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Review Article

Role of PIR-B in Autoimmune Glomerulonephritis

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PIR-B, an inhibitory receptor expressed on murine B cells and myeloid cells, regulates humoral and cellular immune responses via its constitutive binding to the ligand, MHC class I molecules, on the same cells (*cis*) or on different cells (*trans*). Although it has been speculated that PIR-B is important for maintaining peripheral tolerance, PIR-B single deficiency does not cause overt autoimmune diseases. Recently, however, the combination of its deficiency with the Fas *lpr* mutation was found to result in augmented production of autoantibodies such as IgG rheumatoid factor and anti-DNA IgG, leading to glomerulonephritis in mice. Although the precise molecular mechanism for the overall scenario is unclear, PIR-B was found to suppress TLR9-mediated production of naturally autoreactive antibodies by innate B cells or B-1 cells by inhibiting the activation of Bruton's tyrosine kinase. Thus, PIR-B is an important regulator of innate immunity mediated by TLR9 in B-1 cells, which can otherwise provoke autoimmunity when overactivated.

1. Introduction

B cells are continuous producers of natural antibodies with potential reactivities to autologous tissues as well as the on-demand factories of high-affinity antibodies principally against pathogens. Because various self-reactive antibodies are produced in many autoimmune diseases through several elusive mechanisms, a regulatory network for maintaining tolerance to self in B cells while keeping the potential for producing antibodies with specificities and affinities against foreign antigens in sufficient amounts has long been a challenging field for immunologists.

We are now aware of that a series of inhibitory receptors are expressed preferentially on B cells and other immunoregulatory cells such as dendritic cells (DCs). Thus, the roles of these receptors, including that of the paired immunoglobulin- (Ig-) like receptor (PIR)-B [1, 2] in the regulation of B cells, which we will describe in detail in this paper, have been attracting much interest from many researchers. Some intriguing knowledge on the regulatory role of PIR-B in the immune system has already been reported elsewhere [3–5] (Table 1). Therefore, after briefly overviewing PIR-B's novel characteristics revealed in the past several years, this paper will mainly deal with the recent progress of the understanding of PIR-B-mediated immune regulation, particularly focusing on its role in controlling the production of potential autoantibodies in response to activation via innate-immune stimuli.

2. PIR-B Is the MHC Class I Receptor in B Cells and Myeloid Cells

2.1. Recognition of MHC Class I Molecules. In the immune system, there are three types of major histocompatibility complex class I- (MHCI-) recognition molecules. In addition to the well-known T cell receptor (TCR)-CD8 complex of $CD8^+$ T cells and the killer cell receptors on NK cells, mammalian B cells and myeloid-lineage cells possess the third type of MHCI-recognizing inhibitory receptors, which may constitutively regulate these cells. The latter are murine PIR-B and its close relatives or orthologs in humans, the leukocyte Ig-like receptors (LILR)B1 and LILRB2 [3, 4, 6, 7]. In contrast to the former two types of receptors, which target the polymorphic $\alpha 1/\alpha 2$ region of MHCI, LILRB molecules were demonstrated to bind a constant $\alpha 3$ domain and β_2

microglobulin of MHC [8, 9]. PIR-B's mode of recognition of MHCI has not been clarified yet, although it is speculated to be essentially the same as that of LILRB [4].

2.2. Protein Structure. PIR-B is a type I transmembrane glycoprotein comprising six extracellular Ig-like domains, a hydrophobic transmembrane segment, and a cytoplasmic portion with four immunoreceptor tyrosine-based inhibitory motif (ITIM) or ITIM-like sequences (Figure 1). Comparison of the available sequences of PIR extracellular portions from 129/Sv, B10.A, and BALB/c mice indicated fairly high sequence similarity, but multiple substitutions of amino acid residues were observed like in LILR molecules [23], especially in the first four ectodomains [2, 24]. It is not known whether these polymorphisms could modulate the binding of PIR-B to various MHCI and its inhibitory functions.

