

## **TLR-dependent T cell activation in autoimmunity**

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### **Abstract**

Autoimmune disease can develop as a result of a breakdown in immunological tolerance, leading to the activation of self-reactive T cells. There is an established link between infection and human autoimmune diseases. Furthermore, experimental autoimmune diseases can be induced by immunization with autoantigens that are administered together with complete Freund's adjuvant containing killed *Mycobacteria tuberculosis*, which in some cases can be replaced with individual pathogen-associated molecular patterns (PAMPs). Exogenous PAMPs and endogenous danger signals from necrotic cells bind to pathogen recognition receptors, including Toll-like receptors, and activate signaling pathways in innate immune cells and on T cells, leading to inflammatory cytokine production and T cell activation, and this is now considered to be a major factor in the development of autoimmunity.

### **Introduction**

The discovery by Janeway and Mezhitov that innate immune cells sense pathogens through pathogen recognition receptors (PRRs), has revolutionized our understanding of the role of the innate immunity in infection and in driving T cell responses<sup>1</sup>. It has also significantly enhanced our understanding of the mechanisms of immune dysregulation leading to autoimmunity. Organ-specific autoimmune diseases can develop through a combination of hereditary and environmental factors that lead to a failure to regulate adaptive immune responses to self antigens, in particular those mediated by IL-17-producing T helper cells (T<sub>H</sub>17 cells)<sup>2</sup>. The

mechanism is thought to involve a breakdown of immunological tolerance, mainly due to defective regulatory T ( $T_{Reg}$ ) cell function, which leads to the uncontrolled generation of autoantigen-specific antibodies and pathogenic T cells that mediate tissue inflammation<sup>3</sup>.

Inflammatory T cells are induced by pathogen- or commensal- derived molecules, as well as by endogenous stress-induced self molecules. These **microbe-associated molecular patterns (MAMPs)** and **damage-associated molecular patterns (DAMPs)** [G] induce inflammatory T cells either indirectly through the induction of pro-inflammatory cytokine production by innate immune cells, or directly through their binding to PRRs on T cells. Evidence for this is provided by the described association between infections and autoimmune diseases (such as multiple sclerosis and rheumatoid arthritis), which develop following activation of self-reactive B and T cells, or between infections and chronic inflammatory diseases (such as Crohn's disease) which result from uncontrolled innate and adaptive immune responses to normal constituents of the gut microflora.

There are several families of PRRs: Toll-like receptors (TLRs) sense conserved molecules from bacteria, virus and parasites; the cytosolic nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) sense bacterial products; c-type lectin receptors (CLRs) bind  $\beta$ -glucans; and RIG-1-like receptors (RLRs) bind nucleic acids<sup>4-5</sup>. There have been a number of exciting recent discoveries that have linked the activation of PRRs with autoimmune diseases, especially around the capacity of exogenous and endogenous ligands to promote innate inflammatory responses that drive the induction of autoreactive T cells, in particular IL-17-producing  $T_H17$  cells and  $\gamma\delta$  T cells<sup>6-11</sup>. Coupled with the discoveries around the pathogenic role of these IL-17-producing T cells in autoimmunity, this has significantly advanced our understanding of the disease process in many autoimmune disorders. TLRs are a key family of PRRs involved in driving autoimmune inflammation, therefore, inhibitors of TLR binding or signaling are now considered to have great potential as therapeutics for autoimmune and other inflammatory diseases.

This Review article discusses the role of TLRs in organ-specific autoimmune diseases in the context of direct and indirect (through innate immune cell-derived cytokines) activation of auto-reactive T cells either directly or via innate immune cells. In addition, it explores new immunotherapeutic approaches based on inhibiting TLR-induced immune activation.

**PAMPs shape innate and adaptive immune responses** PRRs are expressed by a variety of cell types, especially cells of the innate immune system, such as dendritic cells (DC) and macrophages, where they sense danger or damage signals. The PRR ligands include **pathogen-associated molecular patterns (PAMPs)** [G] — conserved microbial structures, such as lipopolysaccharide (LPS), flagellin, viral and bacterial nucleic acids — but also endogenous danger signals from dead and dying cells, called DAMPs, such amyloid- $\beta$  and saturated fatty acids (BOX 1). The primary function of PRRs is to mediate innate immune responses to pathogens and tumors, but they also have a role in **sterile inflammation** [G] and have been linked with autoimmune and chronic inflammatory diseases<sup>1,4</sup>.

Innate immune cells, especially DCs, direct adaptive immunity, not only by acting as antigen presenting cells (APCs) for naïve T cells, but also by providing the cytokine stimulus (the third signal) for T cell activation<sup>1-2</sup>. Binding of agonists to TLRs promotes DC maturation and production of T cell-promoting cytokines<sup>1</sup>, using distinct signaling pathways (FIG. 1). Activation of NF- $\kappa$ B results in production of the pro-inflammatory cytokines, including tumour necrosis factor (TNF), interleukin-6 (IL-6), pro-IL-1 $\beta$  and pro-IL-18<sup>4-5</sup>. Activation of the interferon regulatory factor (IRF) pathways mediate IL-12p35 and type I interferon (IFN) production, whereas active extracellular signal-regulated kinase (ERK) and p38 MAP kinases are required for IL-23p19 and IL-10 production respectively<sup>5,12-13</sup> (FIG. 1). TLR agonists have potent immunomodulatory activity and can promote adaptive immune responses to co-administered antigens and consequently have been exploited as adjuvants in infectious disease and cancer vaccines and as tumor immunotherapeutics<sup>14-15</sup>. Conversely, because of their ability to activate innate immunity and inflammation, molecules that antagonize TLR ligand binding or inhibit TLR signaling pathways are being developed as therapies against autoimmune and other inflammatory diseases<sup>5</sup>.

Targeting innate cytokines that promote pathogenic T cell responses has become a major focus in drug development against autoimmune diseases (FIG. 2). Whereas much of the initial focus was on IL-12, which is the key driver of pathogenic T<sub>H</sub>1 cells, the discovery of T<sub>H</sub>17 cells and their role in autoimmunity (BOX 2) has shifted the emphasis to the innate regulatory cytokines IL-6, IL-23 and IL-1, which promote the development of T<sub>H</sub>17 cells. It is now recognized that IL-6 and TGF $\beta$  promote differentiation of murine T<sub>H</sub>17 cells from naïve CD4<sup>+</sup> T cells<sup>16-18</sup> and that IL-23 and IL-1 $\beta$  or IL-18 (which are produced via activation of caspase-1 and

the **NLRP3 inflammasome** [G] in TLR-primed DCs<sup>8</sup>) promote expansion and IL-17 production by memory T<sub>H</sub>17 cells<sup>19-20</sup> (**FIG 3**). Moreover, it has been suggested that IL-23 can also induce differentiation of T<sub>H</sub>17 cells<sup>21-22</sup>.

As well as having an indirect role in promoting IL-17 production by T cells, there is increasing evidence that PRR-triggered innate immune cells can act as a direct source of IL-17.  $\gamma\delta$  T cells<sup>6,23</sup>, natural killer T (NKT) cells<sup>24</sup> and **lymphoid tissue inducer-like** (LTi) [G] cells<sup>25</sup> have all been shown to be important sources of IL-17 and associated cytokines. We have demonstrated that IL-1 $\alpha$  or IL-1 $\beta$  can synergize with IL-23 to promote IL-17 secretion from  $\gamma\delta$  T cells without T cell receptor engagement<sup>6,19</sup>.  $\gamma\delta$  T cells secrete IL-17, IL-21 and IL-22 and act in an amplification loop to expand T<sub>H</sub>17 cells<sup>6</sup>. A high frequency of IL-17-producing  $\gamma\delta$  T cells are found in the CNS of mice with experimental autoimmune encephalitis (EAE), where they function with T<sub>H</sub>17 cells to mediate autoimmune inflammation<sup>6,26</sup>.

Although much of the focus on TLRs has center around their role in sensing and responding to infection by the innate immune system, it is now clear that TLRs agonists also have a crucial role in adaptive immunity. Indeed activation of DC and other innate immune cells through PRRs appears to be a critical step in the induction of T cell responses to pathogens, but also in the early inflammatory responses that lead to the development of autoreactive B and T cells that mediate many autoimmune diseases.

### **The link between PAMPs and autoimmunity**

Expression of PRRs has been found in tissues of patients with organ-specific autoimmunity. Furthermore a polymorphism in TLR2 is associated with type 1 diabetes<sup>27</sup> and polymorphisms in TLR4 and TLR9 have been described in patients with Crohn's disease<sup>28</sup>. Mutations in NOD2 have been strongly associated with Crohn's disease, and the synergy between NOD2 and TLR9 activation of inflammatory cytokine production by peripheral blood mononuclear cells is lost in cells from patients with the NOD2 mutation<sup>29</sup>. Moreover, the presence of PAMPs in tissues following infection has been associated with autoimmunity. These observations provide indirect evidence that PAMPs may promote autoimmune and chronic inflammatory diseases.

*Infections precipitate autoimmune diseases.* There is a well established link between infection and autoimmune diseases both in clinical settings and in animal models. This link has been

attributed to either molecular mimicry between pathogen-derived antigens and self antigens or non-specific activation of innate immunity leading to a breakdown in immunological tolerance and the development of self antigen-specific T cell- and antibody-responses.

Transgenic mice expressing a T cell receptor (TCR) specific for a myelin-derived peptide develop spontaneous EAE when housed in conventional, but not specific pathogen free conditions<sup>30</sup>. This suggests that infection may precipitate autoimmunity in mice. Several studies in multiple sclerosis patients have suggested that disease may be triggered or exacerbated by infections with *Chlamydia pneumonia*, human herpes virus 6 or Epstein-Barr virus (EBV). The strongest evidence comes from studies on EBV, as viral latent protein has been found in the brain of patients with multiple sclerosis<sup>31</sup>. In patients with relapsing remitting multiple sclerosis, there is an association between upper respiratory tract infections and relapse<sup>32</sup>. It has also been reported that systemic infections may trigger relapse in multiple sclerosis by bystander enhancement of myelin-specific T cell responses<sup>33</sup>.

Parvovirus B19 is considered to be one triggering factor for rheumatoid arthritis; the virus was detected by PCR in synovial biopsies of 75% of patients with rheumatoid arthritis compared with 17% of patients with osteoarthritis and other arthritides<sup>34</sup>. Furthermore, EBV DNA and RNA were detected in 34% of patients with rheumatoid arthritis compared with 10% of healthy individuals<sup>35</sup>.

