Role of negative regulation of immune signaling pathways in neutrophil function

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Abbreviations

A(A2)AR = adenosine receptor

BM = bone marrow

CEACAM = carcinoembryonic antigen-related cell adhesion molecule

Csk = C-terminal Scr kinase

fMLF = formyl-methionyl-leucyl phenylalanine peptide

GAP = GTPase activating proteins

GEF = guanine nucleotide exchange factor

G-CSF = Granulocyte colony stimulating factor

G-CSFR = Granulocyte colony stimulating factor receptor

GPCR = G protein coupled receptor

GRK = G protein coupled receptor kinase

IBD = Inflammatory bowel disease

ICAM-1 = Intracellular Adhesion molecule-1

IL = interleukin

InsP6 = Inositol hexakisphosphate

ITAM = immunoreceptor tyrosine-based activation motif

ITIM = immunoreceptor tyrosine-based inhibitor motif

JAK = Janus kinase

LFA-1 = lymphocyte function-associated antigen-1

LPS = lipopolysaccharide

LTB_4 = leukotriene B_4

NADPH = Nicotinamide adenine dinucleotide phosphate

NETs = Nuclear extracellular traps

MPO = myeloperoxidase

PI3K = phosphoinositide 3-kinase

PIAS = protein inhibitor of activated STAT

PILRα = paired immunoglobulin-like type 2 receptor-α

PIR-B = paired immunoglobulin receptor

PMN = polymorphonuclear neutrophils

PNCA = proliferating cell nuclear antigen

PtdIns(3,4,5)P3 = Phosphatidylinositol 3,4,5-triphosphate

PtdIns(3,4)P2 = Phosphatidylinositol 3,4-biphosphate

PTEN = Phosphatase and tensin homolog

PTP1B = protein-tyrosine phosphatase 1B

PTPN6 = Tyrosine-protein phosphatase non-receptor type 6; also known as SHP-1 in humans

ROS = reactive oxygen species

SDF-1 = stromal derived factor

SHIP-1 = SH2 domain-containing inositol phosphatase-1

SHP = SH2 domain-containing phosphatase

Siglecs = Sialic acid binding Ig-like lectins

SIRP-α = Signal regulatory protein-α

SLE = systemic lupus erythematosus

SOCS3 = suppressor of cytokine signaling-3

STAT = signal transducer and activator of transcription

WHIM = warts, hypogammaglobulinemia, infections, myelokathesis

Wip-1 =Wild-Type p53-induced phosphatase 1

WT = wild type

Abstract

Polymorphonuclear neutrophils (PMN) play a critical role in host defense against infection and in the resolution of inflammation. However immune responses mediated by PMN must be tightly regulated to facilitate elimination of invading pathogens without inducing detrimental inflammation and host tissue damage. Specific engagement of cell surface immunoreceptors by a diverse range of extracellular signals regulates PMN effector functions through differential activation of intracellular signaling cascades. While mechanisms of PMN activation mediated via cell signaling pathways have been well described less is known about negative regulation of PMN function by immune signaling cascades. Here we provide an overview of immunoreceptor mediated negative regulation of key PMN effector functions including maturation, migration, phagocytosis, ROS release,

degranulation, apoptosis and NETosis. Increased understanding of mechanisms of suppression of PMN effector functions may point to possible future therapeutic targets for the amelioration of PMN-mediated autoimmune and inflammatory diseases.

Introduction

Polymorphonuclear neutrophils (PMN) are the predominant leukocytes in human blood and as such they are key effectors of the innate immune response. The highly motile nature of PMN paired with their ability to identify and rapidly respond to chemoattractant signals makes them critical first responders at sites of infection/inflammation. Following migration into inflamed tissues PMN execute a number of effector functions that facilitate pathogen clearance and resolution of inflammation including phagocytosis, generation of reactive oxygen metabolites, as well as release of microbiocidal granule contents and extracellular traps. However, the considerable array of microbiocidal and pro-inflammatory mediators contained within PMN granules means that excessive PMN accumulation and activation within inflamed tissues can be detrimental to the host [1, 2]. As such, PMN mediated mucosal tissue damage is a pathological consequence of a number of inflammatory disorders including inflammatory bowel disease (IBD), chronic obstructive pulmonary disorder (COPD), chronic peritonitis, sepsis and renal injury [3].

Given the destructive potential of PMN in tissues, maturation, recruitment and inflammatory effector functions of PMN must be tightly regulated processes subjected to both positive and negative regulators. Such control is achieved in part through engagement of PMN cell surface receptors that trigger very diverse signal transduction pathways. PMN express several classes of cell surface receptors including G-protein-coupled-seven-transmembrane receptors (GPCR), integrins, selectins, Fc receptors, cytokine receptors and innate immune receptors including C-type lectins and toll like receptors [4]. While engagement of cell surface receptors activates a range of PMN immune functions, it is also well accepted that PMN express various inhibitory receptors that negatively regulate critical

effector functions. This suppression of PMN activation serves to facilitate host protection from pathogens while avoiding bystander tissue damage and, as such is of critical importance for a successful immune response. Therefore, this mini-review highlights the regulation of PMN behavior through negative regulation of immune signaling pathways.

Negative Regulation of PMN granulopoiesis

The production and maturation of PMN (granulopoiesis) occurs in the bone marrow (BM) through sequential differentiation of hematopoietic stem cells to myeloblast, promyelocytes, myelocytes, metamyelocytes, band cells, and finally mature PMN. At the end of this process PMN leave the BM and begin circulating in the vasculature [5]. Granulocyte colony stimulating factor (G-CSF) is the major cytokine that induces granulopoiesis. However, G-CSF binding to its receptor G-CSFR also recruits several cytoplasmic tyrosine kinases, including members of the Src family kinases that function to negatively regulate PMN release from the BM. As such, triple knockout (KO) mice lacking Hck, Lyn, and Fgr, the major Src kinases expressed in myeloid cells, have an increased proliferative response to G-CSF and more circulating PMN as compared to wild-type mice [6]. The analysis of individual roles for Hck and Lyn revealed that Hck negatively regulates G-CSF-induced proliferation of granulocytic precursors, whereas Lyn negatively regulates the production of myeloid progenitors [6].

It has also been described that the suppressor of cytokine signaling-3 (SOCS3) inhibits G-CSFR signaling by a classical negative feedback loop mechanism. Activation of G-CSFR results in recruitment of SOCS3 which binds directly to tyrosine Y729 on G-CSFR inhibiting JAK/STAT3 activation and thus regulating G-CSF mediated PMN proliferation [7]. In addition to inhibiting G-CSFR signaling, SOCS3 also inhibits IL-6 signaling by binding the

signal transducing receptor subunit gp130, a receptor that is closely related to G-CSFR [7]. Boyle *et al.* showed that mice expressing a truncated SOCS3 protein (lacking the C-terminal SOCS box) had an increased number of colony-forming cells upon exposure to G-CSF and IL-6. In an *in vivo* model of arthritis, truncation of SOCS3 leads to a more florid arthritis when compared with WT mice, suggesting that dysregulated PMN proliferation in the BM is associated with the development of autoimmune disorders [8].

