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Inhibitory receptors on myeloid cells: New targets for therapy?

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ABSTRACT

Immune regulation of cellular activation is a tightly regulated process dictated by a balance of activation and inhibitory signals. Although initially described and characterized on natural killer cells, it has become increasingly apparent that inhibitory receptors are expressed and functional on myeloid cells. These receptors can override signals elicited by activation pathways including cytokine and chemokine receptors, growth factor signaling and more recently innate immune receptor signaling. Inhibitory receptors have key roles in various cellular and pathological processes and are thus potential targets for future therapeutics. In this review, the structure and function of inhibitory receptors will be discussed. Furthermore, utilization of these receptors as pharmacological targets and recent examples of strategies targeting inhibitory receptors will be analyzed.

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Contents

1. Introduction

An intricate network of positive and negative signals mediates regulation of immune responses (Ravetch & Lanier, 2000). These signals are the basis for immune cell homeostasis in health and disease and are often achieved at the cellular level through a combination of signals from cell surface receptors, intracellular signaling intermediates and gene transcription (Daeron et al., 2008).

Historically, studies on the immune system have been largely concentrated on pathways that activate immune cell function in response to antigens and pathogens. However, over the last two decades it has become increasingly apparent that in addition to pathways that stimulate immune cell activation, a counter regulatory system exists that can restrain their action and thereby promote cellular homeostasis (Cooper, 2008). These suppressive signals can regulate

various checkpoints in immune cell function including differentiation, maturation, adhesion, chemotaxis and mediator release.

In 1998, Lanier first set the term "inhibitory receptor superfamily" in his description of receptors that suppress activation of natural killer (NK) cells (Lanier, 1998). The "classical" view of inhibitory receptors is that these receptors inhibit cell activation in *trans*, contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and bind phosphatases for their inhibitory function (Lanier, 1998). In addition, they are capable of inhibiting multiple activation pathways albeit require interaction with an activating receptor (Fig. 1).

Much of the data on inhibitory receptor function has emerged from studies on NK cell function or B and T cell receptor signaling (Moretta et al., 1996; Lanier, 1998; Ravetch & Lanier, 2000; Campbell & Colonna, 2001; Moretta et al., 2002). However it has become noticeable that myeloid cells including macrophages, dendritic cells, neutrophils, eosinophils and mast cells express functional inhibitory receptors that are likely to have key roles in the function of these cells, particularly in settings where these cells are recruited and activated (Munitz & Levi-Schaffer, 2004; Kanazawa et al., 2004; Katz, 2007; Munitz & Levi-Schaffer, 2007). The focus of this review will be on the expression and function of inhibitory receptors on myeloid cells. Basic structure and mechanism of action will be discussed and lessons from in vitro and in vivo studies will be illustrated. Finally, utilizing inhibitory receptors as pharmacological targets for immune suppression will be presented and discussed.

2. Structure of inhibitory receptors

Inhibitory receptors can be broadly divided into two groups, belonging either to the immunoglobulin (Ig) superfamily receptors (IgSR) or the C-type lectin inhibitory receptors (Lanier, 1998; Ravetch & Lanier, 2000; Robinson et al., 2006). The IgSR is characterized by a single V-type Ig-like domain in the extracellular portion and may contain various C-type domains. Myeloid cells express various IgSR including leukocyte immunoglobulin-like receptors/immunoglobulin-like transcript (LIRs/ILTs) (Cella et al., 1997), Leukocyte-associated immunoglobulin-like receptor (LAIR) (Meyaard et al., 1997), gp49B1 (Katz, 2007), inhibitory receptor protein 60 (IRp60)/CD300a (Clark et al., 2009) and sialic acid binding Ig-like lectins (Siglecs) (Crocker et al., 2007) (see Table 1). The term "C-type lectin" indicates a calcium-dependent carbohydrate-binding protein motif, yet many C-type lectin inhibitory receptors posses no obvious calcium binding or carbohydrate specificity (Kanazawa et al., 2004; Robinson et al., 2006). Thus, this term is a structural rather than functional term. The C-type lectin inhibitory receptors also include proteins with a structural domain that was originally identified as a protein fold in the carbohydrate-recognition domain of mannose-binding lectin (Robinson et al., 2006). The prototypical C-type lectin inhibitory receptor is the NK receptor NKG2/CD94 or Ly49 (Held et al., 1996; Brooks et al., 1997). However, myeloid cells express various C-type lectin inhibitory receptors such as mast cell function-associated antigen (MAFA), dendritic-cell-associated C-type lectin 2 (DCAL-2) (Chen et al., 2006) and dendritic cell inhibitory receptor (DCIR) (Kanazawa et al., 2004) (Table 1).

3. Inhibitory signaling: mechanism

The prototype immune inhibitory receptor (either an IgSR or C-type lectin) can be identified by a consensus amino acid sequence, the ITIM, which is present in the cytoplasmic domain of these receptors. The ITIM sequence is composed of 6 amino acids (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/Val), where X represents any amino acid. Inhibitory receptors can express either one or several ITIM domains. Upon engagement with their ligands, the ITIM motifs undergo tyrosine phosphorylation (often by a Src-family kinase), which provides a docking site for the recruitment of cytoplasmic phosphatases having a Src homology 2 (SH2) domain such as SH2-containing phosphates 1 (SHP-1), -2 and SH2-containing inositol phosphatase (SHIP-1) (Long, 1999, 2008) (Fig. 1). These phosphatases are perceived to dephosphorylate (and therefore elicit inhibition) tyrosine residues that provide docking sites for signaling kinases that are recruited by activation receptors. In general it is perceived that optimal inhibition will be achieved with maximal ITIM phosphorylation. Notably, the majority of inhibitory receptors recruit the tyrosine phosphatases SHP-1 or -2 but not the lipid phosphatase SHIP with the exception of FcγRIIB, which primarily recruits SHIP but not SHPs (Fong et al., 1996). In addition, and unlike the majority of immune inhibitory receptors, the inhibitory receptor CD200 lacks an ITIM but contains an NPXY motif in its cytoplasmic domain and can bind proteins with phosphotyrosine-
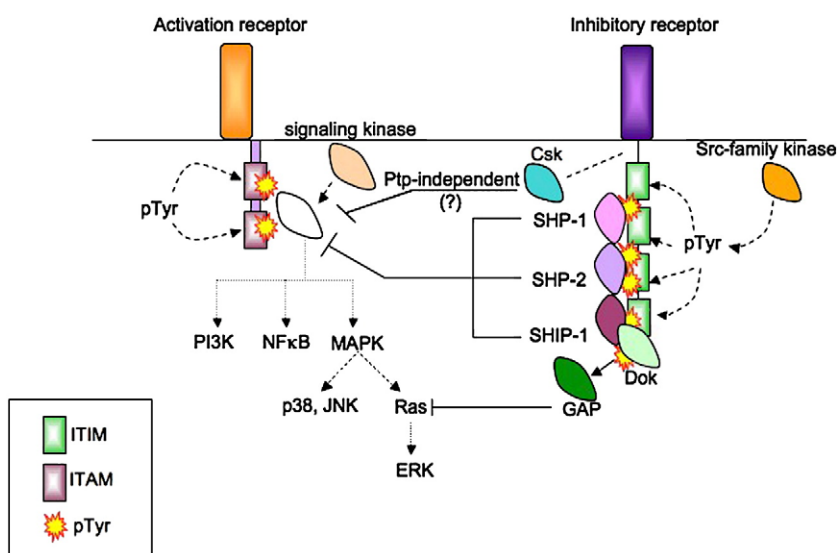
Fig. 1. The mechanism of inhibitory receptor function. Upon binding to their ligand, inhibitory receptors recruit a Src-family kinase (e.g. Lyn, Fgr, Hck) that rapidly phosphorylates their intracellular ITIM domain. Consequently, SH₂-containing phosphatases such as SHP-1, -2 and SHIP bind the receptor and are capable to suppress proximal activation signals mediated by signaling kinases that are recruited to activation receptors (either via ITAMs or independent of ITAMs). Alternatively, several inhibitory receptors can recruit Csk that will suppress immune cell activation independent of SHP binding. In addition, inhibitory receptors can recruit and activate the phosphotyrosine-binding adaptor molecules such as Dok. These can either bind SHIP and mediate inhibition through SHIP activation or independently bind GAP to inhibit the RAS:ERK pathway, Ptp-phosphatase.

