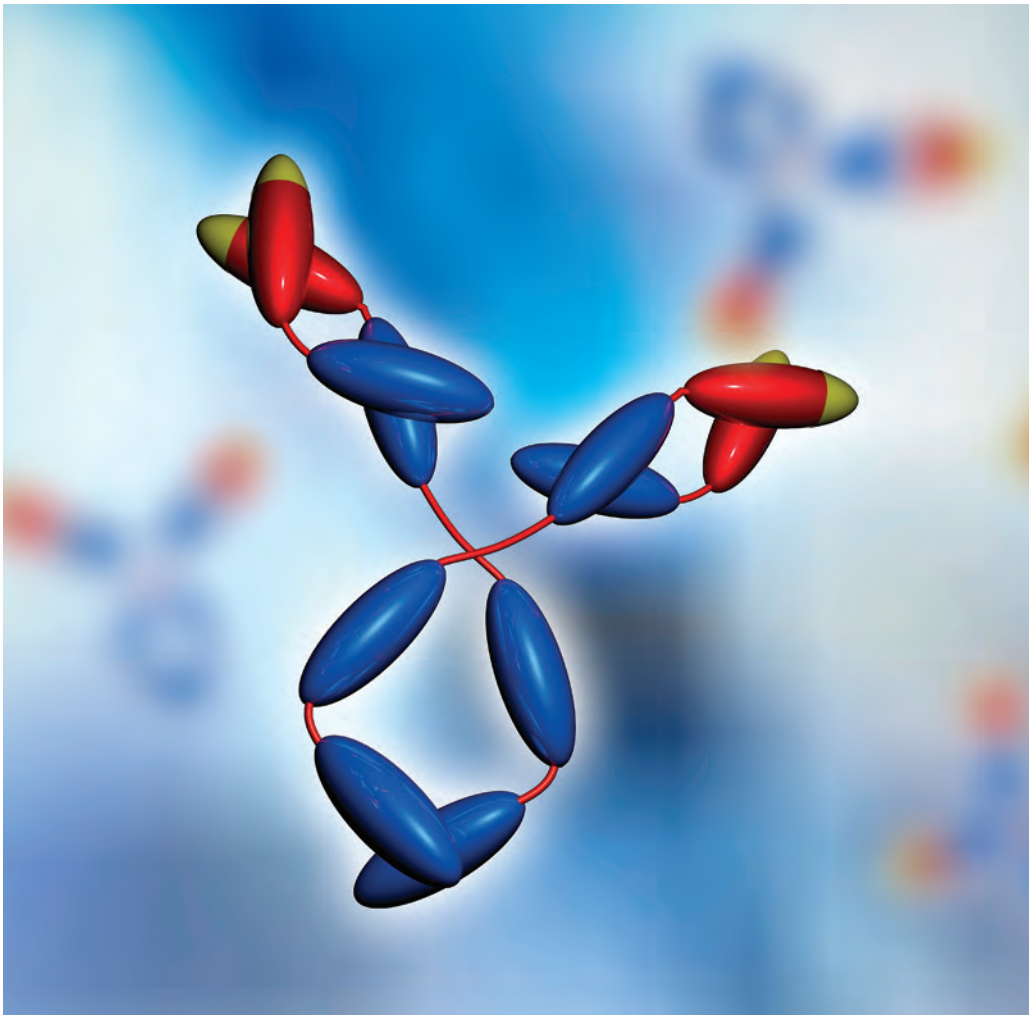
RSC Drug Discovery

Edited by Lyn H Jones and Andrew J McKnight

Biotherapeutics

Recent Developments using Chemical and Molecular Biology



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Preface

Biotherapeutic modalities are often considered beyond the reach of the medicinal chemist. The language and terminology can differ significantly between small and large molecule drug discovery, and yet the aims are essentially the same. We believe chemistry has an essential role in the future success of this exciting area, and this book was conceived as an attempt to illustrate the successful partnership of chemical and molecular biology to enable and advance biotherapeutics (so-called 'chemologics').

The design–synthesis–screen–design cycle, an engine for successful small molecule drug discovery, is not usually a component of biotherapeutic discovery, yet our challenge to this community is that that need not be the case – a deeper molecular understanding should be brought to bear in the biotherapeutics field, such that the empiricism that currently persists can be addressed. The challenge will be to generate knowledge and apply those learnings prospectively to avoid making the same mistakes and accelerate our decision making – the delineation of structure–function or structure–toxicology relationships will find increasing value in the biotherapeutic space. Small molecule drug discovery has already evolved past the period of 'make lots of stuff, screen lots of stuff, and see what pops out!' It is neither inspiring nor cost effective – design strategies are now far more sophisticated, augmented significantly by advances in biophysical techniques, computational sciences and the accuracy of predictive *in silico* tools. Our belief is that these methods will be harnessed to a greater extent in the advancement of biotherapeutic discovery and optimization approaches in the future.

This book approaches the huge area of biotherapeutics from the perspective of improved *molecular design*, which draws from the synergies between chemical biology, medicinal chemistry and molecular biology in particular. Recent developments in these disciplines that have delivered drugs, clinical candidates or significantly advanced biotherapeutic discovery and design will

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be described. A broad range of modalities are highlighted that will appeal to those working in a number of biomolecular areas (oligonucleotides, sugars, proteins and peptides). The chapters, written by an impressive list of world experts in their respective fields, detail a number of diverse therapeutic opportunities, including immunopharmacotherapy, optimized fully human or humanized antibodies, bicyclic peptide phage libraries, synthetic proteins and vaccines, micro-RNA, bacterial toxins, stabilized cyclotides, antibody–drug conjugates, peptide epitope mimicry and synthetic immunology.

Additionally, we believe this book will serve as inspiration for the medicinal chemistry community, particularly when presented with examples of how their expertise can make considerable impact in the biotherapeutics arena. Much has been made of the need to chose the 'best target' in drug discovery, but as much emphasis should then be placed on choosing the 'best modality', really, our approach should be 'modality agnostic'.

Our vision is that all biopharmaceutical chemists, whether in industry or academia, are equipped with capabilities both in small and large molecule drug discovery (and as a minimum can speak the language of, and engage in, 'biotherapies') and we hope this book will help towards that goal. In some ways, this book is a call to the traditional small molecule medicinal chemistry community to ask broader questions of their projects and therapeutic programmes. At the earliest stage of interest in a biological target we should be asking 'what therapeutic modalities shall we apply?' and both chemists and biologists are fundamental to the success of those strategic discussions, as well as the successful prosecution of the programme.

We are extremely grateful to the authors of the chapters in this book. They have not only described their areas of interest and expertise with great skill, but they have also shared compelling insights into the future opportunities for biotherapeutics. We also thank Rosalind Searle and Cara Sutton, RSC Publishing, for their editorial support and encouragement.

Lyn H. Jones and Andrew J. McKnight

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CHAPTER 1

Synthetic Immunology

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1.1 Introduction and Scope

The field of immunology has become increasingly lucid at the level of atoms and molecules. Owing also to advances in synthetic chemistry, the rational design and construction of synthetic systems that perform complex immunological functions – an area termed synthetic immunology – has come within reach. Here we will highlight one facet of synthetic immunology concerned with the development of low-molecular weight (''small''), synthetic molecules that are capable of functionally mimicking biological molecules. It is important to note at the outset that this article does not aim to be comprehensive in scope. At the expense of being all-inclusive, we focus on several specific contributions, which highlight how advances in immunology and chemistry have proven mutually complementary. We have divided this chapter into four subsections: (1) synthetic ligands for pattern recognition receptors, including toll-like receptors (TLRs), NOD-like receptors (NLRs), nuclear family receptors and C-type lectins, (2) synthetic molecules that modulate the complement system,

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(3) synthetic systems for controlling cell–cell communication, including chemokine/cytokine mimetics, and (4) synthetic ligands for modulating adaptive immune processes, including T-cell and B-cell functions.

