SPRINGER BRIEFS IN SPACE LIFE SCIENCES

Alexander Choukèr
Oliver Ullrich

The Immune System in Space: Are we prepared?

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SpringerBriefs in Space Life Sciences

The extraordinary conditions of space, especially microgravity, are utilized for research in various disciplines of space life sciences. This research that should unravel – above all – the role of gravity for the origin, evolution, and future of life as well as for the development and orientation of organisms up to humans, has only become possible with the advent of (human) spaceflight some 50 years ago. Today, the focus in space life sciences is 1) on the acquisition of knowledge that leads to answers to fundamental scientific questions in gravitational and astrobiology, human physiology and operational medicine as well as 2) on generating applications based upon the results of space experiments and new developments e.g. in non-invasive medical diagnostics for the benefit of humans on Earth. The idea behind this series is to reach not only space experts, but also and above all scientists from various biological, biotechnological and medical fields, who can make use of the results found in space for their own research.SpringerBriefs in Space Life Sciences addresses professors, students and undergraduates in biology, biotechnology and human physiology, medical doctors, and laymen interested in space research.The Series is initiated and supervised by Prof. Dr. Günter Ruyters and Dr. Markus Braun from the German Aerospace Center (DLR). Since the German Space Life Sciences Program celebrated its 40th anniversary in 2012, it seemed an appropriate time to start summarizing – with the help of scientific experts from the various areas - the achievements of the program from the point of view of the German Aerospace Center (DLR) especially in its role as German Space Administration that defines and implements the space activities on behalf of the German government.

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Foreword

The Immune System in Space: Are We Prepared? is the title of this new booklet in our series *SpringerBriefs in Space Life Sciences*. In fact, the authors couple their description of the immune system and its function in space and on Earth to the question if humans are prepared – from an immunological point of view, of course – to undertake exploration class missions such as traveling to Mars. Is this a reasonable and valid question? Indeed it is: Since the early days of human spaceflight more than 50 years ago, it is well known that the immune system of astronauts is severely compromised during and after their spaceflights. However, until today, the exact causes and mechanisms for these spaceflight-induced problems are not well understood – in spite of numerous scientific studies.

After a short introduction into the evolutionary history thought to provide some insight for the understanding of the complexity of the immune system, the authors start to tackle the predominant question of the booklet, namely, how space and space-like environmental conditions affect immunity. After describing briefly the interaction between the immune system and various environmental factors and stressors as well as relevant results obtained from spaceflight studies, the authors present in some detail the cellular effects of altered gravity first on the innate immune system and the endothelial barrier (part 3 of Chap. 2) and then on the human adaptive immune system (part 4 of Chap. 2). Here, special attention is given to the T lymphocytes for which – after the pioneering work during the first Spacelab mission in 1983 – a wealth of new information is available from recent space experiments and accompanying ground work. The results from this research may provide new targets for therapeutic or preventive interventions not only for astronauts but also for people on Earth. The chapter closes with a look at the microbial environment of spacecrafts; this is an important aspect, since the combination of an altered microbial flora with a complex immune function can be considered as a significant risk for infectious diseases during long-term space missions.

In Chap. 7, this line of thought is continued with a view on spacecraft contamination monitoring and control. This is mandatory in order to reduce potential hazards for the crew as well as for the infrastructure that is also affected by bio-destructive microorganisms. In order to meet the challenges such as complete autonomy from

Earth during long-term missions, a novel approach called cell-based therapy is proposed for health care in astronauts. In combination with lyophilization of cells, therapeutical human cells could amount to comprehensive treatment and prophylaxis in the future, not only in space but also on Earth. First successful applications are already available in traumata and cancer treatment.

Are we prepared? In the final chapter, the authors summarize the findings of many years of research reaching at the conclusion that – generally speaking – humans are adapted remarkably well to the altered environmental conditions of spaceflight, especially to microgravity. However, in spite of all technical and medical preparations, some risks will remain, when one day in the not-too-far future astronauts will start the greatest journey of mankind, the journey to Mars.

DLR Bonn, Germany
May 2016

Prof. Dr. Günter Ruyters

Preface to the Series

The extraordinary conditions in space, especially microgravity, are utilized today not only for research in the physical and materials sciences—they especially provide a unique tool for research in various areas of the life sciences. The major goal of this research is to uncover the role of gravity with regard to the origin, evolution, and future of life and to the development and orientation of organisms from single cells and protists up to humans. This research only became possible with the advent of manned spaceflight some 50 years ago. With the first experiment having been conducted onboard Apollo 16, the German Space Life Sciences Program celebrated its 40th anniversary in 2012—a fitting occasion for Springer and the DLR (German Aerospace Center) to take stock of the space life sciences achievements made so far.

The DLR is the Federal Republic of Germany's National Aeronautics and Space Research Center. Its extensive research and development activities in aeronautics, space, energy, transport, and security are integrated into national and international cooperative ventures. In addition to its own research, as Germany's space agency, the DLR has been charged by the federal government with the task of planning and implementing the German space program. Within the current space program, approved by the German government in November 2010, the overall goal for the life sciences section is to gain scientific knowledge and to reveal new application potentials by means of research under space conditions, especially by utilizing the microgravity environment of the International Space Station (ISS).

With regard to the program's implementation, the DLR Space Administration provides the infrastructure and flight opportunities required, contracts the German space industry for the development of innovative research facilities, and provides the necessary research funding for the scientific teams at universities and other research institutes. While so-called small flight opportunities like the drop tower in Bremen, sounding rockets, and parabolic airplane flights are made available within the national program, research on the International Space Station (ISS) is implemented in the framework of Germany's participation in the ESA Microgravity Program or through bilateral cooperations with other space agencies. Free flyers such as BION or FOTON satellites are used in cooperation with Russia. The recently started utilization of Chinese spacecrafts like Shenzhou has further expanded

Germany's spectrum of flight opportunities, and discussions about future cooperation on the planned Chinese Space Station are currently under way.

From the very beginning in the 1970s, Germany has been the driving force for human spaceflight as well as for related research in the life and physical sciences in Europe. It was Germany that initiated the development of Spacelab as the European contribution to the American Space Shuttle System, complemented by setting up a sound national program. And today Germany continues to be the major European contributor to the ESA programs for the ISS and its scientific utilization.

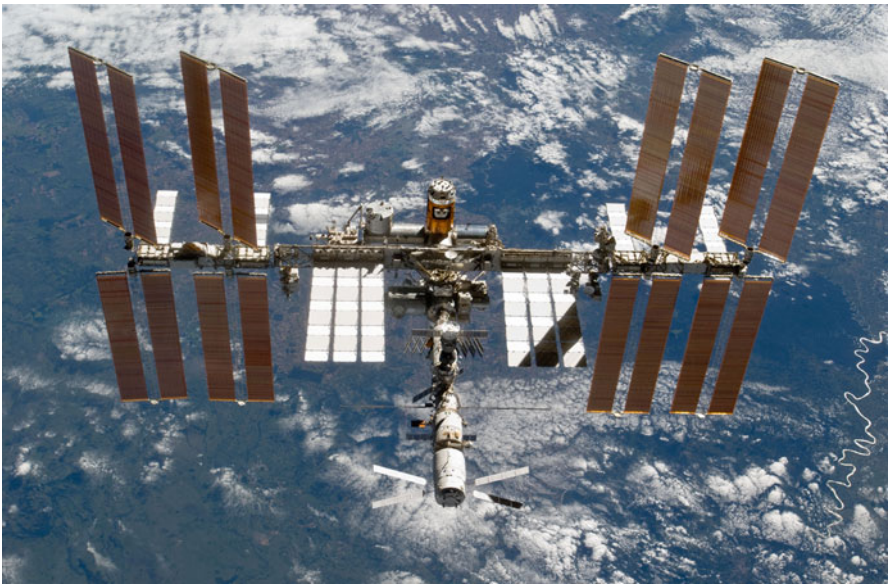
For our series, we have approached leading scientists first and foremost in Germany, but also—since science and research are international and cooperative endeavors—in other countries to provide us with their views and their summaries of the accomplishments in the various fields of space life sciences research. By presenting the current SpringerBriefs on muscle and bone physiology, we start the series with an area that is currently attracting much attention—due in no small part to health problems such as muscle atrophy and osteoporosis in our modern aging society. Overall, it is interesting to note that the psychophysiological changes that astronauts experience during their spaceflights closely resemble those of aging people on Earth but progress at a much faster rate. Circulatory and vestibular disorders set in immediately, muscles and bones degenerate within weeks or months, and even the immune system is impaired. Thus, the aging process as well as certain diseases can be studied at an accelerated pace, yielding valuable insights for the benefit of people on Earth as well. Luckily for the astronauts: these problems slowly disappear after their return to Earth, so that their recovery processes can also be investigated, yielding additional valuable information.

Booklets on nutrition and metabolism, on the immune system, on vestibular and neuroscience, on the cardiovascular and respiratory system, and on psychophysiological human performance will follow. This separation of human physiology and space medicine into the various research areas follows a classical division. It will certainly become evident, however, that space medicine research pursues a highly integrative approach, offering an example that should also be followed in terrestrial research. The series will eventually be rounded out by booklets on gravitational and radiation biology.

We are convinced that this series, starting with its first booklet on muscle and bone physiology in space, will find interested readers and will contribute to the goal of convincing the general public that research in space, especially in the life sciences, has been and will continue to be of concrete benefit to people on Earth.

Bonn, Germany — Prof. Dr. Günter Ruyters
Bonn, Germany — Dr. Markus Braun
July, 2014

DLR Space Administration in Bonn-Oberkassel (DLR)



The International Space Station (ISS); photo taken by an astronaut from the space shuttle Discovery, March 7, 2011 (NASA)

Extravehicular activity (EVA) of the German ESA astronaut Hans Schlegel working on the European Columbus lab of ISS, February 13, 2008 (NASA)

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Abbreviations

5-LOX	5-Lipoxygenase
A1, 2A/B, 3	Adenosine receptors type 1, 2A/B and 3
APO	Apoptosis antigen
ATP	Adenosine triphosphate
BAECs	Bovine aortic endothelial cells
BFU-E	Burst-forming units of erythroid type
CD	Cluster of differentiation
CES	Cultured epidermal sheets
CFU-GEMM	Colony-forming units of granulocyte/erythrocyte/monocyte/megakaryocyte type
CFU-GM	Colony-forming units of granulocyte/monocyte type
CIK cells	Cytokine-induced killer cells
ConA	Concanavalin A
DAMPS	Damage-associated molecular pattern
DC	Dendritic cells
DLR	Deutsches Zentrum für Luft- und Raumfahrt (*German Aerospace Center*)
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleinacid
DNA	Deoxyribonucleic acid
DPG	2, 3-Diphosphoglycerate
EC	Endothelial cells
ECS	Endocannabinoid system
ENose	Electronic nose
FADH2	Flavin adenine dinucleotide
FBI	Federal Bureau of Investigation
FBS	Fetal bovine serum
F-CES	Cryopreserved (frozen) cultured epidermal sheets
FPR	Formyl peptide receptor
g	Earth gravity
GC	Ground control

GVHD	Graft-versus-host disease
GWAS	Genome-wide association studies
HACCP	Hazard analysis critical control point
HARV	High-aspect ratio vessel
HEPA	High-efficiency particulate arrestance
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPCs	Hematopoietic progenitor cells
HS	Human serum
HUVECs	Human umbilical vein endothelial cells
hyp-g	Hypergravity
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
ISS	International space station
KLRK1	Killer cell lectin-like receptor subfamily K, member 1
L-CES	Lyophilized cultured epidermal sheets
LED	Light-emitting diode
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
miRNA	MicroRNA
MSCs	Mesenchymal stem cells
MVOC	Microbial volatile organic compounds
n/a	Not available/applicable
NADH/H+	Nicotinamide adenine dinucleotide
nd	Not determined
NF-kB	Nuclear factor of kappa B
NK	Natural killer cells
NKG2D	Natural killer group 2, member D
Orion MPCV	Orion multi-purpose crew vehicle
PAMPS	Pathogen-associated molecular patterns
PARP	Poly (ADP-ribose) polymerase
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PDB	Phorbol dibutyrate
PHA	Phytohemagglutinin
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PMNs	Polymorphonuclear leukocytes
PRR	Pattern recognition receptors
PSCs	Pluripotent stem cells
PVP	Polyvinylpyrrolidone
RBCs	Red blood cells
RCCS	Rotary cell culture system
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RPE cells	Retinal pigment epithelial cells
RPM	Random positioning machine
RPMI-1640	Roswell Park Memorial Institute-1640 medium
RQ	Respiratory quotient
RWV	Rotating wall vessel
SIRS	Systemic inflammatory response syndrome
STS	Space transport system
TCA	Tricyclic acid cycle
TCR	T-cell receptor
THESEUS	Towards Human Exploration of Space: A EUropean Strategy
TLR	Toll-like receptor
TNF	Tumor necrosis factor
USSCs	Unrestricted somatic stem cells
UV	Ultraviolet
VZV	Varicella zoster virus

Chapter 1
The Immune System in Evolution

Buqing Yi, Manfred Thiel, and Alexander Choukèr

Why and how our immune system functions and sometimes dysfunctions? Immunologists are often surprised by the complexity of the human immune system's performance. A brief exploration of the evolutionary history of the immune system might be able to provide insight for understanding this complexity of our important defense system and its role for human health.

Human immunity works through a complex, orchestrated, and many functional and organ-specific, though always interconnected, approaches. As from the evolution from simple organisms - as known especially from insects with a short life time (e.g. fruit fly) - to highly developed mammals, we know that two major immune system branches have evolved subsequently as a consequence of expanded life times and environmental challenges, the innate immunity and adaptive immunity. The coordinated efforts of the innate and adaptive immune branches normally guarantee an effective host defense against potentially harmful pathogens, to differentiate immune answers between self and nonself and hereby avoiding to harm the host. Innate immunity is the primary line of immune defense and yields an immediate nonspecific response, which is mediated mainly by neutrophils, monocytes, macrophages, dendritic cells (DCs), and natural killer (NK) cells, together with cytokines, defensins, and complement and acute phase reactants such as C-reactive protein (Akira et al. 2006; Medzhitov and Janeway 1997). Adaptive immunity, the so-called secondary line of defense, relies upon B and T lymphocytes which express antigen-specific surface receptors. There are two key components of the adaptive immune

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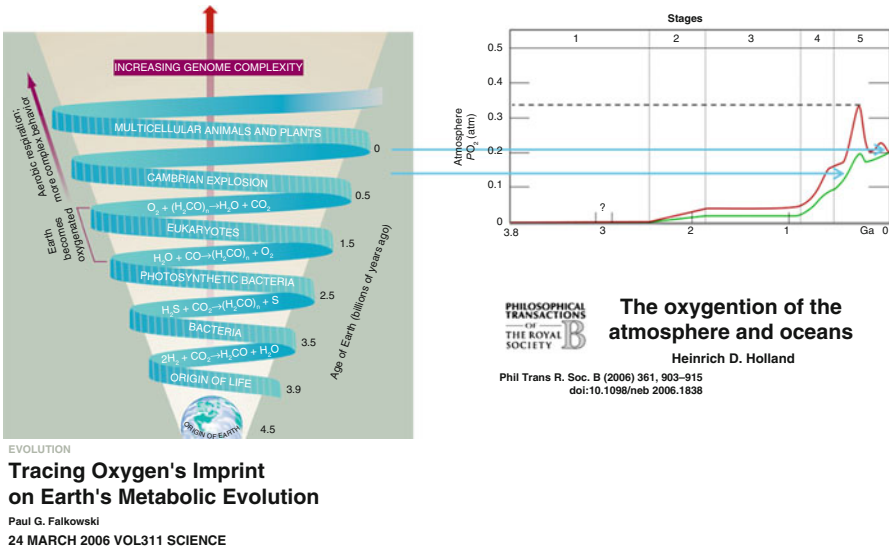

Fig. 1.1 The "cambrian explosion": increase of the diversity and complexity of organisms as paralleled by the increase of oxygen in the atmosphere. Right graph *green and red lines* reflecting the anticipated lower and upper range of the oxygen concentration (cited figures as published by Falkowsky 2006 and Holland 2006)

system: the humoral, antibody-mediated, and depending on B lymphocytes, and the cellular immunity as coordinated by T lymphocytes.

Innate immune mechanisms can be tracked back to almost the lowest level of the evolutionary tree of life, which indicates the importance of innate immunity in life surviving starting from the appearance of single-cell microorganisms on Earth more than 3.5 billion years ago (Kimbrell and Beutler 2001). The following evolution of diverse bacteria, archaea, and eukaryotes proceeded to the development of multicellular organisms (metazoans) that occurred around 600 million years ago. After the "cambrian explosion," oxygen concentration and diversity of organisms had increased, and the diversity in metazoan species offered new host opportunities for microbial pathogens (Fig. 1.1).

On the same timescale, the diversity of microbial pathogens might explain the consecutive and remarkable varieties of innate defense mechanisms in plants and animals. Interestingly, a unifying element of innate immunity exists, which is the use of germline-encoded pattern recognition receptors for pathogens or damaged self-components, such as the Toll-like receptors, nucleotide-binding domain leucine-rich repeat (LRR)-containing receptors, and C-type lectin receptors (Buchmann 2014) [see also Chap. 3, part 3].

Adaptive immunity appeared in vertebrates around 500 million years ago with its unique feature of the somatic development of clonally diverse lymphocytes, each of which has a specific antigen recognition receptor that can trigger its activation. The existence of a highly diverse lymphocyte receptor repertoire allows vertebrates to

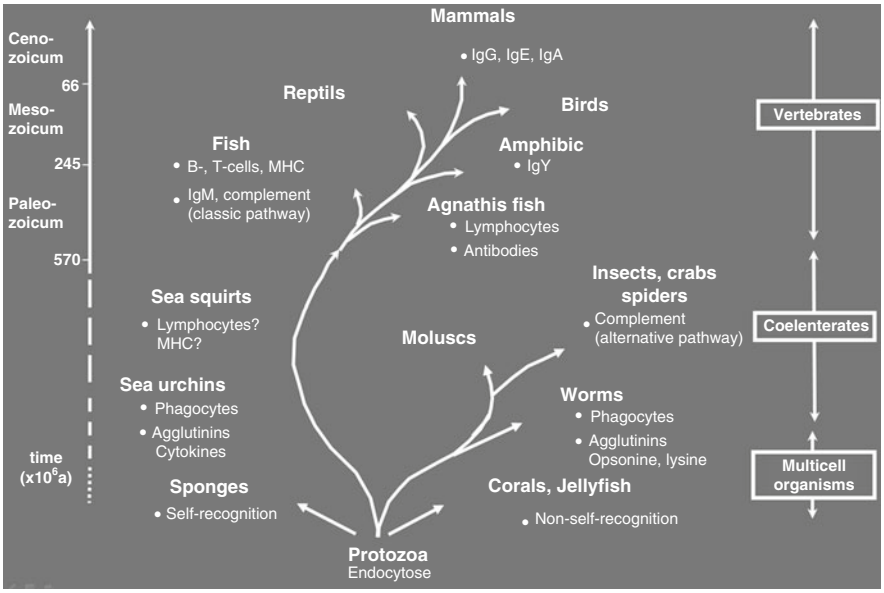

Fig. 1.2 The evolution of the immune system (Compiled after Paul 2003)

recognize almost any potential pathogen or toxin and to mount antigen-specific responses to it (Cooper and Herrin 2010). Activated lymphocytes then engage in population expansion and differentiation into mature effector lymphocytes with cytotoxic and proinflammatory functions or into plasma cells that secrete antibodies. In addition, the population expansion and some long-existing antigen-primed cytotoxic lymphocytes and plasma cells provide protective memory to prevent from potentially detrimental consequences of the next invasion (Cooper and Herrin 2010).

T-cell-related cellular immune responses and B-cell-related humoral immune responses require the involvement of various phagocytic cells, dendritic cells (DCs), natural killer (NK) cells, and other types of innate immune cell and humoral components, but it is difficult to trace the evolutionary history of the extensive network of individual immune cell types like that in other systems such as myogenic cells (Yi et al. 2009, Cooper and Herrin 2010). Moreover, evolutionary processes are continually affecting the immune system. For example, we can see a rather recent evolution of very different types of NK cell receptors in mice and humans, which shared a common ancestor around 65 million years ago (Abi-Rached and Parham 2005). This kind of evolutionary changes increases the difficulty in deciphering some of the steps in the evolutionary history of immunity, for instance, the exact time when DC and NK cells entered the evolutionary scene remains a puzzle.

When reflecting the evolutionary history of immunity (see Fig. 1.2), the conclusion can be drawn that the high complexity of actions and interactions of the innate and adaptive immunity are the result of powerful and long-lasting selection and deselection processes, the increasing complexity, and life span of the organisms,

which over the time has had to increase also the probability to efficiently distinguish between self and nonself and hereby combating pathogens (Flajnik and Kasahara 2010). However, the appearance of an adaptive immune system featuring a big randomly created receptor repertoire expressed by lymphocytes with proinflammatory potential would undoubtedly pose the danger of autoimmunity. Since we need to understand how the individual components of our complex immune system collaborate to activate protective immunity, a more general and "holistic" view is therefore important for the understanding of inflammatory and autoimmune diseases and for designing strategies to alleviate inappropriate or excessive immune responses. The importance of such understanding is of ultimate importance in our civilization in view of the fact that autoimmune and infectious causes of diseases are rising worldwide. The rise in the prevalence of allergic diseases has continued in the industrialized world for more than 50 years (from the American Academy of Allergy, Asthma & Immunology (AAAAI), Milwaukee/MI, USA); autoimmune disease prevalence is rising according to the National Institutes of Health (NIH, Bethesda/MD, USA), as well as the incidence of sepsis is increasing in all areas of the world where epidemiology studies have been conducted (Martin 2012).

It will be of key importance and of special interest how the further evolution and adaption processes of immune cells and immunity as a whole will occur in the coming hundreds and thousands of years. It should be considered also that since the gravitational environment on Earth might represent a key factor in the molecular homeostasis of the immune system and therefore optimal conditions for evolutionary development and adaptation, it has become even more interesting to investigate the "new immune system" when new living conditions occur and challenges are affecting our immune responses and evolution: life under conditions of reduced gravity in the hostile environment of space.

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Part I
How Does Space and Space Like Conditions Affect Immunity?

Chapter 2
The Immune System and Man-Environment Interaction: A General Understanding

Buqing Yi and Alexander Choukèr

Environmental factors have long been known to be able to affect immune responses from both animal and human studies (Glover-Kerkvliet 1995; Monteleone et al. 2012; Rook 2013; Tedeschi et al. 2003). Over the past few decades, many efforts have been made to understand the interaction between various environmental factors, genetic factors, and the development of immune pathologies, such as allergic/autoimmune disease (Andiappan et al. 2014; Lau et al. 2014; Barne et al. 2013; Kauffmann and Demenais 2012; Willis-Owen and Valdar 2009). The environmental factors and stressors related with missions to space include: microgravity, ecologically and environmentally closed systems, prolonged isolation, acute physical strain (such as during launch or landing), radiation, changes in blood sheer forces, as well as other variables that might have not been recognized yet (Sonnenfeld et al. 2003; Gueguinou et al. 2009; Crucian and Sams 2009). These environmental factors could each individually affect immune functions, but they could also be interactive during spaceflight to alter immunity (Gueguinou et al. 2009; Crucian and Sams 2009).

Many studies of gene-environment interaction have indicated that individuals often vary in their susceptibility to environmental influences (Hunter 2005). Among others, two specific genetic polymorphisms, the serotonin transporter gene 5-HTTLPR and the dopamine receptor gene DRD4, have been widely studied. They have long been regarded as "vulnerability genes," since carriers of particular alleles have higher risk of developing certain psychological problems or physiological disorders including inflammatory diseases in the face of adversity. However, more recent evidence indicates that they should more appropriately be treated as "plasticity genes" because carriers of the putative risk alleles seem to be especially susceptible to environmental influences either adverse influences or also favorable ones (Belsky

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and Hartman 2014). For 5-HTTLPR, it has been reported that in the case of Caucasian children under 18 years of age, short-allele carriers are more susceptible than long-allele carriers to both positive and negative developmental experiences (van Ijzendoorn et al. 2012). For DRD4, increased susceptibility has been found in the 7-repeat allele carriers with social circumstances such as maternal positivity and pro-social behavior, contextual stress and support, and several other kinds of environmental influences (Belsky and Hartman 2014).

Although genetic factor plays an important role in deciding reactions to environmental influences, interestingly, a recent systems-level analysis of 210 healthy twins has revealed that the human immune system is mainly "shaped" by environment, with a generally limited influence of genetic factors (Brodin et al. 2015). Environment, often described as combination of multiple "environmental exposures" is defined as "non-genetic" factor in the broad sense. Compared to the fast development of human genome sequencing tools for examining individual susceptibility through genome-wide association studies (GWAS), only a limited number of tools or methods are available so far for performing exposure assessments. Given that autoimmunity, chronic infection, and other chronic diseases develop predominantly from a combination of environmental exposures with restrained genetic background influences, the ability to measure and to describe environmental exposures becomes particularly demanding to understand the effects of specific environmental exposures on human health. Environmental exposures, if we only consider the external factors based on traditional understanding of environment, can be categorized as specific ones and general ones. Specific exposures may refer to radiation, infectious agents, environmental contaminants, air pollutions, diet, lifestyle factors (e.g., tobacco, alcohol), occupation, and medical interventions (Wild 2012). These factors have been the main focus of epidemiological studies seeking a link between environmental risk factors with chronic immune disease. For general exposures, they include the broader social, economic, and psychological influences on each person, for example, social status, education level, financial condition, physiological or psychological stress, geographic environment, and climate (Wild 2012). All these specific and general environmental exposures work together and may to a certain extent formulate the major causes of a large number of human disorders.

For space exploration, space travelers are exposed to many extreme environmental conditions, and for future interplanetary space exploration, such as Mars mission, astronauts can be exposed to a completely strange environment, which means new and more complex combinations of conditions of "environmental exposure." How could these "environmental exposures" affect the human immune system and the health conditions? This is a critical and challenging question waiting for illumination. The main challenge here is to identify, to understand, and to elucidate the interaction between one type of exposure and the corresponding immune responses to that exposure. Knowledge achieved from this aspect can not only imply the link between an exposure and a disorder, but also provide insights into the underlying mechanisms of how an exposure might be applying its effects, which may add to the mass of evidence in allocating causality to an exposure-disease association and shed light on prevention strategies through modulation of specific identified mechanistic

pathways. To investigate interactions between exposure, mechanism, and disease has become one of the emerging directions for biomarker discovery (Vineis and Perera 2007).

Space exploration, as mentioned in this volume, provides many extreme environmental conditions. The capability of addressing the interaction between exposure, mechanism, and health problem might yield innovative insights into how seemingly distinct risk factors, such as psychosocial stress (e.g., Yi 2015; Basner et al 2014), diet that is too salty (e.g., Yi et al. 2015) or too sweet, immune suppression, or immune hypersensitivity, act to produce similar health problems (Terry et al. 2011; Thayer and Kuzawa 2011). With an integrative systems biology approach in this regard, evaluations of psychosocial stress have been reported to be correlated with inflammation and telomere length, contributing evidence of how seemingly unrelated risk factors may act through shared biological pathways (Wild 2012).