During PMN differentiation, progenitor cells committed to hematopoietic lineages interact and migrate along and then across BM while receiving stimulatory and inhibitory signals from stromal marrow cells. It is known that constitutive expression of stromal-derived factor (SDF-1/CXCL12) and its G protein-coupled chemokine receptor (CXCR4) are required for hematopoietic cell adhesion, and PMN retention in the BM [9]. In contrast, release of PMN occurs through CXCL2 (MIP-2) interaction with its receptor CXCR2, or through G-CSFR or Toll-like receptors, all of which appear late during PMN maturation in the BM [10]. Consequently, mature PMN up-regulate expression of CXCR2 and decrease CXCR4 expression, a balance that favors release of BM PMN into the circulation [11]. CXCR2, is a chemokine receptor that has been shown to favor PMN release and as such, *Cxcr2*$^{-/-}$ PMN fail to exit the BM, reproducing a myelokathexis phenotype. G-CSF decreases CXCL12 expression in the BM, decreases PMN CXCR4 expression and, induces BM expression of the CXCR2 ligand CXCL2, ultimately promoting PMN release into the circulation [12]. CXCR4 on the other hand, opposes the role of CXCR2. Therefore mutations causing increased activation of CXCR4 result in increased PMN retention in the BM as seen in the human syndrome WHIM (warts, hypogammaglobulinemia, infections, myelokathexis) [13]. Conversely, the deletion of CXCR4 in myeloid cells promotes a substantial neutrophilia [14]. Therefore, CXCL2-CXCR2 signaling represents a chemokine axis that controls PMN migration out of the BM by opposing CXCL12-CXCR4 signals [11]. PMN release may be

also be mediated by signals through G-CSFR, fMLF receptors or Toll-like receptors, but in the absence of CXCR4, the stimulation of these receptors does not cause additional PMN release from the BM, demonstrating the essential role of CXCR4 signaling for PMN retention in the BM [14].

The adhesion and retention of PMN precursors within the BM depends on β1 and β2 integrins interacting with their receptors expressed on stromal marrow cells. However, the exact mechanisms by which attenuated CXCR4 signaling leads to migration of PMN from the BM are not yet completely understood. The Src family kinases Lyn, Fgr, and Hck have been shown to play an essential role in integrin function and signaling in PMN. Of particular interest, Lyn has been shown to act as a negative regulator of PMN functions and it is an important part of the regulatory network that links chemotactic signals to adhesive responses of hematopoietic cells [15]. Nakata *et al*. showed that Lyn negatively regulates CXCL12-CXCR4 mediated PMN adhesion to BM [16]. Activation of Lyn by CXCL12-CXCR4 inhibits activation of the β2 integrin LFA-1, reducing adhesion to intercellular adhesion molecule-1 (ICAM-1) and allowing PMN to move more freely within the marrow, where they continue their maturation before being released into the circulation [16]. *In vitro* assays showed that PMN deficient in Lyn express normal levels of integrins (including LFA-1), but demonstrate increased attachment to BM stromal cells and decreased migration in response to CXCL12. Most probably this transient Lyn mediated inhibition of CXCR4-β2 integrin activation occurs via inside-out signaling through as yet unidentified pathways. This negative regulation is not solely activated by the CXCR4 receptor as fMLF-induced PMN chemotaxis also requires Lyn to inhibit β2 integrin-dependent cell adhesion to ICAM-1 [16].

GPCRs involved in PMN proliferation and maturation in the BM are regulated by the activity of downstream kinases. As such, members of the GPCR kinase (GRK) and arrestin families induce GPCR desensitization upon agonist stimulation leading to a feedback

mechanism that rapidly uncouples the receptor from its associated G protein. There are seven GRKs known, with GRK2, -3, -5, and -6 being the four most widely expressed members. Studies from *Grk6*-deficient mice suggest GRK6 inhibits CXCL12-induced chemotaxis of PMN [17, 18]. Balabanian *et al*. also described a pivotal role for GRK3 in CXCR4 mediated signaling through internalization and desensitization of CXCR4 in fibroblasts and T cells. CD4+ T cells from WHIM syndrome patients displayed a stronger chemotaxis in response to CXCL12 [19]. However, the role of GRK3 and GRK6 in regulating PMN mobilization from BM into blood in response to CXCL12-CXCR4 has not been fully characterized.

In addition to negative regulation of PMN maturation by protein kinases, granulopoiesis is also suppressed by protein phosphatases. Wild-Type p53-induced phosphatase 1 (Wip1, also known as PP2Cd) is an oncogene that inhibits several p53-dependent tumor suppressor pathways. Expression of Wip1 increases during PMN maturation and, Wip1 deficient mice display increased granulocytic differentiation, proliferation, and BM maturation leading to a severe neutrophilia, suggesting that Wip1 is a negative regulator of both granulopoiesis and PMN homeostasis [20]. Further, in response to G-CSF, BM PMN deficient in Wip1 displayed rapid and increased phosphorylation of p38 MAPK and STAT1 suggesting that Wip1 inhibits p38 MAPK/STAT1 mediated PMN maturation [20]. Another possible explanation for the neutrophilia observed in *Wip1*$^{-/-}$ mice is that Wip1 deficient PMN express higher levels of CXCR2 and lower levels of CXCR4 than wild type mice, thus favoring the release of PMN into the circulation [11].

Signaling events that inhibit PMN trafficking to sites of infection/inflammation

PMN migration to inflamed mucosal tissues requires sequential migration across endothelial [21] and epithelial [1] barriers and is facilitated through a complicated series of protein-protein binding interactions. Trafficking of mature circulating PMN to sites of infection or inflammation is essential for effective defense against invading pathogens and for successful resolution of inflammation. However, given that PMN carry an arsenal of toxic metabolites that can cause damage to host tissues, all steps of the PMN trafficking cascade are highly regulated. While protein kinases such as Syk and Src family members mediate PMN activation through immunoreceptor tyrosine-based activation motif (ITAM) signaling domains, Src family members also activate immunoreceptor tyrosine-based inhibitor motif (ITIM) domains to negatively regulate PMN signal transduction pathways. ITIM phosphorylation by Src family kinases (Hck, Fgr and Lyn) recruits SH2 domain-containing phosphatase (SHP) 1 and 2 as well as SH2 domain-containing inositol phosphatase (SHIP) 1 and 2 that dephosphorylate substrates of ITAM mediated activating signaling pathways in order to down-regulate PMN functional responses. Therefore, several ITIM containing immunoreceptors have been shown to play key roles in negative regulation of PMN trafficking (Fig. 1).

SIRP receptors

Phagocytes, including PMN, express the ITIM containing protein signal regulatory protein α (SIRPα) the counter ligand for the widely expressed membrane receptor CD47. SIRPα also binds soluble surfactant A and D, innate immune system collectins (collagen-containing C-type lectins). While mAbs to SIRPα and soluble CD47 have been shown to decrease PMN transepithelial migration [22], more recently it has been demonstrated that SIRPα mutant mice (which express the extracellular domain of SIRPα but not the

cytoplasmic ITIM signaling domain) have increased PMN migration in a uric-acid induced peritonitis model of sterile inflammation [23].

Siglec receptors

Sialic acid binding Ig-like lectins (Siglecs) are another family of ITIM containing immune receptors expressed by human and murine PMN. In human PMN, engagement of Siglec 5 and Siglec 9 by sialylated ligands induces ITIM phosphorylation, recruitment of SHP-1 and SHP-2 phosphatases and down-regulation of PMN activator signaling pathways [24]. As such, PMNs deficient in Siglec E (the murine paralog of Siglec 9) show accelerated recruitment to the lung upon intranasal administration of LPS. This increase in PMN trafficking is mediated by inhibition of CD11b outside-in signaling downstream of engagement of Siglec E by sialylated fibrinogen [25].

