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Beta2 Integrins Inhibit TLR Responses by Reducing NF-kappaB Pathway Activation

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Abstract

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Although originally considered to be the archetypal cell activation pathway, signals through immunoreceptor tyrosine-based activation motifs (ITAM) are known to be inhibitory and have recently been appreciated to cross-regulate TLR responses. ITAM signaling in myeloid cells is mediated by the ITAM-containing adapters DAP12 and FcR γ , which can associate with many cell surface receptors including the β_2 integrins. However, the ability of these ITAM-containing adapters to dampen TLR responses, the relationship between the β_2 integrins themselves and TLR activity is unclear. We demonstrate here that β_2 integrins are required for negatively regulating TLR activation in macrophages and DCs. β_2 integrin-deficient myeloid cells (*Itgb2*^{-/-}) are hypersensitive

to TLR stimulation and produce more inflammatory cytokines in response to a panel of TLR agonists. *Itgb2*^{-/-} mice also hyperrespond to LPS injection, demonstrating that β_2 integrins control TLR activity in vivo. This hypersensitivity is not due to previously associated functions of β_2 integrins, including changes to IL-10, A20, ABIN-3, Hes-1 or Cbl-b. Instead, β_2 integrin-mediated TLR inhibition directly dampens NF- κ B activation by controlling p65 activation and binding to target promoters. Furthermore, β_2 integrins also control type I IFN synthesis and signaling, perhaps by modulating expression of SOCS-1 via miR-155. Thus, control of TLR responses by β_2 integrins involves multiple, potentially overlapping mechanisms.

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Dedication

This dissertation is dedicated to my wife Jenna Yee, my son Zachary Yee, and my parents, Gene and Po Yee, whose unwavering support and love have made this work possible.

Chapter 1: Introduction

Project Overview

Innate immune cell activation is a critical first step in generating an immune response capable of detecting and resolving infections from an assortment of pathogens. Since their discovery 15 years ago, Toll-like receptors (TLRs) have become the best-studied receptor system responsible for facilitating macrophage and dendritic cell (DC) activation. TLR ligation generates a signal network that involves Toll/IL-1-receptor (TIR)-containing adapters and results in inflammatory cytokine and type I IFN secretion. These mediators, in turn, coordinate other aspects of immunity. These include initiation of the humoral aspects of innate immunity (acute phase proteins, complement), shaping of the T cell response by inflammatory cytokines, or in the establishment of the “antiviral state” following type I IFN signaling.

Although TLR activation is essential for proper immunity, the potency of the TLR-induced response can have severe health consequences if left unregulated. The dangers of sepsis and autoimmunity are normally avoided by inhibitory mechanisms that act to blunt the effects of TLR activation and return the cell to quiescence. Owing to the importance of TLR inhibition, many inhibitory processes have been identified that serve redundant functions to orchestrate TLR blockade. Of these, cross-regulation by separate receptor systems is particularly fascinating given that TLR activity in vivo occurs in the presence of other signals that may compete against or augment cell activation. In this light, immunoreceptor tyrosine-based activation motif (ITAM) signaling must be considered. ITAM-based signals are classically associated with cell activation in T and B

cells, however, in the context of TLR activation in macrophages and DCs, the ITAM-containing adapters DAP12 and FcR γ can have an inhibitory function. Many receptors are known to signal through DAP12 and FcR γ , including the β_2 integrins, which are critical cell adhesion molecules that mediate the migration of leukocytes towards the sites of infection. Beyond their role as adhesion receptors, β_2 integrin signals have also been associated with direct modulation of TLRs, though the nature of this contribution is controversial as a multitude of studies have attempted to link β_2 integrins to both positive and negative regulatory roles. Thus, the connection between β_2 integrins and inflammation is complex and currently unclear.

The goal of this dissertation is to show definitively that β_2 integrins are necessary for inhibiting TLR activation in macrophages and DCs. To accomplish this, we assessed TLR responses in *Itgb2*^{-/-} cells and mice that are deficient for all β_2 integrins. β_2 integrin deficiency resulted in TLR hypersensitivity in vitro and in vivo and we demonstrate here that β_2 integrin signals inhibited NF- κ B pathway activation during both early and late phases of TLR activation. Furthermore, β_2 integrin functions were responsible for blocking “secondary” type I IFN signaling downstream of TLR activity, perhaps through control of the SOCS-1 protein, an inhibitor known to block numerous aspects of TLR activation.

The Innate Immune System

The vertebrate immune system is compartmentalized into two arms that differ in the rapidity of their activation, their cellular makeup and associated effector functions. The components of the innate immune system provide a rapid and potent first line defense against pathogenic insults by generating broad antimicrobial activities. Moreover,

innate immune cell activation is responsible for enabling adaptive immune cellular responses, which use antigen receptors to target specific microbes. Interplay between the innate and adaptive arms of the immune system is crucial for protecting the host against numerous bacteria, viruses, helminthes and fungi.

“Innate immunity” refers to a range of cellular and humoral systems that are evolutionarily conserved and are collectively the vanguard of antimicrobial action. The skin and mucosal epithelia are physical barriers that normally prohibit pathogenic invasion, and, if breached, quickly induce activation of innate immune cells through triggering of germline-encoded pattern recognition receptors (PRR), in particular, the class of PRR known as TLRs. TLRs recognize structural motifs found on microbes termed pathogen-associated molecular patterns (PAMP) such that TLR activation leads to a series of antimicrobial responses, including reactive oxygen and nitrogen species production, inflammatory cytokine and type I interferon synthesis, and complement activation. TLR-induced activity in macrophages and dendritic cells (DC) is of particular interest. In the steady state, macrophages are efficient phagocytes that internalize and recycle cellular debris, an ability that directed during active infection for the destruction of pathogens. DCs sample the local mileu and are considered “sentinels” of innate immunity. Following TLR recognition of foreign material, DCs become potent antigen-presenting cells that migrate to the lymph node (LN) to present antigens to T cells, providing a direct link between innate and adaptive immune systems. Furthermore, macrophages and DCs can rapidly secrete cytokines, chemokines and type I IFNs to coordinate the other aspects of the immune system. Therefore, the study of macrophage and DC effector functions and their regulation provides a better understanding of how

these cells ensure appropriate immunity against the innumerable daily pathogenic insults that we encounter.

Macrophages and Dendritic Cells: Developmental Origins and Function

In 1908, Elie Metchnikoff was awarded the Nobel Prize for his pioneering work in classifying phagocytes in starfish, being the first to identify macrophages and microphages (neutrophils) and correctly predicting that phagocytosis is central to innate immunity by stating, “that the intracellular uptake and digestion of foodstuff also performs a protective role against injurious agents which are either formed by the organism itself or enter the organism from the outside” (1). After subsequent studies by Mackaness and colleagues laid the groundwork for the concept of non-specific “macrophage activation” (2), the heterogenous network of resident macrophages, recruited macrophages, neutrophils and their precursors was compartmentalized into the mononuclear phagocyte system (MPS) (3), which would later include antigen-presenting dendritic cells (DCs) after their discovery by Ralph Steinman and Zanvil Cohn (4, 5). However, despite their shared classification within the MPS, macrophages and DCs differ in numerous ways, with each cell type possessing unique developmental requirements and functional capacities.

Macrophages are found in nearly all mammalian tissues and diverse macrophage populations have been extensively characterized in lymphoid compartments and in non-lymphoid organs such as the liver, lung, gut, skin and kidney (6). Macrophage development occurs in a number of steps. Monocytes develop from precursors in the bone marrow in a Csf-1/M-CSF-dependent manner (7). In mice, circulating blood monocytes can be differentiated based on cell surface marker expression. “Inflammatory”

monocytes ($CX_3CR1^{low}CCR2^+Ly6C^+$) rapidly emigrate to peripheral tissues in response to inflammatory stimuli and can differentiate into splenic red pulp macrophages and alveolar macrophages (6, 8, 9), whereas “resident” monocytes ($CX_3CR1^{hi}CCR2^-Ly6C^{low}$) persist within the bloodstream and are thought to replenish macrophages within the lung and lamina propria under homeostatic conditions (8). Demand for increased tissue macrophage numbers in the steady-state and during injury can also be met by local proliferation of macrophages in situ, and macrophage self-renewal has been reported to occur in response to helminth infection (10).

Macrophages can exist in two active functional states. “Classically” activated macrophages, which are derived in the presence of $IFN\gamma$ and LPS, provide microbicidal reactive oxygen and nitrogen species and secrete inflammatory cytokines, thus associating them with T_H1 -mediated cellular immunity (11). “Alternatively” activated macrophages, in contrast, develop in response to IL-4 and IL-13 signaling, and are important for T_H2 -driven anti-parasitic immunity as well as wound healing (11). Macrophage functions also vary depending on their tissue localization. For example, osteoclasts within the bone marrow promote bone resorption (12), whereas $CD169^+$ macrophages within the lymph node (LN) subcapsular sinus are critical for clearing virally-infected cells from the lymph and in preventing vesicular stomatitis virus (VSV) entry into the central nervous system (13). Despite these variances in function, macrophages are defined by their prodigious ability to phagocytose, and in homeostatic conditions macrophages are the primary cell type responsible for removing cellular debris from apoptotic cells as well as “recycling” an estimated 2×10^{11} erythrocytes per day (11).

While all myeloid cells develop from the common myeloid progenitor (CMP) in the bone marrow, conventional DCs (cDC) in the spleen and non-lymphoid tissues as well as plasmacytoid DCs (pDC) share a proximal progenitor that is known as the common DC precursor (CDP), which lacks monocyte development potential (14, 15). Unlike the development of macrophages which requires Csf-1/M-CSF, DC generation requires Flt3 signaling (15) which also homeostatically maintains DC numbers in lymphoid and non-lymphoid tissues (6). Under inflammatory conditions, circulating monocytes can develop into TNF and iNOS-producing DCs (Tip DC) which are crucial for inhibiting *Listeria* infection in the spleen (16-18), or into antigen-bearing LN DC (19).

The heterogenous nature of DCs is best exemplified by the functional studies of tissue-resident DC subsets in lymphoid tissues. Splenic conventional DCs (cDC) are defined by high surface expression of MHC II and the β_2 integrin CD11c, but can be differentiated based on CD8 α expression. CD8 α^+ and CD8 α^- cDCs are two distinct subsets with unique functional properties. Murine CD8 α^+ cDCs, which are minor population normally found in splenic T cell zones (20), excel at producing IL-12 and cross-presenting antigens on MHC I to CD8 $^+$ T cells to establish an effector CTL response (21, 22). In contrast, CD8 α^- cDCs are potent antigen presenting cells (APCs) that present exogenous antigens on MHC II to stimulate cognate CD4 $^+$ T cells responses (21, 22). Contrasting these populations, CD11c int PDCA-1 $^+$ pDCs are phenotypically distinct from cDCs and, upon microbial nucleic acid stimulation, are able to synthesize large amounts of Type I IFNs (23). Of note, tissue-resident splenic DCs differ from classic “migratory” DCs (dermal DCs, Langerhan cells) that are activated in the periphery and home to the LN to deliver antigens to T cells (23).

