dermis (Calienni et al. 2019). Despite the studies being in the initial phase, the results found show that the use of transferosomes can be an alternative for the topical delivery of Vismodegib for the topical treatment of cancer, being able to optimize the local effects of the drug on the skin and reduce the systemic side effects.

14.3.2.4 Polymerized Particles

These are multifunctional nanostructured drug carriers made from synthetic polymers with the potential to encapsulate drugs, overcoming biological barriers and delivering drugs to specific sites, for instance, solid tumors. The preparation of nanospheres and nanocapsules by polymerization of monomers makes it possible to incorporate drugs, hydrophilic or hydrophobic, and macromolecules such as proteins and peptides (Souto et al. 2012).

Knowing that the extracellular space of tumors is generally slightly acidic, Zheng et al. developed pH-responsive polymeric nanoparticles loaded with doxorubicin to induce selective release of the drug into the tumor tissue. The use of pH-responsive

polymeric nanoparticles was shown to efficiently release the drug into the cytoplasm, the IC50, which is 60 times higher than free doxorubicin in doxorubicin-resistant cell lines (Zheng et al. 2011). In summary, these findings demonstrate the potential use of polymeric nanoparticles to be used in cancer therapy to overcome drug resistance.

Although polymeric nanoparticles have been showing great potential for specific drug delivery, its cytotoxicity has been studied. Those particles may not be biodegradable, they accumulate in human cells, which limits their application (Antonio et al. 2014).

14.3.2.5 Dendrimers

The word "dendrimer" comes from the Greek radical "dendros" which means "tree" and "mere" which means "part of" (Tomalia et al. 1985). Dendrimers are nanocarrier systems composed of poly (amidoamine), which are presented as synthetic polymers in a branched structure, they are used to transport and release the assets. It has proven to be successful technology due to its high solubility, controlled release capacity, and low toxicity (Fox et al. 2018). As a result of its attachment to the macromolecular carrier, the drug-nanotechnology conjugate has largely reduced toxicity compared to the free drug doxorubicin, and, therefore, deleterious side effects are minimized (Fréchet 2002). The conjugation of drugs and targeting moieties such as folic acid, peptides, and monoclonal antibodies in dendrimeric structures has been described as an efficient tool to provide target delivery for therapeutic and diagnostic applications in cancer. Topically, cationic dendrimers have been able to increase skin penetration of 5-FU (Wolinsky and Grinstaff 2008; Venuganti et al. 2011).

14.3.2.6 Microsponges

Polymeric spheres composed of a highly porous network, capable of retaining the compound of interest, releasing it slowly by diffusion, pressure, or volatilization, with an average pore size of 0.25 μm. Microsponge delivery advantages include excellent stability in pH ranges from 1 to 11, improved bioavailability of some drugs, improved stability, and allowance of vehiculation of immiscible compounds in a single formula (Won 1987; Kappor et al. 2014).

The microspheres are open structures, so the compound of interest moves freely from the sponges to the external environment until an osmotic balance is reached. When applied topically, the micro sponges are not absorbed, being retained in the stratum corneum where they slowly start to release the compound of interest according to its absorption by the skin. Consequently, the application of drugs in micro-sponging systems results in a slow and gradual release, allowing the application of the drug in smaller and more widely spaced doses (Kaity et al. 2010).

The Carac® cream consists of 0.5% 5-FU encapsulated in a micro-spherical system based on cross-methyl methyl methacrylate/glycol dimethacrylate and

dimethicone. In addition to the lower dosage, Carac® cream also has a different dosage regimen than conventional 5-FU formulations, being administered once daily, while conventional creams are administered twice daily. Studies show that 0.5% of formulations using the micro sponge system have less systemic absorption and greater skin retention, in addition to less skin irritation and greater patient tolerability (Levy et al. 2001a, b; Loven et al. 2002).

14.3.2.7 Fullerenes

Fullerenes considered nanostructures with spherical shape are composed of carbon atoms. Fullerene particles have been showing activity in photodynamic therapies, antioxidant, neuroprotective, antimicrobial, and antiretroviral action (Fox et al. 2018). Because of the ease to transport multiple assets, it could be a useful alternative to skin cancer treatment by minimizing unwanted side effects (Kazemzadeh and Mozafari 2019). According to Goulart et al. (2015) theoretical studies associating fullerene with anticancer substances have been described in the literature, such as cyclophosphamide, as well as experimental studies, associating fullerenes with 5-FU, in order to create a delivery system for this drug, potentiating its anticancer action and reducing its adverse effects.

14.3.2.8 Metal Nanoparticles

They have been widely studied for their most diverse applications. For example, silver nanoparticles have antimicrobial properties, zinc oxide and titanium dioxide nanoparticles have a protective effect against ultraviolet light, and cerium oxide nanoparticles have a healing effect on the treatment of wounds. On the other hand, gold nanoparticles (AuNPs) stand out for having a unique combination of physical, chemical, optical, and electronic properties for the delivery of drugs (Goyal et al. 2015; Krishnan and Mitragotri 2020). A recent study demonstrated that magnetic nano emulsion loaded with zinc phthalocyanine were able to increase the drug release on the deeper skin layers, showing great potential as a synergic application for SC treatment (Primo et al. 2008).

Gold nanoparticles loaded with conjugated nisin and doxorubicin have been shown to have a potential antitumor effect in vivo in the treatment of murine skin cancer (Preet et al. 2019). Doxorubicin is a type of chemotherapy that acts by slowing or stopping the growth of cancer cells by blocking the enzyme topo isomerase (Tardi et al. 1996). On the other hand, nisin is a cationic antimicrobial peptide produced by *Lactococcus lactis* that has anticancer effects reported in the literature (Joo et al. 2012). The synergistic use of assets is a strategy used to reduce the probability of developing resistant cell lines (Preet et al. 2019).