PIR receptors

The paired immunoglobulin receptor (PIR-B) is another ITIM containing immunoreceptor that functions as a negative regulator of integrin-mediated migration in PMN. Src family kinases Hck and Fgr phosphorylate tyrosine residues of the PIR-B ITIM domain thereby activating it and suppressing PMN activation. As such, treatment of PMN deficient in PIR-B with cytokines (MIP-1α/CCL3, and MIP-2/CXCL2) results in enhanced intracellular calcium mobilization, increased ERK1/2 activation, increased actin polymerization and enhanced migratory capacities [26, 27]. Another PMN inhibitory receptor, PILRα (paired immunoglobulin-like type 2 receptor α), contains two ITIM domains that recruit SHP-1 and SHP-2. PILRα binds *in cis* to PMN expressed sialyloglycoproteins to

negatively regulate chemoattractant-triggered activation of PMN by β2 integrins [28]. Increased activation of Rap1 following fMLF stimulation of *Pilrα*$^{-/-}$ PMN compared to wild type PMN has also been reported, suggesting that PILRα down-regulates GPCR mediated integrin activation. As such, *Pilrα*$^{-/-}$ mice have increased PMN recruitment *in vivo* in a thioglycolate-induced sterile peritonitis model [28].

Src Family Tyrosine Kinases and Phosphatases signal downstream of ITIM domains

In addition to inhibitory signaling mechanisms orchestrated through phosphorylation of ITIM domains, protein phosphatases also counterbalance PMN signaling activation and play a central role in inhibiting PMN migration. Tyrosine phosphorylation is one of the earliest consequences of PMN cell surface receptor activation [29]. The Src family tyrosine kinases have been implicated in a number of GPCR signaling pathways that propagate downstream signals in a positive fashion resulting in activation of PMN processes including migration, phagocytosis and degranulation [30, 31]. However, the role of Src kinases in PMN activation is somewhat controversial in that it has also been reported that Src kinases can also act as negative regulators of PMN function. PMN from double knockout *Hck*/*Fgr* mice displayed significantly higher peak Ca^{2+} flux following stimulation with chemokines including CCL3, CXCL1 and CXCL2 and IL-8 [27]. In addition, double mutant *Hck*$^{-/-}$*Fgr*$^{-/-}$ PMN demonstrated increased ERK1/2 activation, increased F-actin polymerization and increased chemotaxis *in vitro* to CCL3 and CXCL2. Similar to results observed *in vitro*, increased PMN trafficking of *Hck*$^{-/-}$*Fgr*$^{-/-}$ PMN was observed *in vivo* using a thioglycolate induced model of sterile peritonitis, suggesting that Hck and Fgr signal through ERK1/2 to dampen PMN migratory responses to agonists of both CXCR2 and CCR1 [27]. In support of this negative regulation of PMN trafficking by Src kinases, other studies have shown that Lyn negatively regulates

integrin mediated PMN adhesion through ITIM domain phosphorylation of inhibitory immunoreceptors (SIRP1α and PIR-B) and subsequent recruitment of SHP-1. Therefore, reduced tyrosine phosphorylation of both SIRP1α and PIR-B following engagement of β2 integrins in *Lyn*$^{-/-}$ PMN results in reduced recruitment of SHP-1 and a hyper-adhesive/hyper-responsive phenotype [15]. In keeping with this, it has also been reported that PMN and macrophages from mice with a point mutation in *Shp-1,* that reduces expression of SHP-1 by 90%, display a hyper-adhesive phenotype [15, 32]. Abram *et al.* demonstrated that selective deletion of SHP-1 in murine PMN resulted in a hyper-activated PMN phenotype characterized by increased activation of ERK1/2, Src-Family kinases and Syk as well as spontaneous PMN mediated cutaneous paw inflammation [33]. As in mice, increased PMN trafficking to the skin resulting in neutrophilic dermatoses is also seen in humans with a spontaneous mutation in the *Ptpn6* gene [34]. Thus, deficiency of PTPN6/SHP-1 or impaired recruitment of SHP-1 to ITIM containing immunoreceptors in *Lyn*$^{-/-}$ PMN results in hyper-responsiveness, increased integrin ligation and increased PMN migration.

The protein-tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed phosphatase that is activated upon exposure to pro-inflammatory mediators including TNFα. Activated PTP1B can in turn act as a common negative regulator of NFκB, Akt and MAPK activities. In keeping with its role as a negative regulator of immune cell function *Ptp1b* KO mice exhibited higher accumulation of PMN (as measured by myeloperoxidase (MPO) levels and severity of edema) in the ear in response to LPS or Zymosan [35] and in the lungs in response to *P. aeruginosa* [36]. Similarly, in a respiratory syncytial virus (RSV) model of lung inflammation, loss of PTP1B expression resulted in changes in cytokine signaling, elevated PMN accumulation and increased damage and disruption of the epithelial cell barrier [37].

Immune signaling through small GTPases regulates PMN trafficking

PMN trafficking is facilitated by rapid chemokine-induced generation of cellular phosphoinositol phosphate gradients. Agonist-activated phosphoinositide-3 kinases (PI3Ks) trigger activation of lipid second messengers PtdIns(3,4,5)P3 and PtdIns(3,4)P2. These lipids recruit and activate PI3K effectors including guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and Rho family small GTPases. Rho family small GTPases expressed by PMN include Rac proteins (Rac1, Rac2 and RhoG), RhoA and Cdc42. Certain small GTPases are known to negatively regulate PMN migratory responses. In general, the activation of Rho GTPases is positively regulated by GEFs and negatively regulated by GAPs. Thus, RhoA is reported to act as a rheostat suppressing premature PMN activation. In keeping with this, depletion of RhoA in PMN leads to increased PMN chemotaxis towards β2 integrin independent stimuli including CXCL1 [38]. Increased trafficking of RhoA/B deficient PMN was also observed *in vivo* following sterile inflammation induced by thioglycolate [38]. ARHGAP25 is a Rac-specific GAP expressed by PMN, and similar to what was found for RhoA, it has been reported that loss of ARHGAP25 expression leads to a pro-inflammatory phenotype with increased PMN Rac activation and elevated transmigration into inflamed tissues [39]. Bcr and Abr are GAPs that contain binding sites for a number of signal transduction proteins including tyrosine kinases, serine/threonine kinases and scaffolding proteins. Interestingly, increased PMN accumulation in the lungs of double mutant *Abr*$^{-/-}$ *Bcr*$^{-/-}$ mice following LPS injection has been reported [40], suggesting that Bcr and Abr are PMN cell signaling mediators that down regulate PMN trafficking responses.

