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The expanding roles of ITAM adapters FcRγ and DAP12 in myeloid cells

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Summary

The adapter proteins DAP12 and FcRγ associate with a wide spectrum of receptors in a variety of innate immune cells to mediate intracellular signaling pathways when their cognate receptor is engaged. These adapter proteins are coupled to their receptors through charged residues within the transmembrane regions of the adapter and receptor. DAP12 and FcRγ contain specific protein domains (referred to as ITAM domains) that serve as the substrates and docking sites for kinases allowing amplification of intracellular signaling reactions. Recent research has broadened the repertoire of receptors that utilize these adapters for signaling to include not only novel Ig-superfamily members but also cytokine receptors, integrins and other adhesion molecules. Even more amazing, there is abundant evidence that these multifunctional signaling adapters also mediate inhibitory activity, downmodulating signaling from TLRs and other heterologous receptors. In this review, we discuss the newly described receptors that utilize DAP12 and/or FcRγ adapters to modulate innate immune responses.

Keywords

ITAM signaling; immunoreceptors; inhibitory pathways; myeloid cells; TLRs

Introduction

The immunoreceptor tyrosine-based activation motif (ITAM) intracellular signaling pathway represents perhaps the dominant mechanism by which immune cells respond to their environment. ITAM signaling was first recognized to be critical in lymphocyte stimulation by antigens through both B-cell and T-cell receptors (BCR and TCR) (reviewed in (1, 2)). Indeed, signaling through these receptors provides the overall conceptual framework for how ITAM pathways function in immune cells. In this framework, the receptor subunits, which mediate ligand binding, physically associate with different ITAM-containing adapter proteins (the CD79 subunits for the BCR and the CD3 subunits for the TCR) to communicate intracellular signaling. The ITAM itself consists of a highly conserved sequence D/ExxYxxL/I(x₆₋₈)YxxL/I (3). The ITAM-containing signaling adapter

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is coupled to the ligand binding receptor through charged amino acid interactions within the transmembrane regions of each protein (commonly aspartic acid in the ITAM-containing subunit and lysine or arginine in the receptor). Following engagement of the receptor by ligand (for example antigens in the case of the BCR or TCR), Src-family kinases become activated (by a yet unclear mechanism), which in turn phosphorylate the two tyrosine residues of the ITAM. These phospho-tyrosine residues form a docking site for ζ -associated protein of 70 kD (ZAP70) kinase or spleen tyrosine kinase (Syk) through interactions with Src-homology 2 (SH2) domains on the kinases. This induces activation of the kinase, which in turn phosphorylates a number of downstream substrates (for example, LAT in T-cells, SLP-76 in B cells) allowing for formation of a signaling complex that communicates to a number of pathways in the cell (MAPK, PLC γ /Ca²⁺, Vav/Rac or NF- κ B as examples), leading to cellular activation (Fig. 1). Because this pathway was first defined for receptors containing immunoglobulin-like domains (such as the BCR and TCR), it is commonly referred to as the immunoreceptor (or ITAM) pathway. In this review, we define the term immunoreceptor as Ig superfamily or C-type lectin receptors that associate directly with ITAM-adapters through a positively charged amino acid in their transmembrane region.

In myeloid cells, the primary ITAM-containing signaling adapters are DAP12 and the Fc ϵ R γ chain (referred to as FcR γ) (4, 5). These adapters were first defined by their role in signaling downstream of immunoreceptors present in NK cells or myeloid cells, respectively. All ITAM signaling adapters, including DAP12 and FcR γ , are structurally characterized as small (15 – 20 kD) proteins with very short extracellular regions that contain a cysteine residue to allow the molecules to form homodimers through disulfide bonds. Within the cytoplasmic region, both DAP12 and FcR γ have a single ITAM which, when dually phosphorylated, provides a high affinity binding site for the Syk family kinases ZAP-70 and Syk. The ITAM sequences of DAP12 and FcR γ are remarkably conserved through evolution, and related orthologs of these proteins are found in all vertebrates, demonstrating their central role in immune cell signaling. Mutation of either of the ITAM tyrosines (or the respective SH2 domains in ZAP70/Syk kinases) completely blocks the signaling function of all associated immunoreceptors in the cell. Complete deletion of either DAP12 or FcR γ results in loss of cell surface expression of the majority of immunoreceptors to which the adapter pairs, demonstrating that formation of a transmembrane charge bridge stabilizes the receptor at the cell surface.

DAP12 and FcR γ are found in most cells of the innate immune system, including neutrophils, macrophages, various dendritic cell (DC) types, basophils, eosinophils, mast cells and NK cells. Within these cell types, these signaling adapters pair and mediate signaling from a remarkably large repertoire of immunoreceptors (Fig. 1) (6). Originally, both DAP12 and FcR γ were defined as pairing with immunoreceptors that induce cellular activation; for example DAP12 mediates NK cell activation through Ly49D and Ly49H in the mouse (7) or the CD94/NKG2C complex in the human (8), whereas FcR γ mediates macrophage, neutrophil and mast cell activation following engagement of their Fc γ or Fc ϵ receptors (9, 10). The repertoire of immunoreceptors to which these adapters pair to mediate innate immune cell activation has since grown considerably. The ligands for some, but not all, of these immunoreceptors are known, and consist of both protein or carbohydrate structures present on many pathogenic organisms as well as endogenous cellular macromolecules.

A notable development in this field is the relatively recent description of the involvement of DAP12 and FcR γ in signaling pathways mediated by non-immunoreceptors, which we define as those receptors that do not directly associate with these signaling adapters using positively charged amino acids in their transmembrane regions (reviewed in (11-13)). Most of these studies have evolved out of phenotypic characterization of non-immunoreceptor

signaling pathways in innate immune cells derived from DAP12 and/or FcR γ deficient mice. Examples of non-immunoreceptors in myeloid cells that rely on ITAM adapters for signaling include integrins, growth factor receptors and antigen presenting (MHC) proteins. Perhaps even more remarkable, it is now well established that DAP12 and FcR γ serve an inhibitory function in innate immune signaling as well (reviewed in (12, 14)). This discovery also emanates from phenotypic characterization of cells from DAP12 and/or FcR γ deficient mice or from cross-linking of associated receptors with antibodies.

This review will cover the new aspects of both the positive and negative (or inhibitory) regulatory function of these ITAM adapters in a variety of recently described signaling responses.

Positive signaling by ITAM adapters

As described above, both DAP12 and FcR γ were first defined by their ability to transmit signals leading to cellular activation by coupling to Ly49 receptors in NK cells or Ig (Fc γ , Fc ϵ and Fc α) receptors in myeloid cells, respectively. However, the spectrum of receptors to which these molecules couple has now expanded dramatically and includes many non-immunoreceptor type proteins. FcR γ has now been demonstrated to mediate signaling through immunoreceptors such as Mincle (15-18), Dectin-2 (19), and MAIR-IV (20), but even more surprisingly from non-immunoreceptors such as the IL-3 receptor (21) or receptors involved in mycobacterial sensing (22). The spectrum of DAP12-associated receptors is even larger, extending way beyond just activating receptors in NK cells (reviewed in (4)). Included in this list are many immunoreceptors such as TREM-2 (23), MDL-1 (24) and PDC-TREM (25), as well as a surprising number of non-immunoreceptor molecules such as the c-Fms receptor (26, 27). In many cases, signaling from non-immunoreceptors requires both DAP12 and FcR γ , as loss of function is only seen in cells derived from mice lacking both adapters. This is certainly the case in the ITAM-mediated signaling of adhesion receptors such as integrins (reviewed in (28)) and P-selectin glycoprotein ligand (PSGL-1) as well as the involvement of DAP12 and FcR γ in IFN γ priming/activation (29, 30) or cross presentation of particulate antigens by MHC I (31). Whereas most immunoreceptors pair with either DAP12 or FcR γ exclusively, the myeloid and B cell expressed MAIR-II receptor has been shown to pair with both ITAM-containing signaling adapters (32).

Activation by FcR γ -associated receptors – new immunoreceptor associations

While most intensely studied in Fc receptor signaling pathways, it is now clear that the FcR γ adapter is important in other innate immune signaling responses. Indeed, the requirement for FcR γ in signaling through receptors other than FcRs was first realized in 1997 by Watson's group in their studies of the platelet collagen receptor GPVI (33). FcR γ was subsequently shown to associate with a variety of NK and myeloid-expressed receptors, including OSCAR, PIR-A and activating ILT receptors (6). Recently, Sato et al showed that FcR γ associates with the fungal receptor Dectin-2 (19). This receptor is present at high levels on activated DCs and macrophages where it is responsible for binding the hyphal forms of various fungi such as *Candida albicans*, *Microsporium* and *Trichophyton*. Binding of hyphae from these organisms to macrophages activates a tyrosine kinase pathway that leads to pro-inflammatory cytokine release and phagocytic uptake of the hyphae. Dectin-2 is physically associated with the FcR γ chain and crosslinking of Dectin-2 leads to Src-family kinase dependent phosphorylation of FcR γ followed by Syk kinase recruitment in a classical immunoreceptor pathway. Interestingly, the other major fungal receptor on innate immune cells, Dectin-1 does not use an associated adapter like DAP12 or FcR γ but instead has its

own intrinsic ITAM in its cytoplasmic tail with which it initiates intracellular signaling in a Src-kinase/Syk pathway (34).

Macrophage-inducible C-type lectin (Mincle) was first identified as a LPS-induced target of the NF-IL6 (C/EBP β) transcription factor in macrophages. Mincle has a short 21 amino acid cytoplasmic domain with no apparent signaling capacity (35). Recently, Yamasaki et al. showed that Mincle associates with FcR γ in macrophages and that signaling through Mincle is dependent upon this ITAM adapter (18). Cross-linking of Mincle with a mAb induces TNF and MIP-2 (CXCL2) secretion as well as Syk and ERK1/2 phosphorylation in wild-type (WT) macrophages, but not in FcR γ -deficient macrophages. Interestingly, the authors found that Mincle binds a component of snRNPs, spliceosome-associated protein 130 (SAP130). SAP130 is typically a nuclear protein, but is released from late apoptotic or necrotic cells. Consistent with this finding, late apoptotic cells or supernatant from these cells can activate reporter cells expressing Mincle/FcR γ . In contrast, treatment of FcR γ -deficient macrophages with recombinant SAP130 results in less MIP-2 secretion compared to WT cells (18).