A topical application system based on gold nanoparticles loaded with 5-FU covered by cetyltrimethylammonium bromide 5-FU/CTAB-GNPs was developed for the topical treatment of skin cancer. The 5-FU/CTAB-GNPs gel and cream

showed superior anticancer permeability and efficacy when compared to the drug in conventional formulations. The in vivo anticancer efficacy evaluated in mice with A431 tumor showed a reduction approximately seven times greater compared to the control treated with conventional 5-FU formulations. The study demonstrated that the delivery of 5-FU in gold nanoparticles can be an alternative to increase the effectiveness of the drug (Safwat et al. 2018).

14.3.2.9 ISCOMs (Immuno Stimulating Complex)

Immune stimulating complexes with hollow, spherical, cage-shaped structures, consisting of phospholipids, cholesterol, and saponins (triterpene glycosides). It is an antigen carrier matrix used in vaccines (Nevagi et al. 2018).

The source of saponin generally used to produce ISCOMs is derived from the bark of the Molina *Quillaja saponaria* tree. The base of this unique ISCOM structure is the interaction between the saponin and cholesterol molecules, which when combined form stable rings in aqueous solutions after removing detergents. These cholesterol-saponin rings combine to form pentagonal dodecahedra (resembling the shape of a soccer ball).

Cancer vaccines and immunomodulatory agents have shown great potential to eradicate cancer cells. However, the systemic application of these drugs is still a concern due to systemic effect. In this context, ISCOMs have shown great potential to be applied as target delivery tools for systemic treatment of aggressive tumors, including advanced melanoma (Qiu et al. 2017). For instance, Bourquin et al. (2008) and Sokolova et al. (2010) developed CpG oligodeoxynucleotides NPs capable of generating protective antitumoral immunity in a murine model of melanoma.

14.4 Conclusion

Topical cancer treatment options are still limited due to high toxicity and limitations to reach deeper layers of the skin, making surgical excision the most indicated treatment option. Drug-loading nanotechnological systems have been studied for skin cancer treatment. In this chapter, we summarized the main characteristics and applications of nanocarriers for topical skin cancer treatment. In sum, lipid-based vesicles have a great affinity to skin tissue, increasing skin permeation and retention. Polymeric, carbon-based, and metal-based nanoparticles can selectively bind to high amounts of several drug groups with diverse polarity and size. Finally, ISCOMs can provide a very specific target delivery system due to the ability to carry recognition molecules in the surface area.

Several studies using nanocarriers demonstrated the potential to minimize side effects, increase stability, and modulate the distribution profile of cancer drugs. These advances aim to promote greater retention of drugs in the epidermis/dermis

tissues, improving the therapeutic efficacy of topical medications and bringing precision to the treatments.

It is important to point out that most studies are still in preclinical stages, and in order to have nanotechnological options for cancer treatment, large-scale production and in vivo effects must be better understood.

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Chapter 15
Sulforaphane-Loaded Nanomedicines Applications: Trends on Inflammatory Diseases and Cancer Treatment

Mônica Helena Monteiro do Nascimento, Naially Cardoso de Faria, and Daniele Ribeiro de Araujo

Abstract Sulforaphane (SFN), a natural isothiocyanate derivative, has been extensively studied as therapeutic compound. Different cellular pathways were described for explaining its promising pharmacological effects such as anti-inflammatory, antitumoral, and antioxidant. In this sense, several studies have investigated SFN as single or in association with conventional drugs, specially as anti-inflammatory and antitumoral. In this sense, new strategies for delivering SFN have been discussed for overcoming physicochemical and/or biopharmaceutics limitations by using a variety of nanocarriers types such as micelles, polymeric/lipid/inorganic nanoparticles, nanocomposites, and gels. In this chapter, a discussion associating SFN molecular mechanisms of action with its potential pharmacological applications and the main nanocarriers for SFN delivery are provided, highlighting the relationships between biological synthesis, pharmacological aspects, and the new nanotechnological strategies for developing effective and safe pharmacotherapeutic alternatives.

Keywords Sulforaphane · Nanomedicines · Inflammation · Cancer

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Abbreviations

ARE	Antioxidant response element
CSCs	Cancer stem-like cells
EOC	Epithelial ovarian cancer cell
ER	Estrogen receptor
GLS	Glycosinolates
GRR	Glycorafanine
GSH	Glutathione
GST	Glutathione S-transferase
HO-1	Heme-oxygenase-1
ICT	Isothiocyanates
MMPs	Metalloproteinases
NfkB	Nuclear factor kappa B
NQO1	Quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
NSCLCs	Non-small cell lung cancers cells
PC-3	Human prostate cancer cells in culture
PC-3	Human prostate cancer cells in culture
ROS	Reactive oxygen species
SFN	Sulforaphane
TNBC	Triple-negative breast cancer
γGCL	γ-Glutamylcysteine ligase

15.1 Sulforaphane: Biological Synthesis and Metabolism

Sulforaphane (SFN) [1-isothiocyanate-(4*R*)-(methylsulfinyl) butane] (Fig. 15.1a) is a natural compound widely studied since 1980 (Guerrero-Beltrán et al. 2012). It belongs to the group of isothiocyanates (ICT) phytochemicals and is found in abundance in cruciferous vegetables. These plants belong to the *Brassicaceae* family, which has about 350 genera and 3200 species, including broccoli (*Brassica oleracea var. italica*), white cabbage (*Brassica oleracea var. capitata*), cauliflower (*Brassica oleracea var. Botrytis*), Brussels sprouts (*Brassica oleracea var. gemmifera*), watercress (*Nasturtium officinalis*), white mustard (*Sinapis alba*), arugula (*Eruca sativa*), and radish (*Raphanus sativus*) (Fahey et al. 2001, 2015). Among them, broccoli and, in particular, its sprouts, have the greatest potential for extracting SFN (Totušek et al. 2011).