Exposure of humans, animals, and cell cultures to spaceflight conditions has resulted in aberrance of immune responses (Gueguinou et al. 2009; Crucian and Sams 2009). Although cellular immunity has been shown to be primarily influenced, changes in humoral immune responses after spaceflight have also been observed (Gueguinou et al. 2009; Crucian and Sams 2009). Both the innate and adaptive immune systems were affected, characterized by changes in "cytokine production, leukocyte blastogenesis, NK cell and macrophage activity and production, antibody production, and enzyme functions in pathways important for immune functions" (Sonnenfeld 2013). Several recent studies have consistently indicated alterations in neutrophil, monocyte, and lymphocyte populations (cell population numbers and function), altered expression of antibody variable heavy chain genes, and others in response to spaceflight conditions (Gueguinou et al. 2009; Crucian and Sams 2009). However, the question of which of the factors are responsible for the spaceflight-induced alterations of the immune functions has to be elucidated and some of which would be discussed in more detail in the following chapters.

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Chapter 3
The Immune System in Space and Space-Like Conditions: From the Human Study Perspective

Buqing Yi and Alexander Choukèr

It has been around 50 years since the first moon landing of humans, and the next goal for space exploration is manned interplanetary mission to Mars. As discussed above, one of the crucial concerns about human space exploration is the effect of extreme environments and conditions in space on the human immune system. Microgravity, solar and cosmic radiation, chronic stress of prolonged isolation and confinement, as well as the stress of readaptation to Earth environment after return, all adding to the complexity of understanding the effect of spaceflight on human immune functions (Crucian and Sams 2009; Gridley et al. 2009). Multiple studies were performed to investigate the effects of spaceflight on human immunity during and after spaceflight, the results of which indicated that the immune system undergoes a variety of changes after space travel, such as altered leukocyte distribution (Crucian et al. 2008, 2013), altered monocyte and granulocyte function (Kaur et al. 2004, 2005), changes of cytokine production patterns in plasma, and in response to stimulation (Crucian et al. 2000, 2014). Furthermore, reactivation of latent viruses has been repeatedly reported in the crew during short-duration spaceflight (Mehta et al. 2013, 2014; Cohrs et al. 2008; Pierson et al. 2005). Recent investigations on crew members of long-duration space missions have revealed the potential development of the immune dysfunctions into two directions: immune hyperactivity, which may result in risks such as hypersensitivities or autoimmunity and immune hyporeactivity, which means an anticipated increased risk for infectious diseases and viral reactivation (Crucian et al. 2014).

From the clinical aspect, increased susceptibility to infection in astronauts can be dated back to the Apollo era, and there were a surprisingly high number of reported infectious disease incidences or inflammation-related symptoms on board or after

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spaceflight (Mermel 2013). However, most studies about the effect of spaceflight on immunity were performed following short-term spaceflights that lasted less than 15 days (Gueguinou et al. 2009). There is only limited knowledge about the impact of long-term spaceflight on human immunity. Compared with short-term spaceflight conditions, astronauts/cosmonauts face more severe physiological and psychological stressors owing to prolonged exposure to space environment during long-term spaceflight. The effects have been currently studied by the space agency's researchers (i.e., Integrated/Functional Immune by NASA, IMMUNO1 and 2 by ESA-IBMP/Roscosmos).

Several space-related human immunity studies have consistently reported changes in the peripheral blood leukocyte phenotype postflight (Gueguinou et al. 2009; Crucian and Sams 2009). Following landing, highly increased leukocyte numbers including neutrophils, lymphocytes, and most lymphocyte subgroups have been observed. This phenomenon is likely, at least in part, triggered by the landing process which can apply dramatic acute physical stress to human body owing to coexistence of microgravity, hypergravity, and fierce vibration during the landing process. Elevated stress hormones cortisol and catecholamines were often observed immediately following landing, and it is known that the immune system reacts to acute stress by releasing a large number of leukocytes (Dhabhar et al. 2012; Stowe et al. 2013; Meehan et al. 1993).

Neutrophil activation after spaceflight has been recently found following long-duration spaceflight, mainly characterized by a differential expression of adhesion molecules on the cell surface of neutrophils (preliminary, unpublished). Neutrophils are the first to arrive at sites of infection and are critical to the host's defense against bacterial infection, and functional defects of neutrophil cells are involved in poor wound healing and recurring bacterial infection. Clinically, neutrophil activation often indicates potential inflammation signals (Liu et al. 2012; Kolaczkowska and Kubes 2013; Bian et al. 2012). Investigations following short-duration spaceflight also reported changes of neutrophil functions demonstrated by enhanced chemotactic activity after landing, increased neutrophil adhesion to endothelial cells and significantly changed L-selectin expression (Stowe et al. 1999). But interestingly, L-selectin expression on the surface of neutrophils was significantly increased after short-duration spaceflight (Stowe et al. 1999), showing a difference from the findings following long-duration spaceflight. This difference suggests that the activation of neutrophils may result from the accumulative effects of long-duration mission-related influential factors (i.e., microgravity, radiation, or readaptation to earth environment). Accordingly, recall antigen response after long-term spaceflight were seen to be increased in responses to, for example, fungal antigens (Choukèr 2012). Taken together, the immune alterations observed following long-duration spaceflight aggravate immunopathology during the course of inflammatory responses. Such alterations, should they persist during prolonged interplanetary space missions and habitation of moon or Mars, could lead to diseases associated with immune imbalance such as chronic inflammation, autoimmune diseases, and other inflammation-related diseases.

So far it is not yet clearly understood which environmental exposures during spaceflight are majorly responsible for spaceflight-induced alterations in immune

phenotype and immune functional states and how the effects are translated to changes on the genetic, transcriptional, or epigenetic levels. These immune changes may result from physiological deconditioning of the accumulative effects of mixed space influential factors in the long-duration mission. Among all the factors, multiple studies have indicated that microgravity may suppress T-cell proliferation and inhibit T-cell activity (Sonnenfeld 2012; Chang et al. 2012). Interestingly, several other influential factors have been reported to be able to trigger heightened immune responses. For example, the condition of isolation and confinement as a typical chronic stressor are among the major stressors in space, which may potentially induce considerable psychological and physiological modifications. It has been reported that prolonged isolation and confinement acting as chronic stressors could trigger leukocyte phenotype changes and poorly controlled immune responses, and it may even have a long-lasting physiological effect (Yi et al. 2014, 2015a, b). Similarly, altered cytokine production profiles were detected during the isolation of the Antarctic suggesting isolation-related T-cell activation (Shearer et al. 2002; Tingate et al. 1997), although in the Antarctic environment the effects of immune modulation by lower oxygen tension is also acknowledged (Feuerecker et al. 2014). It is also noteworthy that after staying in the closed spacecraft for 6 months, back to Earth environment means exposure to a new set of antigens, and environmental exposures have long been known to be able to affect immune activity as from both animal and human studies (Monteleone et al. 2012; Rook 2013; Tedeschi et al. 2003; Brodin et al. 2015; Wild 2012). Consistent with it, hypersensitive immune responses have been observed after the simulated Mars mission in which no microgravity, radiation, or landing process have been simulated (Yi et al. 2015b). Furthermore, the acute physical stress produced by the landing process can be another contributor to the changes of immune phenotype after return. It is likely that multiple factors, including microgravity, radiation, chronic stress imposed by prolonged isolation and confinement, the landing process, and environmental (re-) exposures after spaceflight, are affecting immune functions with distinctive but interactive mechanisms.

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Chapter 4
Cellular Effects of Altered Gravity on the Innate Immune System and the Endothelial Barrier

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The innate immune system is of essential importance to protect the human body from infection as it recognizes, inactivates, and kills intruding pathogens. It comprises different types of leukocytes, each having specialized functions to dispose pathogens. Their capacities cover phagocytosis, secretion of cytokines to recruit other cells, oxidative burst, and secretion of toxins. During elongated spaceflight, a pronounced immune dysfunction has been observed in astronauts that becomes manifest in an enhanced susceptibility to infections by bacteria, viruses, and fungi (Sonnenfeld 2002). This immunodeficiency has inspired curiosity about possible effects of altered gravity conditions on immune cells, and numerous studies have been performed since the 1970s to address the effects of altered gravity on immune cells as a possible underlying mechanism of space-induced immunodeficiency. This chapter will focus on the effects of altered gravity on the cells of the innate immune system, while the effects on the adaptive immune system are discussed in Chap. 3 [part 4].

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During acute inflammation, leukocytes, especially granulocytes, need to interact highly coordinated with the endothelial cells (ECs) of the vascular system to reach the sites of infection. The vascular endothelium is composed of a layer of closely connected ECs and separates the blood from the surrounding tissue. This endothelium plays a fundamental role in tissue homeostasis as it regulates vasoconstriction/vasodilatation and builds a semipermeable barrier that regulates blood-tissue exchange of plasma, molecules, and cells. ECs have mechanosensory properties; they can react to fluid shear stress (Topper and Gimbrone 1999) and pressure (Fu and Tarbell 2013). Additionally the endothelium builds a physical barrier against pathogens that have entered the circulation and hinders them to infiltrate the surrounding tissues. For leukocytes, the endothelial barrier provides an inducible and highly specific permeability: during inflammation ECs are activated, meaning that the expression pattern of surface molecules is altered which enables leukocytes to roll along and subsequently bind to the endothelium. These changes allow leukocytes to cross the endothelial barrier, a process called diapedesis, and migrate through tissues to the sites of infection (Yuan and Rigor 2010). Junctional complexes between adjacent cells play a major role in leukocyte extravasation and vascular permeability; their composition is modulated dynamically (Aghajanian et al. 2008). Dysfunction of the endothelial barrier is involved in many pathological circumstances such as the extravasation during tumor metastasis, thrombosis, inflammation, diabetes mellitus, trauma, epilepsy, sepsis, and multiple sclerosis (Yuan and Rigor 2010; Reymond et al. 2013). Additionally to the already mentioned immune dysfunction (Sonnenfeld 2002) and the well-known dystrophic effects on muscle and bone, astronauts suffer from cardiovascular issues due to vascular impairment during spaceflight (Convertino 2009). ECs are of central importance for both cardiovascular homeostasis and inflammatory processes. Taking into account that ECs can sense mechanical stimuli and convert them into cellular signals (Feletou et al. 2010; Busse and Fleming 2003), the question arises if ECs are sensitive to gravitational changes and possibly contribute to the physiological dysfunctions observed during spaceflight.

Numerous studies have been conducted to evaluate and to understand the effects of altered gravity on cells of the innate immune system and ECs (Maier et al. 2015). Therefore, the blood of astronauts and participants of parabolic flights has been investigated, and many in vitro studies with isolated cells in real and simulated microgravity have been performed. Various effects of microgravity and hypergravity were observed comprising very basal cellular functions such as proliferation as well as effector functions such as oxidative burst, adhesion, locomotion, and cytokine secretion. Table 4.1 summarizes the effects of altered gravity on cells of the innate immune system and on ECs.

The results obtained in different studies might seem partly conflicting. To interpret the data, it must be kept in mind that they were obtained partly in real microgravity and partly from platforms that provide simulated microgravity, which can only model some aspects of real microgravity. Another source of discrepancies between experimental outcomes may be the use of cell models from different species and the differences between primary cells and cell lines. For ECs, the origin of the cells with respect to aortic or venular location in the vascular system might also have an influence on the experimental outcome. Therefore, results should be interpreted with respect to their particular experimental setup.

Table 4.1 Effects of altered gravity on cells of the innate immune system and endothelial cells

Cell type/focus of investigation	Cell model	Research platform	Findings	Reference
Monocytes/ intracellular signal transduction	U937	Space shuttle flight	The subcellular distribution of PKC was altered in microgravity samples compared to on-board 1 g samples: with increasing g-force, the level of PKC in the nuclear fraction decreased while it increased in the cytosolic fraction. The synthesis of interleukin-1β was decreased in microgravity samples	Schmitt et al. (1996)
	U937 monocytic cells	Spaceflight	The translocation of PKC from the cytosol to the particulate fractions showed an altered as compared to controls on the ground. Additionally enhanced binding of phorbol ester to PKC was observed in-flight, while hypergravity (1.4 g) led to decreased binding	Hatton et al. (1999)
	U937 monocytic cells	Spaceflight	Translocation of the PKC isoforms PKC β II, delta, and epsilon in response to phorbol ester was decreased compared to 1 g but was increased by hypergravity (1.4 g)	Hatton et al. (2002)
	Peripheral blood monocytes	Sounding rocket, spaceflight	In LPS-stimulated cells microgravity led to an impairment of Jun-N-terminal kinase activation compared to on-board 1 g controls. In contrast, activation of p38 MAP kinase was not altered	Verhaar et al. (2014)
	U937 monocytes	Parabolic flight	Microgravity led to enhanced overall tyrosine phosphorylation and activation of c-jun in non-stimulated U937 cells and to decreased overall tyrosine phosphorylation and reduced activation of c-jun in PMA-stimulated cells	Paulsen et al. (2010)
Monocytes/ differentiation	Bone marrow cells, murine	Spaceflight	In a subpopulation of murine bone marrow cells that contained macrophage-like cells, 13 days of spaceflight led to decreased expression of Ly6C, c-Fos, CD44 (high), and Ly6G and an increased expression of F4/80	Ortega et al. (2009)
	Monocytes/ macrophages	International space station	The expression of genes involved in the differentiation process of monocytes into macrophages is altered in microgravity	Hughes-Fulford et al. (2008)

(continued)

Table 4.1 (continued)

Cell type/focus of investigation	Cell model	Research platform	Findings	Reference
Monocytes, proliferation, and cell cycle control	U937 monocytic cells	RWV	Cell proliferation was reversibly decreased in microgravity	Cotrupi and Maier (2004)
	U937	RWV	Microgravity leads to slower growth, a decreased level of cdc25B, alteration in cytokine secretion, and decreased proteasome activity	Maier (2006)
	THP-1 monocytic cells	RCCS	Microgravity for 24 h leads to a decrease in proliferation and to an inhibition of LPS-induced expression of tissue factor mRNA	Yu et al. (2011)
Monocytes, oxidative burst	RAW 264·7, macrophage cell line, murine	RWV bioreactor	4 days of culture in microgravity and stimulation with LPS/IFN-γ on day 2 led to a decrease in the production of nitric oxide of 65 % and to a decrease of cytokine production (TNF-α, IL-6, IL-12) of 80 % compared to 2D cultured cells	Hsieh et al. (2005)
	NR8383 rat alveolar macrophages	Clinostat (2D), parabolic flight, centrifuge	The release of ROS was decreased by real and simulated microgravity and was increased in hypergravity in a rapidly responding and reversible manner	Adrian et al. (2013)
	NR8383 rat alveolar macrophages	Fast-rotating clinostat	Macrophages that were stimulated with zymosan, curdlan, or lipopolysaccharide produced significantly less ROS when exposed to microgravity compared to normal gravity. Reduced phosphorylation of spleen tyrosine kinase (Syk) was observed. The translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) to the nucleus was not altered	Brungs et al. (2015)
Monocytes/ phagocytosis	Monocytes from blood of astronauts	Four space shuttle missions	Spaceflight (5–11 days) decreased the percentage of phagocytizing monocytes and the phagocytic index of monocytes as measured by the ability to engulf bacteria. The expression of the surface markers CD32 and CD64 was altered	Kaur et al. (2005)

Monocytes/ interleukin secretion	Jurkat T cells and THP-1 monocytes	Biosatellite Cosmos 2044	T lymphocytes and monocytes in co-culture respond to T cell activation with normal secretion of IL-2 and IL-1, respectively, under microgravity. In contrast, if cultured separately and stimulated, IL-2 and IL-1 secretion were reduced in microgravity	Limouse et al. (1991)
	Peripheral blood monocytes	Spacelab	Peripheral blood monocytes responded with a strong decrease in interleukin-1 secretion to weightlessness in an experiment with T lymphocytes and monocytes which were stimulated with ConA	Cogoli et al. (1993)
	Human peripheral blood mononuclear cells	Spaceflight (Biorack facility), clinorotation	During clinorotation and real microgravity, PBMCs reacted with a decrease in IL-2 receptor expression to stimulation with anti-CD-3. Upon stimulation with anti-CD-3 (leading to cell-cell contact between T cells and monocytes), the level of synthesized IL-1 by monocytes stayed unaffected during 24 h clinorotation	Hashemi et al. (1999)
	Monocytes from astronaut blood	Spaceflight	In peripheral monocytes from astronauts, reduced levels of CD62L and HLA-DR were measured after 13–16 days of spaceflight. Cells responded to ex vivo LPS stimulation with decreased expression of IL-6, TNF-α, and IL-10 and increased expression of IL-1b. The expression of IL-8 was found to be regulated in either direction, dependent on the space flight mission	Crucian et al. (2011)
	B6MP102, cultured murine bone marrow macrophage cell line	Spaceflight	In a murine bone marrow macrophage cell line LPS-induced secretion of tumor necrosis factor-α and interleukin-1 was higher compared to controls on earth	Chapes et al. (1994)
	RAW264.7 cells and primary mouse macrophages	RCCS	In mouse macrophages cultured for 24 in microgravity, the expression of LPS-induced TNF-α was markedly decreased compared to 1 g culture. Phosphorylation of IKK and JNK and nuclear translocation of NF-kB as well as TNF-α mRNA stability were not altered, but heat shock factor-1 (HSF1), a repressor of TNF-α promoter, was upregulated	Wang et al. (2014)

(continued)

Table 4.1 (continued)

Cell type/focus of investigation	Cell model	Research platform	Findings	Reference
Monocytes/ cytoskeleton and locomotion	J-111, adherent monocyte cell line	RPM	The ability for locomotion was inhibited, and actin, tubulin and vinculin were altered	Meloni et al. (2006)
	Monocytes J-111	International space station	The ability of monocytes to migrate was decreased in microgravity samples compared to 1 g in-flight and ground controls. The cytoskeletal architecture was markedly disrupted, as the distribution of F-actin, β-tubulin, and vinculin structures was changed. For F-actin fibers, quantitative analysis showed a significant reduction	Meloni et al. (2011)
Macrophages	Primary mouse macrophages	RCCS	Microgravity for 24 h led to increased levels of arginase mRNA and protein levels, enhanced expression of C/EBPβ (a transcription factor which is relevant for arginase transcription), and activation of p38. LPS-stimulated primary mouse macrophages reacted with increased levels of IL-6 and decreased levels of IL-12B	Wang et al. (2015)
NK-Cells	NK cells from human blood, in vitro	International space station and clinostat	24 h of real microgravity and clinorotation did not lead to alterations of cytotoxic activity of NK cells toward target cells. The interferon production of NK cells upon binding of target cells in real microgravity did not alter from that of ground controls	Buravkova et al. (2004)
	NK cells from human blood, ex vivo expanded	RWV	Microgravity for 48 h led to a decrease in cytotoxicity which could be counteracted by IL-15 alone or in combination with IL-12. Increased levels of apoptosis and necrosis and decreased expression levels of IFN-γ and perforin were observed. The NK cell surface receptors NKG2A and NKG2D were expressed at reduced levels after exposure to microgravity; expression of NKp30 and NKp44 was not altered	Li et al. (2013)
	Peripheral blood monocytic cells, human	RWV	Natural killer activity and lymphokine-activated killing of PBMCs upon stimulation with IL-2 were not different in cells exposed to microgravity and 1 g controls. Nevertheless, the stimulation-induced upregulation of IL-2 receptor α chain (CD25) was decreased under microgravity, and the secretion of the secondary cytokines of IFN-γ, IL-1β, and TNF-α was reduced	Licato and Grimm (1999)

	NK cells from astronaut blood	Spaceflight	After a 9-day space mission, the number of NK cells in the blood of astronauts was decreased, while it was unchanged after a 16-day mission	Stowe et al. (2003)
	NK cells from astronaut blood	Spaceflight	After 7 days of spaceflight, NK cells of astronauts displayed a decreased killer activity and decreased induced interferon production on day one after return from space	Talas et al. (1983)
	NK cells from astronaut blood	Spaceflight	In a cosmonaut the ability to bind target cells and the percentage of NK cells were decreased after spaceflight of 21 days	Konstantinova et al. (1995)
	NK cells from astronaut blood	Spaceflight	During four space shuttle missions that lasted 10–18 days, no differences in NK cell percentage was observed in the blood of 27 astronauts	Crucian et al. (2000)
Neutrophil granulocytes, PMNs	*Propionibacterium acnes*-induced peritoneal inflammatory cells	Parabolic flight	Superoxide-anion ($O2^{-}$) production by peritoneal inflammatory cells was increased fourfold by microgravity	Fleming et al. (1991)
	Neutrophil granulocytes from blood of astronauts	Space shuttle flight	After 8–15 days of spaceflight, the number of neutrophil granulocytes in the blood of astronauts was increased by 50 %. In an optimal dose response chemotactic assay, the neutrophil granulocytes showed a tenfold decrease after spaceflight indicating an enhanced chemotactic activity	Stowe et al. (1999)
	Polymorphonuclear leukocytes (PMNs) from the blood of astronauts	Space shuttle flight	The number of PMNs in the blood of astronauts was increased after a 16-day space shuttle mission	Stowe et al. (2003)
	Neutrophil granulocytes from blood of astronauts	Space shuttle flight	After spaceflight of 5–11 days, the number of neutrophil granulocytes in the blood of astronauts increased by 85 %	Kaur et al. (2004)

(continued)

Table 4.1 (continued)

Cell type/focus of investigation	Cell model	Research platform	Findings	Reference
	PMNs from the blood of parabolic flight participants	Parabolic flight	In the blood of volunteers, the number of PMNs was enhanced significantly post flight. Whereas the spontaneous production of hydrogen peroxide was not altered, the capability to produce it upon stimulation with fMLP, fMLP, and TNF-α, calcium ionophore or PMA was markedly increased. No differences in the ability of the PMNs to adhere and to phagocyte were detected. IL-8 and granulocyte colony-stimulating factor (G-CSF) were found to be elevated in the cell plasma	Kaufmann et al. (2009)
	PMNs from the blood of parabolic flight participants	Parabolic flight	In PMNs from participants of parabolic flight, the potency of adenosine to control the release of hydrogen peroxide was significantly increased 48 h after the flight compared to the status before flight. This effect was due to an upregulation of the adenosine A2(A) receptor function	Kaufmann et al. (2011)
Dendritic cells	CD34+progenitor cells from peripheral human blood, generated in vitro into dendritic cells	RCCS	Dendritic cells were generated in static culture or in a RCCS. The latter were less in number, had decreased capability for phagocytosis and a decreased density of HLA-DR on their surface, and were less effective in antigen-induced responses	Savary et al. (2001)
Endothelial cells	Human umbilical vein endothelial cells (HUVEC), primary	RWV bioreactor	Microgravity led to a reversible stimulation of cell growth, an enhanced expression of heat shock protein 70, and a decreased level of IL-1 α. Furthermore remodeling of the cytoskeleton and, after several days, a decrease of actin were observed	Carlsson et al. (2003)
	Human umbilical vein endothelial cells (HUVEC), primary	RWV bioreactor, RPM, Centrifuge (MidiCAR3.5 xg)	Microgravity led to enhanced growth, enhanced NO production, while migration was not affected. Remodeling of the actin cytoskeleton was observed with a decrease of the amount of actin protein. Hypergravity (MidiCAR3.5 xg) for 24–48 h led to enhanced migration and enhanced NO synthesis in HUVEC cells. After 96 h the distribution of actin fibers was altered toward a perinuclear gathering, but the amount of actin protein was not changed	Versari et al. (2007)

	Human umbilical vein endothelial cells (HUVEC), primary	Spaceflight	After 10 days of spaceflight 1023 genes were modulated significantly as compared to 1 g ground controls. Modulated genes are involved in oxidative phosphorylation, cell adhesion, cell cycle, stress response, and apoptosis. The secretion of IL-1α and IL-1β was enhanced, and nitric oxide production was not altered	Versari et al. (2013)
	Human umbilical vein endothelial cells (HUVEC), primary	RWV bioreactor	Cells grew faster than control cells for up to 8 days. They produced more prostacyclin and NO than controls (vasodilators). Production of metalloproteinases was not changed; productions of TIMPs was enhanced	Carlsson et al. (2002)
	Human umbilical vein endothelial cells (HUVEC), primary	RPM, In vivo hind limb suspension	24 or 48 h culture in a RPM led to significantly decreased expression of IL-6 and TNF-α gene expression. The gene expression and the surface presence of ICAM-1, VCAM-1, and E-selectin were significantly decreased. The effects were reversible by addition of mechanical loading during the mechanical unloading period. In vivo hindlimb suspension led to an increased expression in eNOS and caveolin-1 and caveolin-2 in mouse aortas. Mechanical unloading also led to cytoskeletal changes: decreased length and width and disorganization of the F-actin network, and perinuclear clustering of the fibers was observed	Grenon et al. (2013)
	Human umbilical vein endothelial cells (HUVEC), primary	Spaceflight	12 days of spaceflight led to cytoskeletal lesions and increased cell membrane permeability. In readapted cells which were cultivated after retrieval, persisting cytoskeletal changes, decreased cell growth, and decreased metabolism were observed	Kapitonova et al. (2012)
	Human umbilical vein endothelial cells (HUVEC), primary	NASA RCCS, parabolic flight	24 h in the RCCS led to significantly increased surface expression of ICAM-1 compared to 1 g controls in TNF-α-activated cells. In real microgravity during parabolic flight, an upregulation of ICAM-1 was observed already after 20 s as compared with in-flight 1 g controls. Simulated microgravity (5 min -24 h) led to changes in the distribution of F-actin and altered clustering of ICAM as shown by immunocytochemistry. ICAM-1 mRNA expression was enhanced compared to the controls after 30 min and 1 h, while it was equal to the control after 24 h. Similar results were obtained for VCAM-1	Zhang et al. (2010)

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Table 4.1 (continued)

Cell type/focus of investigation	Cell model	Research platform	Findings	Reference
	Human umbilical vein endothelial cells (HUVEC), primary	RPM	2-D proteome analysis of cellular secretome revealed that after 96 h simulated microgravity, the secretion of proteins relevant for the regulation of cytoskeleton assembly was altered. IL-1α and IL-8 (pro-inflammatory) secretion was inhibited; RANTES and Eotaxin (leukocyte recruitment) secretion was increased. Secretion of the pro-angiogenic factor bFGF was decreased	Griffoni et al. (2011)
	Human umbilical vein endothelial cells (HUVEC), primary	Spaceflight, ISS	8 days in real microgravity led to enhanced levels of IL-6, sICAM-1, and e-selectin in cell supernatant indicating endothelial activation. The mRNA expression of IL-6, ICAM, and VCAM-1 in the cells was increased	Muid et al. (2010)
	Human umbilical vein endothelial cells (HUVEC), primary	Clinorotation (2D)	In TNF-α stimulated cells 18 h of microgravity led to an increase of ICAM-1 expression but a decrease of e-selectin and VCAM-1. In non-stimulated cells exposure to microgravity also enhanced the expression of ICAM-1 and had no effect on e-selectin and VCAM-1. In a co-culture of HUVECs and lymphocytes, the adhesion of phorbol ester-stimulated lymphocytes to endothelial cells was enhanced by 18 h of clinorotation, while the adhesion of non-stimulated lymphocytes was not altered or even slightly lower	Buravkova et al. (2005)
	Human umbilical vein endothelial cells (HUVEC), primary	Centrifuge, 3 g	In HUVECs hypergravity (3 g) for 48 h led to a shift in the cell cycle distribution toward the G(0)/G(1) phase. Calveolin1 gene expression was enhanced and intracellular distribution of caveolae was increased. COX-2 expression, NO production, and prostacyclin (PGI2) production were upregulated	Spinsi et al. (2003)