Signaling through second messengers and GPCRs regulates PMN trafficking

PI3K mediated generation of PtdIns(3,4,5)P3 and Rac activation at the leading edge of migrating PMN is counter-balanced by the activity of PtdIns(3,4,5)P3 phosphatases, SH2 domain containing inositol phosphatase 1 (SHIP-1) and phosphatase tensin homologue (PTEN) which, convert PtdIns(3,4,5)P3 to PtdIns(3,4)P2 at the back of migrating cells. In keeping with its role as a negative regulator of PMN activation, Subramanian *et al.* demonstrated that in response to chemoattractants (including IL-8, C5a and fMLF), PTEN deficient PMN demonstrated enhanced Akt activation, prolonged F-Actin activation, increased membrane ruffling and increased chemotaxis across transwell support filters [41]. In addition, loss of PTEN led to an increase in PMN recruitment to the peritoneum in response to inflammatory stimulants including thioglycolate and *E. coli*. Further *in vivo* studies used intravital microscopy to show that disruption of PTEN on PMN increased PMN rolling velocity and PI3K dependent PMN recruitment to the cremaster muscle in response to various stimuli including fMLF, TNF-α and CXCL2. Increased PMN emigration efficiency to the cremaster muscle in the more physiologically relevant setting of ischemia/reperfusion injury was also observed in myeloid specific PTEN KO mice compared to WT mice [42]. Enhancement of PTEN null PMN trafficking was also observed in a mouse model where lung inflammation was induced by intra-tracheal instillation of *E. coli* (one of the most common pathogens in neutropenia related pneumonia). Further, specific increases in PtdIns(3,4,5)P3 and Akt phosphorylation as well as increased PMN trafficking to the lungs were also reported in PTEN deficient PMN compared to WT PMN in a neutropenia-associated pneumonia model [43].

Another second messenger-mediated binding interaction that down-regulates PMN trafficking is binding of adenosine to its GPCR (Adenosine Receptor A(2A)AR). Adenosine is an an extracellular messenger that plays a protective role in acute inflammation and promotes wound healing through down-regulation of PMN activity [44]. Activation of

A(2A)AR causes a reduction in PMN motility and as such depletion of A(2A)AR *in vivo* leads to increased PMN trafficking to the airways and increased lung inflammation in response to aerosolized ragweed allergen [45].

Binding of the glucocorticoid-regulated protein Annexin A1 (Anxa1) to its GPCR (formyl peptide receptor type 2/lipoxin A4 receptor, FPR2/ALX) has also been identified as a mechanism for limiting the scope of an inflammatory response. As such, administration of Anxa1 to mice results in potent inhibition of PMN egress from the microcirculation [46]. Corroborating evidence of the inhibitory role of Anxa1 was provided by *in vitro* studies demonstrating inhibition of PMN transmigration across endothelial cells upon treatment with Anax1. Consistent with these data, PMN from Anxa1 deficient mice have increased CD11b/CD18 surface expression and increased chemotactic responses compared to WT PMN [47]. Binding of the anti-inflammatory ligand lipoxin A4 to FPR2/ALX also inhibits PMN chemotaxis, adhesion and transmigration across vascular endothelium [48]. Further evidence for FPR2/ALX acting as an anti-inflammatory GPCR was provided using mice lacking FPR2/ALX. These mice had increased inflammation associated with a marked increase in PMN adherence and emigration in the mesenteric microcirculation following ischemia reperfusion injury and as well as an increased acute response to carrageenan-induced paw edema compared to WT FPR2/ALX expressing PMN [49]. Given the number of studies demonstrating that PMN FRP2/ALX can be targeted to negatively regulate PMN trafficking and PMN inflammatory function, signaling through this GPCR is an area that is currently being investigated for anti-inflammatory therapeutics [50].

Immune signaling by JAK-STAT regulates PMN trafficking

Cytokine stimulation of immune cells activates the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway mediating a range of biological processes including PMN trafficking. JAK-STAT pathways are therefore regulated at many steps through distinct mechanisms including tyrosine phosphatases, protein inhibitor of activated STAT (PIAS) and SOCS. Negative regulation of PMN trafficking by members of the SOCS family has been previously described. Mice with myeloid specific deletion of SOCS3 have elevated STAT 3 activation and more extensive PMN infiltration into the cerebellum and brainstem compared to WT mice in an experimental autoimmune encephalomyelitis model [51]. Similarly, using an *in vivo* model of inflammatory arthritis Wong *et al.* demonstrated that deletion of SOCS3 from PMN results in more pronounced joint inflammation characterized by increased numbers of PMN in the inflamed synovium [52].

Cytokine-mediated suppression of PMN trafficking

Negative regulation of PMN trafficking is also mediated directly by cytokine triggered immune signaling. In response to *Staphylococcus aureus,* human PMN engage IL-20 resulting in modification of actin polymerization and inhibition of a broad range of actin dependent functions. Using an *in vitro* model of PMN migration, Gough *et al*. recently demonstrated that IL-20 actively inhibits PMN transmigration across bronchoepithelial cells in an ERK1/2 dependent fashion [53]. A similar IL-20 mediated inhibition of PMN trafficking has been reported *in vivo* in a mouse model of corneal healing [54].

Immune signaling mediated regulation of PMN Phagocytosis

Following trafficking to a site of infection, PMN are the first immune cells to encounter and subsequently engulf invading pathogens. As such phagocytosis is central to the microbiocidal function of PMN and, as with other PMN effector functions including maturation and migration, the process of PMN phagocytosis is also highly regulated by a variety of intracellular signaling cascades. PMN phagocytosis requires reorganization of the actin cytoskeleton, a process that depends on small GTPases, particularly Rac [55]. There are many studies from transgenic mice, and human PMN demonstrating that most, if not all, PMN functions including phagocytosis are subject to regulation by Rho family small GTPases. To date, two PtdIns(3,4,5)P3 activated Rac GAPs, ARHGAP15 and ARHGAP25, have been shown to drive Rac-inactivation in myeloid cells. It has also been demonstrated that ARHGAP15-deficient PMN display increased Rac-dependent F-actin reorganization as well as increased phagocytosis and pathogen killing [56]. As such, overexpression of ARHGAP25 in an FcγRIIa-expressing cell line (COSphoxFcγR) significantly decreased phagocytosis of serum-opsonized yeast particles. In a promyelocytic cell line (PLB-985) differentiated to a PMN-like phenotype, silencing ARHGAP25 significantly enhanced complement-mediated phagocytosis of opsonized yeast particles. ARHGAP25-mediated increases in phagocytosis were also reported in primary macrophages [57]. These phenotypes suggest a potential role for both of these Rac GAPs in down regulating PMN phagocytosis in order to promote pathogen clearance while minimizing PMN-inflicted tissue damage (Fig. 2).

In addition to F-actin mediated regulation of phagocytosis, suppression of phagocytosis downstream of phosphatase activity has also been reported. PTEN is a phosphatase that negatively regulates PtdIns(3,4,5)P3 therefore acting as a negative regulator of several PMN effector functions including phagocytosis. Li and colleagues reported that PTEN null PMN showed enhanced bacteria killing in a pneumonia model due

to increased phagocytic capacity [43]. Another phosphatase, the ubiquitous tyrosine phosphatase PTP1B has been shown to negatively regulate PMN function. In a *P. aeruginosa* infection model, *Ptp1b*$^{-/-}$ mice displayed enhanced bacterial clearance accompanied by increased PMN infiltration in the lungs, suggesting increased phagocytosis in the absence of PTP1B [36]. However, further analysis dissecting the individual roles of PMN and macrophages in bacterial clearance is necessary in order to conclude PTP1B negatively regulates PMN phagocytosis.