2.3. Expression. PIR-B is expressed on B cells and myeloid-lineage cells including mast cells, macrophages, granulocytes, DCs, and osteoclasts, but not on thymocytes, mature T cells, or NK cells [1, 2, 25, 26]. PIR-B expression takes place mostly in a pairwise fashion with PIR-A, an activating isoform associated with the Fc receptor common γ subunit (FcRγ), as judged on Western blot analysis [25]. In many cases, however, the cell-surface expression of PIR-B occurs more predominantly than that of PIR-A, as judged on flow cytometric analysis of PIR-B- or FcRγ-deficient cells with a specific monoclonal antibody (mAb), 6C1, that targets a shared epitope of PIR-B and PIR-A [10, 14, 25]. In mature naive B cells, PIR-B is exclusive on the surface [10]. The cell surface levels of PIR molecules on myeloid- and B-lineage cells increase with cellular differentiation and activation [25]. The PIR-B level is highest on marginal zone (MZ) B cells, and B-1 cells express higher PIR-B levels than B-2 cells [11, 25].

2.4. Unexpected Role in the Neuronal System. Recently, it was reported that PIR-B and one of its human orthologs, LILRB2, are expressed on neuronal cells [20, 21]. In addition, Atwal et al. [21] have identified three proteins expressed in the central nervous system as the novel physiological ligands for PIR-B and LILRB1. These are neurite outgrowth inhibitor protein (Nogo)A, myelin-associated glycoprotein (MAG or Siglec-4), and oligodendrocyte myelin glycoprotein (OMgp) [21]. This finding suggests a novel notion that PIR-B is not only a receptor of MHCI for immune regulation but also a determinant of neuronal regeneration responding to the multiple ligands in the central nervous system. These issues have recently been reviewed extensively elsewhere [27, 28].

3. Signal Regulation by PIR-B

3.1. Cis/Trans Binding to MHCI. Considering the ubiquitous expression of MHCI molecules on hematopoietic as well as nonhematopoietic cells, the question arises as to whether PIR-B can recognize MHCI on the same cells (in *cis*), on different cells (in *trans*), or both. It has been shown that inhibitory Ly49A expressed on murine NK cells not only binds to its H-2D^d ligand expressed on the potential target cells in *trans* but also is constitutively associated with H-2D^d in *cis* [29]. The *cis* association and *trans* interaction occur through the same binding site. Consequently, the *cis* association restricts the number of Ly49A receptors available for the *trans* binding of H-2D^d on target cells and reduces NK cell inhibition by lowering the threshold at which NK cell activation exceeds NK cell inhibition [30–32]. This notion, however, has not been verified to be the rule for every inhibitory receptor that binds to its physiological ligand expressed in the own tissues.

Our study on mast cell regulation by PIR-B in the context of allergic responses in *Pirb*$^{-/-}$ mice verified that PIR-B on mast cells binds to MHCI molecules in *cis*, as judged on fluorescence resonance energy transfer (FRET) analysis [14]. In addition, FRET revealed that the *cis* interaction was also the case for the interaction between MHCI and LILRB2 expressed on human basophilic leukemia KU812 cells. Interestingly, mast cell responses to stimulation by IgE crosslinking or lipopolysaccharides were suppressed to a significant extent by such a *cis* interaction on the mast cell surface. In both PIR-B and β2-microglobulin, its ligand, deficiency, both the in vitro cytokine responses and in vivo anaphylactic responses were significantly augmented, suggesting the physiological importance of the *cis* interaction [14]. Our analysis of osteoclast precursor cells revealed that the development of osteoclasts is also regulated by PIR-B and that this regulatory mechanism involves the *cis* interaction of PIR-B-MHCI on osteoclast precursor cells [26]. The *cis* interaction between PIR-B and MHCI was found also on other cell surfaces such as that of B cells and DCs [13].