We regret not being able to cover all of the exciting developments that might be classified into the area of Synthetic Immunology. Such areas include: synthetic vaccine development, as this topic is covered elsewhere in this book;[1] synthetic modulators of cellular signaling processes, as the functions of such molecules extend beyond the immune system;[2–5] protein-based and cellular immunotherapies, including therapeutic monoclonal antibodies;[6–9] immunomodulator strategies involving nanoparticles or virus-like particles;[10–13] DNA- and RNA-based therapeutics;[14–16] and strategies for controlling cellular differentiation.[17–20] Lead references to each of these areas are provided for interested readers.

It is our hope that this chapter will serve as a broad-based introduction for biomedical scientists, including chemists interested in extending their activities into the immunological realm, as well as immunologists looking to learn about how modern synthetic chemistry can enhance fundamental biological understanding. Ultimately, we believe that the intellectual perspective residing at the interface between synthetic chemistry and immunology will enable scientific advances that were never before thought possible, thus proving critical to the furtherance of basic biomedical research and patient care.

1.2 Synthetic Ligands for Pattern Recognition Receptors (PRRs)

Pattern recognition receptors (PRRs) are a diverse class of proteins that function canonically as part of the innate immune response. These receptors recognize pathogen-associated molecular patterns (PAMPs), which are widely conserved, repeating motifs found within pathogens and not within hosts, as well as damage-associated molecular patterns (DAMPs), which are host-derived molecules that arise from tissue damage.[21,22] Improper activation of these receptors has been shown to cause hyper-inflammatory disease states, and extensive research efforts have focused on identifying PRR antagonists. Such developments have been reviewed elsewhere.[23–33] In this section we discuss a few select examples of molecules that functionally mimic the natural ligands of PRRs.

1.2.1 Synthetic Mimics of TLR Ligands

Toll-like receptors (TLRs) were originally discovered as important receptors for *Drosophila melanogaster* embryonic development,[34] and were later found to play a critical role in innate immunity in humans.[35] Extensive research during the past two decades has revealed the TLR superfamily to contain more than ten different family members (TLRs 1–13).[22]

TLR2 is capable of binding various bacterial lipopeptides,[22] as well as self-derived ligands such as high mobility group box 1 (HMGB1) and products of lipid oxidation.[36,37] Interestingly, TLR2 preferentially forms heterodimers with other TLR members (e.g., TLRs 1 and 6), thus expanding its ligand binding profile.[38] Indeed, synthetic lipopeptide collections, designed to mimic the acylated amino terminus of bacterial lipoproteins,[39,40] have proven critical to understanding the different adaptor molecules involved in TLR2 function. These studies have led to the understanding that the TLR2 heterodimer formed in response to a given ligand depends both on the arrangement of fatty acyl groups and the sequence of peptide backbones in that ligand.[38,41] For example, synthetic tri-acylated lipopetpides, such as Pam_3CSK_4 (Figure 1.1, Panel A, **1**), selectively engage the TLR2–TLR1 heterodimer, while di-acylated analogs, such as Pam_2CSK_4 (**2**), bind both TLR2–TLR1 and TLR2–TLR6 heterodimers.[40] Modification of the peptide backbone in Pam_2CSK_4 with the decapeptide GDPKHSPKSF provides fibroblast-stimulating lipopeptide-1 (FSL-1, **3**),[42] which is selective for the TLR2–TLR6 complex.[38] A crystal structure of the TLR1–TLR2–lipopeptide provides some insight into the molecular origin of this ligand-dependence.[43] Furthermore, the specific TLR2 heterodimer formed in response to ligand stimulation can have a significant effect on downstream biological outputs. For example, synthetic ligands that induce TLR2–TLR1 heterodimer formation (e.g., Pam_3CSK_4) have been

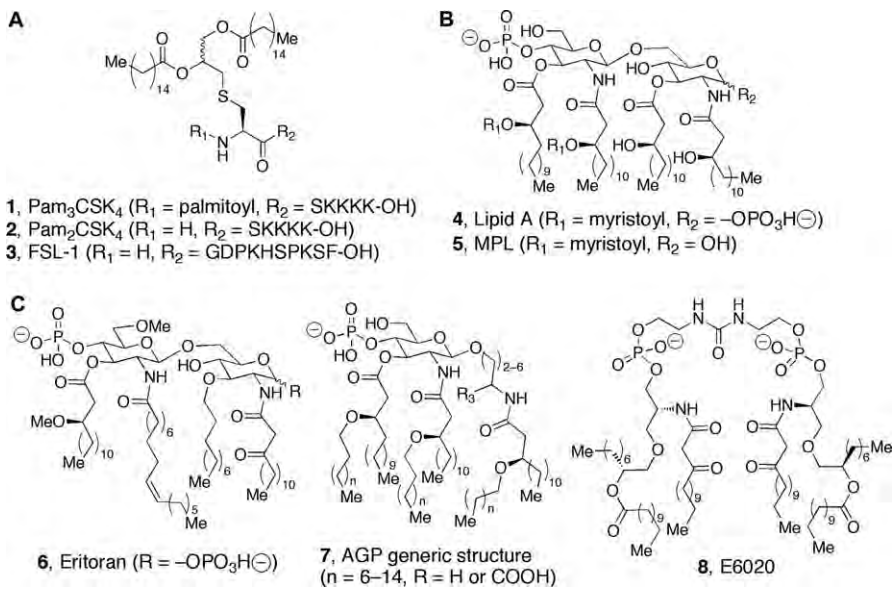

Figure 1.1 TLR 2 and TLR4 ligands. (A) Synthetic ligands capable of agonizing TLR2-containing heterodimers. (B) Lipid A-derived ligands for TLR4 including natural *E. coli* lipid A (4) and monophosphoryl lipid A (5). (C) Lipid A derivatives eritoran (6), aminoalkyl glucosaminide 4-phosphates (AGPs, 7), and non-carbohydrate ligand E6020 (8).

shown to enhance regulatory T-cell (T_{reg}) proliferation to a larger extent than those leading to other heterodimeric TLR2 complexes.[44] Conversely, a small molecule inhibitor of the TLR2–TLR1 complex was recently discovered through a cell-based high-throughput small molecule screen.[45] Efforts to expand the repertoire of TLR2-targeted compounds offer hope of providing chemical insights into immunomodulatory functions, while also delivering useful lead molecules for studying pathogen recognition, cancer progression, and other disease-relevant biological processes.[21,46]

TLR4 recognizes lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell wall. Stimulation of TLR4 leads to rapid induction of various pro-inflammatory processes including cytokine release and immune cell differentiation.[22] TLR4 ligands can serve as useful adjuvants for vaccine development,[47] however activation of this receptor is also associated with autoimmune diseases, neuropathies, and septic shock.[48] Therefore there has been significant interest in identifying both synthetic agonists and antagonists of TLR4.[49,50] Efforts to this end have primarily started from lipid A (Figure 1.1, Panel B, **4**) – the hydrophobic portion of LPS – and have led to the development of numerous synthetic immunomodulators. For example, early studies demonstrated that removal of the anomeric phosphate group in **4** can ameliorate some of the molecule's toxic effects in humans. The resulting compound, monophosphoryl lipid A (MPL, **5**), retains its TLR4 agonist activity and has been approved by the FDA, for use as a vaccine adjuvant.[51] Further investigations into synthetic lipid A analogs have led to a general model relating ligand structure with TLR4-modulating activity: agonists are believed to form "conical" structures in solution – wherein the cross-section of the hydrophobic portion of the molecule is greater than that of the hydrophilic one – whereas antagonists are believed to form "cylindrical" shapes such that hydrophobic and hydrophilic cross-sections are nearly identical.[47] Indeed, this model is supported by extensive structure-activity relationships along with recent crystallographic studies.[52–54] For example, compounds lacking hydrophobic acyl substituents – such as eritoran (Figure 1.1, Panel C, **6**) – serve as receptor antagonists.[55,56] On the other hand, agonist structures have been obtained from replacement of labile phosphate moieties with negatively charged bioisosteres (e.g., sulfate), substitution of ester-branched acyl groups with corresponding alkyl ethers, and removal of the lipid A reducing sugar to afford aminoalkyl glucosaminide 4-phosphates (AGPs, **7**).[50,57] Complete replacement of the lipid A disaccharide with a simple phosphatidylethanolamine-based construct provides agonist E6020 (**8**), which is believed to retain the "conical" conformation and is capable of eliciting TNF-α production from whole blood, as well as IL-6 production and NF-κB activation in cell lines expressing TLR4.[47,58–60] Additional TLR4 modulators have been obtained using "glycocluster" strategies,[61] attaching LPS to magnetic nanoparticles,[62] and other approaches.[63–65] Furthermore, the discovery of TLR4-active compounds structurally unrelated to lipid A, such as paclitaxel, heme derivatives, and opioids, will undoubtedly lead to new opportunities for developing synthetic immunomodulators.

