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Cross-regulation of signaling by ITAM-associated receptors

Lionel B Ivashkiv

Arthritis and Tissue Degeneration Program, Hospital for Special Surgery, Immunology and Microbial Pathogenesis Program, Weill Cornell Graduate School of Medical Sciences, New York, New York, USA

Abstract

An important function of receptors that signal through immunoreceptor tyrosine-based activation motifs (ITAMs) is to regulate signaling by heterologous receptors. This review describes mechanisms by which ITAM-associated receptors modulate signaling by Toll-like receptors (TLRs), tumor necrosis factor receptor family members and cytokine receptors that use the Jak–STAT signaling pathway, and the biological importance of this signal transduction cross-talk. ITAM-mediated cross-regulation can either augment or dampen signaling by other receptors. Conversely, TLRs and cytokines modulate ITAM-mediated signaling, by means including activation of β_2 integrins that are coupled to the ITAM-containing adaptors DAP12 and Fc γ . Integration of ITAM signaling into signaling networks through cross-talk with other signal transduction pathways results in tight regulation and fine tuning of cellular responses to various extracellular stimuli and contributes to induction of specific activation and differentiation pathways.

The ITAM is a conserved signaling motif preferentially used by hematopoietic cells¹. The ITAM motif (consensus sequence YXXLX_{6–8} YXXL/I) is contained in the cytoplasmic domain of transmembrane adaptor molecules that are associated with and transmit signals from various immunoreceptors. ITAM-coupled receptors include the T cell and B cell antigen receptors (TCR and BCR) and Fc receptors (FcRs), which bind immunoglobulins. In lymphocytes, ITAM-containing adaptors transmit antigen receptor signals that lead to cell activation or tolerance, depending on the intensity of receptor stimulation and the presence or absence of co-stimulatory signals². Myeloid cells express approximately 20 ITAM-associated receptors, including FcRs, TREMs, ILTs (also called LILRs), MAIRs, PIR-A, MDL-1, DCAR, OSCAR, CD200 receptors and c-Fms (refs. ^{3,4}). These receptors signal through two main ITAM-containing adaptors expressed by myeloid cells, termed Fc γ and DNAX activation protein-12 (DAP12). Except for FcRs, which bind immunoglobulins and immune complexes, the ligands for myeloid ITAM-coupled receptors are not well defined; they include MHC class I molecules and other, as yet unknown ligands that are expressed on myeloid cells or on tissue cells with which myeloid cells interact. Thus, in myeloid cells ITAM-coupled receptors are constitutively engaged, which leads to tonic low-level ITAM-mediated signaling (reviewed in ref. ⁵). More recently, it has become clear that Fc γ and DAP12 associate with β_2 and β_3 integrins and contribute to signaling by these receptors^{6–8}. Thus, ITAM signaling is also induced by cell–cell and cell–extracellular matrix (ECM) interactions that are mediated by these integrins.

The ligation of ITAM-associated receptors in myeloid cells leads to a signaling cascade that begins with phosphorylation of ITAM tyrosine residues by Src-family kinases, followed by the recruitment and activation of the spleen tyrosine kinase (Syk)^{1,5}. Syk then activates a

Correspondence should be addressed to L.B.I. (ivashkivl@hss.edu).

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signaling cascade leading to downstream activation of NF- κ B and MAPKs, important signaling effectors and inducers of gene expression (Figs. 1 and 2). An important component of ITAM-mediated signaling is activation of phospholipase- $\text{C}\gamma$ (PLC γ), resulting in the generation of diacylglycerol (DAG) and parallel activation of calcium signaling (Fig. 2). DAG activates PKC and contributes to MAPK activation through RasGRP/Ras guanyl releasing protein (RasGRP). Calcium signals activate the phosphatase calcineurin, which activates nuclear factor of activated T cells (NFAT) and calcium-dependent kinases such as calmodulin-dependent kinase (CaMK) and Pyk2. CaMK and Pyk2 contribute to the activation of MAPKs and of calcium-regulated transcription factors such as CREB. Of the major signaling pathways and effectors activated by ITAM-associated receptors, NF- κ B and MAPKs are involved in cell activation and are activated by many other receptors. Direct activation of calcium pathways by ITAM-associated receptors distinguishes them from macrophage-activating receptors such as TLRs and inflammatory cytokine receptors.

