Chapter 7
Emerging Immune Context

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Contents

Abstract Historically, in the microbiological contamination control (MCC) of small molecule drugs (SMDs) and large volume parenterals (LVPs), the adaptive immune response was of little concern. Fever, as a systemic response, as measured first in a rabbit then as correlated in an *in vitro* horseshoe crab blood test, has been the gauge used to monitor endotoxin presence as a contaminant. The differences between biologics and small molecule drugs (SMDs) that came before are detailed in this chapter and the next chapter. Major differences to be borne in mind here include: (i) the size and complexity of biologic molecules that exert their therapeutic influence by interacting with receptors on the *outside* of cells, (ii) the propensity for immune related responses and (iii) the potential for PAMPs to act as co-stimulatory signals to adaptive immunity (i.e. the "second signal" as described in Janeway's 1989 lecture and as, in some cases, synonymous with the adjuvant effect of vaccinology). The latter provides a way to understand the immune stimulating effects of endotoxin that is separate from its historical role as "*only a pyrogen*".

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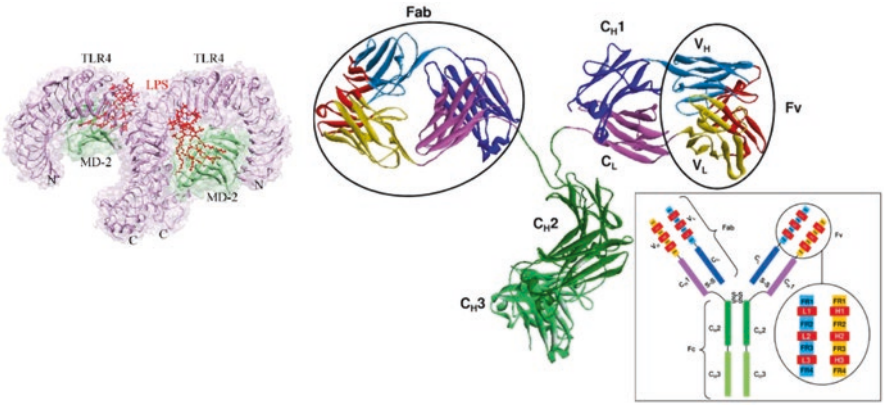


Fig. 7.1 Contrasting the innate **static**, germ-line encoded TLR structure of the TLR4/MD-2 dimer containing endotoxin (*Left*, from Manavalan, Basith and Choi [2]) are the **dynamic**, hypervariable amino acid sequences (red boxes) of an adaptive BCR as the source of antigen binding variability. This hyper variability produces structural variants that can bind a myriad of PAMP antigen structures. The use of chaotic rearrangement points to the need for additional signal(s) to differentiate microbial antigens versus potential self-proteins. From Sela-Culang et al. [3]

7.1 Introduction

The innate immune response occurs quickly and is based upon highly conserved pattern recognition receptors (PRR such as TLRs) whereas the adaptive response is generated in a delayed manner after recognition of an antigen by a lymphocyte receptor (BCR or TCR). Deciphering the interworking's of the innate and adaptive puzzle has greatly accelerated in the last decades and is now a highly complex proposition. There are emerging immunological concepts beginning to impinge upon the long-standing tenants of MCC, including endotoxin detection. The intention here is to paint with a broad brush, simplifying where possible some complex, recent immune concepts as they relate to biologics MCC, as the next chapter will build upon this knowledge to detail the biologics revolution and emerging endotoxin test concerns.

According to Janeway: "*the immune system evolved to discriminate infectious nonself from noninfectious self*" [1]. It has been known for some time that LPS activates both arms of the immune system, however, only recently has it been determined that it is the innate immune system that largely gives "permission" for the adaptive response. It is important to note that neither the rabbit pyrogen test (a 3-hour period of monitoring for temperature rise) nor a *Limulus*-based test can speak to adaptive immune responses. *Limulus*, of course, has no adaptive immune system [although there are some overlapping features, ancient (Chap. 18) to modern (Chap. 20)].

Though this chapter is not specific to endotoxin as a PAMP, true to its historical role, endotoxin is often at the forefront of the discussion. The dual nature of endotoxin can be seen in its proinflammatory and immune stimulatory characteristics in that the two effects can be chemically separated. A "detoxified" endotoxin can be made by changing the structure (and such structures exist naturally) such that the proinflammatory activity is greatly reduced but leaves intact the ability to stimulate the immune system. This is the basis of adjuvant use in vaccinology and LPS has been detoxified as used today as an (FDA-approved) adjuvant in modern vaccines. Janeway called the need for an additional signal the "second signal" or "co-stimulatory signal" that provides the stimulus for adaptive immune activation in protein antigen recognition that was the early clue that lead him to outline the coming revolution in immune understanding (See Appendix I).

7.2 Emerging Immune Context

7.2.1 Immune Reactions Including Fever Are Common in Biologics Administration

A look into the immune reactions that come with biologics therapy serves as the "why" for the exploration of an alternative understanding of underlying mechanisms of immune activation via various PAMP impurities. A significant way in which biologics differ from either LVPs or SMDs is the propensity for fever and other immune reactions. This is partly due to the size of biologic molecules which cannot enter cells but rather interact with them via cell surface signaling molecules (CSSMs). "Immune surveillance tends to focus on large molecules and cellular fragments. Thus, small molecules tend not to elicit an antigenic response" [4]. *In a book about endotoxin, one cannot overstate the importance of the routine occurrence of fever in biologics drug administration.* This is not because this fever is known to be caused by endotoxin, but because, before biologics, the occurrence of fever, though rare, was *the* adverse event most clearly associated with drug contamination events [5]. Without fever as a clear indication of contamination, the identification of drug contamination events is obscured. Today, adverse reactions associated with biologics include fever as well as more serious incidents, including immunogenicity, which is a general term that can include infusion reactions, anaphylaxis, IgE antibody-mediated (allergic) responses, and antidrug antibody formation. As "*fever is common*" in biologics administration, the expectation of fever (or pyrexia or infusion reaction) is stated in the package insert for each biologic. Biologics package inserts often contain instructions for the preadministration of steroids or other antipyretic drugs prior to biologic infusion in anticipation of fever. As per FDA:

> A major problem with protein-based therapeutics is their immunogenicity, that is, their tendency to trigger an unwanted immune response against themselves. One form of immune response is activation of B cells, which produce antibodies that bind to the proteins and

> reduce or eliminate their therapeutic effects. Such antibodies can also cause complications that can be life-threatening. Therefore, a critical part of determining the clinical safety and efficacy of protein-based therapeutic products is measuring their tendency to trigger antibody formation [6].

From Baldo's *Safety of Biologics Therapy* [7]:

> Infusion of many biologics, particularly mAbs, provokes a characteristic infusion syndrome, usually within one or a few hours during/after the first administration. Whereas most reactions are mild to moderate with symptoms often described as "flu"-like with fever, chills, rigors (shaking from high fever), headache, nausea, asthenia, rash, and pruritus, a small number of patients, mostly at the first or second infusion, show potentially fatal symptoms resembling an IgE antibody-mediated reaction with hypotension, cardiac arrest, bronchospasm, and urticaria.

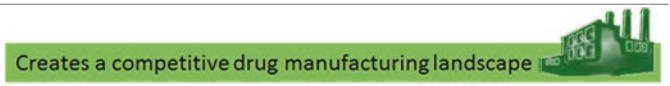
Infusion reactions and associated fever occurrences as referenced above bear similarity to the injection of endotoxin into humans: "…endotoxin doses of 2–4 ng/kg body weight cause flu-like symptoms (fever, chills, myalgia (muscle pain), headache, nausea) and increases in blood TNF and IL-6 levels similar to what is seen in sepsis…" [8]. Historically, fever outbreaks associated with SMD or LVP contamination were few, however, fever invariably pointed to endotoxin.

Today, fever is a characteristic of many life-saving biologic therapies and often the reason for its occurrence is simply not known. Significantly, fever occurrences are not documented as Adverse Drug Reactions (ADRs) as CFR[1] 314.80 only requires reporting to FDA (within 15 days of receipt) of the following: "…individual case safety reports for events classified as 'unexpected' or 'unlabeled' (not detailed in the package insert (PI)) and 'serious'". Thus, fever from a biologic where fever (or pyrexia or infusion reaction) is a stated expectation of the package insert is not an ADR. Given that many biologic drug package inserts recommend pre-administration of anti-fever/pyretic drugs including steroids, the occurrence of fever is routinely encountered. An idea of the prevalence of fever-relevant occurrences listed as "serious and common" in a range of biologic classes of drugs can be seen in a cumulative summary derived from Baldo's tables from multiple chapters as summarized in Table 7.1. Therefore, fever as a historically significant side effect of drug therapy, no longer means what it meant just a few years ago, namely that if fever occurs, then a product is likely contaminated with endotoxin [9].

Many biologics are cytokines, which are "key modulators of the immune and inflammatory responses functioning in an autocrine, paracrine, or endocrine manner stimulating or suppressing cellular activities in infection, innate and adaptive immunity, autoimmunity, inflammation, and malignancy" [7]. Therefore, it is not surprising that many recombinant cytokines produce inflammatory responses including fever, as specific responses including those arising from TNF and interleukins as molecules induced by endotoxin via TLR4 activation. And it should be said that in many cases, especially with biologic cancer drugs (mAbs), that the side effect profiles are better than the toxic SMDs used previously, but lean toward fever. Similarly, vaccines are often microbial-derived proteins and sometimes whole attenuated microorganisms. Vaccines have a long and complicated historical relationship with endotoxin [10].

[1] Code of Federal Regulations.

Table 7.1 Number of biologics molecules per class with specific fever-related adverse reactions listed by Baldo as "*serious and common*"



Class	mAbs Cancer	mAbs Non-cancer	Cytokines	Fusion proteins	Enzymes	Coagulation factors	Vaccines
Baldo table	3.1	3.2	5.2	6.2	9.2	10.1	11.2
Molecules/ Total in class	20/25	11/27	16/23	3/10	15/25	8/22	28/46
Fever-related occurrences per biologics class[a]	80%	40%	69%	30%	60%	36%	70%

[a]Tabulated from (a) fever, (b) pyrexia, (c) infusion reactions, and (d) flu-like symptoms. Fever is not the focus of concern with biologics administration as it has been historically with LVPs and SMDs. Included are all approved biologics as of June 2016 as derived from *Safety of Biologic Therapy*, tables as listed for each drug class, Brian Baldo, Springer, 2017

For biologic drugs, concerns go beyond fever and center around immune reactions which include infusion reactions of various severity: "Severe reactions have been reported for all, or almost all, the mAbs…"[7]. Although not an absolute rule that all fully humanized molecules are better in this regard, in general, fully humanized mAbs display less immunogenicity than those that are chimeric or humanized. However, some expert immunologists believe that common immune reactions should not be *inherent* in biologics therapy.

- "In the absence of adjuvants, proteins are usually not immunogenic, in fact, often they are tolerogenic. That is the basis of allergy immunotherapy…" [11]
- "The unexpected development of immune responses to fully human antibodies and proteins… has become one of the greatest puzzles of the protein therapeutics revolution" [12].
- "Despite the general trend towards 'Humanization', these drugs remain immunogenic in clinical settings, baffling drug developers. In principle, humanized and fully human monoclonal antibodies are 'self' immunoglobulins and should be tolerated" [13].

If some current immune reactions are acknowledged to be brought about by hidden microbial artifacts, as some studies to be discussed here suggest, then it will bring with it the realization that contamination control should update existing tenants. Enormous energy has been expended in the stage of molecule development [14, 15] and in adjusting product formulations and storage conditions [16] to avoid protein sequences and conditions that may bring immunogenicity, including the avoidance of protein aggregate formation. These efforts have benefited biologics drug manufacturing in lessening adverse events for specific molecules. FDA researchers have identified a new potential avenue associated with therapeutic

proteins from a contamination control vantage and that is adjuvanticity: “Most TLR ligands are known to act as adjuvants increasing antigen uptake and presentation, T cell activation and antibody production” [17].

7.2.2 IIRMI as a New Paradigm

An emerging view of potential biologics impurities and/or contaminants originated at FDA and includes the basic premise: *PAMPs that do not provide overt stimulus from a MCC perspective (as in passing an established QC test) may still bring adverse responses from an immunological perspective.* This vantage could include several scenarios: (a) PAMPs may be immune active (adjuvant-like) below the level associated with prototypical testing, (b) PAMPs that are masked by various process constituents may be immune active, (c) PAMP types may differ from the types that can currently be detected, and (d) PAMPs may act synergistically in combination at levels below the current paradigm of detection.

Vertheyli and Wang (FDA) coined the term IIRMI or “*innate immune response modulating impurity*” which refers to such a potential in contrasting conventional contaminants that can be detected by traditional means and at traditional expected levels. TLR activating substances are, of course, the prototypical PAMPs that include LPS, however, from an adjuvant perspective, they do not need to rise to the level of “*fever causing*” or be pro-inflammatory (as pyrogens) to be immune stimulating in recombinant proteins. In this regard the discussion should not be limited to LPS, as CpG DNA (TLR9) [18], RNA [19], Host cell proteins [HCPs, including and porins (TLR2) as discussed in Chap. 4], and flagellin (TLR5) [20] all have adjuvant properties. All of these PAMP types have been found to signal through TLR adapter MyD88 [21]. TLR-derived vaccine adjuvants are selected for their ability to do two things: (i) activate immune receptors and (ii) to do so without overt reaction from a proinflammatory perspective such as a pyrogenic response (fever). This is the basis by which TLR-activating adjuvants are being selected and developed in the vaccine world: “…it is interesting that the less inflammatory compounds, MPL and RC529, have similar effects on the early stages of CD4+ T cell clonal expansion” [22]. Casella and Mitchell [23] relate that “LPS does not generate inflammatory shock in *myd88-/-* mice but it can still… increase expression of major histocompatibility complex II (MHC II) and costimulatory B7 by antigen-presenting cells (APC).” Figure 7.2 gives an overview of the emerging distinction of proinflammatory (i.e. pyrogenic) versus an immune stimulating (adjuvant) activities.

FDA, though not a monolith, is aware of the significance of the sea-change from the concept of endotoxin as merely “pyrogenic” or “proinflammatory” to its status as also potentially “*immunogenic*” or “*immune modulating*” as they have invested in laboratory research and published key studies illuminating the paradigm for almost 10 years now. Other than Janeway’s two signal idea (to be elaborated and from which so much has come) and the ongoing empirical and now rationally devised use

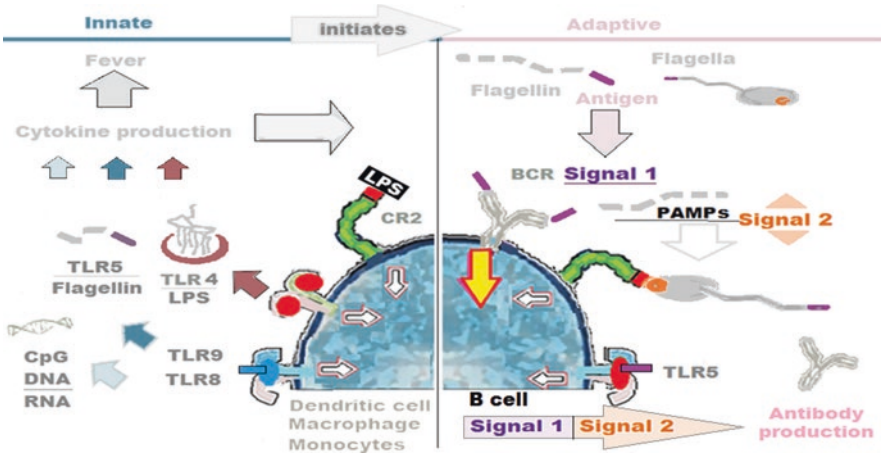


Fig. 7.2 Traditional PAMP proinflammatory activity (left) represents a rapid innate response and contrasts the concept of PAMP initiation of adaptive immune responses (right). At left PAMPs serve as direct ligands for TLRs and transmembrane signaling results in cytokine production. At right PAMPs serve as co-stimulatory (confirmatory) signals for B cell or T cell receptors and act through various non-clonal receptors including TLRs. Signals 1 and Signal 2 remain to be discussed

of vaccine adjuvanticity, this is where the concepts originate as they relate to Pharma MCC. This set of documents also includes an FDA Guidance document that is seldom referenced in endotoxin test circles. The references below are the published IIRMI research from FDA laboratory studies and includes the relevant Guideline from FDA CDER and CBER.

- *Trace Levels of Innate Immune Response Modulating Impurities (IIRMIs) Synergize to Break Tolerance to Therapeutic Proteins, Vertheyli and Wang, PLOS ONE, 2010* [24].
- *Immunogenicity Assessment for Therapeutic Protein Products*, Sect. 5 "Impurities with adjuvant activity", FDA (CDER/CBER) Guidance document, 2014.
- *Detection of Innate Immune Response Modulating Impurities in Therapeutic Proteins, Haile et al., PLOS ONE, 2015* [25].
- *Cell based assay identifies TLR2 and TLR4 stimulating impurities in Interferon beta, Haile et al., Nature Scientific Reports, Sept. 2017* [26].

The Guidance document from above is important, as this chapter seeks to provide background information regarding this specific view. This is the IIRMI-based view (the epitope-based view will be overviewed in the next chapter). The relevant section is cited below:

> **5. Impurities with Adjuvant Activity.** Adjuvant activity can arise through multiple mechanisms, including the presence of microbial or host-cell-related impurities in therapeutic protein products (Verthelyi and Wang 2010; Rhee et al. 2011; Eon-Duval et al. 2012; Kwissa et al. 2012). These innate immune response modulating impurities (IIRMIs), including lipopolysaccharide, β−glucan and flagellin, high-mobility group protein B1

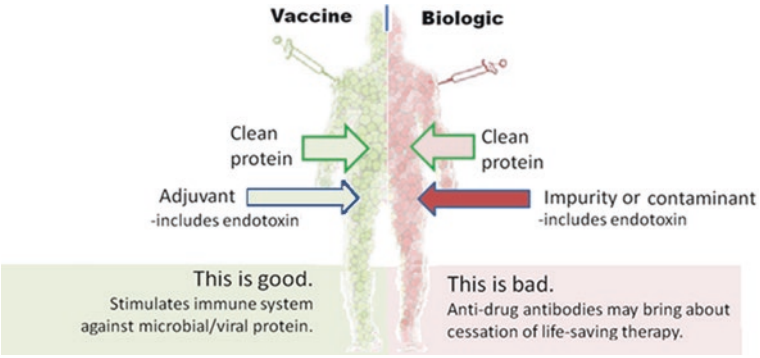


Fig. 7.3 The basic concept of adjuvant use which brings immunogenicity in vaccines and potentially in biologics if microbiological contaminants or impurities are present. The same effect is viewed as good or bad depending upon the recombinant protein therapeutic context. The protein provides signal 1 (antigen) and PAMP provides signal 2 (co-stimulatory) as per Janeway's original description (to be discussed)

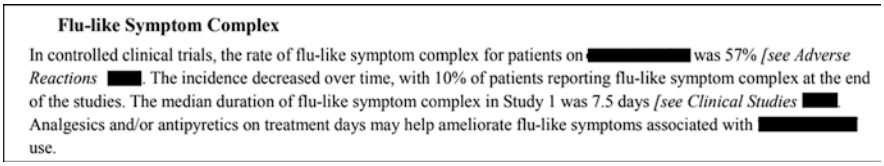
(HMGB1), and nucleic acids, exert immune-enhancing activity by binding to and signaling through toll-like receptors or other pattern-recognition receptors present on B-cells, dendritic cells, and other antigen-presenting cell populations (Iwasaki and Medzhitov 2010; Verthelyi and Wang 2010). This signaling prompts maturation of antigen-presenting cells and/or serves to directly stimulate B-cell antibody production.