Table 1
Expression pattern of inhibitory receptors on myeloid cells.

Inhibitory receptor	Alternative name	Ligand	Myeloid cell expression[a]
Ig-superfamily receptors			
FcγRIIB	CD32B	IgG	MC, Mono, Mac, E, N, DC
ILTs/LIR family			
ILT-2/LIR-1	CD85j		Mono, Mac, DC
ILT-3/LIR-5	CD85k		Mono, Mac, DC
ILT-4/LIR-2	CD85d		Mono, Mac, DC, N
ILT-5/LIR-3	CD85a		Mono, Mac, DC, N, E, B
Gp49B1	LILRB4		MC, Mono, Mac, DC, N, E
PIR-B	LILRB3		MC, Mono, Mac, DC, N, E
LAIR-1	CD305	Collagen	Mono, Mac, DC
Sirp-1α		CD47, SP-A, SP-D	
PILRα		Herpes Simplex Virus 1	Mono
CD200			MC, Mono, Mac
CD300 family			
CD300a	LMIR-1, CLM-1		MC, Mono, Mac, DC, N, E
CD300f	CLM-8		MC, Mono, Mac, DC, N
Inhibitory siglecs			
Human		Sialic acid	
CD33	Siglec-3	"	Mono, Myop
Siglec-5	CD170	"	N, Mono, B
Siglec-6	CD327	"	B
Siglec-7	CD328, p75/AIRM	"	Mono, E
Siglec-8		"	MC, E
Siglec-9	CD329	"	Mono, Mac, N
Siglec-10		"	Mono, E
Siglec-11		"	Mono, Mac
Mouse			
CD33		"	Mono, Mac, N, E
Siglec-E		"	N, Mono, Mac, DC
Siglec-F		"	E
C-type lectin receptors			
DCIR	CLEC4A		DC, Mac, N
DCAL-2			DC
MAFA			MC

This table represents a partial list only of receptors mentioned in this review.
MC—mast cell, Mono—monocyte, Mac—macrophage, DC—dendritic cell, N—neurophil, E—eosinophil, B—basophi, MyoP—myeloid progenitor celll.
[a] The expression data has not been reported for all myeloid cell types.

binding domains (PTBs) such as downstream of kinase (Dok) family members (Barclay et al., 2002; Zhang & Phillips, 2006). Interestingly, Dok family members have been shown to interact with SHP and SHIP molecules and inhibit rat sarcoma (Ras)-Extracellular signal-regulated kinase (Erk) mediated activation (Berg et al., 1999; Robson et al., 2004; Zhang & Phillips, 2006) (Fig. 1).

It is important to note that despite the consistent findings of inhibitory receptors binding SH2 containing phosphatases (i.e. SHP-1, SHP-2, SHIP-1), the precise inhibitory mechanism is not fully understood. Two of the main questions regarding the inhibitor mechanism are:

1) Is there a differential role for SHP-1 and SHP-2 mediated inhibition?
2) Is the inhibitory pathway dependent of phosphatase activity and if so to what extent?

Rationalizing these queries are a few examples. For instance, SHP-1 rapidly associates with the inhibitory receptor gp49B1 after co-ligation of gp49B1 with FcεRI in mast cells. Yet, SHP-1-deficient mast cells exhibited only a partial loss of gp49B1-mediated inhibition of exocytosis, revealing a central, but not exclusive, SHP-1 requirement in the counter-regulatory pathway, which may be SHP-2-dependent (Lu-Kuo et al., 1999). Furthermore, recent studies utilizing a unique structural approach revealed that differential binding of SHP-1 might have a distinctive inhibitory outcome than recruitment and binding of SHP-2. This analysis uncovered that SHP-1 is most active when actually bound to the ITIM receptor whereas SHP-2, which possesses a longer

spacing between its two C-terminal tyrosines, can be active even when unbound to the ITIM (Lu et al., 2001; Zhang et al., 2003). Thus, specific recruitment and consequent binding of phosphatase to the intracellular ITIM may govern the extent and character of inhibition that is elicited by inhibitory receptors.

In addition to the aforementioned studies, recent data highlight a possibility for ITIM-bearing receptors to inhibit even in the absence of SHPs. For example, upon phosphorylation, LAIR-1 can bind the c-Src tyrosine kinase (Csk), which is a negative regulator of Src family kinases and inhibit B cell receptor induced activation even in the absence of SHP-1 and -2 (Verbrugge et al., 2006). The binding of inhibitory receptors to Csk and consequent SHP-independent inhibition is likely a shared phenomenon between inhibitory receptors as the ITIM of immunoglobulin-like transcript (ILT) 2 and signal-regulatory protein α (SIRP-1α) can also bind Csk (Fig. 1) (Sayos et al., 2004). In addition, negative adaptor molecules such as Dok family members may also mediate inhibition that is either mediated via SHIP/SHPs or independently through direct binding of Dok to Ras GTPase activating protein (RasGAP) (Berg et al., 1999; Robson et al., 2004; Zhang & Phillips, 2006).

