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Toll-like receptor signaling in primary immune deficiencies

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Abstract

Toll-like receptors (TLRs) recognize common microbial or host-derived macromolecules and have important roles in early activation of the immune system. Patients with primary immune deficiencies (PIDs) affecting TLR signaling can elucidate the importance of these proteins to the human immune system. Defects in interleukin-1 receptor-associated kinase (IRAK)-4 and myeloid differentiation factor 88 (MyD88) lead to susceptibility to infections with bacteria, while mutations in nuclear factor-κB essential modulator (NEMO) and other downstream mediators generally induce broader susceptibility to bacteria, viruses, and fungi. In contrast, TLR3 signaling defects are specific for susceptibility to herpes simplex virus type 1 (HSV-1) encephalitis. Other PIDs induce functional alterations of TLR signaling pathways, such as common variable immunodeficiency in which plasmacytoid dendritic cell (pDC) defects enhance defective responses of B cells to shared TLR agonists. Dampening of TLR responses is seen for TLRs 2 and 4 in chronic granulomatous disease (CGD) and X-linked agammaglobulinemia (XLA). Enhanced TLR responses, meanwhile, are seen for TLRs 5 and 9 in CGD, TLRs 4, 7/8, and 9 in XLA, TLRs 2 and 4 in hyper IgE syndrome, and for most TLRs in adenosine deaminase deficiency.

Keywords

Toll-like receptors; primary immune deficiency; human immunology; infection

Introduction

Toll-like receptors (TLRs) are expressed broadly by immune and non-immune cells and recognize both microbial and host-derived macromolecules, leading to early detection of infection and other potential dangers.[1] Cells bearing these receptors include B and T cells, dendritic, endothelial and epithelial cells, fibroblasts, and macrophages.[2] TLRs are germline-encoded and designed to bind conserved microbial structures, including cell wall subunits or nucleic acids and host inflammatory mediators. This broad reactively contrasts with the narrow antigen specificity of B and T cell receptors which are shaped through genetic rearrangement.[3] TLR interactions with ligands stimulate innate host defense mechanisms promoting secretion of antimicrobial peptides and cytokines as well as

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expression of adhesion molecules; thus TLRs play a role in the initiation of long-lived adaptive immune responses by promoting antigen-presentation.[4–6] The identification of PIDs impairing TLR signaling has contributed greatly to the understanding of the function of these receptors in humans.[7] By focusing on PIDs due to genetic defects that directly or indirectly impair TLR signaling, and considering information gleaned from associations of TLR polymorphisms with infection, this review examines current understanding of how TLR and related signaling defects lead to susceptibility to infection in humans.

TLRs bridge innate and adaptive immunity

The discovery of TLRs and the identification of these receptors as sensory immunomodulators has been an important advancement in the field of immunology. The novel role of TLRs may explain how long term immunity can be achieved despite the fact that foreign antigen alone may be inadequate to illicit a lasting immune response.[8] In recognition of this, Hoffman and Beutler, who first described TLRs in fruit flies, were awarded the 2011 Nobel Prize in Physiology or Medicine along with Ralph Steinman, who discovered the cell type that utilizes TLRs to initiate adaptive immunity, the dendritic cell (DC). Humans have 10 TLRs, 6 of which are extracellular (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) while 4 are expressed exclusively intracellularly (TLR3, TLR7, TLR8, and TLR9). These receptors have differing biologies in the detection of diverse pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs).[9, 10]

A number of microbial and endogenous molecules have been identified as TLR ligands. TLR1 and TLR6 work in concert with TLR2 to detect di- and triacylated lipoproteins from *Mycoplasma* and other bacteria.[11, 12] Numerous agonists for TLR2 have been reported, including lipoteichoic acid (Gram-positive bacteria), lipoarabinomannan (*Mycobacteria*), zymosan (fungi), as well as a number of envelop antigens from viruses.[13–16] TLR4 is well known for its role as a sensor of lipopolysaccharide from Gram-negative bacteria, and has also been shown to bind heat-shock protein 60, the fusion protein of respiratory syncytial virus, and fungal mannan.[17–20] A major agonist for TLR5 is flagellin, which is conserved among many microbial species.[21] The intracellular TLRs TLR3, TLR7/8, and TLR9 sense double-stranded RNA, single-stranded RNA, and unmethylated (microbial) DNA oligonucleotides respectively.[22–25] The ligand for TLR10 has not yet been identified, though roles for this receptor in the recognition of viral infection and in inflammatory regulation have been suggested.[26, 27] Much of the work elucidating TLR agonists has utilized mouse models or cell lines over-expressing human proteins, which is an important caveat when considering the *in vivo* relevance to human immunity. Thus, PIDs impairing TLR signaling provide insightful models to corroborate findings of TLR function derived from experimental systems.

Overview of TLR function and NF-κB signaling

With the exception of TLR3 and specific modes of TLR4 signaling that utilize Toll/IL-1 receptor (TIR) domain-containing adaptor–inducing interferon-β (TRIF) and the TRIF-related adaptor molecule (TRAM), TLRs utilize myeloid differentiation factor 88 (MyD88)

and the adaptor molecule IL-1 receptor-associated kinase (IRAK) complex to transduce intracellular signals (Fig. 1).[28, 29] Upon binding ligand, TLR homo- or heterodimers (TLR1/TLR2 and TLR6/TLR2) are formed, which positions the intracellular TIR domains of these receptors such that subsequent signaling through adaptor and kinase proteins can occur.[30] The major downstream effects of TLR signaling are activation of mitogen-activated protein kinase (MAPK) and nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) signal transduction pathways; these regulate cell differentiation, proliferation, and survival. Interferon regulatory factors IRF3 and IRF7 are also activated downstream of TLRs, regulating the production of type I interferons.[31–33] Thus, TLRs have the potential to broadly impact the immune response. Quite surprisingly, however, broad immunological impairment is not the norm for PIDs that impair TLR signaling. Infection susceptibility due to genetic disruptions of TLR pathways in humans tends to be narrower than in mice with similar genetic abnormalities.[34] The elucidation of PIDs affecting TLR signaling has highlighted the complexity and biological redundancy within these pathways, as well as indicated that there may be significant differences between human disease and animal models.

NF-κB family members exist in their inactive form within the cytoplasm, either bound to inhibitory kappa B (IκB) proteins in the canonical pathway or sequestered by p100 in the non-canonical pathway.[35] There are five NF-κB family proteins, NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel, which function as transcription factors through the formation of homo- or heterodimers.[36] Canonical NF-κB signaling is mediated by the p50 subunit of NF-κB, while non-canonical NF-κB signaling utilizes p52, which is derived through the processing of p100. TLR activation induces phosphorylation of IκBs by the IκB kinase (IKK) complex, leading to translocation of canonical NF-κB dimers into the nucleus to initiate transcription (Figure 1). In contrast, the non-canonical NF-κB pathway mediates signaling through certain members of the tumor necrosis factor receptor superfamily, including B cell activating factor receptor (BAFF-R), CD40, lymphotoxin beta receptor (LTβR), and receptor activator of NF-κB (RANK).[37]

Human genetic defects impairing TLR and canonical NF-κB signaling

A number of PIDs affecting the canonical NF-κB pathway have been identified, all impairing TLRs (Table 1). These include inherited mutations in *MYD88*, *IRAK4*, NF-κB essential modulator (*NEMO* or inhibitor of NF-κB kinase subunit-γ, *IKBKG*), inhibitor of NF-κB kinase subunit-β (*IKBKB*), and NF-κB inhibitor-α (*NFKBIA*). Additionally, defects of TLR3 signaling, which does not utilize MyD88, have been defined, including genetic defects of *TLR3*, *UNC93B1*, Toll/IL-1R domain-containing adaptor inducing interferon (*TRIF*), *TRAF3*, and TANK-binding kinase 1 (*TBK1*). Homozygous mutations in caspase recruitment domain 11 (*CARD11*) and mucosa-associated lymphoid tissue 1 (*MALT1*) also impair canonical NF-κB activation in lymphocytes, but the impact upon TLR signaling is likely to be minimal, as these proteins exist downstream of antigen receptors rather than TLRs.[38–40]