TLR7/8 are expressed in a variety of immune cells of the myeloid lineage and are intimately involved in anti-viral immunity by triggering the release of type 1 interferons.[66] The first synthetic ligands for TLR7/8 were discovered by 3M scientists in cellular screens of nucleoside analogs for antiviral applications.[67] Interestingly, although these efforts led to the development of the class of imidazoquinoline derivatives – including Imiquimod (Figure 1.2, Panel A, **9**), which was first marketed in 1997 for treating complications of human papilloma virus (HPV) infection[68] – it was not until much later that the mechanism of these compounds as TLR7/8 agonists was confirmed.[69] Over the past decade, derivatives of imidazoquinolines, including resiquimod **(10)** and S-27609 **(11)**,[70] have proven to be effective TLR7-stimulating adjuvants resulting in anti-tumor activity[71] and polarization of immune cells towards T_H1 responses.[70,72] Like single-stranded RNA (ssRNA), which is the natural ligand for TLR7/8, small molecule agonists such as **9**–**11** activate macrophages to produce pro-inflammatory cytokines (IFN, TNF-α and others) via the TLR7 MyD88-dependent signaling pathway.[69] Furthermore, homology modeling studies suggest that both ssRNA and small molecule **10** have overlapping binding sites on TLR8, suggesting that imidazoquinolines are both structural and functional mimics of natural ligands.[68,73,74] Further progress in illuminating the structural details of TLR7/8 interactions with their ligands will undoubtedly lead to improvements in synthetic ligand mimics.

Like TLR7/8, **TLR9** is localized to endolysosomal compartments, recognizes foreign nucleic acids, and induces a type-1 IFN response upon activation.[75]

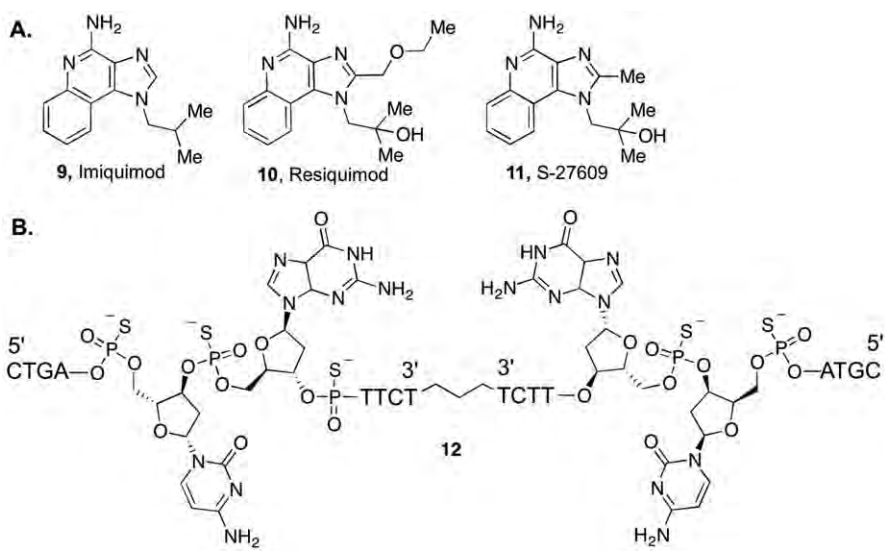

Figure 1.2 Ligands for TLRs 8, 9, and 10. (A) TLR7/8 ligands based upon an imidazilone core structure, including Imiquimod (9), Resiquimod (10) and S-27609 (11). (B) One example of the "immunomer" class of TLR9 ligands.

Unlike TLR7/8, however, the natural ligand for TLR9 is unmethylated CpG oligodeoxynucleotides (CpG DNA), which is present in both bacterial genomes and DNA viruses. Synthetic agonists for TLR9 that take advantage of structural similarities with natural nucleic acid ligands have been developed. For example, having determined that an accessible 5'-end of CpG-containing oligodeoxynucleotides is necessary for immunomodulatory activity of synthetic TLR9 ligands,[76] researchers at Hybridon have developed a class of "immunomers" (**12**) containing short CpG DNA sequences chemically connected through synthetic 3'–3' linkers.[77] Interestingly, both the chemical composition and the length of "immunomer" derivatives were found to impact their immunomodulatory effects.[78–80] Indeed, immunomers containing only 5 or 6 nt in each segment possess immunostimulatory properties in both mice and humans.[81] Also, replacement of phosphate backbone with phosphorothioate motifs,[82,83] or incorporation of 5'-dinucleotides containing non-natural bases,[78] can confer enhanced stability and alter patterns of cytokine secretion in tissue culture systems. Evaluation of synthetic TLR9 agonists in human clinical trials as vaccine adjuvants, and immunotherapies for allergy, cancer, and infectious disease are currently underway.[84]

1.2.2 Synthetic NOD Agonists

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are a family of soluble cytosolic proteins consisting of over twenty members. NLRs are believed to detect cytosolic PAMPs derived from foreign agents as well as danger-associated molecular patterns (DAMPs) from injured host tissue. NLRs promote NF-κB and mitogen-activated protein kinase (MAPK) signaling and complex with the inflammasomes to upregulate pro-inflammatory IL-18, IL-1β, IL-6, TNF, and type I IFN secretion.[85]

NOD1 has been shown to recognize the dipeptide, D-glutamyl-meso-diaminopimelic acid (iE-DAP),[86] present as a component of bacterial peptidoglycan (**13**), which activates the NF-κB pathway, enhancing the production and secretion of several cytokines.[87] Hasegawa and coworkers have chemically synthesized iE-DAP derivatives to identify minimal motifs responsible for NOD1 activation.[88] It was found that the iE-DAP structure serves as a core motif for NOD-1 activation; functionalization of this group with substituted alkyl chains (**14**), benzamides (**15, 16**), or short peptides (**17**) enhances NOD-1 activation. The authors speculate that this enhancement may result from increased hydrophobicity of the molecules compared to the parent compound (**18**), which enhances their interactions with the cell membrane. Notably, compound **14** was found to be several hundred fold more active than the original iE-DAP.[89] Moreover, it was found that iE-meso-DAP stereoisomer showed a 10 to 10,000 fold greater ability to stimulate NOD-1-mediated NF-κB activation as compared to other stereoisomers.[88]

NOD2, a close relative of NOD1, is expressed in several phagocytic cells, including monocytes, granulocytes, and dendritic cells.[90] Its natural ligand is believed to be the MurNAc-L-Ala-D-isoGln muramyl dipeptide (MDP,

Figure 1.3, Panel A, red box),[91] which is found in both Gram-positive and Gram-negative bacteria. In a similar manner to NOD1, NOD2 ligation leads to the secretion of IL-12, IL-8, IL-6, and TNF-α. Almost four decades of research on MDPs have led to the development of numerous analogs,[92] including mifamurtide (Figure 1.3, Panel C, **19**), approved for combination chemotherapy treatments.[93] One of the primary reasons for the development of these synthetic molecules was the potent pyrogenicity and somnogenicity induced by the natural ligand that made it relatively unattractive as an adjuvant. These derivatives of MDP have been shown to have greater biological activity and lack the