	EA.hy926, cell line	RPM	Microgravity led to increase of collagen types I and III. After an initial upregulation with a maximum after 10 min, the expression levels of osteopontin and TGF-β1 had declined to the level of the controls at day 10. After 10 days of microgravity Caspase-3 protein content was higher, and the number of Bax and Bcl-2 proteins positive cells was increased, whereas fewer cells were positive for Fas protein. Microgravity-induced cytoskeletal alterations included changes in α- and β-tubulins and F-actin fibers. Secretion of the soluble factors neurotrophic factor, ET-1, tissue factor, and VEGF was decreased in microgravity after 10 days of microgravity	Infanger et al. (2007)
	EA.hy926, cell line	Parabolic flight, centrifuge	Differential regulation in gene expression of 320 genes was observed upon microgravity, partly up and partly downregulated. The cytoskeletal element β-tubulin underwent cytoplasmic rearrangement Hypergravity (1.8 g) reduced CARD8, NOS3, VASH1, SERPINH1, CAV2, ADAM19, TNFRSF12A, CD40, and ITGA6 mRNAs	Grosse et al. (2012)
	EA.hy926, cell line	RPM	After 5 days of simulated microgravity, genomic analysis revealed differentially regulated genes that are involved in the processes of signal transduction, are angiogenic factors, have a role in cell adhesion, membrane transport, or are enzymes involved in serine biosynthesis	Ma et al. (2013)
	EA.hy926, cell line	RPM	Cells responded with an increase in extracellular matrix proteins and changes in microtubules and intermediate filaments to microgravity. After 4 h of microgravity, and more pronounced after 72 h, morphological and biochemical signs of apoptosis were detected. Induction of apoptosis could be attenuated by the addition of VEGF	Infanger et al. (2006)

(continued)

Table 4.1 (continued)

Cell type/focus of investigation	Cell model	Research platform	Findings	Reference
	EA.hy926, cell line	Parabolic flight, centrifuge	Cells reacted with altered gene expression to microgravity. Regulated genes were involved in angiogenesis, cytoskeleton, extracellular matrix, cell cycle regulation, and apoptosis. Hypergravity in a centrifuge led to downregulation of Pan-actin, tubulin, and moesin proteins. Additionally many genes were up- or downregulated by hypergravity	Wehland et al. (2013)
	Porcine aortic ECs (PAECs)	RPM	Microgravity for 72 h led to a decreased cell number, an upregulation in the expression of proapoptotic genes, and a decrease in the expression of antiapoptotic and proliferative/survival genes. Changes of nucleus shape and dissolution of intracellular organelles was assessed by autofluorescence analysis. Cells lost their ability to respond to angiogenic stimuli	Morbidelli et al. (2005)
	Murine lung capillary endothelial cells (1G11 cells)	RWV bioreactor	Microgravity led to reversible inhibition of cell growth. After 72 h, cells exhibited an upregulation of p21, a decreased synthesis of IL-6, and an increased amount of NO	Cotrupi et al. (2005)
	Human pulmonary microvascular endothelial cells	Clinorotation (2D)	72 h of clinorotation led to a disappearance of desmosome-like junctions and induction of apoptosis (TUNEL-staining, upregulation of BAX and caspase-3 genes, downregulation of Bcl-2 gene, increased protein expression of caspase-3 and caspase-9, decreased protein expression of PI3K and p-Akt). Disruption of actin filament integrity was observed	Kang et al. (2011)
	Bovine aortic ECs (BAEC)	Centrifuge	Exposure to discontinuous hypergravity leads to changes is integrin distribution, cytoskeletal network reorganization, downregulation of proapoptotic signals, and reduced expression of genes involved in inflammation and vasoconstriction	Morbidelli et al. (2009)

	Bovine aortic ECs (BAEC)	Centrifuge	In bovine aortic ECs (BAECs) centrifugation at 3 g led to a transient reorganization of actin fibers in 3 min which was abolished by inhibitors of Rho-kinase and tyrosine kinase. 3 g hypergravity for 1 or 2 h/day enhanced endothelial migration in 5 days	Koyama et al. (2009)
	Bovine aortic ECs (BAEC)	RWV bioreactor	Cells growing as a three-dimensional culture on microcarrier beads for up to 30 days responded to microgravity with enhanced NO production (dependent on the rotation speed). Furthermore a decreased permeability of the cell layer was observed, accompanied with increased expression of the junctional complex proteins ZO-1 and occludin, indicating an enhanced barrier function of the endothelial layer	Sanford et al. (2002)
	Coronary venular ECs	Centrifuge	5 periods of 10 min exposure to 10 g separated by 10 min of recovery at 1 g led to downregulation of proapoptotic genes FADD, Fas, and FAS-L; upregulation of NF-kB; upregulation of genes for cytoskeletal proteins β-actin, α-tubulin, and vimentin; and altered cytoskeletal organization in coronary venular ECs	Monici et al. (2006)

4.1 Monocyte/Macrophage System

Cytokine Secretion As one of the central effector functions of monocytic and macrophageal cells, the secretion of cytokines under the influence of gravitational changes was investigated in numerous studies. Monocytes and T cells in co-culture respond to T cell activation with normal secretion of interleukin (IL)-2 and IL-1, respectively, under real microgravity on a Biosatellite (Limouse et al. 1991). Likewise upon stimulation of peripheral blood monocytic cells (PBMCs) with anti-CD-3 (leading to cell-cell contact between T cells and monocytes), the level of synthesized IL-1 by monocytes stayed unaffected in clinorotation (Hashemi et al. 1999). In contrast, if T cells and monocytes are cultured and stimulated separately, IL-2 and IL-1 secretion was reduced in microgravity (Limouse et al. 1991). Likewise, during an experiment in Spacelab ConA-stimulated peripheral blood monocytes responded with a strong decrease in IL-1 secretion to weightlessness (Cogoli et al. 1993), and in an experiment on a space shuttle flight, the synthesis of IL-1 β by U937 cells was decreased compared to controls (Schmitt et al. 1996). But there are also reports about upregulation of IL-1 secretion upon microgravity exposure in in vitro experiments: in the murine bone marrow macrophage cell line B6MP102, the lipopolysaccharide (LPS)-induced secretion of IL-1 and tumor necrosis factor-α (TNF-α) was higher during spaceflight compared to controls on earth (Chapes et al. 1994). In an ex vivo experiment with peripheral monocytes from astronauts taken after 13–16 days of spaceflight, cells responded to LPS stimulation also with an increased expression of IL-1β (Crucian et al. 2011). In the same study the expression of other cytokines was assessed revealing a decreased expression of IL-6, TNF-α, and IL-10. Expression of IL-8 was found to be regulated in either direction, dependent on the space flight mission (Crucian et al. 2011). Similar results were obtained in a study using a ground-based model of microgravity; in murine macrophageal cells that were cultured in a 3D rotating wall vessel (RWV) for 2 days followed by stimulation with LPS/interferon(IFN)-γ and cultivated for another 2 days, the production of TNF-α, IL-6, and IL-12 was decreased significantly compared to 2D cultured cells (Hsieh et al. 2005). In mouse macrophages cultured for 24 h in a rotary cell culture system (RCCS), the expression of LPS-induced TNF-α was markedly decreased compared to 1 g culture. Phosphorylation of IKK and JNK and nuclear translocation of NF-kB (processes of LPS-induced intracellular signal transduction) as well as TNF-α mRNA stability were not altered upon microgravity, but microgravity led to an upregulation of heat shock factor-1 (HSF1), a repressor of TNF-α promoter (Wang et al. 2014). In a later study under the same conditions, increased levels of IL-6 and decreased levels of IL-12B were measured (Wang et al. 2015). Additionally an increased level of arginase mRNA and protein levels and enhanced expression of C/EBPβ (a transcription factor which is relevant for arginase transcription) were reported (Wang et al. 2015). Taken together the secretion of cytokines is sensitive to real and simulated microgravity, and the nature of the changes is highly dependent on the microenvironment surrounding the cells.

Intracellular Signal Transduction Protein kinase C (PKC) is a central element of many signal transduction cascades. Upon activation, PKC is translocated into different cellular compartments where it transmits the signal by phosphorylation

of other proteins. Several studies provide evidence that PKC signaling is sensitive to altered gravity conditions. In an experiment on a space shuttle flight, it was observed that the subcellular distribution of PKC in U937 was sensitive to gravitational changes: with increasing acceleration, the level of PKC in the nuclear fraction decreased, while the level of PKC in the cytosolic fraction increased (Schmitt et al. 1996). The translocation of PKC from the cytosol to the particulate fractions of U937 cells upon stimulation with phorbol ester happened with an altered kinetic during space flight as compared to controls on the ground. Additionally the binding of a phorbol ester to PKC occurred to an enhanced extend in-flight, while hypergravity (1.4 g) leads to a decreased level of binding (Hatton et al. 1999). Experiments investigating the influence of microgravity on different isoforms of PKC revealed that phorbol ester-induced translocation of PKC beta II, delta, and epsilon to the particulate fraction was decreased during spaceflight compared to 1 g and was increased by 1.4 g. These alterations of PKC signaling have been discussed to be involved in the various changes in gene expression and cell functions induced by altered gravitational conditions (Hatton et al. 2002). In LPS-stimulated peripheral blood monocytes, real microgravity led to an impairment of Jun-N-terminal kinase activation compared to on-board 1 g controls. In contrast, activation of p38 MAP kinase was not altered (Verhaar et al. 2014). In another study simulated microgravity in a RCCS for 24 h led to activation of p38 MAPK in primary mouse (Wang et al. 2015). Weightlessness during parabolic flight led to enhanced overall tyrosine phosphorylation and activation of c-jun in non-stimulated monocytic U937 cells but to decreased overall tyrosine phosphorylation and reduced activation of c-jun in phorbol myristate acetate (PMA)-stimulated monocytic U937 (Paulsen et al. 2010). Thus, the effects of gravitational changes seem to be specific with respect to different signaling pathways and the activation status of the cells.

Phagocytosis In monocytes isolated ex vivo from the blood of astronauts after the return from spaceflight, the capacity to phagocytose was investigated. Spaceflight of 5–11 days decreased the percentage of phagocytosing monocytes and their phagocytic index as measured by the ability to engulf bacteria (Kaur et al. 2005).

Cytoskeleton and Locomotion In many cell types changes in the cytoskeleton upon exposure to microgravity have been observed, for example, in T lymphocytes and osteoblasts (Schatten et al. 2001; Hughes-Fulford 1991). Likewise, gravisensitivity of the cytoskeleton could be shown in macrophageal cells: in the monocytic cell line J-111, the cytoskeletal proteins actin, tubulin, and vinculin were altered in simulated microgravity in a random positioning machine (RPM) (Meloni et al. 2006). This effect could be affirmed in real microgravity when in experiments on the ISS, the cytoskeletal architecture of J-111 monocytes appeared to be markedly disrupted, as the distribution of F-actin, β-tubulin, and vinculin structures were severely changed under microgravity. Additionally a quantitative analysis showed a significant reduction of F-actin fibers. Possibly as a consequence of this disruption of the cytoskeleton, the ability of the cells to migrate was markedly decreased in microgravity samples compared to 1 g in-flight and ground controls (Meloni et al. 2011). Other experimental results point to a gravisensitivity of the adhesion-associated molecules CD62L and intercellular adhesion molecule 1 (ICAM-1) (Crucian et al. 2011; Paulsen et al. 2015).

Oxidative Burst A central effector function of macrophages is the oxidative burst, the release of reactive oxygen species (ROS) that dispose pathogens. Phagocytes and the NADPH oxidase enzyme-triggered oxidative burst reactions are part of the ancient innate immune system and represent the most important barrier for microbes invading the body. In murine macrophageal cells that were cultured under simulated microgravity in a 3D RWV for 4 days and stimulated with LPS/IFN-γ on day 2, the production of nitric oxide (NO) was decreased by 65 % (Hsieh et al. 2005). Using an elegant in vitro experimental setup that enables live measurement of ROS release, it was shown that the release of ROS during the oxidative burst by NR8383 rat macrophages was strongly decreased during parabolic flight and 2D clinorotation and was increased in hypergravity (centrifuge). These effects happen rapidly after alterations of the gravitational condition and are reversible (Adrian et al. 2013). When NR8383 macrophages were exposed to simulated microgravity in a fast rotating clinostat and stimulated with zymosan, curdlan, or LPS, they produced significantly less ROS compared to the samples with normal gravity. Furthermore the phosphorylation of spleen tyrosine kinase (Syk), as an early signaling step required for ROS production, was decreased in the clinorotated samples. At the same time, a later step in ROS production, the translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) to the nucleus, was not altered by simulated microgravity (Brungs et al. 2015). The TRIPLE LUX A ISS experiment (uploaded with SpaceX CRS-6, BIOLAB/COLUMBUS) provided direct evidence that ROS release is highly sensitive to altered gravity, is adapting very fast to an altered gravitational environment, and reacts within a certain range of gravitational forces (Thiel and Ullrich 2015).

Differentiation/Bone Marrow Cell Phenotype One study investigated the effect of real microgravity on the differentiation process of macrophageal cells in bone marrow. In a subpopulation of murine bone marrow cells that contained very granular macrophage-like cells, 13 days of spaceflight led to decreased expression of Ly6C, c-Fos, CD44(high), and Ly6G and an increased expression of F4/80, suggesting that spaceflight leads to an enhanced differentiation compared to ground controls (Ortega et al. 2009). Another study revealed that the expression of genes involved in the differentiation process of monocytes into macrophages is altered by real microgravity (Hughes-Fulford et al. 2008).

Proliferation and Cell Cycle Control Monocytic cells react to ground-based simulated microgravity with decreased proliferation and changes in cell cycle control: a decrease of proliferation was shown in U937 in RWV bioreactor, accompanied by a decreased level of cdc25B (Maier 2006; Cotrupi and Maier 2004). THP-1 cells that were cultured in a RCCS for 24 h also proliferated less than controls. Additionally the percentage of cells in the G0-Gl phase increased, and an inhibition of LPS-induced expression of tissue factor mRNA, a phosphatase involved in cell cycle control, was observed (Yu et al. 2011).

4.2 Natural Killer Cells

Natural killer (NK) cells belong to the cytotoxic lymphocytes and constitute an important link between the adaptive and the innate immune systems. They eliminate cells that are infected by viruses and cells that show tumorous features and thus play an important role in the control of inflammatory processes and tumor surveillance.

Early investigations of the effect of spaceflight on NK cells have revealed that after a 7-day space mission, the NK cells of astronauts displayed decreased killer activity and decreased inducible interferon production on the first day after return from the space mission (Talas et al. 1983). Similar results were obtained with NK cells from an astronaut after a 21-day mission; the cytotoxicity, the ability to bind and lyse target cells, and the percentage of NK cells were decreased after spaceflight (Konstantinova et al. 1995). Decreased numbers of NK cells were confirmed in further missions, and a dependence on the mission duration was suggested. After a 9-day mission, the number of NK cells was decreased, while it was unchanged after a 16-day mission (Stowe et al. 2003). In another study the percentages of NK cells were unchanged after space flight in four space shuttle missions that lasted 10–18 days (Crucian et al. 2000).

Subsequent in vitro experiments with NK cells exposed to real or simulated microgravity lead to partly conflicting results. In studies by Buravkova et al., NK cell exposure to real microgravity during a space mission and to simulated microgravity induced by clinorotation did not result in any alterations of cytotoxic activity toward target cells. Also the interferon production of NK cells upon binding of target cells in real microgravity did not differ from that of ground controls (Buravkova et al. 2004). The authors reason that immune cells do not lose their ability to bind to, recognize, and destroy target cells in vitro in microgravity (Buravkova et al. 2005). In contrast, evidence of a sensitivity of NK cells to microgravity in vitro was found in experiments using the RWV bioreactor as source of simulated microgravity. In these experiments IL-2 induced NK activity and lymphokine-activated killing of PBMCs were not different in cells under simulated microgravity and 1 g controls, but the upregulation of IL-2 receptor α chain (CD25) was decreased under simulated microgravity conditions. Furthermore, the secretion of the secondary cytokines IFN-γ, IL-1 β, and TNF-α was reduced. This suggests that in NK cells IL-2 pathways are differentially regulated in RWV-induced microgravity (Licato and Grimm 1999).

In 2013 Li et al. found that the cytotoxicity of NK cells was decreased upon exposure to simulated microgravity in a 2-D RWV. This effects could be counteracted by (IL)-15 alone or in combination with IL-12. Simulated microgravity led to increased levels of apoptosis and necrosis, and the expression of interferon (IFN)-γ and perforin was decreased. Among the surface receptors of the NK cells, NKG2A and NKG2D were expressed at a reduced level after exposure to simulated microgravity, while expression of NKp30 and NKp44 was not altered (Li et al. 2013).

4.3 Neutrophil Granulocytes/Polymorphonuclear Leukocytes

As the "first-line of defense," granulocytes protect the body from invading pathogens. They bind microorganisms, internalize them, and neutralize them with ROS in the phagosome, or they secrete ROS to kill pathogens extracellularly.

Several studies have shown that the number of neutrophil granulocytes is strongly increased in the blood of astronauts that have attended space flight for 5–16 days (Stowe et al. 1999, 2003; Kaur et al. 2004). The same effect has been observed in the blood of volunteers that were exposed to short-term microgravity during parabolic flight (Kaufmann et al. 2009).

Additionally to the number, also some effector functions of granulocytes have been reported to be affected by microgravity: in an in vitro experiment with murine *Propionibacterium acnes*-induced peritoneal inflammatory cells, the superoxide-anion production was found to be increased fourfold by microgravity during parabolic flight (Fleming et al. 1991). PMNs from the blood of individuals that had undergone parabolic flight showed an increased capability to produce hydrogen peroxide (H_2O_2) upon stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP), fMLP and TNF-α, calcium ionophore (A23187), and PMA. Nevertheless, the spontaneous production of (H_2O_2) was not altered in that experiment (Kaufmann et al. 2009). Analysis of the regulatory mechanism of H_2O_2 release revealed that after parabolic flight, adenosine was more effective in controlling the release of cytotoxic H_2O_2 by primed PMNs and that this was due to an upregulation of the adenosine A2(A) receptor function (Kaufmann et al. 2011). After 8–11 days of spaceflight, granulocytes from the blood of astronauts reacted with a tenfold decrease in an optimal dose response chemotactic assay, indicating an enhanced chemotactic activity (Stowe et al. 1999).

Data regarding the effect of microgravity on further effector functions is scarce. In PMNs from blood of participants of parabolic flight, no differences in the ability to adhere and to phagocyte were detected (Kaufmann et al. 2009).

4.4 Dendritic Cells

Effects of microgravity on dendritic cells are largely unexplored. One study revealed that simulated microgravity affects the in vitro generation of dendritic cells: dendritic cells were generated from CD34+ progenitor cells from peripheral human blood in either a RCCS or in a static culture. Dendritic cell from the RCCS were less in number compared to the static culture; furthermore they had decreased capability for phagocytosis and a decreased density of HLA-DR on their surface and were less effective in antigen-induced responses (Savary et al. 2001).

4.5 Endothelial Cells

The effects of altered gravity on endothelial cells (ECs) are a broad field of research as endothelial functions are severely involved in cardiovascular homeostasis which is disturbed during spaceflight (Convertino 2009). Studies of the effect of altered gravity on many functions of ECs have been conducted. Here we will focus on the properties of ECs which are most directly connected to immunity, namely, the adhesive interaction with leukocytes, the cellular junctions and barrier function, the secretion of soluble substances relevant for the immune response, and gene expression. Additionally, the effects of altered gravity on the cytoskeleton of ECs are addressed. Integrity of the cytoskeleton is a prerequisite for almost all cellular functions as the cytoskeletal network is required not only to sustain the cellular morphology but also as a scaffold for transport of molecules inside the cell, for signaling processes, and for adhesion and migration.

Effects of altered gravity on proliferation, migration, and apoptosis are reviewed elsewhere (Maier et al. 2015).

Adhesion Adhesion of lymphocytes to the endothelium is a central step in immune reaction against intruding pathogens. During inflammation, adhesion is enabled by adjustment of the surface expression of adhesion molecules. Many reports describe the expression of adhesion molecules under altered gravity, while in only a few studies, adhesion could be examined on the functional level.

The data on the influence of simulated microgravity on human umbilical vein endothelial cells (HUVECs) are not very consistent. Grenon et al. found out that the gene expression and the surface presence of ICAM-1, VCAM-1, and e-selectin were significantly decreased after 24 or 48 h mechanical unloading in a RWV. These effects were reversible by addition of mechanical loading during the mechanical unload period (Grenon et al. 2013). In contrast, in a study using the RCCS (24 h), TNF-α-activated HUVECs reacted with significantly increased surface expression of ICAM-1 compared to 1 g controls. ICAM-1 mRNA expression was enhanced compared to the controls after 30 min and 1 h, while it was equal to the control after 24 h. Similar results were obtained for VCAM-1 (Zhang et al. 2010). The effect of microgravity seems to be dependent on the activation state of the cells and can be specific for different adhesion molecules as shown by Buravkova et al: in TNF-α stimulated HUVEC cells exposure to clinorotation for 18 h led to an increase of ICAM-1 expression but a decrease of e-selectin and VCAM-1. In non-stimulated cells exposure to microgravity also enhanced the expression of ICAM-1 but had no effect on e-selectin and VCAM-1 (Buravkova et al. 2005). In the same experiment functional investigation of the adhesion of lymphocytes to endothelial cells revealed that in a co-culture, the adhesion of phorbol ester-stimulated lymphocytes to HUVECs was enhanced by 18 h of clinorotation, while the adhesion of non-stimulated lymphocytes was not altered or was even slightly lower (Buravkova et al. 2005).

In an experiment in real microgravity on the ISS (8 days), HUVECs reacted with enhanced levels of IL-6, sICAM-1, and e-selectin in the culture supernatant and enhanced mRNA expression of IL-6, ICAM, and VCAM-1 indicating endothelial activation (Muid et al. 2010). During parabolic flight 20 s of real microgravity leads to an upregulation of ICAM-1 expression in HUVECs (Zhang et al. 2010). Wide-ranging genomic studies confirmed the regulation of genes involved in adhesion after 10 days of spaceflight in HUVECs (Versari et al. 2013) and after 5 days in a RPM in EA.hy926 (Ma et al. 2013).

Taken together, both promoting and decreasing effects of microgravity on adhesion molecules are found, making it difficult to predict the effect on adhesion. Nevertheless, the only functional study shows enhanced adhesion under microgravity conditions.

One study suggests that also hypergravity impacts adhesion properties of ECs: exposure of bovine aortic endothelial cells (BAECs) to discontinuous hypergravity (5×10 min at $10 \times g$ separated by 10 min at $1 \times g$) in a centrifuge leads to changes in integrin distribution, while adhesion itself was not affected (Morbidelli et al. 2009).

Cellular Junctions and Barrier Function Endothelial layers build a selective barrier for molecules, particles, and cells. Tight junction complexes between the cells of an endothelial layer strongly influence vascular permeability and leukocyte extravasation (Wallez and Huber 2008; Aghajanian et al. 2008). Unfortunately data is rare on the influence of altered gravity on the cellular junctions and the permeability of endothelial layers.

Sanford et al. investigated the functionality of the endothelial barrier in BAECs growing as a three-dimensional culture on microcarrier beads. Simulated microgravity in a RWV for up to 30 days led to a decreased permeability of the cell layer as assessed by measuring the transendothelial passage of particles. In accordance with this, the expression of ZO-1 and occludin, two junctional complex proteins that are located at tight junctions, was increased. These effects indicate an enhanced barrier function (Sanford et al. 2002). Kang et al. observed disappearance of junctions between pulmonary microvascular ECs upon 72 h of clinorotation, which would theoretically lead to decreased barrier function. But the disappearance of junctions was accompanied by the induction of apoptosis and might not be happening in viable ECs (Kang et al. 2011).

Release of Nitric Oxide (NO) NO is produced by ECs and immune cells. Additionally to its function as a toxic defense molecule, NO acts as a mediator of inflammatory responses. In NK cells, it is necessary for the cytotoxic activity and for responsiveness to IL-12 (Bogdan et al. 2000), and it influences the functional activity of other cell types including macrophages, neutrophils, and mast cells (Coleman 2001). NO production by ECs was repeatedly shown to be upregulated in HUVEC under simulated microgravity in a RWV and in a RPM (Versari et al. 2007; Carlsson et al. 2002). This upregulation was confirmed in two other types, BAECs and primary murine lung capillary ECs, which were

incubated in a RWV for several days (Sanford et al. 2002; Cotrupi et al. 2005). Nevertheless in a spaceflight experiment, NO production by HUVECs was found to be not altered after 10 days of microgravity (Versari et al. 2013). Interestingly hypergravity seems to cause also an upregulation of NO synthesis, as shown in two independent experiments with HUVECs: 5 g (MidiCAR) for 24 and 48 h and 3 g for 48 h led to an enhanced production of NO (Versari et al. 2007; Spisni et al. 2003).

Cytokines as Mediators of Inflammation Many studies document a downregulation of pro-inflammatory cytokines upon simulated microgravity. Incubation in a RWV for 24 or 48 h led to significantly decreased expression of IL-6 and TNF-α gene expression in HUVEC cells. The effects were reversible by addition of mechanical loading during the mechanical unloading period (Grenon et al. 2013). In an experiment using the same microgravity platform and the same cell type enhanced expression of heat shock protein 70, and a decreased level of IL-1 α was observed (Carlsson et al. 2003). Decreased synthesis of IL-6 was also observed in primary murine lung capillary ECs after 72 h in a RWV bioreactor (Cotrupi et al. 2005). In HUVECs a 2-D proteome analysis of cellular secretome revealed inhibited secretion of IL-1α and IL-8, while RANTES and eotaxin (leukocyte recruitment) secretion was increased after 96 h in a random positioning machine (Griffoni et al. 2011). In contrast to these rather anti-inflammatory effects, in real microgravity during a 10-day spaceflight, secretion of IL-1α and IL-1β was enhanced in HUVECs (Versari et al. 2013). In a study investigating other soluble factors, secretion of neurotrophic factor, ET-1, tissue factor, and VEGF was shown to be decreased after 10 days in a RPM in EA.hy926 cells (Infanger et al. 2007).