In fresh vegetables, SFN is obtained from the hydrolysis of glycorafanine (GRR), a secondary metabolite of glycosinolates (GLS) family, also called sulforaphane glycosinolate, from the catalytic activity of the enzyme myrosinase (Pérez et al. 2014). When vegetable tissues are processed by cutting, cooking, freezing, or chewing, GLS are exposed to the action of the enzyme myrosinase, which

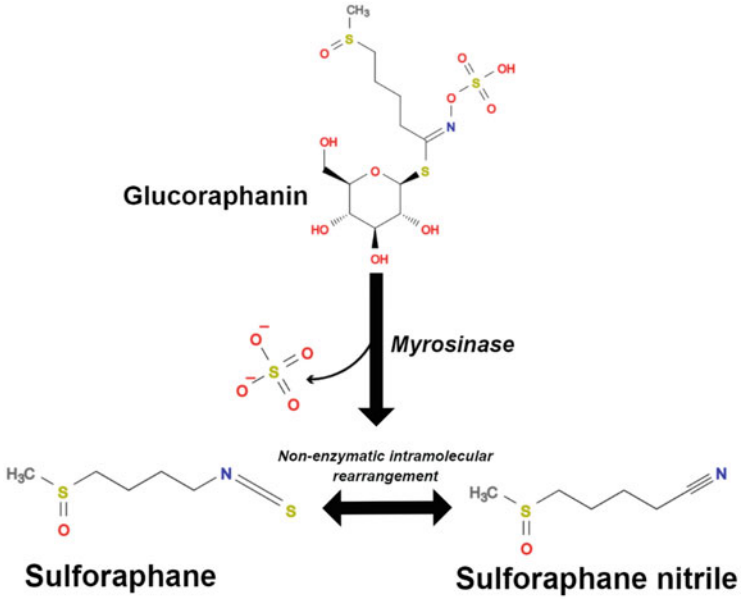

Fig. 15.1 Sulforaphane biological synthesis reaction

hydrolyzes them to isothiocyanates, which are the bioactive compounds (Fig. 15.1). The β-thioglucoside bond is hydrolyzed by myrosinase, producing glucose, sulfate, and a diverse group of aglycone products. The resultant aglycones then undergo nonenzymatic, intramolecular rearrangement to yield isothiocyanates, thiocyanates, or nitriles (Fig. 15.1).

In addition, the human intestinal flora is also capable of converting GLS into isothiocyanates with biological activity, as it has an isoform of the enzyme myrosinase, but hydrolysis in the intestinal tract manages to convert only between 14 and 20% of glucoraphanin in sulforaphane (Fahey et al. 2001; Rungapamestry et al. 2007; Van Eylen et al. 2007).

Some factors, such as basic pH and high temperatures, favor the formation of SFN from GRR, while acidic pH, the presence of ferrous ions and proteins (non-catalytic co-factors of the enzyme myrosinase) increase the nitrile formation of SFN which has no potential activity. However, the main determinant for isothiocyanates production from their precursor GLS is the way the vegetable is cooked. In this sense, the consumption of lightly cooked vegetables over overcooked vegetables is preferable. Additionally, the composition of the meal does not seem to alter the bioavailability of the SFN (Rungapamestry et al. 2007; Williams et al. 2008).

Broccoli is recognized as the best source of SFN, a portion can contain up to 60 mg of the precursor GRR (Rungapamestry et al. 2007). The ideal cooking condition that maximizes the SFN content in broccoli was determined by Pérez et al. (2014) and corresponds to immersion in water at 57 °C for 13 min. In this

condition, the minimum content of GLS and GRR was observed and the mirosinase showed its maximum activity. Fresh young broccoli sprouts contain 128 mg of GLS per gram of fresh weight, in contrast, blanched broccoli contained only 92 mg, cooked broccoli contained 47 mg, and frozen broccoli contained 45 mg per gram of fresh weight (Cieślik et al. 2007; Vanduchova et al. 2019). The determination of SFN from plant tissues or functional foods is based mainly on analysis by high-performance liquid chromatography (Vanduchova et al. 2019).

After ingestion, SFN is formed inside the gastrointestinal tract reversibly binding to thiols, organosulfur compounds that contain a group –SH. Then, they are transported by plasma proteins to cross the plasma membrane, by passive diffusion, entering tissue cells. After internalization, the ITCs will react with glutathione (GSH), forming its conjugate (*S*-(*N*-alkyl/arylthiocarbamyl)-glutathione), this reaction is catalyzed by the enzyme glutathione S-transferase (GST). The glutathione conjugate is released to the outside of cells through carrier proteins or MRPs "multidrug resistance proteins." In the middle extracellular, glutathione conjugated to γ-glutamyl and glycine residues, will be cleaved by the enzyme γ-glutamyl transferase (γ-GT) and dipeptidase giving rise to a cysteine conjugate that will be transported to the liver. Finally, the conjugate of cysteine, under the action of the enzyme N-acetyl transferase, will become mercapturic acid (Yagishita et al. 2019; Langston-Cox et al. 2020). After the formation of mercapturic acid, it is then transported to the kidney, where it will be eliminated (Yagishita et al. 2019; Langston-Cox et al. 2020).

15.2 Cellular and Molecular Mechanisms of Action

In the last years, the interest in extraction, isolation, and characterization of the biological activity of compounds from broccoli have been demonstrated by several published works, with the majority of studies dedicated to the analysis of GLS and related compounds, especially SFN (Singh and Singh 2012; Gupta et al. 2014; Mishra et al. 2019).