The simplistic and traditional view of inhibitory receptor function described the ITIM as an opposing signal to immune receptor tyrosine-based activation motif (ITAM) phosphorylation (Long, 2008). These motifs are present on various adaptor molecules including DNAX activation protein 10 (DAP10) and DAP12 as well as on the Fcγ chain (Wu et al., 2000). Nevertheless, recent data demonstrating negative regulation of inhibitory receptors towards receptors that do not contain ITAMs indicate a broader inhibitory capacity for these receptors (Sinclair, 1999; Dietrich et al., 2000; Brown, 2005). In fact, inhibitory receptors are relatively outnumbered by activation pathways (including cytokine/chemokine receptors, pattern recognition receptors, adhesion molecules, Fc receptors, complement receptors, co-stimulatory molecules, etc.) and a single inhibitory receptor can suppress multiple pathways. For example, CD300a was found to inhibit both G-protein coupled receptor signaling and cytokine receptor signaling (Munitz et al., 2006a). PIR-B can inhibit chemokine receptor activation, integrin signaling as well as innate immune receptor mediated-responses (Pereira et al., 2004; Zhang et al., 2005; Nakayama et al., 2007; Munitz et al., 2008; Torii et al., 2008). Thus, suggesting a relative dominance of inhibitory signaling over activation. Furthermore, this also suggests redundancy as one ITIM-bearing receptor can regulate multiple activation pathways.

4. Ligands for inhibitory receptors

Assuming such an important role for inhibitory receptors in cellular homeostasis, one would predict that a) the ligands for inhibitory signaling would be widely expressed and b) each cell type can express various inhibitory receptors with redundant functions. Early studies in the NK and cytotoxic T cell field have illustrated the "missing self" hypothesis in which NK inhibitory receptors recognize a repertoire of major histocompatibility complex (MHC) class I molecules that prevent cellular activation upon recognition with a "self" cell (Colonna, 1996; Held et al., 1996; Moretta et al., 1996; Lanier, 1998; Ravetch & Lanier, 2000). However, upon viral infection or acquiring a malignant phenotype, MHC molecules are significantly downregulated and therefore the cytotoxic cell "loses" its inhibition thus becoming activated by the activation receptors that are present on its surface. While this paradigm is well understood for cells that are specialized in cell-mediated cytotoxicity, the biological rationale for MHC recognition by inhibitory receptors on myeloid cells is not quite clear (Sinclair, 1999).

As characterization of inhibitory receptors on myeloid cells expanded it became evident that another class of inhibitory recognition that is independent of MHC binding is present on myeloid (and also NK) cells (Lebbink & Meyaard, 2007). For example, the

ligand for gp49B1 has been reported to be the $\alpha V \beta_3$ integrin and collagens have been shown to serve as functional, high affinity ligands for the inhibitory immune receptor LAIR-1 (Castells et al., 2001; Lebbink et al., 2006). Interestingly, even paired immunoglobulin-like receptor B (PIR-B), which was originally identified as a myeloid-specific inhibitory receptor that binds MHC class I molecules, has been recently shown to recognize and bind various bacteria (Takai, 2005; Masuda et al., 2007; Nakayama et al., 2007). Substantiating a role for non-MHC-restricted inhibitory signaling are also recent reports demonstrating that the inhibitory receptor paired immunoglobulin-like type 2 receptor alpha (PILRα) recognizes CD99, which is widely expressed on all leukocytes (Shiratori et al., 2004). Furthermore, ligand(s) for other inhibitory receptors such as IRp60/CD300a are yet to be defined. In fact, CD300a was considered at one point to be a potential NK cell inhibitory molecule, but it failed to bind to HLA-class I molecules (Cantoni et al., 1999; Clark et al., 2009), making it unlikely that MHC class I molecules are its natural ligand. Future studies defining the ligands of myeloid-associated inhibitory receptors are likely to contribute to our understanding of the physiological role for such receptors in immune regulation.

5. Inhibitory receptors: function

All of the abovementioned evidence suggests a broad role for inhibitory receptors in suppressing myeloid cell function. Cells from the myeloid lineage including macrophages, neutrophils and eosinophils respond to different cytokines, chemokines and express differential receptors. Nevertheless, the main pathway of myeloid cell recruitment into the inflamed tissue is similar and may be divided into a few checkpoints (Fig. 2). In general, myeloid cells originate in the bone marrow where they undergo initial differentiation and proliferation and thereafter enter the blood stream. During an inflammatory process, chemokines are secreted which induce the recruitment of these cells into the inflamed area where they are further stimulated, can secrete their specific mediators and/or interact with other cells (Rothenberg, 1998; Gordon & Taylor, 2005). This paradigm highlights multiple checkpoints, which can be potentially regulated by inhibitory receptors and will be discussed below.