Release of anti-inflammatory cytokines during later stages of an immune response also serves to dampen PMN activation and prevent detrimental damage to host tissues. Recently Gough *et al.* found that IL-20 inhibits PMN phagocytosis, degranulation and migration by modifying actin polymerization in an *S. aureus* model of infection [53]. IL-20 signals through two different receptor complexes made up of heterodimers of the common IL-20RB subunit and either IL-20RA (complex type I) or IL-22RA (type II). While PMN from peripheral blood of healthy donors expressed only IL-22RA, exposure to *S. aureus* resulted in increased expression of IL-20RB in IL-8 or TNFα primed PMN *in vivo* and *in vitro*. Following *S. aureus* infection, treatment with IL-20 reduced intracellular bacterial killing in primed PMN, by impairing phagocytosis [53]. IL-20 induced phosphorylation of ERK1/2 but not p38 MAPK, and inhibition of ERK1/2 signaling restored the phagocytic ability of TNFα primed PMN suggesting that IL-20 inhibits PMN phagocytosis in an ERK1/2 dependent fashion.

Small GTPase mediated regulation of PMN ROS production

Following migration into inflamed tissues and phagocytosis, PMN destroy engulfed pathogens in part through the generation of an oxidative burst (reviewed in full elsewhere

[58]) However, given its destructive potential, ROS production by PMN is tightly regulated. PMN ROS production requires complex cytoskeleton rearrangement facilitated by small GTPases of the Rho family (Rac, Rho and Cdc42). As such Rho family GTPases are important negative regulators of the PMN oxidative burst. Adherence of PMN to a variety of extracellular matrix proteins results in a delay in the formation of ROS. This suppression of the oxidative burst represents a protective mechanism to prevent tissue damage by PMN as they move through tissues to reach sites of inflammation. This adhesion mediated regulation of NADPH oxidase is in part mediated by the small GTPase Rac2. It has been reported that activated PMN integrins suppress Rac2 activation and assembly of a functional NADPH oxidase complex. This inhibitory effect is also associated with tyrosine phosphatase mediated decreases in Y174 phosphorylation of the membrane associated Rho/Rac guanine nucleotide exchange factor (GEF), Vav-1 [59]. These data therefore suggest that Vav1 is an important link between G protein-coupled chemoattractant receptors and Rac2 mediated activation of NADPH oxidase. The TNFα induced protein 8 family member TIPE2 also regulates the PMN oxidative burst by binding to and blocking Rac GTPases. As a result of this inhibitory interaction, compared to WT PMN, *Tipe2*$^{-/-}$ PMN exhibited enhanced intracellular and extracellular ROS production in response to stimulation with fMLF or *Listeria monocytogenes* [60]. Bcr is another Rac1/Rac2 GAP implicated in signaling events that down-regulate PMN ROS production and it has been reported that PMN from *Bcr*$^{-/-}$ mice have increased Rac mediated reactive oxygen metabolite production in response to fMLF [61]. Arap3 is a PI3K regulated RhoA GAP that also signals to down-regulate PMN activation. In keeping with this, an increase in β2 integrin/adhesion dependent ROS generation in PMN deficient in Arap3 has been reported [62].Finally, enhanced ROS generation associated with augmented translocation of the gp91^{phox}-p22^{phox} complex, the phagocyte oxidase component p47^{phox} and the atypical protein kinase Cζ to the plasma

membrane has been reported in RhoA/B deficient PMN, further demonstrating the suppressive role of small GTPases on generation of the PMN superoxide burst [38] (Fig. 2).

ITIM containing immunoreceptors regulate PMN ROS generation

Similar to other important PMN effector functions (discussed above), PMN ROS production is also subject to negative regulation by ITIM-domain containing immunoreceptors. The ITIM domain containing inhibitory receptor CD300a is up-regulated by inflammatory stimuli on human PMN and reduces Fc receptor (CD32a) mediated ROS production by inhibiting Ca^{2+} flux [63]. Clec12 is another ITIM-containing C type lectin receptor expressed by PMN that recruits SHP-1 and SHP-2 to negatively regulate Syk signaling. Clec12 is also a negative regulator of PMN ROS production and it has been demonstrated that monosodium urate crystals (MSU)-induced $p40^{phox}$ phosphorylation and subsequent ROS generation is increased in the absence of Clec12 signaling [64]. Glycophorin A, a sialylated erythrocyte membrane glycoprotein, engages PMN via the ITIM-containing inhibitory receptor Siglec 9 to suppress PMN activation and down-regulate the oxidative response [65]. Similarly, murine PMN lacking Siglec E have an enhanced oxidative burst following stimulation compared to WT PMN [66]. The ITIM containing immunoreceptor PIR-B also plays a role in the regulation of PMN activation and inflammatory function with PIR-B deficient PMN displaying increased ROS production compared to PMN from WT mice [26]. Lastly, CEACAM-1 (CD66a) is the only ITIM domain containing carcinoembryonic antigen-related cell adhesion molecule (CEACAM) expressed by PMN. LPS triggers initial phosphorylation of Syk followed by CEACAM-1 mediated recruitment of SHP-1 resulting in inhibition of IL-1β production and negative fine-tuning of the immune response. As such, PMN from *Ceacam-1*$^{-/-}$ mice challenged with LPS exhibit an augmented oxidative burst [67].

Given the requirement for PtdIns(3,4,5)P3 during the oxidative burst, it has been reported that the phosphatase PTEN, which converts PtdIns(3,4,5)P3 back to PtdIns(3,4)P2, is a physiological suppressor of chemoattractant induced PMN ROS release. As such, increased PtdIns(3,4,5)P3 signaling in PTEN deficient PMN corresponds with enhanced Akt phosphorylation, augmented actin polymerization as well as increased fMLF-triggered superoxide production [41]. In addition to the role of Src family kinases in ROS production, it has also been reported that Btk, a member of the Tec-family kinases involved in Toll-like receptor (TLR) signaling, down-regulates the amplitude of the PMN oxidative burst. Therefore, following TLR stimulation PMN from *Btk*$^{-/-}$ mice had increased activation of key signaling molecules involved in NADPH oxidase activation, increased targeting of Rac-2 to the plasma membrane, increased PI3K activation and increased ROS release [68].

Negative regulation of PMN degranulation

PMN granules contain large amounts of antimicrobial peptides, proteases, respiratory burst oxidases as well as membrane bound receptors and adhesion receptors that allow PMN to rapidly respond to and destroy invading pathogens. As tissue-damaging effects of PMN are dependent on the release of microbiocidal PMN granule contents, degranulation is a highly regulated process subject to both positive and negative regulation. Bcr and Abr, are two GTPases-activating proteins (GAPS) with activity towards Rho family GTPases that have been reported to negatively regulate PMN function *in vivo.* Cunnick *et al* showed these two proteins share some overlapping functions while also performing distinct functions [40]. BM PMN from double KO mice (*Abr*$^{-/-}$*Bcr*$^{-/-}$) have elevated levels of activated Rac1 and Rac2 in response to fMLF and cytochalasin B, leading to increased MPO and elastase release compared with WT PMN. However, lactoferrin release, gelatinase release and CD11b

surface expression did not differ between *Abr*$^{-/-}$*Bcr*$^{-/-}$ PMN and WT PMN, indicating that exocytosis of primary granules, but not secondary granules, tertiary granules, or secretory vesicles are negatively regulated by Bcr and Abr [40].