Activation of the Innate Immune System by Pattern Recognition Receptors

Innate immune cells must discriminate between self and non-self to effectively provide the first line of defense against invading pathogens. This is accomplished through germline-encoded PRRs that recognize broadly expressed motifs found on foreign antigens known as PAMPs. PRR activation initiates a strong activation signal in myeloid cells leading to synthesis of antimicrobial proteins and enhancement of antigen presentation, thereby activating adaptive immunity. Because there is overlap in PRR specificity, PRRs work collectively to generate effective innate immunity against a wide range of infectious material. PRRs include RIG-I-like receptors (RIG-I, MDA5, LGP2) that bind to cytoplasmic RNA, NOD-like receptors (NALP1/NLRP1, NALP3/NLRP3) that induce inflammasome formation and IL-1 β release, dsDNA receptors (DAI, IFI16, AIM2) and C-type lectin receptors that are used to generate anti-fungal responses (24). In addition to these PRRs, anti-microbial functions are induced by a large family of TLRs, which are widely distributed among multiple immune cell types, including monocytes/macrophages, conventional DCs and plasmacytoid DCs, B cells, and mast cells, and are also expressed on renal and intestinal epithelium (25). TLR ligand specificity, function and regulation are discussed below.

Toll-like receptors

TLRs in Microbial Recognition

TLRs were first described in drosophila with the identification of Toll, a receptor that controls embryonic development in the fruitfly (26). Toll influences dorsal-ventral polarity by transducing an IL-1-like signal, which led to the hypothesis that homologous receptors could regulate immune cell activation in mammals. The mammalian

homologues of Toll, the TLRs, have been studied extensively since their discovery by Medzhitov and Janeway in 1997 (27). Structurally, TLRs consist of an extracellular leucine-rich repeat domain (LRR) responsible for binding PAMPs and an intracellular TIR domain that initiates and propagates a cell activation signal cascade (25, 28). To date, 10 mouse and 13 human TLRs have been identified which collectively recognize a large variety of microbial structures. Stimulation of TLRs links pathogen recognition to an immune response that includes synthesis of antimicrobial proteins, release of reactive oxygen and nitrogen species, and cytokine production (25).

Bacterial and Fungal PAMP Recognition by TLRs

TLR4 is responsible for detecting the major Gram-negative bacterial outer membrane component lipopolysaccharide (LPS) (29). LPS recognition depends on cooperation between TLR4 and two other proteins, MD-2 and CD14 (25), with GPI-linked CD14 delivering LPS to the TLR4:MD-2 signaling complex (30, 31). TLR4 can also bind to other ligands, including Taxol, viral proteins, stress-induced heat shocks proteins, and components of the extracellular matrix such as fibrinogen and fragments of hyaluronic acid and heparan sulfate (25). TLR2 recognizes an assortment of PAMPs that include lipoteichoic acid and lipoproteins from Gram-positive bacteria (25,32), zymosan from fungal particles, GPIs from *Toxoplasma gondii* (33) and GPI anchors from *Trypanosoma cruzi* (34) and “atypical” LPS from *Leptospira interrogans* and *Porphyromonas gingivalis* (25). TLR2 can complex with either TLR1 or TLR6, with each heterodimer bestowing unique binding properties. For instance, TLR2/1 binds to Gram-negative triacylated lipopeptides and TLR2/6 recognizes diacylated lipopeptides from Gram-positive bacteria and is the dominant heterodimer for zymosan binding (24,

25, 35). TLR5 recognizes flagellin, the principal component of bacterial flagella (36).

Lastly, unknown bacterial structures from uropathogenic bacteria and *Toxoplasma gondii* Profilin are bound by mouse TLR11 (24).

TLR-based Recognition of Nucleic Acid PAMPs

TLRs 3, 7, 8 and 9 recognize nucleic acid that results from viral and bacterial infection. TLR3 specificity encompasses dsRNA that can be found genomically in dsRNA viruses (reoviruses) and dsRNA that derives from replication of ssRNA viruses (influenza A virus) or dsDNA viruses (MCMV, HSV-1), as well as the synthetic dsRNA poly(I:C) (24). TLR7 and TLR8 recognize ssRNA derived from HIV or influenza, especially those ssRNAs high in guanosine and uridine, as well as synthetic imidazoquinolines. TLR9 is structurally similar to TLR7/8 and is essential for binding to unmethylated CpG DNA motifs. Recognition of CpG DNA by TLR9 has been described for HSV-1, HSV-2, MCMV and other pathogens such as *Leishmania major* and *Listeria monocytogenes* (25, 37-39). The ligand specificity of these TLRs can lead to unintentional recognition of host DNA and RNA which may promote autoimmunity (40). However, this danger is minimized in normal conditions by effective compartmentalization of TLRs 3, 7, 8 and 9 into intracellular vesicles, sequestering them away from host nucleic acids.

TLR Compartmentalization and Signaling

In addition to ligand specificity, TLR cell location an important factor in determining the utility of individual TLRs against specific infections, and a review of TLR signaling must be understood in the context of where the activation signal initiates. The most prudent illustration of this is TLR4, which is unique among the TLR family in

that it is the only receptor known to signal from the cell surface as well as from endosomes, leading to different kinetics of pathway activation. TLR4 signaling requires four proximal adapters, MyD88, TRIF, TIRAP and TRAM, that define the major TLR signal axes: “MyD88-dependent” and “TRIF-dependent” (41). TLR4’s use of these adapters differs depending on where it encounters its ligand. When located on the cell surface, TLR4 activation is MyD88-dependent and involves both MyD88 and TIRAP recruitment, the latter of which is responsible shuttling MyD88 to the cytoplasmic portion of TLR4 (25, 41). Further protein activation then culminates in a TRAF6-TAK1 complex, an important junction leading to early phase NF- κ B and MAPK/AP-1 pathway activation and inflammatory cytokine production (25, 41). TLR4 is then internalized in a CD14-dependent manner (30) into early endosomes or Rab11a+, PAMP-containing phagosomes (42), where it associates with the proximal adapters TRAM and TRIF, leading to TRIF-dependent signaling (39, 41). This cascade activates TRAF6-TAK1 with delayed kinetics when compared to MyD88-dependent activation and leads to late-phase NF- κ B and MAPK activity (41). Distinct from MyD88 signals, however, the TLR4 TRIF-induced cascade culminates in IRF3 dimerization to drive IFN β production, demonstrating that TLR4 activates different signal effectors depending on its subcellular localization (41).

All other TLRs with the exception of TLR3 signal through a MyD88-dependent process to lead to inflammatory cytokine synthesis. TLR5 and TLR2/1/6 are activated on the cell surface, though TLR2 may also be internalized into endosomes to induce type I IFN production through TRIF-dependent signaling in response to virus infection (24). In contrast, TLRs 3, 7, 9 are located in and signal exclusively from acidified endosomes (43). TLR7 and TLR9 both activate MyD88-dependent processes that lead to inflammatory

cytokines in macrophages, conventional DCs and pDCs (39). In pDCs, however, an additional MyD88-based pathway exists in downstream of TLR7 and TLR9 that leads to large quantities of type I IFNs (24). Unlike TLRs 7 and 9, TLR3 stimulation in mature DCs invokes TRIF recruitment to the endosome, which in turn activates NF- κ B, MAPK and IRF3 pathways, similar to internalized TLR4 (39).

Positive TLR Regulation

Microbial entry, PAMP recognition and cytokine signaling are known to induce TLR expression. TLR2 upregulation has been noted after *Mycobacterium avium*, *E. coli* and *Haemophilus influenzae* infection (25). Infection with influenza A and Sendai virus also enhances TLR1, 2, 3 and 7 levels in an IFN α/β -dependent process (44). TLR2 expression in RAW264.7 macrophages is increased after treatment with IL-2, IL-15, IL-1 β , IFN- γ and TNF (45). Furthermore, modulation of TLR4 is driven by M-CSF and macrophage migration inhibitory factor (MIF) treatment (46, 47). Thus, during an ongoing immune response these mechanisms feed back and amplify TLR activation and microbial clearance.

Negative TLR Regulation

The potent inflammatory response activated after TLR ligation is critical for microbial clearance. However, if left unchecked, TLR stimulation can have pathological consequences and can lead to sepsis, atherosclerosis (48), asthma (49), rheumatoid arthritis and other autoimmune diseases (40, 50). To maintain the appropriate balance between inflammation and tissue homeostasis, mammalian cells have evolved many strategies to diminish TLR activity. Importantly, these negative regulators act at multiple steps during TLR activation and in many cases possess redundant functions in order to

best return the cell to quiescence (51). For instance, initial TLR stimulation is prevented by soluble decoy receptors. Soluble versions of TLR2 and TLR4 have been identified in humans and mice respectively, and both function in a competitive binding capacity to inhibit ligand binding (52, 53). After TLR stimulation occurs, multiple intracellular inhibitors act to diminish the TLR signaling network. Proteins such as MyD88s, IRAK-M, SARM and SOCS1 are induced after LPS stimulation that block the proximal steps of TLR4 signaling. IRAK phosphorylation is inhibited after synthesis of MyD88s, a splice variant of MyD88, and IRAK-M (51). MyD88s complexes with MyD88 to create a heterodimer that is unable to signal (54), while IRAK-M's mechanism of activity is currently being elucidated and may involve restricting IRAKs to the plasma membrane (55). SARM is a TIR-containing adapter that specifically targets TRIF-induced signals (28). SOCS-1, the first of an 8 member family of cytokine signaling inhibitor molecules (56), inhibits TLR2 and TLR4 signaling by degrading TIRAP and perturbing NF- κ B activation (57). Other intracellular modulators are constitutively expressed, in particular, the TRAF6/NF- κ B inhibitor proteins A20 and ABIN-3 (51, 58) and TOLLIP, another inhibitor of IRAK activation (59).

MicroRNAs (miRNA) have recently been appreciated to be vital regulators of TLR responses. miRNA are small, non-coding RNAs that bind to the 3' UTR region of target mRNAs to inhibit their transcription (60). Each miRNA has multiple targets (60), however, in the context of TLR signaling certain miRNAs have emerged as important posttranscriptional regulators. miR-146a, for example, targets IRAK1 and TRAF6 mRNA and is known to be an effective negative regulator downstream of TLRs 2, 4, 5, 7 and 9

(61, 62). This is in contrast to positive regulators such as miR-155, which promote TLR stimulation by targeting the inhibitors SHIP1 and SOCS1 (63-65).

Cross-regulation by other signaling pathways has also been implicated in TLR inhibition. The PI3Ks are serine/threonine kinases responsible for the conversion of PIP2 to PIP3, ultimately leading to activation of Akt (66). A role for PI3K in TLR regulation was demonstrated by Fukao *et. al.* who demonstrated that PI3K-deficient DCs produce more IL-12 p70 in response to TLR2, TLR4 and TLR9 stimulation (66). Details of the PI3K regulatory system were recently clarified with the finding that B-cell adapter for PI3K (BCAP) is the protein responsible for connecting TLR stimulation to PI3K function (67, 68). BCAP-deficient macrophages are hypersensitive to TLR stimulation due to the absence of BCAP:PI3K p85 subunit binding at the cell membrane, which is critical for PI3K activity (67, 68). As will be outlined in the next section, signaling through immunoreceptor tyrosine-based activation motif (ITAM)-containing adapters represents yet another critical method by which TLRs are inhibited. Clearly, the presence of multiple, redundant inhibitory proteins and networks highlights the importance of TLR inhibition in protecting the host against the deleterious effects of unrestrained inflammation.