6. Negative regulation of myeloid cell differentiation, proliferation and survival by inhibitory receptors

Hematopoeisis is a multi-step process in which the development of each cell along the different cell lineages occurs as a consequence of a sequential effect of growth factors and transcription factors. In this process, the cells can acquire or lose expression of cell surface molecules including lineage markers and inhibitory receptors. For example, the expression of CD33, an inhibitory receptor belonging to the siglec family of receptors, corresponds with the cell commitment towards the myelomonocytic precursors (Dexter & Spooncer, 1987). In fact, CD33 expression is maintained in the myeloid and monocytic lineage, whereas it is lost in mature neutrophils (Vitale et al., 1999, 2001). CD33 is homologous to siglec-7, which is also highly expressed by cells of the myeloid lineage. Flow cytometeric analysis of the expression of CD33 and siglec-7 on cord blood derived CD34$^+$ cells that have been cultured in the presence of stem cell factor (SCF) and granulocyte-macrophage colony stimulating factor (GM-CSF) revealed that all CD34$^+$ cells steadily expressed CD33 as early as day 4 in culture. On the other hand and under similar conditions, siglec-7 expression was gradually acquired and only 50% of the cultured cells expressed siglec-7 by day 10 under the same conditions (Vitale et al., 1999, 2001). Despite differential kinetics in expression, antibody cross-linking of siglec-7 or CD33 were capable to suppress in vitro proliferation of myeloid cell precursors. In fact, cross-linking of both receptors inhibited the proliferation of leukemic cells obtained from chemotherapy-free patients with chronic myeloid leukemia (Vitale et al., 1999, 2001). While this may suggest a broad non-specific capability of inhibitory receptors to suppress proliferation, this does not appear to be the case since CD300a/IRp60, which is expressed in a similar fashion to CD33, does not inhibit CD34$^+$ myeloid cell proliferation or chronic myelogenous leukemia (CML) proliferation (Mingari et al., 2001). Interestingly, examination of the effects of all
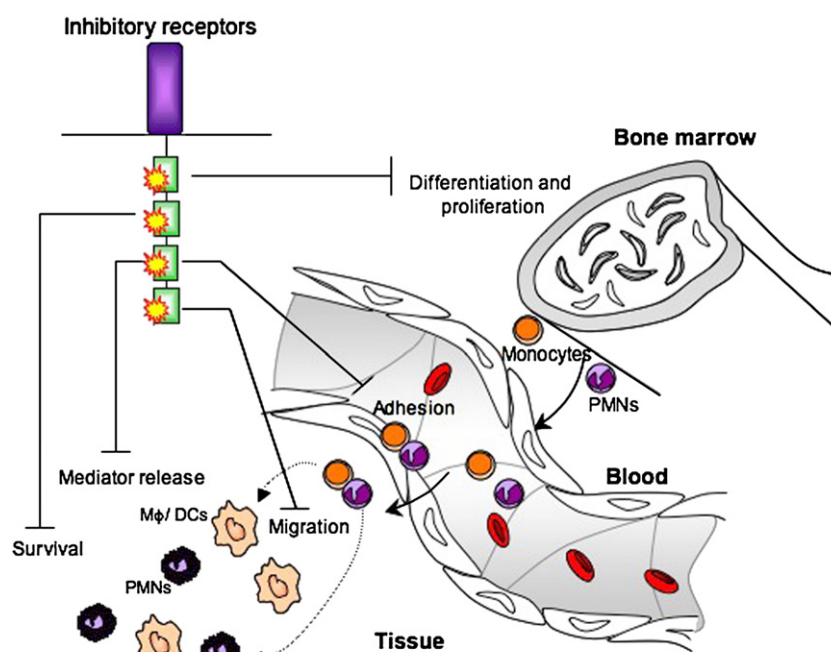
Fig. 2. Cellular checkpoints regulated by inhibitory receptors. Myeloid cells including polymorphonuclear cells (PMN) such as neutrophils and eosinophils and monocytes originate in the bone marrow and enter the blood. Upon inflammatory stimulus or tissue injury these cells transmigrate to the tissue through a process of adhesion and chemotaxis. In the tissue, and under the influence of local cytokines monocytes undergo final maturation to macrophages or dendritic cells (MΦ and DC). All of the aforementioned cellular processes can be regulated by inhibitory receptors.

three receptors (i.e. CD33, siglec-7 and CD300a/IRp60) on the proliferation of acute myeloid leukemic cells revealed that siglec-7 acts to suppress acute myelogenous leukemia (AML) cell proliferation whereas CD33 induces their apoptosis and cross-linking of CD300a/IRp60 has no effect (Vitale et al., 2001; Mingari et al., 2001).

Another line of evidence supporting a role for inhibitory receptors in myeloid cell proliferation and apoptosis arise from studies conducted on LAIR-1. Similar to the previous inhibitory receptors discussed above, engagement of LAIR-1 prevents GM-CSF-induced proliferation of AML cell blasts. LAIR-1 activation blocked the cells in the G_0/G_1 phase of cell cycle and the cells underwent apoptosis within 4 days of LAIR-1 activation (Zocchi et al., 2001). Mechanistically, this latter phenomenon was associated with inhibition of GM-CSF-induced Ca^{2+} mobilization and activation of the AKT1/protein kinase B α (PKBα) pathway (Zocchi et al., 2001). It is important to note that LAIR-1 was capable of inducing its inhibitory effect even on AML blasts that do not express CD33. Thus the inhibitory activity of LAIR-1 is CD33-independent.

Although cells obtained from myeloid leukemia provides a good experimental system to dissect the role of inhibitory receptors on cell proliferation, apoptosis and survival, an important question is whether they can influence these parameters in non-proliferating cells such as mature eosinophils or neutrophils. Recently it has been shown that activation of siglec-8 on human eosinophils by means of antibody cross linking suppressed their survival by initiating mitochondrial injury, reactive oxygen species (ROS) generation, rapid cleavage of caspase-3, -8 and -9 and inducing apoptosis (Nutku et al., 2003, 2005). This effect was even further enhanced in the presence of interleukin (IL)-5, IL-3 and GM-CSF, the hallmark eosinophil "survival cytokines" which seem to prime siglec-8 activity and diminish the need for secondary antibody cross-linking (Nutku et al., 2003; Nutku-Bilir et al., 2008). In contrast to the observations with siglec-8, which actively induced eosinophil apoptosis, cross-linking of CD300a/IRp60 on human eosinophils suppressed their survival in response to the same "survival cytokines" (Munitz et al., 2006a). As such, cross-linking of CD300a/IRp60 inhibited IL-5 and GM-CSF signaling and thus suppressed survival signals rather than actively induce apoptosis.

Supporting this notion was the finding that the inhibitory effect of CD300a/IRp60 was only seen in the presence of IL-5 or GM-CSF and quite interestingly the ability of CD300a/IRp60 to suppress eosinophil activation was proportional to cytokine concentration. Thus, increased concentration of IL-5/GM-CSF enhances the inhibitory effect (Munitz et al., 2003). In the case of CD300a/IRp60 the inhibitory process is likely due to a crosstalk between CD300a/IRp60 and the IL-5/GM-CSF/IL-3 common β chain and involves the recruitment of intracellular phosphatases and consequent inhibition of Janus kinas (JAK) 2 phosphorylation (Munitz et al., 2006a). Unlike CD300a, CD300LF (an additional CD300-inhibitory receptor belonging to the CD300 family of IgS receptors) has been recently shown to actively induce cell death in macrophages in an ITIM-independent caspase-independent fashion (Can et al., 2008). Thus, the molecular basis for the negative regulation of survival by inhibitory receptors may not be dependent on ITIM-activity.

Similar to siglec-8 on eosinophils, siglec-9 ligation can induce neutrophil apoptosis. Interestingly, neutrophils obtained from patients with acute septic shock or rheumatoid arthritis demonstrated increased siglec-9 expression and activity. Mechanistically, siglec-9 was capable to induce an apoptotic (ROS- and caspase-dependent) and non-apoptotic (ROS-dependent) death pathways (von Gunten et al., 2005). The findings that cytokines and/or disease state can increase the inhibitory capability of such receptors require further investigation.