There is abundant evidence that Src family kinases play a pivotal role in PMN degranulation. C-Terminal Scr kinase (Csk) is a regulator of Src kinases that contributes to the control of acute inflammation *in vivo* through phosphorylation of C-terminal inhibitory tyrosine residues [69]. In a mouse model, Csk deficiency in granulocytes (Csk-GEcre), results in hypersensitivity to LPS, increased integrin-dependent adhesion, and exaggerated spontaneous and adhesion-dependent degranulation of secondary granules as measured by lactoferrin release [70]. In Csk deficient PMN, crosslinking of integrins or Fc receptors leads to increased Hck activity that induces hyper-phosphorylation of Syk, paxillin, and cortactin which promotes polymerization and rearrangement of the actin cytoskeleton. This suggests that Csk suppress exocytosis of secondary granules through negative regulation of cytoskeletal remodeling downstream of Fc receptors and integrin activation [70].

Cytokine mediated inhibition of PMN phagocytosis has also been reported. Specifically, exocytosis of tertiary (gelatinase) granules in response to *S. aureus* was inhibited by IL-20 in TNFα-primed PMN [53]. Tertiary granules are more highly associated with actin than primary and secondary granule populations. The regulatory mechanism of IL-20 in TNFα-primed PMN is dependent on ERK1/2 activation and F-actin polymerization. These recent studies highlight the need for increased understanding of negative regulation of PMN activation/resolution of inflammation downstream of host-derived cytokines such as IL-20.

Negative immune regulators of PMN Apoptosis

To achieve and maintain homeostasis, circulating PMN die via constitutive apoptosis and are cleared by macrophages in the liver, spleen and BM. Given the short half-life of PMN, suppression of apoptosis is also essential to facilitate the extended PMN life span observed in the tissues during infection/inflammation. Several agents that prime or activate human PMN including, cytokines, GM-CSF, C5a, LPS, $InsP_6$ and LTB_4 are also reported to prolong PMN survival and down-regulate apoptosis [71]. In particular, PI3K/Akt signaling plays a pivotal role in PMN survival/suppression of apoptosis. Akt-mediated phosphorylation inactivates caspase-9 and stops the pro-apoptotic Bcl-2 family member Bax from associating with mitochondria [72]. In addition, inactivation of the Akt target GSK3β prevents phosphorylation, ubiquitination and proteosomal degradation of the pro-survival Bcl-2 family member Mcl-1 (Fig. 2) [72]. Mechanistically, it has been reported that in apoptotic PMN, accumulated ROS inhibits actin-mediated-PI3Kγ-dependent PtdIns(3,4,5)P3 generation, thus blocking Akt activation and allowing apoptosis to proceed [73]. Proliferating cell nuclear antigen (PNCA) also functions as a PMN anti-apoptotic mediator that binds constitutively to pro-caspases-3, -8 and -9 and blocks their proteolytic processing and activation [74]. Elevated levels of the second messenger cyclic AMP can also prolong PMN lifespan by delaying apoptosis. Though the exact mechanism behind this has yet to be fully elucidated, it has been reported that increased intracellular cAMP levels delay PMN apoptosis via a transcriptionally independent pathway, independent of PI3K activity that involves inhibition of caspase 3 activation and a loss in mitochondrial potential. Another signaling mechanism employed by PMN to delay apoptosis is augmenting production of the second messenger PtdIns(3,4,5)P3 and thus, increasing Akt activation. As such, depleting PTEN increases PtdIns(3,4,5)P3 levels, dramatically delays spontaneous apoptosis of PMN *in vitro* [75], and reduces apoptosis of PMN in inflamed alveolar air spaces [43]. The Src family kinase member Lyn is also an important tyrosine kinase for transducing anti-apoptotic signaling in PMN [76]. ITIM mediated signaling also regulates PMN apoptosis. In particular binding of

CD47 to SIRPα induces ITIM phosphorylation and recruitment of SHP-1 and SHP-2 ultimately resulting in the inhibition of PMN apoptosis [77]. As such, increased PMN surface expression of CD47 is associated with a delay in apoptosis and poor prognosis in non-small cell lung cancer patients [78]. Finally, recent studies have demonstrated that sialylated erythrocyte surface glycans engage the inhibitory immunoreceptor Siglec 9 on circulating PMN to suppress activation and apoptosis [65].

Inhibition of PMN NET release

Neutrophil extracellular trap (NET) release represents an additional PMN extracellular antimicrobial function that can also lead to an alternative form of PMN cell death (NETosis) that is distinct from necrosis or apoptosis. Negative regulation aimed at dampening NET *formation* remains largely unstudied despite recognition of the contribution of NETs to vascular damage and autoimmunity [79, 80]. However, it would be logical to think that ITIM mediated negative regulation of NADPH-dependent ROS production and degranulation of primary granules would also suppress NET formation (Fig. 2). Signal inhibitor receptor on leukocytes-1 (SIRL-1) is an inhibitory receptor, exclusively expressed in myeloid cells, that contains two canonical ITIMs essential for its inhibitory function. While the endogenous ligands for SIRL-1 are yet to be identified, cross-linking of SIRL-1 has been shown to dampen MEK-ERK signaling and suppress spontaneous NET *release* in PMN from individuals with systemic lupus erythematosus (SLE) [81]. Moreover, ligation of SIRL-1 also reduces NET formation in PMN from control donors when incubated with plasma from SLE patients [81]. In addition, NET *formation* induced by opsonized *S. aureus,* and monosodium urate is suppressed by SIRL-1 [82]. In contrast, cross-linking of SIRL-1 does not reduce NET formation when human PMN were exposed to non-opsonized *S. aureus* or to the TLR4

agonist LPS, indicating SIRL-1 regulates NET formation only in response to specific signals. Importantly, phagocytosis and ROS production remain intact following crosslinking of SIRL-1. Therefore, SIRL-1 specifically regulates cellular pathways required for extracellular NET *formation*, but not intracellular microbial killing [82].

The ITIM containing immunoreceptors Siglec 9 and Siglec 5 also deliver inhibitory signals that dampen NET *formation* by human PMN. As discussed above, PMN activation in the circulation is suppressed by erythrocyte sialoglycoproteins interacting with Siglec 9 [65]. In addition, increased concentrations of erythrocytes significantly reduced extracellular DNA release by PMN in response to PMA. Further, this effect was lost when the side chains of the terminal sialic acids were selectively and specifically modified to reduce erythrocyte binding to Siglec 9, proving a specific sialic acid mediated interaction. Incubation of PMN with platelets resulted in inhibition of other PMN functions including apoptosis [83]. However, whether or not platelets can contribute to dampening NET formation through the interaction of their rich sialic acid covered surfaces with PMN Siglec 9 has not yet been reported. Siglec 9 also binds high molecular weight hyaluronan (HMW-HA), which is abundantly expressed in many tissues including synovium, skin, heart valves, etc. While low molecular weight hyaluronan (LMW-HA) is released under inflammatory conditions and can induce pro-inflammatory cytokines, cell proliferation and angiogenesis, HMW-HA mitigates the inflammatory response by binding CD44 and Siglec 9 on PMN. The interaction of HMW-HA with CD44 reduces NET formation induced by PMA. Moreover, the interaction of HMW-HA with Siglec 9 increases SHP-1 recruitment and suppresses not only NET formation, but also apoptosis and oxidative burst by PMA-stimulated PMN as mentioned above [84].