SFN cell signaling pathways are dependent on different molecular targets; however, their best-described mechanism of action is via the Nrf2 pathway (Kensler et al. 2012; Wu et al. 2019; Yagishita et al. 2019; Yang et al. 2020) (Fig. 15.2). Nrf2 is a central transcription factor with a central role on cellular redox process. In unstimulated cells, it is repressed by the protein Keap1, which causes the ubiquitination and subsequent degradation of Nrf2. SFN can interact with the Keap1 protein, disrupting the Nrf2–Keap1 interaction, allowing the nuclear activation and translocation of Nrf2. In the nucleus, Nrf2 binds to the antioxidant response element (ARE), a DNA region that promotes genes encoding antioxidant enzymes, including NAD (P) H: quinone oxidoreductase 1 (NQO1), heme-oxygenase-1 (HO-1), γ-glutamylcysteine ligase (γGCL), and thioredoxin (Vomhof-DeKrey and Picklo 2012) (Fig. 15.2).

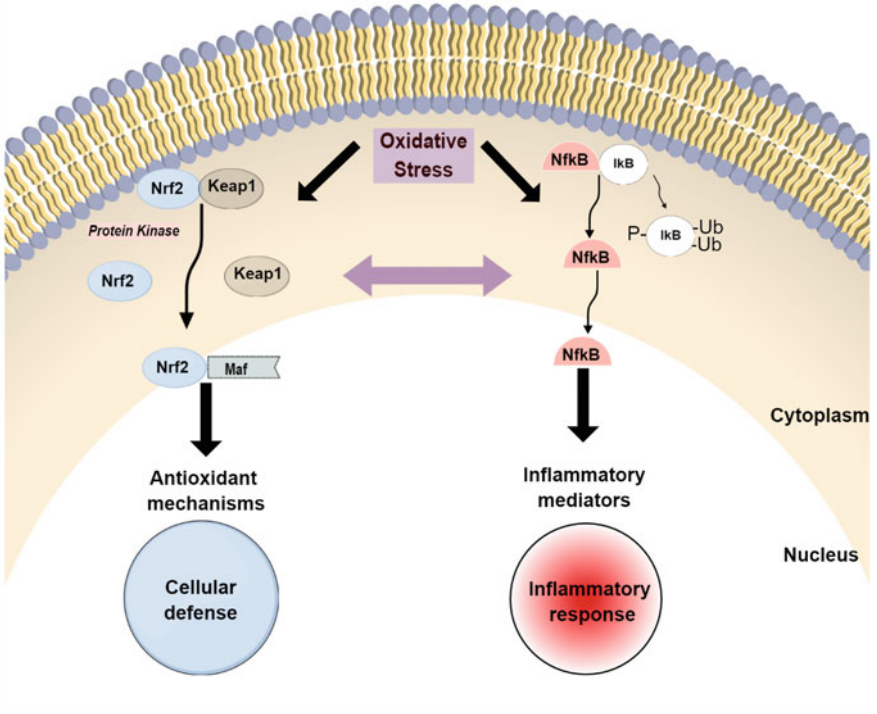

Fig. 15.2 Proposed molecular mechanism for sulforaphane anti-inflammatory activity through the NF-κB pathway

The enhanced transcription of Nrf2 target genes causes a strong cytoprotective response, increasing resistance to carcinogenesis and other diseases that have oxidative stress involved in pathogenesis, including neurodegenerative and chronic inflammatory diseases, such as colitis, atopic dermatitis, osteoarthritis (Nascimento et al. 2021; Kensler et al. 2012). In addition, SFN through the activation of Nrf2 increases the activity of phase II enzymes such as glutathione-S transferase (GST), involved in the elimination of xenobiotic compounds (Guerrero-Beltrán et al. 2012). It is suggested that the induction of phase II enzymes may be one of the main mechanisms by which cruciferous vegetables result in health benefits (Manchali et al. 2012).

Recently, several studies have shown that the SFN also has an anti-inflammatory activity, acting through the NF-κB pathway (Fig. 15.2).

The main mechanisms involved in the regulation of NF-κB signaling by SFN compresses the inhibition of phosphorylation and/or degradation of IkB, phosphorylation of IKK, and nuclear translocation of NF-κB (Fig. 15.2). All these mechanisms are described in the literature, in different cell types (Xu et al. 2005; Kim et al. 2012; Davidson et al. 2013, 2017). In a study using macrophages (cell line RAW 264.7), lipopolysaccharide-induced inflammation (LPS) was attenuated with SFN,

which negatively regulated the activity of the enzymes iNOS, COX-2, and the expression TNF-α (Heiss et al. 2001). Likewise, SFN reduced the synthesis of inflammatory mediators, such as interleukin IL-1β, TNF-α, and IL-6, induced by LPS, in primary microglial and rat astroglial cell co-cultures (Wierinckx et al. 2005). SFN also has anti-arthritic and immunoregulatory activity thus inhibiting synovial hyperplasia and the proliferation of activated T cells (Kong et al. 2010). In addition, it inhibits the expression of metalloproteinases (MMPs), as well as regulates the cell cycle keeping it in the G2/M phase, blocking joint degeneration both in vitro and in vivo (Heiss et al. 2001; Kim et al. 2009; Davidson et al. 2013, 2017).

15.2.1 Therapeutic Applications

This section presents and discusses relevant publications based on the progress in the design of SFN protective effect in a variety of in vivo pathologies as well as in in vitro studies on in vitro/in vivo experimental models, as summarized in Table 15.1.