The adaptive immune response requires the confirmation of clonal receptor activation via a PAMP co-stimulatory signal. The simple contrast of the use of adjuvants in vaccinology versus the emerging paradigm of adjuvant activity of impurity or contaminant PAMPs in biologics production is shown in Fig. 7.3.

7.2.3 Best Proof of Concept

The 2017 reference, by Vertheyli and Wang, is among the most recent FDA IIRMI research, and is the best demonstration of a difficult to prove hypothesis. The hypothesis is that *impurities or contaminants (PAMPs) that do not react in pyrogen or Limulus testing may still impact the biologic drug safety profile*.

Four marketed interferon beta (INF-β) drugs were compared given the knowledge that two of the four are more immunogenic than the other two, based on clinical data. That is to say, two of the four drugs have significant side effects. One of the drugs has a 31% associated fever response and a 57% associated "flu-like symptom" response (see Fig. 7.4). FDA researchers wanted to determine if it was possibly caused by sub-pyrogenic or masked, TLR-activating contaminants including LPS. Discontinuation of life-saving treatment can be a significant issue for specific patients: "Neutralizing ADA (NAb) develop in up to 47% of patients using IFNβ-1b and up to 28% and 6% for those treated with s.c. IFNβ-1a and i.m. IFNβ-1a, respectively" [27].



Flu-like Symptom Complex

In controlled clinical trials, the rate of flu-like symptom complex for patients on [redacted] was 57% *[see Adverse Reactions* [redacted]. The incidence decreased over time, with 10% of patients reporting flu-like symptom complex at the end of the studies. The median duration of flu-like symptom complex in Study 1 was 7.5 days *[see Clinical Studies* [redacted]. Analgesics and/or antipyretics on treatment days may help ameliorate flu-like symptoms associated with [redacted] use.

Fig. 7.4 Package insert verbiage around flu-like symptom complex associated with two of four marketed interferon beta drugs. Product name and identifiers are redacted

Given the knowledge that all TLRs *except TLR3* signal through TLR-adapter molecule MyD88 (see Chap. 20), the researchers reasoned that by using MyD88 knockout (KO) mice, they could determine if TLRs as a group were being triggered in the drug milieu. Also, to avoid the activation of interferon receptors by the drug molecules (as mouse and humans share significant homology), they used murine cells lacking interferon receptors (IFNAR). The same two drugs that showed higher immunogenicity in the clinic activated NF-κB in HEK-293 cells transfected with Toll-like receptors "*in a MyD88 dependent manner*". "Importantly, the IIRMIs in (redacted drug name) induced up-regulation of IL-6, IL-1β, and ccl5 in the skin of IFNAR knock out mice following subcutaneous administration. This indicates that trace level IIRMIs in (redacted drug name) could contribute to the higher immunogenicity rates seen in clinics."

Subsequently, the researchers employed polymyxin B (PMB), which most strongly binds LPS, to confirm via the same HEK-293 transfected cell testing, the presence of LPS that previously tested below detectable levels via LAL (as these are all marketed products). The cell based response was muted by the addition of PMB but was not entirely removed after binding which lead to an additional treatment using a protease (proteinase K as Petsch et al. had used, see Chap. 8), which removed all remaining activity. This latter test demonstrated that an additional (synergistic) contaminant, presumably a host cell protein, was responding through TLR2.

Though the two products indicating endotoxin activity were produced in *E. coli*, the researchers point out that another product, interferon alpha-2b did not induce NF-κB activation, and thus not all proteins produced in *E. coli* can be assumed to contain deleterious microbial artifacts. The presumption is, as it is with all biologics, that processing removes any potential endotoxin. See Fig. 7.5 for an overview of the study methodology. Regarding detection using LAL, they also state that, "Levels of HSA above 0.5% consistent with those in INF-β formulations, interfered with the detection of LPS using the LAL assay". In conclusion, the researchers restate the basic premise of IIRMI theory: "*Despite the broad spectrum of IIRMIs that could be present in therapeutic proteins and peptides, current testing strategies are often limited to the use of the LAL test to measure endotoxin, a PCR test to detect host cell DNA, and ELISA based tests for host cell proteins.*" Note that the complex testing done by the researchers would not be an expectation of routine testing, but could support the development of manufacturing processes to determine if they are achieving the necessary levels of impurity removal.

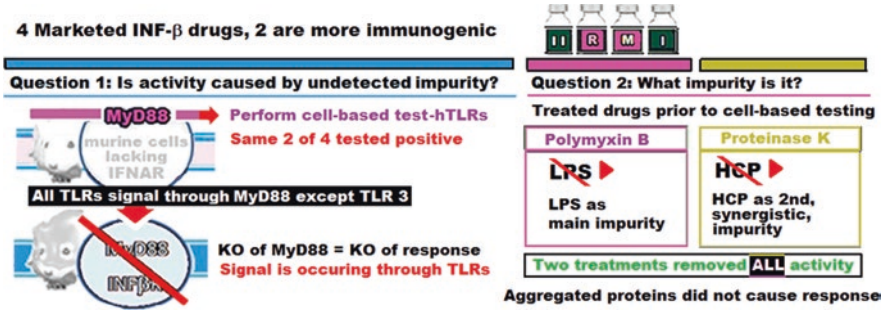


Fig. 7.5 *FDA Research.* Four marketed INF-β drugs, two are known to be more immunogenic than the other two. Since they are marketed drugs they were tested using LAL and passed QC release. FDA researchers showed TLR-activating impurities (IIRMI) present in the two interferon drugs known to be more immunogenic. Researchers tested with INF receptor knock-out (KO) and MyD88 TLR adapter KO mice. Subsequent KO of response indicated signal occurs through TLRs. Major reduction of TLR activation via polymyxin B (PMB) binding implicates LPS. Removal of remaining residual activity by proteinase K treatment implicates a host cell protein (porin, etc.). Therefore, the study provides evidence that low level PAMPs may act synergistically to produce adverse responses that could not be detected by conventional methods

The search for the causes of immunogenicity have been hyper-explored upstream in molecule design [28] such as antibody paratope construction [29] and otherwise in drug development (i.e. glycosylation [30]) and these efforts have improved adverse event prospects but from the IIRMI vantage, a simpler hypothesis of contamination via low level, synergistically active or otherwise masked microbiological contaminants has been put forward.

7.3 Innate Activation of Adaptive Immunity

This section can be viewed as including some limited but necessary background information to support the remaining sections as well as Chap. 8. Endotoxin detection and control has been viewed historically as an effort to preclude the possibility of fever reactions, which are innate immune responses rather than adaptive or antibody-based responses. However, for biologic drugs, fever is common, therefore the relevance of fever has greatly diminished just as the relevance of other immune related responses has overshadowed all other concerns. These concerns center around the triggering of innate and adaptive immune responses that are more worrisome than a simple fever response. These responses, often antibody-based, are not dependent upon causing a fever. The "*emerging immune context*" is to view the potential for covert, not just overt, PAMPs as unwanted process-constituents.

7.3.1 Microbiology Meets Immunology

Verthelyi and Wang[2] have elaborated the "emerging" immune context:

> During the manufacture of therapeutic proteins a few IIRMIs, such as LPS and DNA, are regularly screened-for and have assigned limits or acceptance criteria for product release. Of note, the current guidelines for setting limits on these impurities are not based on their potential impact on product immunogenicity. For example, the current recommendation for endotoxin content in parenteral products… is based on its pyrogenic potential, while the WHO recommendations for DNA content (10 ng DNA/dose) are based on minimizing the risk of DNA integration (USP 85. and [31]). Yet studies using individual TLR 4 or TLR 9 agonists as adjuvants show that concentrations lower than those that may be pyrogenic or lead to significant DNA integration can augment the immune response to co-administered protein antigens [32–34]. Furthermore, as shown in the studies above, the levels of agonists sufficient to stimulate an innate response can be lower when multiple receptors are engaged [35].

Understanding the control of adaptive immunity as triggered by the innate immune system recognition of PAMPs is also fundamental to the understanding of biologic drugs mechanism of action as well as the potential for adverse responses. It is important to see that the weight of control for antibody formation has been found to rest on Toll-Like receptor (TLR) and other PRR activation via PAMPs and is not a separate and unrelated process as historically innate and adaptive immunity have been presented, often with little overlap.

> The innate immune system encompasses a collection of host defenses that range from the non-specific barrier function of epithelia to the highly selective recognition of pathogens through the use of germline-encoded receptors. A common feature of these diverse elements is a rapid and blunt response to infection or tissue destruction (Janeway and Medzhitov, 2002). In contrast, the adaptive immune system uses somatically rearranged antigen receptor genes to create receptors for virtually any antigen. The adaptive immune response is slower but more flexible and is able to combat infections that have evolved to evade innate responses. The adaptive immune system has the capacity to recognize and respond to virtually any protein or carbohydrate imaginable; yet, without the innate immune system to instruct it—in effect, telling it whether, when, how, and where to respond—it is powerless [36].

Historically, the activation of lymphocytes (B cells and T cells) [37] via endotoxin was recognized well before the elucidation of either the receptors of the innate immune system including TLRs or any necessity of precluding such a response as there were no biologics being administered except vaccines [38], which, of course, are intended to provoke an immune response. The control of adaptive immunity (the triggering thereof) by the innate immune system is described in most texts via many specific and tedious examples, rather than as guiding concept. An overarching concept is necessary to build a simple MCC model relevant to microbiological detection and control, particularly to overcome the idea that only systemic pyrogenicity is relevant. The guiding concept of the "second signal" or "co-stimulatory signal"

[2] Division of Therapeutic Proteins, Office of Biotechnology Products, CDER, FDA, Bethesda, MD, USA.

was the epiphany conveyed to many (including Medzhitov) by Janeway's important Cold Spring Harbor lecture in 1989[3]:

> Janeway proposed that the activation of the adaptive immune response is controlled by the evolutionarily older innate immune system and suggested the principles that might underlie this control. His views had already aroused skepticism—but not from the young Medzhitov.[4] "It was a beautifully simple framework that made so much sense", he says. "It was a transformative moment for me because it made sense of a lot of very confusing phenomena" [39].

A wide variety of mammalian Toll-like receptors, 10 human and up to 13 mammalian TLRs, dimerized in "mix and match" fashion into heterodimer and homodimer complexes, detect dozens of microbial artifacts including, arguably the most potent, LPS.[5] The detection of PAMPs by the innate immune system in mammals is dominated by the TLRs. The interaction of the innate immune system with the adaptive can be seen by the simple existence of TLRs on macrophages, dendritic cells, mast cells, eosinophils, neutrophils, B lymphocytes, T lymphocytes, epithelial cells, endothelium, and cardio-myocytes. As per Reynolds and Dong: "TLR1, TLR2, TLR4, TLR5, and TLR6 occur on the surface of both professional and non-professional antigen-presenting cells (APCs) and recognize various bacterial and fungal components" [40]. This is an important overlap connecting the innate and adaptive immune systems.

Endotoxin, as a model PAMP, has two different pathways by which receptor dimer complex (TLR4/MD/LPS) response proceeds. One is the MyD88-dependent pathway which is proinflammatory and the other is the MyD88 independent (TRIF-dependent) pathway which, from a vaccinology perspective, produces interferon which produces adjuvanticity.[6] With LPS sitting in the receptor complex, the TLR4/MD-2 dimer signals across the cell membrane to instruct the production of various sets of cytokines via transmembrane adapter recruitment. Some cytokine sets activated are prototypically proinflammatory (include fever and inflammation) while some other sets are immunostimulatory (low or no fever association) and promote adjuvanticity [23].[7] The vaccine world has many decades worth of empirical experience with the use of LPS as an immune stimulator.

What are the characteristics of PAMPs that are the conserved artifacts of the microbial world that metazoans have developed to signal or alarm of prokaryotic

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[3] *Approaching the asymptope: Evolution and Revolution in Immunology*, Cold *Spring Harbor Symp. Quant Biol*. 1989. 54: 1–13, Janeway, Pillars of Immunology.

[4] "…Ruslan Medzhitov, Professor of Immunobiology at the Yale School of Medicine. In May (2013), he was awarded the first annual Lurie Prize in the Biomedical Sciences from the Foundation for the National Institutes of Health for his work on the immune system; in June (2013), he picked up another inaugural award, this one from the German Else Kröner Fresenius Foundation and worth an eye-watering €4 million."

[5] …with heat-labile superantigen toxins also being very potent.

[6] *Differential induction of the Toll-like receptor 4-MyD88-dependent and –independent signaling pathways by endotoxins*, Zughaier et al., Infection and Immunity, May 2005, pg. 2940–2950.

[7] *Kdo2-Lipid A: Structural diversity and impact on immunopharmacology*, Wang et al., Biol. Rev. 2015.

invasion? Like LPS, they are all highly conserved structures that make up the cell wall and other surface molecules that the bacteria cannot easily change and that are thus integral to the functioning of the cell (peptidoglycan, porins, flagella, RNA, DNA, etc.). If the PAMPs targeted by metazoan receptors could easily evolve away from these structures, then they would not be good targets for metazoan retaliation and such a loss would thwart hard-won immune defenses developed over eons.

7.3.2 Receptors and Markers Overview

Perhaps the greatest difference between modern biologics and previous SMDs is the mechanism of action of biologics that is specific to binding receptors on the surface of various immune cells whereas SMDs often work by entering into cells.[8] Receptor activation and blockage (modulation) is the means of both disease causation and therapeutic intervention via biologic molecules. Receptors are the powerful interactive buttons that exist on the surface of immune cells.

Receptors, also called cell surface signaling molecules (CSSMs), are the means by which immune cells sense their surroundings and differentiate self from non-self as well as respond to infectious invaders and PAMPs from such invaders (*symbols of infection*) such as LPS. They also allow immune cells to pass along the knowledge of infectious interactions, immune cell to immune cell, to conspire (weigh the seriousness of and decide on a response) against potential threats. Markers are proteins identified by monoclonal antibody attachment and used to differentiate various cell types. Markers are used to define various immune cells according to their "origin, growth, differentiation, activation, recognition, migration, and function of the monocyte/macrophage" [41].

The activation of co-receptors (costimulatory and coinhibitory) has become an overarching principle of innate and adaptive immune system interaction in terms of both disease causation and in developing therapeutic strategies. The following is from Bojadzic et al. [51].

> Cosignaling interactions, which can be either costimulatory or coinhibitory, play important roles in regulating the activation of T cells and, therefore, adequate immune responses [42]. These cell surface protein-protein interactions (PPIs) belong to two main families: the immunoglobulin superfamily (IgSF; e.g., CD28–CD80/86, CTLA4–CD80/86, or PD-L1–PD-1) and the TNFR–TNF superfamily (TNFSF; e.g., CD40–CD154, OX40–OX40L, or 4-1BB–4-1BB-L). They are particularly valuable therapeutic targets because their modulation can provide more activation- and antigen-specific effects and, hence, safer and more effective immunomodulatory agents than currently existing ones [43–46]. There are now more than 25 cosignaling pairs in both the IgSF and TNFSF, presenting a large number of possible immunomodulatory targets [47]. The high therapeutic value of these PPIs is illustrated by the fact that two recent rational drug design success stories in immunopharmacology are related to their modulation by biologics (antibodies and/or fusion

[8] However, with the knowledge that has come from biologics therapy small molecule drugs are now also being used to target cell surface receptors.

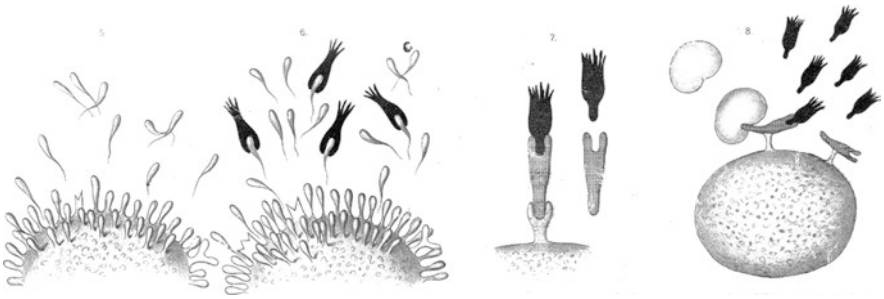

Fig. 7.6 Diagrams illustrating the side-chain theory of Paul Ehrlich. Credit: Wellcome Collection, CC 4.0

proteins). Specifically, inhibition of the binding of TNF to one of its receptors resulted in five FDA-approved anti-TNF biologics (e.g., infliximab) [48], while more recently, several anticancer biologics targeting immune checkpoint (coinhibitory) PPIs, in particular PD-1–PD-L1, have received FDA approval (e.g., pembrolizumab) [49, 50].

Of course, the existence of surface receptors hasn't always been known. Ehrlich proposed the "side-chain" theory toward the end of 1800's and from this the concept of "receptors", as he later called them, the science has since been greatly elaborated. The side-chain theory, Ehrlich first speculated, was the basis of interaction between antibodies and antigens and is shown in Fig. 7.6 as he imagined it.

Contrast Ehrlich's early vision above with a graphic of today's known receptors as shown in Fig. 7.12.

7.3.2.1 CD Markers

CD or "*cluster of differentiation*" is used to distinguish different immune cell types (i.e. "CD34+, CD31−" are cells that express CD34, but not CD31). Combining CD marker designations allows for cell types to be very specifically defined, given the many differentiated immune system cells. CD designated markers have been assigned every few years since 1982 when the first Human Leukocyte Differentiation Antigen (HLDA) workshop was held in Paris. To date over 400 CD surface markers have been assigned (Fig. 7.7). The Ig superfamily members make up 121 of 371 (of a total of 401 CD molecules as there are numerous sub-designations, i.e. 1a, 1b, etc.) as of 2015 (Engel et al.). CD cell surface molecules are shown in Table 7.1 of "CD Nomenclature 2015: Human Leukocyte Differentiation Antigen Workshops as a Driving Force in Immunology":

The CD designation refers to a group of mAbs shown by the statistical method of cluster analysis to recognize a particular cellular-differentiation pattern. The CD nomenclature is also used to name the molecule itself. For example, CD4 designates both the group of mAbs recognizing the CD4 cell surface molecule, as well as the CD4 molecule itself [53].