Amongst the best-described syndromes are autosomal recessive (AR) mutations in *IRAK4* and *MYD88*, which impair signals via IL-1, TLR1, TLR2, TLR5, TLR6, TLR7, TLR8,

TLR9, TLR10, and TIR domain containing adaptor protein (TIRAP)-dependent TLR4 signaling.[41–44] These mutations also impair the activation of MAPK, NF-κB, and downstream cytokines in response to TLR ligands. Despite this seemingly broad immunologic loss, the spectrum of pathogens that cause infections in these patients is surprisingly narrow. Invasive infections (meningitis and sepsis) or non-invasive (cutaneous and respiratory infections), are mostly due to *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.[42, 45] These infections typically first occur before the age of 2 years, and re-occur in most undiagnosed survivors.[46] Prophylactic antibiotics and vaccination are essential, and supplementation with immunoglobulin replacement therapy has been used in many cases. Interestingly, with increasing age, infections become limited to non-invasive forms, allowing for the discontinuation of prophylactic therapy in some patients.[47] The reason for this clinical improvement is unclear. Despite the purported role of IRAK-4 and MyD88 in TLR-driven adaptive immune responses, circulating T cell numbers and T cell proliferation in response to antigen are normal.[46]

Based upon the important immunological role of TLR and canonical NF-κB signaling in host defense, the narrow spectrum of infectious disease in these subjects has been puzzling. Impairment of pneumococcal-specific IgG has been reported in a few subjects with *IRAK4* mutations, but this finding does not always correlate with the occurrence of invasive bacterial infections.[46, 48, 49] More recently, it was reported that circulating $IgM^+IgD^+CD27^+$ B cells, which produce T-independent (TI) IgM responses against bacteria, are depleted in these patients, suggesting that the loss of this B cell subset may be clinically important.[50, 51] Exploring this relationship further, we found that diminished TI IgM responses corresponds with inadequacy of the $IgM^+IgD^+CD27^+$ B cell subset in IRAK-4 and MYD88 deficiencies, which may contribute to the susceptibility to bacterial infections.[44] $IgM^+IgD^+CD27^+$ B cells are thought to be a human corollary to the marginal zone B cell subset in mice, which mount rapid TI antibody responses that are required for host defense against bacteria.[50, 52, 53] The importance of this B cell subset in bacterial infection could explain both the observed susceptibility to bacterial pathogens as well as the observed improvement with age. The TI IgG repertoire, which fully matures around adolescence, may ultimately compensate the impairment of TI IgM, lessening the frequency and severity of infections.[54, 55] Though it is not yet clear why the $IgM^+IgD^+CD27^+$ B cells and TI IgM responses would be more susceptible than other types of B cell-driven immunity to IRAK-4 or MyD88 deficiency, these findings may indicate that this B cell subset is more dependent upon IRAK-4– and MyD88-dependent signaling, perhaps through TACI or TLRs, for its activation and survival. Recently, a systems approach was utilized to further address the question of why IRAK-4 and MyD88 deficiencies predispose to a limited range of pathogens, revealing that while responses to purified TLR agonists are disrupted in these patients, only a narrow spectrum of transcriptional targets were impaired upon exposure to whole bacteria.[56] This approach provides a limited panel of transcriptional candidates that will be tested further in the attempt to explain the confounding pattern of susceptibility in IRAK-4 and MYD88 deficiencies.

Mutations in *IκBα* and *NEMO* also impair MyD88-dependent and TRIF-dependent TLR signaling as well as T cell receptor, B cell receptor, and tumor necrosis factor receptor

activation.[57, 58] Given this broader role of IκBα and NEMO compared to that of IRAK-4 and MyD88, it is not surprising that the infectious susceptibility of these patients is also broader; infections with fungi, mycobacteria, and viruses, in addition to bacteria, are reported.[59] Patients with IκBα and NEMO defects also may develop inflammatory bowel disease-like colitis and/or recurrent diarrhea. Additional manifestations of IκBα and NEMO defects include anhidrotic ectodermal dysplasia (EDA, abnormal teeth, hypohidrosis, and sparse hair), which along with the immunodeficiency syndrome is termed EDA-ID.[60] *IκBα* mutations have been inherited in an autosomal dominant (AD) manner and have hypermorphic expression, as IκB proteins inhibit translocation of NF-κB to the nucleus. *NEMO* mutations are X-linked recessive (XL) and hypomorphic, as NEMO promotes IKK-mediated phosphorylation and removal of IκBs that allow for NF-κB translocation. Highlighting an important role for NF-κB signaling in B cell function, over 80% of patients with EDA-ID do not have measurable TI anti-carbohydrate antibody responses in the serum.[36] While T cell numbers are generally normal, the T cell proliferative response to antigen is impaired in patients with EDA-ID. A wide spectrum of immunodeficiency phenotypes has been associated with *NEMO* mutations, including PID without EDA and infection susceptibility limited to mycobacterial disease.[61–65] Treatment for EDA-ID patients usually includes vaccinations, antibiotic prophylaxis and immunoglobulin replacement therapy.[48, 66] Bone marrow transplant has resulted in immune reconstitution in patients with both the AD and XL forms of EDA-ID, but there are concerns with engraftment and post-transplant complications that may make this intervention particularly challenging.[67–71] Recently, four patients from two consanguineous families were described to have AR defects in IKKβ, which is incorporated with NEMO and IKKα in the IKK complex.[72] They presented with childhood bacterial, fungal, and viral infections along with hypogammaglobulinemia and reduced effector T cell numbers and proliferation despite normal overall B and T cell counts.

TLRs are not the only immune receptors affected by genetic disruptions of canonical NF-κB signaling. The interleukin 1 receptor family (including IL-1R, IL-18R, and IL-33R) also signals through the canonical NF-κB pathway, and genetic defects of IRAK-4, MyD88, NEMO, and IκBα also impair these responses.[47] Likewise, responses to TNF have been shown to be impaired in canonical NF-κB pathway defects. Transmembrane activator and CAML interactor (TACI), which is expressed by B cells, requires MyD88 and the canonical NF-κB pathway.[73] TACI binds BAFF trimers as well as trimers of another B cell-activating cytokine, a proliferation inducing ligand (APRIL), to activate B cells in a T cell–independent manner.[74] While the impact of genetic defects in the canonical NF-κB pathway upon TACI signaling have not been reported, defects in TACI are associated with antibody deficiency as well as a propensity for autoimmunity and lymphoproliferation.[75] While both heterozygous and homozygous mutations in TACI can cause antibody deficiency, only monoallelic mutations appear to increase the risk of autoimmunity and lymphoproliferative complications.[76] Curiously, unaffected family members may harbor the same heterozygous mutations in TACI and have B cell impairment *in vitro*, indicating that the relationship between TACI defects and PID is complex.[77]