Interestingly, some human pathogens, including Group A (GAS) and Group B (GBS) *Streptococcus*, interact with PMN Siglecs through their capsular polysaccharide components to down-regulate PMN activation and increase the rate of bacterial survival [84-87]. GAS

expresses a HMW-HA capsule and GBS displays terminal sialic acids, both of which are able to engage Siglec 9 and block NET formation [84, 86]. GBS also binds to Siglec 5 through β protein expressed in its capsule, resulting in inhibition of NET formation independent of sialic acid binding [85]. *Pseudomonas aeruginosa*, an opportunistic human pathogen, does not synthetize sialic acid but it is able to absorb sialoglycoproteins from host serum that it uses to bind to PMN Siglec 9 thus suppressing PMN ROS release and NET formation [87].

Another family of proteins that play multiple roles in regulating PMN effector functions are the semaphorins. Semaphorins are transmembrane proteins that bind several receptors, i.e. plexins B1/B2 and CD72 [88]. Recently it has been reported that binding of PMN expressed semaphorin 4D (SEMA4D) to its endothelial expressed receptor Plexin B2 results in activation of the SEMA4D intracellular domain, decreased Rac activation and inhibition of anti-PMN cytoplasmic-induced oxidative burst and NET *release* [89].

Conclusions

PMN exert important effector functions in order to provide host defense against bacterial and fungal infections. Activation of PMN functions is initiated by receptor-ligand binding events and modulated by an array of intracellular signaling pathways. Insufficient PMN activity renders the host susceptible to repeated infections, which can be life-threatening. Yet, dysregulated PMN activation can result in excessive collateral damage to the host, as exemplified by a range of inflammatory and autoimmune diseases. Therefore, PMN activation must be tightly regulated to protect the host from pathogen assaults without inducing detrimental inflammation and tissue damage.

This review provides an overview of immune signaling pathways that negatively regulate PMN function with a particular emphasis on PMN regulation through ITIM-bearing inhibitory receptors (Table 1). Despite the existence of a number of studies describing negative regulation of PMN function downstream of cell surface receptor engagement, many questions remain. For example, it is not well understood how single classes of PMN inhibitory receptors can differentially respond to various extracellular stimuli to specifically downregulate discrete PMN effector functions. Similarly, it is yet to be determined how different PMN inhibitory receptors can exert discrete functional responses to the same extracellular signals. While we have focused on suppression of PMN functions by intrinsic pathways it is also known that PMN activation in inflamed tissues is regulated by specialized pro-resolution mediators (secreted by epithelial and immune cells) including resolvins, protectins and maresins which favor deactivation of PMN and the resolution of inflammation. The identification of new drug targets aimed at downregulating PMN activation may be useful for reducing the uncontrolled inflammation that is characteristic of septic shock, chronic inflammatory conditions and autoimmune diseases. Therefore, increased understanding of the mechanisms that negatively regulate PMN function is likely to have profound biologic and pharmacologic relevance.

Authorship

V. Azcutia, C.A. Parkos and J.C. Brazil contributed to writing this review.

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Disclosures

The authors declare no conflicts of interest.

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Figure legends

Figure 1. Schematic representation of ITIM mediated inhibition of PMN trafficking. Phosphorylation of intracellular ITIM domains by Src kinases leads to recruitment of SHP1/2 and SHIP1/2 followed by subsequent inhibition of Syk kinase-mediated PI3K activation, CD18/CD11b signaling, actin polymerization, ERK1/2 activation, Rap activation and Ca^{2+} mobilization, decreased generation of PtdIns(3,4,5)P3 and decreased Akt activation leading to reduced PMN activation and reduced PMN trafficking [16, 26-29, 33-35, 42].

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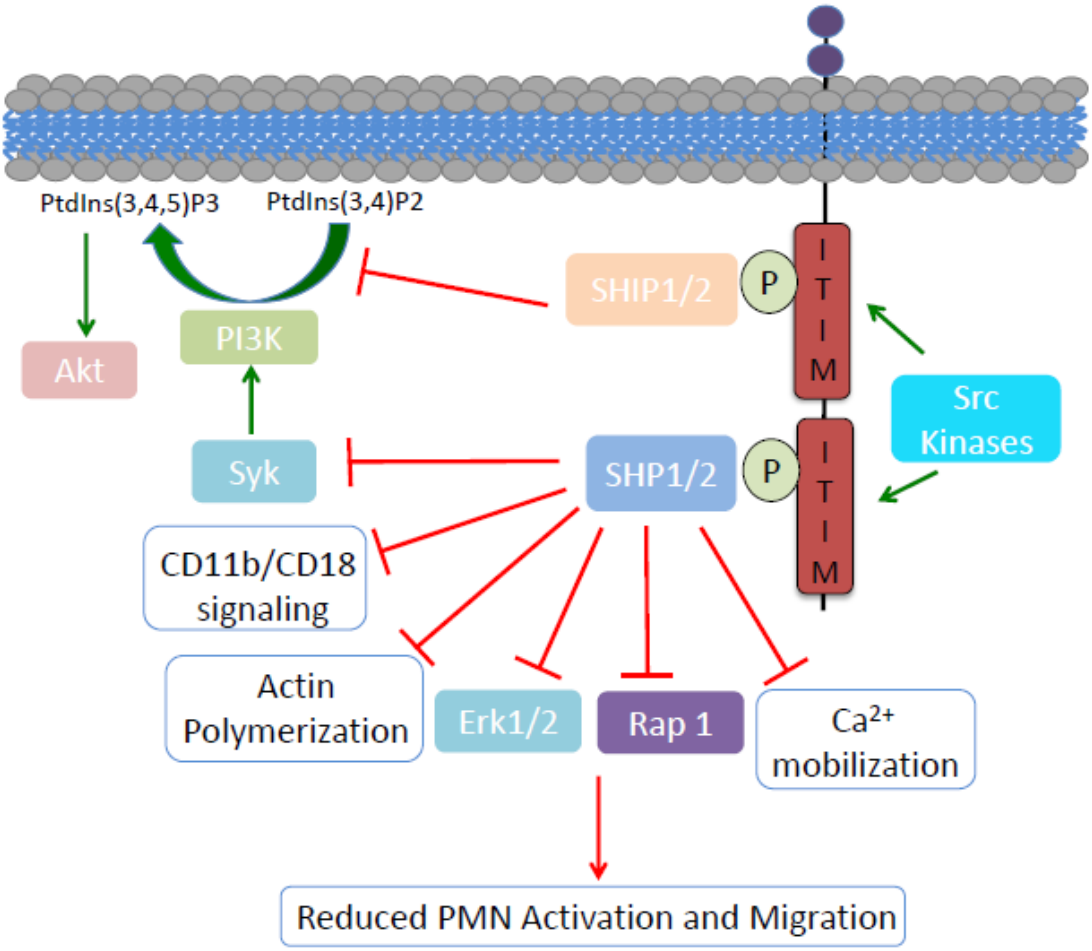

Figure 2. Different effectors negatively regulate PMN functions. GTPase-activating proteins (GAPS), have been shown to suppress PMN functions such as phagocytosis, ROS production, and degranulation by inhibiting the activation of Rac and F-actin polymerization [39, 40, 56, 57, 61]. Vav-1 and TIPE-2 also inhibits Rac activation and therefore, suppress ROS production [59, 60]. Phosphatases such as PTP1B and PTEN negatively regulates phagocytosis [36]. PTEN also regulates ROS production by regulating the availability of PtdIns(3,4,5)P3 [41]. Since it is well described NET formation depends of ROS production it is possible that negative regulators of ROS production are also potentially inhibiting NETosis. C-terminal kinase inhibits degranulation by phosphorylating the C-terminal inhibitory tyrosine residues of Src kinases [69]. PMN apoptosis can be delayed by active Akt promoting the inactivation of caspase-9 and therefore the inactivation of the pro-apoptotic Bcl-2 family member Bax. In addition, Akt has been described to inactivate GSK3β, preventing the degradation of the anti-apoptotic Bcl-2 member Mcl-1 [72].