ITAM-based Signals Control TLR Responses

The ITAM Pathway and Cell Activation

The ITAM pathway is considered the prototypical cell activation cascade, the details of which were first described for TCR and BCR signaling in lymphocytes (69, 70). Many receptor systems are multicomponent, consisting of separate ligand binding and signal transducing subunits. In canonical ITAM signaling, receptor crosslinking results in

phosphorylation of the tyrosines within the conserved ITAM sequence, D/ExxYxxL/I(x₆₋₈)YxxL/I (71), which is facilitated by Src family kinases (Hck, Fgr, Src, Lyn in myeloid cells; Lck and Fyn for T cells (69, 72)). These phosphorylated tyrosines then serve as recruitment sites for Syk family kinases (Syk in myeloid cells, B cells and NK cells; ZAP-70 for T cells and NK cells (73)) which, in turn, initiate a series of phosphorylation events after binding via their SH2 domains. Importantly, this proceeds to the formation of a nucleated complex that centers around the scaffolding proteins SLP-76 or BLNK and the terminal activation of many cell activation pathways, including NF- κ B, MAPK and Ca²⁺-dependent transcription factors such as NFAT (73, 74).

Within this framework is the notion that ITAM-based activation is inhibited by an opposing system centered around the immunoreceptor tyrosine-based inhibition motif (ITIM). ITIM-bearing receptors, including some NK cell KIRs and Fc γ RIIb (75) are able to limit activation by recruiting protein tyrosine phosphatases (PTP) that dephosphorylate the ITAM-containing adapters or other kinases to block the signal (76). Receptors that signal through the ITIM sequence recognize ligands found on the cell surface or in large complexes that also ligate ITAM-bearing receptors (76). In doing so, there is coaggregation of ITAM and ITIM components such that dephosphorylation of the activating components can physically occur, thus resulting in inhibition. This is best illustrated by antibody complex binding to the BCR and inhibitory Fc γ RIIb, demonstrating a functional coupling between ITAM and ITIM signals (76).

Inhibitory ITAM Signaling Through the Myeloid Cell Adapters DAP12 and Fc γ R

Despite their role in facilitating cell activation, signals through ITAMs have an well appreciated ability to also transduce inhibitory signals. DAP12 and Fc γ R are the

only ITAM-containing adapters in macrophages and DCs (74). Both proteins can associate with many receptors and have been described to transduce cell activation signals through NK cell KIR proteins (77) and induce mast cell activation downstream of Fc γ R or Fc ϵ RI ligation (74, 78, 79). There is now considerable evidence that DAP12 and Fc γ R signals can be inhibitory in the context of TLR signaling. An analysis of DAP12-deficient macrophages by Hamerman *et. al.* demonstrated the importance of this adapter in downregulating TLR responses (80). DAP12 KO macrophages displayed a hypersensitivity to TLR stimulation and produced more inflammatory cytokines in response to a panel of TLR agonists and DAP12-deficient mice were more susceptible to endotoxic shock and had increased responses to *Listeria monocytogenes* and *Mycobacterium tuberculosis* (74, 80). DAP12/Fc γ R doubly-deficient DCs were also reported to be hyperactive to TLR stimulation, creating more cytokines and demonstrating an elevated capacity to induce T cell proliferation over WT control cells. (REFERENCE) Together, these results clearly show that DAP12 and Fc γ R signals block TLR stimulation in myeloid cells.

The precise mechanism by which DAP12 and Fc γ R accomplishes this inhibitory activity is still being defined. Hamerman *et. al.* found a requirement for the tyrosines in DAP12 and Fc γ R ITAM residues and noted differences in ERK and Akt activation, demonstrating that ITAM signaling can block TLR signaling directly and may influence other inhibitory pathways such as PI3K (74, 80). A recent study by Pfirsch-Maisonnas and colleagues used Fc α RI-mediated mast cell inhibition, which occurs through Fc γ R (81), to identify a unique intracellular “inhibisome” structure that physically sequesters

activating receptors and SHP-1 phosphatase, resulting in dephosphorylation of the activating receptor and downregulation of the signal network (82).

Of the many proteins known to signal via DAP12 and FcR γ , a handful have been found to deliver an inhibitory signal through these adapters, including TREM-2 in macrophages and DCs (83-85) and ILT7, BDCA-2 and Siglec-H in pDCs (74). Among the other DAP12/FcR γ -utilizing receptors that have not been tested for TLR inhibitory potential, the β_2 integrin family of adhesion proteins are particularly appealing. β_2 integrins are found on all hematopoietic cells and have been implicated in both positive and negative control of inflammation. Importantly, β_2 integrin-dependent processes requires signals through DAP12 and FcR γ in neutrophils and macrophages (86), which may indicate a role for β_2 integrins in inhibiting TLR responses as well. Based on these observations, we hypothesize that TLR activation in macrophages and DCs is limited by β_2 integrin activity.

β_2 integrins

β_2 Integrin Signaling and Function

During an inflammatory event, leukocytes must exit the circulation and home towards the inflamed tissue to mediate the appropriate immune response. Of the numerous proteins that are involved in this process, the β_2 integrins are the principal cell adhesion receptors that control cell trafficking and migration (87). Specifically, β_2 integrins are required for the early step of leukocyte firm adhesion to the activated vasculature and β_2 integrin signals enable transmigration of these recruited leukocytes through the endothelium and into the infected tissues (87, 88). β_2 integrins also play a

central role in other cell-to-cell contacts, including T cell:APC interactions via their ability to sustain the immunological synapse to facilitate T cell receptor (TCR) signal amplification (69).

Structurally, the β_2 integrins are heterodimers composed of an alpha subunit that is a member of the CD11 family, which is complexed to a shared, common β_2 chain, also known as CD18. The four β_2 integrins LFA-1(CD11a:CD18), Mac-1 (CD11b:CD18; CR3), CR4 (CD11c:CD18; $\alpha_x\beta_2$; p150p95) and CD11d:CD18 ($\alpha_D\beta_2$) exhibit differential expression across immune cells and, fittingly, the variation in their subunit composition directs ligand specificity (89). LFA-1, which is expressed on virtually all leukocytes, has a strong affinity for intracellular adhesion molecules (ICAMs)-1, -2, -3 and -5. Mac-1 and CR4 are commonly used markers to define monocytes, macrophages and DCs and both recognize a diverse set of ligands that include extracellular matrix components (fibrinogen, fibronectin, heparin etc.) and activated complement proteins such as iC3b. $\alpha_D\beta_2$ possesses similar recognition specificity as Mac-1 and CR4, however, its expression is restricted to certain tissue macrophage and neutrophil populations, including some splenic red pulp macrophages, and macrophages in the lung, kidney and atherosclerotic plaques (89-91).

Each β_2 integrin may act alone or in concert with other members of the family. For example, individual integrins control defined roles in the “cell adhesion cascade” (87). Activated LFA-1, which possesses a high affinity for ICAM-1, predominates during rolling and firm adhesion onto the endothelium, whereas Mac-1 binding is dispensable (92) for these initial processes. Yet, during extravasation, both LFA-1 and Mac-1 signals cooperate to direct leukocyte migration through the vasculature (93, 94). Once in the

tissues, surface expression of Mac-1 and $\alpha_D\beta_2$ increases and has been proposed to act as a “brake” in cell trafficking via binding to the ECM, thereby modulating the extent to which recruited leukocytes travel within an inflamed tissue (95). The role of individual β_2 integrins in cell trafficking is also tissue-dependent. During neutrophil emigration into the peritoneal cavity LFA-1 signals are dominant, whereas Mac-1 activation is dispensable (92). β_2 integrins also differentially control other cellular processes as well. Targeted deletion of CD11b blocks Mac-1 surface expression without influencing the levels of other integrins (92, 96). CD11b-deficient neutrophils are unable to phagocytose complement-opsonized particles, suggesting that the remaining β_2 integrins cannot compensate for Mac-1 deficiency in this context (96). Clearly, β_2 integrin function is dictated by differences in ligand binding specificity and the availability and conformational state of the ligand in the tissue microenvironment.

β_2 integrins signal bidirectionally in networks that are termed “inside-out” and “outside-in” (89, 97-99). All β_2 integrins exist in “bent”, inactive conformations on circulating leukocytes in the steady state. Chemokine receptor, TLR4 or TCR/BCR stimulatory signals culminate in Rap1 GTPase activation and the movement of adapters such as Talin and RapL to the cytoplasmic tails of the α and β integrin chains (89, 100). This inside-out signal leads to extension of the subunit ectodomains into high-affinity conformations (89). Inside-out signaling also promotes β_2 integrin clustering within lipid rafts, thereby increasing integrin avidity (98).

Ligand binding to the high-affinity, clustered β_2 integrin promotes a distinct signal cascade defined as “outside-in”. Outside-in signaling is responsible for a number of functional responses in myeloid cells and neutrophils, including cell adherence, cell

spreading, degranulation, actin cytoskeletal rearrangements, ROS production, cytokine mRNA stabilization and differentiation (98). Many experiments over the last decade have identified key molecular players involved in potentiating this response, and interestingly, they include the same proteins found in classical ITAM-based signaling, including the cytoplasmic tyrosine kinases (Src family and Syk), DAP12 and FcR γ and downstream effectors including SLP-76 and FAK/Pyk2 (72, 86, 98, 101, 102). Importantly, a deficiency in any of these proteins disturbs β_2 integrin outside-in activation. For instance, ablation of the Src family kinases (SFKs) Hck, Fgr, and Lyn blocks macrophage firm adhesion and cell spreading and neutrophil NADPH oxidase activity (103-105). These functional defects are also phenocopied in DAP12/FcR γ -deficient and Syk-deficient cells (86, 101), though the importance of Syk in β_2 integrin-mediated neutrophil migration differs depending on their tissue localization (106, 107). Interestingly, the short cytoplasmic tail of all β_2 integrins lacks SH2 or SH3 domains and the charged transmembrane residues that are normally associated with the ability to bind SFKs/Syk and DAP12/FcR γ , respectively. Despite this curious observation, the current model of β_2 integrin outside-in signaling resembles that of classical ITAM-based signal transduction. The proximal activation step appears to be activation of SFKs, followed by DAP12/FcR γ phosphorylation and Syk recruitment to the ITAM-containing adapters. Downstream activation then occurs in many other effectors. These include the scaffold protein SLP-76, the Rho GTPase Vav, PLC γ , PKC and PI3K, which together yield MAPK activation, release of Ca²⁺ from intracellular stores and actin polymerization, thus enabling the many functions induced by β_2 integrin ligation.

β_2 Integrins and Leukocyte Adhesion Deficiencies

The ability of β_2 integrins to mediate leukocyte adhesion and migration is central to generating an effective immune response, and the importance of this is evident in patients who suffer from defects in β_2 integrin function, a disease known as Leukocyte Adhesion Deficiency type I (LAD-I). First described in 1979, LAD-I patients suffer from recurrent bacterial infection, impaired wound healing, abnormal neutrophil adhesion and mobility, and defects in neutrophil respiratory burst (108). Experiments conducted by Springer and colleagues identified a deficit in resting and activated levels of all β_2 integrins (109), which was later confirmed to be due to heterogeneous defects in the common β_2 subunit, CD18 (110). LAD-I patients are clinically categorized based on the relative levels of β_2 integrin surface levels. “Severe” LAD-I (<1% of normal expression) can be lethal in early childhood, with patients suffering from potent and frequent bacterial infections. “Moderate” LAD-I (2-10% of normal expression) causes fewer infections and patients may survive into adulthood with appropriate clinical treatment (111). In both cases, LAD-I patients present with a prominent neutrophilia in the circulation owing to a failure of granulocyte emigration, underscoring the importance of β_2 integrin-mediated cell migration in initiating an appropriate inflammatory response.