PIR-B on DCs and MHCI on CD8$^+$ T cells were found to interact in *trans* at the immunological synapse. Moreover, CD8$^+$ T cells were found to be more activated upon interaction with *Pirb*$^{-/-}$ DCs than wild-type cells. This observation was unexpected, because it was known that *Pirb*$^{-/-}$ DCs could not support sufficient CD4$^+$ T cell activation toward Th1 development due to their insufficient maturation in PIR-B deficiency and reduced secretion of IL-12 [10]. Considering the possibility that MHCI molecules on DCs could play a role as a shared ligand for CD8 on T cells and for PIR-B on the DCs themselves, it would be interesting to test the hypothesis that CD8 and PIR-B could be competitive as to binding to MHCI on DCs, and it was demonstrated that this was the case [13]. Surface plasmon resonance analysis revealed that PIR-B and CD8$\alpha\alpha$ compete in binding to MHCI. In vitro and in vivo analyses also verified that *Pirb*$^{-/-}$ DC provoked cytotoxic T lymphocytes (CTLs) more efficiently, leading to accelerated rejection of skin grafts and tumors. Thus, PIR-B regulates CTL triggering by blocking the access of CD8 molecules to MHCI. It remains to be determined whether or not PIR-B on DCs is more prone to bind MHCI in *trans* on CD8$^+$ T cells than the binding in *cis* when an immunological synapse is induced between DC-CD8$^+$ T cells.

3.2. Immune Regulation. Studies on *Pirb*$^{-/-}$ mice during the past 10 years have provided us with an insight into

Table 1: Phenotypes observed in *Pirb*$^{-/-}$ mice and *Pirb* transgenic (tg) mice.

Mice	Cells[a]	Phenotypes[b]	References
Pirb$^{-/-}$	B-2 cells	Enhanced proliferation upon BCR stimulation	[10]
	B-1 cells	Enhanced proliferation and autoantibody production upon CpG stimulation	[11]
	DCs	Impaired maturation	[10]
		Exacerbated graft-versus-host disease	[12]
	CD8$^+$ T cells	Augmented activation	[13]
	Mast cells	Augmented anaphylaxis	[14]
	Macrophages	Augmented cytokine and chemokine signaling	[15]
	Neutrophils	Augmented integrin signaling	[16]
	Eosinophils	Enhanced recruitment in Th2 response	[17]
	Macrophages[c]	Sensitive to *Salmonella* infection	[18]
	Macrophages	Reduced binding of *Staphylococcus aureus*	[19]
	Neuronal cells	Enhanced plasticity of visual nerve connections	[20]
		Enhanced neurite outgrowth	[21]
Pirb tg	Thymocytes	Normal development	[22]
	Mature T cells	Impaired Th1 response	[22]

[a]Cells involved mainly in the observed phenotype.
[b]Major phenotypes observed.
[c]Unidentified, though involving macrophages.