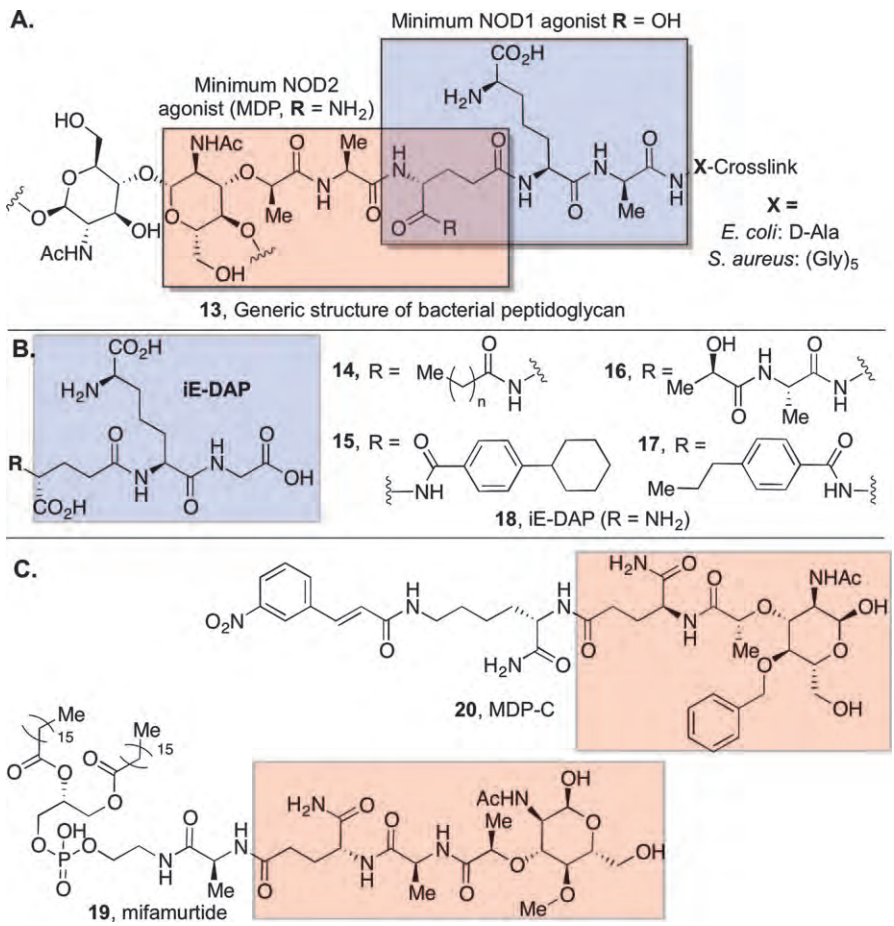

Figure 1.3 Natural and synthetic ligands for NOD 1 and NOD2. (A) Generic structure of bacterial peptidoglycan (13) with boxes indicating the components recognized by NOD1 (blue) and NOD2 (red). (B) Synthetic NOD1 agonists with various substitution on the N-terminus of iE-DAP (blue box). (C) Two NOD2 agonists mifamurtide (19) and MDP-C (20). Red boxes indicate the regions of natural peptidoglycan that are being mimicked.

undesirable characteristics of MDP, and have been extensively discussed elsewhere.[92,94] Other efforts to optimize the biological effects of MDP have led to the identification of MDP-C (**20**), which is both apyrogenic and nonallergenic, yet potently immunostimulatory. Here, Yang *et al.* utilized a novel mesh-bagged gathered-bunch combinatorial chemistry strategy (abbreviated MBGB) to synthesize MDP analogs on solid supports.[95] They produced over 2300 MDP derivatives and tested their ability to stimulate murine macrophages to eliminate tumor cells. This work has resulted in the discovery of the novel compound MDP-C (**20**), which significantly enhances dendritic cell (DC)-mediated, IL-2, IL-12 release and the induction of cytotoxic activity by cytotoxic T-cells (CTLs) *in vitro*. Finally, MDP-C has also been shown to have Hepatitis B Surface Antigen specific antibody response *in vivo*, with minimal pyrogenicity (in rabbits), passive cutaneous anaphylaxis (in rats), and low toxicity (in mice).[96]

Researchers have also explored the use of cocktails of synthetic PRR agonists to stimulate NLRs and TLRs simultaneously. Such a combinatorial treatment may more closely mimic a bacterial infection compared to single agents, and in turn induce a potent immune reaction. Indeed, Tada *et al.* combined two synthetic NOD2 agonists (MDP and N-acetylmuramyl-L-aranyl-L-isoglutamine (MDP-LL)), two NOD1 agonists (FK565 and FK156),[97] and various TLR agonists (TLR4- lipid A, TLR2- Pam_3CSSNA, TLR3- poly (I:C), and TLR9- CpG DNA) to stimulate DC cultures. Combination treatments led to the synergistic upregulation of IL-12 and IFN-γ production by T-cells, indicative of a T_H1-response.[98] Utilizing complex cocktails to mimic the natural repertoire of PRR ligands is likely to prove increasingly important to synthetic immunology strategies in the future.

1.2.3 Peroxisome Proliferator-activated Receptor Gamma

Peroxisome proliferator-activated receptor gamma (PPAR-γ) is a ligand-activated transcription factor that plays an important role in regulating storage and catabolism of dietary fats, and whose stimulation is also associated with various immunosuppressive effects. These include inhibition of DC maturation, $CD4^+$ T-cell differentiation into T_H17 cells,[99] and the suppression of pro-inflammatory cytokine release.[100] Endogenous PPAR-γ ligands are similar to one another in that they are highly lipophilic and contain long, polyunsaturated fatty acid structural motifs (e.g., docosahexanoic acid, arachidonic acid, eicosapentaenoic, and gamolenic acid).[101] Among these ligands, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2, Figure 1.4, **21**), which is formed as a terminal product of the cyclooxygenase-2 pathway, has proven especially useful in studying PPAR-γ.[102] Perhaps the most widely studied synthetic PPAR-γ ligands were not originally developed for their immunomodulatory effects; the class of thiazolidinediones (TZDs) or "glitazones" – which include pioglitazone (**22**), troglitazone (**23**) and others – were discovered from screening efforts in rodent models for insulin resistance and have been used for over 28 years as therapeutics for diabetes.[101] Their mechanisms of action have only recently been shown to involve PPARs.[103] The critical features

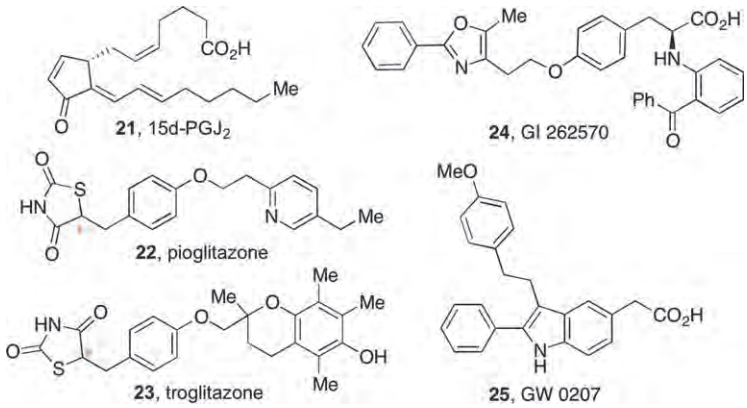

Figure 1.4 Natural and synthetic ligands for PPAR-γ. Red stars in structures 22 and 23 indicates the C-5 position of the thiazolidinedione core, which is prone to epimerization.