The *in vivo* function of Mincle in recognition of dead cells was addressed using a blocking antibody to this receptor and whole body irradiation of mice, which induces rapid thymocyte apoptosis. In response to irradiation, macrophages in the thymus secrete MIP-2, which causes neutrophils to accumulate within the organ. In the presence of blocking anti-Mincle mAb, both MIP-2 secretion by thymic macrophages and neutrophil accumulation is reduced. Blocking Mincle also reduces MIP-2 and TNF release and the accumulation of neutrophils in the peritoneal cavity after the injection of dead cells. These data show that Mincle is an important receptor for recognition of dead cells both *in vitro* and *in vivo*.

Mincle has also been shown to be a receptor for pathogenic and non-pathogenic fungi (15, 16, 18). Wells et al. found that recombinant Mincle binds to soluble extracts from *Candida albicans* and *Saccharomyces cerevisiae*; treatment of macrophages with a blocking anti-Mincle mAb reduces TNF secretion induced by *C. albicans* (15, 16). This group also generated Mincle-deficient mice and found that macrophages lacking Mincle secrete less TNF in response to *C. albicans* compared to WT cells, but show normal TNF production to zymosan, a *S. cerevisiae* cell wall extract. After systemic challenge with *C. albicans*, Mincle-deficient mice manifest higher titers of fungi in their kidneys than WT mice, illustrating the importance of Mincle to the immune response to *C. albicans in vivo* (16). Yamasaki et al. also found that Mincle is a receptor for pathogenic fungi using a functional assay to screen a panel of fungi with Mincle/FcR γ -expressing reporter cells (17). In this assay Mincle specifically recognizes *Malassezia* species, but not species of *Candida*, *Aspergillus* and other fungi. This binding requires calcium and the EPN motif of the carbohydrate recognition domain of Mincle, suggesting that Mincle has true lectin activity. Notably, the binding of Mincle to SAP130, described by the same group, does not depend upon calcium or the EPN motif (18). As expected, Mincle-deficient macrophages produce less MIP-2, TNF, KC and IL-10 than WT macrophages in response to *M. pachydermatis*. Similarly, following intraperitoneal challenge with *M. pachydermatis*, Mincle-deficient mice produce less IL-6 and TNF compared to WT mice. Therefore, in a theme common to other FcR γ and DAP12-associated immunoreceptors, Mincle recognizes both self (SAP130) and foreign (fungal) ligands.

Interestingly, a second C-type lectin receptor, CLEC9A (also known as DNGR-1), has recently been found to mediate activation of CD8 α^+ DCs following uptake of dead cells (36). Signaling through this receptor is required for efficient cross priming of CD8 $^+$ T-cells that recognize neo-antigens derived from necrotic cells. Like Mincle (and TREM-2 as well as TREM1-4, which also recognize dead cell material, see below), CLEC9A signaling is

mediated by the ITAM/Syk pathway. However, in contrast to these other receptors, CLEC9A mediates this signaling through a partial ITAM sequence embedded directly in its C-terminal tail – mutation of the critical tyrosine in the “hem-ITAM” cytoplasmic domain of CLEC9A blocks its ability to signal cross presentation by DCs. The molecular identity of the CLEC9A ligand is unclear and whether this receptor may also recognize pathogen molecules is unknown. Hence, in analogy to Dectin-1 and Dectin-2, innate immune cells use different receptors that either couple to ITAM adapters or contain ITAM sequences within the receptor itself, to mediate inflammatory responses to necrotic cells.

Activation by FcR γ -associated receptors – non-immunoreceptor associations

In addition to the immunoreceptors described above, FcR γ has recently been found to be an important component of the IL-3 receptor signaling pathway (21). This discovery was made through studies of basophil function in FcR γ -deficient mice. Though basophils develop normally in FcR γ -deficient mice, they fail to produce IL-4 when stimulated with IL-3, resulting in poor differentiation of Th2 cells in co-cultures of basophils and T-lymphocytes. The involvement of FcR γ in IL-4 production seems to be selective, as other functions of IL-3, such as the promotion of basophil proliferation, is not affected in FcR γ -deficient mice. Remarkably, these investigators found that FcR γ directly associates with the β c signaling subunit of the IL-3R through transmembrane portions which are distinct from that used for association of FcR γ with Fc ϵ RI or Fc α RI. Mutation of the ITAM of FcR γ ablates its ability to signal IL-4 release following IL-3 stimulation of basophils. These studies demonstrate that ITAM/Syk kinase type signaling responses may be just as important for aspects of cytokine responses as the traditional JAK/STAT pathways that we think of as being the dominant mechanism of cytokine signaling.

The FcR γ to Syk kinase pathway has also been found to be critical in DC and macrophage responses to *Mycobacterium tuberculosis* glycolipids (22). The mycobacterial cell wall component Trehalose-6,6-dimycolate (TDM), or its less toxic, synthetic analogue Trehalose-6,6-dibehenate (TDB) are both potent activators of cytokine release and nitric oxide (NO) production by macrophages. However, both of these analogs fail to induce NO release from FcR γ -deficient and Syk-deficient macrophages, whereas DAP12-deficient cells respond normally. The FcR γ /Syk mediated response to these glycolipids, as well as to a number of fungal agents, seems to be most important in activating the CARD9/MALT1 inflammasome pathway (leading to NF- κ B activation), rather than coupling to the more traditional Ras/Erk pathway as described for most immunoreceptors (37). This novel function of FcR γ -initiated signaling highlights the growing diversity of receptors and pathways in which this ITAM adapter participates. Clearly, definition of the receptors involved in the innate immune cell response to mycobacterial lipids is of paramount importance for vaccine research.

Activation by DAP12-associated receptors – new immunoreceptor associations

There have been many advances in understanding the function of DAP12 and its associated receptors in the activation of myeloid cells. Recently, the myeloid DAP12-associating lectin (MDL-1), also known as CLEC5, has been found to function as a receptor for dengue virus; an MDL-1-Fc fusion protein can directly bind dengue virions as determined by direct ELISA and immunoprecipitation (38). Though MDL-1 is a C-type lectin receptor, binding to dengue is not dependent upon Ca⁺², but is partially inhibited by excess fucose and mannose. This is in contrast to DC-SIGN, another myeloid-expressed C-type lectin receptor, which

also binds the dengue envelope but in a Ca^{+2} -dependent manner that is blocked by mannans and mannose (39, 40). Treatment of human monocyte-derived macrophages with dengue virions induces phosphorylation of DAP12. ShRNA-mediated knockdown of MDL-1 expression in these cells blocks DAP12 phosphorylation and reduces inflammatory cytokine production following dengue virus challenge (38). In contrast, MDL-1 appears to have no role in the infection of macrophages by dengue, which was dependent upon DC-SIGN. Therefore, these two C-type lectin receptors for dengue virus participate in different processes—DC-SIGN in viral uptake and MDL-1 in viral-induced inflammatory mediator production. Blocking MDL-1 in a mouse model of dengue virus infection partially protects mice from lethal dengue virus disease by reducing serum TNF and IP-10 levels. These data show that MDL-1 participates in the inflammatory response to dengue virus and may be an attractive drug target for treating dengue shock syndrome and dengue hemorrhagic fever, two life-threatening diseases associated with secondary dengue virus infection.

MDL-1 has also recently been shown to participate in osteoclast function in mice, through association with both DAP12 and DAP10 (24). DAP10 is a small signaling adapter expressed in myeloid and NK cells. It also uses a negatively charged aspartic acid in its transmembrane to associate with immunoreceptors, but the DAP10 cytoplasmic domain contains a PI-3 kinase binding site rather than an ITAM (4). DAP12-deficient mice have modest osteopetrosis (or bone overgrowth), which is likely due to reduced fusion capacity of bone marrow precursors during formation of multinucleate osteoclasts (41-43). DAP10-deficient mice also have mild osteopetrosis and reduced osteoclast fusion in culture at limiting doses of osteoclast growth factors M-CSF and RANKL (24). Co-immunoprecipitation experiments demonstrate that DAP10 and DAP12 both associate with MDL-1 in osteoclast precursors, suggesting that in these cells, the adapters may heterodimerize. Consistent with pairing of both DAP10 and DAP12 to MDL-1, surface expression of the receptor is decreased in DAP10-deficient or DAP12-deficient osteoclasts, and is absent on DAP10/DAP12 double-deficient cells. Confirming the role of MDL-1 in osteoclast development, knockdown MDL-1 in pre-osteoclasts reduces their ability to generate mature osteoclasts (24).

The idea of dual signaling through DAP10 and DAP12 co-associated receptors has also been suggested in NK cells, where both of these adapters bind to the NKG2D activating receptor. However, biochemical experiments suggest that NKG2D pairs only with one homodimer of each signaling adapter, not heterodimeric DAP10/DAP12 complexes (4). More recently, several groups have shown that DAP10 can associate with and contribute to the function of other NK cell-expressed C-type lectin receptors previously thought only to use DAP12, including Ly49H and Ly49D (44-46). The potential dual utilization of DAP10 and DAP12 by other myeloid innate immune receptors remains to be explored.

Several new studies have added to our understanding of the function of the DAP12-associated TREM-2 receptor. TREM-2 is expressed on tissue macrophages, (including osteoclasts and microglia), bone marrow-derived macrophages and DCs, where it mediates both positive and negative signals in myeloid cells (see below). TREM-2 and DAP12 have also been shown to have both positive and negative effects on osteoclastogenesis *in vitro* (47). Interestingly, TREM-2 associates with Plexin-A1 on DCs and Plexin A1-deficient mice show osteoclast defects similar to DAP12-deficient mice (48). A new study from Helming et al. found that TREM-2/DAP12 also regulates a different form of macrophage fusion into multinucleate giant cells (49). Mature macrophages fuse into multinucleate giant cells *in vivo* during a foreign body response or granulomatous inflammation, such as is seen during mycobacterial infection. This fusion occurs *in vitro* if mouse thioglycollate-elicited macrophages or bone marrow-derived macrophages are cultured in the presence of IL-4 or IL-4 and GM-CSF, respectively. Helming et al. found that both murine and human

monocyte-derived macrophages lacking DAP12 or DAP12 signaling, either by knockout or knockdown, are defective in *in vitro* fusion compared to WT macrophages (49). Generation of multinucleate giant cells *in vivo* during a foreign body response is also reduced in mice lacking DAP12. Knockdown of TREM-2 in mouse bone marrow-derived macrophages severely reduces fusion, showing that TREM-2 is the principal DAP12-associated receptor required for *in vitro* fusion. The authors conclude that TREM-2 and DAP12 allow macrophages to reach a fusion-competent state as they regulate the expression of several proteins known to be involved in macrophage fusion, including DC-STAMP and E-cadherin. Though this observation is consistent with the majority of the work showing a positive role for TREM-2 and DAP12 in osteoclast fusion in mice and humans (41, 42, 50-52), it is not consistent with the fact that TREM-2-deficient bone marrow shows accelerated *in vitro* osteoclastogenesis or necessarily with the bone cysts seen in patients lacking these proteins (see below) (47). Analysis of macrophage and osteoclast fusion in TREM-2-deficient mice will be of future interest as will identification of endogenous TREM-2 ligands that mediate these effects.