AR deficiency of HOIL-1 (*RBCK1*), which is part of the linear ubiquitination chain assembly complex (LUBAC) and functions in the polyubiquination of NEMO and NF-κB activation, was recently described.[78] These patients have susceptibility to invasive bacterial infections as well as systemic autoinflammation and muscular amylopectinosis, in which abnormal glycogen storage results in muscular deposition of glycogen. Notably, TLR signaling is impaired in fibroblasts and immortalized B cells derived from HOIL-1–deficient patients.[78] However, monocytes from these patients are hyper-responsive to IL-1β while fibroblasts were found to be hyporesponsive to the same stimuli. This finding offers possible reconciliation for the concurrent immunodeficiency and hyperinflammatory phenotype seen in these patients, and further demonstrates the marked complexity of NF-κB signaling revealed by human PID. Total B and T cell counts do not appear to be reduced in HOIL-1–deficient patients, though memory B cell numbers and antibody responses to pneumococcal carbohydrates were reduced.[78]

Numerous PIDs affecting the TRIF-dependent pathway of TLR3 signaling have also been described, with mutations affecting TLR3, the endosomal TLR chaperone UNC-93B, TRIF, TRAF3, and TBK1 all resulting in early susceptibility to herpes simplex virus 1 (HSV-1) encephalitis.[79–83] Even compared with the narrow spectrum of infections seen in some other defects of TLR signaling, the extent of pathogens seen in these patients with TRIF pathway defects is remarkably restricted, as infections have been limited to one specific pathogen (HSV-1) and one specific type of infection (encephalitis) in all cases. This suggests that the TLR3/TRIF-dependent pathway has a redundant immunological role in humans, with the exception of a required role in defense against HSV-1 in the central nervous system (CNS).[84] Induced pluripotent stem cell-derived neurons and oligodendrocytes derived from TLR3 or UNC93B deficient patients were found to have impaired type I interferon production and heightened susceptibility to HSV-1 infection compared to controls.[85] Thus, proteins functioning in TLR3/TRIF-mediated signaling may have a unique role in host defense against HSV-1, specifically by promoting protective type I interferon responses in the CNS.

TLR polymorphisms and infection susceptibility

Genome-wide association studies have uncovered numerous polymorphisms within TLRs in association with infectious and inflammatory disease.[86] A complete review of these polymorphisms is beyond the scope of this article, and the reader is directed to excellent publications on this topic for further detail.[86, 87] Below, we will consider single-nucleotide polymorphisms (SNPs) of particular relevance to TLR functions and infectious diseases, demonstrated experimentally or through known PID phenotypes. As is quite clear given the diversity of infections explored and populations tested, any conclusions derived from studies of TLR polymorphisms are potentially limited by small sample sizes, ethnic backgrounds, gender, environment, and polygenic influences.

TLR2 has been demonstrated to bind numerous conserved microbial antigens, including the cell wall from Gram-positive bacteria, *Mycobacteria*, and fungi, as well as envelope proteins from multiple viruses. Supporting the role of this TLR in the initiation of anti-bacterial immunity, the G2258A *TLR2* variant has been correlated with asymptomatic bacteriuria in

women.[88] Other *TLR2* polymorphisms were associated with aggressive periodontitis in one study, but not in another, and SNPs in this gene were also linked with nasopharyngeal bacterial carriage in infants as well as higher rates of Gram-positive bacterial infections in liver transplant patients.[89–92] Supporting the role of TLR2 in the recognition of *Mycobacteria*, multiple reports link *TLR2* SNPs with *Mycobacteria* susceptibility, both in leprosy and tuberculosis, corresponding with impaired TLR2 signaling.[93–98] Additionally, the R753Q *TLR2* polymorphism was associated with cytomegalovirus infection in liver transplant patients in two studies.[99, 100]

Suggestive of its role in recognizing viral nucleic acid, polymorphisms in *TLR3* have been linked to susceptibility to viral infection. One *TLR3* SNP was associated with hepatitis B virus infection.[101] The L412F *TLR3* polymorphism has been linked to both resistance against HIV infection in an Italian population and protection against acute liver transplant rejection in patients with hepatitis C virus (HCV) cirrhosis.[102, 103] A polymorphism in the *TLR3* promoter region was noted to predispose to HCV infection, however another study failed to find an association between two other *TLR3* polymorphisms and chronic HCV infection.[104, 105] While PIDs that impair TLR3 signaling have been exclusively associated with HSV-1 encephalitis, only one *TLR3* polymorphism study has reported an association with HSV infection.[106] In this study there was no association with severity of HSV disease, as the SNPs were found equally in symptomatic and asymptomatic individuals.

As TLR4 was one of the first TLR extensively studied and has the purported role of recognizing Gram-negative lipopolysaccharide as well as other bacterial, fungal, and viral antigens, many studies have looked for associations of polymorphisms in *TLR4* with infection. Infections and sepsis with Gram-negative bacteria, RSV bronchiolitis, and disseminated candidiasis have all been linked to *TLR4* polymorphisms.[107–110] However, other studies have failed to associate the same *TLR4* SNPs with sepsis.[111–113] Similarly mixed results were found for mycobacterial infection, with some studies associating *TLR4* polymorphisms with susceptibility to *Mycobacteria* while others did not.[114–118] Numerous polymorphisms in the *TLR4* promoter region were associated with urinary tract infections (UTIs) and impaired innate immune responses during UTIs, as measured by urinary IL-6, IL-8, and neutrophil numbers in a Swedish cohort.[119]

Other notable TLR SNPs include a TLR polymorphism in the ligand-binding domain of *TLR5* (392STOP) that has been linked with increased susceptibility to *Legionella* pneumonia, impaired IL-6 response to flagellin, as well as an increased risk of UTIs in Caucasian women, but that did not correlate with *Salmonella enterica serovar Typhi* infection in a Vietnamese cohort.[120, 121] Highlighting its putative role in sensing viral nucleic acid, a *TLR7* polymorphism (c.32A>T) was found to be associated with chronic HCV infection and SNPs in *TLR7* and *TLR8* were associated with HCV infection in a Taiwanese cohort.[122, 123]

Functional alterations in TLR signaling

Curiously, altered responses to TLRs are seen in immune deficiencies in which the canonical components of TLR signaling do not bear known mutations (Table 2). Among the

PIDs in this category, common variable immunodeficiency reveals the importance of crosstalk between distinct cell types when responding to shared TLR activators. Other PIDs, including chronic granulmatous disease and X-linked agammaglobulinemia, reveal the complexity of immune regulation, with defects highlighting positive and negative regulators of TLR signaling. While knockout mice have traditionally been used to unravel the complexity of immune pathways, human with PIDs provide opportunities for study of these pathways in context of the human immune system, whether confirming or altering the conclusions from animal models.

TLR signaling in common variable immunodeficiency

Common variable immunodeficiency (CVID), the most common symptomatic PID, is a diagnosis of exclusion given to individuals with hypogammaglobulinemia and recurrent sinupulmonary infections who do not make specific antibodies following vaccination.[124] With patients at increased risk of autoimmune, inflammatory, and malignant complications,[125] CVID encompasses a heterogeneous group of patients, the large majority having no known underlying genetic cause.[124] Given the hallmark of hypogammaglobulinemia, B cells have been heavily studied in this disorder, with defects reported in proliferation, class switch recombination, differentiation, and immunoglobulin (Ig) secretion. In addition, T cell cytopenia, clonality, and defects of (TLR-independent) cytokine production has been described,[126] as have reduced numbers of dendritic cells[127, 128] and *in vivo* increased activation of monocytes.[129]

Our group first reported defective B cell proliferation and Ig secretion in response to CpG motif containing nucleotides,[130] although the mechanism was unknown as this preceded the discovery of mammalian TLR9.[131] Following the characterization of human TLR9 and the discovery of powerful synthetic agonists, our group and others revisited responses of CVID patients to TLR9 stimulation, finding pervasive B cell defects in activation and in cytokine secretion when, notably, patients did not have mutations or variations in *TLR9* (Fig. 2).[132, 133]