Gene Expression The influence of altered gravity on ECs becomes apparent also in the modulation of gene expression. Such alterations were observed in EA.hy926 cells as an effect of 5 days of simulated microgravity in a RPM and applied to genes that encode for angiogenic factors, enzymes that have a role in serine biosynthesis or are involved in the processes of signal transduction, cell adhesion, or membrane transport (Ma et al. 2013). In the same cell type, real microgravity during parabolic flight leads to altered expression of genes involved in angiogenesis, cytoskeleton, extracellular matrix, cell cycle regulation, and apoptosis (Wehland et al. 2013). In HUVECs, 10 days of spaceflight resulted in a significant modulation of genes that are involved in oxidative phosphorylation, cell adhesion, cell cycle, apoptosis, and stress response as compared to 1 g ground controls (Versari et al. 2013). Effects on gene expression were also observed upon hypergravity: exposure of BAECs to discontinuous hypergravity leads to a decrease in the expression of genes involved in inflammation and vasoconstriction (Morbidelli et al. 2009). In EA.hy926 cells hypergravity provided by a centrifuge led to reduced levels of CARD8, NOS3, VASH1, SERPINH1, CAV2, ADAM19, TNFRSF12A, CD40, and ITGA6 mRNAs (Grosse et al. 2012). In a similar study also up- or downregulation of numerous genes was shown (Wehland et al. 2013).

Cytoskeleton Today it is well accepted that the cytoskeleton is central to mechanosensation of physical parameters such as pressure and shear stress, allowing the cell to conduct physical into biochemical signals. It is also discussed to be the primary gravisensitive structure of mammalian cells.

In several independent studies, the effect of simulated microgravity provided by a RWV on HUVECs was assessed. Simulated microgravity led to remodeling of the actin cytoskeleton and, after several days, to a decreased amount of actin protein (Carlsson et al. 2003), Versari 2007 (Versari et al. 2007). In similar experimental setups, the disorganization of the F-actin fibers after 24 and 48 h simulated microgravity could be specified as perinuclear clustering (Grenon et al. 2013), and concomitant changes in clustering of ICAM were observed (Zhang et al. 2010). Griffoni et al. could show with a 2-D proteome analysis of the secretome that after 96 h in a RPM, the secretion of proteins relevant for the regulation of cytoskeleton assembly was altered (Griffoni et al. 2011). Experiments with EA.hy926 cells brought similar results: simulated microgravity in a RPM led to cytoskeletal alterations including changes in α- and β-tubulins, F-actin fibers, microtubules, and intermediate filaments (Infanger et al. 2006, 2007). After 72 h clinorotation, disruption of actin filament integrity was observed in human pulmonary microvascular ECs (Kang et al. 2011).

In only a few studies, the effect of real microgravity on the cytoskeletal architecture was investigated: during parabolic flight, β-tubulin underwent rearrangement and accumulated around the nucleus in EA.hy926 cells (Grosse et al. 2012). After 12 days of spaceflight, cytoskeletal lesions in HUVECs were observed. In the same study readapted cells which were cultivated after retrieval showed cytoskeletal changes that persisted for up to nine passages (Kapitonova et al. 2012).

Less data exists on the effects of hypergravity on the cytoskeleton of ECs. Nevertheless strong effects have been shown which can occur within minutes, and interestingly they are similar to the ones induced by microgravity: after long-term hypergravity of 96 h (3.5 g) in a MidiCAR centrifuge, actin fibers of HUVECs exhibited an altered distribution and tended to gather around the nucleus (Versari et al. 2007). Short-term hypergravity in BAECs (centrifugation at 3 g for 3 min) led to a transient reorganization of actin fibers mediated by RhoA activation and FAK phosphorylation (Koyama et al. 2009).

Exposure of the same cell type to discontinuous hypergravity of 5×10 min at $10 \times g$ separated by 10 min at $1 \times g$ also led to cytoskeletal network reorganization (Morbidelli et al. 2009). The same experiment was performed with coronary venular ECs as an EC cell type from the venular part of the vascular system. In this cell type also cytoskeletal network reorganization was seen, additionally upregulation of genes for the cytoskeletal proteins β-actin, α-tubulin, and vimentin was found (Monici et al. 2006). In EA.hy926 cells hypergravity led to downregulation of Pan-actin, tubulin, and moesin protein (Wehland et al. 2013).

Taken together effects of altered gravity on the cytoskeleton have been observed at the levels of fibers, proteins, regulators, and genes. Although the observations might not always be consistent, the fact that effects have been observed throughout

all used experimental platforms and investigated cell types leads to the assumption that the cytoskeleton is highly sensitive to altered gravity-induced changes.

4.6 Summary

In numerous in vitro and in vivo studies, strong and specific effects of micro- and hypergravity on cells of the immune system and on endothelial cells were revealed. Among the cells of innate immunity, the monocyte/macrophage system is the best studied. An altered gravitational environment has effects on cytokine secretion, intracellular signal transduction processes, phagocytosis, cell migration, cell differentiation, and cell proliferation in the monocyte/macrophage system. Some studies investigated the effect of altered gravity on NK cells, granulocytes, and dendritic cells and reported evidences of altered NK cell activity, cytokine secretion, apoptosis, expression of surface receptors, and the release of hydrogen peroxide. Endothelial cells have well-known mechanosensory properties and gravity-sensitive effects, such as different regulations of adhesion molecules, increased release of nitric oxide, and downregulation of pro-inflammatory cytokines, and different profiles of gene expression have been reported. Therefore, the gravitational environment influences not only basal cellular processes such as cell cycle control but also specific effector functions such as cytokine secretion, oxidative burst, and surface receptor patterns. In general, for all cell systems investigated, there is a lack of functional in vitro studies which could significantly contribute to integrate the knowledge about different effects at the cellular and molecular level. Finally, rearrangement and reorganization of cytoskeletal structures were found in lymphocytes, in macrophages, and in dendritic cells throughout different microgravity platforms. These cytoskeletal changes could contribute to all kinds of pathological conditions observed during altered gravity. Moreover, as the cytoskeleton transduces mechanical stimuli into biochemical signals, it is considered as the primary gravity-responsive element in mammalian cells.

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Chapter 5
Cellular Effects of Altered Gravity on the Human Adaptive Immune System

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5.1 Introduction

During the evolution of life, as the always predominant factor, the Earth's gravitational field has shaped the architecture of all biological systems decisively. It is therefore not surprising that sudden changes in gravity lead to discrepancies in the normal functions of life and of our immune system [see Chap. 1].

According to current knowledge, residence in microgravity strongly influences the human body and leads to a variety of deconditioning symptoms such as bone demineralization, muscle atrophy, reduced performance of the cardiovascular system, altered neurovestibular perception, and a strong deterioration of the immune system (Moore et al. 1996) [see also Chap. 3]. In brief, astronauts showed immune system depression, reduced activation of T lymphocytes, and reactivation of latent

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viruses. (Kimzey 1977; Sonnenfeld and Shearer 2002; Stowe et al. 2001; Mehta et al. 2014).

However, the immune system is not only responsible to defend infections, it is also essential for wound healing, tissue reorganization, and repair. Thus, there are serious reasons to believe that astronauts are exposed to high risk by not only being more susceptible to infections, but also by having poor wound healing and tissue repair. As plans emerge for humans to embark on long-term spaceflights to Mars, Moon, and asteroids in the future, the health risks of a defective regulation of the immunity during spaceflight are important to comprehend. Therefore, the understanding of how gravity affects immune cell function is the key to maintain a proper immune system of astronauts. But which cellular and molecular structures may require gravity for proper function and are thus dependent on the gravity on Earth?

Since the pioneering discovery of Cogoli et al. during the first Spacelab-Mission in 1983, it is well known that proliferative response of lymphocytes to mitogenic stimulation is suppressed in microgravity (Cogoli et al. 1984; Cogoli 1996). Follow-up experiments performed to verify these results demonstrated clearly that factors other than microgravity can be excluded to be responsible for the depressed activation of lymphocytes. Whereas the phenomenological effect of reduced activation of T cells in microgravity is well described and verified (Grove et al. 1995), the fundamental molecular mechanisms remain to be discovered.

For more than 30 years, in vitro experiments with isolated T lymphocytes, the key cell type of the adaptive immune system, have been performed using different research platforms that provide real and simulated microgravity. These experiments have confirmed the effects of altered gravity on the cellular level. Thus, isolated lymphocytes prove to be a suitable biological model system for studying whether and how Earth's gravity is mandatory for cellular and molecular processes in mammalian cells. Numerous experiments in real microgravity of different length have been carried out during manned space missions, on board of orbital and suborbital flights (sounding rockets), and during parabolic flights. Studies using ground-based facilities with the aim to simulate the state of microgravity have supported these experiments in real microgravity. These facilities include fast-rotating clinostats, rotating wall vessel (RWV) bioreactors, random positioning machines (RPMs), and high-aspect ratio vessels (HARVs). As their results are comparable with results of experiments in real microgravity, RWVs and clinostats are recognized and valuable tools for simulating microgravity in suspension cell cultures (Herranz et al. 2013).

These studies in real and simulated microgravity were able to achieve new insights into gravity-sensitive functions in nonactivated and activated T lymphocytes, for example, cell cycle regulation (Thiel et al. 2012), epigenetic regulation (Singh et al. 2010), chromatin modification (Paulsen et al. 2010), differential gene expression (Chang et al. 2012; Thiel et al. 2012), altered microRNA expression profile (Mangala et al. 2011; Girardi et al. 2014; Hughes-Fulford et al. 2015), cell motility (Pellis et al. 1997; Sundaresan et al. 2002), and regulation of programmed cell death (Cubano and Lewis 2000; Lewis et al. 1998; Battista et al. 2012). Also the secretion of cytokines such as interleukin-2 (IL-2) and interferon-gamma (IFNγ) is influenced by gravitational changes (Hashemi et al. 1999; Chapes et al. 1992).

This chapter provides an overview of the results obtained over the last 30 years in in vitro experiments using T lymphocytes performed in space and in ground-based facilities with special emphasis on the used cell culture conditions. These results contribute to our current knowledge of how gravitational changes affect human T lymphocytes in vitro.

5.1.1 Regulation of T Lymphocytes in Real and Simulated Microgravity Experiments In Vitro

Up to now, several in vitro studies have been carried out in order to investigate effects of gravitational changes on isolated T lymphocytes. Representative studies demonstrating cellular and molecular alterations that have been observed in real and simulated microgravity are summarized in Tables 5.1 and 5.2.

5.1.2 T Cell Activation Is Diminished in Microgravity

The first experiments were carried out in terms of studying phenomenological effects of microgravity on isolated T lymphocytes. So far it is well known that microgravity influences T lymphocytes by diminishing the reactivity of T lymphocytes to mitogenic stimulation during spaceflight (Cogoli et al. 1984, 1988; Cogoli and Cogoli-Greuter 1997; Bechler et al. 1986). Experimental studies using the ground-based facilities RWV, clinostat, and RPM yielded the same results (Schwarzenberg et al. 1999; Cooper and Pellis 1998; Hashemi et al. 1999).

Next, the question arose whether binding of the mitogen Concanavalin A (ConA) to the T cell receptor might be changed due to the absence of gravity and therefore leads to reduced T cell activation. But this assumption was disproved in four different experiments on board of sounding rockets (Cogoli-Greuter et al. 1997; Sciola et al. 1999). By means of fluorescently labeled ConA, these experiments showed that binding of the mitogen on T cell receptors was in principal not affected; only a slight delay of patching and capping was observed.

5.1.3 T Cell Function Remains Unchanged Despite Reduced Cell-Cell and Cell-Substrate Interactions

Another factor that could lead to an inhibited T cell function may be the lack of sedimentation under microgravity conditions. This could lead to reduced cell-cell and cell-substrate interactions which in turn could be responsible for the reduced proliferative response to mitogenic stimuli. However, the cultivation of peripheral blood mononuclear cells (PBMCs) in Teflon bags, which show a reduced cell-substrate interaction, had no effect on the proliferation of phytohemagglutinin

Table 5.1 Summary of experiments performed under real microgravity conditions

Year	Author	Cell type	Research platform	Activation	Exposure/activation time	Gravity conditions	Results
2015	Hughes-Fulford et al.	Primary T cells	ISS	ConA/CD28	1.5 h	µg 1 g in-flight 0.5 g in-flight	Suppressed expression of the miRNA miR-21 after 1.5 h T cell activation due to altered gravity. Downregulation of 85 genes associated with T cell signaling. 17 out of the 85 genes were defined as targets of miR-21
2015	Tauber et al.	Primary CD4+ T cells	Parabolic flight	Nonstimulated	20 s	µg 1 g in-flight hyp-g	Suppressed the CD3 and IL-2R-surface receptor expression and reduced phosphorylation of the LAT protein due to hypergravity
2013	Tauber et al.	Primary CD4+ T cells	Sounding rocket	ConA/CD28	6 min	µg 1 g in-flight hyp-g 1 g GC	Dysregulation of several key signal proteins involved in early TCR signaling during the hypergravity phase. No further disturbance of these key proteins in the following microgravity phase
2012	Battista et al.	PBMCs	ISS	ConA	0, 3, 24, and 48 h	µg 1 g in-flight 1 g GC	Increased DNA fragmentation, PARP protein expression, p53 mRNA expression, and calpain mRNA expression. Early increase of 5-LOX activity
2012	Chang et al.	Primary T cells	ISS	ConA/CD28 or CD3/CD28 beads	1.5 h	µg 1 g in-flight	Inhibited transcription of immediately early genes. Disrupted activation of Rel/NF-kB, CREB1, and SRF transcription factors
2012	Thiel et al.	Jurkat cells, $CD4^+$ T lymphocytes	Parabolic flight	PMA or CD3/CD28	20 s	µg 1 g in-flight	Enhanced p21 Waf1/Cip1 mRNA expression due to microgravity being dependent from histone acetylation. Enhanced Tyr15-phosphorylation of cdc2

2010	Paulsen et al.	Jurkat cells	Parabolic flight	PMA or CD3/CD28	20 s	µg 1 g in-flight 1 g GC	Increased p53 phosphorylation in nonactivated as well as in activated cells due to microgravity. Enhanced MEK phosphorylation in activated cells in microgravity compared to 1 g in-flight
2008	Crucian et al.	Whole blood culture of astronauts	Space shuttle and ISS	CD3/CD28	Short- and long-duration space flight	Pre-flight: L-180, L-65, L-10, post-flight: R+0, R+3, R+14, R+30	Shuttle crewmembers: elevated early T cell activation after space flight but decreased percentage of T cell subsets capable of being stimulated to produce IL-2 and IFNγ. ISS crewmembers: reduced early T cell activation after space flight, reduced percentage of T cells capable of producing IL-2, but unchanged IFNγ percentages
2002	Hatton et al.	Primary T cells	Space shuttle	PDBu/ ionomycin or CD3 beads	0, 10, 60 min	µg 1 g in-flight 1 g GC 1.4 g ground centrifuge	Reduced translocation of PKCδ to particular cell fractions in microgravity. Unvarying PKCβII translocation in microgravity compared to 1 g
2001	Schatten et al.	Jurkat cells	Space flight	Nonstimulated	4, 48 h	µg 1 g in-flight 1 g GC 1.4 g ground centrifuge	Occurrence of mitochondria clustering and morphological alterations of mitochondrial cristae. Occurrence of cell divisions during space but also of apoptotic cells. Uneven distribution of mitochondria in cells

(continued)

Table 5.1 (continued)

Year	Author	Cell type	Research platform	Activation	Exposure/activation time	Gravity conditions	Results
2001	Lewis et al.	Jurkat cells	Space shuttle	Nonstimulated	4, 24, 48 h	μg 1 g GC ground vibration samples	Upregulated gene expression of 11 cytoskeleton-related genes in space. Differential expression of genes regulating growth, metabolism, signal transduction, adhesion, transcription, apoptosis, and tumor suppression
2000	Cubano and Lewis	Jurkat cells	Space shuttle	Nonstimulated	0, 4, 24, 48, and 75 h	μg 1 g in-flight 1 g GC 1.4 g ground centrifuge	Increased apoptosis during flight. Time-dependent and microgravity-related release of sFas
2000	Crucian et al.	PBMCs	Space shuttle	PMA/ ionomycin or PMA or PHA	5 h or 24 h	Pre-flight: L-10, post-flight: R+0, R+3	Reduced ability of CD4+ and the CD8+ T cell subsets to produce IL-2 following space flight. Reduced IFNγ production in the CD4+ T cell subset, and unchanged production of IFNγ in the CD8+ T cell subset
1999	Sciola et al.	Jurkat cells	Sounding rocket	ConA	12 min	μg 1 g in-flight 1 g GC	Unaffected binding of ConA to membrane by microgravity and slight retardation of patching. Structural changes of vimentin in microgravity: increasing presence of large bundles in microgravity. No changes in the structure of actin and in the co-localization of actin at the inner side of the cell membrane with ConA receptors after binding of the mitogen

1999	Hashemi et al.	PBMCs, purified T cells	Space shuttle	Okt3/CD28 beads or PDB/ ionomycin or Leu4 or Leu4 beads	24 h	µg 1 g in-flight	Suppressed CD25 and CD69 surface expression after activation
1998	Lewis et al.	Jurkat cells	Space shuttle	Nonstimulated	4, 24, 48 h	µg 1 g in-flight 1 g GC 1.4 g ground centrifuge	Structural changes in the microtubule cytoskeleton: shortened, coalesced filaments lacked normal branching at the cell membrane, and MTOCs were disrupted. Time-dependent increase in the apoptosis-related factor, Fas/ APO-1 in culture medium of flown cells (0 g + 1 g)
1997	Cogoli-Greuter et al.	PBMCs, Jurkat cells	Sounding rocket	ConA	7 min and 12.5 min	µg 1 g GC hyp-g (13 g)	Unaffected binding of ConA to membrane by microgravity and slight retardation of patching and capping. Occurrence of structural changes of intermediate filaments of vimentin as well as of the microtubule network. Occurrence of cell motility and cell-cell-contacts in microgravity
1996	Schmitt et al.	Jurkat cells	Space shuttle	A23187 calcimycin	1 h	µg 1 g in-flight 1 g GC 1.4 g ground centrifuge	G-level-dependent relative distribution of PKC in the cytosolic and nuclear fractions, with cytosolic PKC increasing with increasing g level, whereas nuclear PKC decreased

(continued)

Table 5.1 (continued)

Year	Author	Cell type	Research platform	Activation	Exposure/activation time	Gravity conditions	Results
1996	Cogoli-Greuter et al.	PBMCs	Space shuttle	ConA	46 and 78 h	μg 1 g GC	Aggregate forming of activated cells in microgravity as well as in ground control indicating that cell-cell contacts occur. No decrease of the mean velocity of free cells with increasing exposure time indicating that cell cycle progression is inhibited
1996	Pippia et al.	PBMCs	Space shuttle	ConA	72 h	μg 1 g in-flight 1 g GC 1.4 g ground centrifuge	No rescue of activability by addition of exogenous IL-1 + IL-2, but recovery of IFNγ production in microgravity
1993	Cogoli et al.	PBMCs	Space shuttle	ConA or ConA/ Cytodex beads	52 and 71 h	μg 1 g in-flight 1 g GC	Strong increase of IL-2 production. IL-2R expression was in the normal range with Cytodex beads in microgravity
1992	Bechler et al.	PBMCs	Space shuttle	ConA or ConA/ Cytodex beads	72 h	μg 1 g in-flight 1 g GC	Almost doubled activation in microgravity when bound to Cytodex beads. Increase of IFNγ production by 300 % on Cytodex beads
1992	Chapes et al.	PBMCs	Space shuttle	ConA	24 and 48 h	μg 1 g GC	Higher secretion of IFNγ in space than on the ground
1991	Limouse et al.	Jurkat cells or co-culture of Jurkat + THP-1 cells	Biosatellite	PMA/A23187 calcimycin, or anti-CD3 mAb in the presence of THP-1 cells	24 h	μg 1 g GC	Suppression of IL-2 secretion in microgravity. Occurrence of cell-to-cell contacts in microgravity, leading to normal production of IL-1 and IL-2 compared to ground controls

1985	Cogoli et al.	PBMCs	Space shuttle	ConA	36, 48, 72, and 96 h	µg 1 g in-flight	Reduced mitogenic activation of human lymphocytes by 90% in microgravity
1985	Cogoli et al.	Whole blood culture of astronauts	Space shuttle	ConA	78 h	Pre-flight: L-9, L-2. In-flight: L+3. Post-flight: R+0, R+7, R+13 1 g in-flight	Weakened activation of lymphocytes during and post-flight compared to the pre-flight values. Lack of lymphocyte activation in microgravity compared to 1 g in-flight control
1984	Cogoli et al.	PBMCs	Space shuttle	ConA	71 h	µg 1 g GC	Almost complete absence of mitogenic activation of human lymphocytes in microgravity compared to ground control

Modified from Hauschild et al. (2014)

1 g GC 1 g ground control, *5-LOX* 5-lipoxygenase, *APO* apoptosis antigen, *ConA* Concanavalin A, *FBS* fetal bovine serum, *HEPES* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, *HS* human serum, *hyp-g* hypergravity, *IL* interleukin, *IFN* interferon, *ISS* International Space Station, *PBMCs* peripheral blood mononuclear cells, *PDB* phorbol dibutyrate, *PHA* phytohemagglutinin, *PMA* phorbol-12-myristate-13-acetate, *PKC* protein kinase C, *PARP* poly (ADP-ribose) polymerase, *RPMI-1640* Roswell Park Memorial Institute-1640 medium, *STS* space transportation system, *TCR* T cell receptor, *TNF* tumor necrosis factor, *n/a* not available

Table 5.2 Summary of experiments performed in ground-based facilities in simulated microgravity

Year	Author	Cell type	Research platform	Activation	Exposure/activation time	Gravity conditions	Results
2015	Tauber et al.	Primary $CD4^+$ T cells	Clinostat	Nonstimulated or ConA/CD28	5, 15, 30, and 60 min	1 g sim. μg CC	Reduced expression of CD3 surface receptor and ZAP-70 protein, as well as increased histone H3 acetylation in activated cells after 5 minutes clinorotation. Transient downregulation of CD3 and stable downregulation of IL-2R after 60 minutes clinorotation
2014	Wei et al.	PBLs	RCCS	PHA	72 h	1 g sim. μg	Unchanged number of chromosomes and no structural changes. Enhanced expression of ATR in parallel with the inhibition of cell proliferation. Downregulation of the expression of DNA replication genes and DNA repair genes. Enhanced structural chromosome instability of human PBL cells in simulated microgravity
2014	Girardi et al.	PBLs	RWV	PHA and IL-2	24, 48, and 72 h	1 g sim. μg	Differential expression of 42 miRNAs in RWV-incubated PBLs compared with 1 g controls. miR-9-5p, miR-9-3p, miR-155-5p, miR-150-3p, and miR-378-3p were the most dysregulated correlating with genes involved in immune/inflammatory response, apoptosis, and cell proliferation
2014	Benavides Damm et al.	PBMCs, primary T cells	RPM	ConA (PBMCs) or ConA/CD28 (T cells)	20-22 h	1 g sim. μg, 0.2 g, 0.4 g, 0.6 g	Lymphocyte activation depends on partial gravity exposure. Equally poor activation of cells exposed to 0.2 g and 0 g, but still existing activation in cells exposed 0.6 g equally to the 1 g control. The activation level of cells exposed to 0.4 g was about in the middle of 0.2 g and 0.6 g

2012	Chang et al.	Primary CD4+ T cells	RWV	CD3/CD28 beads	1.5 h	1 g sim. μg	Reduced expression of the immediately early genes cREL, TNF, EGR1, EGR2, and JUNB under RWV conditions
2012	Thiel et al.	Jurkat	Clinostat	CD3/CD28 or PMA	5, 10, and 15 min	1 g sim. μg	Differential protein expression of cell cycle regulatory proteins: enhanced expression of p21 Waf1/Cip1 protein, less cdc25C protein expression, and enhanced Ser147-phosphorylation of cyclin B1 after CD3/CD28 stimulation
2011	Mangala et al.	TK6 human lymphoblastoid cells	RWV	Nonstimulated	72 h	1 g sim. μg	Altered miRNA expression influencing several genes that are involved in the regulation of the NF-kB-related pathway network
2010	Paulsen et al.	Jurkat	Clinostat	PMA or CD3/CD28	5 min	1 g sim. μg	Enhanced phosphorylation of the MAP kinases ERK-1/2, MEK, and p38 and inhibited nuclear translocation of NF-kB, either in nonstimulated or in stimulated cells
2010	Singh at al.	Human T cells	RWV	Nonstimulated	72 h and 7 days	1 g sim. μg	Microgravity-induced epigenetic changes in DNA methylation and chromatin histone modifications (decreased expression of DNMT1 and HDAC1)

(continued)

Table 5.2 (continued)

Year	Author	Cell type	Research platform	Activation	Exposure/activation time	Gravity conditions	Results
2010	Simons et al.	Primary CD4+ T cells	RWV	CD3/CD28	5-90 min	1 g sim. μg	Intact TCR signaling through DAG during PWV exposure. Thus, simulated microgravity might prevent T cell activation more likely by modulating the cellular response to the TCR signal rather than by annulling or limiting the signal itself
2009	Kumari et al.	Human T cells	RWV	Nonstimulated	4 h, 72 h, and 7 days	1 g sim. μg	Decreased expression of DNA repair genes, of cell cycle genes and of anti- and pro-apoptotic genes. Occurrence of DNA damage
2009	Martinelli et al.	PBMCs	Clinostat	PHA	24-48 h	1 g sim. μg	Decreased proliferation and viability after 48 h of rotation in the 3-D clinostat
2009	Simons et al.	PBMCs	RWV	PHA or PHA + PMA	48 h	1 g sim. μg	Recovery of PHA-induced activation of the CD8+ and CD4+ T cell subsets as well as naïve and memory CD4+ T cells due to PMA co-stimulation
2009	Sundaresan and Pellis	PBMCs	RWV	Nonstimulated	24 and 72 h	1 g sim. μg	Downregulation of T cell activation genes DAG kinase, Ser/Thr kinase, and Tyr kinase. Upregulation of HSPA1A (e.g., HSP-70) and downregulation of HSP9A9B (i.e., HSP-90). Upregulation of angiogenic factor PIGf
2006	Morrow	PBMCs	Clinostat	CD3/CD28 beads	2.5-16 h	1 g sim. μg	Continuing existence of Ca++/Cn signaling active, but inhibited PKC pathway since activation of fos and NF-kB is inhibited

2006	Ward et al.	Preliminary activated T cells	RWV	CD3/IL-2	24 h	1 g sim. μg	Changes in the expression of genes belonging to functional categories immune response, cell proliferation and differentiation, protein folding, transport and degradation, as well as apoptosis
2005	Degan et al.	PBMCs, lymphoblastoid cell lines LB and COR3	RPM	ConA	8 or 24 h	1 g sim. μg	Transient occurrence of apoptosis induction, release of sFas, and fluctuation of PARP activity. Decrease in intracellular concentration of ATP. Microgravity exposure might induce a condition of metabolic "quiescence"
2005	Boonyarata-nakornkit et al.	Primary T cells	RPM	ConA/CD28	30 min	1 g sim. μg	Upregulation of 99 genes significantly upregulated during early T cell activation under 1 g condition. No induced gene-expression of those genes in simulated microgravity. 28 % of these genes were component of NF-kB signaling or had evidence for regulation by NF-kB. Blocking of CREB activation by phosphorylation
2005	Risso et al.	Primary T cells	Clinostat	CD69/PMA	48 h	1 g sim. μg	Significant higher calcium concentration in clinorotated activated cells. Prolonged mitochondrial membrane hyperpolarization due to activation since after 20 h followed by depolarization in a fraction of cell population. Inhibition of IL-2 secretion and proliferation of activated cells

(continued)

Table 5.2 (continued)

Year	Author	Cell type	Research platform	Activation	Exposure/activation time	Gravity conditions	Results
2004	Sundaresan et al.	PBMCs	RWV	Nonstimulated	24, 48, 72, and 96 h	1 g sim. μg	Decrease expression of specific calcium-independent PKC isoforms in PBMCs at both the RNA and protein levels. 56 % decrease in phosphorylated PLC-γ1
2002	Galleri et al.	Primary T cells	RPM	ConA/CD28 + Protein G	6, 15, and 30 min	1 g sim. μg	Altered PKC isoform distribution in the three fractions nucleus, cytosol, and plasma membrane
2002	Sundaresan et al.	PBMCs	RWV	PMA	24, 48, 72, and 96 h	1 g sim. μg	Inhibition of locomotion of nonstimulated PBMCs after 24 h with extent of locomotion loss at 72 h. Restoration of locomotion by addition of PMA to the cells
2001	Risin and Pellis	PBMCs, preliminary activated primary T cells	RWV	CD3/IL-2	2, 4, 6, 18, and 24 h	1 g sim. μg	Inhibition of radiation- and activation-induced programmed cell death
1999	Licato and Grimm	PBMCs	RWV	IL-2	2-8 days	1 g sim. μg	Maintenance of cell viability by addition of exogenous IL-2 but no induction of CD25 surface expression or restoration of cytokine (IFNγ, IL-1β, and TNFγ) secretion
1999	Schwarzenberg et al.	PBMCs	RPM	ConA	72 h	1 g sim. μg	Suppressed proliferation of ConA-activated PBMCs of the same order of magnitude as in space after 72 h exposure to RPM

1999	Hashemi et al.	PBMCs, primary T cells	2D-clinostat	PHA or Leu4	3, 24, and 48 h	1 g sim. μg	Reduced proliferation of PHA or Leu4-stimulated PBMCs after 48 h clinorotation. Suppressed expression of CD69 and CD25 activation marker in Leu4 stimulated PBMCs after 24 h clinorotation. Inhibition of cell cycle progression. Slower TCR internalization in simulated microgravity. Restoration of surface CD69 and CD25 expression by PMA co-stimulation of activated PBMCs
1998	Walther et al.	PBMCs	Clinostat and RPM	ConA	1-12 h	1 g sim. μg	Decreased IL-2 and IL-2R gene expression
1998	Cooper and Pellis	PBMCs, primary T cells	RWV	PHA	24-72 h	1 g sim. μg	Suppressed proliferation of stimulated cells and reduced IL-2 secretion. Initially suppressed secretion of IFNγ but recovery after 72 h. Reduced CD25 and CD69 surface expression by over 50%. Restoration of proliferation ability in PHA stimulated cultures using submitogenic concentrated PMA
1997	Pellis et al.	PBMCs	RWV	CD3/IL-2	24 h to 9 days	1 g sim. μg	Hampered locomotion into collagen type I matrix

Modified from Hauschild et al. (2014)

CC cell culture control, *ConA* Concanavalin A, *FBS* fetal bovine serum, *HARV* high-aspect ratio vessel, *HEPES* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, *IL* interleukin, *IFN* interferon, *PBL* peripheral blood lymphocytes, *PBMCs* peripheral blood mononuclear cells, *PHA* phytohemagglutinin, *PMA* phorbol-12-myristate-13-acetate, *PKC* protein kinase C, *PARP* poly (ADP-ribose) polymerase, *RCCS* rotary cell culture system, *RWV* rotating wall vessel, *RPM* random positioning machine, *RPMI-1640* Roswell Park Memorial Institute-1640 medium, *TCR* T cell receptor, *TNF* tumor necrosis factor, *n/a* not available

(PHA) stimulated cells compared with cultures in standard cell culture flask with existing cell-substrate interactions (Cooper and Pellis 1998).