TREM-2 paired with DAP12 has also been found to mediate phagocytosis (53-55). Takahashi et al. originally implicated TREM-2 and DAP12 in the phagocytosis of apoptotic neurons by microglia (55). shRNA-mediated knockdown of TREM-2 or DAP12 in mouse microglia blocks their ability to take up apoptotic neurons, whereas overexpression of TREM-2 induces increases phagocytosis. Hsieh et al. have expanded on this finding by showing that neurons express a ligand for TREM-2 that is increased during the induction of apoptosis, shown by staining cells with a fusion protein of TREM-2 and human IgG1 (53). In fact, several cell lines have increased staining with the TREM-2 Fc reagent after the induction of cell death (53, 56). When non-phagocytic CHO cells are transfected with a TREM-2/DAP12 chimera they can endocytose apoptotic neurons, showing TREM-2 is a bona fide phagocytic receptor for these dying cells (53). One endogenous ligand for TREM-2 has recently been identified as Hsp60, a mitochondrial chaperone which can be released from both healthy and dying cells (57). Hsp60 present on the surface of neuroblastoma N2A cells serves as a ligand for TREM-2/Fc fusion. Moreover treatment of N9 microglial cells with purified Hsp60 increases their phagocytosis of dying cells in a TREM-2-dependent manner. Interestingly, a new DAP12-associated TREM family member, TREM-like 4 (TREM14), has also been shown to bind dying cells. TREM14 is specifically expressed on DCs and certain macrophage populations in the spleen and is most highly expressed on CD8-expressing DCs that efficiently phagocytose apoptotic cells (56). Its ligand is unknown.

TREM-2 has also binds several species of bacteria and fungi (58). Recently, N'Diaye et al. reported that TREM-2 serves as a phagocytic receptor for these microbes (54). Similar to what was shown for apoptotic neurons, transfection of CHO cells with a TREM-2/DAP12 chimera allows these cells to phagocytose *E. coli*, *S. aureus* and *F. tularensis*, but not zymosan, a *S. cerevisiae* cell wall particle. This phagocytosis in CHO cells requires Src and Syk kinase activity as well as the activity of the Rho-family GTPases Rac1 and Cdc42. DAP12-deficient macrophages have reduced phagocytosis of all 3 bacteria tested compared to WT macrophages; expression of the TREM-2/DAP12 chimeric receptor in the DAP12-deficient cells restores their phagocytic activity to WT levels. TREM-2-deficient macrophages or WT TREM-2 knockdown cells also have reduced *E. coli* phagocytosis. Interestingly, Charles et al. found that TREM-2 and DAP12 are involved in regulating the macrophage response to *Salmonella typhimurium* though TREM-2 does not directly bind this organism (59). Collectively, these observations demonstrate that the TREM-2/DAP12 receptor complex binds both endogenous and exogenous ligands to induce phagocytosis. Hence, this dual function of TREM-2 resembles the ability of Mincle to recognize both pathogen and host-derived ligands.

PDC-TREM is another newly described TREM family member that associates with DAP12. Plasmacytoid DCs (PDCs) are specialized for type 1 IFN production in response to viral infection and among the TLRs, they express exclusively TLR7 and TLR9, which sense ssRNA and CpG-containing DNA, respectively. PDC-TREM is specifically expressed on PDCs after stimulation with TLR7 or TLR9 agonists (25). Similar to TREM-2, PDC-TREM associates with Plexin-A1 and participates in signal transduction by the Plexin-A1 ligand, Sema6D. In PDCs stimulated with low levels (0.1 μ M) of CpG, co-stimulation with Sema6D-Ig fusion leads to slow enhancement of IFN α production. But in PDCs that are stimulated with optimum levels of CpG (1 μ M) PDC-TREM expression increases 10-fold to the point where the co-associated PlexinA1 receptor can potentially interact with endogenous Sema6D to mediate production of IFN α . The production of IFN α following optimal CpG stimulation is reduced in DAP12-deficient PDCs, in cells following knockdown of PDC-TREM by shRNA, and in cells treated with a mAb that blocks PDC-TREM/Plexin A1 association. These results suggest that the complex of PDC-TREM/ DAP12/Plexin A1 is required for production of IFN α by TLR agonists in PDCs.

Activation by DAP12-associated receptors – non-immunoreceptor associations

Outside of the immunoreceptor field, there is now compelling evidence that DAP12 plays a central role in signaling through the c-Fms or M-CSF receptor (CSF-1R) in mononuclear cells (26, 27). These studies are particularly revealing because they provide some molecular understanding of the consequences of DAP12 deficiency in both mice and humans. As mentioned above, mice lacking DAP12 develop a modest form of osteopetrosis (41-43). This phenotype is much worse in mice lacking both FcR γ and DAP12 (60, 61), potentially as a result of both poor cell fusion events during osteoclastogenesis and reduced adhesion of osteoclasts to bone matrix through the $\alpha\beta$ 3 integrins leading to poor bone resorption (61). In contrast, humans lacking DAP12 develop polycystic osteodysplasia (bone cysts) leading to spontaneous fractures (62). These patients (which have a syndrome referred to as Nasu-Hakola disease or Polycystic Lipomembranous Osteodysplasia with Sclerosing Leukoencephalopathy (PLOS)) also develop progressive demyelination and CNS gliosis associated with potential defects in microglia function. The involvement of DAP12 in signaling downstream of the CSF-1R may explain these mononuclear cell defects in DAP12-deficient individuals. Mononuclear precursors lacking DAP12 show reduced proliferative responses to M-CSF as well as poor survival at limiting doses of the cytokine (26). This results in a relatively low “fitness” of DAP12 mononuclear cells in competitive repopulation assays in irradiated recipient mice. Otero et al. found that M-CSF-induced signaling through DAP12 in macrophages leads to activation of Pyk2 and the translocation of β -catenin protein to the nucleus where it promotes cell survival and proliferation (26). In osteoclasts, DAP12 is required for coupling M-CSF stimulation to Syk activation, which is also important in osteoclast differentiation and function (26, 27). More importantly, a careful analysis of the DAP12 KO mice reveals that they too have microglial degeneration and apoptosis in the CNS, which would correlate with the neurologic findings in Nasu-Hakola patients (26). The mechanism by which DAP12 is coupled to the CSF-1R is unclear, but mutations within the transmembrane portion of DAP12 block its ability to function in the M-CSF pathway (27). However, the CSF-1R does not have a corresponding charged amino acid with which DAP12 can interact. Thus DAP12 either associates directly with the CSF-1R, through some unusual transmembrane mechanism or via an intermediary protein, such as an immunoreceptor.

Besides signaling in the M-CSF and IL-3 receptor pathways, it is also likely that DAP12 and/or FcR γ have major roles in other cytokine/growth factor pathways. Indeed, we have found that GM-CSF-induced DC differentiation from bone marrow precursors is

dramatically reduced when cells from DAP12/FcR γ double mutant mice are used in comparison with WT cells (Fig. 2A). This defect is likely due to poor activation of Syk kinase in the ITAM-deficient cells, because the same growth impairment is seen in *syk*^{-/-} cells. Cells lacking FcR γ or DAP12 alone have an intermediate growth impairment (data not shown). Similar to the M-CSF pathway, when DAP12/FcR γ -deficient bone marrow cells are cultured in GM-CSF, they show increased rates of apoptosis (Fig. 2B). Whether DAP12 and/or FcR γ are directly associated with the GM-CSF receptor or become phosphorylated following GM-CSF stimulation of DCs remains to be seen, but this is likely as the IL-3 and GM-CSF receptors share the βc signaling subunit. Nevertheless, it seems quite clear that these ITAM adapters play a fundamental role in a number of cytokine signaling pathways.

Activation by both FcR γ - and DAP12-associated receptors

Perhaps one of the earliest examples of the involvement of FcR γ and DAP12 in non-immunoreceptor signaling was the description of their requirement for leukocyte $\beta 1$, $\beta 2$, and $\beta 3$ integrin signaling (63, 64). These studies evolved out of the realization that many of the signaling molecules described as having a central role in leukocyte immunoreceptor signaling were also required for the integrin pathways. This included Src-family and Syk kinases (65, 66) as well as SLP-76 (67) and Vav guanine exchange factors (68). The direct examination of FcR γ and DAP12 deficient cells was definitive; neutrophils and macrophages from these mice show complete blocks in integrin-mediated activation (64). In neutrophils, the lack of integrin signaling in DAP12/FcR γ double mutant cells translates to poor activation of superoxide production, absent release of granule contents and poor cytoskeletal responses following engagement of surface integrins when cells are plated on adhesive surfaces. In macrophages, deficiency of DAP12 alone is sufficient to block integrin signaling responses, resulting in poor adhesive signaling, reduced cytokine production and poor migration, whereas FcR γ -deficient macrophages behave normally (Abram and Lowell, unpublished). In neutrophils, loss of integrin signaling also results in poor phagocytosis of complement opsonized bacteria through the CD18/integrin/complement receptors, but surprisingly does not seem to affect cell migration either *in vitro* or *in vivo*, mainly in response to strong infectious or inflammatory stimuli (Van Ziffle and Lowell, unpublished and (69)). As in the CSF-1R pathway, it remains unclear how the ITAM adapters are coupled to the integrins, whether via direct binding or through an associated immunoreceptor(s), or even through lipid raft co-localization. However, it is clear that in both the CSF-1R pathway and the integrin pathway, the transmembrane arginine residue of DAP12 is required for its signaling capacity, suggesting that an associated immunoreceptor mediates the interaction with integrins.