Commensal microorganisms may also be involved in the development of autoimmune diseases, as suggested by the finding that some species of gut microbiota promote the pathogenesis of inflammatory bowel disease, especially Crohn's disease<sup>36</sup>. Commensal microbes, in particular segmented filamentous bacteria have been shown to promote the induction of T<sub>H</sub>17 cells<sup>9</sup> and as well contributing to intestinal inflammation, these T<sub>H</sub>17 cells can also drive autoimmune arthritis<sup>37</sup>. It has also been reported that commensal bacteria-derived ATP promotes IL-6, TGF $\beta$  and IL-23 production by DC, leading to local activation of naturally occurring and pathogenic T<sub>H</sub>17 cells<sup>38</sup>. However, this was independent of TLR signaling. Conversely, studies in a T cell transfer model of colitis showed that commensal bacteria promote T<sub>H</sub>1 and T<sub>H</sub>17 responses by MYD88-dependant activation of innate immune responses<sup>39</sup>. Although IL-6 was implicated, a role for commensal-induced IL-1 in promoting T<sub>H</sub>17 responses has not been ruled out. These studies point to a role for pathogens and commensal bacteria in promoting or exacerbating autoimmune diseases.

*PAMPS may exacerbate autoimmunity.* In addition to initiating autoimmune and chronic inflammatory diseases, PAMPs may exacerbate these diseases. Infection with *Streptococcus pneumoniae* 7 days after induction of EAE exacerbates autoimmunity in wildtype but not *Tlr2*<sup>-/-</sup> mice<sup>40</sup>. This suggests that pathogens may exacerbate autoimmune diseases via activation of TLRs. Furthermore, PAMPs are present in diseased tissues of patients with autoimmunity. For example, peptidoglycans, which include NLR and TLR2 ligands, has been found in synovial tissue macrophages and DCs isolated from patients with rheumatoid arthritis<sup>41</sup>, in the bowel wall of patients with Crohn's disease<sup>42</sup>, in the brain of primates with EAE<sup>43</sup> and in macrophages and DCs in the brain of patients with multiple sclerosis<sup>44</sup>. Interestingly, the expression of some TLRs (such as TLR2 and TLR3) is enhanced in astrocytes and oligodendrocytes from multiple sclerosis patients, suggesting that increased TLR signaling may occur during ongoing autoimmunity<sup>45</sup>. This in turn may exacerbate autoimmunity. Indeed, treatment of cancer patients with the TLR7/8 agonist imiquimod exacerbates psoriasis<sup>46</sup>.

*Some infections suppress autoimmunity.* In addition to driving autoimmunity, certain infections have also been associated with suppression of autoimmune diseases. Epidemiological data has shown that incidence of multiple sclerosis and type 1 diabetes is increasing in developed countries, and this correlates with a decrease in the number of infections<sup>47</sup>. Conversely, a higher incidence of infection with helminth parasites in rural parts of developing countries is associated with a lower incidence of allergy and autoimmune diseases<sup>48</sup>. The so-called 'hygiene hypothesis' suggests that anti-inflammatory cytokines or T<sub>Reg</sub> cells induced by such infections exert bystander suppression on the immune responses that mediate allergy and autoimmunity<sup>48-49</sup>. This hypothesis is supported by mouse studies showing that helminth infections can suppress autoimmunity through bystander suppression of autoantigen-specific T<sub>H</sub>17 cells, through induction of anti-inflammatory cytokines and T<sub>Reg</sub> cells<sup>50</sup>. It has also been shown that infection of germ-free mice with a single human commensal *Bacteroides fragilis* protected against EAE through T<sub>Reg</sub> cell induction<sup>51</sup>. So, although much of the evidence points to a role for pathogens and commensal bacteria, especially those that induce potent T<sub>H</sub>17 responses, in promoting autoimmune inflammation, chronic infections, especially with parasites that are sustained and dominated by T<sub>Reg</sub> cells, may have the opposing effect of attenuating autoimmunity.

## Role of DAMPs in autoimmunity

While pathogens and their products have been implicated in precipitating autoimmune diseases in humans and can induce experimental autoimmunity in animal models, there is also evidence that DAMPs or **alarmins** [G] released from dead and dying cells can stimulate innate immune responses leading to autoimmunity (FIG. 4). Polly Matzinger first suggested that, as well as responding to PAMPs, APCs can also respond to danger or alarm from endogenous molecules produced by distressed tissues, called DAMPs<sup>52</sup>. The term alarmin was coined by Joost Openheim<sup>53</sup> to describe a group of mediators released by necrotic cells in response to infection or injury that interact with PRRs and activate innate immune cells, including DC and thereby enhance T cell responses. It has been suggested that alarmins are members of a larger family of DAMPs that also includes PAMPs<sup>54</sup>.

Human mobility group box protein 1 (HMGB1) is considered to be a DAMP capable of activating innate immune responses. Although there are reports that it may bind to TLR4 and other TLRs, many of the early studies on HMGB1 and other putative DAMPs were compromised by contamination with PAMPs, especially LPS. However, it has recently been reported that HMGB1 can stimulate innate immune responses by acting as a universal sentinel for nucleic acids<sup>55</sup>. HMGB1 binds to most or all nucleic acids facilitating their interaction with nucleic acid-sensing TLRs (TLR3, TLR7 and TLR9) and cytosolic nucleic acid binding sensors (RIG-1, MDA5, DAI, AIM2) on innate immune cells. Interestingly, HMGB1 concentrations are elevated in the blood of patients with SLE, compared with healthy controls and there was a positive correlation between HMGB1 concentrations and disease activity<sup>56</sup>. This provides indirect evidence that this endogenous DAMP may be involved in the pathogenesis of human autoimmune diseases.

In a murine model of neutrophilic inflammation, Chen *et al* found that intraperitoneal injection of dying cells into mice induced recruitment of neutrophils, which was not associated with HMGB1, but was mediated through MYD88 and IL-1, rather than TLR signals<sup>57</sup>. The neutrophil recruitment and the production of the hepatic enzyme alanine aminotransferase (ALT) that were observed in wild-type mice were absent in IL-1RI<sup>-/-</sup> mice but not in mice defective for a range of TLRs, IL-18R, caspase-1. Moreover, the inflammatory response was blocked by IL-1 $\alpha$ -specific blocking antibody, suggesting that IL-1 $\alpha$  is key alarmin driving sterile inflammation. As

IL-1 $\alpha$  promotes IL-17 production by T cells<sup>19</sup>, it may be an important endogenous activator of pathogenic T cells in autoimmune diseases.

Another alarmin, 15- $\alpha$ -hydroxicholesterene (15-HC), is a derivative of cholesterol and activates microglia via TLR2 and polyADP-ribose polymerase 1 (PARP1). 15-HC is elevated in the serum of patients during the progressive phase of multiple sclerosis and in mice with secondary progressive EAE<sup>58</sup>. TLR2-specific antibody inhibited activation of PARP1 by 15-HC and attenuated the progression of EAE<sup>58</sup>.

Furthermore, human synovial membrane cultures from patients with rheumatoid arthritis were found to express TLR2 and TLR4 and to release endogenous TLR ligands which may contribute to destructive inflammation in the joints of these patients<sup>59</sup>. Finally, it has recently been shown that heterogeneous nuclear ribonucleoprotein (hnRNP), an RNA and DNA-binding protein that is a target of most nuclear antigen-specific T and B cells in systemic autoimmune diseases but also in human rheumatoid arthritis and in pristane-induced arthritis in rats, triggers TLR7- and TLR9- mediated activation of arthritogenic APCs, which activate pathogenic T cells<sup>60</sup>.

Although much of the early studies on DAMPs may be compromised by contamination issues, the more recent studies with knockout mice and molecular defined alarmins suggest that they can promote innate and as a consequence adaptive immune responses that mediate autoimmunity. However, it is still to clear whether this sterile inflammation is sufficient to kick start autoreactive T cell responses, or whether it amplifies responses induced by PAMPs.

### **TLRs in animal models of autoimmunity**

Studies using TLR-defective mice, TLR-specific blocking antibodies, inhibitors of TLR signaling, or TLR agonists in animal models of autoimmune diseases have provided insight into the role of TLR activation in the initiation or exacerbation of autoimmunity (Tables 1 and 2).

*The role of TLRs in EAE.* Actively-induced EAE involves immunization of mice with myelin-derived peptides in complete Freund's adjuvant (CFA). *M. tuberculosis* in CFA contains PAMPs that activate innate immune responses, which in turn promote the pathogenic auto-reactive T cell responses. Reports by Prinz et al<sup>10</sup> and Marta et al<sup>61</sup> showed that mice deficient in MYD88, an adapter protein for IL-1 receptor and all TLRs except from TLR3, are resistant to EAE. This was



associated with reduced IL-6 and IL-23 production by DCs and reduced IL-17 and IFN $\gamma$  production by T cells<sup>10</sup> (Table 1). This suggests that innate immune responses, initiated through TLRs or IL-1R signaling, are required for the induction of experimental autoimmunity.

Mice with a lymphoid cell-specific TLR2 deficiency were less susceptible to EAE and had reduced T<sub>H</sub>17 responses<sup>62</sup> than wild type controls. The reduction in disease severity and IL-17 production was even more dramatic when EAE was induced by adoptive transfer of TLR2-deficient T cells. This suggests that TLR2 signalling in T cells may be important for the induction of EAE. In addition, TLR2 may be involved in the activation of CNS-infiltrating macrophages, microglia or astrocytes during development of EAE<sup>58</sup>.

A study by Prinz *et al.* showed that disease severity was reduced in TLR9-deficient mice, though not as dramatically as in *Myd88*<sup>-/-</sup> mice<sup>10</sup>. Moreover, disease onset was delayed in *Tlr9*<sup>-/-</sup> mice following transfer of MOG-specific T cells from wild-type mice<sup>10</sup>, implicating a role for TLR9-induced innate immune activation in promoting the pathogenic function of activated auto-reactive T cells. Indeed APC activation through TLR9 could break tolerance and facilitate the induction of EAE<sup>63</sup>. Immunization of mice with proteolipid protein (PLP) in incomplete Freund's adjuvant (IFA) induces tolerance due to lack of danger signals provided by the *M. tuberculosis* of the CFA. However, transferred lymph node cells from these tolerized mice induced EAE following stimulation with CpG<sup>63</sup>, providing further evidence of a pro-inflammatory role for TLR9 activation. This is consistent with the demonstration that TLR9 activation may circumvent tolerance and promote intestinal inflammation by suppression of T<sub>Reg</sub> cell conversion<sup>64</sup>.