Furthermore, in experiments with the ground-based facility RWV, the proliferation of T lymphocytes after stimulation of the receptors CD2/CD28 and CD3/CD28 was completely inhibited (Cooper and Pellis 1998). These stimuli activate the cells without required co-stimulatory signals from cell-cell interaction. Moreover, investigations of human PBMCs and Jurkat T cells in real microgravity showed that cell aggregates and thereby cellular interactions occur despite the absence of gravity (Cogoli-Greuter et al. 1996, 1997; Limouse et al. 1991). Therefore, it has been assumed that changes in the signal transduction are responsible for inhibition of T cell function rather than the absence of cell-cell interactions.

5.1.4 Cytokine Pattern Changes Under Microgravity Condition

In addition to the activation of the T cell receptor complex and the co-receptor CD28, also a third signal via the interleukin-2 receptor (IL-2R) is necessary to fully activate the T cell and therefore to trigger proliferation and differentiation into functional effector T cells. Thus, the reduced functionality of T cells in microgravity might also be due to alterations in cellular IL-2 secretion or IL-2R surface expression, resulting in a disability of the positive regulatory feedback loop.

And in fact, experiments with human PBMCs, which were conducted during several space missions, showed that both the IL-2 secretion and IL-2R expression were greatly reduced in microgravity (Cogoli et al. 1993; Pippia et al. 1996; Hashemi et al. 1999). Additionally, clinostat, RWV, and RPM experiments, where PBMCs and primary human T cells have been exposed to simulated microgravity, confirmed these results (Cooper and Pellis 1998; Hashemi et al. 1999; Risso et al. 2005). Further ground-based experiments demonstrated that already the gene expression of IL-2 and its receptor was inhibited in activated T cells (Walther et al. 1998; Boonyaratanakornkit et al. 2005). However, co-stimulation of the cells with submitogenic concentrations of phorbol-12-myristate-13-acetate (PMA) was able to restore the proliferative response and the expression of IL-2R at the cell's surface (Cooper and Pellis 1998; Hashemi et al. 1999).

Activated T cells also produce interferon-gamma (IFNγ) which is a major proinflammatory and regulatory cytokine. IFNγ plays an essential role in relevant immunological processes such as inflammatory reactions, cell-mediated immunity, and autoimmunity. Studies concerning the IFNγ secretion demonstrated that stimulation of PBMCs with ConA during the spaceflight resulted in an increased IFNγ secretion compared to 1 g ground controls (Chapes et al. 1992), whereas a PMA/ionomycin stimulation of samples isolated from astronauts immediately after their landing led to a significant reduction of IFNγ secretion of $CD4^+$ T lymphocytes. In $CD8^+$ T lymphocytes, however, these remained unchanged (Crucian et al. 2000). In addition, comparison of whole blood analyses from astronauts of short-term (Space Shuttle) and long-term missions (International Space Station (ISS)) showed that a short-term stay in space led to a reduction in the percentage of IFNγ producing T

cells, while after long-term missions the proportion of IFNγ producing T cells remained unchanged (Crucian et al. 2008). Lymphocytes in simulated microgravity provided by RWV exhibited an initial reduction of IFNγ secretion. After 3 days, however, normal levels were restored (Cooper and Pellis 1998).

5.1.5 *Microgravity Affects Cytoskeletal Structures and Cell Motility*

The cytoskeleton is an internal filamentous network of different types of cytosolic fibers: actin filaments, microtubules, and intermediate filaments. This cytoskeletal network is responsible for giving a cell its shape and for generating the required forces for cell motility. But also other biological functions such as cell proliferation, survival, and death are influenced by the cytoskeleton. Since the cytoskeleton participates also in transduction of signals from the receptor at the plasma membrane to the nucleus, these cytoskeletal structures play additionally essential roles in maintaining receptor signaling integrity. Thus, further studies set their focus on the cytoskeletal network.

Indeed, several independent experiments performed under real microgravity conditions demonstrated significant changes in the cytoskeletal network. Analyses of T lymphocytes flown aboard of sounding rockets reported altered tubulin and vimentin structures which appeared in thick bundles (Cogoli-Greuter et al. 1997; Sciola et al. 1999). Jurkat T cells flown on board of the space shuttle Atlantis also showed a modified microtubule network (Lewis et al. 1998). The microtubules were coalesced, did not extend to the cell membrane, and the microtubule organizing center was disorganized.

Cell communication and signal transduction processes are not only affected by cell-to-cell interactions, they are also highly influenced by cell motility. Therefore, the cell motility of lymphocytes was observed under microgravity conditions. These studies showed that the cells were motile; however, their motility did not decrease with increasing duration of stimulation (Cogoli-Greuter et al. 1996, 1997). Furthermore, experiments using the ground-based facility RWV exhibited that after 24 h exposure to simulated microgravity, cell motility of PBMCs was inhibited (Pellis et al. 1997; Sundaresan et al. 2002). However, addition of PMA to the cells could restore cell motility (Sundaresan et al. 2002).

5.1.6 *Distribution of Protein Kinase C (PKC) Isoforms to Destined Cellular Fractions Is Dysregulated*

Since the cytoskeleton also has an impact on proper functioning of signal transduction substantially, restructuring of the cytoskeleton induced by microgravity could also result in hampered intracellular localization of signaling molecules. Accordingly, different PKC isoforms are associated with several cytoskeletal fibers.

Upon T cell activation, under normal circumstances, these PKC isoforms are allocated to destined cellular compartments. In two spaceflight experiments with Jurkat T cells and primary human T cells, it could be shown that the relative distribution of certain PKC isoforms to different cell fractions in the in-flight microgravity samples greatly differed from the 1 g ground controls (Hatton et al. 2002; Schmitt et al. 1996). Further, primary T cells exposed to simulated microgravity in an RWV confirmed these results (Galleri et al. 2002). In addition, another RWV experiment revealed that also mRNA expression and protein expression of specific calcium-independent PKC isoforms in PBMCs were inhibited (Sundaresan et al. 2004).

5.1.7 Lack of Gravity Increases the Rate of Controlled Cell Death (Apoptosis)

Another reason for the reduced proliferative response of T lymphocytes in microgravity might be the initiation of the intracellular death program called programmed cell death or apoptosis. In fact, it could be shown by means of biochemical and microscopic investigations that Jurkat T cells exposed to microgravity demonstrated an increased rate of apoptosis (Cubano and Lewis 2000; Lewis et al. 1998; Battista et al. 2012). This was reflected in the release of apoptosis-related factors such as Fas/APO1 in the cell culture medium after about 2 days aboard different space shuttle flights in cell culture medium (Cubano and Lewis 2000; Lewis et al. 1998). Moreover, exposure of lymphocytes to microgravity resulted in increased DNA fragmentation, poly (ADP-ribose) polymerase (PARP) protein expression, as well as multiplied p53 and calpain mRNA. These changes were associated with an early increase of 5-lipoxygenase (5-LOX) activity (Battista et al. 2012). During an experiment that we conducted during the 8th DLR (German Aerospace Center) parabolic flight campaign, we observed an increase in p53 phosphorylation after 20s of real microgravity (Paulsen et al. 2010). Experiments in simulated microgravity, however, did not confirm microgravity-induced stimulation of apoptosis but revealed that radiation and activation-induced programmed cell death in T lymphocytes was inhibited (Risin and Pellis 2001).

5.1.8 Microgravity Does Not Intervene at the Level of Membrane-Proximal Processes of T Cell Receptor Signaling Within the First Minutes

Up to date it has not yet been revealed whether and how gravitational changes affect the T cell signal transduction, in particular the membrane-proximal and cytoplasmic signal transduction cascades as well as the IL-2/IL-2R activation loop. Although some studies suggest that microgravity intervenes at the level of PKC (Hatton et al. 2002; Schmitt et al. 1996), the addition of PMA to cells exposed to simulated

microgravity restored T cell activation (Cooper and Pellis 1998; Simons et al. 2009), surface receptor expression (Hashemi et al. 1999), and cell motility (Sundaresan et al. 2002). In addition, the first activation signals binding, patching, and capping of ConA on the T cell receptors proceed normally (Cogoli-Greuter et al. 1997). Therefore, it is believed that the gravity-sensitive cellular targets are located more likely upstream of the PKC and downstream of the T cell receptor/CD3 complex.

Thus, in recent studies, we investigated the effects of altered gravity on several key elements involved in the early T cell signaling (Tauber et al. 2013, 2015). For this purpose, primary human $CD4^+$ T lymphocytes were examined under real microgravity conditions aboard the sounding rocket MASER-12 (Tauber et al. 2013) and during the 19th DLR parabolic flight campaign (Tauber et al. 2015). Subsequently, we carried out experiments with the same experimental set-up in simulated microgravity using a fast-rotating 2D-clinostat (Tauber et al. 2015). We analyzed the impact of gravitational changes on the key molecules of the early T cell signaling events in both resting and ConA/CD28-activated $CD4^+$ T lymphocytes. We quantified following signaling components: T cell receptor, membrane-proximal signal proteins LAT and ZAP-70, MAPK, IL-2R, and histone acetylation. Table 5.3 gives an overview of the obtained results.

In addition to the microgravity effects, we investigated the influence of hypergravity during the rocket launch and during the climb of the airplane, as well as the influence of the cultivation of the cells in the experimental hardware. The analyses of the protein level after 6 min of real microgravity during the sounding rocket flight showed no obvious effects on the early signal transduction pathway in $CD4^+$ T lymphocytes. Surprisingly, strong effects of the rocket launch could be observed, which often resulted in a significant reduction of the signal molecules. During the parabolic flight experiment, the 20 s hypergravity phase led to a rapid decrease of CD3 and IL-2R surface expression and reduced p-LAT in nonactivated primary T lymphocytes. The subsequent clinostat experiments showed a decreased CD3 surface expression, reduced ZAP-70 abundance, as well as an increased histone H3-acetylation in activated T lymphocytes after 5 min of clinorotation and a transient downregulation of CD3 and, further, a stable downregulation of IL-2R during 60 min of clinorotation.

However, based on these results it can be assumed that gravitational changes do not intervene at the level of the membrane-proximal key proteins within the first 6 min. The initial primary dysregulation of functional T cell activation will probably occur at the level of regulation of gene expression.

5.1.9 Gravitational Changes Induce Alterations in the Gene Expression Profile

The phenomenological characteristics of reduced T cell activation caused by microgravity are now well described. The exact underlying molecular mechanisms, however, are still unknown. So, during the last decade, several studies focused on the

Table 5.3 Overview of qualitative changes of selected proteins involved in T cell activation of primary human $CD4^+$ T lymphocytes induced by altered gravity during the sounding rocket flight of MASER-12, during the 19th DLR parabolic flight campaign, and by 2D-clinorotation

	Signaling molecules					
	CD3	ZAP-70	LAT (pY171)	P-p44/42 MAPK	Acetyl-histone H3	IL-2R
19th DLR parabolic flight campaign						
Nonactivated T lymphocytes						
1 g in-flight vs. 1 g hardware controls	–	–	–	–	–	–
1.8 g hypergravity vs. 1 g in-flight	↓**	–	↓*	–	–	↓**
Microgravity vs. 1 g in-flight	↓*	–	↓*	–	–	↓*
Microgravity vs. 1.8 g hypergravity	–	–	–	–	–	–
MASER-12 sounding rocket						
Controls						
1 g hardware controls vs. cell culture controls	↓**	↓**	↓**	–	↑**	–
Hypergravity (rocket launch) vs. 1 g hardware controls	↓**	↓*	–	↓**	↓**	↓**
Nonactivated T lymphocytes						
Microgravity vs. 1 g in-flight	–	–	–	↓*	–	–
ConA/CD28 activated T lymphocytes						
Microgravity vs. 1 g in-flight	–	–	–	–	–	–
Fast-rotating 2D-clinostat						
Nonactivated T lymphocytes						
Hardware controls vs. cell culture controls	–	–	–	–	–	–
5 min clinorotation vs. 1 g controls	–	–	–	–	↑*	–
ConA/CD28 activated T lymphocytes						
5 min clinorotation vs. 1 g controls	↓**	↓*	–	–	↑*	–
15 min clinorotation vs. 1 g controls	↓*	nd	nd	Nd	nd	–
30 min clinorotation vs. 1 g controls	–	nd	nd	Nd	nd	↓*
60 min clinorotation vs. 1 g controls	–	nd	nd	Nd	nd	↓*

Modified from Tauber et al. (2015)

Hardware control: cells were cultured without changing gravity conditions in the corresponding hardware; *Cell culture control*: optimal culture conditions prior to fixation; *1 g in-flight*: cells were fixed before onset of the parabola during the parabolic flight or cells were reexposed to 1 g at a reference centrifuge aboard the sounding rocket MASER-12; *Hypergravity*: cells were fixed after 1.8 g phase during the climb of the aircraft or after the rocket launch of MASER-12; *Microgravity*: during parabolic flight or suborbital sounding rocket flight; *Clinorotation*: cells were exposed simulated microgravity by means of 2D-clinostats. Cells were immunocytochemically stained, staining was quantified using flow cytometry analysis, and the relative fluorescence intensity was calculated. ↓ indicates downregulation, ↑ indicates upregulation, – indicates no significant difference within the compared groups

nd not determined

*$p<0.05$; **$p<0.01$

investigation of the impact of altered gravity on gene transcription (Thiel et al. 2012; Chang et al. 2012; Boonyaratanakornkit et al. 2005; Ward et al. 2006; Sundaresan and Pellis 2009; Kumari et al. 2009; Lewis et al. 2001).

The evaluations of genome-wide gene expression analyses of T cells revealed that both in real and simulated microgravity, the expression of very early genes, which are primarily regulated by the transcription factors NF-kB, CREB, ELK, AP-1 and STAT, were downregulated in comparison to 1 g controls (Chang et al. 2012; Boonyaratanakornkit et al. 2005). The observed changes in gene expression induced by altered gravity include a number of genes which are associated with responses to cell stress (Sundaresan and Pellis 2009), cell proliferation and differentiation (Ward et al. 2006; Sundaresan and Pellis 2009; Boonyaratanakornkit et al. 2005), cell cycle regulation (Kumari et al. 2009; Thiel et al. 2012), protein folding (Ward et al. 2006), DNA repair (Kumari et al. 2009), transport and degradation (Ward et al. 2006), apoptosis (Ward et al. 2006; Kumari et al. 2009; Lewis et al. 2001), and the cytoskeleton (Lewis et al. 2001). These results show that modulation of gene expression in reduced gravity covers a wide spectrum.

In an RWV study, alterations in the microRNA (miRNA) profiles of human lymphocytes exposed to simulated microgravity for 1–3 days were observed (Girardi et al. 2014). The examinations identified 42 differentially expressed miRNA whereof the upregulated miR-9-5p, miR-9-3p, and miR-155-5p, and the downregulated miR-150-3p and miR-378a-3p were the most dysregulated ones. Further, miRNA-correlated genes whose expression level was also significantly altered by simulated microgravity were investigated. Thus, several miRNA-mRNA pairs, which are involved in biological processes such as immunity and inflammatory response, cell proliferation, and apoptosis, were determined.

In a most recent study, Millie Hughes-Fulford and her team discovered the suppressed expression of the miRNA miR-21 due to altered gravity after 1.5 h T cell activation during spaceflight (Hughes-Fulford et al. 2015). Furthermore, microarray analysis showed that 85 genes associated with T cell signaling were significantly downregulated under microgravity conditions compared to 1 g in-flight controls. Of these gravity-sensitive genes, 17 were defined as targets of miR-21 whereof 5 genes are biologically confirmed targets and are under normal circumstances upregulated in parallel with miR-21. Therefore, it can be assumed that altered gravity influences T cell activation not only by transcription promotion but also by repressing translation via noncoding RNA mechanisms.

Further experimental studies with primary human T cells disclosed microgravity-induced epigenetic changes in DNA methylation and chromatin histone modifications (Singh et al. 2010). Such epigenetic mechanisms regulate and modify the activation of certain genes and therefore lead to differential expression of mRNA. Experiments that we conducted during several parabolic flight experiments (9th, 10th, and 13th DLR and 45th ESA Parabolic Flight Campaign) have revealed an association between microgravity-induced differentially mRNA expression and altered histone acetylation (Thiel et al. 2012).

5.2 Humoral Immunity

Apart from the cellular components, the adaptive immune system also includes the humoral immunity [see also Chap. 1]. However, the humoral immunity has not been investigated to that extent of which the cell-mediated immunity has been investigated. Short-term spaceflight has resulted in no change in levels of plasma immunoglobulins (Voss 1984; Stowe et al. 1999; Rykova et al. 2008), whereas, long-term spaceflight led to different results. Studies of cosmonauts during spaceflight have shown that immunoglobulin G (IgG) levels were unchanged, whereas IgA and IgM levels were in some cases increased (Konstantinova et al. 1993). In another study, immunological investigations comparing the preflight with the postflight situation indicated that the total amounts of serum IgA, IgG, and IgM were unchanged after long-term missions (Rykova et al. 2008). Therefore, the humoral immune responses may not be as sensitive to altered gravity as are cell-mediated immune responses.

5.3 Conclusion

These numerous studies carried out with T lymphocytes in microgravity have clearly shown that already individual cells are sensitive to changes in gravity. In addition, these experiments conducted under real and simulated microgravity conditions contributed greatly to our current knowledge of how changes of the gravitational force affect basic cellular mechanisms. The influence of microgravity on the function of T lymphocytes is reflected in a variety of cellular responses, which can be grouped into different categories displayed in Table 5.4 and Fig. 5.1.

Since the simulation of the microgravity yielded comparable results to real microgravity experiments (Herranz et al. 2013), it was possible to perform a large number of simulation experiments which would not have been feasible in this scale only by space experiments. So far, however, it has not been possible to formulate a generally accepted hypothesis from these various effects and to further locate any possible primary mechanism that underlies the effects of altered gravity on immune cells.

To date, in vitro research has mainly focused on the impact of altered gravity of T helper cells. In a recent in vivo study (Chang et al. 2015), in which the influence of microgravity was examined for tolerance induction, transgenic mice were exposed to microgravity for 15 days during spaceflight. In this experiment, it could be shown for the first time that the immune tolerance is inhibited in space. Moreover, it provides indications of a potential key role of regulatory T cells.

On closer inspections of the studies reviewed in this chapter, the experimental conditions vary widely from study to study. For example, stimuli used for the T cell activation ranged from mitogens over calcium ionophores up to antibodies against

Table 5.4 Overview of the observed effects of altered gravity on human T cells cultured in vitro

Category	Effects
Apoptosis	Time-dependent increase in apoptosis-related factors (Cubano and Lewis 2000; Lewis et al. 1998). Increase of DNA fragmentation, PARP protein expression, and p53 and calpain mRNA levels; early increase of 5-LOX activity (Battista et al. 2012). Increased p53 phosphorylation (Paulsen et al. 2010). Inhibition of induced programmed cell death (Risin and Pellis 2001). Induction of DNA damage (Kumari et al. 2009)
1.1 Cell cycle regulation	Enhanced p21 protein expression, less cdc25C protein expression, and enhanced phosphorylation of cyclinB1 (Thiel et al. 2012)
1.2 Cell motility	Inhibition of PBMC locomotion (Pellis et al. 1997; Sundaresan et al. 2002)
1.3 Chromosomal instability	DNA replication was inhibited, therefore structural chromosome instability was enhanced (Wei et al. 2014)
1.4 Cytokine secretion	Suppressed IL-2 secretion (Cooper and Pellis 1998; Limouse et al. 1991; Risso et al. 2005; Crucian et al. 2000) and increased IFNγ secretion (Chapes et al. 1992). Reduced IFNγ production by $CD4^+$ T cell subset (Crucian et al. 2000). Reduced percentage of T cell subsets producing IL-2 and/or IFNγ during short resp. long-term spaceflight (Crucian et al. 2008). Strongly increased IFNγ (Bechler et al. 1992; Cogoli et al. 1993) and IL-2 (Cogoli et al. 1993) production of cells attached to microcarrier beads. Suppressed IFNγ secretion, but reversible (Cooper and Pellis 1998)
1.5 Cytoskeleton	Structural changes of intermediate filaments of vimentin and of the microtubule network (Lewis et al. 1998; Cogoli-Greuter et al. 1997; Sciola et al. 1999; Schatten et al. 2001)
1.6 Epigenetic changes	Differential DNA methylation and chromatin histone modification (Singh et al. 2010)
1.7 Gene expression	Differential expression of genes involved in DNA repair, cell cycle, cell growth, metabolism, signal transduction, adhesion, transcription, apoptosis, tumor suppression, immune response, cell activation, proliferation and differentiation, protein folding, transport and degradation, cytoskeleton, stress response, and apoptosis (Thiel et al. 2012; Chang et al. 2012; Boonyaratanakornkit et al. 2005; Ward et al. 2006; Sundaresan and Pellis 2009; Kumari et al. 2009; Lewis et al. 2001)
1.8 miRNA expression	Altered miRNA expression influencing genes involved in regulation of NF-kB-related signaling network (Mangala et al. 2011). 42 differentially expressed miRNAs in RWV-incubated lymphocytes whereof miR-9-5p, miR-9-3p, miR-155-5p, miR-150-3p, and miR-378-3p were the most dysregulated (Girardi et al. 2014). Suppressed expression of miR-21 after 1.5 h T cell activation correlating with 17 downregulated genes defined as targets of miR-21 (Hughes-Fulford et al. 2015)
1.9 Mitochondria distribution	Mitochondria clustering and morphological alterations of mitochondrial cristae (Schatten et al. 2001)
1.10 PKC distribution	Altered distribution of PKC isoforms to particular cell fractions (Hatton et al. 2002; Schmitt et al. 1996; Galleri et al. 2002)

(continued)

Table 5.4 (continued)

Category	Effects
1.11 Signaling	Hypergravity-induced dysregulation of several key signal proteins involved in early TCR signaling (Tauber et al. 2013, 2015). Enhanced phosphorylation of the MAP kinases and inhibition of NF-kB translocation into nucleus during simulated microgravity (Paulsen et al. 2010). Higher calcium concentration in CD69/PMA stimulated cells (Risso et al. 2005). Calcium signaling remains active, activation of fos and NF-kB is inhibited (Morrow 2006)
1.12 Surface receptor expression	Reduced surface expression of CD25 and CD69 (Cooper and Pellis 1998; Hashemi et al. 1999). Retarded TCR internalization (Hashemi et al. 1999). Suppressed CD3 and IL-2R-surface receptor expression (Tauber et al. 2015)

Modified from Hauschild et al. (2014)

surface receptors. Moreover, different basal media for culturing of the T cells were used which were supplemented in most cases also with different additives in varying concentrations. In nearly all present studies, fetal bovine serum (FBS) has been added to the T cell culture medium ranging from 10 to 20 %.

However, the chemical composition of serum is highly variable and ill defined. Since it contains a large number of constituents, including biomolecules with a variety of growth-promoting and growth-inhibiting activities, the use of serum in cell culture media has obviously considerable effects on phenotypic and genotypic cell stability. Furthermore, the concentrations of the components vary not only from manufacturer to manufacturer but also from batch to batch. Thus, cell signaling, cell proliferation, and differentiation and of course gene expression are influenced by the varying components in different serum used for different experiments.