DAP12 and FcR γ have also been found to signal in the PSGL-1 adhesion receptor pathway (70, 71). PSGL-1 is primary adhesion receptor on neutrophils that mediates attachment of cells to vascular P- and E-selectins. Engagement of PSGL-1 on neutrophils induces a tyrosine kinase signaling response, which in cooperation with leukocyte integrins (mainly $\alpha L\beta 2$ or LFA-1) initiates slow rolling of cells over inflamed endothelium (72). This signaling response depends on Syk kinase, and in particular the SH2/ITAM binding function of Syk. These observations lead us to ask whether DAP12 and/or FcR γ were involved. Not surprisingly, neutrophils from the double deficient mice show an impaired ability to mediate slow rolling over surfaces coated with E-selectin and ICAM-1 (70). Though this signaling response may reflect the role of these ITAM adapters in the integrin pathway because ICAM-1 is required for slow rolling, the observation that DAP12/FcR γ double deficient neutrophils manifest reduced activation of Syk and p38 MAPK when plated on E-selectin alone suggests that PSGL-1 signaling is the major contributor. In this pathway, it appears that the Src-family kinase Fgr is the primary enzyme that phosphorylates DAP12 and/or FcR γ to initiate the PSGL-1 response.

In the theme of regulation of cytokine responses in innate immune cells by DAP12 and FcR γ , there is clear evidence that these adapters, along with Syk, play a signaling role in IFN γ priming of macrophages (29). Pre-incubation of macrophages with IFN γ dramatically increases their responses to subsequent IFN α stimulation as measured changes in inflammatory cytokine production (TNF γ or IL-1RA) and phosphorylation of STAT1. In part, this is due to increased expression of STAT-1 in IFN γ stimulated cells. However, IFN γ priming of DAP12/FcR γ double mutant macrophages fails to induce dramatic phosphorylation responses to subsequent IFN α stimulation, even though STAT1 expression increases as expected. The downstream pathway from the ITAMs that leads to increased IFN γ priming involves the calcium dependent kinases CaMK and Pyk2 since inhibitors of these enzymes block the IFN γ effect (30). The fact that the enhanced priming effect of IFN γ is dependent on cell adhesion (macrophages kept loosely in suspension have reduced IFN γ priming) suggests that that integrin-mediated signaling through DAP12/FcR γ is the primary pathway that leads to enhanced priming. In this regard, tonic signaling by cell adhesion, mediated through the integrin/ITAM/Syk pathway may serve as a general amplifier of a number of signaling responses besides just the IFN γ response (reviewed in (13)).

ITAM signaling through DAP12/FcR γ is also involved in maximizing antigen presentation responses in DCs (31). DCs derived from DAP12/FcR γ double mutant mice manifest reduced cross-presentation of particulate, but not soluble, antigens to cognate T-cells. This defect does not seem to be mediated by poor uptake of the particulate antigens, but instead reflects some other signaling pathway involving the Vav GEF proteins since DCs lacking these enzymes also have defects in particulate antigen presentation. Indeed, tyrosine phosphorylation of Vav proteins is reduced in DAP12/FcR γ deficient DCs following stimulation with particulate antigens. The relevant receptors that mediate this antigen presentation defect are unclear.

Inhibitory signaling by ITAM adapters

In addition to contributing to activation of myeloid cells, the DAP12 and FcR γ ITAMs also mediate signals that inhibit cell function. There were some descriptions of this type of inhibitory activity by FcR γ -paired Fc receptors in the 1990's (73-75), however in the last 5 years there have been many more receptors implicated in inhibition, including those that associate with DAP12 and/or FcR γ . Generally, this inhibitory activity is seen when the cells are simultaneously activated through a DAP12 or FcR γ -associated receptor and a distinct receptor system, such as Toll-like receptors (TLRs) or Fc receptors, leading to a reduced response when compared to cells activated through the heterologous receptor alone. The inhibition requires association of DAP12 and/or FcR γ with a particular immunoreceptor(s) and is not thought to be mediated through direct association of the ITAM adapters with the heterologous receptor(s). Inhibition of activation by ITAM-containing adapters has also been shown in B and T cells, through Ig α and the CD3 molecules respectively (reviewed in (12)), showing that ITAM-based inhibitory signals are widespread in the immune system. Several different mechanisms for ITAM-based inhibition in myeloid and lymphoid cells have been proposed, and it is as yet unclear if there will be a single mechanism for inhibition, or whether it will vary by cell type, ITAM adapter, and/or inhibited signaling receptor.

Inhibition by FcR γ -associated receptors

One of the earliest descriptions of the inhibition of myeloid cell responses by a receptor associated with an ITAM-containing adaptor was that of the IgE receptor, Fc ϵ RI (75). IgE binds to the α chain of the receptor, which pairs with 2 ITAM-containing chains, Fc ϵ RI β and FcR γ . Torigoe et al. showed that cross-linking of Fc ϵ RI on the RBL mast cell line

simultaneously with a low affinity and a high affinity ligand for the IgE pre-bound to FcεRI resulted in the reduction in mast cell degranulation when compared with cells treated with either the low or the high affinity ligand alone. Cells treated with both the low and high affinity Ags manifest a reduction in the phosphorylation of downstream signaling molecules Syk, Pyk2 and Erk as well as a reduction in the phosphorylation of the receptor ITAMs. In this system, there is no heterologous receptor, the model is that the FcεRI ligated by the low affinity hapten inhibits the signal from the FcεRI ligated by the high affinity hapten.

Around the same time Sutterwala et al. (73) showed that ligating IgG receptors on mouse macrophages with IgG-coated sheep red blood cells (SRBC) inhibits LPS-induced IL-12 p40 production. This inhibition depends upon FcRγ, but not FcγRIII (CD16), suggesting that either FcγRI (CD64) or FcγRIV is mediating inhibition (74). Treatment with IgG-coated SRBC also increases LPS-induced IL-10 production, but IL-10 does not mediate the inhibition of IL-12 p40 production (76). This was the first description of the inhibition of TLR responses by ITAM-containing proteins.

Another Fc receptor that pairs with FcRγ to inhibit myeloid cell function is human FcαRI (CD89), which has no mouse equivalent (77). FcαRI is one of many FcRs for IgA, but is the only one found on leukocytes -- it binds specifically to the IgA subtype found in serum. Over the years, there had been many descriptions in the literature showing that monomeric IgA can inhibit macrophage functions including phagocytosis, chemotaxis, and respiratory burst (reviewed in (78)). In addition, many patients with IgA deficiency have autoimmunity or atopic diseases, supporting the idea that IgA serves to limit the immune response (79). Similar to what had been shown previously for IgA itself, Pasquier et al. found that a Fab fragment of an anti-FcαRI mAb can inhibit IgG-mediated phagocytosis, implicating FcαRI as the receptor mediating IgA-induced inhibition (77). In FcαRI-transfected RBL mast cells, both monomeric IgA and the anti-FcαRI Fab fragment also inhibit IgE-induced degranulation responses. However, this only works when the IgA or the Fab are monomeric; if the IgA or anti-FcαRI Fab fragments are cross-linked with secondary antibody, the inhibitory signal switches to enhanced FcεRI-mediated responses. In this system, the FcαRI-mediated inhibition requires association with FcRγ as well both the tyrosines in the FcRγ ITAM.

By examining the signaling responses downstream of monomeric versus oligomeric engaged FcαRI, Pasquier et al. provided a model for how this ITAM could limit signaling from the FcεRI receptor. Cells treated with monomeric IgA (or anti-FcαRI Fab) have less phosphorylation of the FcRγ ITAM, Syk, LAT and ERK1/2 when compared to cells exposed to oligomeric ligands (anti-FcαRI F(ab)₂ plus rat-anti mouse secondary) in conjunction with FcεRI cross-linking (77). Less Syk is recruited to FcRγ under inhibitory (monomeric) conditions in comparison with activating (oligomeric) conditions, while recruitment of the tyrosine phosphatase SHP-1 is seen only when inhibitory signals are transduced with the anti-FcαRI Fab. These data suggest a model where low avidity cross-linking of FcαRI with monomeric ligand results in weak phosphorylation of the FcRγ ITAM, leading to preferential recruitment of SHP-1, which in turn causes reduced phosphorylation of signaling components downstream of the heterologous activating signal (in this case FcεRI). However, following engagement by high avidity oligomeric ligands, the FcRγ ITAM is fully phosphorylated leading to Syk kinase recruitment, maximal signaling and full cellular activation through the co-engaged heterologous FcεRI (Fig 1) (77, 80).

More recently, the Monteiro laboratory has extended the number of heterologous receptors that can be inhibited by monovalent ligands for FcαRI to include signaling induced by the chemokine MCP-1 (CCL2), the cytokine TNF, and the TLR4 agonist LPS, all in human monocytes (81). Treatment with anti-FcαRI Fab inhibits monocyte chemotaxis to MCP-1

and TNF, as well as downstream signal transduction, in an SHP-1-dependent fashion, consistent with previous findings. To extend these findings to an *in vivo* model, these investigators generated transgenic mice expressing human Fc α RI; treatment of these animals with the anti-Fc α RI Fab ameliorates kidney disease in a passive transfer model of anti-glomerular basement membrane IgG-mediated glomerulonephritis. Taken together these studies clearly show that signaling through Fc α RI/FcR γ with monovalent ligands can inhibit a broad array of macrophage functions (77, 81).

The Monteiro group has also investigated inhibitory signals through FcR γ -associated Fc γ RIII (CD16) in mouse macrophages (82). After caecal ligation and puncture (CLP)-induced sepsis, FcR γ -deficient mice and Fc γ RIII-deficient mice have markedly prolonged survival compared to WT mice -- all WT mice succumb to shock within 70 hours, whereas only 50% of WT mice succumb by 180 hours. This protection is associated with less recovered *E. coli* from the peritoneal cavity, as well as less serum and peritoneal TNF in FcR γ -deficient mice compared to WT mice. *In vitro*, phagocytosis of *E. coli* (but not zymosan or *S. aureus*) is increased in peritoneal neutrophils and macrophages lacking FcR γ compared to WT cells. FcR γ -deficient macrophages are at least as efficient, if not more efficient, as WT macrophages in killing phagocytosed *E. coli*. The authors conclude that the reason for increased survival of FcR γ -deficient mice during CLP is that the peritoneal neutrophils and macrophages clear the bacteria more efficiently and therefore there is reduced systemic TNF and other inflammatory cytokines that mediate shock. The authors suggest a model whereby in WT macrophages recognition of *E. coli* by Fc γ RIII leads to weak FcR γ phosphorylation, recruitment of SHP-1 to the receptor, decreased PI-3 kinase signaling and subsequent impaired function of the scavenger receptor MARCO, all of which cause prolonged exposure of macrophages to *E. Coli* LPS and increased TLR4 responses, hence more TNF leading to shock (82). In FcR γ -deficient cells, this inhibitory function is reversed and *E. coli* phagocytosis through MARCO proceeds robustly.