The consumption of isothiocyanates, especially SFN, through the diet is directly related to the decreased risk of certain types of cancer, including lung, pancreas, ovarian, breast, prostate, colon, and bladder. It can act on multiple pathways: inhibiting growth, and proliferation of cancer cells, inducing apoptosis, inhibiting angiogenesis, and cell cycle as well as metastasis formation (Gupta et al. 2014; Kamal et al. 2020). In addition to acting as a chemopreventive, it also can act as an antineoplastic treatment (Singh and Singh 2012; Aumeeruddy and Mahomoodally 2019; Kamal et al. 2020).

Singh et al. (2005) demonstrate that SFN inhibited the growth of human prostate cancer cells in culture (PC-3), through the administration of 20 μM for 24 h, inducing apoptosis initiated by reactive oxygen species (ROS) generation (Singh et al. 2005). Similarly, Choi et al. (2007) also demonstrate the SFN effect on PC-3 and LNCaP prostate cancer cell lines. The in vitro treatment promoted the inactivation of inhibitors of apoptosis proteins (IAP-family) (Choi et al. 2007).

The effects of SFN treatment have also been evaluated in human bladder cancer T24 cell (Shan et al. 2006). Treatment with 10–40 μM SFN for 24 and 48 h significantly inhibited proliferation in a dose-dependent manner and also induced early apoptosis of T24 cell in a lower level of (5 μM) treatment (Shan et al. 2006).

SFN also inhibited cell growth and death in several human breast cancer cell lines, representative of a wide range of breast tumor phenotypes (MDA-MB231, MDA-MB-468, MCF-7, and T47D cells), by the inhibition of estrogen receptor (ER), EGFR1 and HER2, which are particularly important for the growth of breast cancer (Pledgie-Tracy et al. 2007). Another approach studied the effect of SFN on the inhibition of growth in breast ductal carcinoma (ZR-75-1) cells (Cheng et al. 2019). They demonstrated a cell cycle arrest (G1/S) caused by the downregulation of SERTAD1 gene expression by reducing the CDK4 activity in breast cancer cells (Cheng et al. 2019). Other in vitro and in vivo recent investigations reveal that SFN

Table 15.1 Sulforaphane anticancer and anti-inflammatory cellular and molecular mechanisms described in in vitro and in vivo models

Organ/tissue	Pathology	Cellular and molecular mechanisms	References
Bladder	Cancer	Inhibited the proliferation and induced apoptosis of T24 cells in vitro	Shan et al. (2006)
Breast	Cancer	Inhibition of cell growth (G2-M cell cycle block) and induction of apoptosis in multiple breast cancer cell lines	Pledgie-Tracy et al. (2007)
Breast	Cancer	SFN-paclitaxel-induced apoptosis by inhibiting the overexpression of Bcl-2	Kim et al. (2017)
Breast	Cancer	SFN enhanced the efficacy of doxorubicin in suppressing breast tumor growth	Bose et al. (2018)
Breast	Cancer	Inhibited the proliferation by G1/S arrest in breast carcinoma (ZR-75-1) cells	Cheng et al. (2019)
Breast	Cancer	Triple-negative breast cancer (TNBC) proliferation was suppressed in in vitro and in vivo models	Castro et al. (2019)
Colon	Cancer	Induction of G1-phase cell cycle arrest in HT-29 cells	Shen et al. (2006)
Colon	Cancer	Synergistic cytotoxicity effect with curcumin and dihydrocaffeic acid	Santana-Gálvez et al. (2020)
Digestive	Cancer	Suppression of migration and cell invasion in oral carcinoma	Jee et al. (2011)
Lung	Cancer	Arrest of cell migration and invasion avoiding metastasis of lung cancer	Wang et al. (2017)
Lymphoblastic leukemia	Cancer	Inhibition of lymphoblastic leukemia, inducing cell cycle arrest	Suppipat et al. (2012)
Ovarian	Cancer	SFN induces growth arrest and apoptosis epithelial ovarian cancer cell (EOC) line	Bryant et al. (2010)
Ovarian	Cancer	Inhibition of ovarian cancer progression via cell cycle and apoptosis	Kan et al. (2018)
Pancreas	Cancer	Inhibited human pancreatic carcinogenesis, reducing proliferation and tissue invasion	Li et al. (2013)
Prostate	Cancer	Induced apoptosis in PC-3 cells by ROS generation	Singh et al. (2005)
Prostate	Cancer	Inactivation of inhibitors of apoptosis inducing the death of human prostate cancer cells	Choi et al. (2007)
Cartilage	Rheumatoid arthritis	Pro-inflammatory cytokines reduction and synovial hyperplasia in vitro and in vivo models	Kong et al. (2010)
Cartilage	Osteoarthritis/ rheumatoid arthritis	Inhibition of cytokine-induced metalloproteinase expression in human chondrocytes and synovial cells	Davidson et al. (2013)
Cartilage	Osteoarthritis	SFN-rich diet can provide chondroprotection	Davidson et al. (2017)

(continued)

Table 15.1 (continued)

Organ/tissue	Pathology	Cellular and molecular mechanisms	References
Skin	Atopic dermatitis	Inhibition of IFN-γ and TNF-α-induced production of TARC/CCL17 and MDC/CCL22 in human HaCaT cells by inhibition of NF-κB pathway	Jeong et al. (2010)
Skin	Skin inflammation	Reduced inflammation scores in atopic dermatitis mice model	Wu et al. (2019)

can inhibit malignant cell proliferation and tumor sphere formation of cancer stem-like cells (CSCs) in triple-negative breast cancer (TNBC) model (Castro et al. 2019).