Extensive work has established that high-avidity cross-linking of ITAM-associated antigen receptors and FcRs strongly activates NF- κ B and MAPKs and leads to cell activation (reviewed in ref. ²). This has led to the paradigm that ITAMs and associated receptors mediate activating signals that are opposed by a distinct set of receptors that signal through a cytoplasmic-domain immunoreceptor tyrosine-based inhibitory motif (ITIM)⁹. ITIMs recruit phosphatases including the tyrosine phosphatases SHP-1 and SHP-2 and the inositol phosphatase SHIP that attenuate ITAM-induced signaling by dephosphorylating and thereby inactivating signaling intermediates. One surprise that has emerged from recent work is that ITAMs can also generate inhibitory signals and attenuate signaling by heterologous receptors^{3,5,10,11}. Although specific ITAM-associated receptors may be predominantly inhibitory, several experimental systems show that the same ITAM-coupled receptors can generate both positive and negative signals. For example, DAP12-associated TREMs can either synergize with TLRs in augmenting cytokine production and associated toxicity or, in a different context, can inhibit TLR-induced cytokine production^{12–15}. Fc γ -associated Fc α R (receptor for immunoglobulin A) can activate cells but also cross-inhibits several other receptors, including Fc γ Rs, the tumor necrosis factor receptor (TNFR) and TLRs^{16–18}. Mechanisms by which ITAM-coupled receptors generate inhibitory signals and the molecular basis of the ‘switch’ between pro- and anti-inflammatory ITAM signaling are not well understood. The avidity of receptor ligation may determine the nature of the signal that is generated, and thus ligand density governs the switch in ITAM function^{3,10}.

ITAM-associated receptors typically do not function in isolation; instead, they cooperate with heterologous receptors to achieve cell responses most appropriate for the microenvironment^{1,2,19}. Such cooperation can either augment or dampen signals induced by other receptors. In lymphocytes, antigen receptor-induced signals are integrated with signals from co-stimulatory receptors such as CD28 and cytokine receptors such as the interleukin (IL)-2 receptor to achieve full cell activation. Recent work has highlighted ITAM-mediated cross-regulation of TLRs, cytokine receptors that use the Jak-STAT pathway, and TNF family receptors^{3,5,10,11}. Such cross-regulation can promote cell activation or homeostatic regulation, depending on cell context. Mechanisms of cross-regulation of heterologous receptors by ITAM-mediated signaling and the biological importance of this signaling cross-talk are discussed in this review.

Regulation of TNFR family responses

ITAM-associated receptors have been shown to regulate signaling by the TNFR family members receptor activator of NF- κ B (RANK) and BLYS receptor 3 (BR3, also called BAFFR). RANK is required for differentiation of myeloid cells into osteoclasts—multinucleated cells that are specialized for bone resorption. Osteoclast generation is increased

in conditions of inflammation, and osteoclast-mediated bone lysis contributes significantly to pathogenesis of autoimmune and inflammatory diseases such as rheumatoid arthritis^{20–22}. TNFR family members signal through TRAF adaptor molecules, and RANK uses TRAF6 to activate NF- κ B and MAPK pathways (Fig. 1). These TRAF6-dependent pathways, and in particular MAPK-induced Fos transcription factor, contribute to induction of expression of NFATc1, which functions as a master regulator of osteoclast differentiation and is required for osteoclastogenesis^{21,23,24}. However, RANK-induced TRAF6-dependent activation of NF- κ B and MAPK pathways is insufficient to activate NFATc1 responses, because nuclear translocation, and thus function, of NFATc1 depends on dephosphorylation by the calcium-dependent phosphatase calcineurin. Thus, activation of NFATc1 requires calcium signals that activate calcineurin. In myeloid cells, these calcium signals are provided by co-stimulatory ITAM-associated receptors that activate PLC γ , calcium oscillations and post-translational modification of NFATc1 (refs. 8,21,23,25–28) (Fig. 1). ITAM-associated receptors that have been implicated in providing co-stimulation for RANK signaling in osteoclastogenesis include DAP12-associated TREM-2, SIRP β and $\alpha_V\beta_3$ integrins, and FcR γ -associated PIR-A and OSCAR. Deficiency in DAP12 and FcR γ results in diminished osteoclastogenesis and thus osteopetrosis (high bone mass) in mice^{26,28–30}. DAP12 and TREM-2 are implicated in bone remodeling and osteoclastogenesis in humans^{31–33}. When these ITAM-associated receptors provide a calcium signal that activates NFATc1 nuclear translocation, NFATc1 amplifies expression of its own promoter (Fig. 1), leading to high NFATc1 expression, induction of osteoclast-related genes, and osteoclast differentiation³⁴.