There have also been several studies showing that FcR γ -associated receptors inhibit TLR responses in human plasmacytoid dendritic cells (PDCs) (83-86). Blood dendritic cell antigen (BDCA)-2 was originally described as a C-type lectin-like receptor specifically expressed on human PDCs that when cross-linked with antibody could inhibit IFN α induced by Influenza virus or DNA-containing immune complexes (86). Though BDCA-2 has a small cytoplasmic region of 21 amino acids with no known signaling motifs, cross-linking of BDCA-2 induces protein tyrosine phosphorylation and intracellular calcium mobilization. Cao et al. found that BDCA-2 associates with FcR γ to transduce signals (85). In this study, the authors show that PDCs express many signaling molecules in common with B cells to transduce ITAM signals, including Syk, Lyn, BLK, PLC γ 2 and BCAP. Cross-linking BDCA-2 in PDCs, which potently suppresses TLR7- and TLR9-induced IFN α , TNF and IL-6 secretion, also induces the phosphorylation of Src family kinases, Syk, Vav1, PLC γ 2 and ERK. Remarkably, mAb cross-linking of BDCA-2 up to 4 hours after CpG DNA treatment is able to inhibit PDC cytokine secretion (85), though the BDCA-2 ligand that mediates this effect is unknown.

Cao, Liu and colleagues also have studied Immunoglobulin-like transcript (ILT) 7, which similar to BDCA-2, is specifically expressed on human PDCs and associates with FcR γ (83). Like BDCA-2, mAb mediated cross-linking of ILT7 potently inhibits TLR7 and TLR9-mediated IFN α and TNF production by PDCs. More recently, this group has identified a ligand for ILT7 that can mediate this inhibitory effect (84). They found that several breast cancer and melanoma cell lines express a cell surface ligand for ILT7 that can activate a reporter cell line expressing ILT7/FcR γ . By generating blocking mAbs against these cancer cell lines, they identified the ILT7 ligand as bone marrow stromal cell antigen (BST) 2. Recombinant BST2 binds to ILT7 with an affinity of approximately 1 μ M, causing

inhibition of PDC IFN α secretion induced by CpG DNA or influenza virus. Interestingly, BST2 expression is induced by IFN α treatment in many cell lines and is even expressed on PDCs themselves, suggesting the possibility for a cis interaction between ligand and receptor (84, 87). BST2 is also expressed on mouse PDCs, though there is no direct ortholog of ILT7 in mouse. Though not directly shown by these authors, it is possible that ILT7 or BDCA-2 is inhibiting the function of PDC-TREM following treatment of cells with TLR7 or TLR9 agonists.

Fc γ R has also been implicated in the inhibition of conventional DC function induced by cross-linking of MHC class II molecules (88). Though not heavily studied, it is now well recognized that engagement of Ag loaded MHC II by T-cells can lead to signaling responses in the antigen presenting B cells, monocytes and DCs. MHC II does not have any recognized signaling motifs in its cytoplasmic tail, however in B cells MHC II associates with the BCR ITAM adapters Ig α and Ig β (CD79a and CD79b) to transmit activating signals to the B-cell (89). Engagement of MHC II on DCs by the lymphocyte activation gene (LAG)-3, a CD4-related protein expressed on regulatory T cells, has recently been found to inhibit up-regulation of CD86 expression (88). mAb cross-linking of MHC II mimics the effect of LAG-3 binding, causing reduction of LPS-induced CD86 expression and IL-12 production, as well as diminishing the ability of peptide-pulsed DCs to activate antigen-specific T cells. MHC II cross-linking of Fc γ R-deficient DCs fails to down modulate CD86 expression, suggesting that this inhibitory signal is mediated by ITAM signaling. Similar to inhibition by Fc α RI monovalent cross-linking, down-regulation of CD86 by MHC II requires ERK activity and SHP-1 (88). Low levels of SHP-1 are constitutively associated with Fc γ R and MHC II in DCs, which may mediate the inhibitory effect of mAb engaged MHC II. Overall, these observations suggest a mechanism by which regulatory T-cells, through expression of LAG-3, down modulate DC function. While the observation that recruitment of SHP-1 by MHC II/Fc γ R complexes is consistent with the model proposed by Pasquier et al. in their studies of Fc α RI, there are several surprising points to this modulatory effect of MHC II (mainly the constitutive rather than induced association of SHP-1 with Fc γ R) that require further investigation. Nevertheless, these studies provide an important example of a non-immunoreceptor (MHC II) that mediates inhibitory signaling through Fc γ R.

Inhibition by DAP12 associated receptors

As with Fc γ R, DAP12 and its associated receptors are capable of inhibiting myeloid cell function. We originally described DAP12's inhibitory properties by examination of DAP12-deficient macrophages (90). Both bone marrow and peritoneal macrophages lacking DAP12 display increased inflammatory cytokine production (TNF, IL-6, IL-12 p40) compared to WT cells following treatment with a panel of TLR agonists or with zymosan, which signals through TLR2 and Dectin-1. As a result of loss of this inhibitory pathway, DAP12-deficient mice develop higher serum levels of TNF than WT mice after LPS injection, and as a result are more susceptible to endotoxic shock. The increased response to TLR agonists is, however, protective following bacterial infection, since DAP12-deficient mice have improved clearance of *Listeria monocytogenes* compared to WT mice. DAP12-deficient mice also have increased responses to infection with *Mycobacterium tuberculosis* (L. Lanier, personal communication, (91)).

Loss of DAP12 not only affects macrophage TLR responses, but also results in increased signaling through other macrophage receptor systems. Cross-linking of Fc γ RIII with plate-bound mAb results in the secretion of more TNF and IL-6 from DAP12-deficient macrophages than from WT macrophages (90). Similarly, DAP12-deficient macrophages show enhanced phagocytosis of IgG-opsonized SRBC compared to WT macrophages over a range of particle doses (Fig. 3A). DAP12-deficient macrophages also phagocytose zymosan

through the Dectin-1 receptor more efficiently than WT macrophages (Fig. 3B). Besides inhibiting heterologous immunoreceptor (Fc γ RIII) or C-type lectin receptor (Dectin-1) function, we have found that, we have found that DAP12 can also inhibit signaling through the IFN γ R in macrophages. DAP12-deficient macrophages show significantly higher upregulation of MHC II following low dose IFN γ treatment than do WT macrophages (Fig. 3C). Therefore, similar to inhibition via Fc α RI/FcR γ , DAP12 can inhibit a variety of heterologous signaling pathways in macrophages.

The exact mechanism by which DAP12 inhibits TLR and other signaling pathways in macrophages is still being elucidated. The ITAM residues of DAP12 are required for this inhibitory function, since reintroduction of an ITAM tyrosine mutant of DAP12 into DAP12-deficient macrophages fails to restore normal responses, while re-expression of WT DAP12 functions normally (90). Macrophages deficient in Syk show similarly increased TLR-induced inflammatory cytokine production as DAP12-deficient macrophages, again supporting the role of the DAP12 ITAM in the inhibition of TLR responses. To begin to understand how DAP12 inhibits TLR signaling, we compared signal transduction pathways that are activated downstream of TLRs in WT and DAP12-deficient macrophages. The phosphorylation of ERK1/2 is consistently higher in DAP12-deficient macrophages than in WT cells. In contrast, the phosphorylation of p38 MAPK and JNK and the degradation of I κ B α , a measure of activation of the NF- κ B pathway, are similar between WT and DAP12-deficient macrophages (90). More recently, we have found that LPS-induced AKT phosphorylation is also increased in macrophages that lack DAP12, suggesting an increase in PI3-kinase activity (Fig. 4). These data show that DAP12 regulates multiple signaling pathways important in TLR responses in macrophages. It will be interesting to examine if ERK and AKT phosphorylation are also increased downstream of Fc γ RIII cross-linking in DAP12-deficient macrophages and to fully delineate the inhibitory signaling pathway.

Of the many DAP12-associated receptors expressed in macrophages, TREM-2 is the single receptor that has been shown to be required for inhibition of TLR and FcR-signaling in macrophages (92, 93). Expression of a chimeric receptor composed of the extracellular domain of TREM-2 fused to DAP12, in which the transmembrane aspartic acid was mutated to alanine, in DAP12-deficient macrophages restores normal signaling responses to CpG DNA, zymosan, or mAb cross-linking of Fc γ RIII (92). In contrast, chimeric receptors containing the extracellular region of TREM-1, MAIR-II and PILR β have no effect. Additionally, shRNA-mediated knockdown of TREM-2 increases the CpG DNA- and zymosan-induced TNF production of WT macrophages. Most importantly, macrophages from TREM-2-deficient mice show the same exaggerated TLR-induced inflammatory cytokine production as those from DAP12-deficient mice (93). The nature of the ligand for TREM-2 that mediates inhibitory signaling is not known, though the finding that a TREM-2/Fc chimeric protein binds directly to macrophages would suggest that these cells possess an endogenous ligand (92). Both microbial and endogenous ligands for TREM-2 have been identified (53, 54, 56-58, 94), but whether these mediate the inhibitory signal has not been tested. Together, these studies clearly implicate TREM-2 as the major inhibitory DAP12-associated receptor on mouse macrophages. Further questions of interest include whether the TREM-2/DAP12 receptor complex is also inhibitory in human macrophages and the nature of the TREM-2 ligand that mediates inhibition. Interestingly, TREM-2 also inhibits *in vitro* osteoclast formation in mice -- TREM-2-deficient bone marrow precursors display accelerated osteoclastogenesis in comparison with WT bone marrow cells (47), though this is in direct contrast to what is seen in human *in vitro* osteoclastogenesis assays with TREM-2-deficient monocytes precursors (50, 52) or with knockdown of TREM-2 in RAW cells (51).