Some studies have shown SFN to be effective in preventing ovarian cancer, another important gynecologic cancer-associated mortality. Kan et al. (2018) investigation indicated that SFN effectively suppressed ovarian cancer cells (A2780 and OVCAR lines) proliferation, migration, and cell cycle progression, and also enhance apoptosis (Kan et al. 2018). SFN also inhibited the growth of epithelial ovarian cancer cell (EOC) (MDAH-2774 and SkOV-3 line) in vitro by the modulation of cell cycle regulatory proteins and by increasing apoptosis (Bryant et al. 2010).

SFN was able to regulate the cell cycle and inhibit its proliferation in other types of cancer. Suppipat et al. (2012) investigated in vitro the SFN activity in lymphoblastic leukemia cells, noting that after exposure of 15 μM for 1 day, these cells undergo cell cycle arrest and apoptosis thus preventing their multiplication and invasion to other tissues (Suppipat et al. 2012). Shen et al. (2006) detected the antiproliferative effects of SFN in the human colon carcinoma cell line, HT-29, by blocking the cell cycle at G1 (Shen et al. 2006).

As another important feature, SFN also induces anti-metastatic effects by suppressing cell migration and invasion. Li et al. (2013) studied the hypothesis of SFN acting on the malignant cells of pancreas in vivo (Li et al. 2013). Having verified, that with the administration of a dose between 0–20 mg/kg in mice over a 6-week period, the cell carcinogens were suppressed. SFN also inhibited cell migration and invasion through blockade of miR-616-5p expression and suppression of the epithelial-mesenchymal transition (EMT) process in non-small cell lung cancers (NSCLCs) cells (Wang et al. 2017). Jee et al. (2011) demonstrated that the anti-cell migratory effect of SFN was associated with MMPs suppression of human oral squamous cell carcinoma (Jee et al. 2011).

Nowadays, combination therapy has become the hallmark of different types of cancer treatment due to the disease progression after monotherapeutic treatments. In this context, the SFN has combined effect with other medicinal agents to act synergistically against cancer (Kim et al. 2017; Bose et al. 2018; Aumeeruddy and Mahomoodally 2019; Mangla et al. 2019; Santana-Gálvez et al. 2020). A study by Kim et al. (2017) test the combination of SFN and paclitaxel and observed an increase in the activation of apoptotic signaling pathway members (caspase-3, caspase-8, and caspase-9 and cytochrome c) (Kim et al. 2017). In addition, the combined treatment downregulated the NF-κB signaling pathway, reducing the

protein expression of the apoptosis regulator genes of breast cancer. Bose et al. (2018) determined in a rats model, that SFN reduces DOX cardiotoxicity through Nrf2 activation while enhancing the killing of cancer cells by DOX (Bose et al. 2018). Another approach evaluated the effect of SFN, curcumin (C), and dihydrocaffeic acid (D, a chlorogenic acid metabolite) individually and in different combinations, over the viability of human colon cancer cells (HT-29 and Caco-2) (Santana-Gálvez et al. 2020). The best combination was SFN-D (1:1) since it was both synergistic and significantly more cytotoxic for colon cancer cells than healthy colon cells.

Several studies have shown that SFN exhibits anti-inflammatory activity by inhibiting NF-κB translocation and through the activation of Keap1/Nrf2 pathway, a mechanism that interrupts inflammatory signals to the nucleus (Vanduchova et al. 2019). Some approaches have presented SFN anti-arthritic and immunoregulatory activity (Table 15.1) (Kong et al. 2010; Davidson et al. 2013, 2017; Du et al. 2020). Kong et al. (2010) demonstrated that SFN inhibits synovial hyperplasia, activated T cell proliferation, and the production of IL-17 and TNF-α by rheumatoid arthritis (RA) T cells (Kong et al. 2010). Moreover, in a mouse model, SFN suppressed chronic autoimmune arthritis, inducing apoptosis in the proliferating synovium, at a high dose (200 μM). Another RA study revealed that activating Nrf2 by SFN profoundly inhibited the TNF-α-induced proliferation invasion, and MMPs expression in RA-fibroblast-like synoviocytes (RA-FLS) (Du et al. 2020). In pro-inflammatory cytokine-stimulated osteoarthritis (OA) study, SFN was able to suppress PGE2 or NO production from articular chondrocytes and inhibit proteoglycan and type II collagen degradation (Kim et al. 2012). The chondroprotective effect of SFN was also demonstrated by Davidson et al. (2013). SFN inhibits the expression of key MMPs implicated in OA, prevents inflammation at NF-κB pathway, and protects against cartilage destruction in vitro and in vivo (Davidson et al. 2013). Davidson et al. (2017) also conducted a human study to determine the detection of dietary isothiocyanates (ITCs) in knee joint (OA) patients and identify changes in the joint tissues. They demonstrate that a dietary bioactive with chondroprotective properties reaches the synovial fluid at concentrations with biological impact on the articular joint tissues (Davidson et al. 2017).

SFN has also attenuated other types of chronic inflammatory diseases (Table 15.1). Wu et al. (2019) demonstrated that SFN can reduce the level of inflammation in the skin of the atopic dermatitis (AD) mice model, reducing the accumulation of eosinophils and mast cells in the epithelial tissue (Wu et al. 2019). The effective target of SFN for the treatment of inflammatory skin diseases was also demonstrated by the downregulation of chemokines (TARC/CCL17 and MDC/CCL22) production in human keratinocytes (HaCaT) by inhibition of NF-κB activation (Jeong et al. 2010). Recently, the protective effects of SFN on brain health have been also demonstrated (Table 15.1) (Schepici et al. 2020). Hou et al. (2018) investigated the potential effects of SFN on amyloid-β (Aβ—a striking feature of Alzheimer's disease (AD) oligomer generation) (Hou et al. 2018). In vitro SFN improved cell viability and preserved dendritic length and in vivo SFN improved cognitive deficits, inhibited aggregation, and tau hyperphosphorylation,

as well as reduced the oxidative stress and neuroinflammation. SFN can also exert anti-inflammatory effects, reducing the neuronal damage mediated by microglial activation and reducing the synthesis of inflammatory mediators such as IL-1β, TNF-α, IL-6, and COX-2 (Klomparens and Ding 2019).