Interestingly, the study by Marta *et al.* showed that mice defective in either TLR9 or TLR4 had enhanced IL-6, IL-23 and IL-17 expression and developed severe EAE<sup>61</sup>, suggesting a protective role for these TLRs in this autoimmune disease. In this study mice were immunized once with MOG peptide, whereas in the study by Prinz and colleagues mice were immunized twice with myelin protein, and this may have obscured the regulatory role of TLR9<sup>61</sup>.

Finally, it has been reported that parenteral injection of poly I:C, a TLR3 ligand, suppressed relapsing-remitting EAE and this was associated with IFN- $\beta$  production<sup>65</sup>. Collectively these findings indicate that TLR and IL-1 signalling is crucial for the induction of EAE, but that there may be redundancy between certain TLRs. Moreover, certain TLRs may be pro-inflammatory (TLR2 and TLR9), whereas others (TLR3 and TLR9) may also have a

regulatory role in EAE, depending on the different stages of disease. When compared with knockout mouse studies, studies with TLR-specific antibodies or inhibitors, which can be used to block TLR signalling at different stages of disease, may give a clearer picture of the potential of the role of individual TLR pathways in EAE and other autoimmune diseases.

*The role of TLRs in arthritis.* In most mouse models, arthritis is induced following activation of inflammatory responses by killed bacteria or their products and there is good evidence that this involves activation of PRRs. In the mouse model of collagen-induced arthritis (CIA), which is induced by immunization with collagen and CFA, *M. tuberculosis* in CFA provides a source of PAMPs. *Staphylococcus epidermidis*-derived peptidoglycans (which includes TLR2 and NLR agonists) was also shown to induce acute arthritis in mice<sup>66</sup>. Moreover, zymosan, a polysaccharide from the cell wall of *Saccharomyces cerevisiae*, which binds TLR2 and the CLR dectin-1, has been used to induce experimental arthritis in mice. Zymosan-induced arthritis was found to be dependent on TLR2 activation, as disease was significantly attenuated in *Tlr2*<sup>-/-</sup> mice, but not by treatment with lamarin which blocks the dectin-1 receptor<sup>67</sup>. In addition, injection of immune stimulatory DNA sequences (ISS-ODN) into the joints of rats promoted the development of adjuvant arthritis<sup>68</sup>. This suggests that activation of TLR9 may also precipitate the innate immune responses that drive inflammation in the joint

Spontaneous T cell-mediated arthritis in *IL-1Ra*<sup>-/-</sup> mice was dependant on TLR4 activation by microbial flora; germ-free mice or *Tlr4*<sup>-/-</sup> *Il1ra*<sup>-/-</sup> double knockout mice did not develop arthritis<sup>11</sup>. Furthermore, a TLR4 antagonist, LPS from *Bartonella quintana*, inhibited IL-1 $\beta$  in the joint and attenuated arthritis in CIA and *Il1ra*<sup>-/-</sup> arthritis models<sup>69</sup>. By contrast, *Tlr2*<sup>-/-</sup> *Il1ra*<sup>-/-</sup> mice developed more severe disease, which was associated with increased IFN $\gamma$  production by T cells and reduced T<sub>Reg</sub> cell function<sup>11</sup>, as direct TLR2 activation can induce expansion but transiently disrupt the function of T<sub>Reg</sub> cells<sup>70</sup>. Thus microbiota may promote arthritis in *Il1ra*<sup>-/-</sup> mice by augmenting IFN $\gamma$  and suppressing T<sub>Reg</sub> cell function via TLR2. So, unlike its role in EAE, where TLR2 signalling appears to drive inflammatory pathology, TLR2 may both promote and inhibit inflammatory T cell responses in mouse models of arthritis. However, there is more consistent evidence, from studies with the TLR4 antagonist as well as TLR4-deficient mice, that targeting TLR4 has potential in the treatment of arthritis.

*Other autoimmune diseases.* Further studies with mouse models of autoimmunity reveal that the contribution of TLR signaling to the initiation of autoreactive T cell responses depends on the disease type and experimental conditions. For example, *Myd88*<sup>-/-</sup> mice are resistant to experimental autoimmune uveitis (EAU) and exhibit significantly reduced autoantigen-specific T<sub>H</sub>1 responses<sup>71</sup>, whereas *Tlr2*<sup>-/-</sup>, *Tlr4*<sup>-/-</sup>, and *Tlr9*<sup>-/-</sup> mice develop normal T cell responses and are susceptible to EAU. By contrast, the development of type 1 diabetes in NOD mice is markedly reduced in the absence of TLR2 but not TLR4<sup>72</sup>, and it appears that APCs sense apoptotic  $\beta$  cell death through TLR2 and thereby promotes diabetogenic T cells.

Moreover, TLR2 and TLR9 were shown to have distinct pathogenic roles in a murine model of anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV), a multisystem autoimmune disease where neutrophil myeloperoxidase (MPO) is targeted by autoantibodies<sup>73</sup>. Immunization with MPO in the presence of the TLR2 agonist Pam3Cys enhanced vasculitis by promoting T<sub>H</sub>17-dominated responses and neutrophil recruitment, whereas immunization with MPO and the TLR9 agonist CpG selectively promoted T<sub>H</sub>1 responses and macrophage activation<sup>73</sup>. Finally, topical application of the TLR7/TLR8 agonist imiquimod in mice induces skin lesions in mice resembling psoriasis; this was associated with epidermal expression of IL-23 and IL-17 and enhanced T<sub>H</sub>17 cells in the spleen<sup>74</sup>.

Taken together, the studies in mouse models suggest that TLR agonists may trigger autoimmunity, with the most definitive phenotype seen in *Myd88*<sup>-/-</sup> mice. However, this adapter protein is also used for IL-1 signaling, so it cannot be inferred that the protective effect of MYD88 deficiency is solely due to a loss of TLR-mediated inflammation. Studies in mice lacking a single TLR have revealed less dramatic defects in induction of experimental autoimmunity in a number of models, with perhaps the best evidence supporting a role for TLR2 in EAE and T1D, TLR4 in arthritis. Conversely there is evidence for a protective role for TLR3 in EAE. Finally, there are reports of protective as well as pathogenic roles for TLR9 in EAE and TLR2 in arthritis and further study is required to define the potential of these TLRs as targets in multiple sclerosis and rheumatoid arthritis.

### **Indirect modulation of autoimmune T cell responses by TLRs**

There is growing evidence that activation of PRRs on innate immune by pathogens or PAMPs cells can lead to a breakdown in self tolerance and bystander activation of autoreactive T<sub>H</sub>1 or T<sub>H</sub>17 cells. This supports the link between infection and autoimmunity.

*Activation of pathogenic T cells.* There is evidence that PAMP-induced secretion of pro-inflammatory cytokines from monocytes and DCs promotes the induction and expansion of T<sub>H</sub>17 cells<sup>7,75-76</sup> (FIG. 3). For example, human monocytes stimulated with LPS, zymosan, flagellin or lipoteichoic acid promote IL-17 production by anti-CD3 activated human CD4<sup>+</sup> T cells<sup>75</sup>. The TLR-induced signal was not identified, and addition of IL-6 or TGFβ inhibited IL-17 production in this setting. Furthermore, hyphae of *Candida albicans* selectively promoted T<sub>H</sub>17 cell induction by inducing IL-23 but not IL-12 production from human monocytes and DCs<sup>77</sup>.

TLR3, TLR4 and TLR9 ligands induce MYD88-dependent production of cytokines from DCs, which promotes the differentiation of T<sub>H</sub>17 cells<sup>18</sup>. Moreover, the TLR4 ligand LPS promotes IL-17 production by murine antigen-specific memory T cells through the induction of IL-1 and IL-23 production by DCs<sup>76</sup>. Finally, TLR2 agonists have been shown to promote the differentiation of murine T<sub>H</sub>17 cells *in vitro*<sup>62</sup>.

T<sub>H</sub>17 cells can also be induced independent of TLR and NLR activation, through activation of dectin-1 with the β-glucan curdlan, which promotes IL-6, TNF and IL-23 production by DCs<sup>78</sup>. Similarly, bacterial derived ligands of NOD-2, such as muramyl dipeptide (MDP), have been shown to drive IL-17 production by human memory T cells by stimulating IL-1 and IL-23 production by DCs<sup>7</sup>. Finally, it has been reported that commensal microbiota can promote differentiation of T<sub>H</sub>17 cells in the lamina propria of mice deficient in MYD88 and TLR3, suggesting that TLR and IL-1R signaling are not essential for induction of T<sub>H</sub>17 cells in the gut<sup>79</sup>.

*T cell-mediated autoimmunity.* The induction of experimental autoimmune disease in animal models, such as EAE, EAU and CIA, very often involves immunization with autoantigen in the presence of CFA. The killed *Mycobacterium tuberculosis* in CFA promotes innate inflammatory cytokines that direct the induction of T<sub>H</sub>1 and T<sub>H</sub>17 cells. *M. tuberculosis* has been shown to activate the NLRP3 inflammasome promoting IL-1β and IL-18 production<sup>8</sup>, and to activate TLRs leading to IL-6 and IL-23, which collectively drive the induction of T<sub>H</sub>17 and T<sub>H</sub>1 cells.

There have been several attempts to substitute *M. tuberculosis* with purified PAMPs (Table 2). Unlike PLP in CFA, which induced potent EAE, active immunization with PLP in IFA containing CpG failed to induce EAE, although mild disease was induced following the transfer

of T cells from mice immunized with PLP in IFA with CpG to naïve recipients<sup>80</sup>. This effect was attributed to the fact that *M. tuberculosis* is a more potent adjuvant than CpG for induction of T<sub>H</sub>17 responses. By contrast, immunization of B10.S mice that express a transgenic TCR specific for PLP<sub>139-151</sub> with IFA and CpG induced EAE in 73% mice compared with 33% that developed spontaneous disease<sup>81</sup>. The study demonstrated that activation of APCs via TLR9 can break tolerance and promotes the activation of self-reactive T cells. Interestingly, LPS was unable to break tolerance in this model.

It has been demonstrated that immunization of mice with MOG in IFA supplemented with zymosan can induce but not sustain EAE. Disease induction was associated with the generation of MOG-specific T<sub>H</sub>17 cells, and transient induction of IL-23 by DC<sup>82</sup>. Hansen *et al.* also reported that immunization with MOG in IFA supplemented with high dose zymosan or LPS, but not the TLR3 agonist poly I:C, could induce EAE<sup>83</sup>. The LPS employed was purchased from Sigma; this is alleged to be contaminated with TLR2 agonists, suggesting that its pro-inflammatory effect was mediated by a combination of TLR2 and TLR4 activation. The adjuvant effect of LPS and zymosan were absent in mice deficient for the IL-1R-associated kinase 1 (IRAK1), suggesting that IL-1 or TLR signaling through the MYD88-independent pathway was involved<sup>83</sup>.