of these synthetic ligands that allow binding to PPAR-γ are believed to include the 2,4-thiazolidinedione structural motif, which mimics the head-group of a lipid molecule, and the aromatic, unsaturated, tail, which mediates hydrophobic contacts with the receptor. This structural model is supported by apo- and ligand-bound crystal structures of PPAR-γ.[104,105] Optimization of TZD compounds using direct PPAR-γ binding assays have revealed that only compounds in the (*S*)-enantiomeric series at C-5 (indicated with red stars in Figure 1.4) bind the receptor.[106] Because the thiazolidinedione nucleus is prone to epimerization at this position, next-generation PPAR-γ modulators have incorporated head groups of increasing kinetic stability such as tyrosine derivatives (e.g., GI 262570, **24**),[107] and the achiral GW 0207 (**25**).[108] Explorations into the immunomodulatory activities of PPAR-γ ligands are still at an early stage; future studies that combine efforts of chemists and immunologists will surely provide insight into fundamental and biomedical applications for PPAR-γ ligands.[109]

1.2.4 C-Type Lectin Receptors (CLRs)

C-type lectin receptors (CLRs) comprise a group of membrane-bound Ca^{2+}-dependent carbohydrate-binding proteins found on "professional" antigen presenting cells (APCs) such as macrophages, DCs, and B-cells.[110] Although there are up to 14 different types of CLRs, we deal here with synthetic strategies for modulating only one of these family members – the DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN).[111,112] Like other CLRs, DC-SIGN can recognize glycosylated peptides and proteins,[113] leading to internalization, antigen processing, presentation on MHC class II molecules, and cross-presentation on MHC class I molecules.[114]

Notably, DC-SIGN expression is restricted to DCs, it possesses a dual specificity for both high-mannose and Lewis-type carbohydrates,[115] and functions to maintain immune homeostasis through cell adhesion and intracellular signaling. Interest in identifying synthetic DC-SIGN modulators has arisen due to the protein's intriguing function as a receptor for pathogen entry; binding to DC-SIGN has been shown to promote infections by both viral and bacterial pathogens. Structural studies have provided insight into DC-SIGN–ligand binding, and numerous DC-SIGN modulators have developed. For example, researchers have explored the binding of various carbohydrates to DC-SIGN, most of which bind with relatively low affinity, and other groups have aimed to enhance interaction strength using multivalent ligands. Prost and colleagues have disclosed an approach wherein functionalization of a (−)-shikimic acid core structure with mannose derivatives, followed by bioconjugation with bovine serum albumin (BSA), afforded a ligand capable of stimulating DC-SIGN-mediated endocytosis as well as JNK signaling.[116] Kiessling and coworkers have identified non-carbohydrate quinoxalinone-based DC-SIGN ligands using a high-throughput screening assay, followed by focused medicinal chemistry efforts.[117,118]

In a separate study, Srinivas *et al.* explored whether a synthetic glycotargeting approach could be utilized to induce antigen uptake and cross-presentation by DCs via the MR or DC-SIGN.[119] To achieve this, the authors developed a fully synthetic glycocluster conjugate containing a 25 amino acid peptide conjugated with either Manα-6Man or a mixture of Lewis^a and Lewis^x oligosaccharides (not shown), which targeted both the MR and DC-SIGN receptor. Romero *et al.* specifically chose a melanoma antigen, A27L Melan-A/Mart-1 epitope,[120] to evaluate if the synthetic glycocluster could facilitate the cross-presentation of this antigen to elicit a CTL response, with the aim of developing a vaccine. Interestingly, their synthetic molecule was not only able to bind to both MR and DC-SIGN in SPR experiments, but it was also able to induce internalization into DCs, and to stimulate Melan-A-specific $CD8^+$ T-cells to induce IFN-γ production *in vitro*. This work serves as an interesting example of how synthetic mimics of biomacromolecules can elicit receptor-specific, targeted pro-inflammatory responses.

1.3 Synthetic Systems for Controlling Cell–Cell Communication: Cytokine Mimetics

The immune system relies heavily on intercellular communication to regulate critical surveillance and defense functions. Cytokines are a diverse family of soluble molecules that have been divided into subtypes based on their function, secreting cell type, and presumed target. These subtypes include chemokines, which trigger cell movement or chemotaxis; interleukins, which are key players in transferring information between leukocytes; and interferons, named for their ability to interfere with viral replication. Because of their roles in co-ordinating both pro- and anti-inflammatory processes, cytokines are critical for

maintaining immune homeostasis. Small molecules capable of replicating cytokine functions have been widely sought.[121] Here we discuss several examples of synthetic molecules capable of functioning as cytokine mimics.

Chemokines are a class of small proteins (8–12 kDa) that are primarily responsible for stimulating leukocyte migration, which is critical for their activation, differentiation, and survival.[122] Chemokine receptors are members of the class of G-protein coupled receptors (GPCRs), which contain seven transmembrane-spanning domains and elicit a complex downstream signaling cascade.[122] Here we focus on a few examples of how synthetic approaches to mimic the natural chemokines have led to novel ligands for several chemokine receptors, including CCR1, CCR3, and CXCR3.

CCR1 interacts with natural peptide ligands CCL3 and CCL5, and triggers chemotaxis of monocytes, macrophages, and T-cells.[123] Jensen *et al.* have recently identified two small molecule metal chelators – 2,2-bipyridine and 1,10-phenanthroline (Figure 1.5, **26**) – which can serve as chemokine mimics. When these compounds are complexed to Cu^{2+} or Zn^{2+}, they are capable of inducing G-protein signaling by binding CCR1. Interestingly, the mechanism of this effect was found to involve binding of the metal–chelator complex deep within a cleft in CCR1, leading to antagonism of the CCL5 binding interaction and enhancement of CCL3 association.[124]

CCR3 is found primarily on eosinophils, and is involved in modulating allergic reactions when activated by its natural ligand CCL11/eotaxin.[125] Using a high-throughput cell-based small-molecule screen involving FACS-based detection of changes in cellular morphology, researchers have identified the chemokine mimic CH0076989 (**27**).[126,127] Mechanistic studies have revealed that **27** can promote chemotaxis of a pre-B lymphoma cell line in a CCR3-specific manner, albeit not as potently as CCL11. Follow-up homology modeling studies have suggested that the binding site for **27** on CCR3 is similar to that for the natural ligand (CCL11), yet appears to overlap with prototypical

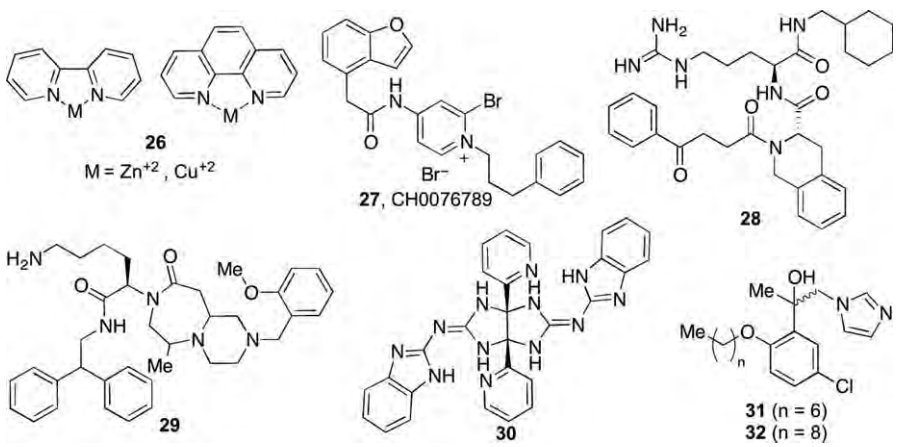

Figure 1.5 Molecules that influence immune cell communication.

antagonist binding regions.[126] Taken together, these findings have indicated that minor changes in the orientation of synthetic ligands can result in major changes in the receptor response. Therefore, these studies have the potential to aid in future structure-based design efforts of both agonists and antagonists of CCR3.