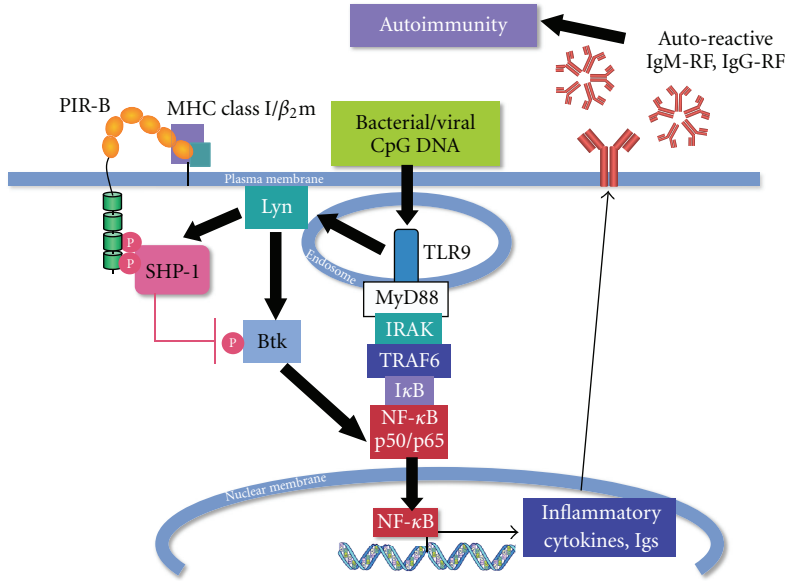

Figure 1: Proposed mechanism for PIR-B-mediated regulation of TLR9-induced autoantibody production in B-1 cells. Unmethylated CpG-harboring DNA of bacterial and viral origin stimulates TLR9 in the endosomal compartments of various cells including B-1 cells via signal relay from MyD88, IRAK, and TRAF6, and then IκB phosphorylation, nuclear translocation of NF-κB, and initiation of the transcription of various mRNAs of innate responses, including those for autoreactive natural antibodies. Excess activation of the TLR9 system is thus potentially harmful, because excessive production of autoreactive IgM and, particularly, that switched to IgG might cause autoimmune disease. TLR9 in B-1 cells is regulated by PIR-B via Btk intersection, in which the phosphorylation of PIR-B is augmented immediately through TLR9-initiated Lyn activation, and the concomitantly enhanced SHP-1 recruitment to the phospho-ITIMs of PIR-B leads to accelerated dephosphorylation of Btk, which then attenuates the phosphorylation level of NF-κB p65RelA. Constitutive association of PIR-B with MHCI in *cis* on the surface of B-1 cells may be crucial for maintaining the TLR9 cascade being not overactivated, and once activated by CpG, immediate early suppression will occur via augmented SHP-1 recruitment to PIR-B-MHCI.

the physiological significance of the MHCI recognition by PIR-B in the immune responses, especially in B cell activation, antigen presentation, humoral immunity, and transplantation (Table 1), most of which have already been described elsewhere [3–5]. This section deals with the recent findings on the PIR-B's functions in bacterial infection, eosinophil signaling, and T cell functions briefly, and in the next section we will focus on its roles in autoimmunity.

3.2.1. Eosinophil Function. Munitz et al. [17] described a positive and negative dual regulatory role of PIR-B in Th2 inflammation, in which PIR-B shows overexpression on eosinophils. Based on the different responses to eotaxin- or leukotriene B_4-dependent chemotaxis of $Pirb^{-/-}$ eosinophils, it was found that extracellular signal-related kinase 1 and 2 or Erk1/2 phosphorylation in response to these stimulators was opposite in eosinophils in vitro. It was proposed that an inhibitory receptor can exhibit dual functionality in distinct cell types after unique cellular signals [17]. This interesting hypothesis will have to be evaluated in other cellular and experimental settings as well as in other receptor systems, which might shift our idea of merely "inhibitory" receptors to more sophisticated "regulatory" ones.

3.2.2. T Cell Development and Function. PIR-B is expressed on immature and mature B cells, and myeloid cells but not on NK cells or T cells. However, besides the speculation that PIR-B should be under the control of B/myeloid-specific transcription factors, it has been unknown why T cells and NK cells preferentially use inhibitory coreceptors such as CTLA-4, PD-1, and KIRs, but not PIR-B. This issue should be considered how PIR-B expression is regulated during prethymic progenitor cells, thymocytes, and mature T cells, referring functional consequences when PIR-B is genetically deleted or expressed by force.

Masuda et al. [33] serendipitously found that PIR immunoreactivity was transiently detected on very early prethymic progenitors of T/NK/DCs, although PIR is not found on thymocytes or mature T cells on flow cytometry and immunohistochemistry with mAb 6C1 recognizing both PIR-A and PIR-B [25]. PIR expression was maintained until the earliest intrathymic stage and then downregulated before the onset of CD25 expression, which is a hallmark of the earliest stage of double negative thymocytes [33], suggesting that PIR-B expression should be down modulated during thymic development. Then, for what is it suppressed? The above findings prompted us firstly to define in more detail the cells at prethymic and intrathymic stages of T cell development in PIR-B deficiency. We found that PIR-B, not PIR-A, is the receptor expressed in the prethymic stage of T-lineage cells. We then examined the hypothesis that PIR-B is not only a regulator of early T cell development but is also detrimental if expressed on mature T cells [22]. Unexpectedly, analysis of the possible influence of PIR-B revealed no abnormalities in the development of immature and mature T cells when PIR-B was expressed ectopically at intrathymic stages or on peripheral T cells. Also, genetic depletion of PIR-B did not cause any obvious alterations in prethymic T progenitors. Interestingly, upon antigenic or allogeneic stimulation, however, peripheral T cells with ectopic PIR-B showed a reduced Th1 response, due to suppression of proximal TCR signaling by constitutive *cis* binding of PIR-B to MHCI on the same cell surface. These results suggest that T cell expression of PIR-B is strictly prohibited at the periphery so as to secure prompt immune responses in urgent situations such as infections by fast-multiplying bacteria. Although it remains to be determined why prethymic early T/NK/DC progenitors express PIR-B, it is possible that mature T cells select a transient inhibitory system like CTLA-4, whose expression is induced upon activation, instead of a constitutive inhibitory system such as PIR-B.