Cooperation of RANK and ITAM-associated receptor signals to activate NFATc1 provides a good example of integration of signals from different receptors to achieve specific cell functions. Recent work has revealed that dual signals from RANK and ITAM-associated receptors are required to activate PLC γ and generate an effective calcium signal^{27,35,36}. Thus, RANK and ITAM signaling are integrated not only distally, at the level of NFATc1 activation (Fig. 1, integration point 1), but also proximally, at the level of PLC γ activation (Fig. 1, integration point 2). Activation of PLC γ requires formation of a signaling complex that includes the BLNK and SLP-76 adaptors, Btk and Tec tyrosine kinases, and PLC γ (refs. 35–38). RANK contributes to PLC γ activation by activating Btk and Tec^{35,36}. RANK-mediated activation of Btk and Tec is independent of ITAM-associated receptors and also independent of TRAF-mediated signaling and instead probably proceeds by a Src kinase-dependent pathway (Fig. 1). ITAM-associated receptors contribute to PLC γ activation by Syk-mediated phosphorylation of BLNK and SLP-76, which promotes assembly of a Btk-, Tec- and PLC γ -containing ‘signalosome’ and Btk- and Tec-mediated phosphorylation and activation of PLC γ . Syk can also contribute to Btk and Tec activation, further underscoring the tight coupling of RANK and ITAM signaling pathways.

There is also evidence suggesting a third level of cross-talk, namely RANK-induced and Src-mediated transactivation of ITAM-associated receptors that leads to increased phosphorylation of DAP12 and FcR γ ITAM motifs and Syk activation^{26,27} (Fig. 1, dotted line, integration point 3). In this model, RANK provides the most upstream signal, which results in TRAF6-dependent activation of NF- κ B and MAPKs, Src-dependent activation of Btk and Tec, and transactivation of ITAMs that results in calcium signaling. The mechanisms that mediate trans-activation of ITAM-associated receptors are not known. This model does not fully accommodate a role for ITAM-associated receptors and their ligands and does not explain the presence of basal DAP12 and PLC γ phosphorylation in osteoclast precursors; indeed, RANK-induced DAP12 and PLC γ phosphorylation seems modest as these signals are readily detected only when basal phosphorylation is diminished by serum and macrophage colony stimulating factor (M-CSF) starvation, and acute RANK-induced calcium fluxes are difficult to measure^{23,26,27}.

An alternative model, not mutually exclusive, is that ITAM signaling is regulated by ligation of ITAM-associated receptors and, in myeloid cells, is tonically active secondary to constitutive engagement of ITAM-associated receptors. These receptors are engaged by mostly unknown ligands expressed on myeloid cells themselves or on interacting stromal and osteoblast-lineage cells. The presence of constitutive ITAM-associated receptor ligation and tonic ITAM and calcium signaling are supported by observations that DAP12 and FcR γ are basally tyrosine phosphorylated, that basal calcium concentrations are elevated, and that Syk and calcium-dependent Pyk2 are basally active in myeloid cells (refs. ^{28,39–41} and K.-H. Parkmin and L.B.I., unpublished data). In this model, low-avidity constitutive receptor ligation and tonic ITAM signaling ‘license’ differentiation of myeloid cells into noninflammatory osteoclasts in response to RANK by providing a signal that activates NFATc1 function. The amplitude of this signal can be further ‘fine-tuned’ by regulation of expression of ITAM-associated receptors and their ligands and by transactivation of ITAMs by RANK. At the same time that this tonic ITAM signaling promotes osteoclastogenesis, it suppresses inflammatory TLR signaling (see below) and thereby suppresses differentiation of inflammatory macrophages. Thus, ITAM-associated receptor signaling is implicated in cell fate decisions in the late stages of differentiation in the myeloid lineage.

With the exception of ECM components that engage DAP12-associated $\alpha_v\beta_3$ integrins and M-CSF and IL-34, which engage c-Fms, ligands for ITAM-associated receptors important in osteoclastogenesis have not been definitively identified. Emerging evidence suggests that TREM-2 can be activated indirectly by ligands for receptors that interact with TREM-2. For example, TREM-2 associates with plexin A receptors and TREM-2–DAP12 signaling is activated by the plexin A1 ligand Semaphorin 6D (ref. ⁴²). Indirect activation of ITAM signaling by Semaphorin 6D is important not only in osteoclast differentiation but also in regulating cytokine production and antigen-presenting function of dendritic cells (which express TREM-2 and plexin A1). In addition, TREM-2–DAP12 signaling is activated in myeloid dendritic cells by PD-L2 (also called B7-DC), a molecule important in mediating effects of dendritic cells on T cell phenotype⁴³. Thus, ITAM signaling can be triggered by various receptors and ligands important in immune responses, and in turn, ITAM signaling modulates the function of the TNFR family member RANK and myeloid cell activation and differentiation. It is clear that ITAM-associated receptors are enmeshed in complex signaling networks, and a systems approach may be helpful in dissecting their regulation and function.