Therefore, the comparability between the various studies that have been carried out, in order to obtain an overall picture and to locate possible fundamental primary microgravity-induced mechanisms, is not reliable. Nowadays, this lack of standardization has to be regarded as unacceptable. Maintaining high-level standards is of fundamental importance for ensuring good scientific practice in order to maximize reproducibility, reliability, acceptance, and successful implementation of results. Moreover, since scientific research in the area of gravitational science is extremely expensive and elaborate, their resources should be spent wisely. Therefore, in order to achieve the highest level of reliability and comparability of the results, gravitational-related immunobiological research should benefit to a large extent from the latest technology for the standardization of cell and tissue cultures and the development of chemically defined media.

The knowledge of the effects of gravitational changes on T cell regulation and the identification of gravity-sensitive cell responses will help to understand the molecular mechanisms of the inhibited immune cell function in altered gravity and thus new targets for therapeutic or preventive interventions with respect to the immune system of astronauts during long-term space missions may be developed (Ullrich and Thiel 2012).

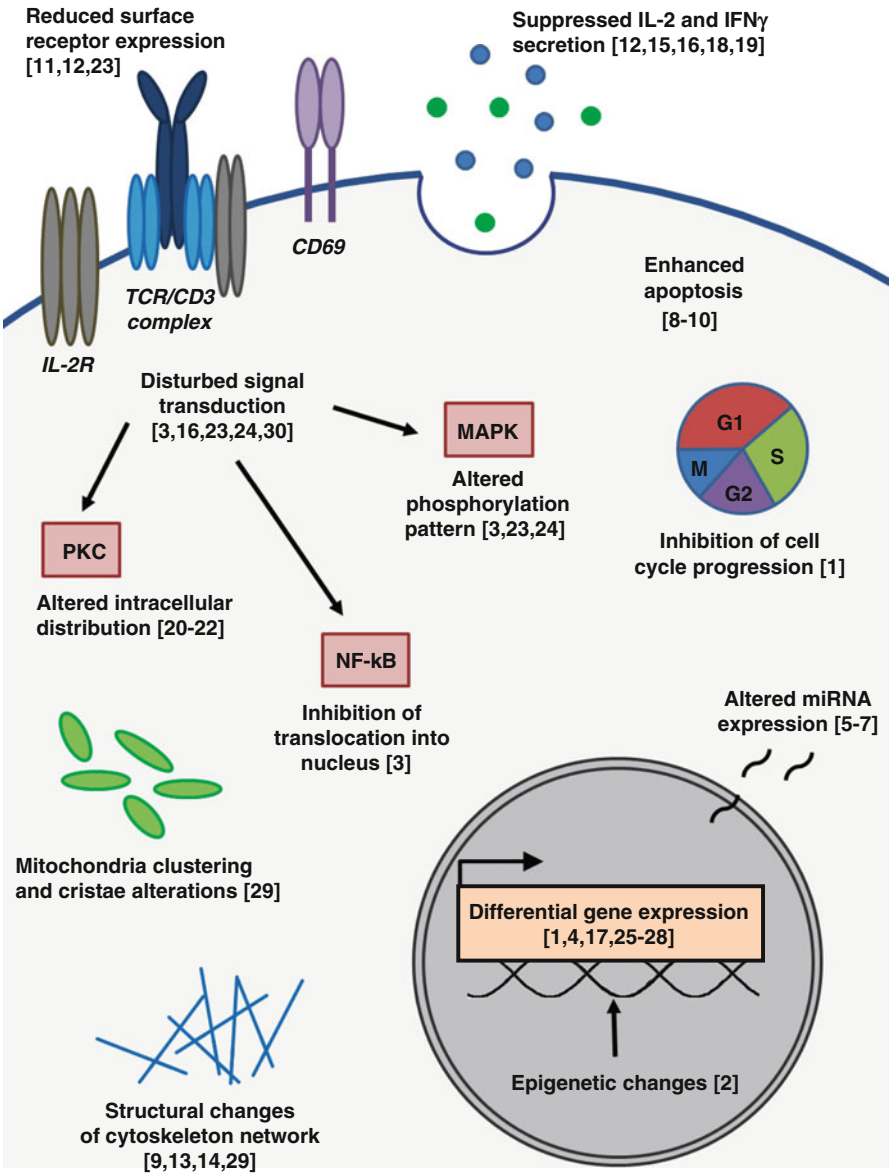

Fig. 5.1 Schematic summary of the influence of microgravity on T lymphocyte function (Modified from Hauschild et al. 2014). *1* Thiel et al. (2012), *2* Singh et al. (2010), *3* Paulsen et al. (2010), *4* Chang et al. (2012), *5* Mangala et al. (2011), *6* Girardi et al. (2014), *7* Hughes-Fulford et al. (2015), *8* Cubano and Lewis (2000), *9* Lewis et al. (1998), *10* Battista et al. (2012), *11* Hashemi et al. (1999), *12* Cooper and Pellis (1998), *13* Cogoli-Greuter et al. (1997), *14* Sciola et al. (1999), *15* Limouse et al. (1991), *16* Risso et al. (2005), *17* Boonyaratanakornkit et al. (2005), *18* Crucian et al. (2000), *19* Crucian et al. (2008), *20* Hatton et al. (2002), *21* Schmitt et al. (1996), *22* Galleri et al. (2002), *23* Tauber et al. (2015), *24* Tauber et al. (2013), *25* Ward et al. (2006), *26* Sundaresan and Pellis (2009), *27* Kumari et al. (2009), *28* Lewis et al. (2001), *29* Cogoli et al. (1985a, b), *30* Simons et al. (2010)

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Chapter 6
Spacecraft Microbiology

Beatrice Astrid Lauber, Olga Bolshakova, and Oliver Ullrich

6.1 Introduction

During spaceflights, the immune system is one of the most affected systems of the human body (Ullrich and Paulsen 2011). To determine the medical risks of long-term spaceflights and to develop prophylactic and therapeutic arrangements, it is important to know the microbial flora on board of a spacecraft or space station and its specific factors influencing this microflora. It is well known from several space missions that crew members suffered from bacterial and viral infections like influenza, *Pseudomonas aeruginosa*, and B streptococci. Also on long-term habitation on space station Mir and ISS, astronauts suffered from acute airway infections, conjunctivitis, and dental infections, and also reactivation of the Epstein-Barr virus was observed. An overview of microbial infections, pathogens, and general observations is given in Table 6.1. Figure 6.1 shows the variables impacting the risk of infections and their transmission during space travel, on which the following headings are related to.

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Table 6.1 Microbiological situation aboard of spacecrafts and space stations

Microbial pathogens, infections, and colonizations	Spacecraft
Occurrence of conjunctivitis, dental infections, acute respiratory infections (Ball and Evans 2001)	Mir
Occurrence of different bacterial and viral infections with influenza, *Pseudomonas aeruginos*a, B streptococci (Sonnenfeld 2002)	Apollo
Reactivation of Epstein-Barr virus (Pierson et al. 2005)	Space Shuttle
Changes in intestinal (Lencner et al. 1984), oral (Brown et al. 1976), and nasal (Decelle and Taylor 1976) microflora	Skylab Apollo Soviet Cosmonauts
Decrease of apathogen and increase of pathogen bacteria in nasal flora (Nefedov et al. 1971)	Soviet Cosmonauts
Breathing air, *Staphylococcus* sp., *Aspergillus* sp., *Penicillium* sp.; water, *Sphingomonas* sp., *Methylobacterium* sp.; surfaces, *Staphylococcus* sp., *Aspergillus* sp., *Cladosporium* sp. (Novikova et al. 2006)	ISS
Gram-positive and gram-negative microorganisms, Actinomyces and fungi; potable water, evidence of DNA pathogenic microorganisms (La Duc et al. 2004)	ISS
Activation of opportunistic pathogens, increased density of aerobic gram-negative bacteria and staphylococci on skin, in upper airways and colon (Ilyin 2005)	Salyut, Mir
58 forms of bacteria, 36 forms of molds and yeasts, significant percentage of pathogenic microorganisms, many of them with biodestructive properties (Viktorov et al. 1992)	Mir
Condensed water: *Serratia liquefaciens*, *Yersinia enterocolitica*, *Pseudomonadaceae*, *Stenotrophomonas maltophilia* (Harada 2001)	Mir
Condensed water: *Escherichia coli*, *Serratia marcescens*, amoeba resembling *Acanthamoeba* or *Hartmannella species* (Ott et al. 2004)	Mir
108 forms of bacteria, 206 forms of fungi, among them pathogenic and material destructive forms (Novikova 2004)	Mir
Expansion of *Penicillium chrysogenum* (Viktorov et al. 1998)	Mir
Potential biodegradation of polymers (Novikova 2004)	Mir
Higher caries incidence in astronauts (*Streptococcus mutans*) (Cheng et al. 2014)	ISS

ISS International Space Station, *DNA* deoxyribonucleic acid

6.2 Microbial Colonization of Spacecrafts

In an orbiting spacecraft, airborne microorganisms (and dust) do not settle due to the absence of gravity, and thermodiffusion or electrostatic forces gain in importance. This results in a more persistent (bio)aerosol and higher microbial contamination level in cabin air, and thus a continuous active removal of the aerosols from the air is necessary (Van Houdt et al. 2012).

On board of the Soviet space station Mir, several microbial studies were established during its operating time from 1986 to 2001 (Table 6.1). In one study, 58 forms of bacteria and 36 forms of mold and yeast forms were found, of which a significant part were pathogen microorganisms (Viktorov et al. 1992). Fungi types with material destructive properties were also identified. Another study found 108 types of bacteria

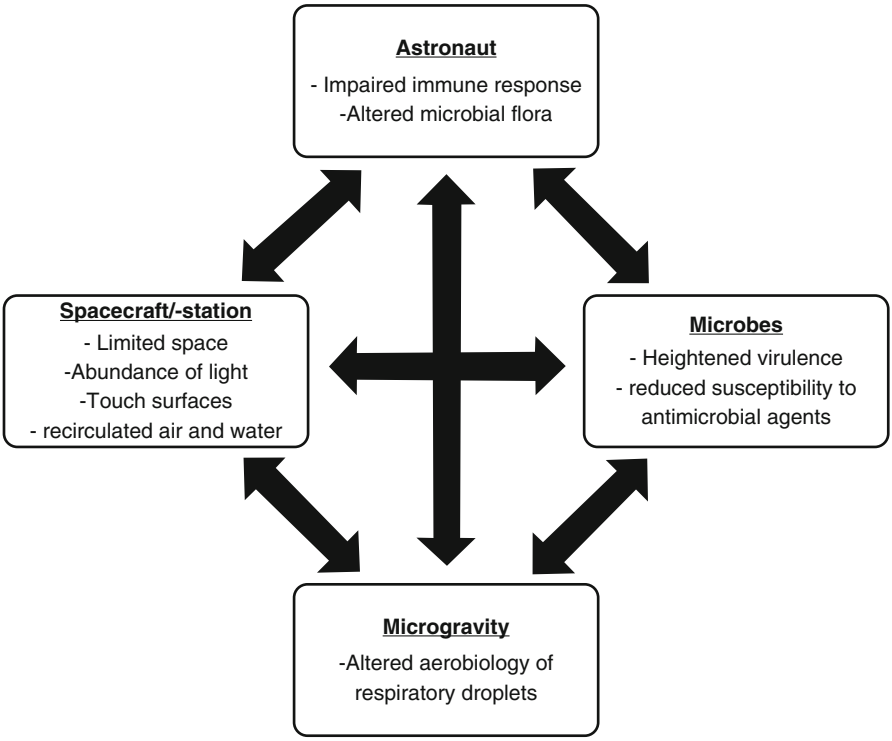

Fig. 6.1 Variables impacting the risk of infections and their transmission during space travel (Modified from Mermel 2012)

and 206 types of fungi, again with many pathogen and/or material destructive types among them (Novikova 2004). Condensed water was contaminated with *Serratia liquefaciens*, *Yersinia enterocolitica*, and *Stenotrophomonas maltophilia* and even radioresistant bacteria (Ott et al. 2004). Examination of optically hazy condensed water from behind instrumental panels aboard of the Mir revealed enterobacteria, *Escherichia coli*, *Serratia marcescens*, *Legionella* sp., spirochetes, protozoa, and mites (Ott et al. 2004). Bacteriofungal associations primarily resided on surfaces and structural materials of space interiors and equipment which gather anthropogenic organic compounds and air condensate enough to allow a full vegetative cycle and reproduction of heterotrophic microorganisms and molds (Novikova 2004). The microbial loading dynamic did not have linearly progressing character within the isolated environment of the Mir, but it was a wavy process of alternations of the microflora with changes of the dominating species (Novikova 2004). Fluctuating alterations in solar activity, degree of radiation, and gradients of magnetic fields can be considered parameters capable of initiating quantitative variations in the microflora of the space station (Novikova 2004). Also the ISS is by now severely colonized by microorganisms (Table 6.1): a 6-year study about the microbial environment on board of the ISS revealed that *Staphylococcus* sp., *Aspergillus* sp., and *Penicillium* sp. within the breathing air, *Sphingomonas* sp. and *Methylobacterium* sp. within the

Fig. 6.2 Mold on panel (ISS) (Ott et al. 2004)

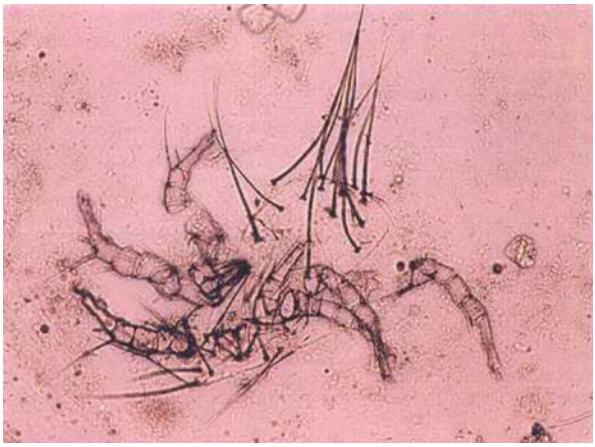

Fig. 6.3 Dust mites, free condensate (Mir) (Ott et al. 2004)

water, and *Staphylococcus* sp., *Aspergillus* sp., and *Cladosporium* sp. on surfaces were dominating, respectively (Novikova et al. 2006). Examinations with cultivation-independent verification procedures revealed many gram-positive and gram-negative microorganisms, *Actinomyces* and fungi (Ullrich and Paulsen 2011). Even within the drinking water, pathogenic microorganisms were found. The identified spectrum of bacterial and fungal species of the ISS was very similar to the spectrum on board the Mir (Table 6.1). Additionally, the main species of bacteria and fungi as found on 15-year-old Mir are the same as those on board of the space shuttle (Pierson 2001). It must be assumed that each and every spacecraft or station is microbially contaminated with comparable spectra as soon as it was in contact with human beings. This leads to the conclusion that the primary source of the contamination is neither the spacecraft nor the food or water brought along but mainly the endogen flora of the astronauts (Ullrich and Paulsen 2011) (Figs. 6.2, 6.3, 6.4, and 6.5).

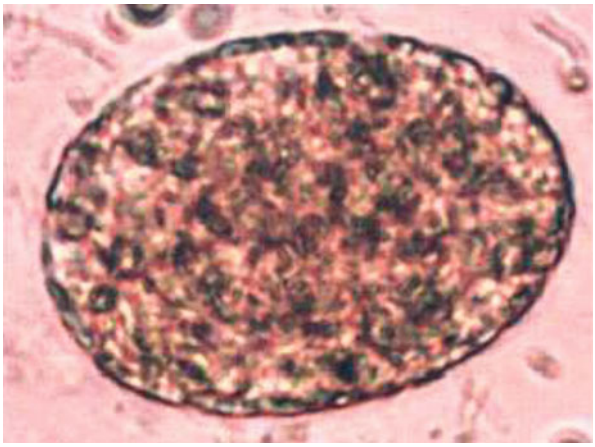

Fig. 6.4 Amoeba, free condensate (Mir) (Ott et al. 2004)

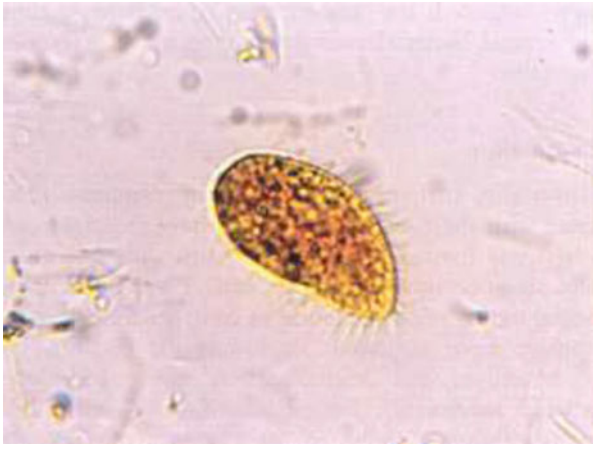

Fig. 6.5 Ciliated protozoa, free condensate (Mir) (Ott et al. 2004)

6.3 Alterations Within the Microflora of Crew Members

Investigations on crew members of the Apollo and Skylab missions and also on Soviet cosmonauts revealed that there were fundamental changes in the intestinal, oral, and nasal microflora under spaceflight conditions. Within the nasal flora, a decrease of apathogen and an increase of pathogen bacteria were found (Nefedov et al. 1971). The causation of this change can be found on the one hand by the crew members themselves, because under the condition of the isolation on board, an obviously considerable mutual exchange of microorganisms occurs between them. This exchange is not only affecting the upper respiratory tract but also intestinal

Table 6.2 Effects of space flight conditions on microorganisms

Effect	Spacecraft/Mission
Increased growth rate of *Chlamydomonas monoica* (Van den Ende and Van den Briel 1997)	Foton 1
Shortened lag phase of *Escherichia. coli* (Bouloc and D'Ari 1991)	STS-65, IML-2
Increased virulence of *Salmonella typhimurium* (Wilson et al. 2007)	STS-115
Faster growth rate, increased virulence, and raised resistance of *Salmonella typhimurium* (Nickerson et al. 2000)	Simulated microgravity
Increased virulence and resistance against antibiotics, tetracycline resistance of coliform bacteria (Klaus and Howard 2006)	Several
Development of resistant *E. coli* (Tixador et al. 1992)	Salyut 7
Faster growth and raised resistance against antibiotics of *E. coli* (Tixador et al. 1985)	STS-61-A, Spacelab D1
Severe increase of denseness of the cell wall of *Staphylococcus aureus* (Lapchine et al. 1986)	STS-61-A, Spacelab D1
Significantly increased mutation rate of bacterial ribosomal genes (Fukuda et al. 2000)	Mir
Enhanced acid tolerance ability, modified biofilm architecture and extracellular polysaccharide distribution of *Streptococcus mutans*, increase of proportion of *S. mutans* within dual-species biofilm (Cheng et al. 2014)	Simulated microgravity
Adoption to anaerobic mode of growth with denitrification of *Pseudomonas aeruginosa* (Crabbe et al. 2011)	ISS

STS Space Transport System, *ISS* International Space Station

bacteria (Taylor and Sommer 2005). The intestinal flora changes a lot, and after 2 weeks of space flight, the amount of detectable bacteria from the gastrointestinal tract is decreasing significantly (Taylor and Sommer 2005). The food on board could be a further reason, because the continuous consumption of sterilized, dehydrated nourishment leads to a rapid decrease in the amount of bifidobacteria and lactobacilli and is therefore promoting the expansion of resistance against antimicrobial drugs and the infection with opportunistic agents (Taylor and Sommer 2005). The construction of spacecraft and components of space stations under clean room conditions is followed by a settlement of the flora brought in by the crew. This is proven by the fact that microorganisms in the air and on surfaces are derived from the crew members (Makimura et al. 2011).

6.4 Raised Resistance Under Space Flight Conditions

Space flight conditions seam to alter the properties of many microorganisms (Table 6.2): on board of spacecrafts, an enhancement of the microbial proliferation, an altered microbial flora, an increased virulence, and a decreased effectiveness of antimicrobial drugs can be reported (Juergensmeyer et al. 1999; Leys et al. 2004). The alteration of susceptibility or resistance to antibiotics is very different,

and the resistance effect is quickly lost upon return to earth. Each bacterial species responds differently to the suite of antibiotics, frequently becoming less resistant but occasionally even more resistant to antibiotics (Juergensmeyer et al. 1999). Bacteria seem better to be able to protruse stressors like changes in osmolarity, pH, temperature, and antimicrobial substances in absence of gravity (Rosenzweig et al. 2010). In weightlessness, a thickening of the cell wall of bacteria could be observed, which showed reversible after returning to terrestrial environment. The decreased stress on surfaces of microorganisms in microgravity can directly alter gene expression and affect physiological functions. In *Salmonella typhimurium*, for example, mechanisms associated with microgravity are mediated by the RNA chaperone. Hfq is a global transcriptional regulator, which plays an important role in the translation in answer to "envelope stress" and environmental stress (Wilson et al. 2007; Crabbe et al. 2011) (see also chapter 1). This chaperone is evolutionary highly conserved and could absolutely be one of the basic principles of the molecular mediation of changes in gravity on cells. Hfq even represents the first spaceflight-induced regulator acting across bacterial species (Crabbe et al. 2011). In addition to the influence of gravity acting on microorganisms on board of a spacecraft, also high doses of cosmic rays do cause an increase in mutation frequency (Horneck et al. 2010). In general it can be said that space conditions may significantly increase the mutation frequency of certain genes in microorganisms (Su et al. 2013). Spaceflight conditions therefore lead most likely to increased proliferation and selection of bacteria that are better adapted to microgravity and to the special environment of a spacecraft or space station (Juergensmeyer et al. 1999; Leys et al. 2004). In addition to these processes of adaptation, the bacterial phenotype trained in weightlessness seems to be particularly resistant to environmental influences. Unlike human cells, such as cells of the immune system (Ullrich and Thiel 2012), bacteria seem to be well prepared for a life under space conditions.

6.5 Material Damage Due to Microbial Contamination

Not only human health is affected by the microbial flora on board, but also the spacecraft, equipment, and different materials can be colonized or even degraded or inhibited in function by fungi or bacterial biofilms. An overview is summarized in Table 6.1, contaminations and control mechanisms in the chapter about contamination monitoring and control. Among the proven microorganisms on the Mir were many species with biodestructive properties that significantly damaged the cabin interior, the plastic seals, cables, and lighting (Novikova 2004; Van Houdt et al. 2012; Viktorov et al. 1992). For example, an expansion of *Penicillium chrysogenum* was observed, a material degrading, and biodestructive fungus (Viktorov et al. 1998). Thin biofilms, which are able to degrade many materials occurring on the ISS, are mostly formed at interfaces (Gu et al. 1998). Bacteria organized in biofilms show a very solid resistance against antibiotics (Mah and O'Toole 2001).

6.6 Conclusion

The ultimate target and attraction to explore the universe remain in human beings to discover and experience space, despite the benefits of using robots. The spaceship or space station that will be the home for a quite long time for future astronauts and the understanding of its microbial environment play a crucial role in making any space intention a success (Nicogossian and Gaiser 1992).

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Part II
The Upcoming Venues and New Perspectives

Chapter 7
Spacecraft Contamination Monitoring and Control

Beatrice Astrid Lauber and Oliver Ullrich

Spacecrafts and space stations are exceptional work and living places with unique conditions for the astronauts and equipment including high working pressure, defined diet and restricted hygienic practices, microgravity, and radiation. All of these influence the microflora on board and within humans. Special circumstances need special actions to keep people healthy and spacecraft and space station free from pathogens as far as possible.

7.1 Contamination Monitoring and Control

The possibly affected and contaminated materials and parts of the environment are the air within the spacecraft; all surfaces; the water, if it is for drinking or for cooling or for cleaning; the food that is taken aboard; and, of course, the human beings of the crew. A summary of these affected objects, affecting contaminations and control mechanisms, is shown in Table 7.1.

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Table 7.1 Affected objects, contaminations, and control mechanisms

Object/ spacecraft	Agents	Control/monitoring	Pro/contra
Air			
Mir	Staphylococci, micrococci, and coryneform bacteria: highest occurrence: *Staphylococcus*, *Bacillus*, *Corynebacterium* (Novikova 2004)	System "Potok 150-MK" designed to remove aerosol particles and microorganisms from air	Inactivation using electrostatic pulses and charged ions followed by filtration filters in general have to be replaced periodically
Mir ISS	Opportunistic pathogens: *Staphylococcus aureus*, *S. capitis*, *S. haemoliticus*, *Flavobacterium meningosepticum*, *Escherichia coli*, *Serratia marcescens*, *Streptococcus sp*., *Bacillus cereus* (Novikova 2004) atmosphere recycling	Russian Ecosphere kit included air sampler SAS (FBI product) to collect air by way of aspiration-sedimentation, and Petri dishes with nutrient media Veggie Space Hardware (Massa et al. 2013)	Cheap when established, additional food supply
ISS	Comparable	High-efficiency (HEPA) filters, pleated woven filters for Russian segment (Van Houdt et al. 2012)	Do not inactivate microbial cells
ISS	Comparable	POTOK 150 MK Russian air filtration and disinfection systems (Van Houdt et al. 2012)	Inactivation using electrostatic pulses and charged ions followed by filtration
ISS	Bacterial and fungal cultures in general	ENose	Already tested and established
Surfaces			
Mir	Dominated: *Penicillium*, *Aspergillus*, *Cladosporium sp*.: *Penicillium expansum*, *Penicillium chrysogenum*, *Cladosporium cladosporioides, Aspergillus sp*. of group *A versicolor*, *Aspergillus versicolor, Aspergillus niger* (Novikova 2004)	Molds: amenable to drying-out, rubbing dry, use of sanitary means	Must be repeated
ISS	*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis, Enterococcus faecium* (Guridi et al. 2015)	AgXX (microgalvanic elements formed by silver and ruthenium with an electroplated silver coating applied onto a V2A stainless steel surface)	Antimicrobial effect superior to that of conventional silver coatings, avoids toxic side effects of high levels of silver ions on eukaryotic cells, very long service life, no external energy used

Table 7.1 (continued)

Object/ spacecraft	Agents	Control/monitoring	Pro/contra
ISS	Biofilm-forming bacteria like *Staphylococcus* and *Bacillus* bacterial species and Penicillium and *Aspergillus* fungal species predominant (Van Houdt et al. 2012)	Either quaternary ammonium compound (supplied by USA) or mixture of hydrogen peroxide and quaternary ammonium compound (supplied by Russia) (Van Houdt et al. 2012)	When cleaning fails repeatedly, removal and replacement of the contaminated surfaces is final countermeasure
ISS	General	Humidity condensate is collected and purified (Van Houdt et al. 2012)	Keeps surfaces dry and water is recycled (e.g., for drinking)
Space vehicles	General	Adenosine triphosphate (ATP) analysis for detection purposes (Birmele et al. 2011)	Fast, no dilutants required
All	General	Preflight: heat, radiation, chemicals (depending on surface-cleaning method compatibility) (Van Houdt et al. 2012)	
All	General	Antimicrobial surface properties (Van Houdt et al. 2012)	May reduce adherence and biofilm formation
Water			
ISS	Dominant: *Methylobacterium*, *Ralstonia*, *Sphingomonas*, *Pseudomonas spp*. (Van Houdt et al. 2012)	Supply of ground-supplied water	Very expensive
ISS	Contamination of water	Addition of silver pre- and in-flight or addition of iodine, which is removed and replaced by silver before consumption	Quality check every month on board and collected for preflight tests
ISS	Contamination of water	UV-A (plus titanium dioxide) LEDs for disinfecting potable water systems (Birmele et al. 2011)	Effective
Food			
ISS	Food commensals (Van Houdt et al. 2012)	Sterilization	Production and packaging rigorously tested and controlled preflight

(continued)

Table 7.1 (continued)

Object/ spacecraft	Agents	Control/monitoring	Pro/contra
ISS	Food commensals	Hazard Analysis Critical Control Point (HACCP) management system (Van Houdt et al. 2012)	
ISS	Food commensals	Gamma irradiation (Mermel 2012)	
Human			
Mir and ISS	*Staphylococcus* (*S. aureus*), *Micrococcus*, *Enterobacter*, *and Bacillus species Aspergillus* and *Penicillium species* → conjunctivitis, infections of upper respiratory tract (Ball and Evans 2001)	Antibiotics broad range	Facilitate emergence and dissemination of antibiotic resistance genes and alter genetic makeup of bacterial populations limited diagnostic feasibility on board
Mir and ISS		Tetrapyrrole dyes such as porphyrins, phthalocyanines, and bacteriochlorins are able to accumulate in and be selectively retained by abnormal cells and by bacteria → Activation of these molecules with visible light in the presence of oxygen leads to the destruction of the target tissue (Taylor and Sommer 2005)	Effective, no resistance, effective against multidrug-resistant bacteria and biofilms
Mir and ISS		Bacteriophages as therapeutic agents (Taylor and Sommer 2005)	Very specific, but not yet well established. Resistance can emerge
Mir and ISS		New antimicrobials (antibiotic efflux pump inhibitors and drugs addressing virulence) (Klaus and Howard 2006)	Specific, increased antibiotic susceptibility, no resistance
Mir and ISS		Hfq (RNA-binding protein) as new drug target (Su et al. 2013)	Specific, addresses virulence
Mir and ISS		Robust vaccination program preflight (Mermel 2012)	

FBI Federal Bureau of Investigation, *HEPA* high-efficiency particulate arrestance, *ENose e*lectronical nose, *ATP* adenosine triphosphate, *UV* ultraviolet, *LED* light-emitting diode, *HACCP* Hazard Analysis Critical Control Point, *RNA* ribonucleic acid

7.1.1 Air

Airborne microorganisms can be dispersed through routes like talking, coughing, sneezing, and movement, and can cause irritation of mucous membranes, respiratory infections, and allergic diseases. Additionally, the aerobiology of respiratory droplets is altered in microgravity. In spacecrafts or space stations, the air has to be filtered continuously to keep microbial levels remaining below thresholds and to prevent the spread of microorganisms through aerosols.