3.2.3. Bacterial Infection. The influence of PIR-B deficiency was examined in infections with bacteria such as *Staphylococcus aureus* and *Salmonella enterica* [18, 19]. $Pirb^{-/-}$ mice were more susceptible to *Salmonella* infection than wild-type C57BL/6 mice [18]. The susceptibility was in part due to the fact that $Pirb^{-/-}$ macrophages failed to control intracellular replication of *Salmonella* in vitro, possibly due to imbalance between PIR-A and PIR-B in the protective immune response to bacteria [18]. Nakayama et al. [19] found that PIR-B, and human LILRB1 and LILRB3 act as cell surface receptors for *S. aureus* and *Escherichia coli* among various pattern recognition receptors including Toll-like receptors (TLRs), scavenger receptors, and lectins expressed on the innate immune cell surface. In mouse bone marrow-derived macrophages, PIR-B masking by mAb or genetic deletion of PIR-B caused an enhanced TLR-mediated inflammatory response to bacteria [19]. It remains unclear whether such inhibition of TLR signaling by PIR-B is beneficial or harmful as to innate and adaptive responses to bacterial infections in vivo. Nonetheless, the observations in this study suggest that PIR-B plays multifaceted roles by recognizing multiple ligands, including bacterial cell surface component(s) in addition to MHCI and neuronal ligands. In line with these observations, Munitz et al. [34] found very recently that PIR-B has an inhibitory role in macrophage activation during intestinal inflammatory responses, such as dextran sodium sulfate-induced colitis, and in response to *E. coli*.

4. TLR9 Regulation in B-1 Cells and Autoimmunity

4.1. B-1 Cells and Natural Antibodies. Setting aside the reason for their expression, many inhibitory receptors are preferentially expressed on B cells. These include the IgG Fc receptor FcγRIIB [35, 36], a carbohydrate receptor, CD22 [37, 38], the CD100 receptor CD72 [39], a sialoglycoprotein receptor, Siglec-G [40], a carbohydrate receptor, CD45 [41], and the MHCI receptor PIR-B. CD45 is a phosphatase itself, and all other receptors described above recruit SH2 domain-containing tyrosine phosphatase (SHP)-1 and/or SHP-2, or inositol phosphatase (SHIP) upon stimulation [40].

In mice, there are four subsets of B cells. Two subsets of mature naive B cells termed follicular B cells or

conventional B cells ($B220^{+}CD23^{high}CD21^{low}$) and marginal zone (MZ) B cells ($B220^{+}CD23^{low}CD21^{high}$) are located primarily in secondary lymphoid organs such as the spleen [42]. Follicular B cells participate in the responses to T cell-dependent antigens and in the production of high-affinity IgG antibodies, while MZB cells participate in the response to blood-borne bacterial antigens. On the other hand, two other subsets of mature naive B cell populations reside mainly in the peritoneal and pleural cavities. These include B-1a ($B220^{low}IgM^{high}CD11b^{+}CD5^{+}$) and B-1b ($B220^{low}IgM^{high}CD11b^{+}CD5^{-}$) cells. Therefore, conventional B cells are often called B-2 cells. Although the precise roles of these B-1 cell populations are still not well understood, B-1b cells are mostly responsible for the adaptive immune responses to T-independent antigens and exert a memory function, while B-1a cells spontaneously produce natural IgM antibodies with broad specificities, thereby contributing to innate immunity [43, 44].