A fruitful area for future research is investigation of ITAM-mediated regulation of other members of the TNFR family. A recent report showed that tonic ITAM signaling by the BCR modulates signaling by the TNFR family member BLYS receptor-3 (ref. ⁴⁴). Tonic BCR-mediated ITAM signaling induces expression of the noncanonical NF- κ B pathway factor p100, which serves as a substrate for activation by BR3 and promotes cell survival. Noncanonical NF- κ B signaling is important in mediating cellular responses to various TNFR family members, including RANK^{45,46}. Thus, it is likely that ITAM-associated receptors regulate various TNFR family members by several mechanisms.

Integration with TLR responses

Many microbial products and complex inflammatory stimuli contain ligands for both ITAM-associated receptors and TLRs and activate the two receptor systems in tandem. For example, the yeast cell wall preparation zymosan activates cells simultaneously through TLR2 and through the Dectin-1 receptor for β -glucans, which signals through a variant ITAM motif^{47–49}; nucleic acid-containing immune complexes simultaneously stimulate macrophages and dendritic cells through FcRs and TLR9 (ref. ⁵⁰). Linked activation of these receptors results in synergistic activation of inflammatory cytokine production and enhances antigen-presenting functions. This synergy may be particularly important under conditions of low ligand

concentration or may function to achieve specific outcomes—for example, zymosan-induced production of IL-23 and the capacity to promote T_H-17 differentiation⁵¹. Ligation of ITAM-associated receptors and TLRs by ligands that are not physically linked also increases cell activation. For example, DAP12-coupled TREM-1 synergizes with TLR4 *in vitro* and *in vivo* to increase cytokine production and potentiate endotoxemia¹⁵. In addition, β_2 and β_3 integrins, which signal in part through DAP12 and FcR γ , augment TLR-induced cell activation and inflammatory cytokine production^{52–54}.

TLRs activate myeloid cells and induce inflammatory cytokine production through downstream signaling molecules NF- κ B, MAPKs and IRFs⁵⁵. The molecular basis of synergistic induction of cytokine production by ITAM-associated receptors and TLRs seems to be enhanced activation of NF- κ B and MAPKs (Fig. 2); as yet, there is no evidence supporting a distinct ITAM-activated pathway that directly activates cytokine genes. Increased activation of NF- κ B and MAPKs may occur by several mechanisms—dual activation of signaling molecules upstream of NF- κ B and/or MAPKs by distinct signals emanating from ITAMs and TLRs. For example, ITAM-associated receptors and TLRs both activate the signaling adaptor CARD9 (refs. ^{51,56–60}) (Fig. 2). ITAM-associated receptors activate CARD9 by a Syk-dependent pathway that leads to assembly of a CARD9-Bcl-10-MALT1 signaling complex and to downstream NF- κ B activation. In contrast, TLRs activate CARD9 through RIP2, leading to downstream MAPK activation. It is not yet clear why activation of CARD9 by ITAM-associated receptors and TLRs leads to, respectively, NF- κ B and MAPK activation and why the role of CARD9 in TLR signaling seems cell-type and context dependent. Synergy may occur secondary to additive activation of NF- κ B and MAPKs by distinct signaling pathways emanating from ITAM-associated receptors and TLRs (Fig. 2). One example is activation of a reactive oxygen burst (ROI production) by Dectin-1 and of a TGF- β -activated kinase (TAK1)-mediated signal by TLR2 that work together to superactivate NF- κ B⁴⁸ (Fig. 2). ROI generation is dependent on PKC, which can also activate NF- κ B through the adaptor CARMA1 (Fig. 2); this pathway is used in a cell type-specific manner. Recent evidence suggests that Dectin-1 also modulates TLR-mediated NF- κ B activation by inducing acetylation of the NF- κ B p65 subunit by a Raf-1 dependent pathway⁶¹. TLRs activate MAPKs through TAK1- and CARD9-mediated pathways, whereas ITAM-associated receptors activate MAPKs through DAG- and calcium-mediated pathways (Fig. 2). Thus, additive and integrated activation of MAPKs by these distinct pathways results in enhanced activation of downstream signaling molecules and transcription factors. Direct interactions between ITAM-associated receptors and TLRs can augment signaling (Fig. 2, top). β_3 integrins interact with TLR2 and augment TLR2-induced signaling and gene activation, possibly by facilitating presentation of microbial ligands to TLR2 (ref. ⁵³). In addition to direct receptor interactions, ITAM-associated receptors can control the function of TLRs. β_2 integrins augment TLR4 signaling by generating phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5) P₂), which serves to recruit the signaling adaptor TIRAP (also called Mal)⁵⁴. Integrins can also modulate signaling by controlling the formation, trafficking and disassembly of microclusters of signaling molecules⁶². It is not known which, if any, of the mechanisms by which integrins augment TLR signaling are dependent on DAP12 or FcR γ —integrins activate distinct signaling pathways through signaling motifs present in their cytoplasmic domains, and these ITAM-independent signals may function to modulate TLR responses.