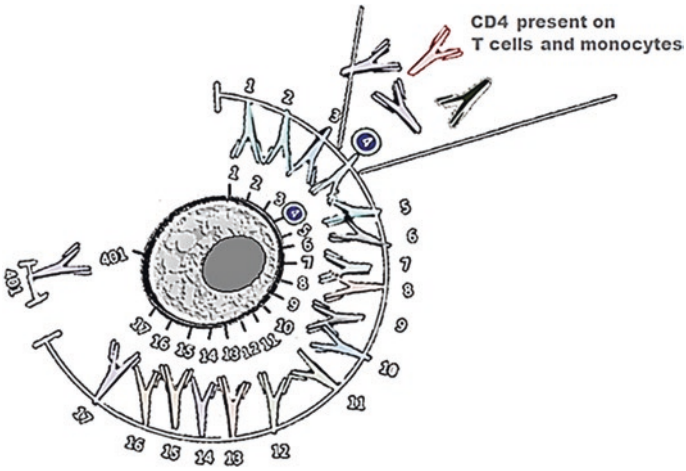

Fig. 7.7 Leukocyte cell (with nucleus) shows CD markers occurring in sequence of discovery on the same cell which, of course, they do not occur, as they help "differentiate" various cells by their presence or absence. CD markers are assigned to leukocyte cell surface molecules. According to Engel et al. "The CD nomenclature is also used to name the molecule itself. For example, CD4 designates both the group of mAbs recognizing the CD4 cell surface molecule, as well as the CD4 molecule itself". Leukocytes are white blood cells: lymphocytes, granulocytes, monocytes, and macrophages

Shown in Table 7.2 are some selected/familiar cell-surface markers and signaling molecules pulled from the Engel et al. reference (Table 1). These include TLR molecules (TLR4 is CD284) with an associated CD number as well as Fc molecules (FcγRs and a C-type lectin). TLR4 is rarely referred to as CD284 since it is so well known as TLR4.

In terms of which CD marker(s) resides on which of the various immune cells, and the vast accumulating numbers of markers and receptors present, it is natural to wonder where on T lymphocytes or B lymphocytes all the various receptors are located. At least for the TCRs, it appears to be the case that they are located on the microvilli, finger like projections, that connect the T cell with B cells and APCs [54]. See Fig. 7.8.

> The surfaces of T cells are not flat; rather, they contain finger-like protruding structures, microvilli, whose potential role in the immune response remains unknown. In this study, we mapped the location of the major T-cell immune response signaling molecule, the TCR, as well as two prototypical adhesion receptors, L-selectin and CD44, and a protein tyrosine phosphatase, CD45, in relation to the 3D surface architecture of T cells. The key finding of this work is that TCR assemblies are highly clustered on microvilli, both in resting lymphocytes and in recently activated effector T cells; disruption of microvilli structures results in disruption of TCR microclusters on the cell surface. This discovery immediately points to a potential role for microvilli in the recognition process inherent in the immune response, a role that has not been suggested before in the literature. (Jung et al.)

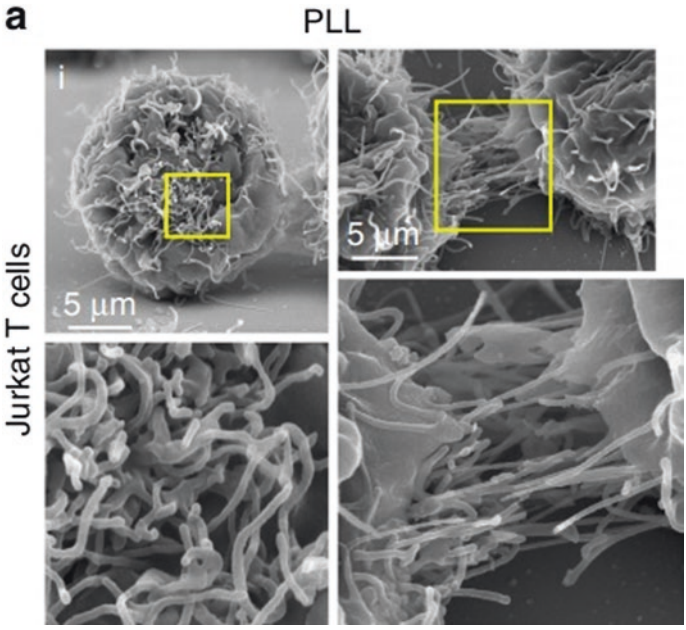

Fig. 7.8 T cells generate microvillus-originated particles upon TCR stimulation. (**a**) T cell microvillus polarization toward antigen-bearing B cells at the early stage of IS (immune synapsis). SEM of resting single (left) or two adjacent (right) Jurkat T cells on PLL (poly-L-lysine (PLL)-coated surface) [55]. CC 4.0. Only (a) shown

Table 7.2 Selected CD designated molecules from CD Nomenclature 2015 (Engel et al.). One can see that many receptors with common names also have corresponding CD nomenclature designations (i.e. TLR4 = CD284)

CD	Other names	Gene family	Gene name	Gene #
CD16	CD16a, FcγRIIIA	Ig superfamily	FCGR3A	2214
CD16b	FcYRIIIA	Ig superfamily	FCGR3B	2215
CD23	FcεRII, BLAST-2	C-type lectin family	FCER2	2208
CD281	TLR, TIL	TLR family	TLR1	7096
CD282	TLR 2, TLR2, TIL4	TLR family	TLR2	7097
CD283	TLR 3, TLR3	TLR family	TLR3	7098
CD284	TLR 4, TLR4	TLR family	TLR4	7099
CD286	TLR 6, TLR6	TLR family	TLR6	10,333

In this way we can envision the on-going communication between immune cells in terms of antigen receptor (TCR/BCR) and co-stimulatory and co-inhibitory receptor signals exchanged via interconnecting microvilli. Just as the pictures of porins (Chap. 4) formed an *a priori* background for reimaging the microbiological state of the Gram negative cell, this picture may help form such a redefining image for lymphocytes and their receptors from an individual perspective.

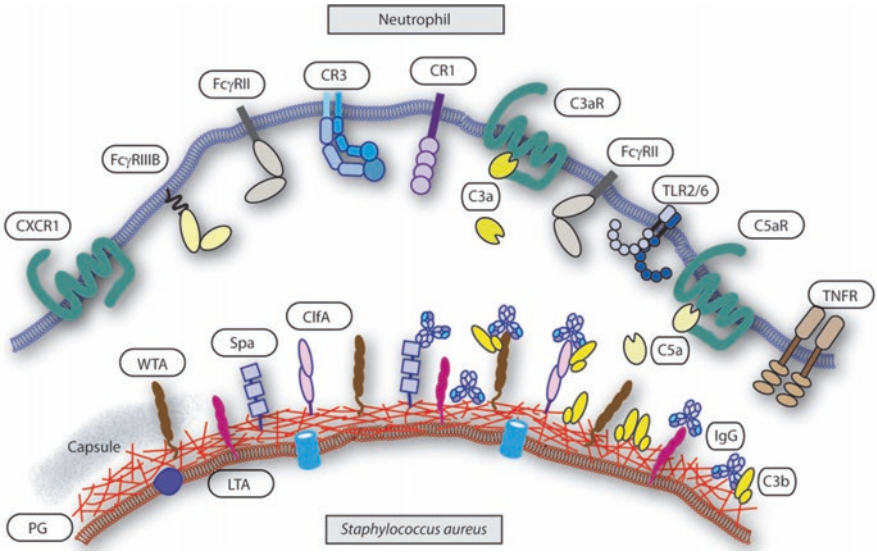

Fig. 7.9 Interface of neutrophil and *S. aureus*. Several groups of receptors mediate neutrophils recognition of *S. aureus* upon opsonization and others are involved in activation or priming of phagocytosis. Targets on the S. aureus surface are the cell wall components peptidoglycan (PG), wall teichoic acid (WTA), lipoteichoic acid (LTA), capsule ("gray area"), and representative associated proteins clumping factor A (ClfA) and protein-A (Spa). Targets are decorated with serum derived opsonins IgG (binding with their Fab part) and C3b. Note the reverse Fc-dependent association of IgG with Spa. Receptors on the neutrophil surface involved in recognition of the opsonized *S. aureus* are FcγRII and FcγRIII for IgG, and CR1 and CR3 for C3b (and iC3b). Examples of receptors on the neutrophil involved in priming or activation of phagocytosis are complement receptors C3aR for C3a and C5aR for C5a, CXCR1 for il-8, and TNFR for TNFα. The heterodimer TLR2/TLR6 represents a common pattern recognition receptor for bacterial lipoproteins. (From van Kessel, Bestebroer and van Strijp [52])

Maria Shipkova and Eberhard Wieland [56] overview the co-stimulatory molecule families and divide them into those that are "constitutively expressed on naïve and activated T cells" and those that are "expressed or upregulated only upon T cell activation". This is an important distinction for the next sections: "Whereas CD28 is constitutively expressed on naïve and activated T cells, the other co-stimulatory molecules are expressed or upregulated only upon T cell activation".

7.3.2.2 Clonal Versus Non-clonal Receptors

There are only two types of clonal receptors: T cell and B cell receptors. These receptors are produced by non-germline encoded rearrangements (of a germ-line encoded small set) to be very specific and diverse and are "clonal" because once activated by antigen and confirmed by a co-stimulatory receptor signal they are cloned to make many identical copies to combat specific molecules associated with

infection. A single B cell contains only one type of surface-bound B cell receptor (BCR) per B cell and when this version is activated it is subsequently cloned and sent out in soluble form (antibodies) to bind whatever antigen sequence it was that activated the membrane bound BCR. The clonal receptors undergo somatic (non-germ cell) hypervariable rearrangement. The antigen-binding structures are not germ-line conserved to specifically fit PAMPs as are the TLRs and other non-clonal receptors. A single B cell "…contains up to 120,000 B cell antigen receptor (BCR) complexes on its cell surface" [57].

The TLRs are the most well-known as well as the best characterized example of non-clonal receptors that have been evolutionarily preserved to respond to specific microbial PAMPs. These are invariant receptors (other than polymorphisms) because they are germ-line encoded proteins that have developed over the eons, are not rearranged, and are selected for their survival utility by their repeated interaction with microbial byproducts. Other non-clonal receptors include a myriad of "CD" designated protein receptors, many of which are involved in the immune cell conversation. It should be emphasized that in addition to TLRs and other PRRs (including complement receptors) the myriad of various CD designated receptors are all non-clonal type receptors:

> These signals, which bound molecules (e.g., B7, CD40L and FasL) are recognized by non-clonal receptors expressed on lymphocytes (CD28, CD40, cytokine and homing receptors, etc.). Non-clonal receptors arise over evolutionary time, have genetically encoded specificities, their ligands contain certain semantic information defined by natural selection and, accordingly, ligation of these receptors induces a cellular response corresponding to the semantics of their ligands. We suggest that these signals are induced by pathogens and carry the information about the features of pathogens required to induce an appropriate immune response [58].

It is important to view these non-clonal receptors as often "*induced by pathogens*" and as such they serve as co-stimulatory signals. The utility of clonal and non-clonal receptors working together and providing an integration of the innate and adaptive response can be seen in Fig. 7.10.

7.3.2.3 Fc Receptors

Human leukocyte Fc receptors (FcR) are also non-clonal receptors and bind the Fc conserved region of antibodies to facilitate the phagocytosis of bacteria and particulate matter. These non-clonal receptors are able to bind the Fc end (tail) of the antibody "Y" while the Fab (clonal) ends bind microbial or viral derived antigens. This allows the phagocytic uptake of Fab bound antigen as shown in Fig. 7.11. See also Fig. 20.11.

There are three types of IgG Fc receptors (IgA and IgE also have Fc receptors):

(a) *FcγRI* (CD64) has high affinity for IgG and is "expressed on monocytes, macrophages, and dendritic cells, and is induced on neutrophils and eosinophils following their activation by IFNγ and G-CSF (granulocyte colony-stimulating factor)." (Roitt)

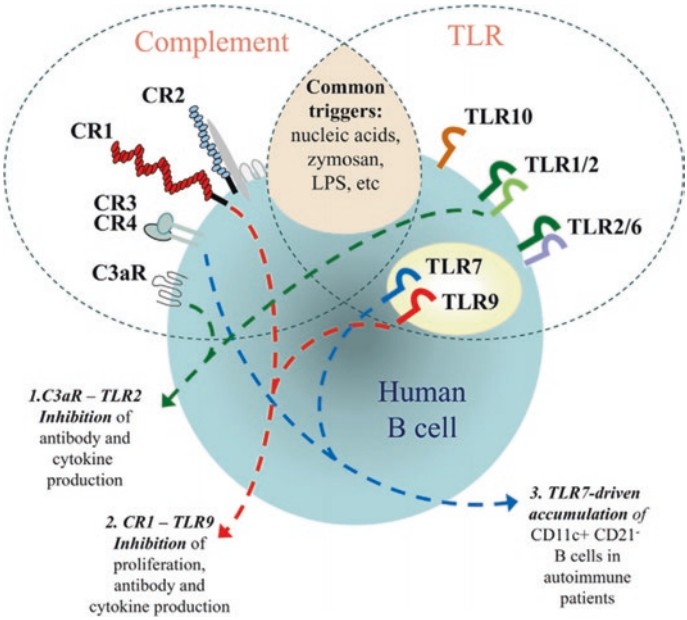

Fig. 7.10 Expression and crosstalk of complement receptors and Toll-like receptors in human B lymphocytes. The complement system and TLRs are key elements of innate defense mechanisms, which can be triggered by several common damage or pathogen associated molecular patterns – such as nucleic acids, zymosan and LPS. The crosstalk between TLRs and CRs on human B cells has been demonstrated in three studies so far. 1. Fischer and Hugli [59] demonstrated the direct, suppressive effect of C3a on antibody and cytokine production by B cells stimulated by *Staphylococcus aureus* Cowan strain I (SAC), which contains a TLR2-active lipopeptide beside SpA [60]. 2. We found that the simultaneous engagement of CR1 and TLR9 inhibits BCR-triggered B cell functions [61]. 3. Rubtsov et al. have shown that TLR7 induces accumulation of a CD11c + CD21− B cell subpopulation in patients with autoimmune disease, which might play a role in presentation of autoantigens [62]. (From Kremlitzka et al. [63])

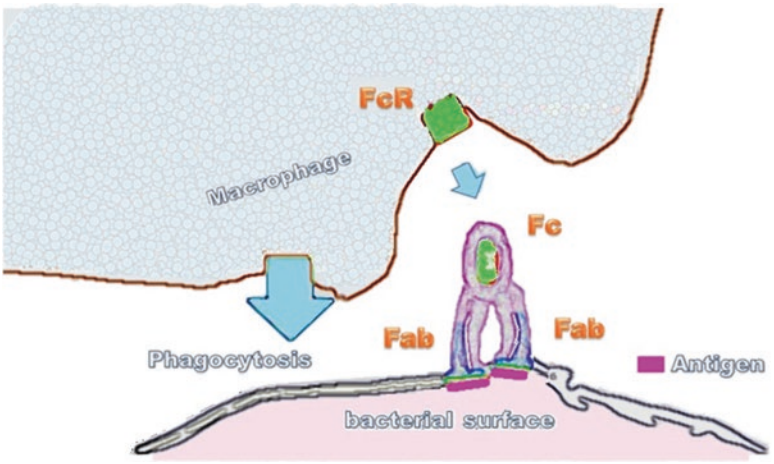

Fig. 7.11 Cartoon of Fc receptor (FcR) juxtaposed with antibody Fc where Fabs are attached to antigen. Besides Fc receptors, phagocytic receptors include scavenger receptors, Dectin-1, and complement receptors

(b) *FcγRII* (CD32) has high affinity for antibody-coated target cells to trigger phagocytic cells
(c) *FcγRIII* (CD16) is mainly responsible for antibody-dependent cell cytoxicity (ADCC) by NK cells and the clearance of immune complexes from the blood via macrophage.

The Fc receptors (FcR) are a literal bridge between innate and adaptive immunity as Fc receptors (non-clonal) on phagocytic cells attach to the Fc region of antibodies (clonal) that have attached to microbes via complement, LPS, pentraxins, C-type lectins (which bind to glycosylated PAMPs including β-glucans), and nucleic acids to facilitate their uptake via phagocytosis. See Chap. 20 for a continuation of this discussion.

7.3.3 PAMP Activation of Adaptive Immunity

Janeway proposed *the very idea of a Pattern Recognition Receptor (PRR)* in 1989 (not so long ago!) and predicted that innate PRRs contain the geometric pattern information necessary to identify and respond to PAMPs like LPS [64].

> Janeway's proposal of a microbial sensor eliciting an innate immune response that was subsequently interpreted by lymphocytes, the key cells of the adaptive immune system, as "permission" to mount a response when they recognized an antigenic substance was enormously influential [65].

7.3.3.1 The Second Signal

Janeway's logic in predicting the necessity of PRR control over adaptive (clonal) responses was that, in contrast to germ-line encoded (hard-coded) PRRs such as TLRs, the adaptive immune system, given the hyper-variability-generating system used to make millions of different antibody variants by shuffling small sets of germ-line genes, can only "guess" at non-self infectious structures.[9]

> The binding properties of antibodies are determined by the sequences of their corresponding B cell receptors (BCRs). These BCR sequences are created in "draft" form by VDJ recombination, which randomly selects and deletes from the ends of V, D, and J genes, then joins them together with additional random nucleotides. If they pass initial screening and bind an antigen, these sequences then undergo an evolutionary process of mutation and selection, "revising" the BCR to improve binding to its cognate antigen [66].

Though not developed to bind specific structures as are TLRs (as antibodies are "anticipatory"), this adaptive "guesswork" is so prolific that it is all but certain to hit a target. When it does hit a target, it needs direction from PRRs to ensure that the target is non-self. Thus, the adaptive structures, membrane-bound antibody receptors

[9] Though there are some complex methods of weeding out self-reactive versions.

on B or T lymphocytes require the context provided by PRRs, including TLRs such as TLR4, otherwise deleterious autoimmune binding events would be much more frequent. PRR confirmatory signals are called "co-stimulatory" or "co-inhibitory" signals:

> Because the gene shuffling mechanisms used to generate diversity among TCRs and BCRs can end up recognizing self, the cells of the adaptive immune system require instruction by the cells of the innate system as to whether an immune response should be mounted to a particular antigen (or not). This is a critical role… [67]

Fierz describes the diversity generating process similarly,

> …the semantic information is conveyed by the non-clonal recognition system (PRRs), because randomly created receptors cannot carry semantic content as they would not *know* in advance what antigen they will recognize and what type of response they will have to induce [68].