15.2.1.1 Sulforaphane and Their Therapeutic Associations: Trends on Nanomedicines for Cancer Treatment

Nanomedicine-based pharmacotherapy has been widely studied as innovative strategy for SFN delivery, especially for cancer treatment. In fact, its promising anticancer effects have driven efforts to overcome some limiting physicochemical properties such as chemical stability and low bioavailability (Tian et al. 2015; Wang and Bao 2021). In general, recent reports describe the development of new delivery systems, considering different routes of administrations and positions, but main innovations are related to their association with other drugs such as currently used anticancer therapies (docetaxel and cisplatin) and non-conventional drugs (acetylsalicylic acid, curcumin). This section will discuss the development and the main results obtained from those studies. Some of them are summarized in Table 15.2.

In the last years, several nanocarriers have been designed for SFN delivery, including polymeric, metallic, and lipid nanoparticles, micro and nanoemulsions, gels, and carbon dots. Among the most reported strategies authors propose the treatment of pancreatic cancer by oral route, which is considered an important factor to increase patient compliance. In this sense, Grandhi et al. (2013) reported the synthesis of solid lipid nanoparticles composed of stearic acid, as lipid phase, and poloxamer 188 as emulsion stabilizer, for encapsulating acetylsalicylic acid, curcumin, and SFN (Grandhi et al. 2013). The whole system chemopreventive effects were studied by *N*-nitrosobis-induced pancreatic cancer animal model, being effective at lower doses compared to other therapies, as well as reduced adverse reaction to the treatment. In another report, the same drug triad was used for avoiding pancreatic cancer progression by encapsulating them in a similar nanocarrier. However, chitosan was used as a stabilizer agent instead of poloxamer 188 to achieve best in vivo performances due to its positive charges, especially regarding bioadhesion to the small intestine and reduced uptake by the reticuloendothelial system (Thakkar et al. 2016). The use of non-steroidal anti-inflammatory drugs in association with SFN was also reported by the same authors. Ibuprofen was encapsulated into solid lipid nanoparticles with different lipid compositions, such as tripalmitin, stearic acid, and Compritol, stabilized by poloxamer 188 or tween 80. In this case, the ibuprofen-loaded nanoparticles and SFN coadministration showed synergistic effects by inhibiting the viability of human pancreatic cells (Thakkar et al. 2015).

In a more recent study, the association of curcumin and SFN was assessed by developing an ethosomal nanogel for skin cancer treatment. Although the study

Table 15.2 Summary of formulations sulforaphane (SFN)-loaded nanocarriers systems, their composition, and main results aiming cancer treatment

Nanomaterial	Composition	Main results	References
Carbon dots	SFN-conjugated carbon dots with thiourea groups	Enhanced targeting and imaging of epidermal growth factor receptor-overexpressing lung cancer cells	Lu et al. (2019)
Lipid nanoparticles	Nanostructured lipid carriers (Precirol®, ATO5, and Transcutol®)	Tamoxifen-SFN-coencapsulated nanoparticles showed increased intestinal permeability, oral bioavailability, and reduced in vivo toxicity	Mangla et al. (2020)
Metallic nanoparticles	Iron oxide–gold core-shell nanoparticles	Induction of apoptosis in human breast cancer cells (MCF-7) with decreased expression of Bcl-2 and Bcl-x_L	Manjili et al. (2016)
Metallic nanoparticles	PEGylated gold-coated iron oxide nanoparticles	SFN-curcumin co-loaded metallic nanoparticles evoked apoptosis in breast cancer cells	Danafar et al. (2017a)
Metallic nanoparticles	Gold nanoparticles	Enhanced cytotoxicity for B16-F10, MCF-7, SW-620, and Caco-2 cells and permeation across intestinal barrier	Soni and Kohli (2019)
Metallic nanoparticles	Tellurium flower-like nanoparticles	In vitro significant reduction of breast cancer cells viability and in vivo pancreatic accumulation	Krug et al. (2020)
Micelles	Monomethoxypoly (ethylene glycol)–poly(*ε*-caprolactone)	Enhanced cytotoxicity against human breast cancer cells (MCF-7)	Danafar et al. (2017b)
Micelles	Poly caprolactone–polyethylene glycol–poly caprolactone	Cytotoxic effects in MCF-7, 4T1 and MCF10A cells mediated by apoptotic events via BCL-2. SFN-loaded micelles evoked reduction in tumor dimensions and prolonged the drug mean residence time	Kheiri Manjili et al. (2017)
Nanocomposites	Silk fibroin in cerium-oxide-carbon dots	Theranostic strategy with enhanced efficacy and imaging in lung cancer cells	Passi et al. (2020)
Nanogel	Ethosomal gel	Enhanced efficacy against B16-F10 murine tumor cells for skin cancer treatment	Soni and Kohli (2019)
Peptide nanoparticles	Prolamin-based nanoparticles stabilized by sodium caseinate and propylene glycol alginate	SFN-encapsulated for colon-specific delivery showed controlled-release rate in simulated gastrointestinal fluid	Wang and Bao (2021)
Polymeric nanoparticles	Poly-lactide-*co*-glycolide-hyaluronic acid-nanoparticles	Docetaxel-SFN dual delivery was cytotoxic in docetaxel-resistant breast cancer stem cells and reduced β-catenin expression	Huang et al. (2016)