Although agonists for individual TLRs, especially TLR2 and TLR9, may activate the innate immune responses that drive activation of self-reactive effector T cells, it appears that these responses are optimally driven by signals from two TLRs or a TLR and an NLR or CLR. This may reflect the need for priming and boosting stimuli. For example, production of mature active IL-1 $\beta$  requires priming with a TLR ligand to induce pro-IL-1 $\beta$  expression, and a second signal from a PAMP or DAMP to activate the NLR inflammasome complex and associated caspase-1, which cleaves the pro-IL-1 $\beta$  into its active form. Consistent with this, *M. tuberculosis* can provide both stimuli<sup>8</sup>, which may explain why it is more effective than individual TLR agonists in inducing experimental autoimmunity.

*Modulation T<sub>Reg</sub> cells in autoimmunity.* In addition to activating effector T cells, TLR agonists can indirectly or directly modulate the function of T<sub>Reg</sub> cells. There is evidence that TLR activation can block T<sub>Reg</sub> cell responses, thereby breaking tolerance to self antigens<sup>84-86</sup>. IL-6 production by TLR-activated DCs can inhibit the suppressive function of T<sub>Reg</sub> cells<sup>85</sup>.

Furthermore, IL-1 and IL-6 production by TLR-activated mature DCs is required to reverse T<sub>Reg</sub> cell anergy induced by immature DCs<sup>87</sup>. However, it has also been reported that TLR4 and TLR9 agonists can promote IL-10-secreting T<sub>Reg</sub> cells that inhibit CD8<sup>+</sup> and CD4<sup>+</sup> effector T cells<sup>13,88</sup>. This may be a strategy to limit immunopathology during infection, but may also control sterile inflammation and prevent the development of autoimmunity. The expansion of inducible T<sub>Reg</sub> cells by TLR agonists appears to be mediated through TLR-induced production of IL-10, TGFβ and other immunosuppressive molecules from innate immune cells<sup>13</sup>. Indeed these regulatory mechanisms may prevent the induction of infection-driven autoimmunity in most individuals, but their failure or attenuation, perhaps by environmental factors, may increase the risks of developing autoimmune diseases.

### **Direct modulation of T cells via TLRs**

Whereas it is well established that TLR signaling in innate immune cells can indirectly promote T cell differentiation and expansion through DC maturation and regulatory cytokine production, T cells also express TLRs and evidence is emerging that TLR signaling in CD4<sup>+</sup> and γδ T cells can promote cytokine secretion or regulate their function (FIG 2).

*Modulation of effector CD4<sup>+</sup> T cells.* TLR2 signaling in T<sub>H</sub>1 cells promotes their proliferation and IFNγ production<sup>89</sup>. Moreover, stimulation with CpG restored the proliferation of highly purified naïve T cells from *Pkcθ*<sup>-/-</sup> mice in response to CD3-specific antibody stimulation<sup>90</sup>. Protein kinase C θ (PKCθ) is involved in T cell differentiation and PKCθ-deficient mice are resistant to experimental autoimmune myocarditis (EAM). Susceptibility to EAM was restored by *in vivo* administration of CpG<sup>90</sup>, suggesting that TLR ligands may act directly on T cells to induce autoimmunity *in vivo*. Finally, stimulation of CD4<sup>+</sup> T cells with Pam3Cys induced their proliferation and IL-17 production, which was enhanced by IL-23. By contrast, *Tlr2*<sup>-/-</sup> mice were less susceptible to EAE and loss of TLR2 in T<sub>H</sub>17 cells reduced their ability to transfer EAE<sup>62</sup>.

*Modulation of CD8 T cells.* CD8<sup>+</sup> T cells are found in CNS lesions of patients with multiple sclerosis, where they appear to be involved in myelin degradation and neuronal damage<sup>91</sup>. Although the mechanism of self-reactive CD8<sup>+</sup> T cell activation in autoimmune diseases is unclear, it may involve cross-presentation of autoantigens and bystander stimulation by PAMPs.

It has been demonstrated that CD8<sup>+</sup> T cells express TLRs<sup>92-93</sup>. Stimulation of with TLR2 agonists enhanced CD3-induced proliferation of murine CD8<sup>+</sup> T cells by lowering the threshold for co-stimulatory signals<sup>94</sup>. The TLR9 agonist CpG has been shown to promote IL-8 release by purified human CD8<sup>+</sup> T cells<sup>93</sup>. Thus TLR agonists may promote the activation of self-reactive CD8<sup>+</sup> T cells in certain autoimmune diseases.

*Modulation of  $\gamma\delta$  T cells.* It has been reported that  $\gamma\delta$  T cells express TLR1, TLR2 and the CLR dectin-1, and secrete IL-17 *in vitro* following stimulation with TLR1 and TLR2 or TLR2 and dectin-1 ligands, particularly in the presence of exogenous IL-23<sup>23</sup>. More recently it has been shown that IL-17-producing  $\gamma\delta$  T cells express higher levels of TLR1, TLR2, TLR4, TLR6 and TLR9 than IL-17-secreting CD4 T cells<sup>62</sup>. We have found that highly purified naive  $\gamma\delta$  T cells produce IL-17 in response to IL-1 $\beta$  and IL-23<sup>6</sup>, or to PAMP-activated DCs in the absence of stimulation with CD3- or CD28-specific antibodies. However, highly purified  $\gamma\delta$  T cells failed to respond directly to TLR agonists alone<sup>8</sup> (C. E. Sutton and K. H. G Mills, unpublished observations). It is thus possible that certain subsets of  $\gamma\delta$  T cells are already differentiated in the periphery and produce IL-17 in response to local IL-23<sup>95</sup> or to IL-23 together with IL-1 that are induced by PAMP- or DAMP-activated innate immune cells<sup>6</sup>.

*Modulation of T<sub>Reg</sub> cells.* Murine T<sub>Reg</sub> cells express TLR4, TLR5, TLR7 and TLR8<sup>96</sup> and there is evidence that TLR agonists can bind to them and modulate their function. Synthetic and natural ligands for TLR8 are capable of reversing the suppressive function of human T<sub>Reg</sub> cells<sup>84</sup>. Furthermore, persistent TLR activation can overcome the suppressive effect of T<sub>Reg</sub> cells and abrogate tolerance to tumour cells<sup>86</sup>.

However, there are also reports that TLR agonists can directly enhance T<sub>Reg</sub> cell function. It has been demonstrated that LPS can induce proliferation and enhance the suppressive function of T<sub>Reg</sub> cells<sup>96</sup>. Human T<sub>Reg</sub> cells express TLR5, and stimulation with flagellin and CD3-specific antibody enhances expression of forkhead box P3 (FOXP3) and their suppressive function<sup>97</sup>. The TLR2 ligand Pam<sub>3</sub>Cys induces proliferation of murine T<sub>Reg</sub> cells and although this was associated with a temporary loss of T<sub>Reg</sub> cell suppressive activity, T<sub>Reg</sub> cell function was restored following removal of the TLR agonist<sup>70</sup>. It has also been reported that a TLR2 agonist enhanced proliferation of T<sub>Reg</sub> cells and transiently suppressed FOXP3 expression, but also rendered

CD4<sup>+</sup>CD25<sup>-</sup> effector cells resistant to suppression by T<sub>Reg</sub> cells<sup>98</sup>. Interestingly, it was recently reported that polysaccharide A from the gut commensal *Bacteroides fragilis* binds to TLR2 on CD4<sup>+</sup> T cells promoting IL-10 production and the expansion of T<sub>Reg</sub> cells, which, in turn, inhibit T<sub>H</sub>17 cells. This suggests that TLR2 activation of T cells may promote tolerance, at least in the mucosa<sup>99</sup>. Most of the evidence to date suggests that TLR activation of T cells is primarily indirect and mediated through TLR-induced activation of APCs. However, T cells do express TLRs and binding of TLR agonists to T cells can contribute to their activation, and this applies to both effector and regulatory T cells. Some of the strongest evidence is around TLR2 activation of T<sub>Reg</sub> cells, so from this perspective blocking TLR2 may attenuate T<sub>Reg</sub> function and might therefore be more applicable to treating cancer than autoimmunity. However, studies in knockout mice have suggested that TLR2 may be a good therapeutic target in certain autoimmune diseases. Therefore, further study is required to decipher the relative contribution of direct versus indirect activation of effector and regulatory T cells by TLRs in different autoimmune diseases.

### **TLRs and other PRRs as drug targets for autoimmunity**

The increasing evidence that the innate and adaptive immune responses that mediate autoimmune diseases are driven by PAMP and DAMP binding to TLRs and other PRRs has motivated the assessment of TLR-specific blocking antibodies or inhibitors of TLR signaling in pre-clinical models of inflammatory and autoimmune disorders. Proof-of-principle has been provided by studies with TLR knockout mice. For example, experimental arthritis is attenuated in *Tlr4*<sup>-/-</sup> mice and this is associated with lower T<sub>H</sub>17 responses, suggesting that TLR4 may be a good upstream target for preventing IL-17 production in rheumatoid arthritis<sup>11</sup>. Indeed it has already been shown that a TLR4 antagonist can treat arthritis in two mouse models<sup>69</sup>.

A synthetic TLR4 antagonist, a lipid A mimetic CRX-526, has been shown to inhibit the development of DSS-induced colitis or colitis in multidrug resistant gene1a-deficient mice<sup>100</sup>. Similarly, a rat TLR4-specific monoclonal antibody, 1A6, reduced inflammation in DSS-induced colitis in mice<sup>101</sup>. A humanized TLR4-specific antibody has been developed by NovImmune and is currently undergoing preclinical evaluation as a potential therapy for inflammatory bowel disease. A lipid A antagonist, Eritoran<sup>TM</sup> (E5564) from Eisai Pharmaceutical, which interferes with the formation of the TLR4/MD-2/LPS complex is an effective inhibitor of LPS-induced



shock in mice<sup>102</sup>, but did not meet its primary endpoint in patients with severe sepsis in a phase III clinical trial (<http://www.eisai.com/news/enews201108pdf.pdf>).