CXCR3 is expressed primarily on T-cells and regulates chemotactic responses to natural ligands CXCL9, CXCL10, and CXCL11.[128] CXCR3 agonists have shown benefits in therapeutic applications such as wound healing and anti-tumor activity through enhancement leukocyte chemotaxis.[129–131] Using a calcium mobilization assay in CXCR3-transfected HEK293 T-cells, Stroke *et al.* screened an encoded combinatorial library of tetrahydroisoquinolines and piperidinyl diazepanones to search for chemokine mimics. These studies have led to the discovery of several CXCR3 agonists (**28** and **29**) capable both of inhibiting CXCL10 and CXCL11 binding, and promoting T-cell chemotaxis.[132] Follow-up experiments have revealed these small molecule agonists can bind three critical residues (D112, D195, and E196) within an intrahelical pocket in CXCR3, thus mimicking the Pro-Arg-Val sequence (positions 37–39) in CXCL10.[133] These molecules represent both structural and functional mimics of chemokines, and may provide effective alternatives to recombinant CXCL10 and CXCL11 tumor therapies.[130,131]

The cytokine Granulocyte-Colony Stimulating Factor (**G-CSF**) is a potent regulator of survival, proliferation, differentiation, and activation of granulocytes. G-CSF binds to the G-CSF receptor (CD114), which results in activation of the JAK/STAT signaling pathway, transcriptional activation of interferon responsive genes, and enhancement of cell proliferation and differentiation.[134,135] Indeed, recombinant G-CSF or filgrastim (marketed by Amgen under the brand name Neupogen®) is used clinically to replenish the loss of leukocytes in response to chemotherapy or bone-marrow transplantation.[136] Synthetic mimics of G-CSF have the potential to provide effective, low cost, and non-immunogenic alternatives to such treatments. To identify G-CSF mimics, one group has developed a high-throughput screening assay utilizing a myeloid-derived murine cell line with a JAK/STAT pathway-dependent luciferase reporter construct. Execution of this screen has led to the identification of the non-peptidic small molecule SB247464 (**30**); this compound has been shown to elicit JAK/STAT signaling, and increase the neutrophil count in an immunosuppressed neutropenic mouse model (BDF-1).[137] Notably, the two-fold rotational symmetry in **30** is believed to be critical to its ability to mimic G-CSF function in inducing oligmerization and activation of CD114.[137–139] Interestingly, although human G-CSF can activate both murine and human G-CSFR, SB247464 has only been found to stimulate murine, but not human, CD114. To address this deficiency, another group performed a high-throughput cellular screen of 10,000 synthetic compounds for their ability to stimulate proliferation of a human G-CSF sensitive BAF/B03 cell line (hematopoetic B-cell line).[140] These studies have identified imidazole derivatives SSCL02446 (**31**) and SSCL02448 (**32**), which have been shown to stimulate STAT3 signaling and increase neutrophil counts *in vivo* in neutropenic rats.[140]

1.4 Synthetic Molecules that Modulate the Complement System

The complement system is an integral part of humoral immunity, serving as a cytotoxic effector system against a variety of pathological stimuli. As shown in Figure 1.6, the complement pathway involves a network of proteins that act in concert to regulate immune cell responses, clear infections and cellular debris, and lyse antibody-opsonized targets.[141] The activation of complement, either through the classical, lectin, or alternative pathways,[142,143] leads to a cascade of enzymatic reactions, culminating in complement-mediated pro-inflammatory signaling, phagocytosis, or lysis of targets.[144] As shown in Figure 1.6, the responses of the complement system are also negatively regulated by various control proteins, including C1-INH, factor H, C4BP, CD46, and CD55. Dysregulation of any of these components can lead to the development of diseases including glomerulonephritis, pancreatitis, psoriasis, rheumatoid arthritis, and asthma.[141,145]

A molecular-level understanding of the complement cascade has facilitated the development of synthetic molecules capable of mimicking the endogenous regulators. For example, in order to develop mimics of complement factor H–related protein 1 (CFHR1) – a human plasma protein that inhibits the activity of C5 convertase – researchers have developed an assay to monitor the C5 cleavage in the presence of a library of aptamers.[146] This screening protocol has led to the identification of C5C6 (Figure 1.7, **32**), a specific inhibitor of C5 esterase that can prevent the formation of C5a and

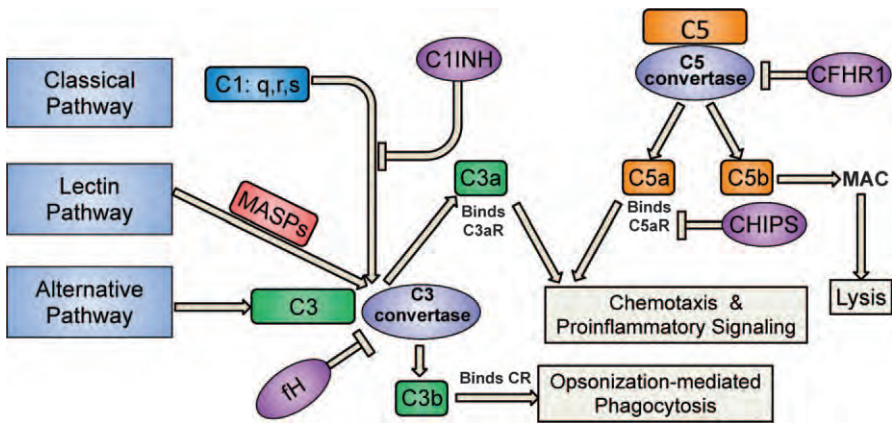

Figure 1.6 Schematic representation of the complement cascade. The endogenous complement inhibitors are colored in purple. MASPs: mannan-binding lectin serine proteases; C1: complement component 1; C3: complement component 3, C5: complement component 5; CFHR1: complement factor H–related protein 1; CHIPS: chemotaxis inhibitory protein Staphylococcus aureus; fH: factor H.

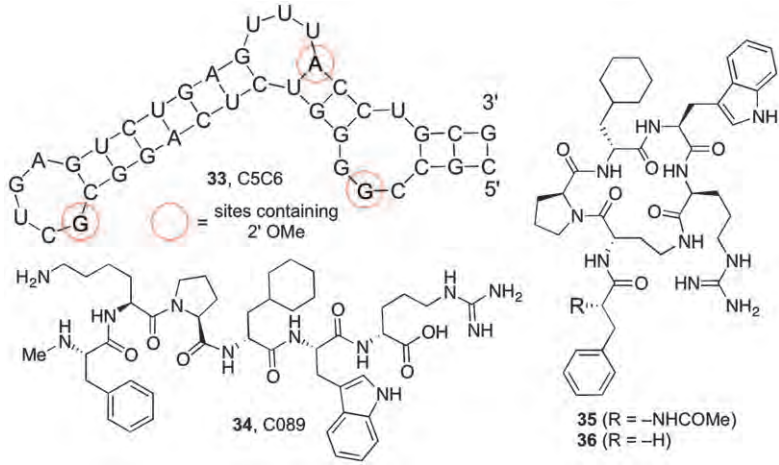

Figure 1.7 Synthetic complement modulators. C5C6 aptamer (32) inhibits C5 convertase (sites containing 2'-OMe indicated with red circles). C089 (34) and cyclic derivatives 35 and 36 inhibit C5.

C5b, much like CFHR1. The three 2'-OMe substitutions in **33** have proven critical for increasing its serum stability and enabling maximal inhibition of complement-mediated hemolytic activity of sheep erythrocytes.[147] Subsequent truncation of C5C6, followed by conjugation to PEG and capping with an inverted nucleotide at the 3' end, have provided ARC1905, an aptamer derivative with improved pharmacokinetic properties compared to **33**.[148] This molecule has shown clinical promise as a treatment for age-related macular degeneration (AMD) and is an excellent example of a fully synthetic, modified aptamer capable of emulating a natural complement-inhibiting protein.