The overall picture emerging is that ITAM-associated receptors augment TLR signaling through several mechanisms, thereby allowing regulation over a broad amplitude of response. Recent research indicates that subcellular localization of receptors can be important in cross-talk between ITAM-associated receptors and TLRs. Activated BCRs traffic to an autophagosome-like compartment and recruit TLR9-containing endosomes to autophagosomes by a phospholipase D-dependent mechanism, and subsequent BCR-TLR interactions result in hyperactivation of MAPKs⁶³. ITAM signaling may also modulate the

function of other pattern-recognition receptors, as Syk-mediated signaling is required for dendritic-cell activation by, and high-affinity interaction with, monosodium urate crystals that precipitate gout attacks⁶⁴. As monosodium urate crystals subsequently activate Nod-like receptors, leading to inflammasome activation and caspase-1-mediated processing of IL-1, these findings suggest a role for ITAM signaling in modulating Nod-like receptor and inflammasome signaling and function. Thus, it seems that ITAM-associated receptors cooperate with many receptors that sense and respond to infectious pathogens and inflammatory stimuli.

Inhibition of TLR responses

ITAM-associated receptors can also dampen TLR signaling. Constitutive ligation of DAP12-associated TREM-2 by as yet unknown macrophage- and dendritic cell-expressed ligands leads to tonic low-level ITAM signaling that attenuates cytokine production induced by TLRs^{12–14,65}. This negative regulation of TLR responses is physiologically important, as DAP12-deficient mice show increased cytokine production and increased toxicity in the D-galactosamine-potentiated endotoxemia model¹³. However, the role of DAP12 in mediating cytokine production and inflammation *in vivo* is complex, as DAP12 deficiency is protective in LPS-induced endotoxemia and in the cecal puncture and ligation model of bacterial peritonitis¹⁵. The mechanisms by which TREM-2 and DAP12 inhibit TLR signaling have not been identified, although DAP12 seems to regulate TLR-induced activation of the kinase ERK. Insight into negative regulatory mechanisms has been provided by findings that low-avidity ligation of Fcγ-coupled FcαR (receptor for immunoglobulin A) by monovalent ligands cross-inhibits signaling by FcγRs, CCR2, TNFRs and TLRs^{16–18}. In contrast, high-avidity multivalent ligation of FcαR using antibodies or immune complexes activates inflammatory signaling. A molecular explanation for these observations is suggested by the findings that low-avidity FcαR engagement recruits SHP-1, which dephosphorylates and inactivates heterologous receptors and signaling intermediates. In contrast, high-avidity ligation recruits Syk and activates positive signaling¹⁷. These findings have led to a model whereby the activating versus inhibitory function of an ITAM-coupled receptor is determined by avidity of ligation and the resulting pattern of partial or complete ITAM phosphorylation and differential recruitment of signaling molecules. However, this model cannot explain all of the reported observations—for example, the inhibition of TLR9 responses in plasmacytoid dendritic cells by high-avidity antibody-mediated cross-linking of Fcγ-associated ILT7 (ref. ⁶⁶). These findings point to alternative and complementary mechanisms by which ITAM-associated receptors inhibit TLRs, such as inhibition of TLR signaling by the ITAM-activated phosphatase calcineurin³⁹ or by PLCγ-mediated dephosphorylation of PtdIns(4,5)P₂ (ref. ⁵⁴), leading to diminished TIRAP recruitment to the plasma membrane. These various inhibitory mechanisms have been discussed in recent reviews^{3,5,10,11}.