TLR2 and nucleic acid binding TLRs are also major targets for a number of inflammatory and autoimmune diseases. EAE is attenuated in TLR2<sup>-/-</sup> mice, suggesting that the TLR2 signaling pathway may be a drug target for the treatment or prevention of multiple sclerosis<sup>62</sup>. An antibody to TLR2 developed by Opsona Therapeutics has been shown to be effective in a murine model of myocardial ischemia reperfusion injury<sup>103</sup>, and a humanized version of the antibody (OPN-305) has recently entered phase I clinical trials.

Antagonists of TLR7, TLR8 or TLR9, including CpG 52364 (Pfizer), IRS-954 (Dynavax) and IM 3100 (Idera) have also shown efficacy in mouse models of SLE and other autoimmune diseases and have recently entered clinical trials<sup>5</sup>. These studies provide evidence that inhibition of TLR signaling in response to endogenous DAMPs is a safe therapeutic approach in humans and has the potential to treat inflammatory diseases that involve sterile inflammation.

**Conclusions and perspectives** Epidemiological studies have linked infection with the development or exacerbation of autoimmune disease. Furthermore, enhanced expression of TLRs and their agonists in diseased tissues provides indirect evidence that innate immune responses mediated through TLRs and other PRRs are involved in the development of autoimmune inflammation. More convincing evidence is provided from mouse models where defects in expression or signalling via TLRs are associated with attenuation of the innate and adaptive immune responses that mediate autoimmunity. Conversely, administration of exogenous TLR agonists to mice can promote or exacerbate autoimmunity.

In the absence of infection, sterile inflammation can be initiated by the release of DAMPs or alarmins from necrotic cells (FIG. 4). It has recently been suggested that pathogens and commensal microorganism-derived MAMPs bind to PRRs, but do not by themselves trigger host immune responses<sup>104</sup>. In order to overcome homeostatic immune regulation, innate immune cells require activation with MAMPs together with DAMPs released from host cells following injury or pathogen-induced cell death<sup>104</sup>. This is consistent with the association between infections and autoimmunity, but it is not compatible with the view that commensal bacteria alone can precipitate autoimmune diseases, unless the second signal for innate activation is provided by injury-induced cell death.

Unless there is preferential expression of individual TLRs or their agonists in the affected tissues, there is likely to be redundancy between different TLRs, and between TLRs and other PRRs. Nevertheless, there is already evidence from mouse models that blocking a single TLR, especially TLR2, TLR4 or TLR9, may be sufficient for the inhibition of the innate responses that promote inflammation and autoimmunity. However, this effect may be disease-dependent as, for example, TLR4 blocking ameliorates arthritis and TLR2 inhibition dampens EAE.

Evidence from phase III clinical trials indicates that drugs that broadly inhibit innate immune responses or completely block the induction or function of effector T cells are likely to significantly compromise host protective immunity. For example, psoriasis patients treated with an IL-12p40-specific antibody, ustekinumab<sup>105</sup> (which blocks T<sub>H</sub>1 and T<sub>H</sub>17 responses), or gouty arthritis patients treated with an IL-1 $\beta$ -specific blocking antibody, canakinumab (FDA Briefing document

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2011;

<http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/ArthritisDrugsAdvisoryCommittee/UCM259596.pdf>) had a higher incidence of infections, including serious infections, than controls. Although these new drugs have significantly improved efficacy over existing therapies, more selective inhibition of specific pathways involved in inflammation in a particular tissue or specific to a particular disease may not involve the same risks. If a single TLR is proven to have a dominant role in a particular autoimmune disease, it is possible that targeting TLRs, especially an extracellular TLR with an inhibitory antibody, may provide a safe and effective therapeutic approach for certain inflammatory and T-cell mediated autoimmune diseases.

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**Box 1. Toll-like Receptors – sensors of microbial molecules and danger signals.**

Human Toll-like receptors (TLRs) are evolutionary conserved homologs of *Drosophila* Toll protein involved in embryonic development in *Drosophila*. TLR are expressed on the cell surface or intracellularly in many cell types, especially cells of the innate immune system, where they function to respond to infection or damage. TLRs are members of the pathogen recognition receptor (PPR) family that sense conserved exogenous molecules from pathogens, called pathogen-associated molecular patterns (PAMPs). However they also bind molecules from commensal bacteria and endogenous damage-associated molecular patterns (DAMPs) from dead and dying cells. Ligands for TLRs include proteins, lipopeptides and nucleic acids from bacteria, viruses, fungi, parasites, human cells and synthetic molecules.

**Examples of pathogen-derived, endogenous and synthetic TLR agonists**

TLR	Expressed	Pathogen-derived agonist	Endogenous agonists	Synthetic agonists
TLR1/2	Extracellular	Bacteria: peptidoglycan, lipoproteins, LTA Fungi: Zymosan		Pam <sub>3</sub> CSK <sub>4</sub>
TLR2/6	Extracellular	Bacteria: lipoproteins	veriscan	MALP-2
TLR3	Intracellular	Virus: dsRNA	mRNA	Poly I:C
TLR4	Extracellular	Bacteria: LPS Virus: RSV fusion protein Fungi: mannan Protozoa: glycoinositolphospholipids	Saturated fatty acids, $\beta$ -defensins, oxLDL and amyloid- $\beta$ <sup>1</sup>	Lipid A derivatives
TLR5	Extracellular	Bacteria: flagellin		
TLR7/8	Intracellular	Virus: ssRNA	Self RNA	Imiquimod, R-848
TLR9	Intracellular	Bacteria: CpG DNA Virus: CpG DNA Protozoa: CpG DNA, hemozoin	Self DNA	CpG-ODN
TLR11	Extracellular	Uropathogenic bacteria Protozoa: profilin-like molecule		

<sup>1</sup>Oxidized LDL and amyloid- $\beta$  bind CD36 and a TLR4-TLR6 heterodimer. CpG-ODN, CpG-containing oligodeoxynucleotides; dsRNA, double stranded RNA, oxLDL, oxidized low-density lipoprotein, LTA, lipoteichoic acid; MALP-2, mycoplasma macrophage-activating lipopeptide-2, RSV, respiratory syncytial virus; ssRNA, single stranded RNA; TLR, Toll-like receptor.

## **Box 2. Role of T<sub>H</sub>17 and T<sub>H</sub>1 cells in autoimmunity**

Prior to the discovery of T helper 17 (T<sub>H</sub>17) cells, it was thought that T<sub>H</sub>1 cells were the primary pathogenic cell population in T cell-mediated autoimmune diseases. However, mice deficient in interferon- $\gamma$  (IFN $\gamma$ ) or interleukin-12 (IL-12) were found to have increased susceptibility to experimental autoimmune encephalitis (EAE) or collagen-induced arthritis (CIA)<sup>106-108</sup>. It was subsequently shown that *Il17*<sup>-/-</sup> mice have reduced susceptibility to EAE<sup>109</sup>, and that myelin-specific T<sub>H</sub>17 cells from mice with EAE expanded *in vitro* with IL-23 can transfer EAE to naïve mice<sup>110</sup>. Furthermore, mice defective in IL-23, which is required for expansion of T<sub>H</sub>17 cells, are resistant to the induction of EAE and CIA<sup>106,111</sup>. Although there is considerable plasticity in T cell subtypes, it now appears that T<sub>H</sub>17 cells are a functionally distinct population of CD4<sup>+</sup> T cells that are pathogenic in many autoimmune diseases<sup>2</sup>.

There is evidence that IFN $\gamma$  may suppress IL-17 production by T<sub>H</sub>17 cells<sup>21</sup>. This provided an explanation for the failure of IFN $\gamma$ -specific blocking antibody therapy in multiple sclerosis and for the more severe EAE in *Ifng*<sup>-/-</sup> mice<sup>108</sup>. However, there is evidence from cell transfer studies that both T<sub>H</sub>1 and T<sub>H</sub>17 cell subsets have a pathogenic role in autoimmune inflammation, perhaps acting at a different stage of disease or recruiting different effector cells<sup>112</sup>. It has also been reported that transfer of T<sub>H</sub>1 cells induced experimental autoimmune uveitis (EAU) in naïve recipients, which was not inhibited by treatment with IL-17-specific blocking antibodies<sup>113</sup>. Furthermore, IFN $\gamma$  exacerbates disease in patients with multiple sclerosis<sup>114</sup> and IFN $\gamma$  promotes IL-1 $\beta$  production by human macrophages<sup>115</sup>, which enhances IL-17 production by T<sub>H</sub>17 cells. Finally, IL-17 and IFN $\gamma$  double positive CD4<sup>+</sup> T cells have been found in the gut of patients with Crohn's disease<sup>116</sup>. It appears that T<sub>H</sub>17 and T<sub>H</sub>1 cells or IL-17<sup>+</sup>IFN $\gamma$ <sup>+</sup>CD4<sup>+</sup>T cells may have pathogenic roles in autoimmune and chronic inflammatory diseases, but their precise are still not fully resolved.

**Table 1. Effect of TLR deficiency or inhibition on autoimmune diseases in animal models**

Model	TLR	Inter-vention	Innate response	T cell response	Disease	Ref
<b>EAE</b>						
MOG +CFA	MYD88	KO	↓IL-6, IL-23	↓IL-17	Resistant	61
MOG+ CFA	TLR4/ TLR9	KO	↑IL-6, IL-23	↑IL-17	Enhanced	61
MOG+ CFA	MYD88	KO		↓IFN $\gamma$	Resistant	10
MOG+ CFA	TLR9	KO			Reduced	10
MOG+ CFA	TLR2	KO			normal	10
MOG+ CFA	TLR2	KO		↓IL-17	Reduced	62
T cell transfer	TLR2	KO T cell		↓IL-17, IFN $\gamma$	Abrogated	62
<b>Arthritis</b>						
Zymosan	TLR2	KO			Reduced	67
IL-1Ra <sup>-/-</sup>	TLR2	KO		↑IFN- $\gamma$ , IL-17 ↓Foxp3	Enhanced	11
IL-1Ra <sup>-/-</sup>	TLR4	KO		↓IL-17	Reduced	11
IL-1Ra <sup>-/-</sup>	TLR9	KO			No effect	11
IL-1Ra <sup>-/-</sup>	TLR4	Antagonist			Reduced	69
CIA	TLR4	Antagonist	↓IL-1 $\beta$		Reduced	69
<b>EAU</b>						
IRBP+ CFA	MYD88	KO		↓IFN- $\gamma$ , TNF- $\alpha$	Resistant	71
IRBP+ CFA	TLR2, 3,4,9,	KO			normal	71,117

CIA, collagen-induced arthritis; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; IRBP, interphotoreceptor retinoid binding protein; KO, knock out, MOG, myelin oligodendrocyte glycoprotein; TLR, Toll-like receptor