Intriguingly, both naïve CD27- and memory CD27+ B cells from patients showed reduced expression of TLR9 transcript and protein, suggesting a possible cause for the defective responses.[132] Previous work had shown that TLR9 responsiveness increases following treatment with interferon alpha (IFN-α)[134] and that, among peripheral blood mononuclear cells (PBMCs), plasmacytoid dendritic cells (pDCs) secrete high levels of IFN-α following TLR9 stimulation.[135] When pDCs from subjects with CVID were examined, they were similarly found to have profound defects, with greatly reduced secretion of IFN-α following TLR9 stimulation, in spite of comparable expression of TLR9.[132] A follow up report showed that supplementation of exogenous IFN-α restored TLR9 responsiveness of B cells, but only for subjects with less severely reduced frequency of class switched (CS) memory B cells.[136] This was consistent with reports of higher levels of CS memory cells associated with better clinical course.[137] In addition, pDCs and B cells from patients were found to be defective in responding to TLR7 and TLR7/8 stimulation,[8] similarly showing rescue of the B cell phenotype with exogenous IFN-α, allowing B cells of patients with retained CS memory cells to proceed to class switch recombination and Ig secretion.[136, 138]

Of note, TLR signaling appears otherwise intact in CVID. IFN-α secretion by pDCs following stimulation through TLR3 mirrored controls,[138] as did upregulation of activation markers on pDCs, conventional DCs, monocytes, and NK cells following stimulation through TLR2, TLR4, or TLR9.[139] Furthermore, normal secretion of TNF-α, IL-6, and IL-12 from PBMCs stimulated through TLR1, TLR2 TLR3, TLR4, TLR5, TLR7, or TLR9 was seen in patients,[138] as were levels of intracellular IL-12 in myeloid DCs stimulated through TLR9.[128] Interestingly, a recent report did not find defects in pDC production of IFN-α following TLR9 stimulation,[128] finding similar levels of intracellular IFN-α when gating on pDCs in PBMC cultures, unlike earlier studies examining isolated pDCs.[132, 138] This discrepancy suggests either increased fragility of CVID pDCs, a pDC defect in IFNα secretion, but not intracellular accumulation, or else raises the possibility of correction of the pDC defect by a PBMC component. A tantalizing hint supporting involvement of additional cell types comes from a report of modest gains in TLR9-driven IgG secretion with the addition of exogenous retinoic acid,[140] a vitamin A metabolite produced by some myeloid cells.[141, 142] Collectively, these data support a view of functional aberrations of TLR7 and TLR9 signaling in pDCs and B cells of subjects with CVID, and highlight the importance of TLR-mediated cross talk between different cells in optimizing responses to shared activators.

TLR signaling in chronic granulomatous disease

Chronic granulomatous disease (CGD) is caused by genetic mutations in components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system that produces reactive oxygen species (ROS) leading to the respiratory burst of phagocytes. In the absence of a functional system, neutrophils are incapable of killing phagocytosed bacteria and fungus, leaving patients at increased risk of recurrent pyogenic infections.[124] Other immune aberrations in patients include inflammatory disorders, largely restricted to GI tract, that are thought to be independent of infection.[124, 143] The most common form of CGD is caused by mutations in X-chromosome *CYBB*, affecting males but largely sparing their carrier mothers, and inactivating both copies of autosomal components of NADPH oxidase will similarly cause CGD.[124] The first report assessing TLRs in CGD focused on patient neutrophils, finding them to secrete increased levels of TNF-α and IL-8 following LPS stimulation.[144] Similar findings were later reported in PBMCs, with increased TNF-α and IL-6 secretion following treatment with TLR2 or TLR4 agonists (Fig. 3, in blue).[145] This study further showed that the TLR induced activation of NF-κB was independent of ROS,[145] strongly suggesting that ROS are needed for appropriate dampening of responses to these TLRs. The following year, microarray analysis of patient and control monocytes provided further support that the immune system of CGD patients excessively responded to TLR4 stimulation, with additional hypersensitivity seen at baseline as well.[146] The authors linked the absence of ROS to increased expression of NF-κB subunits, seen both basally and following stimulation, suggesting that this loss endowed patient cells to produce higher levels of proinflamatory cytokines.[146] As such, the defects in NADPH oxidase may result in increased TLR2 and TLR4 inflammatory responses contributing to the GI inflammation seen in these patients.

Further assessment of TLRs in CGD found that TLR5 and TLR9, but not TLRs 1, 2, or 4, were expressed at lower levels by patient neutrophils (Fig. 3, in blue).[147] These changes were independent of infectious history, and were seen in the mRNA transcript as well as in intracellular and, for TLR5, cell surface protein expression.[147] Of note, TLR5 expression correlated with the extent of NADPH oxidase function, and inhibition of this system in neutrophils from healthy controls reduced the expression of TLR5 and TLR9 in these cells.[147] As such, NADPH oxidase or resultant ROS may be important for proper expression of TLR5 and TLR9 in neutrophils, with absence resulting in impaired responses to stimulation by these TLR ligands and less efficient phagocytosis.[147] The NADPH oxidase system was further shown to be important in the context of TLR9 stimulation in B cells, with defects in long term antibody memory responses recently reported in CGD patients.[148] The authors linked deficient ROS production in B cells to reduced frequencies of memory B cells in patients, with a milder phenotype seen in carriers of CGD mutations (Fig. 3, in blue).[148] In the setting of previous measles vaccination, authors demonstrated the functional implications of defective NADPH oxidase by showing that patients with CGD had age-dependent reductions in serum anti-measles antibody levels, concomitant with reduced induction of measles-specific antibody secreting cells (ASCs) downstream of TLR9 activation.[148] While as earlier study found influenza-specific ASCs to be similar between patents and controls,[149] some participants in this earlier study were on immunosuppressive therapy that could have altered their antibody responses. This study did not assess expression of TLR9 in CGD B cells, thus it remains unknown whether NADPH oxidase might promote TLR9 expression in B cells, as it does in neutrophils.[147] If this were the case, reduced expression of TLR9 might link the defects in NADPH oxidase and ROS production to promotion of long-term B cell memory. In concert, these results highlight the importance of NADPH oxidase and ROS in regulating TLR signaling, and further demonstrate the this regulation can be TLR specific, with NADPH promoting responses downstream of TLRs 5 and 9[147, 148] yet dampening responses downstream of TLRs 2 and 4.[144–146] Future work to understand the mechanism remains, yet these results suggest that modulation of ROS might be useful, whether to possibly ameliorate CGD autoinflammatory disorders or possibly as a way to treat antibody based autoimmune syndromes.[150, 151]

TLR signaling in X-Linked agammaglobulinemia

X-Linked agammaglobulinemia (XLA), the first human PID described, was reported in 1952 by Col. Bruton.[152] Brutons tyrosine kinase (*BTK*), a member of the Tec family of tyrosine kinases, was identified in 1993 as the gene mutated in XLA,[153] and the critical dependence of B cells on BTK activation downstream of the B cell receptor (BCR) was reported shortly thereafter.[154, 155] A role for Btk in other cells was quickly identified with a report that Xid mice, which have a point mutation in *Btk* and a milder phenotype than human patients, had disrupted microfilarial clearance.[156] With BTK expression found in all hematopoietic cells with the exception of T cells, study of BTK functions in myeloid cells were undertaken, including studies of its role downstream of TLR signaling. However, studies of freshly isolated PBMCs from patients found conflicting results, reporting either defective[157] or increased[158] cytokine secretion following activation through various TLRs. Through mechanistic studies, BTK has been shown to interact with various pathways, both activating

and inhibitory, such that a deficiency could cause either suppression or hyperactivation of immune responses. As such, these discrepant outcomes may reflect the preferential activation characteristics of the different cells, agonists, and putative inhibitors used in the various reports, with both informing the understanding of human TLR signaling pathways.