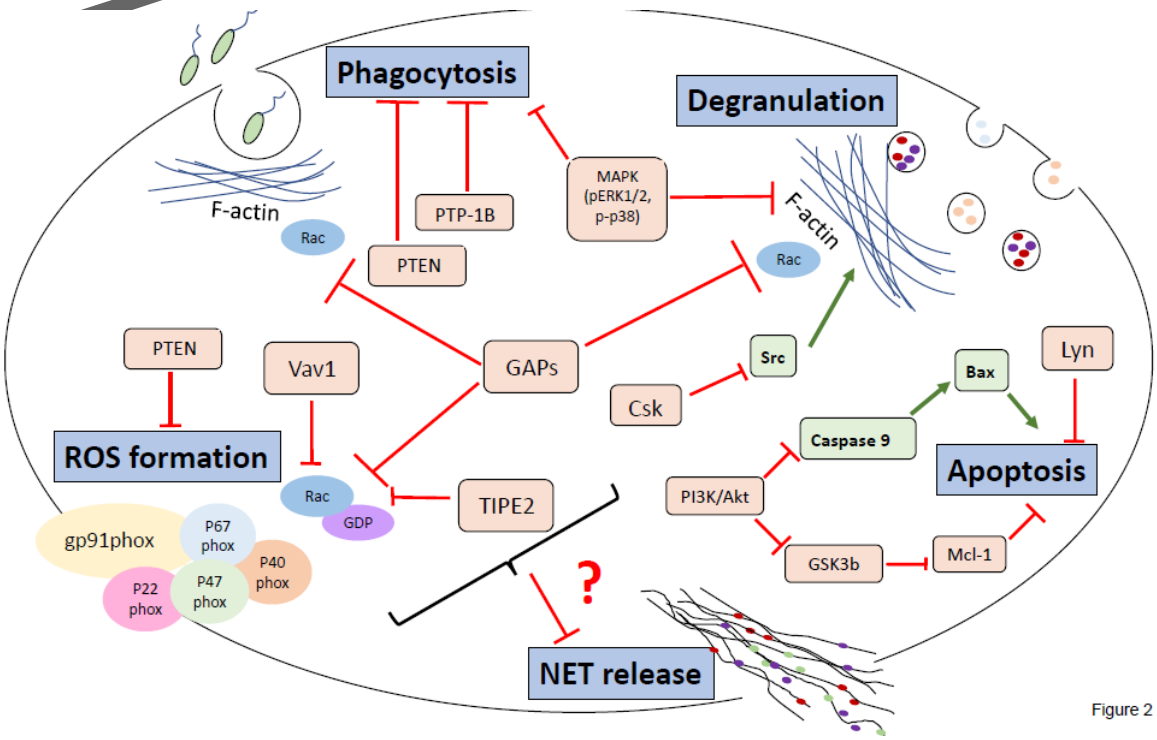

Figure 2

Effector	PMN Function	Gene	Reference
Kinases:			
Csk	Degranulation	*Csk*	70
Akt	Apoptosis	*Akt, Pkb,*	72, 73, 75
Src family kinases:			
Hck	Granulopoiesis, Trafficking	*Hck*	27
Fgr	Trafficking	*Fgr, Src2*	27
Lyn	Trafficking, Apoptosis	*Lyn, Jtk8*	6, 15,16, 76
Small GTPases and regulators:			
RhoA/B	Trafficking, ROS	*Rhoa, Arha; Rhob, Arhb*	38
Vav1	ROS	*Vav1*	59
Tipe2	ROS	***Tnfaip8l2***	60
Arap3	ROS	***Arap3****, Centd3*	62
ARHGAP25	Trafficking, Phagocytosis	*Arhgap25*	39, 57
ARHGAP15	Phagocytosis	*Arhgap15*	56
Abr	Trafficking, Degranulation	*Abr*	40
Bcr	Trafficking, Degranulation, ROS	*Bcr*	40, 61
Phosphatases:			
Wip1 (PP2Cd)	Granulopoiesis	*Wip1, Ppm1d*	20
SHP-1	Trafficking, ROS, Apoptosis, NETosis	*Ptpn6 Hcp, Ptp1c*	15, 24, 32-34, 64, 67, 77, 84
SHP-2	Trafficking, ROS, Apoptosis	*Ptpn11, Ptp2c*	24, 66, 77
PTP1B	Trafficking, Phagocytosis	*Ptpn1, Ptb1b*	35-37
PTEN	Trafficking, Phagocytosis, Apoptosis	*Pten Mmac1, Tep1*	41-43
Cytokines and suppressors:			
IL-20	Trafficking, Phagocytosis	***Il20*** *ZCYTO10*	53, 54
SOCS-3	Granulopoiesis, Trafficking	*Socs3*	7, 8, 51, 52

Orthologue in mouse	Ligand	PMN Function	Gene	References

Inhibitory Receptors:					
PILRα	*ND*	CD99-like protein HSV-1: glycoprot B	Trafficking	*Pilra*	28
LILRB3	PIR-B	MHC-I and class I like molecules, Angiopoietin-like Protein 2	Trafficking, ROS	*Lilrb3 Pirb*	15, 26, 27
SIRP-α		CD47, SF-A, SF-D	Trafficking, Apoptosis	*Sirpa*	15, 22, 23, 77
Siglec 5	Siglec F	Sialic acid	NETosis	*Siglec5*	85
Siglec 9	Siglec E	Sialic acid, HMW-HA	NETosis, ROS, Apoptosis	*Siglec9*	25, 65, 66, 83, 84, 86, 87
SIRL-1	Not in mouse	*ND*	NETosis	*Vstm1*	81, 82
CD300a		Phosphatidylserine Phosphatidylethanolamine	ROS	*Cd300a Cmrf35h*	63
CEACAM1 (CD66a)	CEACAM1P	CEACAM1	ROS	*Ceacam1 Bgp, Bgp1*	67
CLEC12a	Clec12a	Uric acid	ROS	*Clec12a Dcal2*	64
Receptors w/o ITIM domains:					
CXCR4	CXCR4	CXCL12	Granulopoiesis	*Cxcr4*	9, 12-14
CD47 (IAP)	CD47	SIRP-α, -γ, TSP-1, -2	Apoptosis	***Cd47***	78
A(2A)AR	A(2A)AR	Adenosine	Trafficking	***Adora2a*** *Adora2*	44, 45
FPR2/ALX	FPR2	Lipoxin A4, Annexin A1, serum amyloid A, Resolvin D1, formylated peptides.	Trafficking	***Fpr2***	48-50
Semaphorin4D	Semaphorin4D	Plexin B1/B2, CD72	NETosis	***Sema4D***	89

ND: not determined

Table 1: Effector molecules and inhibitory receptors found on PMN. Top table shows effector molecules playing a role in negative regulation of PMN functions. Bottom table shows inhibitory receptors found on PMN. First column shows the most common names found on human PMN inhibitory receptors. The second column shows the orthologues found

in mouse PMN. All inhibitory receptors possess at least one immunoreceptor tyrosine- based inhibitory motif (ITIM). Other receptors without an ITIM domain are able to suppress PMN functions.