**Table 2. Induction or modulation of autoimmune diseases by TLR agonists in animal models.**

Model	TLR	Agonist	Intervention	Innate response	T cell response	Disease	Ref.
<b>EAE</b>							
MOG+ CFA	TLR+ dectin-1	Zymosan	MOG + IFA + zymosan	↑IL-6, IL-23,	↑IL-17	Induces (transient)	82
MOG+ CFA	TLR4/ TLR2	LPS/ Zymosan	MOG + IFA + TLR agonist s.c.			Induces	83
MOG+ CFA	TLR4	LPS	MOG + IFA + LPS		↑IL-17, IFN- $\gamma$	Mild	62
B10.S PLP Tg	TLR9	CpG	PLP + IFA + CpG			Enhances	81
PLP+ CFA	TLR9	CpG	CpG, IFA + PLP immunization		↑IFN- $\gamma$ , low IL-17	None	80
T cell transfer	TLR9	CpG	CpG+PLP immunization			Mild	80
LN cell transfer	TLR9	CpG	CpG activation of LN cells from PLP +IFA primed mice	↑IL-12	↑IFN- $\gamma$	Induces	63
PLP+ CFA	TLR3	Poly I:C	i.p. days 5, 7, 9	↑IFN- $\beta$		Reduces	65
<b>Arthritis</b>							
Adjuvant arthritis	TLR9	ISS-ODN	Injection into joint		↑IFN- $\gamma$	Induces	68
	TLR2	PGN	Immunization			Induces	68
CIA	TLR4	LPS	Type II collagen + LPS i.p.	↑IL-12, IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$		Induces	118
Cell transfer from pristine-primed	TLR 7,9	Nucleic acids	Spleen cells stimulation with heterogenous ribonucleoproteins	↑IL-6, TNF- $\alpha$		Induces	60
Cell transfer from pristine-primed	TLR3 TLR9 TLR7	polyI:C, ODN, imiquimod	Spleen cells stimulation with TLR agonists			Induces	60

IL-1ra <sup>-/-</sup>	TLR4	LPS	i.p. injection			Enhanced	<sup>11</sup>
IL-1ra <sup>-/-</sup>	TLR2	Pam3Cys	i.p. injection			Enhanced	<sup>11</sup>
<b>Other</b>							
Antibody-associated vasculitis	TLR2	Pam <sub>3</sub> CSK <sub>4</sub>	Immunization myeloperoxidase + TLR agonist		↑IL-17	Induces	<sup>73</sup>
Antibody-associated vasculitis	TLR9	CpG	Immunization myeloperoxidase + TLR agonist		↑IFN-γ	Induces	<sup>73</sup>
Psoriasis	TLR7/ TLR8	Imiquimod	Topical application	IL-23	↑IL-17	Induces	<sup>74</sup>
EAU	TLR4	LPS	IRBP+CFA+LPS	↑IL-6, IL-1β, TNF-α, IFN-γ,	↑IFN-γ, IL-17	Enhances	<sup>117</sup>
Myocarditis	TLR9	CpG	CpG i.p. PKC-θ <sup>-/-</sup> mice before/after immunization with myosin+CFA		↑IL-17, TNF-α	Induces	<sup>90</sup>

CIA, collagen-induced arthritis; CFA, complete Freund's adjuvant; CpG-ODN, CpG-containing oligodeoxynucleotides; IFA, incomplete Freund's adjuvant; DSS, dextran sulfate sodium; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; KO, knock out, LPS, i.g. intra-gastric; ISS-ODN immunostimulatory sequence-containing oligodeoxynucleotides; i.p., intra-peritoneal; i.r., intra-rectal; IRBP, interphotoreceptor retinoid binding protein, lipopolysaccharide; LTA, lipoteichoic acid; LN, lymph node, MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; s.c., subcutaneous; Tg, transgenic; TLR, Toll-like receptor; TNBS, trinitrobenzene sulfonic acid.

## Figure Legends

**Figure 1. Bacteria-induced signaling in innate immune cells leading to T cell-promoting cytokine production.** With the exception of Toll-like receptor 3 (TLR3), each of the TLRs employ myeloid differentiation primary response protein 88 (MYD88) as an adapter protein. In addition, TLR4 and TLR2 utilize MYD88-adapter-like (MAL), whereas Toll-interleukin-1 resistance (TIR) domain-containing adaptor-inducing interferon- $\beta$  (TRIF) and TRIF-related adapter molecule (TRAM) are required for TLR3- and TLR4-mediated type I interferon (IFN) production. The figure illustrates some of the signalling pathways induced in a dendritic cell in response to a Gram-negative bacterium. Lipopolysaccharide (LPS) from the bacteria binds TLR4 in complex with MD2 and this complex initiates signalling by recruiting the adapter proteins MYD88, MAL, TRIF and TRAM. MYD88 associates with interleukin (IL)-1 receptor associated kinase 1 (IRAK1) and IRAK4 and recruits tumor necrosis (TNF) receptor-associated factor 6 (TRAF6). This complex recruits transforming growth factor- $\beta$ -activated kinase 1 (TAK1) leading to phosphorylation of I $\kappa$ B and activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and consequent transcription of a range of genes coding for pro-inflammatory cytokines, including TNF $\alpha$ , interleukin 6 (IL-6), pro-IL-1 $\beta$  and pro-IL-18. In addition, TLR agonists activate the interferon regulatory factor (IRF) pathways, leading to IL-12p35 and type I IFN production. TLR-induced activation of TAK-1 also results in phosphorylation of mitogen activated protein kinases (MAPK), including p38 and ERK. Phosphorylation of ERK promotes transcription of IL-23p19 and IL-23 production. Phosphorylation of p38 activates cAMP response element-binding (CREB) leading to IL-10 production. Bacterial molecules, also bind intracellular nucleotide-binding oligomerization domain (NOD)-like receptor (NLRs), such as NOD-1 or NOD-2, which activate NF- $\kappa$ B and MAP kinases. Activation of NLRs, such as NLRP3, the associated inflammasome complex and caspase-1 facilitates the processing of pro-IL-1 $\beta$  and pro-IL-18 into mature cytokines. IL-12 promotes T helper 1 (T<sub>H</sub>1) cell differentiation, IL-10 induces regulatory T (T<sub>Reg</sub>) cells and IL-1, IL-6, IL-18 and IL-23 promote T<sub>H</sub>17 cell differentiation or expansion.

**Figure 2. Effector cells and drug targets in autoimmunity.** Toll-like receptor (TLR) agonists activate innate immune cells, such as dendritic cells (DC), promoting release of inflammatory cytokines, including interleukin 12 (IL-12), which induces T<sub>H</sub>1 differentiation, and IL-6, which



works with transforming growth factor- $\beta$  (TGF- $\beta$ ) to promote differentiation of  $T_H17$  cells.  $T_H17$  cells are further activated by IL-1 and IL-23, but also by IL-17 and IL-21 produced early in the immune response by  $\gamma\delta$  T cells and other innate lymphoid cells (ILC). Interferon- $\gamma$  (IFN $\gamma$ ) produced by  $T_H1$  cells activates macrophages, promoting release of inflammatory mediators, such as IL-1 $\beta$ , tumor necrosis factor (TNF), matrix metalloproteinases (MMPs) and reactive oxygen species (ROS), which mediate tissue damage. IL-17 induces chemokine production, especially macrophage inflammatory protein (MIP)-2/IL-8, which recruits neutrophils to the site of inflammation. Finally, granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by both  $T_H1$  and  $T_H17$  cells activates DC and macrophages and appears to be essential for the development of autoimmunity. Biological response modifying drugs that are in use or in pre-clinical or clinical evaluation target many of the cytokines or the intracellular signaling molecules in innate cells or T cells.

**Figure 3. Indirect and direct activation of T cells by TLR agonists.** Pathogen-associated molecular patterns (PAMPs), bind to pathogen recognition receptors (PRRs), including Toll-like receptor (TLR), but also NOD-like receptors (NLR), C-type lectin receptors (CLR), and RIG-I-like receptors (RLR). TLR activation promotes co-stimulatory molecule (CD80 and CD86) and major histocompatibility complex (MHC) expression (maturation) of immature dendritic cells (iDC) making them capable of activating naïve T cells. TLR-activated DC and macrophages (Mac) act as a source of immunomodulatory cytokines for directing differentiation of T helper 1 ( $T_H1$ ) or  $T_H17$  cells. DC and macrophage-derived interleukin 12 (IL-12) promotes differentiation of naïve T cells into  $T_H1$  cells either directly or via interferon- $\gamma$  (IFN $\gamma$ ) production from natural killer (NK) cells. Activated  $T_H1$  cells secrete further IFN $\gamma$ . IL-6 and transforming growth factor- $\beta$  (TGF $\beta$ ) promote differentiation of naïve T cells into  $T_H17$  cells. IL-1 $\beta$ , IL-1 $\alpha$  or IL-18 in synergy with IL-23 activate  $T_H17$  cells, inducing their proliferation and IL-17 secretion.  $\gamma\delta$  T cells also secrete IL-17, IL-21 and IL-22 following stimulation with IL-1 $\beta$ , IL-1 $\alpha$  or IL-18 with IL-23 released by PAMP activated DC or macrophages; this does not require association between the DC and  $\gamma\delta$  T cell or stimulation through the T cell receptor engagement. IL-21 and IL-17 produced by  $\gamma\delta$  T cells stimulate further differentiation of  $T_H17$  cells. There is also some evidence that TLR agonists may act directly on  $CD4^+$  T cells and  $\gamma\delta$  T cells, promoting

proliferation and cytokine production. However, in the case of T<sub>H</sub>17 cells, this appears to require co-operation with IL-23.

**Figure 4. Infection, PAMP- and DAMP-mediated activation of inflammatory responses.**

Optimum activation of inflammatory responses in innate immune cells, such as dendritic cells (DC) and macrophages (MØ) appears to require two signals, which can be derived from exogenous microbe-derived or endogenous host-derived molecules. For example, interleukin (IL)-1 $\beta$  or IL-18 production requires priming of the cell through a Toll-like receptor (TLR) to promote production of the pro-form of the cytokines and the processing of these cytokines is mediated by caspase-1 induced by a second signal provided by a pathogen-associated molecular patterns (PAMP) or a damage-associated molecular pattern (DAMP). Three, non mutually exclusive, models of inflammatory responses involving PRRs are illustrated. a) PAMPs from pathogens or commensal bacteria including TLR agonists, but also ligands for NOD-like receptors (NLR), C-type lectin receptors (CLR) and inflammasome activators that mediate induction of inflammatory cytokines in response to infection. b) DAMPs or alarmins released from dead or dying cells include TLR agonists and inflammasome activators, such as ATP, that mediate induction of inflammatory cytokine production from tissue in response to injury or stress. IL-1 $\alpha$  which is released from necrotic cells is also a key alarmin driving sterile inflammation. c). Microbe-associated molecular patterns (MAMPs) from pathogens or commensal bacteria including TLR-, NLR-, and CLR- agonists and inflammasome activators activate innate, in combination with DAMPs released from pathogen-infected or damaged host cells mediate induction of inflammatory cytokines in response to pathogens.