The first reports using human cells found decreased TLR responsiveness with mutated BTK, proposing a role for the kinase in activating proinflammatory pathways. After stimulation with TLR2 or TLR4 agonists, XLA monocytes were found to secrete less TNF-α and IL-1β relative to cells from healthy controls, a defect not seen for secretion of IL-6, IL-8, and IL-10 (Fig. 3, in pink).[157, 159] BTK phosphorylation, a sign of activation, was reported following TLR stimulation,[157, 160] making a link between TLR pathways and BTK more likely, and a mechanism of direct interaction was proposed when overexpression and yeast two-hybrid studies showed BTK interactions with TIR domains of several TLRs as well as their signal adaptors Mal (also known as TIRAP) and IRAK-1.[160] Signaling studies of patient PBMCs found normal IκBα phosphorylation and degradation, however, thereby strongly suggesting that BTK was not involved in the canonical NF-κB signaling cascade downstream of TLRs.[157, 159, 161] Instead, patient PBMCs were found to have lower levels of phosphorylated p38, linking BTK to activation of MAPK pathways.[157, 159] To determine whether BTK could phosphorylate p38 directly, authors overexpressed BTK in monocyte derived macrophages (moMs), showing increased cytokine secretion and p38 phosphorylation in cells overexpressing btk.[157, 159] Interestingly, the M-CSF used to mature moMs strongly induced the expression of Tec, a closely related tyrosine kinase, which might have contributed to restoring LPS induced TNF-α secretion in patient cells (Fig. 3, in pink).[157] As such, while overexpressed BTK can activate MAPK pathways downstream of TLR activation, this function might be more generally attributable to Tec family kinases. Several studies have similarly reported decreased cytokine secretion in monocyte derived DCs (moDCs) of XLA patients.[162, 163] While these studies did not examine signaling, they nevertheless suggest that the involvement of Tec family members in promoting cytokine secretion downstream of TLR activation might be a general hallmark of these pathways.

Other studies, meanwhile, found increased cytokine secretion in the absence of BTK, in line with elevated levels of TNF-α, IL-1β, and IL-6 in patient serum.[164] Our group found primary patient monocytes to secrete increased levels of TNF-α and IL-6, but not IL-10, when stimulated through TLR4 or TLR7/8, while patient myeloid DCs (mDCs) showed higher secretion of all three cytokines (Figure 3, in green).[161] In line with these findings, a study examining PBMCs from 13 XLA patients found increased secretion of TNF-α, IL-1β, IL-6 and IL-10 in response to LPS.[158] Increases in cytokine secretion were similarly seen among LPS stimulated moDCs, with one study reporting increased IL-10 but similar IL-12 levels,[165] and another study reporting increases of IL-6.[166] TLR hyperresponsiveness was not seen in pDCs, however, as these cells responded to TLR7/8 or TLR9 stimulation by secreting IFN-α to levels resembling controls.[132, 161] As such, TLR7/8 responses were perturbed in some cells but not others, suggesting that BTK modulates TLR signaling without being directly downstream of these receptors. As patient monocytes showed increased cytokine secretion yet comparable intracellular cytokine levels, BTK could instead work to slow signaling kinetics.[161] In agreement, TLR4 signaling was intact in the setting of increased responsiveness, inducing comparable IκB, p38, and ERK phosphorylation,[161]

findings also seen in another study of XLA patients that reported unchanged intracellular cytokine levels (without commenting on secreted cytokines).[167] Work in Xid mice and cell lines treated with LFM-13A, a non-specific BTK inhibitor,[161] showed that BTK could phosphorylate Mal, inducing its degradation.[168] While LPS stimulated XLA cells showed a trend towards reduced Mal degradation,[161] this mechanism is expected to be specific for TLRs 2 and 4[168] and thus cannot account for increased responses to TLR7/8 agonists. An alternative mechanism, for which there is only indirect evidence, involves BTK dampening TLR signaling by enhancing PI3K activation, a known function of BTK downstream of BCR activation (Fig. 3, in green).[169] Downstream of TLRs, PI3K can be activated by TIR containing B cell adaptor for PI3K (BCAP), a novel inhibitory TLR signaling adaptor.[170] Intriguingly, a BCAP-deficient murine macrophage cell line showed enhanced early cytokine secretion following TLR stimulation,[170] reminiscent of BTK deficient cells from XLA patients. While it is not known whether BTK amplifies TLR-dampening PI3K responses in myeloid cells, or whether BTK might even phosphorylate and activate BCAP in these cells, such roles would explain the increased responsiveness to TLR agonists seen in XLA.

Lastly, in concordance with a view of a functional modulation of TLR signaling, studies of neutrophils from subjects with XLA found normal activation and oxidative burst induction following TLR stimulation.[167, 171] These findings are perhaps expected as patients on Ig replacement are not at increased risk of overwhelming infections, instead having increased risk of chronic and low grade infections, largely viral in nature.[172, 173] As such, XLA represents a PID with cell-type restricted alterations in responses to TLR agonists.

TLR signaling in hyper IgE syndrome and adenosine deaminase deficiency

While the functions of TLR signaling have been extensively studied for each of the above PIDs, aberrations downstream of TLRs have also been reported hyper IgE syndrome (HIES) and adenosine deaminase (ADA) deficiency leading to severe combined immunodeficiency (SCID). Patients with HIES have recurrent skin and pulmonary abscesses, often without signs of inflammation, as well as with chronic eczema, eosinophilia, and increased serum IgE.[124] The more common AD form of the disorder is caused by a dominant negative (DN) mutation in one copy of signal transducer and activator of transcription 3 (*STAT3*).[174] Prior to identification of the genetic cause of HIES, two studies assessed TLR function in small groups of patients in order to determine whether TLR defects underlie this disorder. Instead of defective TLRs, both studies found increased responsiveness to TLR2 or TLR4 stimulation, with the first study reporting a trend towards increased TNF-α and IL-12 secretion from PBMCs of six patients[175] and the second study finding increased TNF-α and IL-12 secretion from whole blood of four patients (Fig. 3, in orange).[176] A later study of 25 patients confirmed these findings, showing increased TNFα secretion from patient PBMCs following TLR2 or TLR4 stimulation.[177] While these early studies did not report *STAT3* as a causative gene, the phenotype of TLR hyper-responsiveness was later confirmed in patients with DN-STAT3 mutations.[178] While studies directly examining the mechanisms underlying TLR hyper responsiveness in HIES patients have not been performed, Stat3 deficient murine immune cells have been reported to have higher secretion of Th1 cytokines, such as TNF-α, after TLR stimulation.[179, 180] With rising interest in blocking STAT3 in tumor

immunotherapy, as reviewed in,[181] AD-HIES patients serve as a cautionary reminder of the importance of determining optimal levels of STAT3 inhibition.