 β_2 Integrin Mutant Mice: Models of LAD-I and Human Psoriasis

Two strains of loss-of-function pan- β_2 integrin mutant mice have been generated, each obtained through manipulation of the common β subunit, CD18. *Itgb2*^{hypo/hypo} mice bear a hypomorphic variant of CD18 and display a mild myeloid hyperplasia reminiscent of human LAD-I (112). *Itgb2*^{hypo/hypo} cells retain only 2-16% of normal WT β_2 integrin expression and neutrophil emigration into the peritoneal cavity is hampered in these

animals as a result (112). Of particular interest, *Itgb2^{hypo/hypo}* mice on the PL/J background present with skin lesions resembling human psoriasis (113-115), and importantly, analysis of the inflamed skin revealed a profound increase in TNF-producing F4/80+ macrophages (113). Macrophage depletion (113) or specific inhibition of NF- κ B within macrophages (116) resolves the psoriaform lesions, suggesting a clear role for β_2 integrins in dampening inflammatory responses in myeloid cells to prevent autoimmune pathologies. It should be noted, however, that these findings are complicated by a number of factors, including residual β_2 integrin signaling and defects in homing to the skin by T cells, which are classically considered to be the initiating cell type in psoriasis (114, 117, 118).

In contrast to CD18 hypomorphic mutant mice, *Itgb2^{-/-}* animals lack all surface β_2 integrin expression. Originally created on a mixed 129/Sv and C57BL/6 genetic background, these β_2 integrin-null mice have a phenotype closely resembling that found in human patients with LAD-I, including neutrophilia, myeloid hyperplasia-driven splenomegaly, peripheral lymphadenopathy, chronic dermatitis and perinatal lethality (119). *Itgb2^{-/-}* animals suffer from spontaneous mucocutaneous bacterial infections, unlike CD18 hypomorphs, and have impaired neutrophil emigration towards sites of infection (119). Breeding *Itgb2^{-/-}* mice onto a pure C57BL/6 genetic background suppresses some of these phenotypes, particularly eliminating neonatal lethality and recurrent bacterial infection (Yee and Hamerman, unpublished observations). In removing these complications, we believe that these animals are an ideal model for elucidating the functional role of β_2 integrins in modulating inflammatory responses in macrophages and DCs.

Direct Activation of Innate Immune Responses by β_2 Integrins

Beyond facilitating cell adhesion, we and others have hypothesized that β_2 integrins may have a direct role in cross-regulating myeloid cell TLR responses. Perhaps owing to the complexity of β_2 integrin signaling, however, the exact relationship between β_2 integrin ligation and cell activation remain unclear, and numerous studies have identified both positive and negative regulatory roles for β_2 integrins in this context. For instance, many studies position β_2 integrins as inducers of immune cell activation. In vitro experiments conducted by Medvedev and colleagues demonstrated that Mac-1 and CR4 (CD11c/CD18) activity was responsible for TNF release following group B streptococcus infection in CHO cells (120). These observations were reinforced by other findings showing that β_2 integrins positively controlled cytokine release in human and mouse monocytes/macrophages after infection with group A/B streptococci and *Escherichia coli* (Mac-1) or *Listeria monocytogenes* and *Staphylococcus aureus* (LFA-1 and CR4) (121, 122). The ability of β_2 integrins to potentiate a pro-inflammatory cell program has been closely studied for Mac-1, which has been reported to be a critical component of the LPS recognition complex that also includes TLR4 and CD14 (123). Specifically, CD11b/Mac-1-deficient macrophages (*Itgam*^{-/-}) are unable to synthesize IL-6 after LPS treatment due to their inability to localize the TLR signaling adapter TIRAP to the plasma membrane, demonstrating that Mac-1 directs TLR signaling (124). Supporting this, a mutation of the Mac-1 binding motif within fibrinogen renders mice susceptible to *S. aureus* infections by directly blocking antimicrobial function, though the cellular mechanism underlying this observation remains to be explored (125). Therefore,

multiple avenues of study demonstrate that β_2 integrins can promote inflammation in diverse settings.

β_2 integrins as Potential Inhibitors of Inflammation

The above findings are contrasted by many reports demonstrating that β_2 integrins dampen leukocyte activation and inflammation. Deletion of $\alpha_D\beta_2$, a β_2 integrin found on a subpopulation of splenic red pulp macrophages, provides a survival advantage in response to systemic *Plasmodium berghei* infection, while accelerating mortality from *Salmonella typhimurium*-induced sepsis, arguing that $\alpha_D\beta_2$ potentiated an inhibitory signal (91). In addition, activation of β_2 integrins through divalent cation treatment (89) reduces DC and macrophage antigen presentation, inhibiting these cells from driving T cell proliferation (126). Antibody blocking experiments by these authors demonstrated that Mac-1 was the principal β_2 integrin responsible for this effect (126), though CD11b- and CD11d-deficient splenocytes have been reported to retain WT levels of antigen presentation ability (127). A more physiological approach towards studying β_2 integrin function and antigen presentation was employed by Podgrabinska and colleagues, who analyzed human DC activation in the presence of activated endothelial cells expressing ICAM-1 (128). Plating of DCs onto monolayers of activated lymphatic endothelial cells (LECs) reduced CD86 expression in TNF-stimulated DCs and inhibited antigen presentation via a Mac-1- and CR4-dependent process (128). Strangely, antigen presentation inhibition was only observed for immature DCs, perhaps suggesting that β_2 :ICAM-1 contacts between DCs and LECs are important for maintaining T cell tolerance in the steady state, but may have a lesser role in suppressing DC activation during active infection (128).

A regulatory role for β_2 integrins has also been observed in a number of autoimmune disease models. Genetic deletion or antibody blockade of CD11b enhances dextran sodium sulfate (DSS) and trinitrobenzene sulfonic acid (TNBS)-induced experimental colitis by inhibiting macrophage and neutrophil infiltration into the colon (129, 130). CD11b deficiency also reduces oral-induced tolerance to foreign antigen by limiting IL-6-driven T_H17 generation (131), which may also be true for *Itgal*^{-/-} (CD11a KO) animals that present with high serum IL-17 levels (122). Additionally, skin exposure to UV is protective against dinitrofluorobenzene (DNFB) contact hypersensitivity via complement activation (132). This immunosuppression is largely induced by iC3b fragment generation, which in turn binds Mac-1, and *Itgam*^{-/-} (CD11b KO) mice display elevated DNFB-induced ear thickness even after UV treatment (132).

A strong association exists between CD11b/Mac-1 and control of Systemic Lupus Erythematosus (SLE) in humans. SLE is a chronic systemic autoimmune disease characterized by autoantibody production and tissue destruction within the kidney, brain and skin (133-135). SLE pathogenesis has a strong genetic basis, with susceptibility associated with immunoregulatory genes, including the HLA class II alleles DRB1*0301 and DRB1*1501 (136), and variants of *IRF5*, *STAT4* (137, 138), *TNFAIP3* (A20) (139) and *PTPN22* (140). Strikingly, three independent studies published in 2008 (133-135) followed by two additional studies in 2009 (141, 142) reported a strong association between *ITGAM*, the gene encoding CD11b, and SLE. By using genome-wide association studies (133, 134, 141, 142), a directed candidate gene approach at the 16p12.3-q12.2 chromosomal region (135), and direct sequencing (142), these studies identified nonsynonymous SNPs in *ITGAM* that are linked to SLE in patients of European and

European American (rs1143678 and rs11574637) (133-135), African American (rs1143678 and rs11574637) (135), Hispanic (141), and Chinese and Thai (rs1143683 and rs1143679) (142) ancestry. Intriguingly, SLE pathogenesis is also associated with aberrant TLR activation. In the current model, DNA- or RNA-containing immune complexes created from inefficiently cleared apoptotic cells activate TLR7 and TLR9 on pDCs and TLR9 on B cells to induce type I IFN, inflammatory cytokine, and autoantibody production, thus inducing autoimmunity (40). It is tempting to speculate that inhibitory CD11b signals can control TLR activation in healthy individuals, and that inhibitory potential is lost with the aforementioned *ITGAM* SNPs. However, whether these SNPs confer loss-of-function or gain-of-function mutations is currently unknown and remains an open area of study.

Recent work has identified a negative regulatory role for β_2 integrins in TLR activation itself (143-145). Wang and colleagues plated human monocytes onto fibrinogen-coated plates as a means to trigger high avidity β_2 integrin outside-in signals. In doing so, fibrinogen-stimulated cells diminished TLR and Type I IFN activation when compared to non-adherent cells (143). The mechanism of this activity stemmed from de novo synthesis of signaling inhibitors, including A20, ABIN-3, Hes1, SOCS3 and IL-10, which were all induced by fibrinogen in the absence of TLR activation. Importantly, fibrinogen-driven inhibitor expression was dampened in *DAP12*^{-/-}, *Syk*^{-/-}, and *Itgb2*^{hypo/hypo} mice, suggesting that the β_2 integrin signaling axis was responsible for TLR inhibition (143). Han *et. al.* has also implicated Mac-1 in negative control of TLRs (144). *Itgam*^{-/-} (CD11b KO) peritoneal macrophages produced more inflammatory cytokines in response to TLR3, TLR4 and TLR9 and *Itgam*^{-/-} mice were more susceptible to endotoxic shock

(144). In addition to finding a requirement for Syk in this process, these authors linked CD11b activation to phosphorylation of the E3 ubiquitin ligase Cbl-b (146), and demonstrated that Cbl-b could direct degradation of MyD88 and TRIF in HEK293 cells following LPS treatment (144). In a follow-up study Bai *et al.* reported that CD11b-deficiency also enhances TLR9-mediated CD8⁺ T-cell priming by DCs (145). In this setting, *Itgam*^{-/-} DCs secrete elevated levels of IL-12 p70 due to a defect in miR-146a synthesis. miR-146a is able to target and downregulate Notch-1, which itself is an inducer of IL-12 p70 expression (145, 147). Thus, in WT settings, these authors propose that Mac-1 induces miR-146a expression, which in turn limits Notch-1, leading to a block in IL-12 p70 production in DCs (145).

Unfortunately, there are fundamental difficulties in analyzing the data presented in these findings. In the case of Wang *et al.*, fibrinogen is a non-specific β_2 integrin agonist as it can also bind to TLR4, $\alpha_v\beta_3$ integrins, $\alpha_5\beta_1$ integrins, $\alpha_{11b}\beta_3$ integrins, and ICAM-1 (125, 148). Furthermore, the CD11b-Cbl-b-MyD88/TRIF signaling axis identified by Han *et al.* was elucidated in HEK293 cells, which are different biologically from the cells normally associated with TLR responses in vivo, including myeloid cells and B cells. Lastly, as Bai *et al.* state with regards to CD11b blocking DC cross-priming through miR-146a modulation, “because single miRNA might tune protein synthesis from thousands of genes by direct or indirect effects, there may be other molecules or signaling pathways that are targeted by miR-146a in CpG-ODN-stimulated DCs,” (145). Indeed, miR-146a targets other important molecules necessary for TLR signaling and cell activation, including IRAK1/2 and TRAF6 (62), though changes in these proteins were not explored. This dissertation not only aims to demonstrate unequivocally the ability of

β_2 integrins to block TLR responses, but also to define the biochemical and molecular changes that enable β_2 integrin signaling to disturb TLR activation. As such, we also address previously proposed mechanisms in pan- β_2 integrin-deficient macrophages in Chapter 3: Results.