Several innate interfacing receptors are involved in endotoxin detection, especially TLR4 and complement receptors (CRs), which are highly responsive to LPS. Of relevance to biologics MCC, it is apparent that a patient need not have a fever to generate an anti-drug antibody (ADA) or neutralizing drug antibody (NDA) responses to injected therapeutic proteins and the generation of antibodies can be facilitated by the presence of TLR ligands, especially LPS, at levels below the occurrence of fever. This comes in the form of a co-stimulatory signal or "second signal". Vaccinology has long utilized an analogous concept by adding adjuvants made from microbial and viral PAMPs to induce immunogenicity. As can be seen in Fig. 7.12, the immunological knowledge surrounding the immune responses via various receptors, co-stimulatory and co-inhibitory, has advanced exponentially since Janeway's initial observations. The number of receptors that can provide the "co-stimulatory" or "co-inhibitory" signals as provided by PAMPs is a large and growing list. The figure below from Zhu, Yao, and Chen shows seven different immune type cells (labeled near the nucleus) and shows the various initial or primary signals (green arrow) as well as many different but also overlapping co-stimulatory (red arrow) and co-inhibitory (black T) receptors.

Such a figure demonstrates the current adaptive immue complexity, but here the focus is on expressing a "*simple model*" suitable to guide overarching methods. Though the mammalian host receptors and corresponding microbial surface PAMPs shown in Fig. 7.9 show a neutrophil interfacing a Gram-*positive* bacterium (*S. aureus*), the receptors are largely overlapping in terms of Fc receptors, complement receptors, chemokine receptors, TLRs, TNF receptors, etc. with PAMPs (including LPS) on Gram-*negative* bacteria. It is good to view the totality of receptors from a Gram positive PAMP engagement perspective here as LPS should be acknowledged often as "*one of many*" PAMPs. The "*one of many*" view has been greatly glossed-over historically in the MCC of SMDs because LPS has been by far the most critical PAMP (potent, prevalent, enduring, etc.), however, with the advent of biologics there is now an indication that synergy of LPS with other PAMPs may play a more important role.

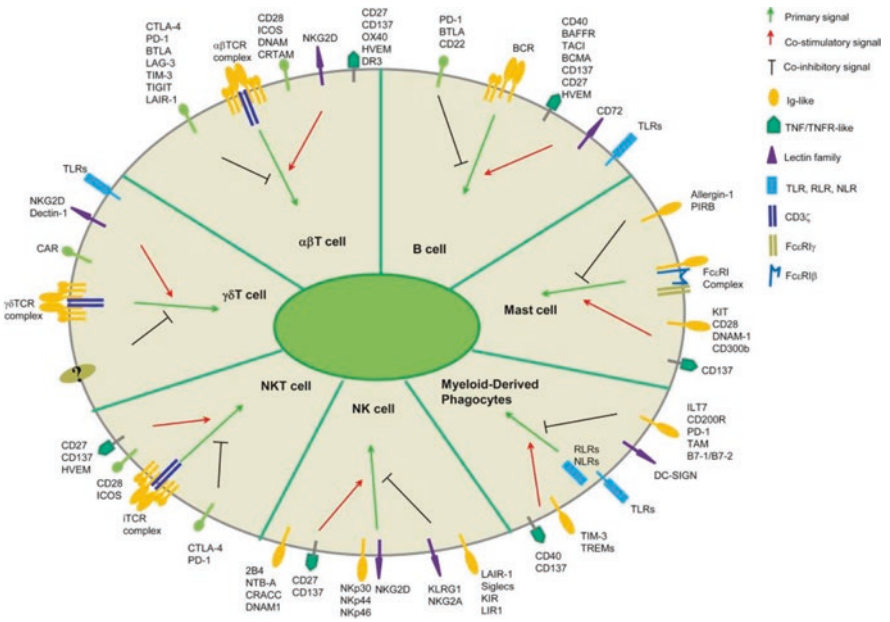

Fig. 7.12 Cell surface signaling molecules in the control of immune responses. Primary signal and co-signals (co-stimulatory or co-inhibitory) are defined differently in each immune cell type. TLR, Toll-Like Receptor; RLR, RIG-like Receptors; NLR, NOD-like Receptor. "B cell" receptor section (top right) shows initial signal and potential 2nd signals. **Initial signal:** BCR, **Co-inhibitory:** PD-1, BTLA, CD22. **Co-stimulatory:** CD40, BAFFR, TACI, BCMA, CD137, CD27, HYEM, CD72, TLRs. (From Zhu, Yao and Chen [69])

7.3.3.2 Licensing the Second Signal

In speaking of the "*second signal*", the concept of "*licensing*" of that signal is important to highlight because it seems much less obvious relative to direct PAMP activation, in that the non-clonal receptor presence on APCs are activated by PAMPs. The idea of "*licensing*" is, therefore, the transfer of adaptive immune system activation "*permission*" from a PAMP to an antigen presenting cell (APC) in the sense of conveying upon it the "power" of adaptive activation. It extends the second signal idea to that of PAMP activation via a "*licensed*" immune cell or cytokine signal as produced by PAMP interaction which is (at least) one step away from direct PAMP activation as occurs via a PRR/TLR. In T-cells there is also "signal three" (that includes the production of cytokines that provide directionality to Tc and Th cells) [70]. The cytokine-priming function by signal 3 primarily determines the nature of the T cell responses generated [71]. Rather than gaining permission directly from a PAMP (such as LPS), the APC has presented a "licensee" in the form of a non-clonal receptor (in Fig. 7.13 it is B7) that serves the co-stimulatory purpose.

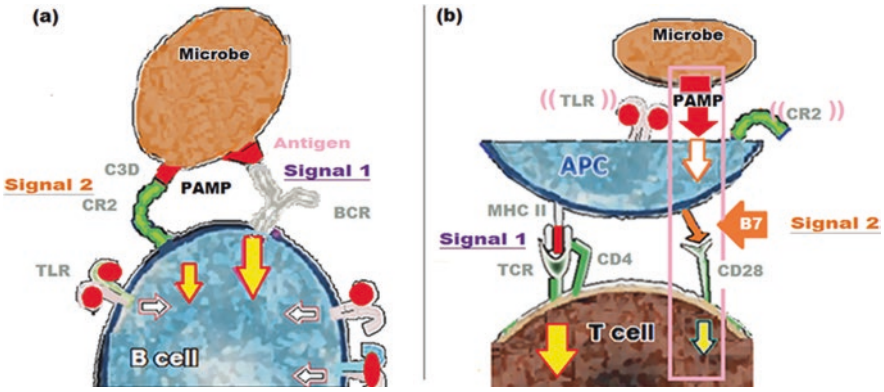

Fig. 7.13 Direct versus licensed co-stimulation where in (**a**) co-stimulatory signal derives directly from receptor for PAMP (complement receptor or TLR as shown also in Fig. 7.10) whereas in (**b**) the co-stimulatory signal is licensed from PAMP to B7 to activate CD28 for T cell co-stimulation (as also shown in Janeway's Fig. 7.15). Co-stimulatory signals also occur via cytokines (pink parenthesis) which are PAMP activated and provide additional directionality (called signal 3). Red indicates direct PAMP activation (though processed through MHC II for TCR). Orange indicates co-stimulation as licensed from a PAMP, here via B7

7.4 A Simple Immunological Model for MCC

Janeway's observation was a straightforward proposition that has been wildly borne out, indeed it has become an organizing principle for all of immunology, but the details and variety of its expression have also become much more complex as applied to a myriad of specific immune cell and PAMP specific interactions. As it turns out, the "*second signal*" (co-receptor) that represents the innate permission for a lymphocyte to respond to an antigen BCR/TCR can be given in many ways that includes directly as given by a PAMP (i.e. BCR and Complement receptor on B cell) or as "*licensed*" to an APC to present to a B cell or T cell: "A DC that has been properly activated for this purpose is referred to as a DC 'licensed' for cross-priming" (Thaiss et al [72]).

As Janeway explained it, a "*second signal*" (co-stimulatory or co-inhibitory) is needed from the non-clonal PRRs to confirm or deny permission to B and T cell receptor responses and thus, ultimately, to antibody production. He thought this because the clonal type receptors (TCR and BCR) contain hyper-variable or random CDRs (paratopes) that are referred to as "anticipatory" in nature and therefore cannot be expected to "*know*" what is self and what is non-self without direction from PRRs (including TLR4). Looking back at quotes from Janeway's 1989 Cold Spring Harbor lecture, his ideas were very prescient and today read like an outline for discoveries that have occurred since 1989. See Appendix I for a longer list of Janeway's more recently borne out predictions from 1989. The most important of the concepts are summarized in Fig. 7.14.

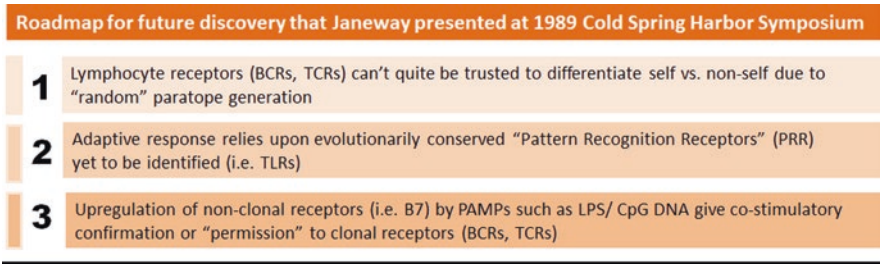

Fig. 7.14 Concepts proposed in Janeway's Cold Spring Harbor Symposium lecture in 1989 that were subsequently confirmed

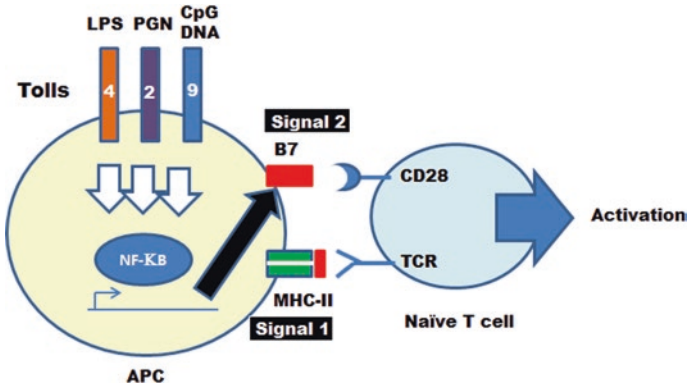

Fig. 7.15 Various PAMPs recognized by cognate pattern recognition receptors expressed on APCs induce the expression of B7 molecules, which signal the presence of pathogens and allow activation of lymphocytes specific for antigens derived from the pathogens. Shown are lipopolysaccharide recognition by TLR-4, peptidoglycan recognition by TLR-2, and the recently reported role of TLR-9 in the recognition of CpG DNA. (Derived from Figure 1 of Janeway's paper (2001) [73])

At the time Janeway delivered the Cold Spring Harbor lecture (1989), the development of the adaptive immune system had been largely defined in broad terms, whereas the existence of innate receptors (including Toll-like receptors) had yet to be discovered. It is interesting that the much more complex clonal mechanisms of adaptive immunity were deciphered before the evolutionarily older germ-line encoded PRRs (especially Toll-like receptors) were found (by Janeway and Medzhitov, 2001). The reason for this may have been related to the early empirical knowledge gained from adaptive immune treatments including vaccines and antitoxins that had been discovered just prior to the turn of the century, 1900's (Chap. 2).

A simple model of the important concept (innate activation of adaptive immunity) provides non-immunologists charged with MCC activities with a view of microbiological control that is relevant for biologics drug manufacturing from a non-pyrogen vantage. Janeway's model put forward is shown in Fig. 7.15. A pro-

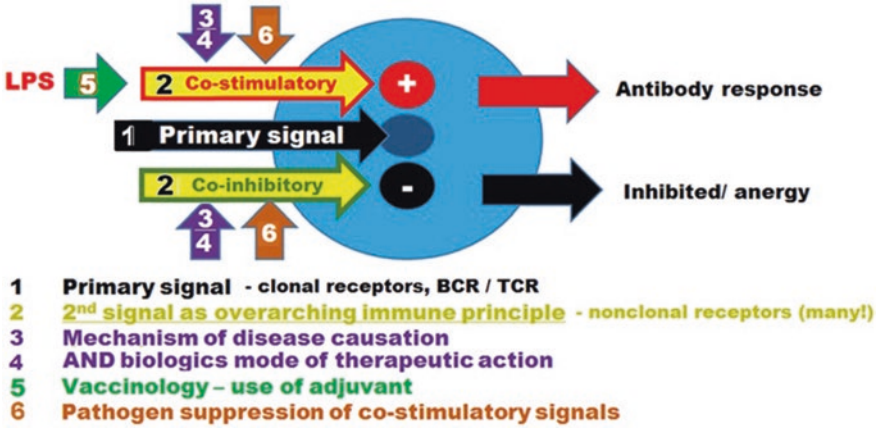

Fig. 7.16 A simple model that informs MCC is shown where an antigen signal is confirmed or denied by a second signal (costimulatory or co-inhibitory). The simple model contrasts the pyrogen-only model that has been used for 40 plus years (Fig. 7.17). Derived and expanded from Zhu, Yao and Chen. The "*second signal*" model adds explanatory power, listed below the graphic, in a manner that the *"endotoxin as only a pyrogen"* model cannot

posed simple model is shown in Fig. 7.16 and the associated list adds "explanatory power" that the pyrogen model cannot encompass.

The need for a second signal serves as a guiding principle in the vast majority of adaptive immune interactions but can be overcome by some particularly strong immune activators (including LPS). Polyclonal or indiscriminate activators of adaptive immune responses are called mitogens:

> In most cases, the engagement of TCRs by pMHCs[10] is not sufficient to fully activate a naïve Th[11] or Tc[12] cell, and signal 2 in the form of costimulatory signaling is required. Occasionally, a Tc cell will encounter a pMHC (usually derived from a virus) that delivers such a strong signal 1 that costimulation is not required; this response is then independent of both costimulation and Th cell help [74].

On T cells alone, the number of known co-stimulatory and co-inhibitory receptors has exploded in number and interrelated complexity as seen in Fig. 7.18. Bugeon and Dallman [76] have asked: "Why are there so many costimulatory molecules?" The answer they have offered is that either this level of redundancy suggests that co-stimulation is critical to survival, or, "alternatively, the different ligands may mediate subtly different effects through binding to the different receptors." Likely, both are true and speak to the level of sophistication that the mammalian immune system has developed. Clark and Kupper [77] summarize the signaling hierarchy as follows:

[10] pMHC are peptides presented by major histocompatibility complex.

[11] T helper cells (or CD4 cells).

[12] Cytotoxic T cell (or CD8+ T-cell).

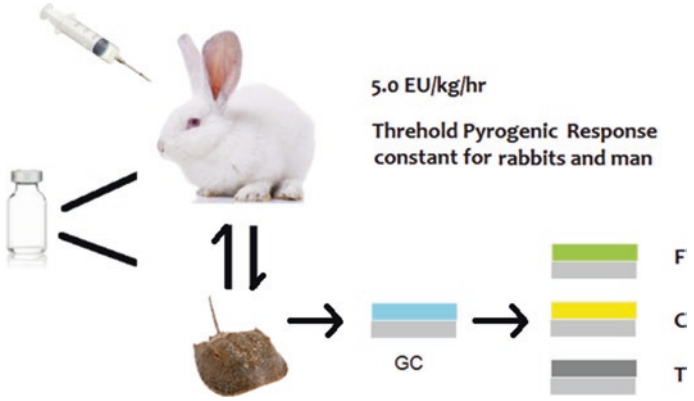

Fig. 7.17 The historical model of the mammalian response to endotoxin, "*endotoxin as a pyrogen*", as used successfully for decades to prevent fever in LVP and SMDs as based upon the systemic level fever response or a fraction thereof as detected by *Limulus*-based methods including, GC Gel Clot, C chromogenic LAL, T turbidimetric LAL, F fluorescent rFC

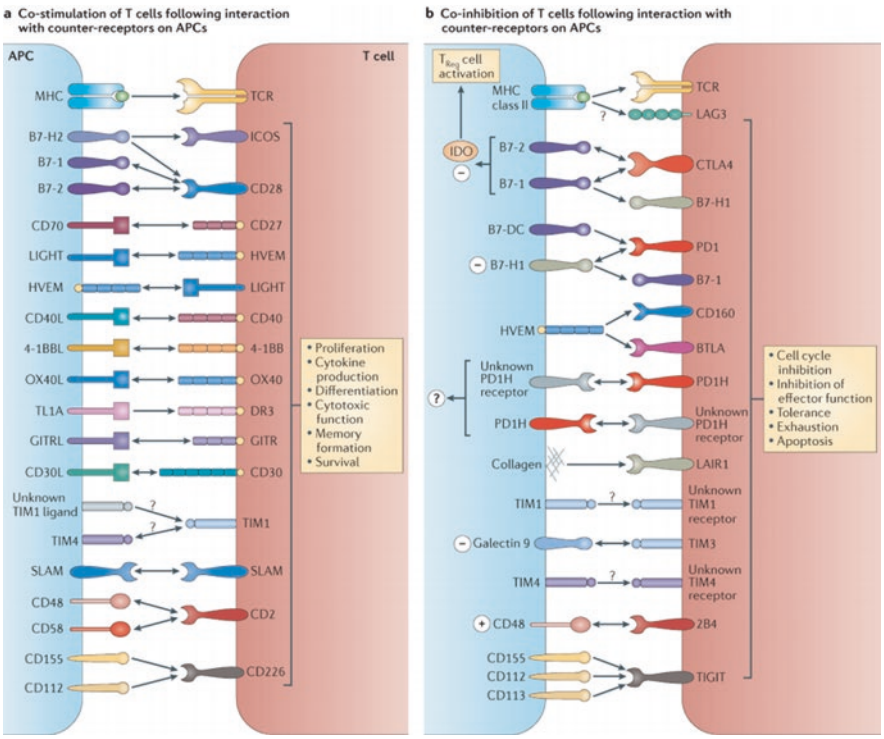

Fig. 7.18 Co-signaling interactions in T cells (**a**) Co-stimulatory molecules deliver positive signals to T cells following their engagement by ligands and counter-receptors on antigen-presenting cells (APCs). Several co-stimulatory molecule interactions are bidirectional. (**b**) Co-inhibitory molecules deliver negative signals into T cells. Cytotoxic T lymphocyte antigen 4 (CTLA4) is involved in bi-directional interactions: it inhibits T cell function after binding B7-1 and B7-2, and CTLA4-bound B7-1 and B7-2 may induce the expression of indoleamine 2,3-dioxygenase (IDO), which acts in trans to suppress activation of conventional T (TCon) cells and promote the function of regulatory T (TReg) cells. (From Chen and Flies [75], Figure 1a, b)

> It is now becoming recognized that dendritic cells pass on a remarkable amount of information to T cells about the type of insult that prompted their maturation (Kapsenberg, 2003). This information affects whether a T cell will respond to antigen, how it will respond, and likely where it will go to respond.