A hallmark of signal transduction is the induction of feedback inhibitory pathways and molecules that limit cell activation^{67,68}; such feedback inhibitors have the potential to cross-inhibit heterologous receptors. For example, ITAM-associated receptors induce suppressors of cytokine signaling (SOCS proteins)^{69,70} in a manner dependent on NF-κB and MAPKs^{70,71} and high-avidity ITAM-associated receptor ligation, in distinction to the low-avidity ligation-dependent inhibitory mechanisms discussed above. In addition, FcγRs synergize with TLRs to induce production of the anti-inflammatory cytokine IL-10, which can cross-inhibit TLR responses^{72,73}. FcγR-induced and ERK-mediated phosphorylation of histone-3 on Ser10 promotes increased chromatin accessibility at the *Il10* locus, which facilitates recruitment of TLR-induced transcription factors^{74,75}. A similar ERK-mediated mechanism is likely to explain the large amounts of IL-10 that are produced by dendritic cells in response to zymosan⁷⁶. The induction of signaling inhibitors by ITAM-mediated pathways is an important

mechanism for suppressing inflammation, and it will be important to fully characterize the inhibitors that are induced and proximal signaling events required for their induction.

Regulation of cytokine Jak–STAT responses

Integration of signaling by ITAM-associated receptors and interferon (IFN) receptors that augments activation of transcription factor STAT1 and expression of downstream inflammatory target genes was recently reported^{40,41}. Enhanced IFN receptor signaling was observed under conditions of tonic ITAM signaling generated in part by integrin-mediated cell adhesion. Tonic ITAM signaling maintains constitutive activity of a signaling pathway dependent on DAP12 and Syk that leads to basal calcium-dependent activity of calmodulin-dependent kinase (CaMK) and Pyk2 (Fig. 3). Pyk2 relays the ITAM signal by interacting with and amplifying the activation of Jak kinases associated with IFN receptors. In addition to integrins, FcγRs play a positive role in enhancing IFN-α signaling⁷⁷, and thus various ITAM-associated receptors have the potential to augment IFN signaling. Furthermore, integration of DAP12 and IL-4 signaling, and activation of FcRγ by the IL-3 receptor, have recently been demonstrated^{78,79}, suggesting more extensive integration of signaling by ITAM-associated receptors and receptors for various cytokines that use the Jak–STAT pathway.

Early *in vitro* experiments demonstrated that, in contrast to tonic ITAM signaling, high-avidity ligation of TCRs inhibits signaling by the IL-2 and IL-4 receptors, which share a common γ signaling subunit (γ_c), and by IFN-α and IL-6 receptors^{80,81}. The physiological importance of such inhibition is supported by the recent *in vivo* demonstration of a phenomenon termed ‘coreceptor tuning’⁸². In ‘coreceptor tuning’, TCR ligation inhibits signaling by the IL-7 receptor, which uses γ_c, and thereby suppresses IL-7-induced expression of the co-receptor CD8. This mechanism ensures that CD8⁺ T cells do not express amounts of CD8 high enough to lead to autoreactivity. Cell surface expression of cytokine receptors is not affected by TCR cross-linking. Instead, activation of receptor-associated Jak kinases, the most proximal step in the Jak–STAT signaling pathway, is inhibited by PKC- and ERK-dependent pathways^{80,81}. Inhibition of Jaks is induced rapidly in the absence of *de novo* protein synthesis and thus occurs by a direct pathway.

Additional studies in myeloid cells have shown rapid and direct inhibition of IL-10 receptor (IL-10R) and IFN-α receptor (IFNAR) signaling after high-avidity ligation of FcγRs or receptors for zymosan^{69,83,84}. Like inhibition of cytokine signaling in T cells, inhibition of IFNAR signaling in macrophages is PKC dependent. Activation of PKC inhibits IFN-α-induced Jak phosphorylation by inducing recruitment of the phosphatase SHP-2 to the IFNAR receptor complex. IL-10R signaling is also inhibited by a PKC-dependent mechanism, but in this case cell surface expression IL-10R is down-regulated, probably by internalization. Thus, rapidly induced, direct inhibition of cytokine signaling by ITAM-coupled receptors is mediated by PKC and targets cytokine receptors and proximal steps in Jak–STAT signal transduction. The negative role of PKC in inhibiting cytokine signaling contrasts with its positive role in augmenting TLR signaling. Sustained activation of FcγRs also leads to delayed inhibition of IFNγ signaling that seems to be independent of ITAM signaling and is mediated by internalization of the IFNγ receptor⁸⁵. Another main mechanism of inhibition of cytokine signaling occurs through induction of expression of SOCS proteins, which interact with phosphotyrosine residues in receptor cytoplasmic domains, compete with STATs for binding to receptor docking sites, inactivate Jak catalytic activity and target receptors for proteasomal degradation⁷⁰. High-avidity ligation of ITAM-coupled receptors induces SOCS3 expression^{69,70,80}, and it is likely that SOCS3 contributes to inhibition of cytokine signaling at later time points, after engagement of ITAM-associated receptors.