It has been suggested that innate B cells, including MZ B, B-1a, and B-1b ones, can be potentially harmful for maintaining peripheral tolerance, because B-1a cells, in particular, continuously produce large amounts of IgM natural antibodies weakly reactive to autologous tissues, but this remains controversial. The natural IgM antibodies can be switched to IgG, without BCR stimulation in response to pathogen-associated molecular patterns (PAMPs) such as bacterial cell wall components, and hypomethylated CpG motif-containing DNA of bacterial and viral origin [42, 45]. Some PAMPs are recognized by TLRs expressed on the cell surface or in endosomal compartments. Murine B cells of all subsets express several TLRs [42]. Therefore, excess stimulation of the TLR pathway in B cells such as in B-1a ones is supposed to be connected with provocation of autoimmunity. In fact, B-1 cells express a set of TLRs, including TLR4, TLR7, and TLR9 [42, 46]. Several lines of evidence indicate the importance of the regulation of TLR signaling in B-1 cells, which prevents over-stimulation of TLRs so as not to evoke overproduction of natural antibodies including potentially harmful autoantibodies [46, 47]. Such regulatory mechanisms for TLRs in B-1 cells may include inhibitory receptors, and we speculated that PIR-B could play a role here.

Interestingly, in $Pirb^{-/-}$ mice the peritoneal B-1a cell population significantly increases, particularly with aging, compared to in wild-type C57BL/6 mice [10]. However, it has not been determined how the B-1a cell compartment is regulated by PIR-B or what the physiological or pathological consequence of the expanded B-1a cells is in the contexts of infection and autoimmunity. We found that PIR-B on B-1 cells suppresses TLR9 signaling via Bruton's tyrosine kinase (Btk) dephosphorylation, which leads to attenuated activation of NF-κB p65RelA and blocks the production of natural IgM antibodies, including anti-IgG Fc autoantibodies or rheumatoid factor (RF) in particular [11].

4.2. Autoimmune Glomerulonephritis. Initially we observed that $Pirb^{-/-}$ mice were grossly normal and survived well, at least to 50 weeks of age, without any abnormalities in the histology of their tissues including glomeruli. However, $Pirb^{-/-}$ mice with the *lpr* mutation of Fas ($Pirb^{-/-}Fas^{lpr}$ mice) were markedly short lived, because of a deficit in renal function due to the development of glomerulonephritis with the depositing of IgG, IgM, and C3. While the levels of IgM- and IgG-RF were high in Fas^{lpr} mice, these levels were further elevated in $Pirb^{-/-}Fas^{lpr}$ animals. On the other hand, the serum levels of anti-dsDNA and -ssDNA autoantibodies were also high in Fas^{lpr} mice, but they were not elevated further in $Pirb^{-/-}Fas^{lpr}$ mice, indicating that PIR-B deficiency has a marked impact on the augmentation of RF production in vivo. The peritoneal B-1a cell and splenic plasma cell populations were also increased in $Pirb^{-/-}Fas^{lpr}$ mice. From these data, it was concluded that PIR-B deficiency in combination with Fas^{lpr} renders mice susceptible to the development of autoimmune glomerulonephritis, which is accompanied by significantly elevated RF production with robust production of other autoantibodies including those against self DNA.

4.3. TLR9 Signal in B-1 Cells. RF is one of the autoreactive natural antibodies reacting with the Fc portion of IgG, whose primary role is believed to be as the first line defense against infections. It is also a marker of rheumatoid arthritis and other connective tissue diseases such as systemic lupus erythematosus and infectious diseases. However, the pathogenicity of RF in autoimmune diseases has not been established sufficiently. Like other natural autoreactive antibodies such as those reactive with phosphatidylcholine, the main source of RF is considered to be B-1 cells [48]. To determine the precise source, the RF levels in sera from B6 and $Pirb^{-/-}$ mice were examined in the absence of Fas^{lpr}, which might have a great influence on the RF level.