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## Glossary

**Microbe-associated molecular patterns (MAMPs):** Molecular patterns that are found in pathogens and commensal microorganisms, but not in mammalian cells.

**Damage-associated molecular pattern molecules (DAMPs):** Molecular patterns that are found in mammalian cells and are released as a result of cellular stress, cellular damage and non-physiological cell death. DAMPs are released from the degraded stroma (for example, hyaluronate), from the nucleus (for example, high-mobility group box 1 protein, HMGB1) and from the cytosol (for example, ATP, uric acid, S100 calcium-binding proteins and heat-shock proteins). Such DAMPs are thought to elicit local inflammatory reactions

**Pathogen-associated molecular patterns (PAMPs):** Molecular patterns that are found in pathogens but not in mammalian cells. Examples include terminally mannosylated and polymannosylated compounds (which bind the mannose receptor) and various microbial components, such as bacterial lipopolysaccharide, hypomethylated DNA, flagellin and double-stranded RNA (all of which bind Toll-like receptors).

**Alarmins:** Endogenous molecules rapidly released by dead or dying host cells in response to injury or infection that activate innate immune responses, recruiting immune cells and activating dendritic cells to prime T cell responses. They are distinct from but elicit similar responses to those induced by exogenous pathogen-associated molecular patterns (PAMPs). Examples of alarmins include HMGB-1 and heat shock proteins that can bind to TLRs and other PRRs, but also inflammatory cytokines, such as IL-1 $\alpha$ , released from necrotic cells.

**Sterile inflammation:** Inflammation, characterized by leucocyte recruitment, not involving infection but precipitated by activation of innate immune cells with endogenous mediators released from host cells (alarmins or danger-associated molecular patterns; DAMPs) following tissue injury and necrotic cell death.

**NLRP3 inflammasome:** Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing (NLRP) are a family of cytoplasmic proteins that can form high molecular weight signaling complexes, termed inflammasomes. The NLRP3 inflammasome, contains the adaptor molecule, apoptosis-associated speck-like protein containing a CARD (ASC) and recruits pro-caspase-1, leading to its autocatalytic activation. Active caspase-1 catalyzes the cleavage of pro-IL-1 $\beta$ , pro-IL-18 and pro-IL-33, resulting in the secretion of biologically active forms of these cytokines. The NLRP3 inflammasome mediates innate immune responses to exogenous bacteria, and environmental molecules, such as alum and asbestos and endogenous molecules, such as ATP and amyloid- $\beta$ .

**Lymphoid tissue inducer-like (LTi) cell:** Innate lymphoid cells that constitutively express CD4 ROR $\gamma$ t, IL-23 receptor, aryl hydrocarbon receptor, and CCR6, but not CD3, NK1.1, CD11b, Gr1, CD11c or B220, found in the spleen and lamina propria and are an early source of IL-17 and IL-22 in host defence and an important source of IL-22 in intestinal homeostasis.

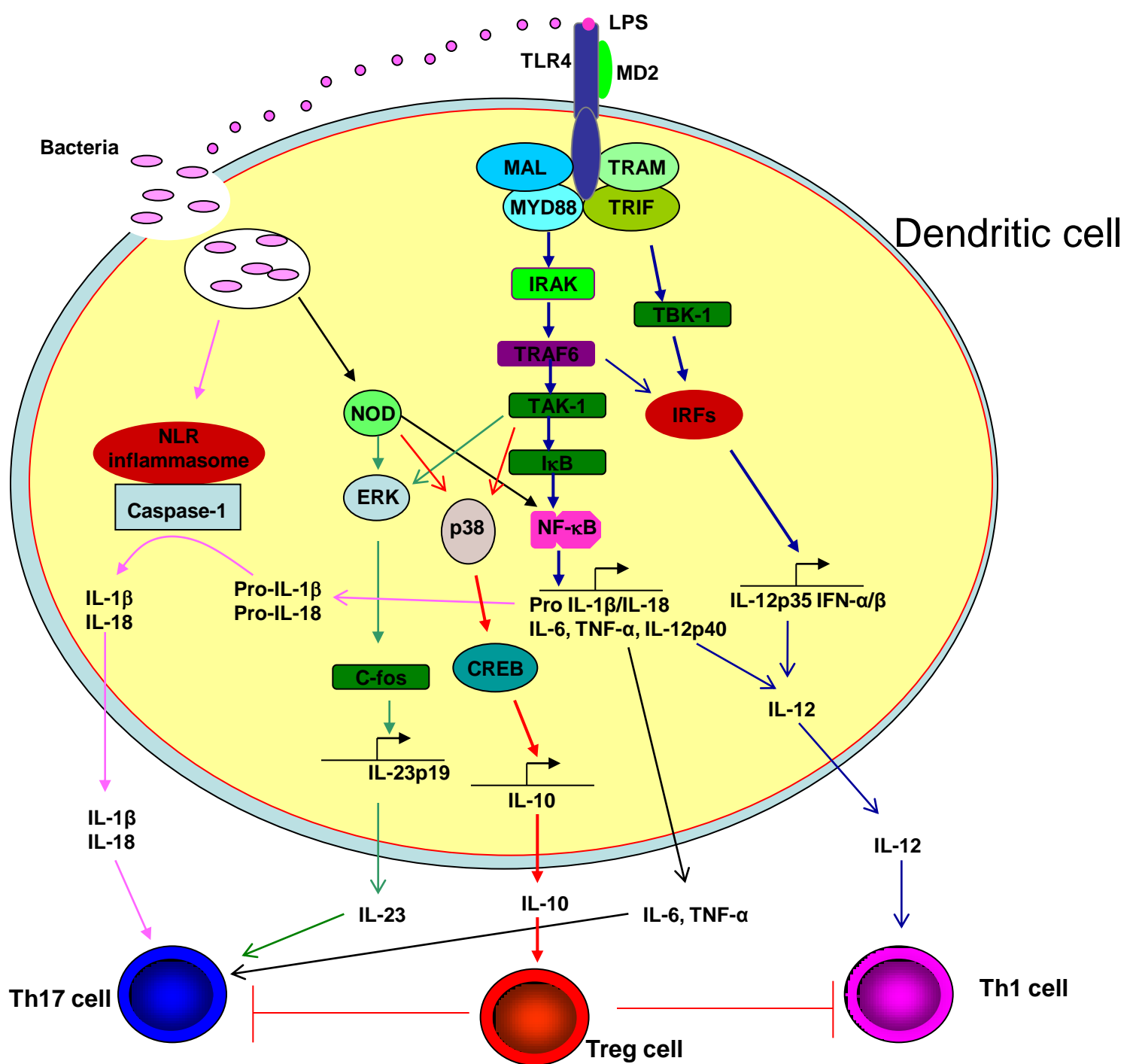
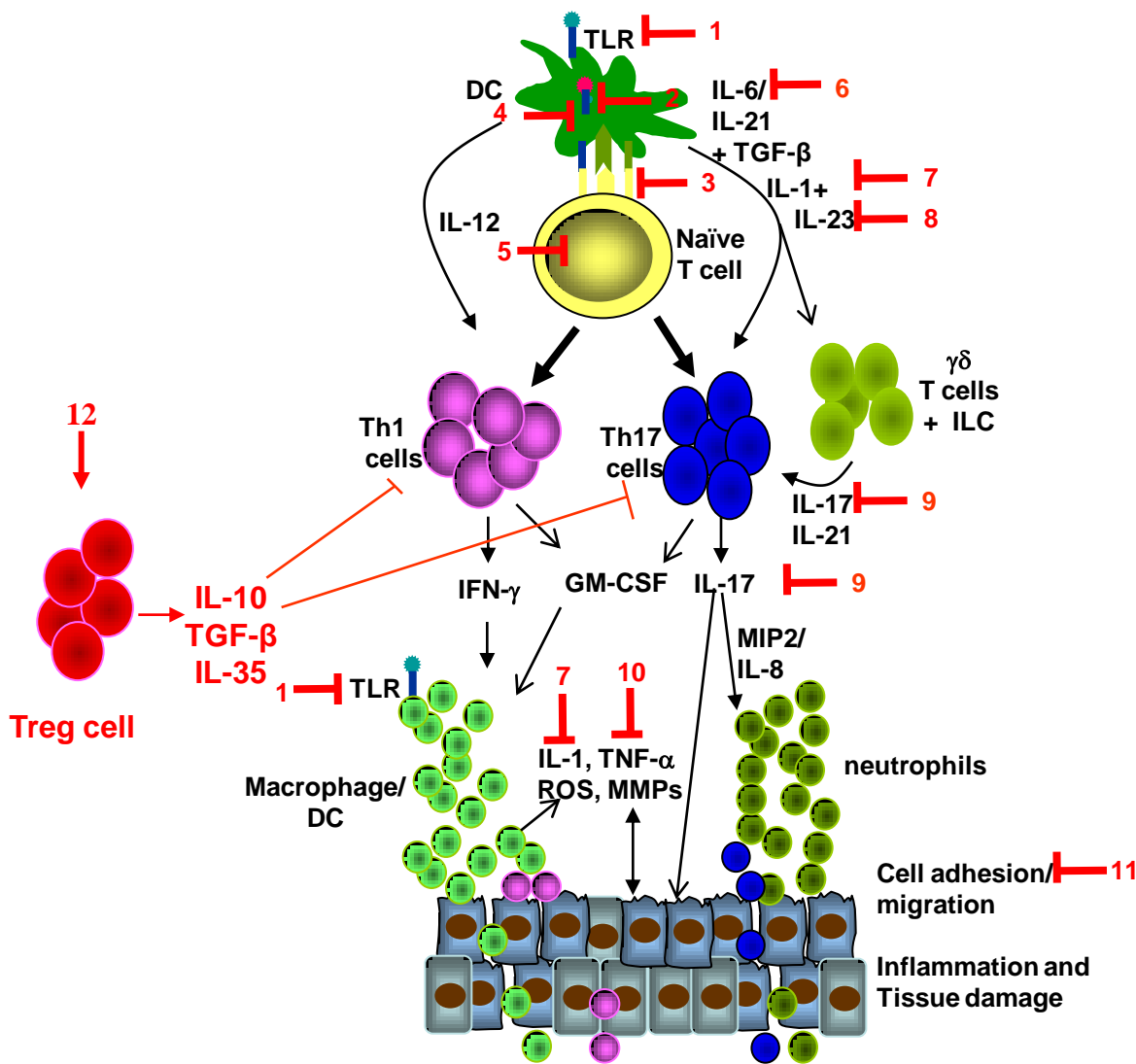