Inhibitory receptors can also negatively regulate myeloid cell differentiation. The potent activity of these receptors in this process may be illustrated by recent observations demonstrating a key role for PIR-B and CD300LF in osteoclast formation and development (Chung et al., 2003; Mori et al., 2008). Both PIR-B and CD300LF

were downregulated in receptor activator for nuclear factor κ B ligand (RANKL)- and macrophage colony-stimulating factor (M-CSF)-induced osteoclast formation. In fact, deletion of PIR-B lead to accelerated osteoclastogenesis and forced expression of CD300LF prevents osteoclastogenesis induced by RANKL and transforming growth factor (TGF)-β1 (Chung et al., 2003; Mori et al., 2008).

Of note, the aforementioned LAIR-1 was implicated in myeloid cell differentiation as well. In vitro studies have shown the LAIR-1 was capable to inhibit GM-CSF-induced monocyte differentiation into dendritic cells. Interestingly, this effect was specific to LAIR-1 since stimulation of FDF03, a highly similar inhibitory receptor, had no effect (Fournier et al., 2000). Collectively, these data suggest non-redundant roles and mechanisms for inhibitory receptor function in the regulation of myeloid cell proliferation, survival and differentiation. Nevertheless, there is currently limited amount of data regarding the functions of these receptors in bone marrow-derived cells and addressing their function in vivo.

7. Inhibition of cell migration

Being the sentinels of host defense, myeloid leukocytes respond to perturbations in immune homeostasis by rapid mobilization into the site of injury or infection. The directed migration of these cells is often coordinated in response to chemokines. These small (~10–20 kDa) secreted proteins induce the migratory effect through interactions with heterotrimeric GPCRs on the responding cell. In response to chemokine stimulation, GPCRs induce a signaling cascade that is accompanied by Ca^{2+} mobilization and activation of Ras:ERK-dependent pathways (Cotton & Claing, 2009). These events are often associated with interactions of the GPCR with Src-family kinases (Rozengurt, 2007). Recent data indicate a direct cross talk between GPCR signaling and inhibitory receptor signaling. Zhang et al., have shown that the kinases feline sarcoma viral (Fgr) and hemopoietic cell kinase (Hck), which are responsible for the baseline ITIM phosphorylation of the inhibitory receptor PIR-B act as negative regulators of neutrophil and dendritic cell migration (Zhang et al., 2005). While the exact mechanism is not fully understood, it appears that under baseline conditions, the ITIMs in PIR-B are phosphorylated by Hck and Fgr leading to association of PIR-B with SHP-1 and -2 and subsequent dephosphorylation of yet unknown targets leading to cell arrest. Upon binding of chemokines to GPCRs, PIR-B is dephosphorylated, the inhibitory effect of SHP-1 dismantled and therefore inhibition of migratory responses is decreased. Indeed, neutrophils and dendritic cells that lack PIR-B or SHP-1 display enhanced chemokine signaling and functional responses, as do Fgr and Hck deficient cells (Brown, 2005; Zhang et al., 2005). We have recently shown that PIR-B may actually have a dual role in the regulation of eosinophil and neutrophil migration. In response to chemokine signaling PIR-B serves as a negative regulator of GPCR signaling via interactions with SHP-1 and -2. However, upon stimulation with leukotriene B$_4$ (LTB$_4$), an arachidonic acid metabolite that induces cell migration (also via GPCR signaling) PIR-B serves as a co-activator of cell migration (Munitz et al., 2008). In attempt to define the downstream molecular events that may cause this duality, biochemical analysis revealed that PIR-B differentially interacts with Src-family kinases or kinase adaptor molecules to either suppress or co-activate migratory responses (Munitz et al., 2008) (Fig. 3).

In human cells, we have recently shown that antibody cross-linking of IRp60/CD300a on eosinophils suppresses their response to eotaxin-induced migration and inhibits eotaxin-induced Ca^{2+} mobilization and ERK and p38 activation (Munitz et al., 2006a). Similarly, it has been reported that activation of dendritic cells with a ligand to the inhibitory receptor LIR-1/ILT-2 (a human PIR-B orthologue) inhibits their response to the chemokine C–C ligand 5 (CCL5) (Wagner et al., 2008).
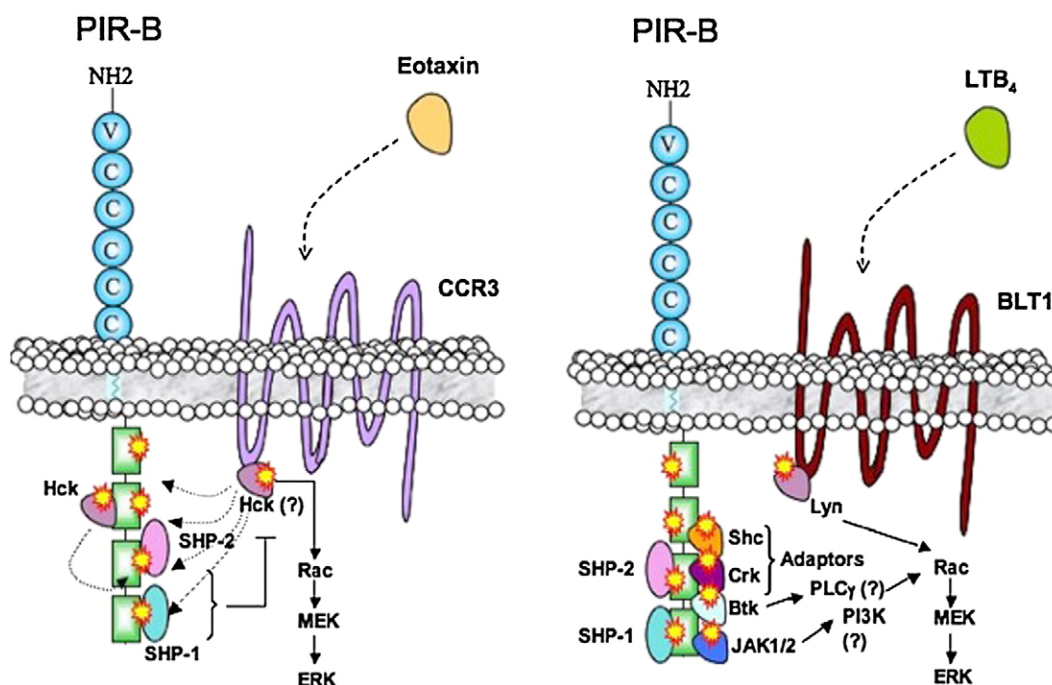
Fig. 3. The molecular basis for the dual role of PIR-B. PIR-B has been recently shown to possess a dual function in respect to eosinophil (and neutrophil) chemotaxis. Upon stimulation of eosinophils with eotaxin that binds the CCR3 receptor, PIR-B undergoes rapid tyrosine phosphorylation and recruits the phosphatases SHP-1 and -2. This leads to inhibition of GPCR-induced Erk activation and therefore PIR-B has an inhibitory role. In contrast, upon stimulation of eosinophils with the arachidonic acid metabolite LTB$_4$ (via the BLT1 receptor), PIR-B is capable to recruit various kinases and adaptor molecules inclusing Btk, JAK1/2, Shc and Crk. Consequently leading to activation of ERK signaling and thus PIR-B serves as an co-activator for LTB$_4$-induced signaling.