See also Corse, Gottschalk and Allison [78] who describe three conditions of activation: potency, density, and duration of contact (T-cell-APC). The desire is to find utility in overarching concepts relevant to microbial control and adaptive immunity. What is not discussed in this chapter but is important also to understand in context of signal 2 is the complexity of Signal 1, especially in T cell responses as discussed in Chap. 8.

7.4.1 Explanatory Power of a Simple Model

A "simple model" for MCC purposes that includes basic adaptive immunological concepts adds explanatory power to biologic drug manufacturing by viewing potential contaminants, including endotoxin, from an immunological versus strictly microbiological vantage. Such a model contributes understanding to a surprisingly wide range of topics relevant to biologics manufacture, therapy, and control that the *"endotoxin as pyrogen"* model cannot speak to, including:

(i) Modulation of co-stimulatory/co-inhibitory receptors is an inherent mechanism of disease causation
(ii) Modulation of co-receptors *is also the mode of action of many biologics molecules (especially mAbs)*
(iii) Action of adjuvant usage in vaccinology, specifically the need for adjuvants when purified microbial or viral proteins cannot generate the needed immunogenicity [79].
(iv) Suppression of host immunity by pathogens via modulation of (co)receptors

The point of the model, least it be missed in the tedious elaboration of the details of its occurrence, is that a number of PAMPs may serve as the second signal to stimulate lymphocytes [80–83] and thus may interfere with the immune modulating activity of biologics therapeutics, including potentially contributing to the generation of antibodies against drug proteins. This activity is not synonymous with pyrogenic activity but is more akin to the use of an adjuvant in vaccinology. According to this rationale, although a lymphocyte can't know what next year's microbial surface structures will be, it does apparently "*know that it doesn't know*" and thus requires cues from the "hard-wired" PRRs including TLRs. Antibody diversity thus produced serves to swamp incoming microbial artifacts (epitopes) with potential binding sequences (paratopes) and thus "matches" the microbial world structure for structure (paratope to epitope) and in a similarly rapid manner as the rate of microbial mutation.

7.4.2 *Co- Receptor Modulation Is Inherent in Disease Causation as Well as the Mode of Action of Biologics Molecules*

These two topics, disease causation and biologics mechanism of action, could have been (laboriously) teased apart into two sections, however, they are so intertwined that in effect they must be discussed together. Medzhitov [84], 20 years later, summarized the two signal concept that was popularized by Janeway's 1989 lecture.

> Charles Janeway's unique contribution was in developing a new synthesis that placed many immunological phenomena in a clear biological context. First, he proposed that the costimulatory signal required for lymphocyte activation was inducible (on antigen-presenting cells [APCs])… Second, the costimulatory signal was suggested to be inducible by conserved microbial products, thus placing the activation of adaptive immunity under the control of pathogen sensing mechanisms. This also explained the adjuvant properties of certain microbial stimuli. The fact that many microbial molecules, such as LPS, had immunostimulatory properties was known before, but it was not clear why some microbial molecules have these properties, including adjuvant activity, whereas others do not. Neither was it known how these microbial structures exerted their adjuvant effects. Janeway suggested that the actual detection of infection was mediated by the receptors of the innate immune system, rather than the antigen receptors. Specifically, he proposed that innate immune system determined the origin of antigens recognized by T and B cells and instructed the latter to initiate the response if antigen was of microbial origin. Pathogen sensing, in turn, was proposed to be mediated by a set of germline-encoded pattern recognition receptors that detect conserved products of microbial biosynthetic pathways (known as pathogen-associated molecular patterns [PAMPs]). Janeway further pointed out that this form of immune recognition must be evolutionarily related to the immune systems of invertebrates, which lack adaptive immunity. Finally, most adjuvants were suggested to work in part by mimicking microbial infections, by triggering the receptors of the innate immune system and inducing costimulatory signals, thus "tricking" the adaptive immune system into action. These and other ideas provided an elegant explanation of many fundamental aspects of the functioning of the immune system. Remarkably, all of them turned out to be fundamentally correct.

The most obvious means of disease causation associated with the second signal is seen in **infection**. Attanasio and Wherry give a good account of the role of costimulation in infection.

> A prominent role for inhibitory receptors during infections has emerged largely from the study of persisting infections where immune function becomes suppressed, facilitating pathogen persistence. Exhaustion of T cells was first described in chronic LCMV[13] infection where T cells are persistently stimulated and develop a series of defects, particularly in the ability to mediate effector functions, proliferate, and acquire memory T cell properties (Fuller and Zajac 2003; Moskophidis et al. 1993; Wherry et al. 2003; Zajac et al. 1998).

Below several examples will be given of B cell and T cell co-receptor activation and inhibition. The best characterized set of costimulatory/coinhibitory receptors are T cell resident receptors CD28/CTLA4 which bind ligands CD80/CD86. Both the costimulatory receptor (CD28) and the coinhibitory receptor (CTLA4) bind to both ligands CD80/CD86 (B7.1 and B7.2). The modulation of CD28/CTLA4 has served

[13] LCMV is Lymphocytic Choriomeningitis Virus.

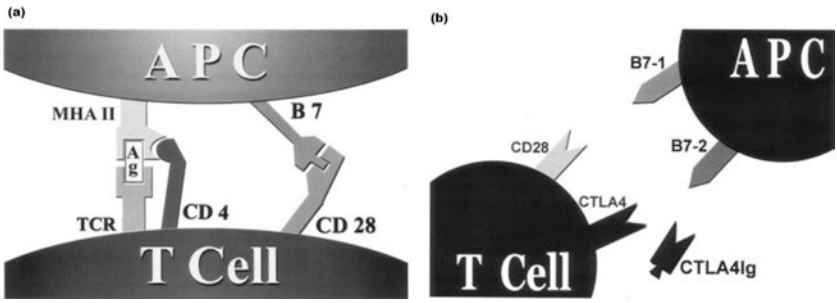

Fig. 7.19 (**a**) Two signals govern the T cell response to antigen. The first signal occurs when the T cell receptor (TCR) binds an antigenic peptide (Ag) in the context of major histocompatibility complex (MHA) molecules on antigen-presenting cells (APC). The second signal involves other receptor-ligand pairs on the surface of T cells and APC, such as CD28 on T cells and B7 on APC. (**b**) One strategy designed to block T cell costimulation is based on the structural similarity (homology) between CD28 and CTLA-4. CTLA-4 binds tightly to the B7 ligands for CD28. Therefore, a fusion protein composed of the extracellular domain of CTLA-4 linked to the constant region of an immunoglobulin molecule (CTLA-4Ig) can bind to B7–1 and B7–2 and, by so doing, prevent them from signaling T cells via CD28. APC antigen-presenting cells. (From Daikh et al. [85])

as the basis for understanding both disease progression and biologics therapy in infection, transplant rejection, autoimmunity and cancer. The details of CD28/CTLA4 competition for receptors shows the tug-of-war occurring between stimulatory and inhibitory signaling. The most basic interactions are shown in Fig. 7.19.

> The inhibitory receptor CTLA-4 is a structural homolog of the costimulatory receptor CD28 and shares with CD28 the same binding partners B7-1 (CD80) and B7-2 (CD86), but binds with greater avidity and affinity (Collins et al. 2002). CTLA-4 is a covalent homodimer (Lindsten et al. 1993; Linsley et al. 1995); its higher avidity for B7 ligands results from the binding of each CTLA-4 homodimer to two divalent B7 molecules, leading to the formation of a stable CTLA-4-B7 structure on the cell surface. This contrasts with the monovalent binding of B7 molecules by CD28 [86].

The greater avidity and affinity of the inhibitory co-receptor (than the co-stimulatory receptor) seems to suggest the immune motto: "*first do no harm*" with autoimmune disease, cancer, and transplant rejection being violations of that motto. The CD28–B7 costimulatory pathway has been found to be important in **infection and transplant rejection** and therapeutic utility has been gained by blocking the pair using abatacept and belatacept [87]. Drug scientific names have been bolded throughout the discussion below, at least at first mention.

> **Abatacept** and **belatacept** are both CTLA-4-Ig fusion proteins that bind to both CD80 and CD86 on the surface of APCs, thereby blocking both CD28 co-stimulatory signals as well as CTLA-4 co-inhibitory signals. By contrast, anti-CD28 (dAb[14]) and scFVs[15] each bind selectively to co-stimulatory CD28, inhibiting its binding to CD80 and CD86 while leaving intact the binding of the CTLA-4 co-inhibitor with these ligands. (Ford, Adams, and Pearson)

[14] dAb is domain antibody.

[15] svFV is single-chain variable fragment.

Interestingly, in kidney transplant rejection LPS has been hypothesized to play a more direct co-stimulatory role in activating B cells [88]. See also Quintana et al. [89].

> The identification of LPS as the immunogen immediately suggests a mechanism for the observed clonality of the B-cell clusters that infiltrate kidney transplants. The B-cells in these clusters may become clonal because they have a selective advantage for replication in that LPS can engage both the BCR and Toll4 receptors and is, thus, capable of costimulation of cells bearing both receptors. (Grover et al.)

B cell responses[16] are classified as T-independent (TI) or T-dependent (TD) depending on whether the antigen type requires help from a T-cell or not. TD antigens are proteins that come via presentation on MHC class II molecules. TI antigens are divided into type I and type II.

> The former (Type I) are mitogenic stimuli such as LPS, CpG, or poly-IC that elicit polyclonal B cell activation via Toll-like receptors, whereas the latter (Type II) are polysaccharides that engage the B cell receptor and thus induce antigen-specific B cell responses [90].

According to Bluestone [91], "more than 80 diseases with an **autoimmune** etiology have been identified." Co-stimulation and co-inhibition signals are thus an important model for understanding disease occurrence as well as for the subsequent development of treatments.

> Costimulation plays an essential role in autoimmunity. In mice, for example, blocking CD40:CD154 costimulation shuts down a variety of autoimmune diseases, including T1D in nonobese diabetic mice, experimental autoimmune encephalitis (the mouse equivalent of MS), and mouse models of RA. Similarly, blocking CD28/CD86 pathways can inhibit a number of autoimmune syndromes in small animals, as well as in humans. Together the TCR and costimulatory antagonist have developed as part of a new drug arsenal for the treatment of autoimmune diseases. (For more information, see [92]); programmed death (PD)-1/PD-L1; and B and T lymphocyte attenuator (BTLA)-4. (Bluestone)

Underlying co-receptor dysfunction in autoimmunity disease causation may often include polymorphisms: "Polymorphisms in CTLA4 are associated with human autoimmune diseases [93], consistent with the critical role of CTLA-4 inhibitory signals in tolerance" [86].

Chittasupho et al. [94] lists 14 costimulatory molecules by which immunological signaling has been found to involve important levers for controlling the immune system. Complement dysfunction has been found to exacerbate autoimmune disease. Complement receptor 2 (CD21) which is active on B cells is fundamental to B cell response as a costimulatory receptor and has been shown to contribute to autoimmune disease as a second signal. As shown below, CR2 is composed of 15 CCP domains. As discussed in Chap. 18, CCP domains are also called Sushi domains (5 such domains occur in *Limulus* Factor C) and are, therefore, an ancient protein domain common in complement and blood coagulation proteins as a product of evolutionary descent. See the mechanism of CR2 receptor complex costimulation of BCR in Fig. 7.20.

[16] as only plasma cells from activated B cells produce antibody.

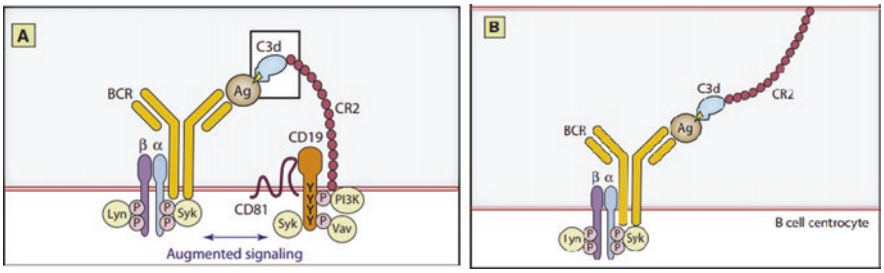

Fig. 7.20 Complement as a bridge linking the innate and adaptive immune systems – the molecular adjuvant role of antigen-linked C3d. (**a**) Co-ligation of the BCR with the CR2; CD19; CD81 complex leads to augmented signaling when naïve B cells first encounter antigen and initiate the process leading to their clonal expansion. The white-boxed area indicates the key binding interaction between CR2(CCP1–2) with a C3d (TED) domain that is covalently bound (yellow triangle) to the antigen recognized by the BCR of this particular B cell. (**b**) CR2 present on FDC may also capture C3d-opsonized antigen and present this antigen to previously primed B cell centrocytes in the germinal centre of the lymph node. **a** and **b** changed from vertical to horizontal layout. (From Carroll and Isenman [95])

Dempsey et al. [96] showed (1996) that complement is integral to B cell activation where the combination of an antigen, (they used chicken egg lysozyme) combined with one, two or three copies of CCP (Sushi) resulted in lowering the amount of antigen needed for B cell response. With one CCP a ten-fold reduction of antigen was required for a B cell activation whereas with the addition of two or three CCP molecules attached, reduction of the activation threshold of 100 and 1000-fold were gained respectively, thus indicating the powerful costimulatory ability of CR2 for BCR activation. DeFranco [97] called the combination of antigen and CR2 a "*two-headed antigen*" in that it typifies the two signals required for lymphocyte activation (BCR/antigen as signal one and CR2/CCP as signal two where CR2 is activated complement that is bound to a PAMP). Anti-CR2 antibodies have been shown to reduce arthritic effects in mice [98]. CR2 binds a variety of bacterial ligands including LPS [99] and several associated with TLR9 (such as CpG DNA and CpG ODN) and has been associated with SLE, as stated previously, which has been treated with an anti-CR2 mAb.

> CR2 generally interacts with many ligands. Bacterial CpG DNA/CpG motif/ CpG ODN in B cell activation in particular largely depends upon TLR9 signaling. B cell surface CR2 and cell surface/cellular TLR9 play a coordinated role in binding, delivering and internalizing, subsequent co-localization may be a vital process that leads pro-inflammatory cytokines generations via NF-κB mediated signaling. Importantly, IFN-α and IL6 as major cytokines play a role in SLE is partially blocked by **anti CR2 inhibitory mAb 171** indicated that CR2 partially involved in recognizing this foreign CpG DNA/CpG motif/ CpG ODN. TLR9 predominantly plays a role which is known to be involved in multiple cell signaling systems in recognizing pathogenic foreign substance in B cell activation [100].

Complement attachment to microbial surfaces (here C3d) and subsequent detection by CR2 presents a variation of the simple model as Cd3 is acting as the licensed receptor for the PAMP (while attached to the PAMP!) but is of mammalian origin

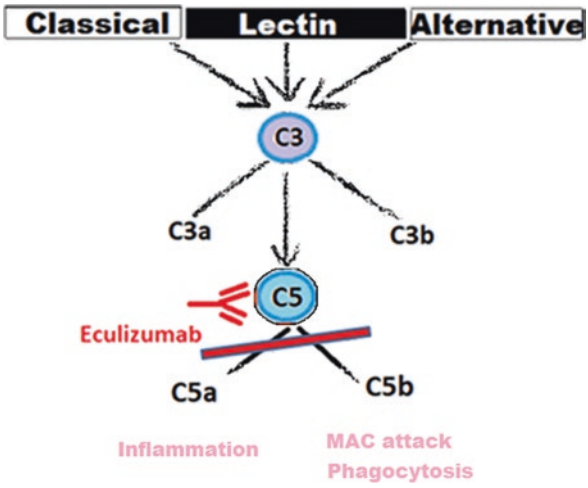

Fig. 7.21 The relationship of the three complement pathways and the blocking of complement activation via eculizumab. (Derived from Baldo [7] and Barnum [103], pg. 176)

(as an opsonin). However, Cd3 cannot attach to CR2 unless it has been activated by the PAMP (in this case electrostically) [101]. Kieslich and Morikis [102] discuss the "dual-functionality" of Cd3:

> …the electrostatic "hot-spots" of C3d have evolved to optimize its dual-functionality (covalently attaching to pathogen surfaces and interaction with CR2), which are both necessary for the formation of B-cell receptor complexes.

Complement factor C5 has been successfully targeted with a mAb to treat several interrelated autoimmune diseases. The relationship of the three complement pathways and the blocking of effects using **eculizumab** is shown in Fig. 7.21.

> …the humanized anti-C5 antibody (**eculizumab**) is widely used to treat atypical hemolytic uremic syndrome and paroxysmal nocturnal hemoglobinuria [104]. In addition, eculizumab was shown to be safe and well-tolerated in a phase 1 trial in patients with systemic lupus erythematosus [105]. These encouraging data suggest that anti-C5 therapy, as well as inhibition of the C5a–C5aR1-axis, could prevent complement-mediated injury in human EBA,[17] and attenuate skin inflammation due to decreased leukocyte recruitment and activation by C5a [106].

It is interesting that the development of **eculizumab** included the use of both IgG2 (Fab) and IgG4 (Fc) regions as IgG2 does not bind Fc and IgG4 does not bind C1q to activate the complement cascade [7].

In yet another example of co-receptor modulation of disease with subsequent intervention from various biologics, specifically in **various cancers**, the inappropriate activation of co-inhibitory surface receptors has been found to contribute to

[17] "Epidermolysis bullosa acquisita (EBA) is an antibody-mediated blistering skin disease associated with tissue-bound and circulating autoantibodies to type VII collagen (COL7)" Mihai et al.

T cell silencing rather than activation against cancer cells (underlined emphasis added).

> Coinhibitory molecules expressed by tumor cells, immune cells, and stromal cells in the tumor milieu can dominantly attenuate T-cell responses against cancer cells. Today, a variety of coinhibitory molecules, including cytotoxic T lymphocyte–associated antigen-4, programmed death-1, B and T lymphocyte attenuator, LAG3, T-cell immunoglobulin and mucin domain 3, and CD200 receptor, have been implicated in immune escape of cancer cells. Sustained signaling via these coinhibitory molecules results in functional exhaustion of T cells, during which the ability to proliferate, secrete cytokines, and mediate lysis of tumor cells is sequentially lost [107].

New cell technologies such as CAR T (chimeric antigen receptor T-cell) [108] are being developed to overcome these mechanisms of tumor escape of immune surveillance.