Deficiency of ADA, which accounts for 11% of newborn-screening detected SCID cases,[182] is a T cell–negative, B cell–negative, and NK cell–negative form of SCID with AR inheritance. In addition to deaminating adenosine, ADA metabolizes lymphotoxic dATP and *S*-adenosyl homocysteine,[183–185] and enzyme replacement therapy (ERT) can prevent recurrent infections in patients.[186] Bone marrow transplant and/or gene therapy (BMT/GT) remains the preferred therapy as it fully corrects immune defects.[187] In contrast, B cells from patients undergoing ERT still showed defects in tolerance reminiscent of IRAK4- and MyD88-deficient patients,[188] prompting study of how ADA modulates TLR signaling in B cells. Using ADA inhibitors, human B cells were found to have defective phosphorylation of Syk, BTK, and PLCγ2 downstream of TLR signaling, with cells failing to upregulate activation markers including CD86 following TLR stimulation.[188] Murine studies have shown that adenosine can block TLR4-mediated phosphorylation of IκB in splenic B cells,[189] either directly or through anti-inflammatory effects of cyclic adenosine monophosphate (cAMP), which similarly accumulates in the absence of ADA (Fig. 3, in purple). As the signaling pathways of adenosine and cAMP are complex, the findings summarized in an extensive review are presented here.[190] Both adenosine receptor agonists and cAMP generally dampen TLR signaling, reducing TLR4 induced TNF-α and IL-12, yet increasing IL-10 secretion. Similar modulation was seen downstream of TLR2, with adenosine agonists reducing TNF-α secretion. Agonist-specific modulation was noted for TLR3- and 5-dependent IL-1β, IL-6, and IL-10 secretion, however, making the effects of adenosine stimulation less conclusive. In context of the defects noted in patients undergoing ADA ERT,[188] cell-intrinsic ability to degrade adenosine is likely important for fine-tuning TLR signaling, with further study needed to clarify the underlying mechanism.

Concluding remarks

Recent years have seen a dramatic increase in the identification of genetic causes for PIDs, leading to the elucidation of many essential immunologic processes in humans.[191, 192] PIDs impairing TLR signaling have illustrated the significance of these pathways for a surprisingly narrow spectrum of infectious diseases in humans. Further research utilizing these PIDs as model systems to test TLR function will undoubtedly expand our understanding of these important receptors and related signaling pathways in humans, and may ultimately explain why defects in these vital immune regulatory proteins manifest as a remarkably small variety of infections clinically. Likewise, further efforts to delineate novel defects of TLR and related signaling pathways will continue to enhance our understanding of human immunology, specifically at the crossroads between innate and adaptive immunity. Additional PIDs, meanwhile, reveal the complexity of TLR regulation, both in terms of collaboration between cell types and in fine-tuning the intensity and duration of responses to TLR stimulation within a responding cell. Further study of such deficiencies could help identify the significant pathways intersecting with TLR signaling, with such understating potentially providing targeted treatment options for PIDs as well as for more common autoimmune and inflammatory disorders.

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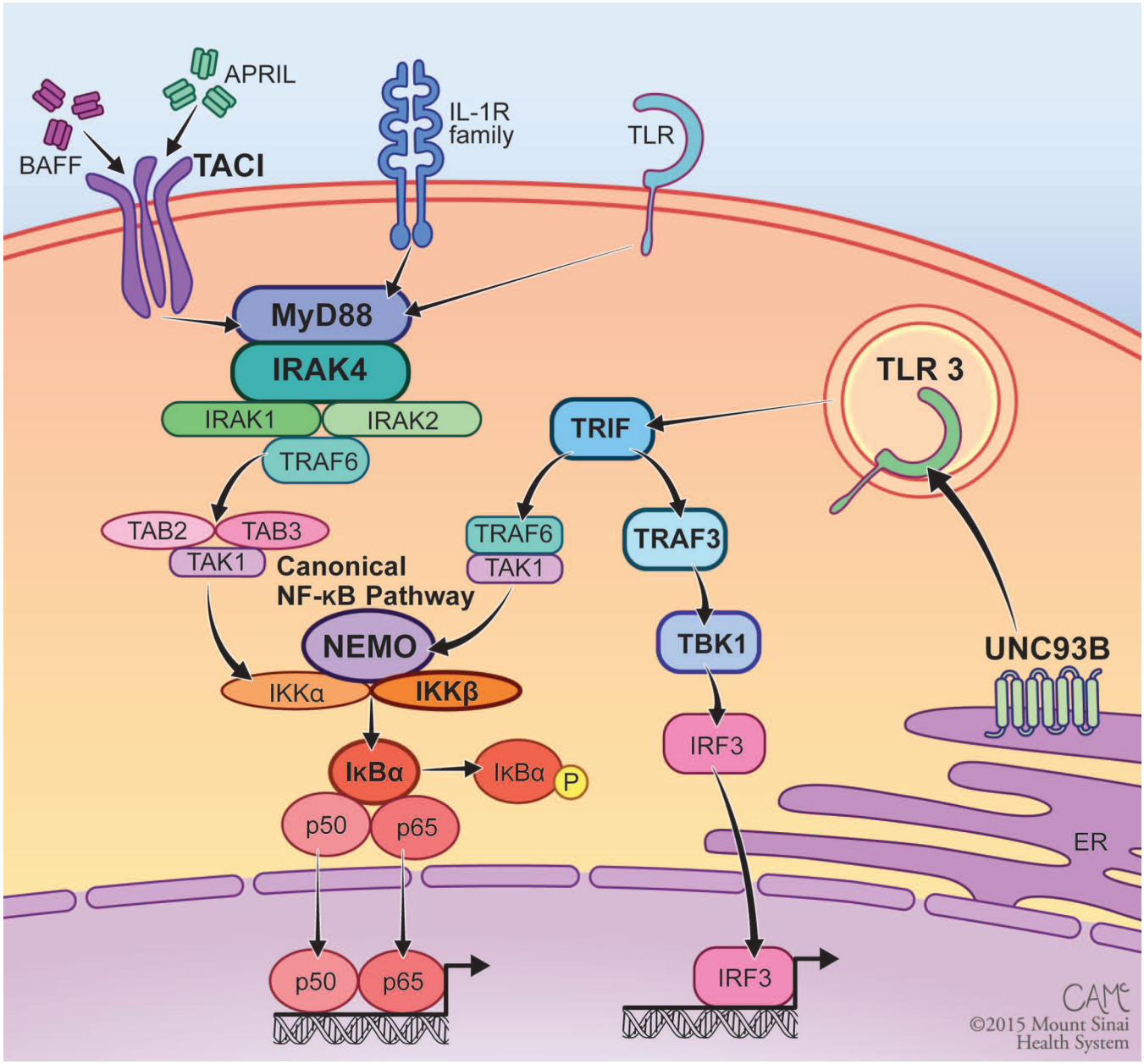

Figure 1.

TLR and NF-κB signaling disrupted by PID. The canonical NF-κB pathway is utilized by numerous immunologic receptors, including the IL-R family, TLRs, and TACI. Signal transduction is mediated through an IKK complex that results in phosphorylation and degradation of IκBα, allowing for nuclear localization of NF-κB. TLR3 signals through TRIF to both activate the canonical NF-κB pathway and induce type I interferon production through IRF3. Genes in which mutations cause known PID are in bold type. Figure courtesy of Courtney A. McKenna © 2015 Mount Sinai Health System.