(continued)

Table 15.2 (continued)

Nanomaterial	Composition	Main results	References
Polymeric nanoparticles	Poly-L-glutamic acid–cisplatin conjugates	Cisplatin-SFN-nanoparticles showed enhanced cell internalization, tumoral accumulation, and antitumor effects	Xu et al. (2019)
Solid lipid nanoparticles	Stearic acid stabilized by poloxamer 188	Association of acetylsalicylic acid, curcumin, and SFN-encapsulated in solid lipid nanoparticles with synergistic antitumor activity in pancreatic cancer animal model	Grandhi et al. (2013)
Solid lipid nanoparticles	Stearic acid, Compritol 888 ATO, or tripalmitin stabilized by poloxamer 188, tween-80	SFN-ibuprofen-loaded solid lipid nanoparticles showed synergistic cytotoxic effects in in vitro pancreatic cancer cells	Thakkar et al. (2015)
Solid lipid nanoparticles	Stearic acid stabilized by chitosan	Acetylsalicylic acid, curcumin, and SFN-encapsulated with low toxicological profile and enhanced intestinal bioadhesive properties for pancreatic cancer treatment	Thakkar et al. (2016)

reports mainly physicochemical aspects, promising antitumor effects were achieved after B16-F10 cell treatment (Soni et al. 2020).

In other reports, the association of SFN with conventional anticancer therapy has also shown promising results. For example, SFN-docetaxel co-loaded PLGA-hyaluronic acid polymeric nanoparticles were studied for avoiding the initiation and progression of breast cancer, including possible metastasis episodes. In this in vitro study, breast cancer stem cells with recognized docetaxel-resistant phenotype were treated with both drugs docetaxel and SFN, where SFN-loaded nanoparticles induced more pronounced cytotoxic effects than that compared to non-encapsulated drugs and, additionally, reduced the expression of β-catenin. In a complementary way, in vivo tests revealed an enhanced antitumor efficacy by SFN and docetaxel-loaded nanoparticles (Huang et al. 2016).

The synergistic effects of SFN with tamoxifen were also investigated for breast cancer therapy. In an attempt to avoid the extensive tamoxifen first-pass metabolism, Mangla et al. (2019) developed nanostructured lipid carriers with different stabilizers (poloxamer 188 or tween 80) for promoting tamoxifen permeation across the intestinal barrier and, simultaneously, inhibit P-glycoprotein efflux transporter activity (Mangla et al. 2019). Those strategies improved the tamoxifen uptake by cancer cells and increased their sensitivity to SFN, explaining the synergism between both therapeutic agents. Subsequently, another study with a similar strategy also reported a possible optimization of dosing and administration frequency, associated with reduced tamoxifen toxicity, when compared to non-encapsulated drugs (Mangla et al. 2020).

Innovative alternatives to overcome conventional drug limitations were also emphasized by other authors (Xu et al. 2019). The cisplatin chemosensitivity restoration was their main purpose when synthesizing poly-L-glutamic acid–cisplatin conjugates associated with SFN. The increased nanoparticles' cellular internalization was able to modulate the glutathione depletion, which promoted the cisplatin capability for DNA binding, resulting in enhanced cell death effects by apoptosis in breast cancer cells.

In addition to therapeutic associations, new SFN-loaded nanocarriers have been reported, especially considering the development of hybrid systems with multifunctional properties. One of the main strategies refers to the design of metallic nanoparticles, for example, gold-coated iron oxide nanoparticles functionalized with thiolated-polyethylene glycol–folic acid, as reported by Manjili et al. (2016). Physicochemical characterization techniques revealed the synthesis of a stable system able to induce apoptosis mechanisms in MCF-7 human breast cells cancer, such as decreased expression rate of anti-apoptotic genes (Bcl-2 and Bcl-x_L). In a similar study, other authors reported the considerable cytotoxic effects of SFN-loaded tellurium flower-like nanoparticles in two breast cancer cells lines (MCF-7 and MDA-MB-231) when compared to normal cells (MCF-10A) (Krug et al. 2020).

Another recent innovation is the use of versatile nanocarrier systems applied to theranostic purposes. Passi et al. (2020) described multifunctional materials based on SFN-loaded silk fibroin and their further association with cationic cerium oxide nanoparticles and carbon dots (Passi et al. 2020). In fact, the whole system multiple functions are resulting from the association among green fluorescence emission, antioxidant and anticancer activity attributed to carbon dots, cerium oxide nanoparticles, and SFN-loaded silk fibroin, respectively. This multifunctional nanocomposite efficiently reduced the reactive oxygen species levels and allowed better resolution fluorescence images from both tumoral (A549) and normal (L132) lung cells. In a similar report, SFN-carbon dots conjugates functionalized with thiourea groups were developed for targeting and imaging epidermal growth factor receptor-overexpressing lung cancer cells (Lu et al. 2019).

15.3 Conclusion

Nanomedicines have been described as one of the most promising alternatives for overcoming physicochemical and biopharmaceutical limitations of a variety of drugs. These advantages are especially useful for improving the pharmacological effects of conventional therapeutics. On the other hand, phytochemicals, such as SFN, have been proposed as new pharmacotherapy, which expands the research for the treatment of some diseases such as chronic inflammatory processes and cancer. Since polytherapy is the gold-standard treatment, dose adjustments, changes in routes of administration, and possible side effects are factors that must be considered. In this sense, several nanocarriers (micelles, organic and inorganic nanoparticles, nanocomposites, etc.) exert an essential role for developing more

effective and safe formulations. In the case of SFN, its incorporation into nanosystems evoked an improvement in cytotoxic and anti-inflammatory effects, with special attention to elucidating the molecular mechanisms involved.

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