Overall, ITAM-associated receptors seem to augment cytokine signaling under conditions of tonic ITAM signaling that occur under physiological conditions of homeostasis. Such amplification of cytokine signaling confers on macrophages the capacity to respond to low concentrations of cytokines produced under physiological conditions and to respond rapidly and strongly to cytokines produced by changes in the environment, such as challenges produced by stress or infection. In contrast, strong ligation of ITAM-associated receptors that by itself activates macrophages attenuates cell responses to cytokines and thereby prevents excessive activation and associated toxicity.

Regulation of ITAM signaling

Cross-talk among signaling pathways allows for bidirectional regulation. For example, IFN- γ induces expression of Fc γ RI and TNF induces expression of PIR-A, thereby amplifying signaling by ITAM-mediated pathways^{86,87}. In addition, TLRs induce expression of ZAP70, a tyrosine kinase closely related to Syk, and thus can augment ITAM-dependent signaling⁸⁸. Notably, TNF modulates signaling in response to immune complexes by increasing expression of ‘activating’, ITAM-containing Fc γ RIIA while decreasing expression of ‘inhibitory’, ITIM-containing Fc γ RIIB, thereby altering the balance of activatory and inhibitory signals^{89,90}. Several other cytokines regulate expression of ITAM-coupled receptors, including IL-4 and IL-10, and we have found extensive remodeling of the pattern of ITAM-associated receptor expression during maturation of human monocytes to macrophages (L.B.I., unpublished data). These cytokine- and differentiation-induced changes in expression of ITAM-associated receptors will affect the avidity of receptor ligation and also will alter the pattern of ligands to which cells are responsive, and thus will qualitatively change cell responses to the environment.

Less is known about direct mechanisms that regulate ITAM signaling, such as the RANK-induced transactivation of ITAM phosphorylation discussed above. Recently, a direct association of the IL-3 receptor with Fc γ R has been demonstrated; this association couples IL-3 to the activation of Syk and channels IL-3 signals into ITAM-mediated pathways⁷⁹. Activation of Pyk2 by cytokines and Jaks^{91,92} (Fig. 3) suggests that cytokine signaling also modulates farther downstream components of ITAM signaling pathways. Association of several more cytokine receptors with heterologous receptors or signaling adaptors, such as IL-15R with DAP10 (ref. ⁹³), G-CSF receptor with integrin $\alpha_9\beta_1$ (ref. ⁹⁴), and IFNAR with TAM receptors⁹⁵, suggests that direct association of receptors is a common mechanism that underlies cytokine receptor and ITAM-associated receptor cross-talk.

In addition, TLRs and cytokines potentially can regulate integrin-induced ITAM-dependent and ITAM-independent signals. TLRs and cytokines activate integrins to assume a high-affinity ligand-binding conformation by a process termed inside-out signaling^{37,96} (Fig. 4). Inside-out signaling is now appreciated to result in increased adhesion to ECM, cell spreading and enhanced cell–cell interactions. This increased affinity of integrin–ligand interactions will lead to increased avidity of integrin ligation, which will in turn generate an integrin-mediated ‘outside-in’ signal. In the case of β_2 and β_3 integrins, such outside-in signals will include signaling through associated DAP12 and Fc γ R, with downstream activation of Syk and calcium signaling (Fig. 4). In support of this model, TNF-induced activation of Syk that is indirect and mediated by β_2 integrins has been reported⁹⁷. Additional direct interactions between integrins and TLRs, as exemplified by β_3 integrin–TLR2 interactions⁵³ and β_2 integrin–TLR4 interactions⁵⁴, are also likely to modulate ITAM-mediated signaling.

Overview and biology

Many canonical signaling pathways are conserved across several cell types and are capable of acting in isolation to elicit cellular responses. For example, the conserved core Jak–STAT

signaling pathway, which operates in many cell types, proceeds to the nucleus in a linear, unbranched manner and elicits a relatively focused cellular response by selectively activating downstream effector molecules and target genes. In contrast, ITAM-mediated signals, which operate only in hematopoietic cells, proceed in a branching manner and activate several signaling cascades and second messengers (Fig. 2); in addition, ITAM signals are typically not sufficient in isolation to induce full cellular activation or differentiation. What are the benefits of a system in which a receptor can activate several branching pathways but by itself is not capable of eliciting cell activation or differentiation?