Given the augmented RF production and enlarged B-1a cell population in $Pirb^{-/-}Fas^{lpr}$ mice, our attention next turned to the characteristics of $Pirb^{-/-}$ B-1 cells. $Pirb^{-/-}$ B-1 cells proliferated particularly well upon stimulation with CpG (TLR9 ligand) compared to wild-type ones, indicating an important role of TLR9 in B-1 cell activation and its regulation by PIR-B. Total IgM, IgM-RF, and IL-10 release was also more elevated in $Pirb^{-/-}$ B-1 cells than in wild-type ones. While B-1a and B-1b cells express PIR-B molecules more abundantly on their surface than B-2 cells [25], the expression of PIR-A, an activating counterpart of PIR-B, was not detected on B-1a cells [11], suggesting the presence of a PIR-B-mediated inhibitory signaling that specifically regulates TLR9.

Like in mast cells and splenic B cells stimulated by IgE receptor or BCR crosslinking, respectively, CpG induced augmented PIR-B phosphorylation in B-1 cells, which was accompanied by an increase in SHP-1 recruitment, whereas such events were not observed in TLR9-deficient cells, which led the conclusion that CpG-mediated PIR-B phosphorylation occurs downstream of TLR9 [11]. Preceding studies indicated that, in splenic B cells, Lyn is the major Src family kinase that phosphorylates PIR-B [49]. However, it is not known whether or not this is also the case in peritoneal B-1 cells. The phosphorylation level of Lyn was

indeed augmented as early as 5 min after CpG stimulation of B-1 cells regardless of the presence or absence of PIR-B. However, augmented phosphorylation of Lyn was not observed in TLR9-deficient B-1 cells. Also, Lyn and PIR-B were co-immunoprecipitated from a macrophage lysate after stimulation with CpG. These results indicate that Lyn is rapidly activated downstream of TLR9 and strongly suggest that Lyn is a candidate, if not the sole, Src family kinase that phosphorylates PIR-B.

What are the downstream signal cascades, such as the NF-κB and MAP kinase pathways, of TLR9 modulated by PIR-B in B-1 cells? MAPK and Erk were significantly augmented after CpG stimulation, but their levels and kinetics were comparable in normal and *Pirb*$^{-/-}$ B-1 cells. On the other hand, p65 NF-κB phosphorylation was found to be markedly augmented in *Pirb*$^{-/-}$ B-1 cells after CpG stimulation, indicating that the NF-κB pathway could be the target step located most downstream of regulation through Lyn-mediated PIR-B phosphorylation (Figure 1).

Then, which molecule mediates the PIR-B-SHP-1-initiated inhibitory signaling pathway to the TLR9-NF-κB cascade? In a B cell line, IIA1.6 cells, Syk and Btk were found to be the substrates for SHP-1 recruited to PIR-B ITIMs upon stimulation of BCR [50]. Examination of the possible involvement of these two molecules in the TLR9 pathway revealed that the phosphorylation status of Syk upon CpG addition to B-1 cells did not show enhancement of the phosphorylation, while Btk phosphorylation was markedly augmented after CpG addition in both wild-type and *Pirb*$^{-/-}$ B-1 cells, and the augmentation was higher in *Pirb*$^{-/-}$ B-1 cells, indicating that Btk is a major substrate for SHP-1 (Figure 1). These results indicate that Btk phosphorylation is dependent, at least partly, on a Src family kinase, most likely Lyn, and that NF-κB p65RelA phosphorylation is also a critical event downstream of Src family kinase activation. The molecular mechanism for the TLR9-initiated Lyn activation is currently not known. Although SHP-1 is a critical regulator of type I interferon production by macrophages and DCs through inhibition of IRAK1 activation downstream of TLR4 [51], augmented IRAK1 activation was not observed in CpG-B stimulated *Pirb*$^{-/-}$ B-1 cells [11], suggesting that B-1 cells preferentially utilize Btk as a key intersecting molecule for the inhibitory circuit (Figure 1).

Recent studies have shown that Btk is an important mediator of the TLR9 cascade in addition to MyD88 and TRAF [52, 53]. Btk is required for NF-κB activation, participating in the pathway leading to increased phosphorylation of p65RelA activated by TLR8 and TLR9 [53, 54]. As is well known, Btk is a critical kinase for the development and function of B cells including B-1 cells by ensuring BCR signaling, as has been demonstrated in immunocompromised *Xid* mice and human X-linked agammaglobulinemia, in which Btk is dysfunctional [55, 56]. Btk deletion in mice induced defective development of B cells including B-1 ones and their function [57]. The finding that Btk is a critical link between the TLR9 cascade and the PIR-B-mediated regulatory loop, particularly in B-1 cells expressing abundant PIR-B [11], provides an insight into the mechanism underlying the crosstalk between the innate immune system and ITIM-harboring receptors.