It is important to note that inhibitory receptors have been also shown to regulate myeloid cell adhesion and integrin signaling (Pereira et al., 2004). Thus, negative regulation of adhesive properties of myeloid cells by inhibitory receptors may also add to the overall suppressive activity that has been observed and reported from in vivo chemotaxis studies. However, in vitro findings clearly point to a direct effect of inhibitory receptors in suppression of chemokine receptor signaling that is independent of integrin signaling. Supporting this, increased Ca^{2+} mobilization (a process that is negatively regulated by inhibitory receptors) in response to chemokine stimulation is independent of integrin signaling and results from direct stimulation of phospholipase C (PLC) β by the βγ subunit of stimulated Gi heterotrimeric G proteins (Brown, 2005). Taken together these results clearly demonstrate a key role for inhibitory receptors in regulation of myeloid cell migration.

8. Inhibitory receptors in innate immune inflammatory responses

Following recruitment, myeloid cells are important in coordinating inflammatory responses. Not only do they generate proinflammatory agents in the course of the phagocytic activities or release toxic mediators, but they also produce cytokines that modulate the inflammatory response including IL-6, TNF-α, IL-1β, IL-8, GM-CSF and others (Taylor & Gordon, 2003).

Innate immune signaling components have key roles in multiple diseases including autoimmune, metabolic and allergic diseases (Ravetch & Lanier, 2000). Experimentation in the innate immune field has been able to delineate a complex network of receptors (e.g. Toll-like receptors, nucleotide-binding oligomerization domain (NOD) family receptors) and intracellular adaptor molecules and kinases that are readily available for the immediate response of the host cell (Akira et al., 2006). Given the role of myeloid cells in innate immunity, one would assume that their innate functions could be counter regulated by inhibitory receptors. Indeed, accumulating data

indicate a role of inhibitory receptors in the regulation of innate immune inflammatory responses.

A common phenomenon to various inhibitory receptors is that proinflammatory mediators can regulate the expression of inhibitory receptors. Thus, proinflammatory mediators can influence the "threshold" of immune cell inhibition either by upregulating or downregulating inhibitory receptor expression. Stimulation of human neutrophils with lipopolysaccharide (LPS) and GM-CSF increases the cell surface expression of IRp60/CD300a as a result of the rapid translocation of an intracellular pool of the receptor to the cell surface (Alvarez et al., 2008). Intradermal injection of LPS increases the expression of gp49B1 on mouse bone marrow and blood neutrophils (Zhou et al., 2003). Furthermore, the levels of PIR-B on osteoclasts and macrophages are also upregulated by TNF-α and LPS (Ochi et al., 2007 and Munitz et al., unpublished data). In contrast, stimulation of neutrophils and macrophages with LPS downregulated the expression of the inhibitory receptors DCIR and SIRP-1α (Richard et al., 2003; Kong et al., 2007). The expression patterns of these ITIM-bearing receptors in response to inflammatory stimuli suggest an important role for these receptors in the control of inflammation.

In support of this concept, various reports have demonstrated an in-vivo role for inhibitory receptors in innate immune responses. It has been recently shown that gp49B1 is a negative regulator of LPS induced activation of neutrophils (Zhou et al., 2003; Katz, 2007). Intradermal LPS injection in mice deficient in gp49B1 resulted in significantly more intravascular thrombi, occluding venules in comparison with wild type mice. In addition, neutrophil accumulation was increased in the vascular thrombi and in the ear of the gp49B1 knockout mice. It is interesting to note that this effect is due to a specific inhibition of neutrophils responses to LPS by gp49B1 but not mast cell responses. In fact, the increased pathology that was observed in the absence of gp49B1 was prevented following neutrophil depletion (Zhou et al., 2003; Katz, 2007). Another example for such a role comes from SIRP-1α. Mice that have been transferred with

SIRP-1α-deficient macrophages were highly susceptible to endotoxic shock, developing multiple organ failure and exhibited a remarkable increase in mortality. Of note the inhibitory activity of SIRP-1α was mainly due to the recruitment of SHP-2 and prevention of LPS-induced SHP-2:IKK complex formation (Kong et al., 2007). Similar to SIRP-1α, PIR-B has been recently shown to regulate macrophage function in response to bacterial activation. Upon binding and recognition of bacterial by various receptors, macrophages are activated and secrete large amounts of pro-inflammatory cytokines. Interestingly, PIR-B deficient mice display increased susceptibility to *Salmonella* infection (Torii et al., 2008). Furthermore, PIR-B has been also shown to bind gram-positive and gram-negative bacteria such as *S. aureus* and *E. coli* and to suppress macrophage activation in response to *S. aureus* activation in vitro. This result is particularly interesting as it indicates that inhibitory receptors may serve as pattern recognition receptors to bacterial products (Nakayama et al., 2007). Although it is assumed that inhibitory receptors operate mainly via recruitment of phosphatases and subsequent dephosphorylation of signaling targets an additional mechanism for suppression of inflammatory responses by inhibitory receptors has been proposed. Recently, it has been shown that siglec-9 and -5 can enhance (rather than inhibit) IL-10 production in response to multiple TLR ligands in a process that is dependent on tyrosine phosphorylation of intracellular ITIMs (Ando et al., 2008). The ability to enhance IL-10 production is not specific for siglecs as PIR-B was also described to increase IL-10 production in response to *S. aureus* stimulation (Nakayama et al., 2007). Thus, inhibitory receptors may regulate immune activation and inflammation via an-IL-10 dependent pathway.

In fact, and in addition to the aforementioned inhibitory receptors, FcγRIIB, a well-established and characterized inhibitory receptor (Daeron et al., 2008) can suppress innate immune responses via binding to IgG. In fact, passively immunized mice with Abs to LPS to reversed proinflammatory cytokine production, and the transfer of macrophages whose FcγR had been cross-linked (and thereby activated and display increased IL-10 production and decreased IL-12) could rescue mice from lethal endotoxemia (Gerber & Mosser, 2001; Grazia Cappiello et al., 2001; Kane & Mosser, 2001). Thus, in-vivo activation of the macrophage FcγR, can be exploited to prevent inappropriate inflammatory cytokine responses. Indeed, elegant studies in human patients with active visceral leishmaniasis indicate that IgG (the ligand of FcγR) can cause a novel form of immune enhancement due to its ability to induce IL-10 production from macrophages (Miles et al., 2005).