> For years, the foundations of cancer treatment were surgery, chemotherapy, and radiation therapy. Over the last two decades, targeted therapies like **imatinib (Gleevec®)** and **trastuzumab (Herceptin®)** -drugs that target cancer cells by homing in on specific molecular changes seen primarily in those cells—have also cemented themselves as standard treatments for many cancers.
>
> But over the past several years, immunotherapy—therapies that enlist and strengthen the power of a patient's immune system to attack tumors—has emerged as what many in the cancer community now call the "fifth pillar" of cancer treatment [109].

One can see an even more direct cause of disease in mutations / polymorphisms in the co-receptors as important mechanisms of regulating the immune response, especially as immune surveillance is needed to keep cancer cells from surviving and proliferating.

> Tumor-specific T-cell response is beneficial to limiting the development of cancer, which is influenced by costimulatory and coinhibitory signals [110]. As one of the best characterized costimulatory molecules, CD28 competes with CLTA-4 (coinhibitory molecules) for B7 binding to enhance T-cell proliferation [111]. Therefore, CD28 gene mutations may break the balance between costimulatory and coinhibitory molecules and change the susceptibility of cancer. Recently, the association between CD28 rs3116496 polymorphism and cancer risk has been widely investigated, such as cervical cancer [112], non-small-cell lung cancer [113], colorectal cancer [114], BC (breast cancer) [115], and renal cell carcinoma [116]. Only two studies evaluated the role of CD28 rs3116496 polymorphism in BC risk (Isitmangil et al. 2016; Chen et al. 2013) [117].

An elaboration of five different mechanisms of B cell depletion, all involving the binding of CD20, by Rituximab is given in Chap. 20, Fig. 20.11.

Two example figures (Figs. 7.22 and 7.23) demonstrate the utility of using mAbs (all but one shown are mAbs, as anakinra is a slightly modified version of h-interleukin 1 receptor antagonist) to block co-receptor signals for the relief of autoimmune diseases such as rheumatoid arthritis and lupus (SLE). Finetti and Baldari [119] list over 40 prospective (Tables 7.3 and 7.4) and 7 approved (Table 7.5) therapies. The "*mechanism of action*" columns in both tables show many of the resident costimulatory and coinhibitory molecules being used as pharmacological targets in both drug development and as already used therapeutically in approved drugs.

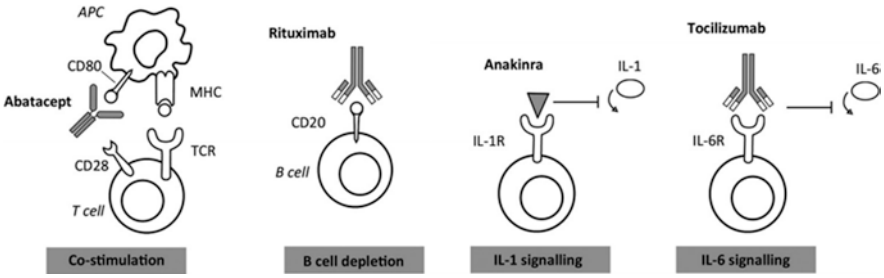

Fig. 7.22 Non-TNF biologic agents approved for the treatment of rheumatoid arthritis. Schematic outlining the main binding sites and mechanisms of the biologic agents other than TNF inhibitors approved for the treatment of RA. **Abatacept** is a fusion protein of the extracellular domain of CTLA-4 and the Fc region of IgG1. **Rituximab** is a chimeric monoclonal antibody targeting the protein CD20. **Anakinra** is a recombinant version of human IL-1Ra. **Tocilizumab** is a humanized monoclonal antibody targeting the IL-6 receptor a protein. APC, antigen presenting cell; MHC, major histocompatibility complex; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; TCR, T-cell receptor [118]

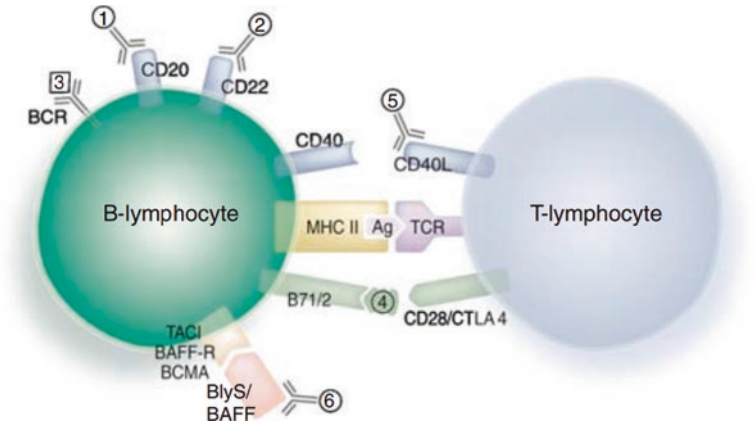

Fig. 7.23 B-lymphocyte-specific therapeutic targets in SLE (Systemic Lupus Erythematosus). B-lymphocyte depletion (1) **rituximab**, (2) **epratuzumab**; reduction of dsDNA titers, (3) **LJP 394/abetimus**; blockade of T-cell costimulation (4) CTLA4-Ig (**abatacept and belatacept**), (5) **IDEC131 and BG9588**; blockade of B-cell stimulation, (6) **belimumab**. Bold added to drug names. (From Bhat and Radhakrishnan)

Table 7.3 Summary of IS (immunological synapse) targeting agents waiting for FDA approval or withdrawn from the market and their target diseases. Clinical trial number (NCT), phase and status have been omitted (see original)

Drug	Mechanism of action	Target disease	Drug	Mechanism of action	Target disease
TRX-518	Anti-GITR mAb	Melanoma or other solid tumors	Varilumab	Anti-CD27 mAb	Ovarian or breast cancer
BMS-986156		Solid tumors			
MK-4166		Advanced solid tumors	CP-870893	Anti-CD40 agonist mAb	Solid tumors
INCAGN01876		Advanced or metastatic solid tumors			Metastatic melanoma
GWN323		Advanced malignancies and lymphomas			Pancreatic carcinoma
MEDI 1873	GITRL fusion protein	Advanced solid tumors	BMS-936561		Renal cell carcinoma or B-cell lymphoma
Utomilumab	Anti-4-1BB niAbs	B-cell lymphoma			
		Advanced ovarian cancer			
		Breast cancer			
		Acute myeloid leukemia			
		Advanced malignancies			
Urelumab		B-cell non-Hodgkins lymphoma	APX005M		Non-small cell lung cancer or metastatic
		Advanced/metastatic colorectal cancer or head			Melanoma
		And neck cancer			Melanoma carcinoma head and neck cancer
		Muscle-invasive urothelial carcinoma of the bladder			Pediatric CNS tumors
					Esophageal and gastroesophageal junction
		Multiple myeloma			Cancers
		Metastatic malignant tumors			Pancreatic adenocarcinoma
		Glioblastoma	ADC-1013		Advanced tumors
		Melanoma			

(continued)

Table 7.3 (continued)

Drug	Mechanism of action	Target disease	Drug	Mechanism of action	Target disease
ARGX-110	Anti-CD70 mAb	Advanced malignancies	JNJ-64457107		Advanced tumors
		Untreated acute myeloid leukemia or high			
		Risk myelodysplastic syndrome	SEA-CD40		Advanced tumors
		Nasopharyngeal carcinoma	R07009789		Metastatic solid tumors
BMS-936561		Renal cell carcinoma or B-cell lymphoma			Pancreatic carcinoma

From Finetti and Baldari [119]

Table 7.4 Summary of IS (immunological synapse) targeting agents waiting for FDA approval or withdrawn from the market and their target diseases. Clinical trial number (NCT), phase and status have been omitted (see original)

Drug	Mechanism of action	Target disease	Drug	Mechanism of action	Target disease
Muromonab	Mouse anti-human CD3ε mAb	Type 1 diabetes	TSR-022	Anti-TIM-3 mAb	Advanced solid tumors
		Metabolic syndrome	MBG453		AML patients or high risk MDS patients,
		Giant cell myocarditis			Advanced solid tumors
		Ulcerative colitis	IMP321	Soluble version of LAG3	Breast cancer
		Graft versus host disease			Renal cell carcinoma
					Melanoma
Teplizumab	Humanized anti-human CD3ε mAb	Type 1 diabetes	OMP-31 M32	Anti-TIGIT mAb	Locally advanced or metastatic solid tumors
Otelixizumab	Humanized anti-human CD3ε mAb	Type 1 diabetes			
		Arthritis, rheumatoid	JTX-2011	Anti-ICOS mAb	Advanced solid tumors
Foralumab	Human anti-human CD3ε mAb	Acute renal transplant rejection	GSK3359609		Advanced solid tumors
		Crohn's disease	MEDI-570		T-cell lymphoma follicular variant or
		Crohn's disease			Angioimmunoblastic T-cell lymphoma
		Inflammatory bowel disease	9B12	Anti-OX40 mAb	Advanced cancer
Etrolizumab	Humanized anti-α4β7 and anti-αEβ7 integrin mAb	Ulcerative colitis	MOXR0916		Advanced or metastatic solid tumors
					Urothelial carcinoma

(continued)

Table 7.4 (continued)

Drug	Mechanism of action	Target disease	Drug	Mechanism of action	Target disease
Efalizumab	Humanized anti-CD11a mAb	Psoriasis	PF-04518600		Kidney cancer
					Advanced or metastatic carcinoma
		Arthritis, arthralgia			Acute myeloid leukemia
		Type 1 diabetes mellitus	MEDI6383		Advanced solid tumors
		Dermatitis, atopic			
		Rheumatoid arthritis	MEDI0562		Advanced solid tumors
		Hidradenitis Suppurativa			Ovarian cancer
		Uveitis, macular edema			Head and neck squamous cell carcinoma (HNSCC) or melanoma
		Lichen planus			
Alefacept	Fc fusion protein with extracellular portion of LFA-3	Psoriasis	INCAGN01949		Advanced or metastatic solid tumors
			GSK3174998		Advanced solid tumors
		Atopic dermatitis	MEDI6469		Metastatic cancers
		Kidney transplantation			
		Graft versus host disease			
		Lymphoma			
		Alopecia Areata			
		Atopic asthma			
		Wegener's granulomatosis			
		Urticaria			
		Scleroderma			
		Relapsing Polychondritis			

From Finetti and Baldari [119]

Table 7.5 FDA approved drugs targeting the IS (immunological synapse) and target diseases. Clinical trial number (NCT), phase, and side effects have been omitted (see original)

Drug	Mechanism of action	Target disease	Drug	Mechanism of action	Target disease
Natalizumab	Humanized anti-α4-integrin mAb	Multiple sclerosis	Belatacept	Fc fusion protein with extracellular domain of CTLA-4, higher affinity compared with Abatacept	Transplantation
		Crohn's disease			Rheumatoid arthritis type 1 diabetes
		Acute ischemic stroke			
Vedolizumab	Humanized anti-α4β7 integrin mAb	Ulcerative colitis	Nivolumab	Human anti-PD-1 mAb	Glioblastoma Multiforme
					Severe sepsis
					Mesothelioma
		Crohn's disease			Melanoma
		Inflammatory bowel disease			Hepatitis C
					Solid tumour
Abatacept	Fc fusion protein with extracellular domain of CTLA-4	Rheumatoid arthritis	Pembrolizumab	Humanized anti-PD-1 mAb	Melanoma
		Ulcerative colitis			Lung cancer
		Multiple sclerosis			Breast cancer
		Graft versus host disease			Solid tumour
		Systemic lupus	Ipilimumab	Human anti-CTLA-4 mAb	Melanoma
		Erythematosus			Lung cancer
		Type 1 diabetes			Prostate cancer
		Giant cell arteritis and Takayasu's arteritis			Renal cell cancer
					Pancreatic cancer
		Atopic asthma			Gastric and gastro-esophageal junction cancer
		Wegener's Granulomatosis			
		Urticaria			Lymphoma
		Scleroderma			Breast cancer
		Relapsing Polychondritis			Urothelial carcinoma
		Lupus nephritis			Acute myeloid leukemia
					HIV
					Head and neck cancer

From Finetti and Baldari [119]

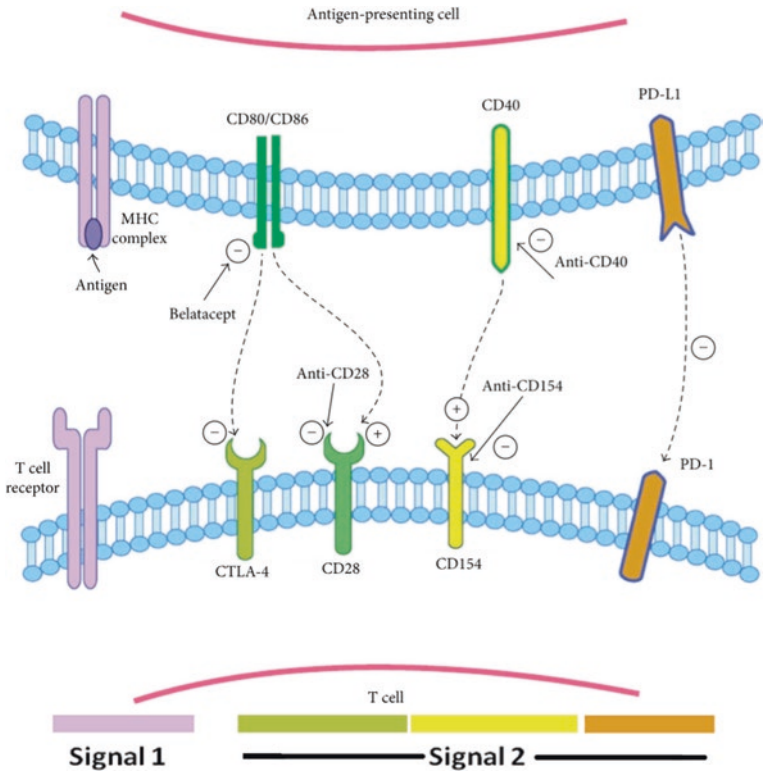

Fig. 7.24 Co-stimulation pathways in T cell regulation. Upon MHC-antigen interaction with the TCR, costimulation pathways can augment or suppress the activation of the T cell. From left to right, CD28 is activated by CD80/CD86. CTLA-4 coinhibitor competes with CD28 for binding to CD80/CD86. CTLA-4Ig and belatacept work by taking advantage of their higher affinity to CD28 over CD80/CD86 and thereby block CD80/CD86 activation of CD28. CD154 and CD40 are other potent activators of T cells; monoclonal antibodies against either of these surface proteins have potential for application in transplant immunosuppression. PD-1 is expressed on T cells, and interaction with PD-1 ligand (PD-L1) produces a suppressive signal to the T cell. (From Samy et al. [120], CC 4.0.) The signal 1 and signal 2 color key has been added below the figure

Given the complexity of multiple costimulatory and multiple coinhibitory receptors on each T cell, the signaling is believed to be the result of the combined overall signal. For this reason Zhu, Yao and Chen described the effect as a "tide" of signals. The complex cross-currents can be viewed in Figs. 7.24 and 7.25 and serve to contrast the accumulating complexity of receptor interaction with the simplicity of Fig. 7.19.

Figure 7.24 shows additional important costimulatory and coinhibitory pathways that have been recently identified and the most current therapeutics have begun to

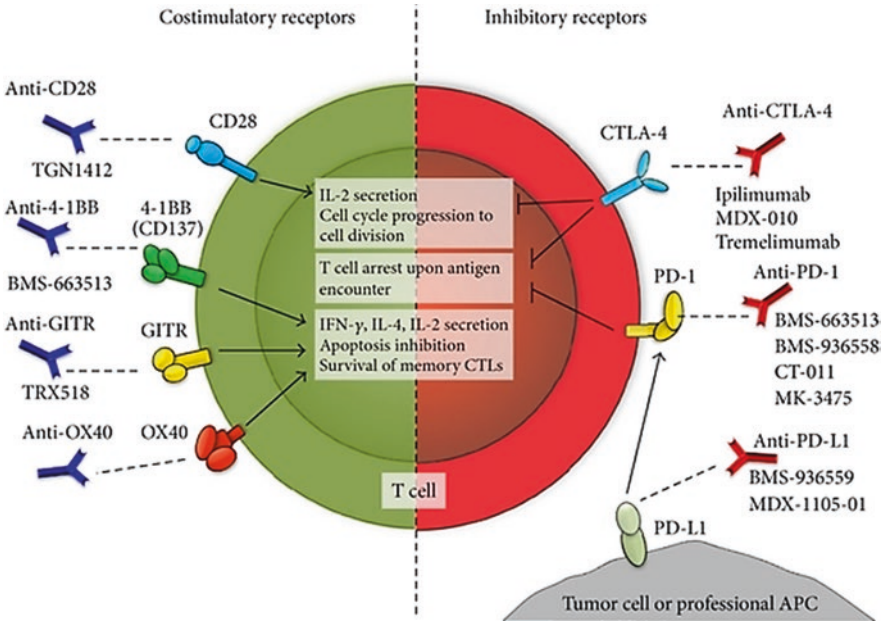

Fig. 7.25 Novel therapeutic approaches targeting the immunological synapse to enhance antitumor immunity. Left. Costimulatory molecules either constitutively expressed (e.g., CD28) or inducible (e.g., 4-1BB, GITR, and OX40) that have or are being considered as targets for antitumor immunotherapy due to their positive effects on T cells after engagement (central white boxes). Right. Inhibitory molecules that have been shown to play roles in the suppression of antitumor T cells and that are expressed either at the surface of T cells (e.g., CTLA-4, PD-1) or at the surface tumor cells and professional APCs (e.g., PD-L1). Blunt arrows indicate the physiological processes (white boxes) that are affected upon the engagement of these molecules. The names of different monoclonal antibodies in present or past clinical evaluation are indicated below each antibody. (From Gonzalez et al. [121])

utilize these additional pathways to modulate immune responses in addition to the initial CD28/CTLA-4 and CD80/CD86 pathways.

Inhibition of the interaction between PD1 and PD-L1 (Fig. 4b, not shown but can be seen in 7.24 with the substitution of tumor cell for APC) can enhance T cell responses in vitro and mediate (preclinical) antitumor activity [122]. Antibodies targeting PD1 or PD-L1 have reached the clinic and include **pembrolizumab** (previously named as **lambrolizumab**; anti-PD1) and **nivolumab** (anti-PD1) [123]. In early phase I trials, PD1-PD-L1 axis blockade alone has yielded promising results in a variety of cancer types; in melanoma, the anti-PD1 antibody nivolumab has shown sufficient clinical responses which are often durable, with some patients remaining free from disease progression for many years [124]. The anti-PD-L1 antibody **atezolizumab** has induced therapeutic responses in patients within a broad range of human cancers, which included lung, colon, head and neck, and gastric cancers in addition to melanoma and renal cell carcinoma. Thus far, both pembrolizumab and nivolumab have been FDA approved for the treatment of melanoma and NSCLC, while nivolumab has been also approved for the treatment of renal cell carcinoma [125]…

The most striking contrast of the agents that target the PD1-PD-L1 axis to the therapies that block CTLA-4 (**ipilimumab**) is the favorable toxicity profile of the PD1-PD-L1 blocking agents [126]. (Farkona, Diamandis and Blasutig) [127].