TREM-2 has also been shown to have a negative regulatory effect on microglia, the resident tissue macrophage of the brain. Takahashi et al. found that not only do TREM-2 and DAP12 function positively to regulate the phagocytosis of apoptotic neurons, as discussed above, but that they also negatively regulate transcription of the genes encoding the inflammatory mediators TNF, IL-1 β and iNOS (55). Recently, two papers have highlighted the inhibitory role of TREM-2 *in vivo* in the CNS disease experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis (94, 95). Treatment of mice with a blocking antibody to TREM-2 results in exacerbation of EAE as measured by clinical score (95). In contrast, treatment of mice with GM-CSF derived myeloid cells overexpressing TREM-2 (by lentivirus gene transduction) show an improvement in clinical score as compared with those that received control cells (94). Taken together, these studies demonstrate that TREM-2 is a potent inhibitor of autoimmune inflammatory disease in the EAE model. Interestingly, high levels of a soluble form of the TREM-2 receptor is found in the cerebrospinal fluid of multiple sclerosis patients while patients with non-inflammatory neurological diseases have lower levels of the receptor (96). This finding may suggest that production of soluble TREM-2 interferes with the inhibition of inflammation by membrane associated TREM-2 in multiple sclerosis patients, possibly by blocking access to TREM-2 ligands.

DAP12-associated receptors on both mouse and human PDCs have also been found to inhibit TLR responses, similar to the effects of ILT7/FcR γ and BDCA-2/FcR γ (87, 97). The DAP12-associated receptor, NKp44, which functions as an activating receptor for NK cell mediated cytotoxicity paradoxically functions to inhibit production of cytokines by human tonsillar PDCs (97). Similar to what had been reported for BDCA-2 cross-linking, cross-linking of NKp44 with plate-bound mAb inhibits the CpG DNA-induced secretion of IFN α , MIP-1 α (CCL3) and MIP-1 β (CCL4). Similar to TREM-2, NKp44 has been described to bind a variety of microbial and endogenously-derived ligands (98-104). The TREM family was originally identified through homology with NKp44 and the genes encoding these proteins in humans are closely linked on chromosome 6 (105).

In mouse PDCs, Siglec-H is a DAP12-associated receptor that can inhibit CpG DNA-induced IFN α secretion (106, 107). Siglecs are sialic acid-binding receptors belonging to the Ig superfamily that predominantly have cytoplasmic ITIMs. In contrast, Siglec-H has a transmembrane lysine and small cytoplasmic region consistent with DAP12 association; indeed, DAP12-deficient PDCs, but not those lacking FcR γ or DAP10, fail to express Siglec-H. Supporting a role for Siglec-H in modulating splenic PDCs in mouse, cells from DAP12-deficient mice produce significantly more IFN α after CpG DNA treatment than WT cells, while *in vivo* administration of CpG DNA results in higher serum amounts of IFN α in DAP12-deficient mice than WT mice (107). These data suggest that Siglec-H on PDCs, similar to TREM-2 on macrophages, recognizes an endogenously-expressed ligand, possibly on PDCs themselves and disruption of this signal by removal of DAP12 results in increased TLR responses.

Inhibition by both FcR γ - and DAP12-associated receptors

During our studies of inhibition of macrophage responses by TREM-2 and DAP12, we were surprised to find that, unlike DAP12-deficient macrophages, DAP12-deficient bone marrow-derived DCs show very modestly increased TLR responses compared to WT cells, despite the fact that bone marrow-derived DCs express abundant amounts of both DAP12 and TREM-2 (90). We hypothesized that FcR γ may also play a role in inhibition of TLR responses in DCs; indeed, DCs lacking both DAP12 and FcR γ have significantly increased TLR responses in comparison with WT DCs as measured by production of TNF, IL-6, and IL-12 and up-regulation of MHC II, CD80 and CD86 (108). Singly DAP12-deficient or

FcR γ -deficient DCs have increased responses that are typically intermediate between the DAP12/FcR γ -deficient DCs. Consistent with the role of ITAM signaling in inhibiting TLR responses in DCs, Syk-deficient DCs also have increased inflammatory cytokine production and maturation compared to WT DCs (108). Furthermore, DAP12/FcR γ -deficient and Syk-deficient DCs both induce more antigen-specific T cell proliferation than WT DCs. These data show that similar to macrophages and PDCs, conventional DC populations have constitutive signaling through ITAM-bearing adapters that inhibits TLR responses. We are currently investigating the DAP12- and FcR γ -associated receptors that participate in these inhibitory signals in DCs.

Summary

The extensive involvement of the ITAM adapters DAP12 and FcR γ in multiple signaling pathways in myeloid cells is now well established. This signaling involves a growing array of immunoreceptors and, surprisingly, many non-immunoreceptors as well, such as integrins and cytokine receptors. In the case of signaling through immunoreceptors, the ITAMs appear to be absolutely critical; deficiency of DAP12 and/or FcR γ ablates signaling. The same is true for integrins, but in the case of cytokine responses, the ITAM signaling seems to modulate and/or be additive to the traditional signaling pathways used by these receptors. In this regard, the molecular details of how either FcR γ or DAP12 couple to non-immunoreceptor proteins remains obscure. Whether these adapters are directly associated with integrin and/or cytokine receptor complexes, or modulate signaling through co-association with other immunoreceptors, needs to be defined. More remarkable is the clear demonstration of the contribution of DAP12 and FcR γ to inhibitory signaling in innate cells. In the case of downmodulation of TLR responses, although the upstream receptor to which DAP12 couples in macrophages is known -- TREM2 -- how the TREM2/DAP12 association mediates an inhibitory signaling is unclear. The current hypothesis in the FcR pathway involves ligand affinity (high affinity ligands mediate activation signaling while low affinity ligands are inhibitory), but that remains to be demonstrated in other inhibitory responses through DAP12 and FcR γ . Overall, it is clear that cross-talk between different receptor signaling pathways can function to allow immune cells to rapidly amplify responses to low levels of antigen, pathogen molecules or cytokines, while also potentially dampening responses to mediate immune tolerance or limit immunopathology. Deciphering the disease related contexts for the dual signal regulation by DAP12 and FcR γ will be critical in design and application of therapeutics that target ITAM signaling.

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References

1. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. *Annu Rev Immunol.* 2009; 27:591–619. [PubMed: 19132916]
2. Monroe JG. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. *Nat Rev Immunol.* 2006; 6:283–294. [PubMed: 16557260]
3. Reth M. Antigen receptor tail clue. *Nature.* 1989; 338:383–384. [PubMed: 2927501]
4. Lanier LL. DAP10- and DAP12-associated receptors in innate immunity. *Immunol Rev.* 2009; 227:150–160. [PubMed: 19120482]
5. Nimmerjahn F, Ravetch JV. Fc γ receptors as regulators of immune responses. *Nat Rev Immunol.* 2008; 8:34–47. [PubMed: 18064051]

6. Humphrey MB, Lanier LL, Nakamura MC. Role of ITAM-containing adapter proteins and their receptors in the immune system and bone. *Immunol Rev.* 2005; 208:50–65. [PubMed: 16313340]
7. Smith KM, Wu J, Bakker ABH, Phillips JH, Lanier LL. Cutting Edge: Ly49D and Ly49H associate with mouse DAP12 and form activating receptors. *J Immunol.* 1998; 161:7–10. [PubMed: 9647200]
8. Lanier LL, Corliss B, Wu J, Phillips JH. Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity.* 1998; 8:693–701. [PubMed: 9655483]
9. Howard FD, Rodewald HR, Kinet JP, Reinherz EL. CD3 ζ subunit can substitute for the γ subunit of Fc ϵ receptor type I in assembly and functional expression of the high-affinity IgE receptor: evidence for interreceptor complementation. *Proc Natl Acad Sci U S A.* 1990; 87:7015–7019. [PubMed: 1698288]
10. Kiener PA, et al. Cross-linking of Fc γ receptor I (Fc γ RI) and receptor II (Fc γ RII) on monocytic cells activates a signal transduction pathway common to both Fc receptors that involves the stimulation of p72 Syk protein tyrosine kinase. *J Biol Chem.* 1993; 268:24442–24448. [PubMed: 8226994]
11. Abram CL, Lowell CA. The expanding role for ITAM-based signaling pathways in immune cells. *Sci STKE.* 2007; re2
12. Hamerman JA, Lanier LL. Inhibition of immune responses by ITAM-bearing receptors. *Science STKE.* 2006; re1
13. Ivashkiv LB. Cross-regulation of signaling by ITAM-associated receptors. *Nat Immunol.* 2009; 10:340–347. [PubMed: 19295630]
14. Turnbull IR, Colonna M. Activating and inhibitory functions of DAP12. *Nat Rev Immunol.* 2007; 7:155–161. [PubMed: 17220916]
15. Bugarcic A, Hitchens K, Beckhouse AG, Wells CA, Ashman RB, Blanchard H. Human and mouse macrophage-inducible C-type lectin (Mincle) bind *Candida albicans*. *Glycobiology.* 2008; 18:679–685. [PubMed: 18509109]
16. Wells CA, et al. The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to *Candida albicans*. *J Immunol.* 2008; 180:7404–7413. [PubMed: 18490740]
17. Yamasaki S, et al. C-type lectin Mincle is an activating receptor for pathogenic fungus, *Malassezia*. *Proc Natl Acad Sci U S A.* 2009; 106:1897–1902. [PubMed: 19171887]
18. Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat Immunol.* 2008; 9:1179–1188. [PubMed: 18776906]
19. Sato K, et al. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor γ chain to induce innate immune responses. *J Biol Chem.* 2006; 281:38854–38866. [PubMed: 17050534]
20. Nakano T, et al. Activation of neutrophils by a novel triggering immunoglobulin-like receptor MAIR-IV. *Mol Immunol.* 2008; 45:289–294. [PubMed: 17543387]
21. Hida S, et al. Fc receptor γ -chain, a constitutive component of the IL-3 receptor, is required for IL-3-induced IL-4 production in basophils. *Nat Immunol.* 2009; 10:214–222. [PubMed: 19098920]
22. Werninghaus K, et al. Adjuvanticity of a synthetic cord factor analogue for subunit *Mycobacterium tuberculosis* vaccination requires FcR γ -Syk-Card9-dependent innate immune activation. *J Exp Med.* 2009; 206:89–97. [PubMed: 19139169]
23. Klesney-Tait J, Turnbull IR, Colonna M. The TREM receptor family and signal integration. *Nat Immunol.* 2006; 7:1266–1273. [PubMed: 17110943]
24. Inui M, et al. Signal adaptor DAP10 associates with MDL-1 and triggers osteoclastogenesis in cooperation with DAP12. *Proc Natl Acad Sci U S A.* 2009; 106:4816–4821. [PubMed: 19251634]
25. Watarai H, Sekine E, Inoue S, Nakagawa R, Kaisho T, Taniguchi M. PDC-TREM, a plasmacytoid dendritic cell-specific receptor, is responsible for augmented production of type I interferon. *Proc Natl Acad Sci U S A.* 2008; 105:2993–2998. [PubMed: 18287072]
26. Otero K, et al. Macrophage colony-stimulating factor induces the proliferation and survival of macrophages via a pathway involving DAP12 and β -catenin. *Nat Immunol.* 2009; 10:734–743. [PubMed: 19503107]