Chapter 2: Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories. CD18-deficient (*Itgb2^{-/-}*) mice (119) were backcrossed 6 generations against C57BL/6 mice and were provided by Dr. Clifford Lowell (University of California, San Francisco). CD11a-deficient (*Itgal^{-/-}*) and CD11b-deficient (*Itgam^{-/-}*) animals were purchased from Jackson Laboratories (92, 94). Cbl-b-deficient (*Cblb^{-/-}*) mice were backcrossed 12 generations against C57BL/6 and were provided by Dr. Phil Greenberg (University of Washington) (149). All animals were housed in specific-pathogen-free facilities and all experiments were performed in accordance to protocols approved by the Institutional Animal Care and Use Committee at the Benaroya Research Institute.

Cell culture

Bone marrow cells were flushed from femurs and tibias, followed by erythrocyte lysis in ACK buffer (Lonza). For macrophages, bone marrow cells were plated onto a 10 cm petri dish (Fisher Scientific) using 10 ml of bone marrow macrophage growth medium, which consisted of DMEM medium supplemented with 10% FBS (Sigma), 2mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Lonza), Penicillin/Streptomycin (Gibco) and 10% CMG14-12 cell conditioned media as a source of CSF-1 (150). Bone marrow-derived DCs were grown in DC medium, which consisted of RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 10 mM HEPES, Penicillin/Streptomycin and 10 ng/ml GM-CSF (Peprotech). For both macrophages and DCs, an additional 10 ml of growth medium was added after 3 days of

culture. Day 6 DCs were isolated from culture by magnetic bead enrichment for MHCII⁺ cells. Cells were treated with anti-FcγRII/III (2.4G2) followed by staining with anti-MHC II-biotin (M5/114.15.2/eBioscience), anti-biotin microbeads (Miltenyi biotech) and sorting with MACS columns according to the manufacturer's instructions. The purity of CD11c⁺ cells was >90% in WT cultures. Bone marrow-derived macrophages and DCs were used at day 6 of culture.

Peritoneal macrophage isolation

Mice were injected i.p. with 3% thioglycollate broth and peritoneal cells were isolated by lavage with Cell Dissociation Buffer (Invitrogen) 5 days after injection. Macrophages were purified by magnetic bead enrichment using anti-F4/80-biotin (BM8/eBioscience) followed by incubation with anti-biotin microbeads and then sorted by MACS according to the manufacturer's instructions. F4/80⁺ macrophages were cultured in DMEM medium supplemented with 10% FBS (Sigma).

TLR stimulation and ELISA

Macrophages or DCs were plated onto 96 well tissue culture-treated plates at 5×10^4 cells/well and allowed to rest for 16 hours prior to stimulation. TLR agonists were added to triplicate wells at the indicated concentrations for up to 24 hours. Supernatants were collected and the amount of IL-12 p40, IL-12 p70, IL-6, TNF or IL-10 were assessed by ELISA (eBioscience). TLR agonists used were as follows: *S. Minnesota* R595 Ultra Pure LPS (List Biological Laboratories), CPG DNA (ODN1826, Invivogen), and zymosan particles (Sigma). CpG DNA and zymosan particles were incubated in 10 μg/ml polymixin B for 1 hour prior to use.

Flow cytometry and intracellular cytokine staining

Macrophages were stimulated with LPS for up to 8 hours in the presence of 10 $\mu\text{g/ml}$ Brefeldin A for the final 2 hours of stimulation. Macrophage FcRs were blocked with 2.4G2 for 10 minutes followed by surface staining with anti-F4/80 (BM8/eBioscience). Peritoneal cells were also surface stained with anti-Siglec F (E50-2440/BD Biosciences) and anti-Gr-1 (RB6-8C5/eBioscience). Macrophages were then fixed and permeabilized using BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences). Intracellular cytokine staining was performed using anti-IL-12 p40 (C15.6/Biolegend), anti-TNF (MP6-XT22/eBioscience), and anti-IL-6 (MP5-20F3/eBioscience). Flow cytometry analysis was conducted using an LSR2 (BD Bioscience) running FACSDiva software (BD Bioscience). All flow cytometry analyses were conducted using FlowJo software (Treestar).

LPS injection

Mice were injected with 1 $\mu\text{g/g}$ LPS i.p. in PBS and blood was collected at indicated time points. Serum concentrations of IL-12 p40, IL-6, TNF and IL-10 were determined by ELISA.

Gene expression analysis

Total RNA was isolated using the RNeasy Plus kit (QIAGEN) and reverse transcribed with Superscript III reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed in triplicate wells using the Power SYBR Green PCR master mix (Applied Biosystems) and reactions were run on a 7900HT Real-Time PCR System (Applied Biosystems). All data were normalized to GAPDH endogenous control. Oligonucleotide primers for GAPDH and A20 were previously described [20]. Primer

sequences were as follows: *Hes1*: 5'-tgccagctgatataatggaga-3' and 5'-ccatgataggctttgatgact-3'; *TNIP3* (ABIN-3): 5'-tccttgcttcccaggacat-3' and 5'-ttcttcttggtggagcacact-3'; *Irak3* (IRAK-M): 5'-tcgacagattacagtgcacaa-3' and 5'-ggctattcctatcaatacgt-3'; *Bcl2a1a* (Bfl-1): 5'-ttccagtttggcagaat-3' and 5'-tcaaactctttatgaagccatctt-3'; *Gadd45b*: 5'-ctgcctcctggtcacgaa-3' and 5'-ttgctctgctctcttccaca-3'; *Nos2* (iNOS): 5'-gggctgtcacggagatca-3' and 5'-ccatgatggtcacattctgc-3'; *Cxcl1*: 5'-agactccagccacactccaa-3' and 5'-tgacagcgcagctcattg-3'; *Cxcl2*: 5'-aaaatcatccaaaagataactgaacaa-3' and 5'-ctttggttctccggtgagg-3' and 5'-ctttggttctccggtgagg-3'; *Cxcl10*: 5'-gctgcegtcattttctgc-3' and 5'-tctcactggcccgtcatc-3'; *Il12b* (IL-12 p40): 5'-gattcagactccaggggaca-3' and 5'-tggttagcttctgaggacaca-3'; *Tnf*: 5'-gtcaggttgctctgtctca-3' and 5'-tcagggaaagagtctggaaag-3'; *Il6*: 5'-aggcataacgcactaggttt-3' and 5'-agctggagtcacagaaggag-3'; *Ifnb*: 5'-gcactgggtggaatgagactattg-3' and 5'-ttctgagcatcaactgacaggtc-3'

Chromatin Immunoprecipitation (ChIP)

Macrophages were stimulated with 1 ng/ml LPS for up to 8 hours and cells were washed, collected, and fixed by adding formaldehyde to a final concentration of 1% with shaking (37% HCHO in 10-15% methanol; Fisher). Crosslinking was stopped after 10 minutes by addition of glycine to a final concentration of 125 mM and incubated for 10 minutes. Macrophages were then washed 3 times with ice cold PBS and spun down, and pellets were flash frozen in a dry ice/ethanol bath and kept at -80°C until further analysis. To isolate nuclei, macrophages were first resuspended in Cell Lysis Buffer (10 mM HEPES pH 7.9, 0.5% IGEPAL-30, 1.5mM MgCl₂, 10 mM KCl) and kept on ice for 25 minutes, vortexing every 5 minutes. Nuclei were then centrifuged at 4°C and resuspended

in Nuclear Lysis Buffer (50 mM Tris pH 8.0, 10mM EDTA, 1% SDS), followed by sonication in a 4°C water bath to create fragments between 200-800bp in length. Sonicated samples were then precleared with Protein A Dynabeads (Invitrogen) for 30 minutes at 4°C and supernatants were collected by magnetic separation. The supernatants were then diluted 1:10 in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl) and incubated with 2 µg of anti-p65/RelA (Santa Cruz) or anti-c-Rel (Santa Cruz) overnight at 4°C. Immunocomplexes were then collected with Protein A Dynabeads and washed with Low Salt buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1), High Salt buffer (Same as low salt but with 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% Sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl) and 2 times with TE buffer. Complexes were extracted with Elution buffer (1% SDS, 0.1M NaHCO₃) and protein:DNA crosslinks were reversed by treating with RNase A and Proteinase K at 65°C. DNA was then purified (MoBio UltraClean PCR kit) and analyzed by qPCR. Normalization was accomplished by subtracting Ct values from precleared “input” chromatin. The primer sequences for the *Il12b* promoter are: 5'-ctttctgatggaaacccaaag-3' and 5'-ggggagggaggaacttctta-3'.

Western blot

Macrophages were stimulated with indicated concentrations of LPS for various times and lysed in lysis buffer containing 1% Triton X-100, protease inhibitors (mammalian protease inhibitor cocktail, Sigma) and 1mM sodium orthovanadate (Sigma). Lysates were separated by Tris-bis SDS-PAGE gels (Invitrogen) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). Rabbit antibodies specific for

I κ B α , phospho-p42/44 ERK, phospho-p38, SOCS-1 and β actin were from Cell Signaling. Rabbit anti-MyD88 was from Biovision. An HRP-conjugated donkey anti-rabbit IgG was used as a secondary (GE Healthcare). Antibody binding was detected by the Immobilon chemiluminescence system (Millipore). Densitometry analysis was conducted using ImageJ software (NIH).

Statistical analysis

Student's unpaired t-test was used to measure statistical significance between two groups and one-way ANOVA with Dunnet's multiple comparison test was used to determine statistical significance between multiple groups against WT control. All statistical analyses were performed by Prism 5 (Graphpad Software).

Chapter 3: β_2 Integrins Inhibit TLR Signaling by Reducing NF- κ B Pathway

Activation

Introduction

Innate immune cell activation depends on the activity of Toll-like receptors (TLRs) that bind conserved molecular features expressed on invading pathogens (151). Upon encountering microbes, macrophages and dendritic cells (DCs) respond to TLR stimulation by inducing antimicrobial and antiviral programs that result in the rapid synthesis and secretion of inflammatory cytokines and type I interferons. In turn, this potent response must be restrained to spare host tissues from the deleterious effects of exaggerated inflammation. This is accomplished by a variety of inhibitory mechanisms, including cytoplasmic effectors that block TLR signaling directly as well as secreted negative regulators, which work together to limit the severity of the immune response (51).