More recently, the drugs that modulate specific co-receptors for cancer therapy have become known as "immune checkpoint inhibitors" [128] and the actual modulation activity is being referred to as immune "checkpoint blockade" [129]. From Fig. 7.25 one can gain further appreciation for the on-going efforts to discover immune "modulatable" co-receptors, here for anti-cancer target discovery.

The biotechnology revolution is described in the next chapter and includes many examples of therapeutic treatment mechanism of action, but here it suffices to see that this revolution proceeds by producing immune modulating drugs, often monoclonal antibodies, that are able to modulate CSSM receptors and thus achieve desirable therapeutic outcomes. In the figures above, one can view where specific mAbs bind to specific non-clonal receptors (and in some cases the BCR itself) to ameliorate various disease states. The simple model of co-stimulatory and co-inhibitory signaling that includes the potential for PAMP effects on such receptors is not encompassed by the historical pyrogen model. A final depiction of the ongoing advancement of the "two signal" paradigm is shown below in Fig. 7.26.

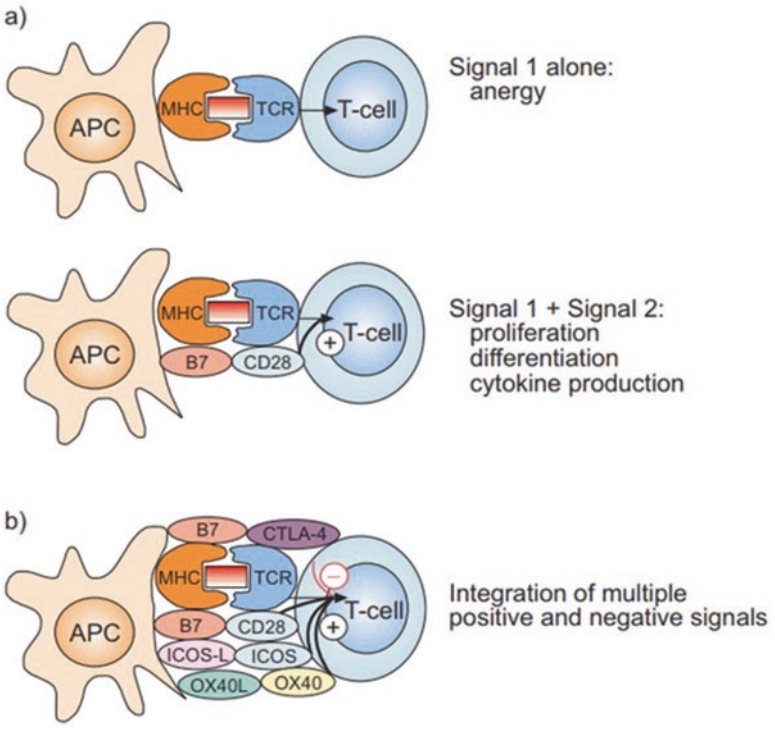

Fig. 7.26 (**a**) Historic view and (**b**) modern concept of co-stimulation. APC: antigen-presenting cell; MHC: major histocompatibilty complex; TCR: T-cell receptor; CTLA: cytotoxic T-lymphocyte antigen; ICOS: inducible co-stimulator; L: ligand. Reproduced with permission of the © ERS 2019: European Respiratory Journal 29 (4) 804-812; DOI: 10.1183/09031936.00094506 Published 30 March 2007

7.4.3 Action of Adjuvant Usage in Vaccinology

The adjuvant effect fits the model of a second signal type (co-stimulatory) activation (particularly for TLR agonists) where the bacterial or viral protein supplies the first signal (antigen for BCR or TCR activation). The necessity of adjuvant usage in vaccinology points to a couple of different important points as it relates to MCC and biologics manufacture (which are worthy of repeating): (i) subunit vaccines, which are clean recombinant proteins, are not all that different from biologics as manufactured recombinant proteins, but are predominately proteins from bacterial and viral microorganisms rather than mAbs (human IgG) or human replacement enzymes or cytokines (etc.), and the fact that they often need help via the addition of PAMPs (adjuvants) to produce immunogenic responses (even though they are foreign proteins) should inform MCC where immunogenic responses are produced to human proteins and (ii) the mechanisms of adjuvant activation via TLRs are the same costimulatory mechanisms that impurities and contaminants use as interpreted by mammalian immune systems. A short summary of the modern usage of LPS as an adjuvant is given by Bergmann-Leitner and Leitner [130].

> …the detoxification of LPS, resulting in Monophosphoryl lipid A (MPL®) [23] (or its commercially available equivalent, MPLA), a TLR4 agonist, which is safe for use in humans and a component of AS04™. The latter is used in the human Papilloma Virus vaccine Cervarix® (the first FDA-approved vaccine with an adjuvant other than alum). The lower toxicity is a result of much weaker signaling through the MyD88 signaling pathway of TLR4. This signaling cascade activates transcription factors (predominantly NF-κB) associated with inflammatory gene products. Signaling through TLR4's second signaling cascade, the TRAM/TRIF (Toll IL-1 receptor domain-containing adaptor-inducing IFNβ) pathway is preserved after binding of MPL [131]. TRIF-signaling is associated with a Type I IFN response which is required for the induction of a strong adaptive immune response.

Janeway viewed the requirement for adjuvants as associated with vaccine proteins as an important initial clue to the overall mechanism of immune activation in that a confirmatory signal (costimulatory) from innate receptors is needed in addition to antibody receptor recognition of antigen to activate lymphocytes.

7.4.4 Suppression of Host Immunity by Pathogens Via Modulation of Receptors

Another key to viewing pathogens and PAMPs as inducing costimulatory receptors in APCs is the converse of this, which is the suppression of host immunity by nullifying the ability to activate these signals or in activating coinhibitory responses by the pathogens themselves. In the arms race of pathogen versus host, bacteria and virions have learned to modulate the co-receptors present on mammalian immune cells. Even with the evolutionary advantage of adaptive immunity and regardless of how large the antibody diversity that can be formed turns out to be (a very large but fuzzy number), *it is not enough in every case* to counteract the ability of some microbial structures, that can employ hypervariable modularity of surface structures

as well as the incorporation of decoy host structures, to escape consistent antibody detection [132]. This is true for the HIV, HTLV-1[18] and Hepatitis virus particle surface structures. In the simplest of terms, they take away tiny pieces of the host and incorporate it into their being. Thusly, they are able to enter in as imposters and navigate through the sophisticated web of defenses.

> HIV-1 selectively incorporates the MHC class II glycoprotein within its membrane and uses it to accelerate entry into human T lymphocytes (Bastiani et al. 1997; Cantin et al. 1997). HTLV-1 displays the complement regulatory proteins CD59 and CD55[19] on its surface to evade complement-mediated lysis (Spear et al. 1995) [133].

In an exaggerated example of costimulatory receptor hijacking by a microbial pathogen, *Staphylococcus aureus* and *Streptococcus pyogenes* have been found to short circuit the CD28/B7 connection along with TCR (MHC-II) activation by binding directly to the molecules and producing a constant "on" signal that results in the damage done by superantigens (T cell hyperactivation), commonly referred to as "toxic shock" syndrome [134]. Researchers have recently used "peptide mimetics of the B7-2" molecule to "attenuate superantigen-mediated induction of inflammatory cytokines in human PBMCs, and protect mice from toxin challenge" Levy et al. [134]. Note that this instance covers three different categories: disease causation (toxic shock), pathogen evasion (*Staph* and *Strep*), and therapeutic mode of treatment (B7–2 peptide mimetics).

From a historic vantage, the longstanding human pathogen, *Mycobaterium tuberculosis* (Mtb) "is one of the most successful pathogens in the history of mankind. There are numerous reports indicating the role of mycobacteria in downregulating the expression of CD80, CD86, and CD40 on APCs [135, 136]. A recent study showed, albeit for BCG,[20] that MHC-II, CD80, CD86, and CD40 are downtuned during chronic phase of infection" Schreiber et al. [137].

> Besides mycobacteria, many other pathogens can exploit CD80/CD86-CD28/CTLA-4 pathways for their persistence. *H. pylori* causes chronic infection in the gut resulting in peptic ulcers. Further, it is known to induce the expression of CTLA-4, resulting in the anergy of T cells and poor clearance of the bacteria [138]. *Yersinia pseudotuberculosis* decreases CD86 expression on B cells and impedes the function of both B cells and T cells [139]. *S. typhi* is known to suppress ICAM-1 and as a consequence reduces the antigen uptake by APCs and inadequate T-cell response [140, 141]. *H. pylori* diminishes the expression of CD40L on T cells and therefore employs CD40/CD40L pathway for its survival. Furthermore, it upregulates PDL-1 expression on gastric epithelial cells and inhibits the activation of T cells recruited to gastric mucosa [142].
>
> It has been reported that *M. leprae* obstructs CD28/B7 signaling pathway for rendering antigen-specific T cell unresponsive in lepromatous leprosy patients [143]. Recently, the importance of CD80/CD86 in controlling mycobacterial infection has been demonstrated in CD80/CD86 double knockout mice [144]. The down-modulation of CD80/CD86 in chronic phase of the infection suggests that mycobacteria may actively exploit this pathway to anergize the T cells (Kahn et al.).

[18] Human T-lymphotropic virus, a human retrovirus known to cause certain cancers.

[19] CD59 and CD55 are human proteins.

[20] "The only licensed vaccine against TB, Bacillle Calmette-Guerin (BCG), is effective at preventing disseminated disease in infants but confers highly variable efficacy against pulmonary TB in adults." Why don't we have an effective tuberculosis vaccine yet?, Tamara Davennea and Helen McShane, EXPERT REVIEW OF VACCINES, 2016 VOL. 15, NO. 8, 1009–1013.

The manipulation of mammalian immune systems via viral attack involves widespread attempts to modulate co-stimulatory signals.

> Like many other intracellular pathogens, HIV efficiently exploits the costimulatory molecules to override the immune responses. Its infection is associated with decreased expression of CD40L on CD4+ T cells [145]. Upon activation, CD4+ T cells from individuals with progressive disease show very little upregulation of CD40L, which corroborates with their inability to help APCs and failure to induce IL-12 in DCs [146].
>
> Measles, herpes, and hepatitis C viruses (HCV) retard the expression of CD80, CD86, CD25, CD83, and CD40 that leads to poor CD8+ T-cell priming [147–149]. In addition, Herpes virus suppresses ICAM-1 on APCs, thereby obstructing immunological synapse with T cells [150] (Kahn et al.).

In a very strange case, another virus incorporates LPS binding molecules from the host (including CD-14, MD-2, LBP and TLR4) into its surface protein repertoire. In effect, the virus binds LPS and provokes cytokines (mis)directed against LPS rather than eliciting an appropriate host response to viral infection (i.e. interferons).

> We find that the viral envelope contains the mammalian LPS-binding factors CD14, TLR4, and MD-2, which, in conjunction with LPS-binding protein (LBP), bind LPS to the virus and augment transmission (Wilks et al. 2015).

Camouflage-type epitopes present another immune evasion technique. They can sterically prevent host antibody from attaching to conserved epitope structures. Alternatively, viral species can simply change surface structures faster than antibodies can be raised against them.

> Development of antibody cocktails is of particular interest for highly mutable pathogens such as HIV, which can evolve escape variants even during treatment with a highly potent, broadly neutralizing antibody. A mixture of antibodies targeting distinct epitopes is expected to protect against a broader range of circulating strains, while simultaneously reducing the risk of escape variants [151].

7.5 Specific Ways Adaptive Immune Context Differs from Microbiological Context

As Medzhitov alluded to, it seems impossible now to understand the immune component of disease or biologics therapy without these very basic concepts that includes the activation of adaptive immunity by the innate arm of immunity. The inclusion of immunological context shouldn't be viewed as a disruption of the status quo, but rather the presentation of an opportunity to increase process and product knowledge relevant to microbiological control where warranted (i.e. during manufacturing development). Pasquale et al. [152] give a good overview of PRR / PAMP involvement in the activation of innate and subsequent adaptive immunity.

Complete adjuvant responses from many PAMPs has not been thoroughly, or in many cases even preliminarily, characterized and endotoxin is no exception. Unlike the "endotoxic conformation" that has been laboriously worked out for LPS, there is no established "adjuvant conformation", although some forms have been found and utilized for vaccine adjuvant systems.

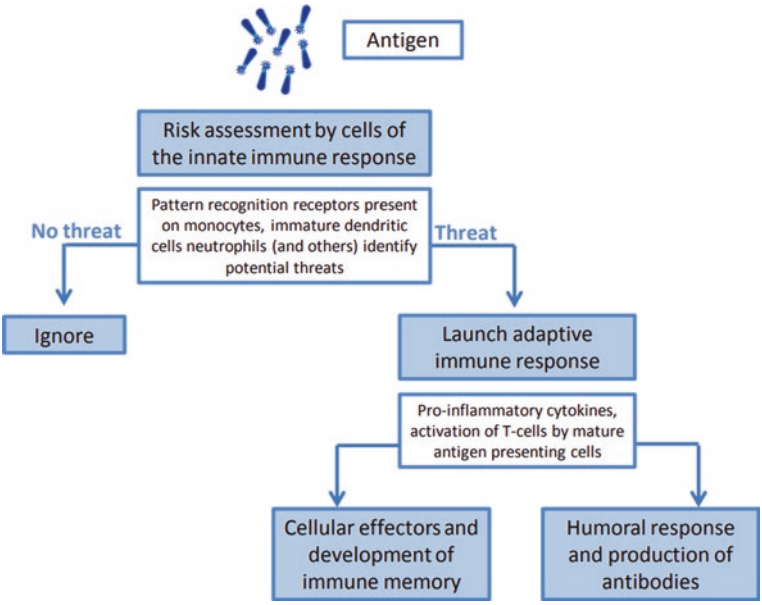

Fig. 7.27 The role of the innate immune response. From Pasquale et al. CC 4.0

Ribi and co-workers established that removal of the phosphate group from the reducing end sugar of the lipid A disaccharide decreased the toxicity of the molecule 100- to 1000-fold without appreciably affecting the immunostimulating activity. The resulting derivative, which had only one phosphate group, was called monophosphoryl lipid A. Myers et al. subsequently determined that removal of an ester-linked fatty acid group from the 3-position further reduced the pyrogenic properties without substantially affecting the adjuvant properties. The resulting 3-O-deacylated monophosphoryl lipid A (MPL), which is isolated and structurally derivatized from LPS of *Salmonella minnesota* R595, has proven to be a safe and effective vaccine adjuvant [153].

Figure 7.27 overviews the potential effects of PAMPs on promoting proinflammation (pyrogenicity) as well as the activation of the adaptive immune system.

The structure of MPLA (hexa-acyl) and P-MPLA (penta-acyl) derivation and production is given in Fig. 7.28.

7.5.1 Synergistic Contaminants

The idea that contaminants or impurities can act synergistically is not really a MCC precept, except perhaps in terms of biofilm where there is recognition that the sum is greater than the parts. Microbiologically, a contaminant can produce toxins, harbor endotoxin, or, as a living cell, can bring infection, yet in terms of producing artifacts that act in concert to conspire to increase the threat to the host, other than sheer number of infecting organisms, this is not a microbiological concept. Synergy is an immunological concept and was demonstrated by Verthelyi and Wang in the

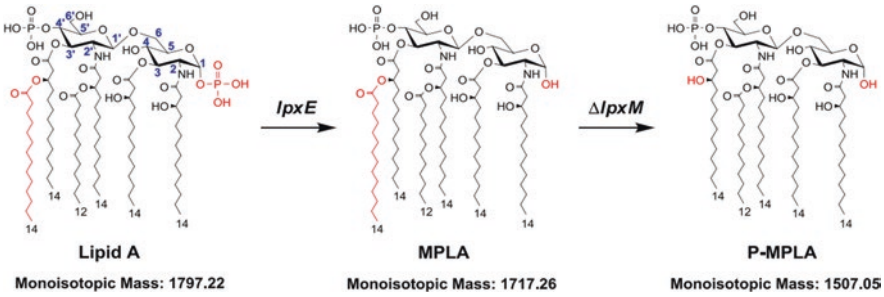

Fig. 7.28 The strategic diagram of structural modification of *E. coli* lipid A. The numbers that specify the glucosamine ring positions and the fatty acid chain length of lipid A are indicated. The gene lpxE encodes LpxE, which removes the phosphate group from the 1-position of lipid A. The gene lpxM encodes LpxM, which adds a secondary tetradecanoyl residue at 3′-position of lipid A. Therefore, expression of lpxE in *E. coli* changes the structure of lipid A to monophosphoryl lipid A (MPLA); expression of lpxE and deletion of lpxM in *E. coli* changes the structure of lipid A to pentaacylated MPLA (P-MPLA). (From Han et al. [154])

first IIRMI study of 2010 and will be described below. The presence of multiple low-level TLR-activating contaminants appears to be capable of magnifying the overall signal. This is like an engine firing on multiple cylinders versus a single cylinder, though a single cylinder can produce an overwhelming response as well as seen in endotoxin and TLR4. Brodin describes the synergistic behavior of immune system components:

> Complex adaptive systems are characterized by multiple different components interacting and giving rise to behaviors by the system as a whole, which are not predictable from observations of individual components alone [155].

Verthayli and Wang demonstrated the synergistic activity of two known adjuvants/contaminants, LPS and CpG DNA.

> As shown in figure 5 (depicted here as Fig. 7.29), administration of ovalbumin alone or together with trace or suboptimal amounts of a single TLR-agonist induced very low levels of IgG antibodies. In contrast, mice that received the same dose of ovalbumin together with 10 ng of LPS plus 500 ng of CpG ODN had significantly increased (p,0.05) IgG antibody responses to ovalbumin 3 weeks post treatment. The same group showed significantly higher antibody titers following re-exposure 4 weeks after priming (p,0.005). Indeed, the antibody levels in these mice were significantly higher than those immunized with ovalbumin together with either 5 mg of CpG ODN or 1 mg of LPS (p,0.05).

Note that mice are notoriously more resistant to endotoxin than man [156]. Importantly, the synergistic effect showed the ability to "*break tolerance to self*" in that once the adjuvant effect has exacerbated the immune milieu with the therapeutic protein, it can also lead to the depletion of the natural corresponding protein.