27. Zou W, Reeve JL, Liu Y, Teitelbaum SL, Ross FP. DAP12 couples c-Fms activation to the osteoclast cytoskeleton by recruitment of Syk. *Mol Cell*. 2008; 31:422–431. [PubMed: 18691974]
28. Abram CL, Lowell CA. The ins and outs of leukocyte integrin signaling. *Annu Rev Immunol*. 2009; 27:339–362. [PubMed: 19302044]
29. Tassioulas I, et al. Amplification of IFN- α -induced STAT1 activation and inflammatory function by Syk and ITAM-containing adaptors. *Nat Immunol*. 2004; 5:1181–1189. [PubMed: 15467722]
30. Wang L, et al. 'Tuning' of type I interferon-induced Jak-STAT1 signaling by calcium-dependent kinases in macrophages. *Nat Immunol*. 2008; 9:186–193. [PubMed: 18084294]
31. Graham DB, et al. An ITAM-signaling pathway controls cross-presentation of particulate but not soluble antigens in dendritic cells. *J Exp Med*. 2007; 204:2889–2897. [PubMed: 17984307]
32. Nakahashi C, et al. Dual assemblies of an activating immune receptor, MAIR-II, with ITAM-bearing adapters DAP12 and FcR γ chain on peritoneal macrophages. *J Immunol*. 2007; 178:765–770. [PubMed: 17202337]
33. Poole A, et al. The Fc receptor γ -chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J*. 1997; 16:2333–2341. [PubMed: 9171347]
34. Goodridge HS, Underhill DM. Fungal recognition by TLR2 and dectin-1. *Handb Exp Pharmacol*. 2008:87–109. [PubMed: 18071656]
35. Matsumoto M, et al. A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages. *J Immunol*. 1999; 163:5039–5048. [PubMed: 10528209]
36. Sancho D, et al. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature*. 2009; 458:899–903. [PubMed: 19219027]
37. Gross O, et al. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature*. 2009; 459:433–436. [PubMed: 19339971]
38. Chen ST, et al. CLEC5A is critical for dengue-virus-induced lethal disease. *Nature*. 2008; 453:672–676. [PubMed: 18496526]
39. Pokidysheva E, et al. Cryo-EM reconstruction of dengue virus in complex with the carbohydrate recognition domain of DC-SIGN. *Cell*. 2006; 124:485–493. [PubMed: 16469696]
40. Tassaneetrithep B, et al. DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med*. 2003; 197:823–829. [PubMed: 12682107]
41. Faccio R, Zou W, Colaianni G, Teitelbaum SL, Ross FP. High dose M-CSF partially rescues the Dap12^{-/-} osteoclast phenotype. *J Cell Biochem*. 2003; 90:871–883. [PubMed: 14624447]
42. Humphrey MB, et al. The signaling adapter protein DAP12 regulates multinucleation during osteoclast development. *J Bone Miner Res*. 2004; 19:224–234. [PubMed: 14969392]
43. Kaifu T, et al. Osteopetrosis and thalamic hypomyelinos with synaptic degeneration in DAP12-deficient mice. *J Clin Invest*. 2003; 111:323–332. [PubMed: 12569157]
44. Coudert JD, Scarpellino L, Gros F, Vivier E, Held W. Sustained NKG2D engagement induces cross-tolerance of multiple distinct NK cell activation pathways. *Blood*. 2008; 111:3571–3578. [PubMed: 18198346]
45. Orr MT, et al. Ly49H signaling through DAP10 is essential for optimal natural killer cell responses to mouse cytomegalovirus infection. *J Exp Med*. 2009; 206:807–817. [PubMed: 19332875]
46. Tassi I, Le Friec G, Gilfillan S, Takai T, Yokoyama WM, Colonna M. DAP10 associates with Ly49 receptors but contributes minimally to their expression and function in vivo. *Eur J Immunol*. 2009; 39:1129–1135. [PubMed: 19247984]
47. Colonna M, Turnbull I, Klesney-Tait J. The enigmatic function of TREM-2 in osteoclastogenesis. *Adv Exp Med Biol*. 2007; 602:97–105. [PubMed: 17966394]
48. Takegahara N, et al. Plexin-A1 and its interaction with DAP12 in immune responses and bone homeostasis. *Nat Cell Biol*. 2006; 8:615–622. [PubMed: 16715077]
49. Helming L, et al. Essential role of DAP12 signaling in macrophage programming into a fusion-competent state. *Sci Signal*. 2008; 1:ra11. [PubMed: 18957693]
50. Cella M, Buonsanti C, Strader C, Kondo T, Salmaggi A, Colonna M. Impaired differentiation of osteoclasts in TREM-2-deficient Individuals. *J Exp Med*. 2003; 198:645–651. [PubMed: 12913093]

51. Humphrey MB, et al. TREM2, a DAP12-associated receptor, regulates osteoclast differentiation and function. *J Bone Miner Res.* 2006; 21:237–245. [PubMed: 16418779]
52. Paloneva J, et al. DAP12/TREM2 deficiency results in impaired osteoclast differentiation and osteoporotic features. *J Exp Med.* 2003; 198:669–675. [PubMed: 12925681]
53. Hsieh CL, et al. A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. *J Neurochem.* 2009; 109:1144–1156. [PubMed: 19302484]
54. N'Diaye EN, et al. TREM-2 (triggering receptor expressed on myeloid cells 2) is a phagocytic receptor for bacteria. *J Cell Biol.* 2009; 184:215–223. [PubMed: 19171755]
55. Takahashi K, Rochford CD, Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J Exp Med.* 2005; 201:647–657. [PubMed: 15728241]
56. Hemmi H, et al. A new triggering receptor expressed on myeloid cells (Trem) family member, Trem-like 4, binds to dead cells and is a DNAX activation protein 12-linked marker for subsets of mouse macrophages and dendritic cells. *J Immunol.* 2009; 182:1278–1286. [PubMed: 19155473]
57. Stefano L, et al. The surface-exposed chaperone, Hsp60, is an agonist of the microglial TREM2 receptor. *J Neurochem.* 2009
58. Daws MR, Sullam PM, Niemi EC, Chen TT, Tchao NK, Seaman WE. Pattern recognition by TREM-2: binding of anionic ligands. *J Immunol.* 2003; 171:594–599. [PubMed: 12847223]
59. Charles JF, et al. The innate immune response to *Salmonella enterica* serovar Typhimurium by macrophages is dependent on TREM2-DAP12. *Infect Immun.* 2008; 76:2439–2447. [PubMed: 18391000]
60. Koga T, et al. Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. *Nature.* 2004; 428:758–763. [PubMed: 15085135]
61. Mocsai A, et al. The immunomodulatory adapter proteins DAP12 and Fc receptor γ -chain (FcR γ) regulate development of functional osteoclasts through the Syk tyrosine kinase. *Proc Natl Acad Sci U S A.* 2004; 101:6158–6163. [PubMed: 15073337]
62. Paloneva J, et al. Loss-of-function mutations in TYROBP (DAP12) result in a presenile dementia with bone cysts. *Nat Genet.* 2000; 25:357–361. [PubMed: 10888890]
63. Abtahian F, et al. Evidence for the requirement of ITAM domains but not SLP-76/Gads interaction for integrin signaling in hematopoietic cells. *Mol Cell Biol.* 2006; 26:6936–6949. [PubMed: 16943434]
64. Mocsai A, Abram CL, Jakus Z, Hu Y, Lanier LL, Lowell CA. Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat Immunol.* 2006; 7:1326–1333. [PubMed: 17086186]
65. Lowell CA, Fumagalli L, Berton G. Deficiency of Src family kinases p59/61hck and p58c-fgr results in defective adhesion-dependent neutrophil functions. *J Cell Biol.* 1996; 133:895–910. [PubMed: 8666673]
66. Mocsai A, Zhou M, Meng F, Tybulewicz VL, Lowell CA. Syk is required for integrin signaling in neutrophils. *Immunity.* 2002; 16:547–558. [PubMed: 11970878]
67. Newbrough SA, et al. SLP-76 regulates Fc γ receptor and integrin signaling in neutrophils. *Immunity.* 2003; 19:761–769. [PubMed: 14614862]
68. Gakidis MA, et al. Vav GEFs are required for β 2 integrin-dependent functions of neutrophils. *J Cell Biol.* 2004; 166:273–282. [PubMed: 15249579]
69. Hirahashi J, et al. Mac-1 signaling via Src-family and Syk kinases results in elastase-dependent thrombohemorrhagic vasculopathy. *Immunity.* 2006; 25:271–283. [PubMed: 16872848]
70. Zarbock A, Abram CL, Hundt M, Altman A, Lowell CA, Ley K. PSGL-1 engagement by E-selectin signals through Src kinase Fgr and ITAM adapters DAP12 and FcR γ to induce slow leukocyte rolling. *J Exp Med.* 2008; 205:2339–2347. [PubMed: 18794338]
71. Zarbock A, Lowell CA, Ley K. Spleen tyrosine kinase Syk is necessary for E-selectin-induced α L β 2 integrin-mediated rolling on intercellular adhesion molecule-1. *Immunity.* 2007; 26:773–783. [PubMed: 17543554]
72. Urzainqui A, et al. ITAM-based interaction of ERM proteins with Syk mediates signaling by the leukocyte adhesion receptor PSGL-1. *Immunity.* 2002; 17:401–412. [PubMed: 12387735]