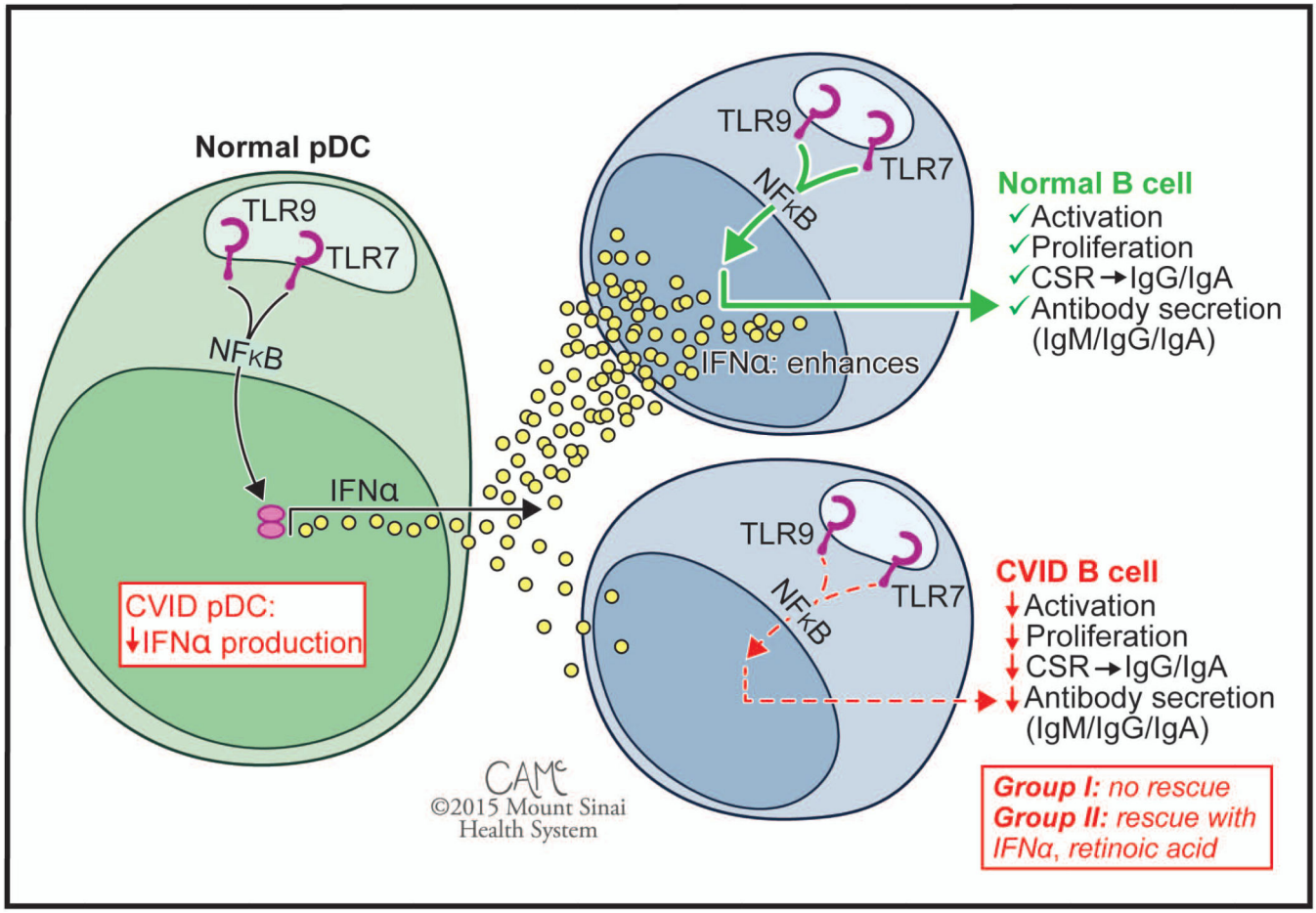

Figure 2.

Cell type specific functional defects of TLR signaling in. CVID. While the normal pDC response to stimulation with TLR7 and TLR9 agonists is secretion of high levels of IFN-α, pDCs of subjects with CVID show defective responses. Similarly, while normal B cells are sensitive to stimulation with TLR7 and TLR9 agonists, patient B cells show defective responses. The B cell defect is correctable in less severely affected Group II patients with the addition of exogenous IFN-α or retinoic acid, but these fail to rescue B cells from Group I patients. Patients are subdivided into Group I (<0.55% SWMB, associated with non-infectious complications) and less severely affected Group II (>0.55% SWMB, associated with better clinical course). CVID, common variable immunodeficiency; SWMB, class switched memory B cells. Figure courtesy of Courtney A. McKenna © 2015 Mount Sinai Health System.

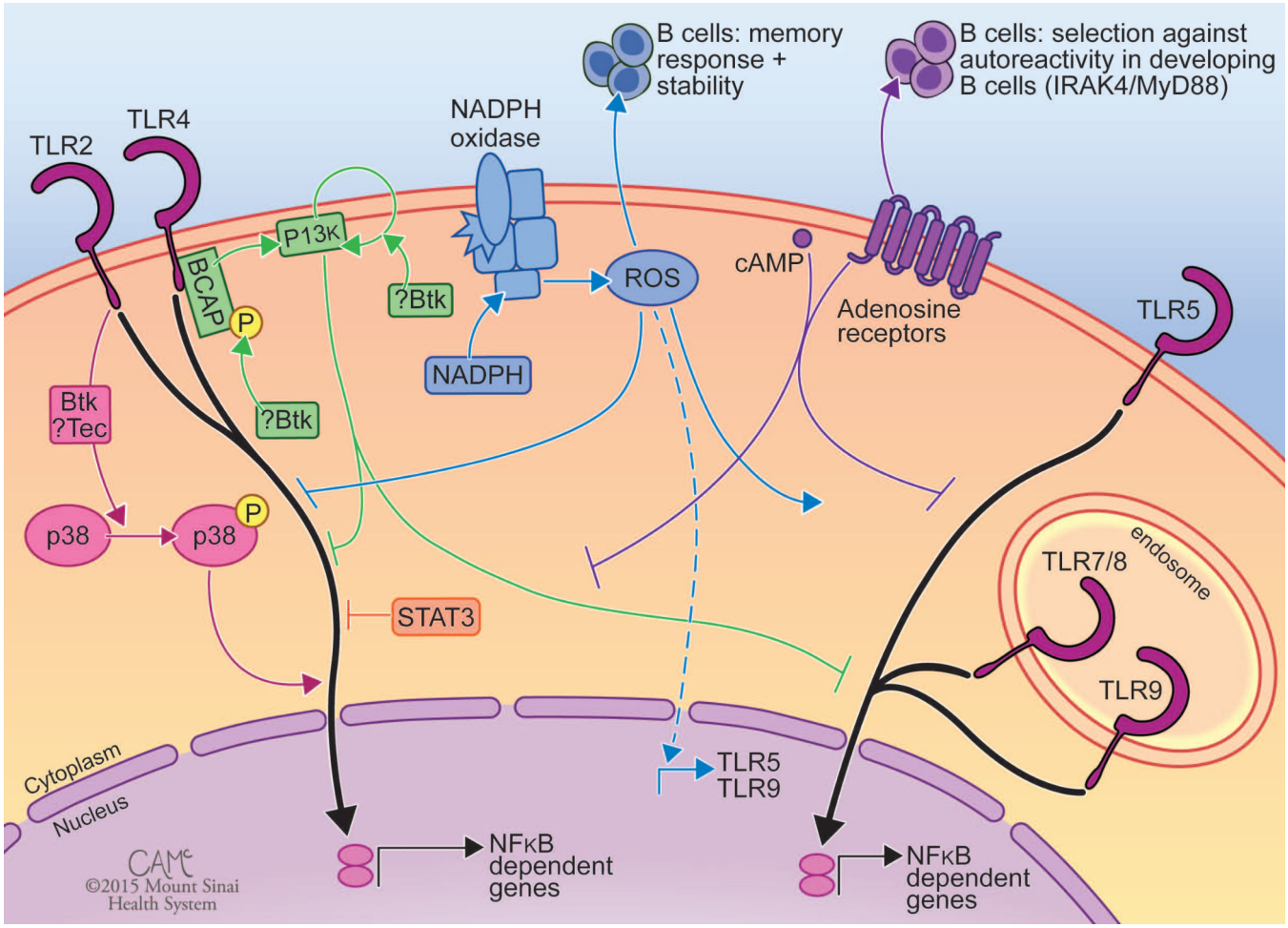

Figure 3.