There are new approaches to grow vegetables on spacecrafts. The goal of the "Veggie Space Hardware" concept is to grow healthy plants that can provide crew for food production and atmosphere recycling (Massa et al. 2013). This is a prospective approach with two benefits, one for the air and the other for the food supply.

The ENose (Ryan et al. 2004) is an electronic gas sensor system to detect microbial pollution online. Microbial contaminations produced by bacteria or fungi can be recognized in a qualitative and quantitative manner. Tests can be done at any place of the ISS to measure pollution. There is an additional, so-called target-book, with different fixed material probes like aluminum, platinum, and isolation material (of electronic devices and of the ISS in general) to test for settlement of bacterial or fungal colonies on these materials. The target-book can be periodically sent back to earth for analysis. MVOCs (microbial volatile organic compounds), produced by metabolism of biological cultures, can be detected and used to generate an olfactory fingerprint.

The ENose was first tested on the STS-95 (Ryan et al. 2004) and then successfully improved and supported by DLR and used on the ISS.

7.1.2 Surfaces

Many microorganisms are able to adhere to most surfaces and form biofilms. This process promotes persistence and resilience of microbial contamination and may have major implications for many industrial activities (Van Houdt et al. 2012). Equipment and utensils should be resistant to thermal, mechanical, and chemical factors and surfaces should exhibit antimicrobial properties. A rational design of spacecraft and space station integrating and respecting both functional and health-related criteria is fundamental. A very new approach, for example, is surface coating based on microgalvanic elements formed by silver and ruthenium with surface catalytic properties (AgXX®) (Guridi et al. 2015). An advantage of this new method lies in avoiding the toxic side effects of high levels of silver ions on eukaryotic cells. Additionally, the antimicrobial effect of the thin AgXX® coatings (3–5 μm) is not dependent on any substance released from the coating material; therefore, the lifetime of AgXX® is only limited by mechanical destruction of the coating. AgXX® was successfully tested on the ISS (Clauß-Lendzian et al. 2015).

Other approaches, not yet tested in space, but in the field of industry, could also be interesting and useful for spacecraft settings in the future. An example from food industry is polyvinyl acetate, which seems to be ideal as matrix-like carrier material for antimicrobial substances and forms a clear film on the coated surface. Especially benzoic acid and sorbic acid are accredited for food packaging and plastic and additionally offer a wide range of antimicrobic properties (against molds, many bacteria and yeasts). The antimicrobial substance is mixed with the fluid polyvinyl acetate and the mixture is used to coat technical surfaces (Sandmeier and Kensbock 2008). A new technology for antimicrobial furniture comprises a biozidal substance including at least one molybdenum-containing compound and a metal oxide. This coating can be used prospectively for furniture in hospitals, kitchens, bathroom, or plumbing unit floors or other technical surfaces. Molybdenum-containing compounds are not toxic to eukaryotic cells (Guggenbichler and Walter 2015).

7.1.3 Water

Microbial contamination of drinking water is a well-known hazard, both from a health perspective and for microbial-mediated corrosion, and is often accompanied by biofilm formation (Van Houdt et al. 2012). Biofilm formation increases persistence of pathogens and increased resistance to disinfectants. Water is recycled and cleaned with filters and addition of silver or with UV-A exposure (Birmele et al. 2011).

7.1.4 Human

Astronauts are vulnerable through their own commensals, through air pollution and through pathogens in drinking water, on surfaces, and even in their food. They can suffer from microbial infections, including conjunctivitis and acute respiratory and dental infections. In addition, injury and trauma, such as lacerations and open fractures, are likely to occur on long missions and will require prophylactic administration of antibiotics to prevent serious wound infection (Klaus and Howard 2006). Known problems are multidrug-resistant bacteria and biofilms. Additionally, antibiotics in turn influence the composition of intestinal and respiratory microflora in a delicate way. Besides unclear factors of pharmacodynamics, it is also expected that the bioavailability of drugs in weightlessness is less present (Lathers et al. 1989). Antibiotic therapy according to terrestrial standards will therefore be possible only with difficulty under spaceflight conditions. Antimicrobial prophylaxis and therapy should therefore follow new approaches. Conceivable would be the use of photosensitive molecules like porphyrins, phthalocyanines, and bacteriochlorins, which, in presence of visible light and oxygen are activated and destroy target tissues like topic infections or even biofilms (Taylor and Sommer 2005). Newer antimicrobials

that inhibit antibiotic efflux pump activity, or modifying a drug so that it is not recognized by the efflux pump, might also increase antibiotic susceptibility, as addressing virulence might do. Additionally, resistance might not develop as quickly as it does for current drugs, because virulence factors are not usually necessary for survival (Klaus and Howard 2006). Also, a well-planned preflight vaccination program may support health (Mermel 2012).

7.2 Conclusion: The Future Space Habitat

In respect to human risk, the importance of monitoring is lower when fast return of the crew is possible, but very important for long-duration missions. Considering economics, the importance is high, as significant losses are linked to up- and download of replacement hardware and water supplies, which were lost for consumption due to contamination.

The THESEUS disciplinary reports (THESEUS Cluster 4 Report 2012) constructed by THESEUS expert groups debate and summarize problems and approaches of human space exploration. One of the goals must be to acquire better knowledge on microbial community and ecosystem dynamics and microbial cell evolution over time in confined manned habitats in space. Another task is to develop efficient materials and methods to prevent environmental microbial contamination and to develop adequate environmental contamination monitoring (prediction, detection, identification) systems for use in space (THESEUS Cluster 4 Report 2012).

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Chapter 8
Cell-Based Therapy During Exploration Class Missions

Liliana E. Layer and Oliver Ullrich

8.1 Unrestricted Somatic Stem Cells from Umbilical Cord Blood

During long-term exploration missions as planned with the Orion MPCV (Fig. 8.1), illnesses of the cardiovascular system should be taken into account. In fact, in-flight cardiac arrhythmia was already observed during the Apollo missions in the early 1970s (Hawkins and Zieglschmid 1975; Reitz et al. 1995). Irreversible myocardial damage is often the result of a myocardial infarction and due to the limited capacity of the damaged myocardium for self-repair and tissue regeneration (Pfeffer 1995). Currently, there is no therapy, except for cardiac transplantation, to replace the damaged myocardium with functioning, contractile tissue. In 2004, a multipotent stem cell population showing high proliferative potential was isolated from human umbilical cord blood. These cells were termed unrestricted somatic stem cells (USSCs) (Kogler et al. 2004). The intramyocardial injection of USSCs into pigs after myocardial infarction was shown to lead to engraftment of the transplanted cells in the infarct region and significantly improve ventricular function (Kim et al. 2005). These results suggested that the transplanted USSCs may have the potential to differentiate into mature cardiomyocytes and contribute to neovascularization.

Considering therapeutic application of USSCs in space exploration missions, further studies and development will be necessary to find other application means than intramyocardial injection of the cells and solve the potential immune rejection in the case of allogenic transplantation.

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Fig. 8.1 Illustration of the Orion MPCV in low Earth orbit during the Exploration Flight Test 1 on December 5, 2014 (Photo credit: NASA)

8.2 Adult Pluripotent Stem Cells Derived from Peripheral Blood Monocytes

In 2003, a yet unknown subset of human peripheral blood monocytes was found acting as pluripotent stem cells (PSCs) (Zhao et al. 2003). These cells, exhibiting a fibroblast-like morphology and hematopoietic stem cell markers including CD14, CD34, and CD45, were successfully induced to differentiate into macrophages, T lymphocytes, epithelial cells, endothelial cells, neuronal cells, and hepatocytes. The physiological function of these PSCs in our bodies is still unknown. However, due to their potential, they could facilitate tissue repair by replacing damaged somatic cells. This hypothesis was corroborated by a study in 2002, in which transplantation patients that underwent chemotherapy or radiation treatment received blood preparations enriched for CD34-positive cells (Korbling et al. 2002). These cells were shown to populate different tissues and differentiate into cells belonging to distinct lineages. This observation could be attributable to cells within the preparations belonging to the PSC type.

The ability to derive adult PSCs from an easily accessible source such as peripheral blood is an advantage over USSCs. This allows autologous transplantation from cell preparations performed before crew departure from Earth and thus circumvents potential immune rejection of the graft cells. Furthermore, PSCs derived from peripheral blood can be expanded *in vitro*, yielding high numbers of stem cells for transplantation. This makes adult PSCs valuable candidates for the treatment used to replenish immune cells that have been eradicated by cancer therapy or of damaged neuronal tissue like in spinal cord injury, stroke, or Parkinson's disease.

8.3 Cytokine-Induced Killer Cells

Cytokine-induced killer (CIK) cells are differentiated from peripheral blood mononuclear cells (PBMCs) by stimulation with a cocktail of interferon-gamma, anti-CD3 monoclonal antibody, and interleukin-2 (IL-2) in a stepwise, time-dependent *ex vivo* culturing process (Schmidt-Wolf et al. 1991, 1993). Following infusion, CIK cells are capable of migrating to the tumor tissue, recognizing it and killing the tumor cells via the natural killer group 2, member D (NKG2D, also termed killer cell lectin-like receptor subfamily K, member 1 (KLRK1) in humans) receptor-ligand-mediated release of perforin (Verneris et al. 2004; Karimi et al. 2005). The mechanism of recognition is major histocompatibility complex (MHC)-independent and relies on NKG2D ligand expression on the target cells (e.g., MICA, MICB and ULBP1-4), which is found on solid as well as hematological tumors (Groh et al. 1999; Salih et al. 2003; Pende et al. 2002). Therefore, a clinically relevant property of CIK cells is their reduced alloreactivity across major HLA-barriers that leads to a reduced risk for graft-versus-host disease (GVHD) (Sangiolo et al. 2008).

Several clinical trials have been conducted using CIK cells for the treatment of hematological malignancies (Leemhuis et al. 2005; Introna et al. 2007; Laport et al. 2011) and solid tumors (Schmidt-Wolf et al. 1999; Jakel et al. 2014). The results were very encouraging, albeit not providing sufficient evidence for the use of CIK cells as monotherapy. However, in the majority of cases, the adjunctive therapy with CIK cells along with conventional treatment was superior to conventional therapy alone. Furthermore, studies showed that after CIK cell application, the levels of CD3+, CD4+, CD3+ CD8+, CD3+ CD45RO+, and CD3+ CD56+ cell populations and the CD4+/CD8+ ratio in the patients' blood increased, indicating a boosting effect of cellular immunity induced by CIK cell transfusions (Jakel et al. 2014).

The combination of CIK cells with oncolytic vaccinia viruses led to even higher therapeutic efficiency due to synergistic effects (Thorne et al. 2006). Additionally, targeted delivery of vaccinia virus to the tumor by CIK cells can help to overcome the localized tumor immunosuppressive environment and increase immune cell infiltrates (Thorne et al. 2010).

Taken together, CIK cells represent a promising approach to in-flight cancer treatment. Moreover, since CIK cells have proven very effective in the erradication of residual cancer cells following conventional therapies as mentioned in the studies above, they could represent a reliable and almost side-effects-free way of cancer prevention during long-term space missions, if administered on a regular basis.

8.4 Lyophilization of Cells for Long-Term Storage of Human Cell Products

Storage of human cells for research or therapeutic purposes is traditionally conducted by freezing them in the presence of permeating cryoprotective agents like dimethyl sulfoxide (DMSO), glycerol, or ethylene glycol. This method has proven

very effective, but apart from being energetically too expensive for long-term space exploration missions and depending on freezing units that may fail, the cryoprotectants are often toxic to the cells and must be washed out immediately after thawing. An interesting alternative to storing cells frozen is the lyophilization (freeze-drying) and the possibility of storing them at ambient temperature subsequently (Fig. 8.2). Many plants and organisms are naturally capable of surviving lyophilization and have provided clues as to how this can be accomplished (Crowe et al. 1992). Most anhydrobiotic organisms accumulate high concentrations of disaccharides (mostly trehalose or sucrose) in their cells and tissues during drying (Crowe et al. 1984, 1992; Clegg 1965, 2001). Since trehalose is impermeant, several procedures have been investigated to introduce it into cells: electroporation (Shirakashi et al. 2002; Tsong 1991), harnessing channels in the plasma membrane (e.g., the ATP-stimulated P2-purinergic pore (Buchanan et al. 2005; Elliott et al. 2006) and α-hemolysin (Chen et al. 2001; Eroglu et al. 2000; Russo et al. 1997)), genetically engineering cells to synthesize their own trehalose (Guo et al. 2000), and thermally responsive Pluronic-based nanocapsules (Zhang et al. 2009). A more physiological way to introduce trehalose into cells is based on fluid-phase endocytosis including the disaccharide in the cell culture medium. It was proven to work efficiently for platelets (Wolkers et al. 2001) and mesenchymal stem cells (MSCs) (Oliver et al. 2004; Zhang et al. 2010). This biologically general process can most likely be transferred to many other cell types and was already shown to be applicable to several cell lines (Oliver 2012).

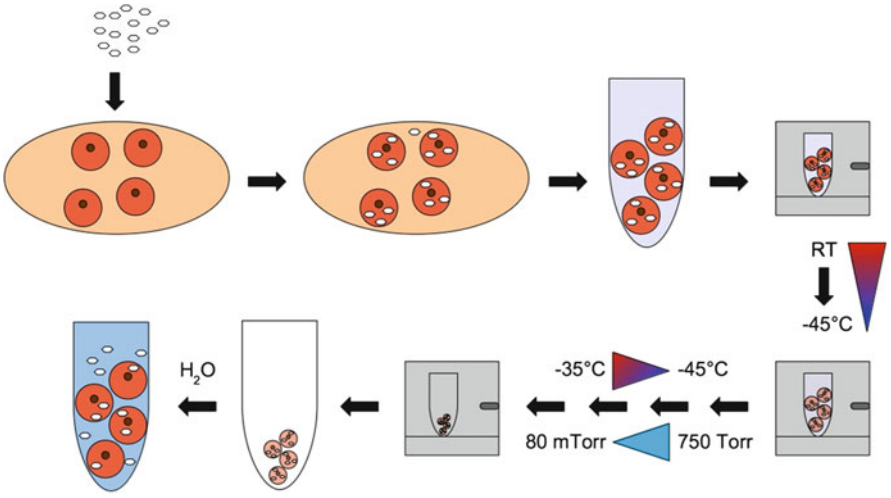

Fig. 8.2 Lyophilization process of cells as described in Buchanan et al. (2010). Trehalose is added to the cell culture medium and taken up by the cells. Subsequently, the cells are transferred to a lyophilization medium containing lyoprotectants and placed into a freeze-dryer. Then, the cells are frozen and lyophilized by the sublimation of water. As a result, lyophilized cells are obtained that can be resuscitated by the addition of water

In the following sections, a brief summary will be given presenting therapeutically relevant cells and tissues that have been successfully lyophilized and could be of interest in cell-based therapy during exploration class missions.

Blood Platelets Freeze-drying of therapeutic blood products was already used in World War II for the preservation of blood plasma as resuscitation fluid and for the restoration of coagulation deficiencies. However, blood plasma is cell-free. Successful dehydration and rehydration of cell-containing preparations is obviously much more complicated. Later in 1956, proagulant properties of a lyophilized preparation from extracted platelet suspension were reported (Klein et al. 1956). Finally, in 2001, the protection for lyophilization of human blood platelets was shown (Wolkers et al. 2001) and they were able to survive the desiccated state at room temperature for up to 2 years (Wolkers et al. 2002). Freeze-drying tolerance was conferred to the platelets by rapid uptake of trehalose at 37 °C. Analysis by Fourier transform infrared spectroscopy demonstrated that the membrane and protein components of platelets after freeze-drying and rehydration were very similar to those of fresh platelets (Wolkers et al. 2001).

Lyophilized blood platelets could be of interest for the treatment of coagulation deficiencies or severe traumata during space exploration missions.

Red Blood Cells Red blood cell (RBC) units are administered routinely to patients suffering from a wide range of acute and chronic conditions, for example, traumatic bleeding and anemia. These illnesses should definitely be considered when planning basic medical treatment for long-term space missions. Unfortunately, the shelf life of conventional, hypothermically stored RBC products is short, because of rapid depletion of adenosine triphosphate (ATP) and 2, 3-diphosphoglycerate (DPG) (Holovati et al. 2009). RBCs were already successfully lyophilized applying intracellular trehalose by osmotic shock reaching recovery rates of about 55 % (Satpathy et al. 2004; Török et al. 2005). By making use of synergistic effects of liposomes and trehalose for lyoprotection, recovery rates of even 70 % could be achieved (Kheirolomoom et al. 2005), and by using electroporation for intracellular trehalose delivery, recovery rates of 70.9 % were reached (Zhou et al. 2010). Employing a new radio frequency lyophilization device, a lyoprotectant solution containing trehalose and human serum albumin, as well as a rehydration solution with dextran, survival rates of RBCs were further increased to 75 % (Arav and Natan 2012). Despite these successes, lyophilized RBCs are still not being used regularly for therapeutical purposes.

Hematopoietic Progenitor Cells Hematopoietic progenitor cells (HPCs) contain committed progenitors and give rise to all blood cell types including lymphoid (T cells, B cells, NK cells, dendritic cells) and myeloid cells (monocytes/macrophages, neutrophils, megakaryocytes, granulocytes, eosinophils, erythrocytes) (Kondo et al. 2003). HPCs can be isolated from bone marrow, peripheral blood, or umbilical cord blood and have been shown valuable in the treatment

of a variety of diseases where bone marrow transplantations became necessary (Bhatia et al. 2005; Nademanee et al. 1994; Hadzantonis and O'Neill 1999; Voermans et al. 2001). Due to the high radiation exposure that is expected during a mission to Mars, in-flight bone marrow transplantations should be considered.

HPCs isolated from umbilical cord blood were loaded with trehalose using the endogenous purinergic cell surface receptor P2Z and lyophilized (Buchanan et al. 2010). Differentiation and clonogenic potential of the cells after single lyophilization steps and after complete lyophilization and subsequent storage for 4 weeks at 25 °C were assessed. Cells reconstituted immediately after lyophilization produced 40 % colony forming units of granulocyte/monocyte type (CFU-GM) relative to fresh HPCs, 40 % erythroid burst forming units (BFU-E), and 82 % colony forming units of granulocyte/erythrocyte/monocyte/megakaryocyte type (CFU-GEMM). Cells reconstituted after 28 days at room temperature produced 35 % CFU-GM relative to unprocessed controls, 26 % BFU-E, and 82 % CFU-GEMM. These studies demonstrate a high retention of functionality of HPCs after lyophilization and storage for 4 weeks at ambient temperature. Therefore, lyophilized HPCs can be regarded as a very promising approach to cell-based therapy using products with extended storage potential.

Mesenchymal Stem Cells from Bone Marrow MSCs are multipotent stem cells that can proliferate and differentiate into multiple lineages. Therefore, they are ideal stem cells for tissue engineering and candidates for the clinical treatment of various diseases including ischemic cardiomyopathy (Hua et al. 2015), bone degeneration (Asatrian et al. 2015), and spinal cord injury (Forostyak et al. 2013).

Fluid-phase endocytosis was employed to load MSCs with trehalose and polyvinylpyrrolidone (PVP) was applied as additional protectant (Zhang et al. 2010). PVP can inhibit sucrose crystallization and stabilize the glassy structure of sugar (Zeng et al. 2001). In that study, MSCs undergoing lyophilization had a recovery rate of up to 69 %. Unfortunately, the proliferation ability of rehydrated MSCs could not be shown. Nevertheless, due to their multipotency, MSCs are promising candidates for cell-based therapies and their potential in that field is currently being explored by many research groups worldwide.

Microencapsulated Human Retinal Pigment Epithelial Cells Microencapsulation is employed to prevent immunological rejection of therapeutic cells by the host allowing the bidirectional transfer of nutrients and cytokines across the capsule membrane at the same time. This concept has been implemented using alginates (Read et al. 2001; Joki et al. 2001; Cirone et al. 2004), hyaluronic acid (Bae et al. 2006), and polyethylene glycol (Wilson et al. 2008) as microencapsulation polymers. Therapeutic cell encapsulation has been proposed as potential treatment in many diseases, such as diabetes (Lim and Sun 1980; Elliott et al. 2007),

cancer (Lohr et al. 2001; Joki et al. 2001), Huntington's disease (Bloch et al. 2004), Parkinson's disease (Stover et al. 2005), cardiac recovery after infarct (Zhang et al. 2008), and retinal degenerations (Sieving et al. 2006).

Retinal pigment epithelial (RPE) cells are interesting candidates for cell encapsulation, because they produce dopamine (important in the treatment of Parkinson's disease) and neurotrophic proteins (Ming et al. 2009). Apart from that, normal RPE cells do not proliferate and remain functional for their entire lifetime, supporting long-term therapy. A genetically engineered and encapsulated RPE cell line (ARPE-19) was freeze-dried in polycation-coated alginate microcapsules and lyoprotective solutions (Wikstrom et al. 2012). The cells retained their viability and structural integrity during lyophilization and subsequent reconstitution.

Astronauts have reported having visual light flashes on orbit (Hughes-Fulford 2011), and space shuttle environment and simulated microgravity were shown to induce retinal degeneration (Tombran-Tink and Barnstable 2005; Roberts et al. 2006) by mitochondrial oxidative damage (Mao et al. 2013) [see also Chap. 3, part 3]. Therefore, lyophilized microencapsulated RPE cells constitute a promising treatment of retinal degenerations caused by spaceflight, if a feasible mode of application can be found.

Cultured Epidermal Sheets Cultured epidermal sheets (CES) have been used to treat cutaneous wounds such as burns and ulcers (Rheinwald and Green 1975; Green et al. 1979; Green 2008). When used as allogenic graft, the transplanted cells are not permanent, but serve as temporary wound dressing, releasing proteins involved in the proliferation and migration of keratinocytes and fibroblasts (Gurtner et al. 2008; Tamariz-Dominguez et al. 2002; Santoro and Gaudino 2005). Allogenic CES prepared from a cell bank have the advantage of being available much faster in an emergency case than autologous CES prepared from patients' skin biopsies. However, in order for the allogenic CES to be readily available, they have to be stored for some period of time without compromising their biological potential. To be considered for trauma treatment during space exploration class missions, storage should not be energy consuming.

CES were prepared from cultured keratinocytes, lyophilized and applied in wound and ulcer treatment (Jang et al. 2013; Navratilova et al. 2004; Slonkova et al. 2004). In all studies, the effectiveness of lyophilized CES (L-CES) could be proven. Apart from this, Jang et al. (Jang et al. 2013) could also show that L-CES, like fresh CES, consisted of three to four well-maintained epidermal layers, as shown by the expression of keratins, involucrin, and p63. They did not observe any differences in the epidermal layer or protein expression between L-CES and cryopreserved CES (F-CES), and both CES were comparable to fresh CES. In a mouse study, wounds treated with L-CES or F-CES completely healed by day 10, while untreated wounds did not heal by day 14 (Jang et al. 2013). These results clearly prove the usefulness of lyophilized CES in wound treatment and make a clinical application already possible.

8.5 Conclusion and Outlook

As early as during the Apollo missions, multiple changes of body functions were observed: vestibular disturbances, in-flight cardiac arrhythmia, reduced postflight orthostatic tolerance, postflight dehydration, and weight loss. Furthermore, a significant decrease in red blood cell mass and negative in-flight balance for nitrogen and a significant loss of calcium and bone were discovered (Hughes-Fulford 2011). During the Skylab missions, osteoporosis was found to occur on the longer missions (Vogel 1975) and the lymphocytes of astronauts were shown to be heavily compromised (Kimsey 1977). Studies revealed that microgravity strongly compromises immune cell function (see also Chaps. 4 and 5), which is currently considered the main reason for dysregulation of immune cell function during spaceflight. These health risks pose serious obstacles when planning long-term space exploration missions. Therefore, reliable treatments have to be identified and further developed to overcome the limiting nature of the human body. The adoption of cell-based therapies is promising with respect to effectiveness, safety, range of application, and ease of use. The majority of the health issues in space were already addressed by research and clinical trials in the field of cell-based therapies. In combination with lyophilization, to guarantee low cost and reliable storage of cell products, therapeutical cells could amount to comprehensive treatment and prophylaxis in the future – not only in space, but also on Earth.

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Chapter 9
Metabolic Control: Immune Control?