73. Sutterwala FS, Noel GJ, Clynes R, Mosser DM. Selective suppression of interleukin-12 induction after macrophage receptor ligation. *J Exp Med*. 1997; 185:1977–1985. [PubMed: 9166427]
74. Sutterwala FS, Noel GJ, Salgame P, Mosser DM. Reversal of proinflammatory responses by ligating the macrophage Fcγ receptor type I. *J Exp Med*. 1998; 188:217–222. [PubMed: 9653099]
75. Torigoe C, Inman JK, Metzger H. An unusual mechanism for ligand antagonism. *Science*. 1998; 281:568–572. [PubMed: 9677201]
76. Gerber JS, Mosser DM. Reversing lipopolysaccharide toxicity by ligating the macrophage Fcγ receptors. *J Immunol*. 2001; 166:6861–6868. [PubMed: 11359846]
77. Pasquier B, et al. Identification of FcαRI as an inhibitory receptor that controls inflammation: dual role of FcRγ ITAM. *Immunity*. 2005; 22:31–42. [PubMed: 15664157]
78. Monteiro RC, Van De Winkel JG. IgA Fc receptors. *Annu Rev Immunol*. 2003; 21:177–204. [PubMed: 12524384]
79. Jacob CM, Pastorino AC, Fahl K, Carneiro-Sampaio M, Monteiro RC. Autoimmunity in IgA deficiency: revisiting the role of IgA as a silent housekeeper. *J Clin Immunol*. 2008; 28(Suppl 1):S56–61. [PubMed: 18202833]
80. Pinheiro da Silva F, Aloulou M, Benhamou M, Monteiro RC. Inhibitory ITAMs: a matter of life and death. *Trends Immunol*. 2008; 29:366–373. [PubMed: 18602341]
81. Kanamaru Y, et al. Inhibitory ITAM signaling by FcαRI-FcRγ chain controls multiple activating responses and prevents renal inflammation. *J Immunol*. 2008; 180:2669–2678. [PubMed: 18250479]
82. Pinheiro da Silva F, et al. CD16 promotes Escherichia coli sepsis through an FcRγ inhibitory pathway that prevents phagocytosis and facilitates inflammation. *Nat Med*. 2007; 13:1368–1374. [PubMed: 17934470]
83. Cao W, et al. Plasmacytoid dendritic cell-specific receptor ILT7-FcεRIγ inhibits Toll-like receptor-induced interferon production. *J Exp Med*. 2006; 203:1399–1405. [PubMed: 16735691]
84. Cao W, et al. Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction. *J Exp Med*. 2009 in press.
85. Cao W, et al. BDCA2/FcεRI gamma complex signals through a novel BCR-like pathway in human plasmacytoid dendritic cells. *PLoS Biol*. 2007; 5:e248. [PubMed: 17850179]
86. Dzionek A, et al. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *J Exp Med*. 2001; 194:1823–1834. [PubMed: 11748283]
87. Blasius AL, Giurisato E, Cella M, Schreiber RD, Shaw AS, Colonna M. Bone marrow stromal cell antigen 2 is a specific marker of type I IFN-producing cells in the naive mouse, but a promiscuous cell surface antigen following IFN stimulation. *J Immunol*. 2006; 177:3260–3265. [PubMed: 16920966]
88. Liang B, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol*. 2008; 180:5916–5926. [PubMed: 18424711]
89. Lang P, et al. TCR-induced transmembrane signaling by peptide/MHC class II via associated Ig-α/β dimers. *Science*. 2001; 291:1537–1540. [PubMed: 11222857]
90. Hamerman JA, Tchao NK, Lowell CA, Lanier LL. Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. *Nat Immunol*. 2005; 6:579–586. [PubMed: 15895090]
91. Divangahi M, et al. Critical negative regulation of type 1 T cell immunity and immunopathology by signaling adaptor DAP12 during intracellular infection. *J Immunol*. 2007; 179:4015–4026. [PubMed: 17785840]
92. Hamerman JA, Jarjoura JR, Humphrey MB, Nakamura MC, Seaman WE, Lanier LL. Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12. *J Immunol*. 2006; 177:2051–2055. [PubMed: 16887962]
93. Turnbull IR, et al. Cutting edge: TREM-2 attenuates macrophage activation. *J Immunol*. 2006; 177:3520–3524. [PubMed: 16951310]
94. Takahashi K, Prinz M, Stagi M, Chechneva O, Neumann H. TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. *PLoS Med*. 2007; 4:e124. [PubMed: 17425404]

95. Piccio L, et al. Blockade of TREM-2 exacerbates experimental autoimmune encephalomyelitis. *Eur J Immunol*. 2007; 37:1290–1301. [PubMed: 17407101]
96. Piccio L, et al. Identification of soluble TREM-2 in the cerebrospinal fluid and its association with multiple sclerosis and CNS inflammation. *Brain*. 2008; 131:3081–3091. [PubMed: 18790823]
97. Fuchs A, Cella M, Kondo T, Colonna M. Paradoxical inhibition of human natural interferon-producing cells by the activating receptor NKp44. *Blood*. 2005; 106:2076–2082. [PubMed: 15941912]
98. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol*. 2001; 31:2680–2689. [PubMed: 11536166]
99. Byrd A, Hoffmann SC, Jarahian M, Momburg F, Watzl C. Expression analysis of the ligands for the Natural Killer cell receptors NKp30 and NKp44. *PLoS One*. 2007; 2:e1339. [PubMed: 18092004]
100. Esin S, et al. Direct binding of human NK cell natural cytotoxicity receptor NKp44 to the surfaces of mycobacteria and other bacteria. *Infect Immun*. 2008; 76:1719–1727. [PubMed: 18212080]
101. Hecht ML, et al. Natural cytotoxicity receptors NKp30, NKp44 and NKp46 bind to different heparan sulfate/heparin sequences. *J Proteome Res*. 2009; 8:712–720. [PubMed: 19196184]
102. Herschkovitz O, et al. Characterization of the recognition of tumor cells by the natural cytotoxicity receptor, NKp44. *Biochemistry*. 2007; 46:7426–7436. [PubMed: 17536787]
103. Ho JW, et al. H5-type influenza virus hemagglutinin is functionally recognized by the natural killer-activating receptor NKp44. *J Virol*. 2008; 82:2028–2032. [PubMed: 18077718]
104. Vieillard V, Strominger JL, Debre P. NK cytotoxicity against CD4+ T cells during HIV-1 infection: a gp41 peptide induces the expression of an NKp44 ligand. *Proc Natl Acad Sci U S A*. 2005; 102:10981–10986. [PubMed: 16046540]
105. Allcock RJ, Barrow AD, Forbes S, Beck S, Trowsdale J. The human TREM gene cluster at 6p21.1 encodes both activating and inhibitory single IgV domain receptors and includes NKp44. *Eur J Immunol*. 2003; 33:567–577. [PubMed: 12645956]
106. Blasius A, Vermi W, Krug A, Facchetti F, Cella M, Colonna M. A cell-surface molecule selectively expressed on murine natural interferon-producing cells that blocks secretion of interferon-alpha. *Blood*. 2004; 103:4201–4206. [PubMed: 14695235]
107. Blasius AL, Cella M, Maldonado J, Takai T, Colonna M. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. *Blood*. 2006; 107:2474–2476. [PubMed: 16293595]
108. Chu CL, Yu YL, Shen KY, Lowell CA, Lanier LL, Hamerman JA. Increased TLR responses in dendritic cells lacking the ITAM-containing adapters DAP12 and FcRγ. *Eur J Immunol*. 2008; 38:166–173. [PubMed: 18081038]

Figure 1. Activating and inhibitory pathways initiated by DAP12 and Fcγ associated receptors
 The left hand panel indicates the downstream signaling pathways that lead to activation of cellular responses when DAP12 and/or Fcγ associated receptors are engaged with high affinity/high valency ligands. The receptors associated specifically with either DAP12 or Fcγ that are discussed in the text are listed, with the immunoreceptors indicated in black font and the non-immunoreceptors in gold font. Dually associated receptors are indicated in blue font. For a more exhaustive list of adapter-associated receptors see Lanier (4) and Nimmerjahn (5). Based on the model of Monteiro (80) the potential mechanism of inhibitory function of DAP12 and Fcγ associated receptors is shown on the right side. These functions would become manifest under low affinity/avidity (ie monovalent) ligand interaction with the receptor(s), which would induce weak phosphorylation of the DAP12 and/or Fcγ ITAMs, leading to primarily SHP-1 phosphatase recruitment. SHP-1 blocks downstream pathways from other DAP12/Fcγ associated receptors or from completely heterologous receptors, such as the TLRs. The inhibitory function is indicated by blue and red lines terminating in rosettes. Whether the same model of low affinity/avidity interactions, leading to SHP-1 recruitment also mediate the trans-inhibitory function of the DAP12/Fcγ associated receptors on TLRs (and other receptors) remains unclear, and hence is indicated by a question mark.

Figure 2. DAP12/FcR γ or Syk-deficient dendritic cells show reduced proliferative responses and increased apoptosis to GM-CSF stimulation

(A) 1×10^6 WT or mutant bone marrow cells were cultured in the presence of 10 ng/ml GM-CSF (Peprotech) for the indicated days and number of CD11c⁺ cells was determined by flow cytometry and hemacytometer counts of viable cells. Results are the mean $\pm$ SD of 3 wells. (B) WT or mutant bone marrow cells were cultured as in A. At the indicated days, the number of CD11c⁺ apoptotic cells was determined by annexin V staining and flow cytometry.

Figure 3. DAP12-deficient macrophages have increased phagocytic capacity and are hyperresponsive to IFN γ stimulation

(A) Bone marrow macrophages were grown as described (90). WT and DAP12-deficient bone marrow derived macrophages (2×10^5 cells) were incubated with AlexaFluor488-labeled, IgG-opsonized SRBCs for 20 minutes. Unbound SRBCs were removed by washing in cold PBS, then bound but extracellular SRBCs were lysed by incubation of the macrophages in water for 1 min, followed by PBS wash and fixation in 4% paraformaldehyde in PBS. The percentage of macrophages that had phagocytosed the labeled SRBCs was measured by flow cytometry. (B) WT and DAP12-deficient bone marrow derived macrophages (2×10^5 cells) were incubated with FITC-labeled zymosan particles for 20 minutes. Unbound zymosan was removed by washing in PBS, then bound but extracellular zymosan was removed by incubation with proteinase K (2.5 units/ml) for 20 minutes at 25°C. Phagocytosis of zymosan was measured by flow cytometry. (C) Day 6 WT and DAP12-deficient bone marrow derived macrophages (2×10^5 cells) were incubated with the indicated concentrations of rmIFN γ for 48 hours at 37°C, blocked with 2.4G2 mAb and then stained with FITC-labeled anti-MHCII mAb, followed by flow cytometry.

Figure 4. DAP12-deficient macrophages display increased activation of Akt following TLR9 stimulation

WT and DAP12-deficient bone marrow-derived macrophages were incubated with 25 nM CpG oligonucleotides (CpG ODN 1826, Invivogen) for the indicated time (min). Cells were then lysed and 5 μ g of cytoplasmic extract for each time point was subjected to western blotting with polyclonal anti-phospho Akt antibody. The blot was then stripped and reprobed with an antibody to total Akt to confirm equal loading.