Although the majority of the studies on inhibitory receptors in innate immunity examine their role in TLR-dependent activation, inhibitory receptors can also suppress non TLR-dependent pathways. Recently CD24-deficient mice have been shown to exhibit increased susceptibility to danger- but not pathogen-associated molecular patterns (Chen et al., 2009). CD24 can associate with high mobility group box 1 (HMGB1), heat shock protein 70 (Hsp70), and heat shock protein 90 (Hsp90) and negatively regulates their pro-inflammatory activity resulting in inhibition of nuclear factor kappa B (NFκB) activation. The inhibitory effect of CD24 is mediated at least in part via association with Siglec-10 or Siglec-G in human and mice, respectively (Chen et al., 2009).

Importantly, inhibitory receptors belonging to the c-type lectin family of receptors have been also shown to possess innate immune roles by suppression of dendritic cell function via receptors such as DC-SIGN (Relloso et al., 2002; Gagliardi et al., 2005; den Dunnen et al., 2009). However the role of such receptors has been recently reviewed elsewhere and will thus not be included in this chapter (Geijtenbeek & Gringhuis, 2009).

Taken together, these studies indicate that inhibitory receptors utilize distinct mechanisms that are capable to suppress innate immune responses in a cell-specific manner. Future studies are likely to shed light on the exact mechanism by which these receptors operate and how they regulate specific innate components.

9. Activating ITIMs

The involvement of inhibitory receptors in the negative regulation of myeloid cell function highlights them as potential targets for therapy. However, when taking into account their inhibitory role, one should also consider another side for these receptors. Recent data has suggested that ITIM-bearing receptors can actually mediate activation and ITAM-bearing receptors could induce inhibition (Barrow & Trowsdale, 2006; Pinheiro da Silva et al., 2008). Although the subject of activation through ITIMs is not fully understood, there are now various reports on reciprocal activity for such receptors. As mentioned previously, we have recently shown that in response to LTB₄ stimulation, PIR-B can serve as a docking site for various adaptor kinases that can promote cellular activation. In fact, our data support a model in which following LTB₄ activation PIR-B can interact with several activating kinases, such as JAK1, JAK2, Shc, and Crk (Fig. 4) (Munitz et al., 2008). Similar to PIR-B, the inhibitory receptor SIRP-1α, was recently reported to stimulate nitric oxide production in macrophages via the Jak/STAT and PI3K/Rac1 pathways, thereby suggesting an activating role for SIRP-1α in a similar molecular mechanism to that of PIR-B (Alblas et al., 2005). Furthermore, the inhibitory receptors TREM-like transcript-1 (TLT-1) and cholecystokinin (CCK) receptor have been also shown to enhance cellular activation (Barrow & Trowsdale, 2006). For example TLT-1, amplifies FcεRI-mediated calcium signaling and CCK (via recruitment of SHP-2 to its ITIM) activates the AKT pathway (Vatinel et al., 2006).

Thus, although it appears that the primary role of inhibitory receptors is to indeed suppress cellular activation, under specific circumstances they can also induce cellular activation.

10. Inhbitory receptors as future therapeutic target: lessons from allergy

As a therapeutic approach to suppress inflammation, one can target activation pathways and inhibit them. Numerous approaches have used this strategy and several biological agents targeting cytokines, angiogenic factors, adhesion molecules and kinase inhibitors are either in development, clinical trials or in actual use. However, the findings that inhibitory receptors display key roles in dampening immune cell activation highlights them as a potential target for therapeutic intervention as well. In this set-up, the anticipated drug will act to enhance inhibition rather than to suppress activation.

One of the best examples for usage of inhibitory receptors as therapeutic targets comes from studies in the allergy field. Several groups including ours have utilized inhibitory receptors for anti-allergic treatment in an overall similar approach of bispecific-antibodies or -fusion proteins (Zhu et al., 2002; Tam et al., 2004; Zhu et al., 2005; Bachelet et al., 2006; Munitz et al., 2006b; Saxon et al., 2008). Notably, the use of similar strategy by three independent groups reveals the necessity to consider designing molecular targets that will display cell-specific regulation. Allergic responses are initiated by mast cell degranulation in response to allergen cross-linking of FcεRI-bound IgE (Fig. 4A). Thus, specific targeting of mast cells (but not general suppression of other myeloid cells) may yield a potent anti-allergic therapeutic approach. Saxon et al., have undertaken two approaches targeting inhibitory pathways in mast cells and basophils (Fig. 4B) (Zhu et al., 2002, 2005). One platform uses negative signaling to drive non-allergen-specific suppression of allergic reactivity. The other platform uses a similar negative pathway but is actually allergen-specific (Fig. 4B). These two drug modalities can be used either to chronically treat allergic patients independent of their allergenicity or specifically "tailored" for the patient to distinctively target an antigen. In general, both platforms (termed GE2 and GFD respectively) are based on a fusion protein that inhibits FcεRI mediated responses by cross-linking it to FcγRIIB (GE2) or cross-links FcγRIIB with Feld1 a well known cat

allergen. Studies utilizing these reagents have shown promising results in-vivo (Zhu et al., 2002, 2005).

We have previously described that IRp60/CD300a is a potent negative regulator of mast cell and eosinophil responses (Bachelet et al., 2005; Munitz et al., 2006a). In attempt to inhibit mast cell and eosinophil functions we designed bispecific antibody fragments that

are capable to recognize IgE or CCR3 (as mast cell and eosinophil specific molecules) and CD300a/IRp60 (Fig. 4C). In vivo administration of these antibody fragments in murine models of allergic peritonitis, passive cutaneous anaphylaxis and a chronic model of established allergic eosinophilic airway inflammation was capable to suppress the allergic response and even reverse the inflammatory process and associated remodeling (Bachelet et al., 2006; Munitz et al., 2006b). Similar to this approach, Tam et al. have designed a bispecific antibody against IgE and FcγRIIB that inhibits antigen-induced histamine release by human mast cells and basophils in-vitro (Tam et al., 2004).