Fig. 1



**Targets for biological response modifying drugs in autoimmune diseases**

1. Antibodies/antagonists to TLRs (e.g. OPN305)
2. Inflammasome/ caspase-1 inhibitors (e.g. Pralnacasan, VX765)
3. Antibodies/Inhibitors of Ag presentation or T cell activation (e.g. CAMPATH-1, Abatacept)
4. MEK kinase inhibitors (e.g. ARRY-438162)
5. JAK kinase inhibitors (e.g. CP-690550, INCB018424)
6. Antibodies to IL-6/IL-6R (e.g. Tocilizumab)
7. Antibodies / antagonists to IL-1β (e.g. Anakinra, Riloncept, Canakinumab, XOMA052)
8. Antibodies to IL-23p40 /IL-12p19 (e.g. CNTO-1275 (ustekinumab), ABT-874, SCH 900222, STA-5326)
9. Antibodies / inhibitors of IL-17/IL-17R (e.g. AIN457, LY2439821, AMG-827)
10. Antibodies / antagonists to TNF-α (e.g. Infliximab, Enberel, Humira)
11. Inhibitors of immune cell egress from lymph nodes or cell migration, adhesion or sequestration (e.g. Tysabrai, Fingolimod)
12. T reg cell induction or transfer (pre-clinical and phase I trials)

Fig. 2

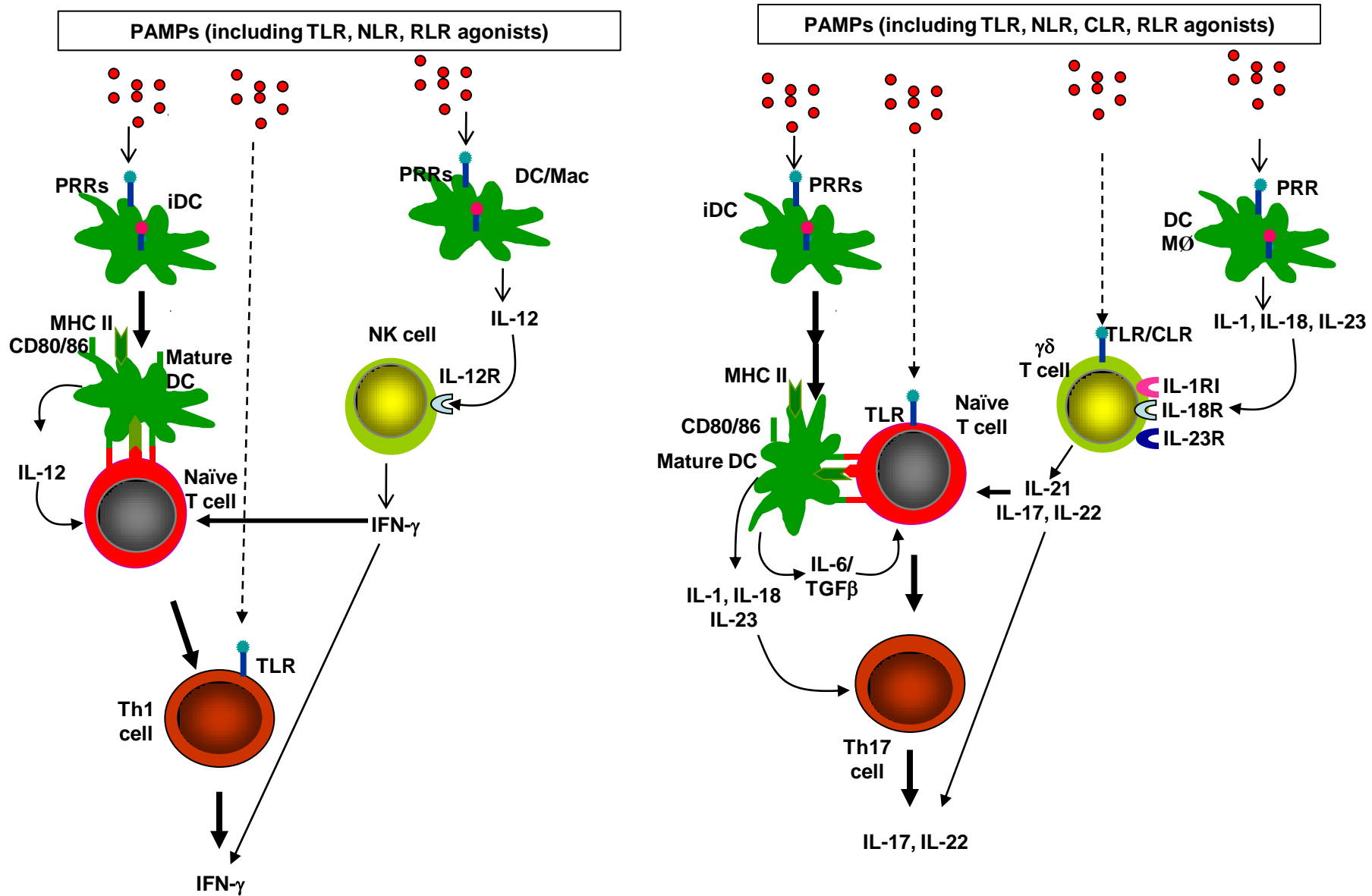
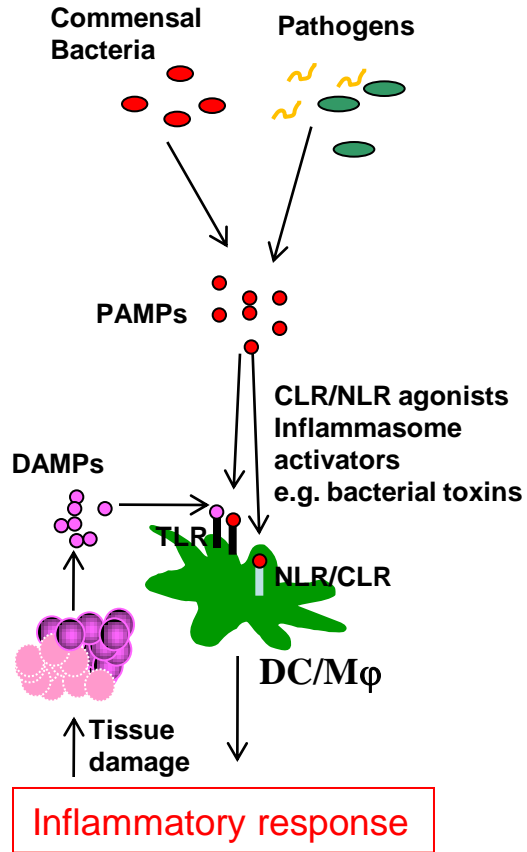
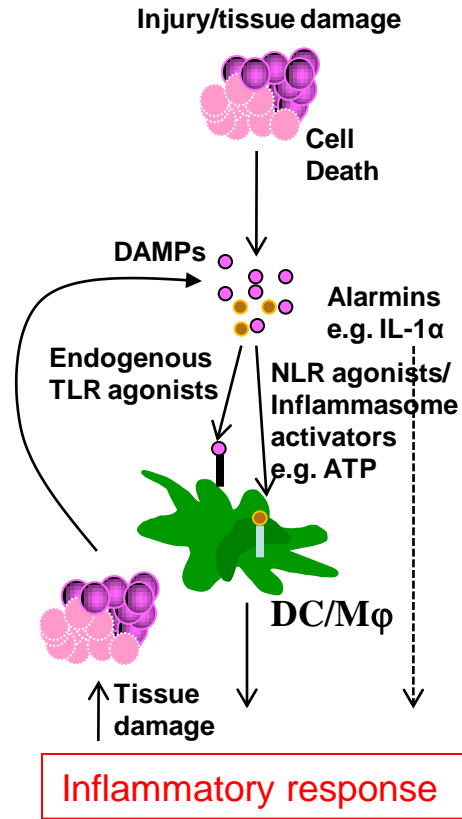


Fig. 3

### A. Infection/PAMP



### B. Damage/DAMP



### C. Microbe/MAMP and danger/DAMP

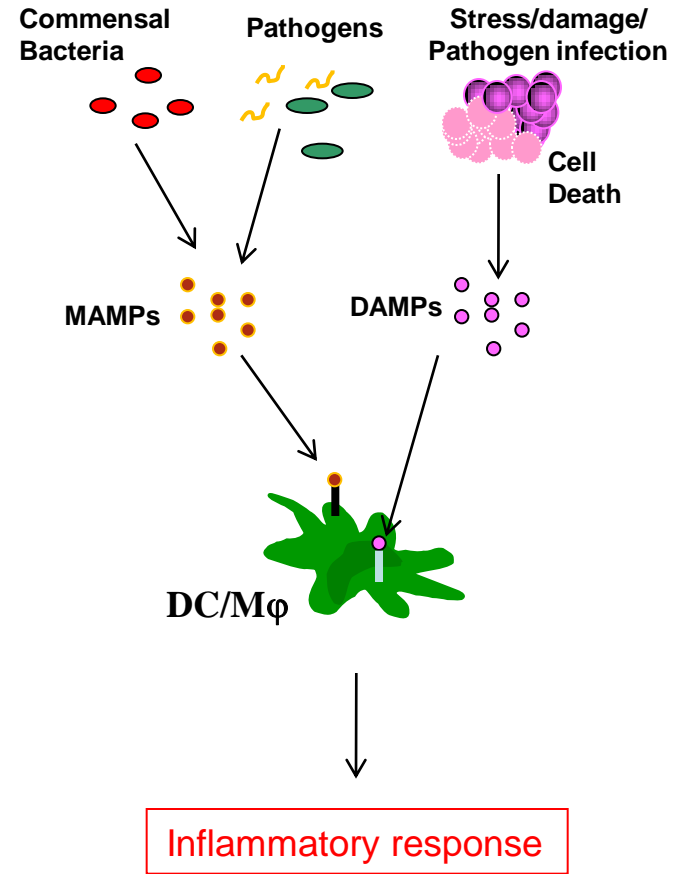


Fig. 4