Although originally considered as an archetypal cell activation pathway, signals through immunoreceptor tyrosine-based activation motifs (ITAM) display functional heterogeneity and have been recently appreciated to cross-inhibit TLR responses (73, 152). ITAM signaling in myeloid cells is mediated by the ITAM-containing molecules DAP12 and FcR γ , which act as signaling adaptors for an extensive collection of cell surface receptors (74, 153, 154). Following ligand binding by paired receptors, ITAM signaling via DAP12 and FcR γ in myeloid cells proximally activates Src-family kinases and Syk kinase to enable downstream signals that are predominantly associated with cellular activation, including inducing NF- κ B and MAPK pathways, and prompting the release of intracellular Ca²⁺ stores (74). However, depending on the identity of the

associated receptor and other undefined parameters, ITAM-based signaling can also induce inhibitory responses. For example, triggering of the DAP12-coupled TREM-2 receptor can dampen TLR activation in macrophages (83). In addition, TREM-2 and/or DAP12-deficient macrophages and DCs produce more inflammatory cytokines in response to TLR stimulation (80, 84, 155, 156), demonstrating that these adaptor molecules can transduce signals attenuating TLR activation.

During an inflammatory response, leukocytes in the blood adhere to the activated vascular endothelium through the use of integrins. In particular, members of the β_2 integrin family facilitate leukocyte firm adhesion, thereby allowing for cell extravasation into the tissues (87). In doing so, β_2 integrins not only mediate cell migration, but also influence cell functions through signals transmitted through β_2 integrin activation. This “outside-in” signaling pathway requires ITAM signals from DAP12 and FcR γ , and also involves early effectors such as the Src family kinases and Syk in neutrophils and macrophages (86, 98). Because β_2 integrins signal through ITAM adaptors in myeloid cells, we hypothesized that β_2 integrin signaling may also inhibit TLR responses. There have been conflicting reports in the literature regarding the influence of β_2 integrin signaling on TLRs, with some studies demonstrating that β_2 integrins can promote TLR-induced inflammation (120, 123, 124), whereas others have reported negative roles for these integrins in TLR response (143, 144). Therefore, the nature in which β_2 integrins interface with TLR activation and cytokine secretion is complex and unclear.

To better define the contribution of β_2 integrins to regulation of TLR signaling, we have examined inflammatory responses in the absence of all β_2 integrins. Here, we demonstrate that deletion of all β_2 integrins rendered myeloid cells hypersensitive to TLR

stimulation in vitro and in vivo, showing an inhibitory role for β_2 integrins in TLR responses. Furthermore, we examined potential direct and indirect mechanisms by which β_2 integrins caused this inhibition, and found that β_2 integrins have a direct effect on $\text{I}\kappa\text{B}\alpha$ degradation that was pronounced in β_2 integrin-deficient cells through both early and late phases of TLR stimulation, thus implicating β_2 integrin signals in inhibiting NF- κB pathway activation.

Results

β_2 integrin-deficient macrophages hyper-respond to TLR stimulation.

The four β_2 integrins, LFA-1 ($\alpha_L\beta_2$), Mac-1 ($\alpha_M\beta_2$), CR4 ($\alpha_X\beta_2$) and CD11d-CD18 ($\alpha_D\beta_2$) are heterodimers that consist of distinct CD11 alpha subunits in association with the common beta chain, CD18 (β_2), which is encoded by the *Itgb2* gene (89). To examine whether β_2 integrin signaling regulates TLR responses, we compared the cytokine secretion profiles of bone marrow-derived macrophages from wild-type (WT) and *Itgb2*^{-/-} mice, which are deficient in CD18 and thus are unable to express any of the β_2 integrins on the cell surface (Figure 3.1A) (119). Despite the inability of *Itgb2*^{-/-} bone marrow-derived macrophages to express Mac-1, these cells exhibited surface F4/80 expression and upregulated MHC II in response to IFN γ treatment (Figure 3.1), demonstrating that they were *bona fide* macrophages. Furthermore, β_2 integrin-deficient macrophages presented with either similar or slightly lower levels of TLR2, TLR4 and TLR9 and the C-type lectin receptor Dectin-1 (Figure 3.2)

To determine how β_2 integrin signals influence TLR activity, we stimulated *Itgb2*^{-/-} bone marrow-derived macrophages with a panel of TLR agonists, including LPS

(TLR4), CpG B DNA (TLR9) and zymosan (TLR2). Zymosan is a complex yeast particle that, in addition to signaling through TLR2, also signals through Dectin-1. After 24 hours of activation, *Itgb2*^{-/-} bone marrow-derived macrophages secreted more IL-12 p40 and IL-6 than did WT control cells (Figure 3.3). In contrast to IL-12 p40 and IL-6, TNF secretion by *Itgb2*^{-/-} macrophages was more similar to that of WT cells, though some increases were observed (Figure 3.3).

We investigated the kinetics of inflammatory cytokine secretion after LPS treatment and found that the induction kinetics for IL-12 p40 and TNF release were similar between *Itgb2*^{-/-} and WT macrophages. Yet, 12 hours after stimulation, IL-12 p40 secretion was greatly enhanced in *Itgb2*^{-/-} macrophages compared to WT, while the levels of TNF were comparable between both populations of macrophages throughout the course of the experiment (Figure 3.4). To ascertain whether the increase in cytokine levels from *Itgb2*^{-/-} macrophages was due to β_2 integrins controlling cytokine secretion, the synthesis of IL-12 p40 and TNF was assessed by intracellular cytokine staining. We observed a larger population of IL-12 p40-producing macrophages in the absence of β_2 integrins, such that at 8 hours after stimulation the percentage of IL-12 p40-positive cells was approximately twice what was seen for WT macrophages, whereas there was little difference in TNF production (Figure 3.5). Therefore, β_2 integrin ablation results in increased TLR responses from bone marrow-derived macrophages, most strongly affecting IL-12 p40 and IL-6 production, with modest effects on TNF protein synthesis.

We also examined whether β_2 integrins inhibit TLR responses in thioglycollate-induced peritoneal macrophages to determine whether β_2 integrins inhibit TLRs in an inflammatory macrophage population. Because β_2 integrins contribute to cellular

infiltration into the peritoneal cavity (94, 157) and as *Itgb2*^{-/-} present with a profound neutrophilia (119), we were unable to procure a pure F4/80⁺Gr-1^{+/-neg} macrophage population, even after 5 days post-injection (p.i.) unlike in WT mice (Figure 3.6). Moreover, F4/80⁺ FACS-isolated *Itgb2*^{-/-} macrophages were unable to survive normal cell culture procedures (data not shown). To minimize complications that elevated neutrophil levels may have on macrophage responses, we isolated D5 p.i. peritoneal macrophages by magnetic bead enrichment (Figure 3.7) and assessed inflammatory cytokine production by intracellular cytokine staining. F4/80^{high} *Itgb2*^{-/-} peritoneal macrophages showed increased TLR4 responses over WT cells (Figure 3.8). *Itgb2*^{-/-} peritoneal macrophage IL-6 production showed the largest increase over WT cells with a smaller increase in IL-12 p40 production and the percentage of TNF-producing peritoneal macrophages was not affected by β_2 integrin deletion, mirroring what we observed in bone marrow-derived macrophages. Thus, these data demonstrate that, in addition to bone marrow-derived macrophages, β_2 integrins also negatively regulate TLR-induced IL-12 p40 and IL-6 production in inflammatory macrophage populations.

β_2 integrins are required for TLR inhibition in vivo.

To identify the contribution of β_2 integrins to inhibiting TLR responses in vivo, we injected WT and *Itgb2*^{-/-} mice with LPS i.p. and measured inflammatory cytokine levels in serum up to 4 hours after injection. The kinetics for TNF, IL-12 p40 and IL-6 induction were similar between WT and *Itgb2*^{-/-} mice, with the peak serum concentration of each cytokine occurring at the same time for both genotypes (Figure 3.9). However, differences in the magnitude of cytokine production were observed. Serum IL-12 p40 levels were dramatically increased in *Itgb2*^{-/-} mice such that by 4 hours post-injection,

Itgb2^{-/-} animals had approximately 3 times as much serum IL-12 p40 as WT controls. *Itgb2*^{-/-} mice also presented with elevated serum IL-6 and TNF in response to LPS injection, though these differences were not significant (Figure 3.9). These data show that *Itgb2*^{-/-} mice hyper-respond to LPS injection to produce more inflammatory cytokines, thus demonstrating that β_2 integrins inhibit TLR responses *in vivo*.

β_2 integrin-mediated TLR inhibition does not involve changes in IL-10 or intracellular TLR inhibitor expression.

TLR stimulation in macrophages results in secretion of the anti-inflammatory cytokine IL-10, which acts in an autocrine or paracrine manner to dampen TLR activation (158). Interestingly, culture of human macrophages on fibrinogen-coated plates induces IL-10 expression, as well as the expression of proteins such as A20, Hes-1 and ABIN-3, which are known to inhibit TLR signaling (143). Fibrinogen is a β_2 integrin ligand and plating of human macrophages onto fibrinogen-coated plates presumably induces a β_2 integrin signal, though other receptors may also be engaged (125, 159-161). To examine whether the TLR hypersensitivity of *Itgb2*^{-/-} macrophages was due to deficiencies in these inhibitors, we analyzed their expression and function after TLR stimulation.

Itgb2^{-/-} macrophages were not hampered in their ability to produce IL-10. *Itgb2*^{-/-} macrophages secreted similar amounts of IL-10 at higher LPS doses, but synthesized more IL-10 than WT macrophages at limiting doses of LPS and at all doses of CpG DNA tested (Figure 3.10A). These results were mirrored in *Itgb2*^{-/-} mice, which produced IL-10 at equivalent levels to WT controls when challenged with LPS i.p. (Figure 3.10B). Furthermore, *Itgb2*^{-/-} macrophages did not have defects in their response to IL-10. Treatment of macrophages with IL-10 prior to stimulation with 1.0 ng/ml LPS, a

concentration that yielded similar IL-10 secretion between WT and *Itgb2*^{-/-} cells (Figure 3.10A), reduced cytokine production in both populations of macrophages to a similar degree (Figure 3.11). These data indicate that neither defects in IL-10 production nor the response to IL-10 can explain *Itgb2*^{-/-} macrophage TLR hypersensitivity. Moreover, the increased TLR response of *Itgb2*^{-/-} macrophages is not due to deficiencies in ABIN-3, A20, Hes-1 or IRAK-M expression, as would be hypothesized by the data presented by Wang and colleagues (143). *Itgb2*^{-/-} macrophages did not have reduced amounts of these inhibitors before or after stimulation. Instead, *Itgb2*^{-/-} cells expressed more ABIN-3, A20 and Hes-1 after TLR stimulation than WT control cells (Figure 3.12), demonstrating that these intracellular TLR inhibitor pathways remain intact without β_2 integrin signals and arguing against a role for these inhibitors downstream of β_2 integrin activation.

The discrepancy between these data and that of Wang *et. al.* may be due to their use of fibrinogen, which has many receptors beyond β_2 integrins (125, 159-161). Since *Itgb2*^{-/-} macrophages are the ideal system to test β_2 integrin-dependent functions, we plated WT and *Itgb2*^{-/-} cells onto sheep and mouse fibrinogen-coated plates and assessed IL-12 p40 secretion following LPS stimulation. Fibrinogen plating was able to suppress IL-12 p40 production in both WT and *Itgb2*^{-/-} macrophages, showing that the inhibitory mechanism employed by fibrinogen is independent of β_2 integrin function (Figure 3.13).

CD11a, CD11b and Cbl-b are dispensable for TLR inhibition.