One advantage of a requirement for integration among heterologous receptor systems is that cells are not likely to be inappropriately activated by one receptor system, as activation requires two or more signals from the environment. Such regulation is particularly important for immune cells that need to interpret signals in the context of ‘danger’ and adopt an appropriate response. The TCR, BCR and FcRs are involved in recognition of foreign antigens and pathogens, and it is important that cells do not become activated in the absence of further evidence of infection, such as that provided by pathogen-induced cytokines or by sensing of pathogens by TLRs. It is also important to avoid inappropriate cell fate decisions—for example, differentiation of osteoclasts in soft tissues where they are not necessary and could be harmful²⁰. Another advantage of integrating ITAM pathways into signaling networks is that this confers upon ITAM-associated receptors the capacity to ‘fine tune’ the amplitude and qualitative nature of cellular responses to heterologous receptors. In this formulation, proposed by M. Colonna¹⁹, ITAM-associated receptors sense the environment and modulate cellular responses to ensure they are appropriate for that environment.

ITAM signaling activates several DAG- and calcium-dependent signaling molecules, such as PKC, CaMKs, Pyk2 and calcineurin-NFAT, that are not strongly activated by other immune cell signaling pathways. It is precisely these PLC γ - and calcium-dependent molecules that have been implicated in cross-talk between ITAM-mediated and other signaling pathways. Thus, calcium signaling plays a prominent role in connecting signaling networks in immune cells.

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Figure 1.

Integration of RANK and ITAM signaling to activate NFATc1. RANK signaling induces NFATc1 gene expression by way of TRAF6 and transcription factors NF- κ B and AP-1 and contributes to induction of calcium signaling by activating Btk and Tec tyrosine kinases and transactivating ITAM-associated receptors. ITAM-associated receptors TREM-2 and SIRP β are activated by ligands constitutively expressed on osteoclast precursors; OSCAR and PIR-A, by ligands expressed on stromal and osteoblast lineage cells; β_3 integrins, by extracellular matrix; and c-Fms by M-CSF and IL-34, which are secreted by various cell types; some of these receptors can also be superactivated after stimulation of RANK by its ligand RANKL. ITAM-associated receptors cooperate with RANK to induce calcium signaling, which activates calcineurin (Cn), which in turn dephosphorylates (de-P) and induces nuclear translocation of NFATc1. NFATc1 is a master regulator of osteoclast differentiation that autoamplifies expression of its own promoter, activates expression of osteoclast-related genes and drives osteoclastogenesis. Specific integration points in RANK and ITAM signaling discussed in the text are labeled 1, 2 and 3. InsP $_3$, inositol-1,4,5-trisphosphate; IKK, I κ B kinase.

Figure 2.

Integrated signaling by ITAM-associated receptors and TLRs leads to enhanced activation of NF- κ B and MAPKs. ITAM-associated receptors and TLRs activate distinct signaling pathways that converge on common signaling effectors such as NF- κ B and MAPKs. Signaling pathways can also converge on upstream signaling molecules, such as CARD9. Integrated and additive activation of signaling molecules by different signaling pathways leads to enhanced signaling and amplified cellular responses. Cn, calcineurin; IRAK1/4, interleukin 1 receptor-associated kinases 1 and 4; MyD88, myeloid differentiation factor adaptor molecule.

Figure 3.

Integration of ITAM and cytokine Jak-STAT signaling. Low-avidity ligation of ITAM-associated receptors leads to tonic calcium signaling that maintains basal activity of a CaMK-Pyk2 pathway that amplifies Jak activity and enhances downstream activation of STAT1. Cytokine receptors and Jaks also activate Pyk2, and Pyk2 can thus relay signals between ITAM-associated receptors and cytokine receptors bidirectionally. ITAM-containing DAP12 also modulates cell responses to IL-4. Cytokines such as IFN-γ, IFN-α, IL-4, IL-10 and IL-27 regulate expression of ITAM-associated receptors and STAT1, and thus cytokine and ITAM-associated receptor signaling pathways are tightly integrated. Adapted from *Nat. Immunol.* **9**, 186–193 (2008).

Figure 4.

Indirect activation of calcium signaling by TLRs and TNFR through integrins. Engagement of TLRs and TNFRs activates signaling pathways that induce a conformational change in integrins (in a process termed inside-out signaling), leading to high-affinity ligand binding and associated high-avidity ligation of integrins. High-avidity integrin ligation results in outside-in signaling that activates integrin-mediated signaling pathways (which in the case of β₂ and β₃ integrins includes activation of DAP12- and FcRγ-mediated signaling).