As mentioned, inhibitory receptors can interact with various inhibitory adaptor proteins such as Dok-1 (Fig. 1) (Berg et al., 1999; Saxon et al., 2004; Robson et al., 2004; Zhang & Phillips, 2006). Interestingly, activation of mast cells with nanomolar concentrations of dexamethasone, upregulates the expression and association of Dok-1 with RasGAP (Hiragun et al., 2005). Thus, it is possible that low-dose glucocorticosteroid treatment will potentiate inhibitory receptor-based therapeutic approaches that are aimed to cross-link (and thereby activate) inhibitory receptors.

Collectively, these independent and timely reports highlight the vast potential and possibilities that inhibitory receptors may have as future therapeutic targets including inflammatory, autoimmune, allergic and malignant disorders. However, when targeting these receptors one should take into account various factors including cell-specificity, general immune-suppression and the inflammatory context, which may cause ITIMs to co-activate rather than inhibit.

11. Concluding remarks

Since the description of the first inhibitory motif in FcγRIIB (Daeron, 1995), the number of ITIM-bearing receptors has noticeably increased. A recent protein sequence analysis that is based on sequence context (i.e. predictions of signal peptide, transmembrane helices and protein domains) it is now estimated that 109 proteins express ITIMs and may represent novel inhibitory receptor candidates (Staub et al., 2004). Currently, there is still need to enhance our understanding on the role of such receptors in various aspects of myeloid cell biology and to define these pathways capable of inhibiting their functions. Identification of the in-vivo ligands and definition of receptor:ligand interactions using gene targeted mice is likely to shed light on the potential of these receptors to serve as therapeutic targets. Future studies will enable us to:

A) Gain insight into the molecular mechanisms involved in myeloid cell cellular inhibition/activation.
B) Provide us with an opportunity to suppress myeloid cell function in different experimental settings and thereby gain valuable insight into the role of myeloid cells in health and disease.
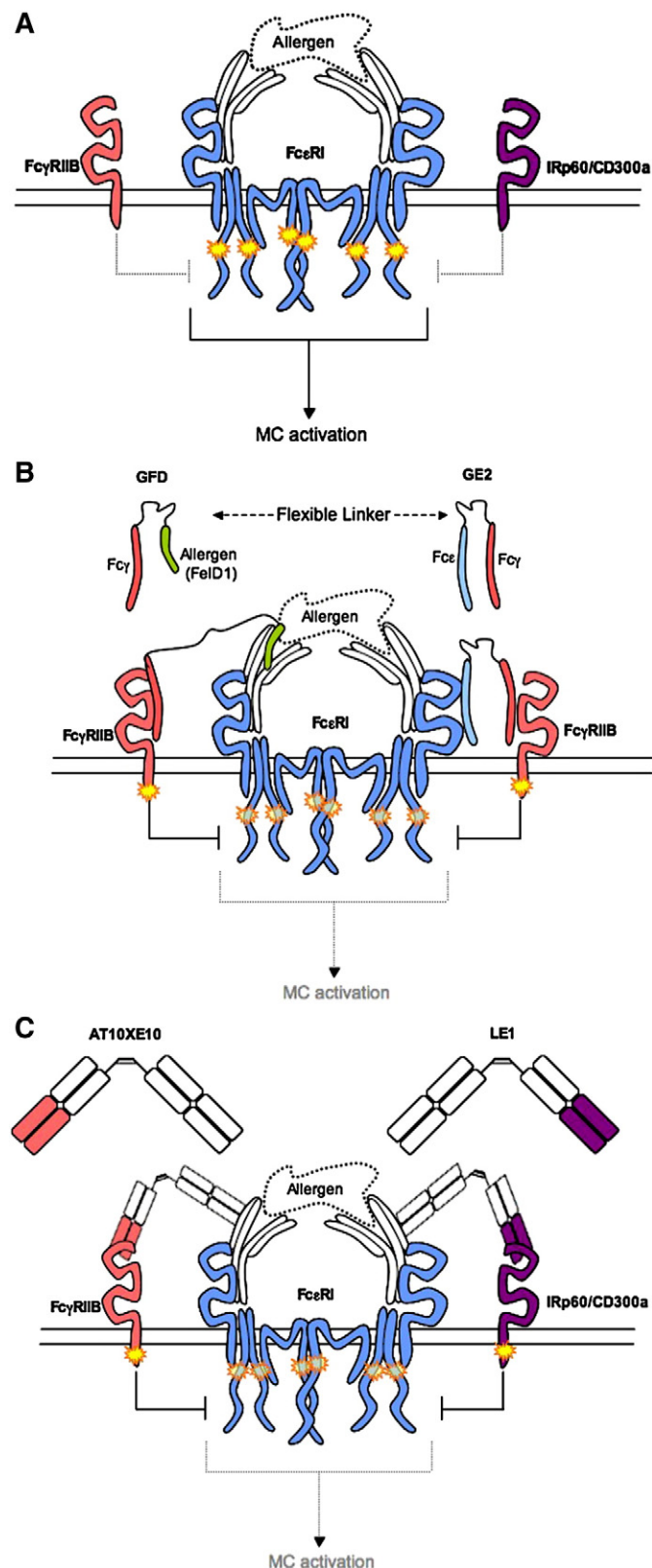C) Provide us with novel tools to combat detrimental myeloid cell functions in disease states.

Fig. 4. (**A**) The allergic/inflammatory response is triggered by cross-linking of Fcε by allergen-bound IgE. In the absence of inhibitory signals, the FcεRI receptor chain ITAMs are phosphorylated and mediate mast cell activation and initiation of inflammation. (**B**) In attempt to utilize the ability of FcγRIIB to inhibit IgE-mediated activation of mast cells and basophils, a platform of two fusion proteins has been designed. A bispecific fusion protein consisting of the Fc portion of IgG (Fcγ) and the Fc portion of IgE (Fcε) were linked (GE2). This fusion protein cross-links FcγRIIB to FcεRI and activates the inhibitory mechanism of FcγRIIB. Similarly, another fusion protein linking a major cat allergen (FelD1) to Fcγ was designed. This fusion protein mimics allergen binding to FcεRI portion and induces allergen-specific cellular inhibition. (**C**) An additional approach that targets inhibitory receptors as anti-allergic drugs utilizes chemically conjugated bispecific antibodies that cross-link FcεRI-bound IgE to IRp60/Cd300a (LE1) or FcγRIIB (AT10XE10) and therefore activate the inhibitory mechanism of IRp60/CD300a or FcγRIIB.

As the rates of diseases involving expansion, recruitment and activation of myeloid cells is currently on the rise, studies that are focused on pathways that inhibit rather than activate are likely to prove key in the regulation of allergic, autoimmune, inflammatory and metabolic diseases. These may represent novel pathways for future therapy.

Acknowledgments

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