> Tolerance to low-abundance self proteins in sera is incomplete and may be overcome when these proteins are presented in the context of adequate adjuvants [157–159]. Using a model established by Ryan et al. [160], we determined whether the addition of low levels of LPS and/or CpG DNA was sufficient to induce a breach in tolerance and the induction of a neutralizing response to erythropoietin. Balb/c mice were treated with recombinant Human Erythropoietin (rhuEPO)

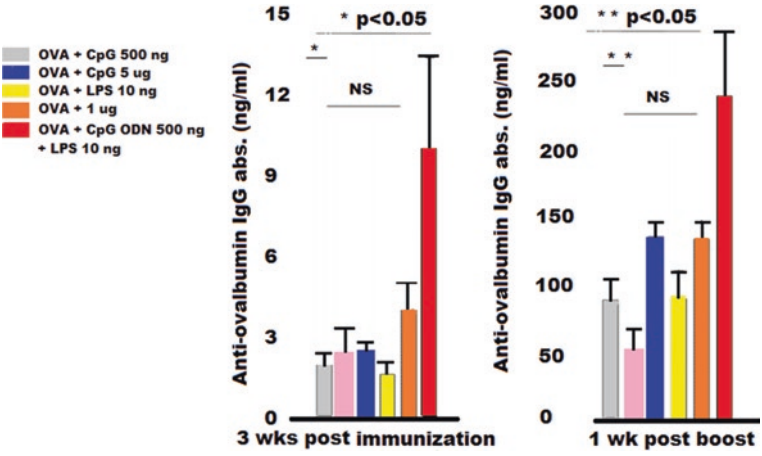

Fig. 7.29 Trace levels of IIRMIs increase protein immunogenicity in vivo. Balb/c mice (3–6-week-old; 5 mice/group) were immunized and boosted i.p. with human ovalbumin alone (5 mg/mouse) or mixed with the stated amounts of LPS and/or CpG ODN. Mice that received saline were used as controls. Mice were tail-bled before the immunization and weekly thereafter. Antibodies (IgG) to ovalbumin assessed by ELISA. Statistical analysis: ANOVA. * = p,0.05, **p,0.005, NS: Not significant. (Adapted from Verthelyi and Wang [35])

alone or together with low levels of LPS and/or CpG ODN on days 0, 14 and 62, followed by weekly hematocrit measurements. As shown in figure 6 (not shown), the hematocrit of untreated mice remains constant over time (51±3%). Despite having only 80% homology with mouse [161], rhuEPO was active in mice, eliciting a reproducible increase in the hematocrit 1 week after each treatment (15.5, 13.9 and 13.6% increase over baseline after the 1st, 2nd and 3rd dose respectively). Addition of low levels of CpG ODN (50 or 500 ng), LPS (100 pg) or the combination (50 ng CpG ODN +100 pg LPS) did not modify the response. In mice treated with rhuEPO together with LPS (10ng) there was evidence of reduced hematocrit following the 2nd inoculation. In contrast, mice treated with rhuEPO in combination with 500 ng of CpG ODN plus 10 ng of LPS showed reduced response to rhuEPO following one treatment as evidenced by increases in hematocrit of 23, 7 and 1.5% over baseline after the 1st, 2nd and 3rd inoculations respectively, followed by a pronounced reduction in hematocrit (2%, 27% and 28% respectively relative to baseline; p,0.001). The reduction in the hematocrit lasted for over 30 days following the 2nd inoculation. This data shows that the rhuEPO augments the hematocrit of mice for about 7 days. The prolonged anemia observed in mice treated with rhuEPO plus LPS and CpG ODN suggests that the inoculations led to a break in tolerance to the endogenous (mouse) EPO, which is needed to maintain the hematocrit stable. Together these data suggest that in mice, the presence of low levels of impurities that can trigger TLR 4 and 9 may foster a break in tolerance to an essential endogenous non-redundant growth factor.

7.5.2 Relevant Levels Are Not Necessarily Pyrogenic Levels

The Schwarz et al. [162] study from 2014 that used research-grade, not therapeutic proteins, manufactured as reagents for various biotechnology purposes may provide some clues as to adaptive immune reactivity relevant levels. These proteins were "naturally" contaminated by their respective production methods and the endotoxin

Table 7.6 Impurities measured in proteins from different suppliers evaluated by LAL (Schwarz et al.)

	Impurities according to datasheet	Impurities according to LAL test	Impurities at 100 ng protein
PBS/0.1%BSA	–	<0.1 EU	–
DC medium without FBS	–	<0.1 EU	–
DC medium with 10% i.a. FBS	–	<0.1 EU	–
Recombinant protein 1, supplier 1	<1 EU (<0.1 ng/μg protein)	1.4 EU (0.14 ng/μg protein)	0.14 ng
Recombinant protein 1, supplier 2	<1 EU (<0.1 ng/μg protein)	<0.1 EU (0.01 ng/μg protein)	<0.001 ng
Recombinant protein 2, supplier 1	<0.1 EU (<0.01 ng/μg protein)	0.32 EU (0.025 ng/μg protein)	0.003 ng
Recombinant protein 3, supplier 1	<0.1 EU (<0.01 ng/μg protein)	0.25 EU (0.025 ng/μg protein)	0.003 ng
Recombinant protein 4, supplier 3	<1 EU (<0.1 ng/μg protein)	<0.1 EU (0.01 ng/μg protein)	<0.001 ng

levels existed, as measured by the Schwarz team, some slightly and some exceeding by several-fold, the specified manufacturer limits. Table 7.6 shows the endotoxin content of various *reagent grade* proteins.

> To analyse whether these low levels of contamination have an effect on immune cells, we stimulated the monocytic cell line THP-1, primary human monocytes, *in vitro* differentiated human monocyte-derived dendritic cells, and primary human CD1c$^+$ dendritic cells (DCs) with very low concentrations of lipopolysaccharide (LPS; ranging from 0.002–2 ng/ml). We show that CD1c$^+$ DCs especially can be activated by minimal amounts of LPS, equivalent to the levels of endotoxin contamination we detected in some commercially available proteins. Notably, the enhanced endotoxin sensitivity of CD1c$^+$ DCs was closely correlated with high CD14 expression levels observed in CD1c$^+$ DCs that had been maintained in cell culture medium for 24 hours.
>
> To assesses whether these small amounts of LPS are capable of activating NF-κB-signaling, we generated a highly LPS-responsive cell system modified from Peters and colleagues [163] by co-transfecting HEK293 cells with expression plasmids encoding the LPS receptor subunits TLR4, CD14 and MD-2 along with an NF-κB luciferase reporter plasmid. These cells were exposed to different concentrations of recombinant protein 1 from suppliers 1 and 2, as well as to different amounts of LPS. As shown in Figure 2 (here shown as Table 7.6**),** the recombinant protein from supplier 1 induced an increase in NF-κB activity, whereas the protein from supplier 2 did not activate NF-κB (even at 400 ng/ml, twice as high as the maximum protein concentration tested from supplier 1). Interestingly, the protein from supplier 1 induced NF-κB activation in the same range as 0.02 ng/ml LPS. Of note, this LPS concentration is approximately equivalent to the amount of contamination in 100 ng of recombinant protein, as measured in the LAL test above. This experiment clearly shows that even the small amounts of endotoxin contamination found in commercially available recombinant proteins are sufficient to activate NF-κB in a highly sensitive cell system.

Thus Schwarz et al. demonstrated that small amounts of LPS are capable of activating NF-κB-signaling and equate to sub-pyrogenic levels as shown in Fig. 7.30.

The "EU" is defined as a level of 1/5th the endotoxin (*E. coli*, EC2) needed to bring about a TPR level as historically defined by Griesman and Hornick (in man and rabbits) to be approximately 1 ng/kg. Therefore, an EU equates to 0.2 ng/kg

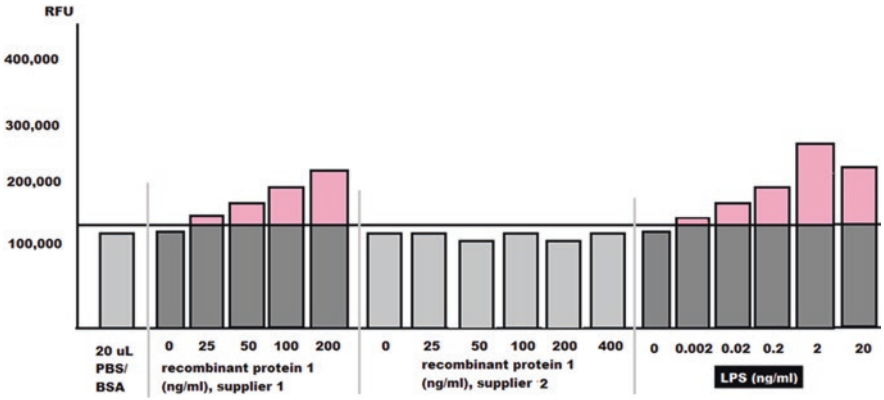

Fig. 7.30 NF-κB activation in HEK cells. Derived from data generated by Schwarz et al. Activation of NF-κB by endotoxin impurities in commercial (not therapeutic) preparations of a recombinant protein. NF-κB activation in HEK293 cells transfected with an NF-κB luciferase reporter plasmid and plasmids encoding LPS-receptor components (TLR4, CD14, MD-2) is shown. Cells were exposed to recombinant protein 1 from supplier 1 or 2, LPS, or solvent, in the amount stated. 20 hours after induction, luciferase activity was measured

(*E. coli*) of patient weight to bring about the first occurrences of fever (threshold response).

7.5.3 Time Is a Factor

The above study, Fig. 7.29 [35], shows that time is a factor in terms of the body's recognition and response to the adjuvant effect. It took up to 3 weeks for mice to respond to the synergistic effects of low level contaminants (LPS and CpG). This shows a significant difference between innate and adaptive immune responses. Endotoxin can be injected into a rabbit and a quick fever response (within 3 hours) will be produced. This is because Toll-like receptors are present and waiting for such events. On the other hand, adaptive responses have several thresholds governing their occurrence and response. One is the presence and recognition of antigen (Ovalbumin was used above), a second is the presence of a costimulatory adjuvant (here, LPS and CpG), and thirdly, time is needed to construct responding antibodies against the identified intruder.

7.5.4 What Is the Meaning?

Microbiologically, according to context, a solution or sample either contains living organisms or their non-living by-products (PAMPs) at a level to elicit a pyrogenic/proinflammatory response or they do not. However, from an immunological

perspective the same microbial by-product can serve to stimulate adaptive immune responses while giving little proinflammatory reaction. This has been seen to be true for protein aggregates, particulates (visible and subvisible), HCPs, nucleic acids, and for LPS and other PAMPs. So, the simple question should be put forward for biologics: "*Does this drug contain anything that may be immunologically relevant besides the therapeutic protein*?" This is not the same question as the pyrogen model asks: "*Does this drug cause fever as arising from microbial pyrogens, especially endotoxin?*"

There is a large infrastructure built around the current paradigm of microbiological contamination control (the current "state of the art"). There are official public and private institutions that decide what standards must be used and what concepts must be enforced for the public good. Companies that develop and manufacture drugs do so tirelessly and to the betterment of us all. Those providing the "rails and ties" for testing, test and reagent suppliers, are also deeply ingrained in the commercial aspects of support. The application of immunological concepts to the domain of microbiological contamination control will need to form over time and via consensus. The most obvious impediments to being able to transition to a more encompassing IIRMI model for biologics microbiologic control from the current simplistic pyrogenic model includes the following:

1. The relevant PAMP levels and the circumstances applicable to adaptive immune responses are not well defined from a control perspective.
2. There are few opportunities for the detection of synergistic PAMP's that are not LPS. That is to say, more PAMP types need to be detected routinely at sensitive levels (see Chaps. 4 and 16).
3. The continued widespread adherence to the "*pyrogen only model*".

The IIRMI view, if extrapolated to the full measure of eventual consequences, may present additional concerns, some of which may or may not come to pass:

- Currently, there is no way to readily detect (non-proinflammatory) structures for many PAMPs. However, some low reactive structures are the ones being targeted for use as immune stimulants.
- Non-biologic drugs given in combination with biologics may have the potential of providing adjuvant type contaminants (IIRMI) if they were produced according to lesser quality standards (as biologics have amassed additional quality requirements).
- Protein or formulation masking may provide shelter from detection for various PAMPs. The next chapter discusses various endotoxin masking effects relevant to biologics drug manufacture and endotoxin removal.
- If a product produces little or no unwanted immune reactions, then likely an immunological context will be viewed as unnecessary or dormant viewpoint, however, for a developmental, clinical or marketed compound that exhibits a poor adverse response profile (and many marketed products do), then the IIRMI view provides additional avenues to pursue.

The FDA studies described here point to an immunological context that goes beyond the previous purely microbiological control concepts historically utilized for LVPs and SMDs. Drug manufacturers looking to protect *multi-billion-dollar* biologics markets and provide medicines with competitive adverse reaction profiles will want to preclude as many PAMP types in manufacturing processes as possible, and down to levels below those associated with pyrogenic responses. This will include potential synergistic contaminants that may interact with LPS (where the technology exists to do so). Manufacturing development is the place to do it. Such efforts may best be viewed from the emerging IIRM vantage. At the very least, these concepts may help serve to blunt simplistic impulses to limit endotoxin and other PAMP detection and control efforts to a "*fever or no fever*" interpretation that encompasses only a very rudimentary view of innate immunity and ignores adaptive immunity altogether.

7.6 Milestone Publications in Bridging Innate and Adaptive Immunity

Listed here are some landmark papers of interest that revolve around the discovery of the connectedness of mammalian innate and adaptive immune responses.

The first presentation of the idea of non-clonal recognition in lymphocyte activation.

- **Immune activation of B cells: evidence for 'one nonspecific triggering signal' not delivered by the Ig receptors**, Coutinho, A. & Moller, G., *Scand. J. Immunol.* 3, 133–146 (1974)

Conceptualization of "pattern recognition receptors" in the detection of PAMPs

- **Approaching the asymptote? Evolution and revolution in immunology**, Janeway, C. A. Jr., *Cold Spring Harb. Symp. Quant. Biol.* 54, 1–13 (1989)

Discovery of first human TLR (TLR4) and activation of TLR4 to induce expression of cytokines and costimulatory molecules (B7.1) thus linking innate and adaptive immunity.

- **A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity**, Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A. Jr., *Nature* 388, 394–397 (1997)

Three papers contributing to the initial discovery of LPS as TLR4 activating ligand.

- **Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene**, Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. & Beutler, B., *Science* 282, 2085–2088 (1998)
- **Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4)**, Qureshi, S. T., Lariviere, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P. & Malo, D., *J. Exp. Med.* 189, 615–625 (1999)

- **Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product**, Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. & Akira, S., *J. Immunol.* 162, 3749–3752 (1999)

Demonstration of TLR gene polymorphism as influencer of human physiologic response.

- **TLR4 mutations are associated with endotoxin hyporesponsiveness in humans**, Arbour, N. C., Lorenz, E., Schutte, B. C., Zabner, J., Kline, J. N., Jones, M., Frees, K, Watt, J. L. & Schwartz, D. A., *Nat. Genet.* 25, 187–191 (2000)

Demonstration of critical function for TLRs in immune cell maturation and induction of adaptive immune responses.

- **Toll-like receptors control activation of adaptive immune responses**, Schnare, M., Barton, G. M., Holt, A. C., Takeda, K., Akira, S. & Medzhitov, R., *Nat. Immunol.* 2, 947 (2001).

Demonstration that immunoglobulin G2a-chromatin immune complexes synergistically engage antigen receptor and TLR9 to lead to antibody production.

- **Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors**, Leadbetter, E. A., Rifkin, I. R., Hohlbaum, A. M., Beaudette, B. C., Shlomchik, M. J. & Marshak-Rothstein, A., *Nature* 416, 603–607 (2002)

Determination of crystal structure of bound ligands (LPS PAMPs) in TLR4 complex activation

- **Crystal Structure of the TLR4-MD-2 Complex with Bound Endotoxin Antagonist Eritoran**, Ho Min Kim, Beom Seok Park, Jung-In Kim, Sung Eun Kim, Judong Lee, Se Cheol Oh, Purevjav Enkhbayar, Norio Matsushima, Hayyoung Lee, Ook Joon Yoo, and Jie-Oh Lee, October 2007, *Cell* 130(5): 906–17

Appendix I

Foretelling of subsequent immune discoveries (Janeway's 1989), paraphrased for brevity from: *Approaching the asymptote*: Evolution and Revolution in Immunology, *Cold Spring Harb Symp Quant Biol*. 1989. 54: 1–13, Janeway, Pillars of Immunology.

a	Infectious agents are highly variable in structure, and their short generation time allows them to alter their structure quickly. This is especially true of their protein structure, which can diversify remarkably even during an infection within an individual…

b	Burnet realized the necessity for the immune system to develop mechanisms to generate an essentially open and unlimited repertoire of receptors, since evolutionary selection could not provide the immune system with receptors that would recognize the hemagglutinin molecules of next year's strain of virus.
c	…T cell receptors derive from a separate and unique set of rearranging gene segments… these two sets (TCR and BCR) of rearranging genes arose only once and by duplications they diverged…
d	…the ability to deliver the **second signal** is induced on host APCs by infectious agents as the result of a distinct type of immunological recognition specific for microorganisms, but not resulting from clonally distributed receptors.
e	I raise the possibility that the **second signals** arose prior to the development of specific antigen recognition. Under this construction, **second signals** may be viewed more as positive initiators of immunity than as late adaptations to avoid autoimmunity.
f	Why do we need to use adjuvants? …it seems likely that these substances are required to provide two attributes not found in soluble proteins. The first is effective antigen uptake into macrophages, thus increasing ligand density in the form of peptides derived from the foreign protein bound to class I MHC molecules. The second is the provision of costimulatory activity, induced in macrophages and/or B cells by the bacterial constituent of the adjuvant.
g	If effector mechanisms used by lymphocytes in contemporary vertebrate immune systems derived from primitive immune systems lacking the rearranging receptor gene families that allow for clonal selection, how was effector function regulated in primitive organisms? The most likely possibility is that primitive effector cells bear receptors that allow recognition of certain pathogen-associated molecular patterns (PAMPs) that are not found in the host. **I term these receptors *pattern recognition receptors*.**
h	I argue that PAMPs are still an important part of vertebrate immune systems… I propose that these pattern recognition systems activated effector functions of primitive immune systems prior to the development of rearranging gene families and continue to play a role in host defense today.
i	What kinds of ligands or patterns should such non-clonally distributed receptors recognize? I think it likely that such receptors will recognize general structural patterns in molecules found in many microorganisms, but not in the multicellular organisms… The pattern recognized should be the product of a complex and critical enzymology in the microorganism. Complex cell wall carbohydrates or LPS are likely ligands.
j	The well-known responses of B cells to polyclonal B-cell activators (also called mitogens), **such as LPS**, presumably represent the action of non-clonally distributed pattern recognition receptors on the B cell.
k	…lymphocyte-activating signals derived from other host cells, evolved prior to the development of rearranging receptor genes. The substances that trigger second-signal expression, such as LPS, do so very rapidly and do not require recognition by rearranging, clonally distributed receptors… Such receptors must be distributed non-clonally on all APCs.

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