Itgb2^{-/-} bone marrow-derived DCs were also hypersensitive to TLR stimulation and secreted more inflammatory cytokines than WT control DCs (Figure 3.14). Because these results generally phenocopied our observations in *Itgb2*^{-/-} macrophages, we reasoned that a β_2 integrin shared between both cell types could inhibit TLR activation

such as LFA-1 (CD11a/CD18) or Mac-1 (CD11b/CD18) (89). *Itgal*^{-/-} (CD11a-deficient) and *Itgam*^{-/-} (CD11b-deficient) macrophages were examined to determine if either LFA-1 or Mac-1 were required to inhibit TLR signals. Neither *Itgal*^{-/-} nor *Itgam*^{-/-} bone marrow-derived macrophages demonstrated increased cytokine production over that of WT macrophages following TLR stimulation, suggesting neither LFA-1 nor Mac-1 alone inhibit TLR activation (Figure 3.15). CD11b-deficient thioglycollate-elicited peritoneal macrophages also did not show increased TLR responses and, in fact, *Itgam*^{-/-} peritoneal macrophages had reduced TLR responses as evidenced by a lower percentage of IL-12 p40-producing cells than seen for WT macrophages after 6 hours of TLR stimulation (Figure 2.15B). Similar to what we found in macrophages, *Itgal*^{-/-} and *Itgam*^{-/-} bone marrow-derived DCs had no increases in TLR-induced inflammatory cytokine production (data not shown). These data reveal that neither CD11a or CD11b act singly to diminish TLR activation.

Cbl-b is dispensable for β_2 integrin-mediated inhibition.

Signals through the β_2 integrin Mac-1 have been suggested to activate Cbl-b, an E3 ubiquitin ligase that can inhibit inflammatory responses in vivo (144). The proposed model suggests that CD11b signaling causes Cbl-b to ubiquitinate and degrade MyD88 and TRIF, thereby attenuating TLR responses. However, little is known about the ability of Cbl-b to regulate TLR responses specifically in macrophages. Therefore, we evaluated how Cbl-b deficiency influenced inflammatory cytokine production in these cells. *Cblb*^{-/-} bone marrow-derived macrophages and *Cblb*^{-/-} thioglycollate-elicited peritoneal macrophages were not hypersensitive to TLR stimulation and produced equal or lower amounts of inflammatory cytokines in response to LPS, CpG DNA and zymosan

treatment (Figure 3.16), indicating that Cbl-b is dispensable for limiting TLR activity in macrophages. The model proposed by Han *et. al.* would also predict that β_2 integrin-deficient macrophages would have less MyD88 degradation after TLR signaling (144). Stimulation with 10 ng/ml LPS led to similar MyD88 degradation in WT and *Itgb2*^{-/-} macrophages, suggesting that β_2 integrins do not inhibit TLR responses by inducing MyD88 turnover (Figure 3.17). Furthermore, we were unable to detect MyD88 degradation in WT or *Itgb2*^{-/-} macrophages treated with a lower dose of LPS (1 ng/mL), with which we observed elevated inflammatory cytokine production in β_2 integrin-deficient cells (data not shown). Interestingly, *Itgam*^{-/-} and *Cblb*^{-/-} macrophages also retained the ability to degrade MyD88 following LPS stimulation (Figure 3.17). These data reveal that a CD11b-Cbl-b inhibitory mechanism is not required for dampening TLR responses in macrophages.

β_2 integrin deficiency enhances NF- κ B activation in macrophages.

The increase in A20 and ABIN-3 expression in *Itgb2*^{-/-} macrophages (Figure 2.12) led us to theorize that β_2 integrins may dampen cytokine production by modulating the NF- κ B pathway directly. qPCR analysis revealed the LPS-induced levels of most of the NF- κ B-dependent genes examined (162) were elevated in *Itgb2*^{-/-} macrophages over WT controls, with significant differences found throughout the course of the stimulation. *Itgb2*^{-/-} macrophages responded to TLR4 stimulation by synthesizing more anti-apoptotic (Bfl-1, GADD45 β) and chemokine gene transcripts (CXCL1, CXCL2, CXCL10), as well as inflammatory cytokine mRNAs as expected (Figure 3.18), indicative of a global increase in NF- κ B activity without β_2 integrin-mediated inhibition. A curious exception

to this gene expression profile was that of iNOS, which directs the antimicrobial nitric oxide response, the synthesis of which was not higher in *Itgb2*^{-/-} than WT macrophages (Figure 3.18).

To assess a role for NF- κ B pathway activation downstream of β_2 integrin signaling, we used the IKK α/β chemical inhibitor BAY 11-7082 to inhibit NF- κ B activity prior to TLR stimulation. We reasoned that because β_2 integrin ablation intensifies NF- κ B activation, *Itgb2*^{-/-} macrophages would be more refractory to NF- κ B inhibition when compared to WT cells. While blocking NF- κ B activity reduced IL-12 p40 and IL-6 secretion in WT and *Itgb2*^{-/-} macrophages, WT macrophages were more sensitive to NF- κ B blockade than *Itgb2*^{-/-} macrophages (Figure 3.19A). BAY 11-7082 treatment blocked IL-12 p40 and IL-6 production in WT macrophages after LPS stimulation by 90% and 70% respectively, and *Itgb2*^{-/-} macrophages demonstrated a 40% reduction in cytokine levels (Figure 3.19B), thus strengthening the notion that *Itgb2*^{-/-} cells have elevated NF- κ B activation following TLR stimulation

We also sought to assess changes in the pathway itself. In canonical NF- κ B pathway activation, NF- κ B subunits are retained in the cytoplasm by I κ B α , which is phosphorylated and degraded after TLR stimulation, allowing NF- κ B proteins to enter the nucleus and enable transcription (163, 164). Thus, the amount and duration of I κ B α degradation can be used to gauge NF- κ B activity. For the early phase NF- κ B response, WT and *Itgb2*^{-/-} macrophages responded to TLR stimulation with similar initial kinetics for I κ B α degradation. I κ B α protein was decreased as early as 5 minutes after LPS stimulation, with the lowest amounts found at 15 minutes for both WT and *Itgb2*^{-/-} macrophages (Figure 3.20). I κ B α was then resynthesized in WT cells such that a stable

level was reached by 30 minutes. However, the resynthesis of I κ B α was delayed in *Itgb2*^{-/-} macrophages when compared to WT. By 60 minutes of LPS stimulation, the level of I κ B α in *Itgb2*^{-/-} macrophages was approximately 50% of WT, and I κ B α intensity in *Itgb2*^{-/-} macrophages did not reach WT levels until 120 minutes after stimulation (Figure 2.20) demonstrating that early NF- κ B pathway activation was increased in the absence of β_2 integrins. TLR stimulation also results in MAPK pathway activation and it is possible that β_2 integrins deliver an inhibitory signal by blocking phosphorylation of MAPKs. Notably, we did not observe enhanced levels of ERK1/2 or p38 phosphorylation after TLR stimulation in *Itgb2*^{-/-} macrophages, and instead noted decreases p38 activation at these early time points (Figure 3.20).

Differences in I κ B α levels were also observed during late phase NF- κ B activation. Both WT and *Itgb2*^{-/-} macrophages displayed reduced I κ B α levels by 2 hours after LPS (Figure 3.21A and 3.21B). However, in the absence of β_2 integrin signals, I κ B α degradation was more potent at this time point for *Itgb2*^{-/-} cells and these low I κ B α protein levels were maintained throughout the course of the experiment. In contrast, WT macrophages exhibited slower and weaker I κ B α diminishment that did not reach the low amounts seen in *Itgb2*^{-/-} cells until 8 hours (Figure 3.21A and 3.21B). I κ B α expression is also controlled by NF- κ B activation and resynthesis of I κ B α serves a crucial negative feedback role for the NF- κ B pathway (165). The prolonged reduction in I κ B α levels observed in *Itgb2*^{-/-} macrophages was not due to suppressed or defective I κ B α synthesis following TLR stimulation as I κ B α mRNA expression was elevated in *Itgb2*^{-/-} cells when compared to WT controls (Figure 3.21C).

The contribution of individual NF- κ B subunits has been well studied for IL-12 p40 transcription. c-Rel is absolutely required for IL-12 p40 synthesis after LPS stimulation (166), while p65/RelA controls the intensity of IL-12 p40 transcription (167). Because of this, we analyzed NF- κ B activation directly by assessing NF- κ B subunit binding to the promoter of *Il12b*, which encodes IL-12 p40, by chromatin immunoprecipitation (ChIP) assay. Interestingly, p65/RelA promoter occupancy was elevated in *Itgb2*^{-/-} macrophages after 8 hours of TLR4 stimulation, while c-Rel binding remained similar between both cell populations (Figure 3.22), demonstrating a direct effect of β_2 integrins on transcription factor binding to the *Il12b* locus. Taken together with our gene expression data and signaling analyses, these observations clearly show that β_2 integrins suppress inflammatory cytokine production by blocking NF- κ B pathway activation.

β_2 integrins inhibit type I IFN signaling by influencing SOCS-1 expression.

In addition to inflammatory cytokine production, recognition of nucleic acids by TLR3, TLR7 and TLR9 and LPS by TLR4 signals for production of type I IFNs, which signal in an autocrine or paracrine manner to induce an activation program referred to as the “antiviral state” (25, 168). This cell response revolves around synthesis of IFN-stimulated genes (ISG) that collectively work to obstruct viral replication, either by directly blocking virion generation within the cell or by inducing apoptosis to remove cell replication machinery (168).

Fibrinogen-derived signals have been implicated in control of type I IFN signaling and production (143, 169). However, given fibrinogen’s promiscuous binding specificity these studies cannot address directly whether these effects are mediated

through ITAM-based signals, despite these authors' assertion that β_2 integrins are responsible (143, 169). As such, we tested type I IFN production and signaling in *Itgb2*^{-/-} macrophages to definitely assess how β_2 integrins influence this pathway. *Itgb2*^{-/-} macrophages expressed significantly more IFN β mRNA after TLR4 stimulation than did WT cells (Figure 3.23A) with a corresponding increase in expression of the ISG CXCL10 after LPS treatment (Figure 3.18). Because CXCL10 synthesis can also occur as a direct result of TLR stimulation, to isolate how β_2 integrins regulate ISG production and type I IFN signaling we stimulated WT and *Itgb2*^{-/-} macrophages with recombinant IFN β directly. In this context, *Itgb2*^{-/-} cells, expressed significantly more Oas-1 and Mx-1 at 6 hours after IFN β stimulation (Fig 3.23B), arguing for elevated type I IFN sensitivity following β_2 integrin deletion.

The type I IFN receptor (IFNAR) is a heterodimer of IFN α/β R1 (IFNAR1) and IFN α/β R2 (IFNAR2) subunits. IFNAR1/2 activation transduces a signal via the JAK-STAT pathway in which activated JAKs target and phosphorylate STAT1 and STAT2, which translocate into the nucleus to enable ISG expression (170). We observed increased surface IFNAR1 levels in *Itgb2*^{-/-} macrophages, though IFNAR2 remained unchanged between *Itgb2*^{-/-} and WT cells (Figure 3.24A). We also detected differences in STAT activation following IFN β stimulation. The induction kinetics of STAT activation were the same between both cell populations, however, we noted prolonged STAT1 and STAT2 phosphorylation in *Itgb2*^{-/-} macrophages as compared to WT cells (Figure 3.24B and 3.24C). This was evident beginning at 4 hours into the stimulation when WT cells were diminishing STAT activity, whereas β_2 integrin-deficient macrophages retained

activation until at least 6 hours (Figure 3.24B and 3.24C), demonstrating that β_2 integrins control proximal IFN β signaling.