Several naturally-occurring peptides and proteins have been identified, which are capable of enabling pathogens to evade the human complement response.[149] For example, the Gram-positive bacteria *Staphylococcus aureus* secretes a chemotaxis inhibitory protein called CHIPS. CHIPS binds to the N-terminus of C5a and abrogates the binding of C5a to the C5a receptor (Figure 1.6, C5aR or CD88,).[150] With the goal of developing novel anti-inflammatory compounds, researchers have identified several synthetic molecules that mimic the function of CHIPS. In one such example – called the chemotaxis inhibitory construct protein of *S. aureus* (CHOPS)[151] – researchers took into account critical structural features of CHIPS (an α-helix and three β-strands), as well as the spatial orientation of its binding to C5aR. To this end, a D-Pro-Gly was incorporated into CHOPS, which helped maintain the helical structural features, resembling the motifs found in CHIPS. Using isothermal titration calorimetry, researchers then found CHOPS to have an affinity for C5aR of 3–4 μM. Although this value is three-orders of magnitude lower than

the affinity of CHIPS for C5aR, CHOPS has served as an important lead compound for further ligand-design efforts.[151]

An alternative mechanism for synthetically mimicking the action of CHIPs has been to develop C5a mimetics that block the C5a–C5aR interaction.[152] C5a has two binding sites that enable it to bind and activate the C5aR; one of these resides at the core inter-helical loops of C5a and contains positively charged residues,[153,154] and the other comprises an eight-residue motif at the protein's C-terminus, which itself is sufficient to agonize C5aR.[155] Researchers have utilized this octapeptide agonist as a basis for developing linear (**34**, C089) and cyclic (**35**, **36**) synthetic antagonists of the C5a–C5aR interaction.[156] These molecules have exhibited inhibitory effects on neutrophil chemotaxis and cytokine production from macrophages both *in vitro* and *in vivo*.[157] These non-immunogenic, synthetic molecules have the potential to serve as starting points for effective anti-complement therapeutics, capable of treating a range of diseases including arthritis, ischemia-reperfusion injuries, and sepsis.[146,158]

Factor H (fH) is an inhibitory protein that is highly abundant in human plasma, and prevents C3 convertase formation by binding to components C3b and C3d.[159] Factor H is recruited to endothelial cells by polyanionic ligands such as sulfated heparin, dermatan sulfate, and glycosaminoglycan, thus protecting these cells from the alternative complement pathway.[160] Therefore, functionalization of biomaterials with synthetic structures capable of recruiting fH has served as a useful strategy for preparing complement-compatible materials.[145] For example, using phage-display-based screening technologies, Wu *et al*. have identified a hexapeptide termed 5C6, which is capable of binding fH without interfering with its complement-inhibitory properties.[161] Immobilization of 5C6 on pegylated polystyrene or glass surfaces, using thiol-maleimide bond-forming reactions, prevented complement fixation in erythrocyte hemolytic assays.[162] This research has provided an interesting and potentially useful approach to the production of biocompatible materials.

1.5 Mimicking and Hijacking Antibody Function

Antibody proteins bridge the innate and adaptive wings of the immune system; they are produced in response to immunogenic epitopes, yet they function by activating innate cytotoxic processes. Antibody-based therapeutic approaches have blossomed over the past decade, in part because they exploit both innate and adaptive features; they can be generated against wide-ranging disease-relevant epitopes, and they can also activate endogenous immune effector mechanisms, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). The development of synthetic systems capable of emulating – and improving upon – the function of antibody molecules has represented an exciting focus of synthetic immunology research in recent years.

For example, with the goal of emulating the adaptive immune system's ability to rapidly identify highly selective epitope-targeting motifs, Kodadek and colleagues have developed a systematic small-molecule synthesis and screening protocol. This process involves construction of oligomeric, bead-based small molecule libraries using split-and-mix synthesis methods, followed by execution of protein- and/or cell-binding selections directly on synthesis resins.[163–166] Because small molecule ligands identified through this approach are both potent and selective – in analogy to natural antibody proteins – they have been termed "antibody mimics."[167,168] Indeed, using their streamlined synthesis-screening protocol, the Kodadek lab has identified nanomolar "antibody mimics" against both soluble and cell-surface-bound targets such as cholera toxin,[169] VEGF receptor,[170,171] orexin receptor,[172] phosphoproteins,[173] and other systems.[174–179]

Researchers have also explored the development of novel cytotoxic agents that are capable of exploiting the innate effector functions of antibodies. For example, attachment of synthetic targeting motifs to intact antibodies or immunoglobulin constant (Fc) domains has led to the development of novel anticancer agents for targeting the endothelin receptor,[180] or $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins.[181–184] Bioconjugation reactions leading to the assembly of these chimeric agents can also be performed *in vivo,* potentially providing new approaches for *in situ* self-assembly of targeted chemotherapeutic agents.[185] Another strategy has been to develop small molecules that hijack antibodies already present in the human bloodstream, and recruit them to disease-relevant cell-surface targets. Indeed, humans possess endogenous antibodies that recognize simple, low-molecular weight haptens such as 2,4-dinitrophenyl, α-gal trisaccharide, and others. By attaching these antibody recognition motifs to high-affinity chemical groups that recognize disease-associated targets, it has been possible to develop bifunctional molecules that exploit the innate cytotoxic properties of antibodies to clear pathologic cells (Figure 1.8). These synthetic agents – called antibody-recruiting molecules (ARMs) – have been used to target cancers,[186–196] bacteria,[197–201] and viruses,[202–204] and are currently in clinical development. A review summarizing ARM-based strategies has recently appeared in the literature.[184]

Overall, by mimicking and/or exploiting the properties of antibody proteins, researchers hope to develop more effective, safer and less costly alternatives to current immunotherapeutic strategies.

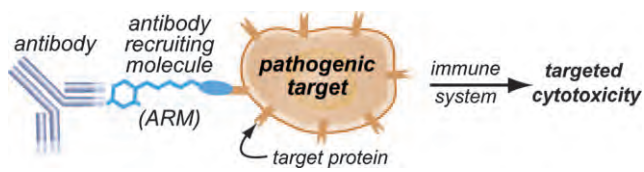

Figure 1.8 Schematic diagram representing the concept of antibody recruiting molecules (ARMs). ARMs are bifunctional molecules capable of bindig to pathogenic target and simultaneously recruiting antibody molecules, this eliciting antibody-dependent immune responses.

1.6 Synthetic Molecules that Modulate T- and B-cell Responses

Immune responses to foreign invaders frequently result in a series of molecular and cellular events that endow a host with long-term immunity (Figure 1.9). This process – termed adaptive immunity – begins with the uptake and processing of non-self molecular motifs by DCs, macrophages, B-cells, and other professional antigen presenting cells (APCs). APCs then present antigens to T-cells, and when accompanied by the appropriate pro-inflammatory signals, the activation and proliferation of antigen-specific B- and T-cells