Quirin Zangl and Alexander Choukèr

9.1 The Essence of Metabolism

Metabolic challenges under the condition of space have been reported from the very beginning of human spaceflight as by the effects on the muscular and skeletal system. The causes and the consequences for metabolism, which includes "construction" (anabolism) and "destruction" (catabolism) of energy depots and tissues on the organic level, respectively, are not well understood because of their complex orchestrated network of endo-, auto-, and paracrine pathways in the regulation of the cell metabolic functions. Such metabolic and inflammatory causes are, for example, considered to be strongly contributing to the degeneration of the musculoskeletal system, as observed during spaceflights (Smith et al. 2015).

All metabolic changes result from substrate and enzyme interactions at the cellular and subcellular levels. Here, the recurrent pathways use "downstream" products of carbohydrate-, fat-, and protein-metabolism to finally confluence into the high-energetic reduction equivalents nicotinamide adenine dinucleotide (NADH/H^+) and flavin adenine dinucleotide ($FADH_2$). Together with oxygen, they are converted into the ubiquitary cellular source of energy, adenosine triphosphate (ATP) in the mitochondria. To produce ATP, products of intermediate metabolism enter the Krebs cycle and deliver electrons for reduction equivalents NADH/H^+ and $FADH_2$. Finally, these equivalents are oxidized by oxygen while delivering energy for the creation of the proton gradient over the inner mitochondrial membrane. The establishment of the proton gradient is regulated by four distinct enzymes (mitochondrial "complexes 1-4"), located in the inner membrane and known as the *electron transport chain*. The backflow of protons into the mitochondrial matrix is used by ATP-synthase (mitochondrial complex 5) for ATP synthesis (Mitchell 1961). For this

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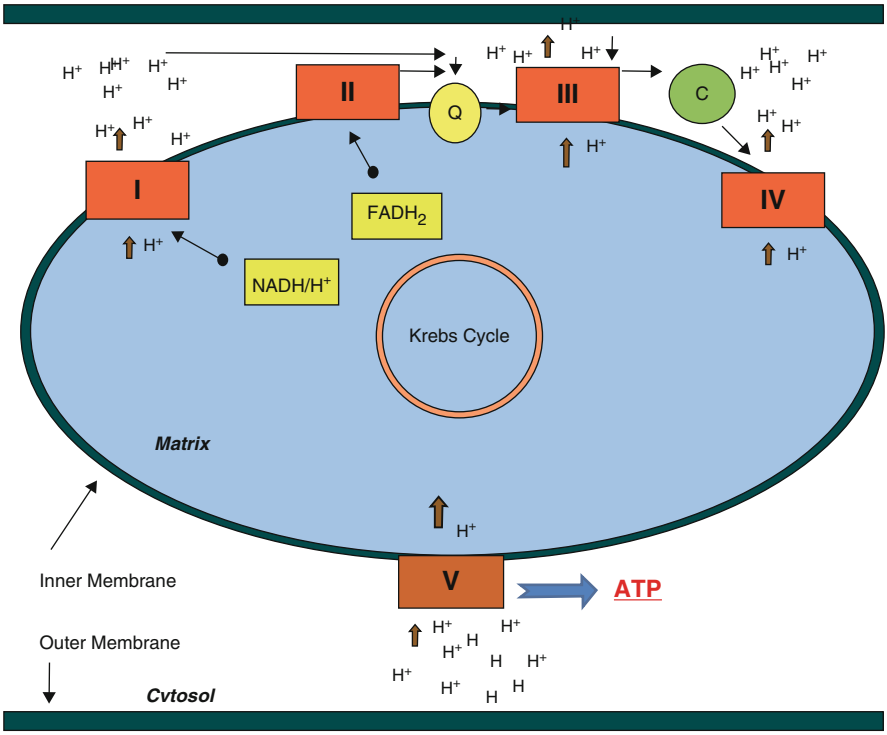

Fig. 9.1 Overview of ATP-(adenosine triphosphate) synthesis in mitochondria: cytochrome c (Cyt), nicotinamide adenine dinucleotide (NADH/H+), flavin adenine dinucleotide ($FADH_2$), and Ubichinon (Q). Enzymes of electron transport chain (*I*, *II*, *III*, *IV*) and ATP-synthase (*V*): I-NADH-Q-oxidoreduktase; II-succinatdehydrogenase; III-Q-cytochrom-c-oxidoreduktase; IV- cytochrom-c-oxidase; V-ATP-synthase. H+: protons

reason, and besides many other physiological functions (Galluzzi et al. 2012), mitochondria are considered the metabolic "heart" of the cells, tissues, and organs [for an overview, see Fig. 9.1].

Besides well-known lethal effects of poisons such as cyanides that block the respiratory chain, there is only very little knowledge about clinically applicable pharmacological substances that affect mitochondrial function directly and exclusively. In contrast, side effects of commonly used drugs on mitochondria are better established, but to the best of our knowledge, until now, *mito-drugs* do not exist (Parikh et al. 2009). However, manipulation of cellular metabolism on the mitochondrial level could be a unique and direct pathway to control and manipulate cell functions and thereby modulate organ-functioning under stressful situations, like lowered oxygen content in the living atmosphere (hypoxia), microgravity, disease states of individuals including inflammatory processes and altered nutritional supply, as all those are related to the challenges man has to face to during exploration-class space missions in the future.

9.2 Homeostasis, Oxygen, and Metabolic Derangements

Understanding the cellular, the organs' and the entire organisms' metabolic adaptation to environmental changes (stressors) in space is inherently multidisciplinary and complex and the metabolic adaptation during long-term space and exploration-class missions needs to be understood. Especially, the effects of gradual G forces as on Moon or Mars together with the effects of lowered oxygen tension (hypoxia) are a matter of concern. These additional environmental stressors will affect the space crew further, since reduced oxygen content is considered to be implemented on such missions and in future habitat designs for various operational and technical reasons.

Both, changes in gravity and living atmospheres can become key elements affecting the cells' metabolic states (Heer et al. 2001). Thus "metabolic control" has become more critical during such missions since it can mitigate unfavorable changes of the cells energy metabolism, the homeostasis. Homeostasis (Greek: ὁμοιοστάσις-balance) is the property of a system in which input and output variables are regulated in a way that internal conditions remain stable and tissue-specific requirements can be realized on a cellular level. Here, there are many actuating variables, like the pH, electrolyte distribution, water distribution, membrane potential, and temperature, which have to be adjusted exactly by energy-consuming biochemical reactions to enable homeostasis also of the immune cells. Mitochondria are the cellular components providing the energy for maintaining homeostasis. The function of these organelles is related to the use of oxygen since more than 90 % of the whole bodies' oxygen consumption takes place in the mitochondria (Ernster and Schatz 1981). During basal metabolism, the oxygen yield is almost complete; experiments have shown that oxygen consumption in Complex 4 (cytochrom-c-oxidase, the actual place of oxygen consumption) cannot be increased more than 16–40 % (Gnaiger and Kuznetsov 2002; Boveris and Britton 1973; Gnaiger et al. 1995; Nolana et al. 2010). In contrast, increasing evidence demonstrates that, during critical situations like systemic inflammatory response syndrome (SIRS), additional donation of oxygen can boost the immune response and further aggravate potential disease states (Strewe et al. 2015a; Zangl et al. 2014; Marconi et al. 2014; Saugstad 2005; Deulofeut et al. 2006; Deuber and Terhaar 2011; Kallet and Matthay 2013; Pagano and Barazzone-Argiroffo 2003; Deng et al. 2000; Garner et al. 1989; Rodríguez-González et al. 2014). This can be well explained by evolution of life on Earth since adaptation mechanisms were predominant to low oxygen concentrations (Hochachka 1998; Fisher and Burggren 2007; Kasting et al. 2003) while hyperoxic conditions probably did never exist in Earth history (Kasting et al. 2003) [see also Chap. 1]. To date, a good demonstration for such adaptation to lower oxygen levels is the intrauterine development of each individual life. Every fetus is subjected to oxygen partial pressures far below the reference areas after birth, though enough oxygen and energy are provided to enable the development of all organs. During those most complex steps of life-development, arterial partial pressures are low and remain between 18 and 26 mmHg, which corresponds to approximately 25 % the worth

adults have (Martin et al. 2010). So the evading question remains, if and how hypoxic environments together with gravitational changes enable mitochondria to maintain energy supply for the (immune-)cellular homeostasis, and where a potential threshold of lowered oxygen tension acceptance will be identified and defined for such missions?

9.3 Mitochondria and Immune Control

Mitochondria play multiple roles and have a critical impact on the regulation of innate and adaptive immune responses. They are important in their functions as bioenergetic organelles – as stated above – and in their biosynthetic functions, and also as immune cell signaling elements (Weinberg et al. 2015).

Biosynthetic functions include key steps of anaplerosis, which is the replenishment of lacking but needed components to realize reaction chains of metabolism. To create the "closed loop" of the citrat cycle (TCA, see figure 9.1), mitochondria have to deliver essential components like acetyl-Co-A, which can also further modify proteins (Hensley et al. 2013). Another molecule, which is substituted in an anaplerotical way, is α-ketoglutarate, also used for further immune-signaling (Wellen and Thompson 2012). Also, reactive oxygen species (ROS) are mostly generated inside mitochondria. ROS from mitochondria play a crucial role in the regulation of transcription via NF-kB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells), a specific transcription factor of almost all cell types in animals. Through the tight interaction between mitochondria and NF-kB, hundreds of immune genes that are involved in regulating cell growth, differentiation, development, and apoptosis, are regulated (Chandel et al. 2000, 2001). Further influences of mitochondria on immune cells beyond energy supply are the proper induction of antiviral signaling (Reikine et al. 2014), T-cell activation (Sena et al. 2013), CD 4+ T-cell differentiation (Berod et al. 2014), and regulation of CD 8+ T-cell memory formation (MacIver et al. 2011). There might be possible interactions between the antiviral immune functions and the energetic state of the mitochondria, especially under deviant oxygen conditions like hypoxia, which are not well understood today.

The role of mitochondria as *signaling elements* is based on the endosymbiotic theory, which postulates, that mitochondria and bacteria share the same origin (Nass and Nass 1963). New insights into the most severe forms of systemic inflammation, sepsis and SIRS, have helped to understand the pathology of the inflammation and the role of mitochondria and bacteria: The two clinical entities of sepsis (induced by bacterial components in blood) and SIRS (the immune system's monotonous-systemic answer to any kind of lesion) are triggered by activation of pattern recognition receptors (PRR) by the innate immune system (Takeuchi and Akira 2010). In such inflammatory condition of sepsis, PRR identify pathogen-associated molecular patterns (PAMPS) from bacteria as the molecular inductors of inflammation. During SIRS, however, damage-associated molecular patterns (DAMPS), directly liberated from damaged mitochondria, activate the innate immune response via

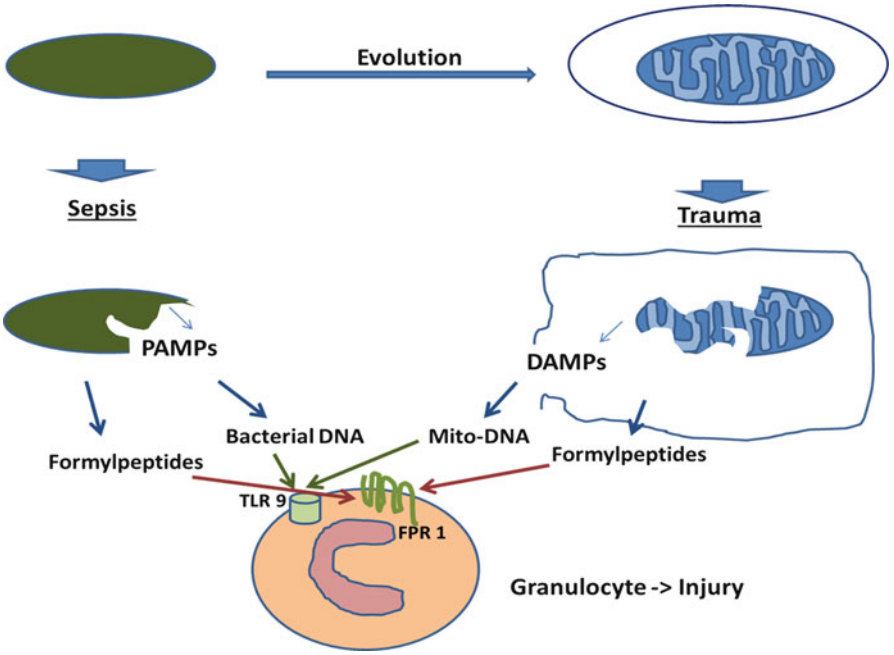

Fig. 9.2 PAMPs and DAMPs in the inflammatory response. Similar to the release of bacterial DNA (deoxyribonucleic acid) following sepsis, the mitochondrial DNA released by severe trauma can also act through the toll-like receptor-9 (TLR9) to activate neutrophils. Similarly, formylated peptides released from bacteria and mitochondria activate the formyl peptide receptor-1 (FPR1) and attract neutrophils by the process of chemotaxis to sites of inflammation and injury. In both cases, the outcome may be acute lung injury, which is part of the systemic inflammatory response syndrome (SIRS). *DAMPs* damage-associated molecular patterns, *PAMPs* pathogen-associated molecular patterns (redraw after: 2010 Nature Publishing Group (Calfee and Matthay 2010))

PRR (Vargas-Parada 2010). Both components, PAMPS from bacteria and DAMPS from mitochondria, confluence into a "crossover" activation of immune cells through the toll-like receptor-9 [TLR9] and formyl peptide receptor-1 [FPR1] on neutrophilic granulocytes (see Fig. 9.2), resulting in detrimental consequences for patients (Zhang et al. 2010)

Thus, the integrity and operational capability of mitochondria are of fundamental importance for immune functions: if homeostasis could not be balanced, malperformance of immune functions with insufficient reactions to pathogens can result. Further decrease of mitochondrial metabolism can result in increased ROS release with the result of direct cellular damage by liberated radicals. If malperformance of mitochondrial metabolism ensues, the breakdown of ATP-production and activation of apoptotic pathways with consecutive cell death would be the result (Wang and Youle 2009). In the case of total metabolic breakdown, direct induction of SIRS by mitochondria can occur. Therefore, both mitochondrial integrity and functionality are the basis of adequate immune answers. The oxygen thresholds for mitochondria to perform sufficient ATP production are not well

established; in vitro experiments showed good metabolic performance, even under hypoxic conditions (Gnaiger et al. 2000). The well-known records of mountain climbers in the Himalayas demonstrate that acclimatization and training enable life with 25 % of the above-mentioned values, though adverse effects on immune functions were observed depending on altitude and exposition time. Currently, interspace agency and polar institute research projects in the high Antarctic plateaus are conducted to investigate such effects in a systematic manner reflecting space-mission-relevant atmospheric conditions and exposition times (Pagel and Choukèr 2016).

9.4 Approaches and Benefit of Metabolic Control During Spaceflights

Obviously, there are many factors in the artificial environment of a spaceflight that can negatively affect the maintenance of homeostasis [see Chaps. 1 and 2]. If a fast, cheap, reversible, and safe method for the (down-)regulation of cellular metabolic activity at the mitochondrial level would exist, the below-mentioned problems could positively be influenced and also related immune responses be controlled, accordingly. The pathways of such an approach include the understanding of the metabolic control that can either include direct mitochondrial targeted drugs (such as adenosine) or the regulation by variation of the oxygen concentrations delivered to the mitochondria. Ultimately, the control of the immune cells' metabolisms and the reduction of the metabolic rate of the entire organism as such could lead to the induction of hibernation. Hibernation is an emerging scientific field for biology, human and life sciences in general and can become an interesting application for space. It is known, from animals and clinical studies in humans that some effects of "tissue hibernation" effects can be elicited by the preconditioning of organs. Preconditioning seems to have strong biological similarities to physiological states as elicited in hibernation and reduces tissue energy consumption and preserves the energy charge of the organ. Thereby, it evokes tolerance to further reduced nutritional supply as characterized by dampened expression of genes, the functions of which influence glucose metabolism, protein turnover, cell cycle, regulation, and ion-channel abundance. These features together mimic hibernation and hypoxia tolerance, suggesting the existence of a conserved endogenous genomic program of physiological adaptations to oxygen limitation that improve survival (Stenzel-Poore et al. 2003; Heldmeier et al. 2004).

Cells' metabolic states do inherently involve signaling through purines and their receptors. Adenosine is one of the key molecules that sense lack of oxygen and high-energy phosphates. Either cellular stress (hypoxia, reduction of tissue energy charge) can result in the production of adenosine and its binding to four different adenosine (A1, A2A, A2B, and A3) receptor sites and thereby regulate intracellular cAMP levels (Chouker et al. 2012; Abbracchio et al. 2009; Jinka et al. 2011). But also stress hormones (see Chap. 2), which are released in space, such as endocannabionoids (ECS) (Strewe et al. 2015b), are candidate ligands that can be involved

in cellular signaling related to metabolic control. Endocannabinoids are rapid-acting, lipid-signaling molecules that bind to endogenous endocannabinoid receptors. They play a critical role in the integration of adaptive responses of the organism to aversive environmental conditions including emotional and physical stress and are immune-regulatory (Hill et al. 2008; Dlugos et al. 2012). Moreover, endocannabinoid receptors are found on the mitochondrial membranes of cells, indicating a direct control of mitochondrial functions (Bénard et al. 2012).

9.5 Summary

The complexity of requirements during human spaceflights have led to developments in various scientific fields, especially in medicine. Knowledge regarding organ performance during critical situations, like degeneration of musculoskeletal system, severe illness, reduced nutritional support, and hypoxia is steadily increasing. A potential target point to influence such critical conditions is to modulate the highly preserved subcellular metabolism in mitochondria. Hypoxic conditions, stimulation with external and internal adenosine (or similar [ant]-agonists), and cannabinoids may help to reduce cellular metabolism and consecutively reduce resources and enable a higher mission success. The use of such pharmacological approaches can become a promising tool to mitigate immune- and metabolism-related risks and offer also new avenues to "metabolically shield" the human from the stressors that occur in such long-duration exploration missions.

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Part III
Summary

Chapter 10
The Immune System in Space: Are We Prepared? Conclusions, Outlook, and Recommendations

Alexander Choukèr and Oliver Ullrich

Humans have been traveling to space for more than half a century and have adapted remarkably well to the altered gravity environment. However, several limiting factors for human health and performance in microgravity have been clearly identified (Comet 2001) and substantial research and development activities are required in order to provide the basic information for appropriate integrated risk management, including efficient countermeasures and tailored life support systems (Horneck and Comet 2006). In particular, serious concerns arose whether spaceflight-associated immune system weakening ultimately precludes the expansion of human presence beyond Earth's orbit (Guéguinou et al. 2009).

The Apollo missions were the first to show significant changes in multiple biological systems: vestibular disturbances, in-flight cardiac arrhythmia, reduced postflight orthostatic tolerance, postflight dehydration, and weight loss. Furthermore, a significant decrease in red blood cell mass and negative in-flight balance for nitrogen and a significant loss of calcium and bone were discovered (Hughes-Fulford 2011). During the Skylab missions, osteoporosis was found to occur on the longer Skylab missions (Vogel 1975) and the lymphocytes of astronauts were shown to be heavily compromised (Kimsey 1977). In the years and decades to follow, studies have shown that microgravity strongly compromises immune cell function, which is currently considered the main reason for dysregulation of immune cell function during spaceflight. The results of space-related clinical and fundamental studies indicate that

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both short- and long-duration spaceflight could largely trigger immune dysfunction, which may exacerbate immunopathology during the course of inflammation and result in altered resistance to infection or cancer or to altered hypersensitivity reactions, yielding severe clinical manifestations that could endanger the host.

Numerous studies carried out with T lymphocytes, cells of the monocyte-macrophage system and endothelial cells in microgravity have clearly shown that individual cells are sensitive to gravity. These experiments, conducted under real and simulated microgravity conditions, have contributed greatly to our current knowledge of how gravitational forces affect basic cellular mechanisms. However, it has not been possible so far to identify a generally accepted primary mechanism from these various effects that underlies the effects of altered gravity on immune cells.

The multitude of cellular and molecular responses to the new gravitational environments have been obviously less ordered than the responses to other environmental changes. This came not as a surprise, since life evolved on Earth in constant gravitational force for 4.8 billion years and, therefore, little or no genetic memory of life responding to gravitational force changes can be expected. Therefore, studying the adaptive processes in cells to altered gravity will clearly increase our understanding of the role of gravity in evolution on Earth. Whereas immune system alterations seem to persist during long-duration spaceflight (Crucian et al. 2015), rapid adaption mechanism could be observed at the cellular level.

To understand these adaptation processes, we tried to summarize individual cellular and molecular effects on a timescale. But we quickly realized that this effort was a “mission impossible”: Experimental conditions varied widely from study to study, from types and concentrations of stimuli to cell culture conditions, using different media and supplements. Finally, nearly all studies used chemically undefined medium supplements, often in different concentrations. In the future, research in gravitational biology of the immune system should benefit from the latest technology for the standardization of cell and tissue cultures and the development of defined conditions at all levels, including stimuli and media.

The health risks pose serious obstacles when planning long-term space exploration missions. Therefore, after a thorough estimation of the indirect stress-related (through neural and hormonal changes) and direct (microgravity, radiation) effects of spaceflight – and the holistic approach to understand the intrinsic and extrinsic loops (see Chap. 2) – reliable treatments have to be identified and further developed to overcome the limiting nature of the human body.

The venues for the identification of the causes will build up on several pillars: investigations in human and with ex vivo onboard analyses of cell responses, using single cell analyses and genetic and protein analyses within an integrated Omics approach. Here, harmonizing of the technical tools and arrays between the agencies and researchers involved needs to be assured and together with sharing such data within the ISS partners, an increase of the number of subjects investigated and scientific impact will be assured. Moreover, these functional and molecular data have to be brought into the context of the duration of the mission and the changes of the other organ systems´ functions and microbial composition, for example, by the analysis of microbiota composition in the gut before, during, and after flight in and

correlation to immune activity and environmental conditions, including the degree of oxygenation or the content of carbon dioxide.

Preconditioning and metabolic control can be two general and efficient tools to adapt to new environmental challenges and to reduce metabolic activities. By definition, preconditioning presents a stressful but nondamaging stimulus to cells, tissues, or organisms to promote a (transient or even permanent) adaptive response so that stress response resulting from subsequent exposure to a harmful stimulus (stressor) is reduced. These benefits aim to target the preservation of energy in the cell, and hence the cell homeostasis, and to increase resistance to a following/secondary damaging impact. Since several types of preconditioning such as pharmacological, thermal ischemic, and especially hypoxic preconditioning have been shown their efficacy, they can be applied to humans as to other biological systems to counteract the unwanted effects of spaceflight on the immune system and other organ systems and to thereby increase resistance and mission success. To which degree the understanding of preconditioning effects and modulation of mitochondrial functions can be used with other tools and conditions to induce even permanent status of hypometabolism (torpor/hibernation) needs to be identified.

The adoption of *cell-based therapies* is promising with respect to effectiveness, safety, range of application, and ease of use. The majority of the health issues in space were already addressed by research and clinical trials in the field of cell-based therapies. In combination with lyophilization, to guarantee low cost and reliable storage of cell products, therapeutical cells could amount to comprehensive treatment and prophylaxis in the future – not only in space, but also on Earth.

The knowledge of the effects of gravitational changes on immune cell regulation and the identification of gravity-sensitive cell responses will help to understand the molecular mechanisms of inhibited immune cell function in altered gravity and provide new targets for therapeutic or preventive interventions with respect to the immune system of astronauts during long-term space missions (Ullrich and Thiel 2012). Those studies may clarify whether and to which extent gravity is involved in normal cell function, how cell function is impaired by altered gravity, and how cells adapt to the new situation. Finally, *knowing the cellular and molecular mechanisms* is an invaluable requirement for a better risk assessment and development of in vitro tests for medical monitoring. For these endeavors, *standard protocols of cell and tissue cultures* should enable cross-study analysis, especially at the timescale of adaptation.

The rearrangement/reorganization of *cytoskeletal structures* was found in lymphocytes and in dendritic cells (DCs) and throughout different microgravity platforms. Supposing that the cytoskeleton is the central gravisensitive element, it possible that the observed alterations have indirect effects on all kinds of cellular functions via intracellular signal transduction and transcriptional pathways. Thus, these cytoskeletal changes can contribute to all kinds of pathological conditions observed during altered gravity conditions.

Testing and validation of such new approaches will require onboard immune function tests, and on-ground spaceflight analogue studies might be able to provide more information to understand the underlying mechanisms and to produce corresponding mitigation strategies to prepare for the coming interplanetary space explorations (Pagel and Choukèr 2016). Complementary to the “golden standard” of the

real exposition to spaceflight (ISS, or sounding rocket, Bion capsules), the important elements of such understanding will be based on the use of high-fidelity ground-based facilities for estimation of either indirect stress-related effects, as investigated in bed rest facilities and in isolation/confinement studies, as well as in scenarios to evaluate the gravitational or radiation-depending damaging effects, for instance by using hypergravity centrifuges for cells, animals, as well as microgravity simulators such as Clinostats, random positioning machines, and rotating wall vessels with and without concomitant radiation effects. Since research in the area of gravitational science is extremely expensive and elaborate, resources should be spent wisely. Thus, in order to achieve the highest level of reliability and comparability of the results, gravitational-related immunobiological research should benefit to a large extent from the latest technology for the standardization of cell and tissue cultures and the development of chemically defined media. In addition, and as a bridging element, the use of experimental animal facilities (e.g., of rodents, as well as amphibians) in space should be used more extensively and in an internationally coordinated fashion.

As interplanetary space exploration and a mission to Mars are contemplated, it is critical to improve our understanding on how immune dysfunctional states occur and to which pathology they can lead. This will be the prerequisite to target new preventive and therapeutic countermeasures to mitigate such risks.

New and innovative approaches have been initiated and will be applied in the future and in space and will, more than before, be based on the strong interaction between the clinical understanding of stress-related maladaptations and a cell-based state-of-the-art molecular approach. This more holistic strategy using new technologies and experimental tools in challenging environments will help us better understand the complexity of immune interactions on the organ and cellular level, for Earth as in space. This knowledge will help enable the ultimate goal of sending man to outer space, and to bring him back safely.

Especially since exploration-class deep space missions are characterized by high radiation exposure, confinement, limited clinical care, and the impossibility of an evacuation to Earth in case of emergency, such missions will be always missions into the unknown. Even if we should have unlimited research resources and endless time to prepare, it is impossible to assess and monitor all possible medical aspects, to elucidate all scientific aspects, and to exclude all risks. Perhaps, our current knowledge will turn out to be incomplete or wrong someday. Maybe, despite all efforts and despite all "modern" science, we could have overlooked something relevant. For preparing exploration-class missions, we should focus, trust, and rely on the crew: highly skilled and professional astronauts with solid scientific and technological backgrounds. Exploration-class missions should be equipped with all scientific, technological, and medical devices and tools to analyze and to solve problems that might occur during the mission: from high-end point-of-care-testing systems (POCT), highly flexible analysis and monitoring systems up to the possibility of efficient cell-based therapies on board. But finally, no one can guarantee 100 % that our knowledge is sufficient to foresee and counteract all facets of the biological reality, and no one can guarantee that we are prepared for everything. Someday, everything will depend on the few astronauts who will commence on the greatest journey of mankind, prepared for the worst and hoping for the best.

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