4.4. Autoimmunity and PIR-B: Questions Remaining. How are the observations on TLR9 signaling in B-1 cells relevant to the prevention of autoimmunity? In this context, several feedback regulators for TLR9 activation have been identified. These include SOCS1 [58], ATF3 [59], IRF-4 [60], and SHP-1 [51]. For example, SOCS1-mediated feedback regulation seems to occur after several hours of TLR9 activation followed by inflammatory cytokine induction [58]. It is very characteristic that the PIR-B-mediated suppression of the TLR9 cascade takes place at several minutes after CpG addition to B-1 cells. Therefore, PIR-B in the TLR9 of B-1 cells does not seem to be a feedback regulatory element but an immediate-early regulator just like the one in mast cell regulation after IgE receptor crosslinking [14, 61] and in B-2 cells upon BCR stimulation [10, 62, 63].

Can RF produced by B-1 cells trigger or exacerbate glomerulonephritis in *Pirb*$^{-/-}$*Fas*^{lpr} mice? CD5^{+} B-1 cells or B-1a cells are considered to be involved in some autoimmune diseases in which RF is frequently detected or RF production is directly coupled to the disease [64–66]. Also, RF^{+} B cells are effectively activated via the IgG2a-chromatin immune complex and synergistic stimulation of TLR9-mediated signaling [67]. It is additionally known that RF can bind to anti-dsDNA autoantibodies and form immune complexes [68]. Also, it has been reported that RF itself exhibits cryoglobulin activity and causes glomerulonephritis without any other additional factors [69]. Therefore, glomerulonephritis in *Pirb*$^{-/-}$*Fas*^{lpr} mice exhibiting markedly and specifically elevated IgM- and IgG-RF production suggests that these anti-IgG Fc autoantibodies worsen the disease, presumably by accelerating the deposition of IgG autoantibodies against glomerular basement membranes.

Do other TLRs, such as TLR4 and TLR7, in B-1 and B-2 cells possess a similar regulatory system involving PIR-B to that of TLR9? In this regard, it is of note that TLR4-mediated cytokine production by mast cells is also negatively regulated by PIR-B [14], although the mechanism has yet to be elucidated. Therefore, it is likely that some TLR-mediated activation cascades in B cells could be linked to the PIR-B-SHP-1-mediated suppression in addition to the case of TLR9. On the other hand, TLR4 response in macrophages was not affected by PIR-B [19, 34]. The reason why TLR4-induced responses are regulated differentially in the differential cell populations is not known currently. However, one may speculate that different cells may be differently dependent on any TLR4-initiated, PIR-B-mediated regulatory pathway such as the Lyn-PIR-B-SHP-1-Btk axis identified in B-1 cells [11]. Such regulation of an innate immune response via TLRs by constitutive and activation-dependent immediate-early suppression of PIR-B could be promising for preventing autoimmunity.

5. Concluding Remarks

In recent studies on *Pirb*$^{-/-}$ mice, there were two important observations. Firstly, the TLR9 system in B-1 cells is

regulated by PIR-B via the Btk intersection, at which PIR-B phosphorylation is immediately augmented by TLR9-initiated Lyn activation, and the concomitant enhancement of SHP-1 recruitment to augmented phospho-ITIMs of PIR-B leads to Btk dephosphorylation, which then attenuates the phosphorylation level of NF-κB p65RelA. Secondly, excessive production of RF together with other autoantibodies by B-1 cells can be linked to glomerulonephritis with IgG immune complex deposition. Considering these observations, it is becoming increasingly important for manipulation of the PIR-B-mediated inhibitory system in the regulation of autoimmune disease while maintaining the integrity of TLR9-mediated microbial sensing, in such cases as rheumatoid arthritis. Understanding of the PIR-B system in B-1 cells may lead to the development of novel and effective ways of controlling autoimmune diseases.

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