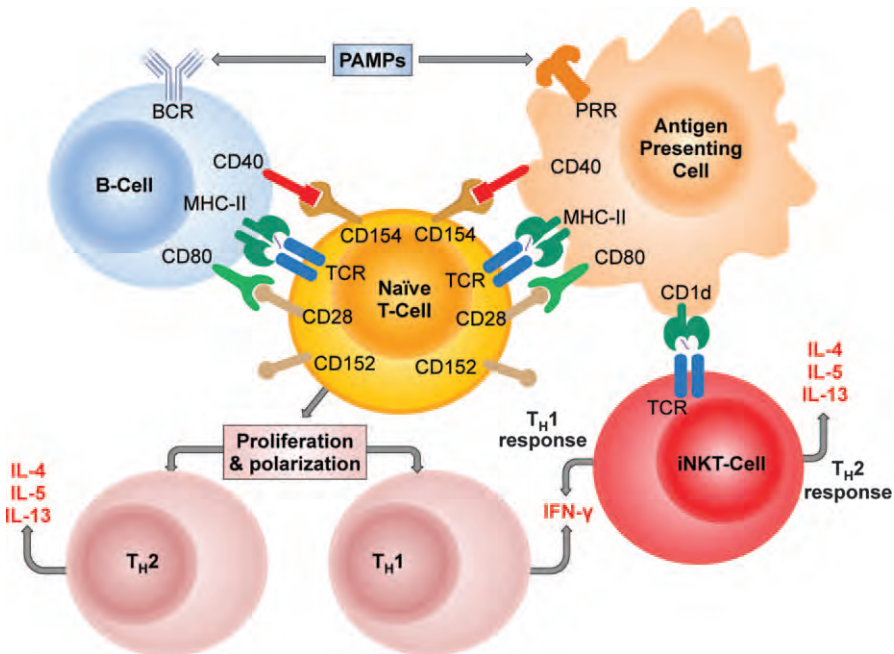

Figure 1.9 Summary of components of the adaptive immune system. Following an infection, pathogen associated molecular patters (PAMPs) are recognized and internalized by pattern recognition receptors (PRRs) on the antigen presenting cells, for subsequent presentation of antigenic epitopes on major histocompatibility complex class II (MHC II) proteins. These MHC II complexes are recognized by specific naïve T-cell receptors (TCRs). Costimulatory signals mediated by CD40 and CD80 of the antigen presenting cells, and CD154 and CD28 on the CD4+ T-cells lead to T-cell proliferation and polarization to T helper cells (T_H1 and T_H2), which then secrete inflammatory cytokines (labeled in red). CD152 regulates this proinflammatory response by inhibiting the interaction between CD28 and CD80. Invariant natural killer cells (iNKTs) are a subset of T-cells whose TCRs recognize lipidated molecules presented by CD1d on antigen presenting cells to elicit either T_H1 or T_H2 cytokine responses.

ensues. B-cells differentiate into plasma cells, capable of secreting antigen-specific antibody molecules that give rise to humoral immunity, while T-cells can differentiate into variants of helper T-cells or cytotoxic T-lymphocytes (CTLs), responsible for antigen-specific cell-mediated immunity. Synthetic approaches to emulate, stimulate and/or suppress adaptive immune responses have been explored, and we highlight a selected few in this section.

With the goal of developing synthetic molecules to promote adaptive immune responses against *Trypanosoma cruzi* – the causative agent of Chagas disease – Fournel *et al.* have developed synthetic molecules that mimic the CD40 ligand (CD154).[205] CD154 is expressed on activated T-cells, while its receptor is present mainly on APCs. The CD40–CD154 interaction is critical for regulating B-cell proliferation and memory cell development, as well as "licensing" DCs to present antigen to cytotoxic T-cells.[206] To mimic natural CD154, researchers developed synthetic structures called miniCD40Ls (Figure 1.10, Panel A). These compounds contain a rigid, symmetrical, trimeric scaffold comprised of either β^3-tripeptide (**37**) or D,L-α-hexapepetide (**38**) coupled to a peptide sequence derived from CD40 ligand (CD154).[205] This trimeric structure reflects X-ray crystallographic findings, which indicate that CD154 presents itself to CD40 as a trimer.[207] Indeed, compound **37** has proven functionally effective in stimulating mouse B-cell proliferation and maturation of a DC cell line, and confirmatory surface plasmon resonance (SPR) experiments have revealed that **37** and **38** binds CD40 trivalently. Furthermore, exposure of *T. cruzi*-infected mice to miniCD40Ls leads to increases in IFN-γ production by lymphocytes, proliferation of B-cells, maturation of DCs, and reversal of *T. cruzi*-mediated immunosuppression, leading to effective control over infection *in vivo*.[208]

An interesting strategy for modulating B-cell receptor (BCR) activity has recently been disclosed by Kiessling and colleagues. Because crosslinking of cell-surface BCRs can induce B-cell activation without assistance from T-cells, these researchers have developed hapten-functionalized polymers that can display BCR ligands in a multivalent fashion (**39**).[209] Exposure of hapten-specific B-cells to these functionalized polymers leads to BCR clustering and production of anti-hapten antibodies *in vitro*. This research has also provided insights into the molecular requirements for B-cell activation and lays a foundation for a novel immunization protocol that could prove useful when extended to living organisms.[210,211]

Invariant natural killer cells (iNKTs) are a class of T-cells involved in the recognition of lipid antigens, which are presented on APCs by CD1 family members – a class of APC-expressed glycoproteins related to MHC I.[212] Derivatives of the natural product agelasphin (Figure 1.10, Panel B, **40**), have proven useful in understanding and mimicking CD1-mediated antigen presentation. Agelasphin and derivatives such as KRN7000 (α-GalCer, **41**) can be taken up by scavenger receptors (SR) on macrophages and DCs, which are then presented to iNKT-cells bound to CD1d. This activation event can lead to the production of both T_H1 (IFN-γ) and T_H2 (IL-4, IL-5, and IL-13) cytokine responses *in vitro*.[213–215] More recently, synthetic, crystallographic,[216] and computational studies[217] have led to a more detailed understanding of the

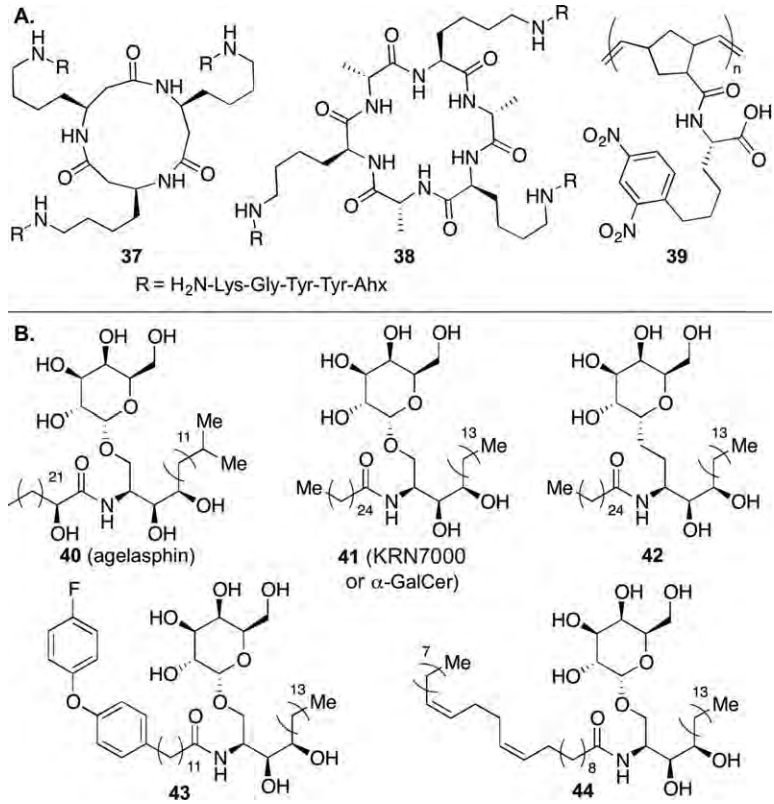

Figure 1.10 Molecules that modulate the function of B-cells (Panel A) and iNKT-cells (Panel B).

CD1d-glycolipid interaction.[218] Importantly, synthetic studies have resulted in the generation of α-GalCer analogs capable of polarizing iNKT-cells to produce either T_H1 or T_H2 cytokine responses.[218–222] In one example, altering only the connectivity between the glycan and lipid portions of ligands, as in C-linked compound **42**, has led to increases in T_H1 cytokine production, and enhanced activity *in vivo* against melanoma metastases in mice.[223] T_H1 responses can be further enhanced by functionalization of one acyl chain with arenes, leading to the optimized structure **43**. Compound **43** has been found to elicit protection against bacterial (*Sphingomonas casulata* and *S. aureus*) and viral (Japanese encephalitis virus) infections in mouse models.[224] Conversely, alterations in the length and saturation of an acyl chain in α-GalCer derivatives were sufficient to elicit potent T_H2 responses.[225] For example, the molecule C20:2 (**44**) was found to reduce IFN-γ levels and increase IL-4 and IL-13 production in mice, conferring protection from type 1 diabetes.[226] These examples illustrate the ability to utilize synthetic derivatives of a natural molecule to fine-tune the adaptive immune response.