The functional alterations in TLR signaling seen in various PIDs highlight crosstalk with regulatory pathways. TLR signaling is shown in black, with interacting pathways shown in color. Pink: Some studies of XLA patients demonstrate a role for BTK and related kinase Tec in activating MAPK pathways and amplifying responses to TLR2 and TLR4. Green: Other studies of XLA suggest a role for BTK in activating or amplifying PI3K signaling to dampen TLR responses. Blue: As illustrated by CGD, NADPH oxidase–induced ROS are important in dampening inflammation following TLR2 and TLR4 activation, promoting expression of TLR5 and TLR9 (dotted line) and responses to TLR5 and TLR9 agonists, and long term memory responses in B cells. Orange: AD-HIES patients indicate that STAT3 can dampen TLR2 and TLR4 signaling. Purple: Findings from ADA-SCID patients conjecture a role of cAMP and adenosine accumulation in inhibiting TLR signaling. CGD, chronic granulomatous disease; NADPH = nicotinamide adenine dinucleotide phosphate. ROS, reactive oxygen species; XLA, X-linked agammaglobulineima; BTK, Bruton's tyrosine kinase; MAPK, mitogen activated protein kinase; BCAP, B cell adaptor for PI3K; PI3K, phosphoinositide 3 kinase. AD-HIES, autosomal dominant hyper IgE syndrome; STAT3, signal transducer and activator of transcription 3; ADA, adenosine deaminase; SCID, severe combined immunodeficiency; ERT, enzyme replacement therapy; cAMP, cyclic adenosine

monophosphate. Figure courtesy of Courtney A. McKenna © 2015 Mount Sinai Health System.

Table I

PIDs due to genetic defects of NF-κB and TLR signaling

Genetic defect	Inheritance	Typical infections	Autoimmune features	Immunological features
MyD88 dependent				
IRAK-4, MyD88	AR	Invasive and recurrent bacterial infections, most commonly *S. pneumoniae*, *S aureus*, and *P. aeruginosa*	None reported	Impaired TLR and IL-1R responses, reduced $IgM^+IgD^+CD27^+$ B cells, impaired TI IgM
NEMO (IKKγ)	XL	Bacterial, fungal, mycobacterial, and viral infections	Arthritis, hemolytic anemia, inflammatory bowel disease-like colitis	Impaired TLR and IL-1R responses, reduced T cell proliferative responses, impaired TI antibodies
IKKβ	AR	Bacterial, fungal, and viral infections	None reported	Hypogammaglobulinemia, reduced CD45RO T cells, impaired T cell proliferative responses
IκBα	AD	Recurrent bacterial infections, pneumocystis pneumonia, chronic mucocutaneous candidiasis	Colitis, recurrent diarrhea	Impaired TLR and TNF-α responses, reduced T cells
HOIL-1	AR	Invasive bacterial infections	systemic autoinflammation, muscular amelopectinosis	Impaired TLR responses, monocytes hyper responsive to IL-1β while fibroblasts are hyperresponsive, reduced memory B cells, impaired TI antibodies
MyD88 independent				
TBK1, TLR3, TRAF3, TRIF, UNC-93B	AD or AR (TLR3, TRIF) AD (TBK1, TRAF3) AR (UNC-93B)	HSV-1 encephalitis	None reported	Impaired TLR3 stimulation of type I interferon
Other				
TACI	AD with incomplete penetrance	Recurrent bacterial infections	Autoimmunity and lymphoproliferation in monoallelic patients	Hypogammagobulinemia, IgA deficiency, reduced isotype-switched memory B cells

AD, autosomal dominant; AR, autosomal recessive; XL, X-linked recessive; TI, T independent

Table 2

Changes reported for TLRs in PIDs with functional alterations of TLR signaling

Immunodeficiency	Genetics	Phenotype	Cells: relevant TLRs	Details
Common variable immunodeficiency	Unknown mutations; variable inheritance	Decreased responses	pDCs: TLR7 and TLR9	Reduced secretion of IFN-α
			B cells: TLR7 and TLR9	Reduced TLR9 expression; reduced upregulation of costimulatory molecules; reduced proliferation; reduced cytokine secretion; reduced immunoglobulin secretion
		Normal responses	pDCs: TLR3	Normal upregulation of costimulatory molecules and secretion of IFN-α
			Monocyte, NK, and DCs: TLR2, TLR4, TLR9	Normal upregulation of costimulatory molecules
			PBMCs: TLR1, TLR2, TLR3, TLR4, TLR5, TLR7/8, and TLR9	Normal secretion of TNF-α, IL-6, and IL-12
Chronic Granulomatous Disease	NADPH oxidase mutations; XL or AR	Increased Responses	Neutrophils: TLR4	Increased TNF-α and IL-8 secretion
			PBMCs: TLR2 and TLR4	Increased TNF-α and IL-6 secretion
		Decreased Responses	Neutrophils: TLR5 and TLR9	Reduced expression of TLR5 and TLR9; reduced phagocytosis of *S. aureus*
			B cells: TLR9	Antigen specific long term memory recall responses reduced
X-Linked agammaglobulinemia	Btk mutations; XL	Decreased responses (some studies)	Monocytes: TLR2 and TLR4	Decreased secretion of TNF-α and IL-1β (but not IL-6, IL-8, and IL-10)
			PBMCs: TLR2 and TLR4	Reduced levels of phosphorylated p38
			Monocyte derived macrophages: TLR4	Overexpressing btk restored defective TNF-α secretion (related kinase Tec strongly induced by culturing media)
			Monocyte derived DCs: TLR2, TLR3, TLR4, TLR7/8	Decreased DC maturation; decreased TNF-α (but not IL-6 or IL-12) secretion in one study; decreased TNF-α and IL-6 in response to TLR8 (but not other TLRs) in a second study
		Increased responses (some studies)	Monocytes and DCs: TLR4 and TLR7/8	Increased secretion of TNF-α and IL-6; increased secretion of IL-10 (DCs but not monocytes); intracellular cytokine levels similar to controls
			PBMCs: TLR4	Increased secretion of TNF-α, IL-1β, IL-6 and IL-10
			Monocyte derived DCs: TLR4	Increased IL-10 (but not IL-12) secretion in one study and increased IL-6 secretion in another
		Normal responses	pDCs: TLR3, TLR7/8, and TLR9	Normal secretion of IFN-α
			Neutrophils: TLR4 and TLR7/8	Normal activation; normal oxidative burst
Hyper IgE syndrome	STAT3 mutations; AD	Increased Responses	PBMCs: TLR2 and TLR4	Increased TNF-α and IL-12 secretion
			Whole blood: TLR2 and TLR4	Increased TNF-α and IL-12 secretion

Immunodeficiency	Genetics	Phenotype	Cells: relevant TLRs	Details
Adenosine deaminase deficiency	ADA mutations; AR	Decreased responses	B cells: TLRs	Defective exclusion of autoreactive B cells during development (resemble IRAK4 or MyD88 deficient)
			B cells: TLR7 and TLR9	ADA inhibitors in healthy donor cells reduced upregulation of costimulatory molecules including CD86 (but not CD69); reduced phosphorylation of signaling components
		Increased responses	Various: TLR2 and TLR4	Adenosine agonists or cAMP in healthy donor cells reduced TNF-α and IL-12 secretion yet increased IL-10
		Altered	Various: TLR3 and TLR5	Adenosone agonist-specific effects of increasing or decreasing IL-1β, IL-6, and IL-10 secretion in healthy donor cells

AD, autosomal dominant; AR, autosomal recessive; XL, X-linked recessive; pDC, plasmacytoid dendritic cell; DC, conventional dendritic cell; PBMCs, peripheral blood mononuclear cells; NADPH, nicotinamide adenine dinucleotide phosphate; Btk, Bruton's tyrosine kinase; STAT3, signal transducer and activator of transcription 3; ADA, adenosine deaminase.