Because the differences in STAT activity could not be readily explained by elevated IFNAR expression, we sought to identify other mechanisms by which β_2 integrins could control early IFNAR signaling events, specifically by assessing SOCS protein levels. Intracellular SOCS proteins are well-studied cytokine signaling inhibitors that act by recruiting the ubiquitin-conjugating complex and promoting protein degradation (56). SOCS-1, in particular, diminishes type I IFN signaling through its interaction with IFNAR1 and JAK2 and IFN α 4-stimulated *Socs1*^{-/-}*Ifng*^{-/-} fibroblasts display increased STAT1 phosphorylation and prolonged ISG expression, phenocopying β_2 integrin-deficient cells (56, 171). *Itgb2*^{-/-} macrophages exhibited reduced SOCS-1 expression at baseline and after TLR4 stimulation when compared to WT, a finding that is consistent with increased IFNAR signaling (Figure 3.25A and 3.25B). Furthermore, this deficiency was coupled with an increase in miR-155 mRNA levels in *Itgb2*^{-/-} cells. miR-155 in immune cells is considered a pro-inflammatory microRNA for its ability to target and downregulate transcription of TLR inhibitors, including SOCS-1(65). Thus, these observations suggest that β_2 integrins inhibit type I IFN signaling by modulating SOCS-1 levels via miR-155 expression.

Discussion

A variety of cell surface receptors use ITAM-containing adapters to relay external signals and enable appropriate cellular changes, including the β_2 integrins, which signal via DAP12 and FcR γ (86, 152). Yet while signals through DAP12 and FcR γ have been clearly shown to block inflammation (80, 85, 155), defining the connection between the

β_2 integrins themselves and inflammatory processes has proven difficult due to conflicting data showing both positive and negative regulatory roles for this family of adhesion molecules (120, 123, 124, 143-145). We have clarified how β_2 integrin activation influences TLR responses by using macrophages and DCs derived from the *Itgb2*^{-/-} mouse, which lack all β_2 integrin surface expression. *Itgb2*^{-/-} macrophages and DCs produced more IL-12 p40 and IL-6 in response to stimulation with a variety of TLR agonists and *Itgb2*^{-/-} mice generated more inflammatory cytokines after LPS injection than did WT control animals, demonstrating that β_2 integrins are essential for inhibiting TLR activity in vitro and in vivo.

While these phenotypic findings are consistent with other studies reporting a suppressive role for β_2 integrins, our use of *Itgb2*^{-/-} myeloid cells provided a useful system with which to test various aspects of TLR regulation and to define the molecular requirements for β_2 integrin-mediated TLR inhibition. To this end, we have identified a novel role for β_2 integrins in inhibiting both early and late NF- κ B pathway activation downstream of TLR ligation. Early NF- κ B activity occurs shortly after TLR stimulation and is due directly to the MyD88-induced signaling cascade, whereas late NF- κ B signaling stems from newly synthesized TNF operating in an autocrine manner (163). By using I κ B α degradation as a readout, we identified changes in NF- κ B activation between WT and *Itgb2*^{-/-} cells. Without β_2 integrin inhibitory signals, macrophages presented with extended and more potent I κ B α degradation at both early and late time points, consistent with elevated gene expression for a number of NF- κ B-dependent genes. Importantly, we noted that the affected genes encompassed both “primary response” (*Tnf*, *Cxcl1*, *Cxcl2*) and “secondary response” (*Il12B*, *Il6*) genes that encode for inflammatory

cytokines, chemokines and anti-apoptotic functions (172). Furthermore, we found that p65/RelA binding to the *Il12b* promoter was specifically elevated in β_2 integrin-deficient macrophages, demonstrating a direct requirement for the β_2 integrins in controlling activation of particular NF- κ B subunits.

These data are consistent with observations made in the *Itgb2*^{hypo} mouse on the PL/J background, which suffers from a chronic inflammatory skin disease similar to human psoriasis (116). Macrophages are required for maintenance of this disease and selective disruption of NF- κ B activation in macrophages improves the psoriaform lesions in *Itgb2*^{hypo} mice (113, 116). While these results suggest a connection between β_2 integrins and NF- κ B regulation, they are complicated by the ongoing disease of the animals and the presence of residual β_2 integrin signaling in all cell types. However, by using myeloid cells isolated from healthy *Itgb2*^{-/-} mice on a C57Bl/6 genetic background, we have avoided these issues and have clearly revealed a role for β_2 integrins in blocking the NF- κ B pathway, demonstrating that β_2 integrin signaling can directly inhibit TLR signaling.

An important unresolved issue is how β_2 integrins can influence the “upstream” and “downstream” components of NF- κ B activation. I κ B α turnover is controlled by the IKK complex, which consists of IKK α , IKK β and IKK γ /NEMO, all of which serve different functions in pathway regulation (165). Our I κ B α degradation data suggests that IKK β activity, which is responsible for phosphorylating I κ B α to direct it towards degradation (165), is increased in *Itgb2*^{-/-} cells. It is also formally possible that these results may also reflect suppressed inhibition of NF- κ B, potentially through a blockade of

IKK α , which acts to stabilize I κ B α in the cytoplasm (162, 173). We have found that β_2 integrin ablation specifically enhances NF- κ B without a corresponding elevation in MAPK phosphorylation shortly following TLR stimulation, and in fact, p38 activation was much lower in *Itgb2*^{-/-} cells. We believe that this is due to β_2 integrin outside-in signals initiating MAPK activation (86), which is absent in *Itgb2*^{-/-} cells. However, we cannot discount the possibility that this also reflects changes in TLR signaling components that direct MAPK activation specifically, such as the E2 ubiquitin ligase Ubc13 (174). Furthermore, the augmented late-phase NF- κ B activity found in *Itgb2*^{-/-} cells may be due to an improved ability of these macrophages to respond to TNF, though this remains to be tested.

Notably, stimulation of *Itgb2*^{-/-} macrophages allowed us to test the requirements of specific inhibitory mechanisms that have been previously associated with β_2 integrin activation (143, 144). *Itgb2*^{-/-} macrophage TLR hypersensitivity could not be attributed to deficiencies in intracellular TLR signaling inhibitors including ABIN-3, A20 and Hes-1, or in changes to IL-10 production or signaling (143). Interestingly, *Itgb2*^{-/-} macrophages presented with higher TLR-induced levels of some of these inhibitors than WT cells, likely owing to enhanced NF- κ B activation. The differences between our results and those of Wang *et. al.* may be due to our use of plastic petri dishes to induce β_2 integrin signals instead of plate-bound fibrinogen, which itself is known to bind to additional receptors (125, 159-161). Indeed, fibrinogen's ability to dampen TLR activity in macrophages may be at least partially β_2 integrin-independent as we found that inflammatory cytokine secretion was suppressed in *Itgb2*^{-/-} macrophages similar to WT cells after plating onto fibrinogen-coated plates. In addition, the disparities in inhibitor

mRNA expression may also be due to our resting of *Itgb2*^{-/-} macrophages overnight prior to TLR stimulation such that baseline expression levels may mimic those created after prolonged fibrinogen exposure.

In attempting to identify the specific β_2 integrins required for TLR inhibition, we found that deletion of Mac-1 alone is insufficient to render myeloid cells hyperresponsive to TLR stimulation. This was a surprising finding given that Mac-1 activation has been proposed to regulate TLR signaling by inducing Cbl-b activity, leading to degradation of MyD88 and TRIF (144). Cbl-b is a potent negative regulator of inflammation (146, 175) and it is known to modulate TLR4 activity in neutrophils by facilitating TLR4-MyD88 binding (176). However, we found that Cbl-b is not required to dampen TLR activation in macrophages. *Cblb*^{-/-} macrophages were not hypersensitive to TLR stimulation and Cbl-b deficiency did not change the kinetics of MyD88 degradation, as would be predicted based on the model proposed by Han *et al.* through experiments in HEK293 cells. Thus, our data suggest that inhibiting TLR4 does not require a CD11b-Cbl-b-MyD88 regulatory axis in primary macrophages. Deleting LFA-1 was also not sufficient to cause hypersecretion of inflammatory cytokines in macrophages. We theorize that one or more integrins shared between both cell types are responsible for TLR inhibition and that compensatory integrin signaling is able to block TLR responses in *Itgal*^{-/-} or *Itgam*^{-/-} myeloid cells.

Itgb2^{-/-} macrophages were also hypersensitive to type I IFN stimulation, exhibiting prolonged STAT1 and STAT2 phosphorylation and synthesizing more ISGs in response to IFN β treatment. Consistent with this, we found reduced levels of SOCS-1 protein and a corresponding increase in miR-155 expression in β_2 integrin-deficient cells.

Curiously, we noted diminished SOCS-1 protein levels at baseline as well. SOCS-1 is considered part of a classic negative feedback loop that is induced upon stimulation (56). In this classification and based on our data showing similar induction kinetics for STAT phosphorylation, it is reasonable to assume that SOCS-1 is effective at diminishing type I IFN signaling at later time points but has little inhibitory activity during the early parts of IFNAR activation. Furthermore, SOCS-1 has a well-defined ability to inhibit p65/RelA activation in the cytoplasm and nucleus through distinct mechanisms (57, 177), and, as such, SOCS-1 may also be the “upstream” molecule responsible for dampening NF- κ B activation observed here, though this remains to be tested. If true, β_2 integrin function would involve SOCS-1 as the central molecule responsible for controlling TLR signaling and “secondary” IFNAR activation (Figure 3.26), indicating that the β_2 integrins block TLR activation at multiple steps.

Chapter 4: Concluding Remarks

The ITAM-containing adapters DAP12 and FcR γ retain the paradoxical ability to facilitate cell activation or inhibition depending on the identity of the paired receptor, the strength of ligand binding and other unknown factors (178). Here, we demonstrate unequivocally that the β_2 integrins, which utilize DAP12 and FcR γ to initiate outside-in signals, are essential for TLR signal diminishment. Earlier attempts at linking β_2 integrins to control of inflammation were complicated by caveats placed by their experimental design, which have been addressed in previous chapters. We successfully avoided these complications by a) using cells derived from *Itgb2*^{-/-} mice, b) analyzing TLR responses in multiple myeloid populations deficient in β_2 integrins, and c) testing numerous inhibitory mechanisms associated with TLR inhibition. In doing so, we clearly demonstrate that β_2 integrin deletion results in TLR hypersensitivity due to changes in NF- κ B pathway induction and SOCS-1 expression. *Itgb2*^{-/-} mice were also acutely sensitive to LPS treatment and had to be sacrificed prematurely (data not shown), highlighting the importance of β_2 integrins in blocking TLR-induced inflammation in vivo.

These data have important implications for therapeutics aimed at controlling β_2 integrin activation. The conventional wisdom in designing targeted therapeutics has been based on β_2 integrin antagonism. Humanized anti- β_2 integrin monoclonal antibodies (Genentech: Erlizumab; Icos: Rovelizumab) have been employed in an effort to stem inflammation following myocardial infraction or psoriasis by inhibiting leukocyte egress. Unfortunately, these treatments have had severe clinical consequences and have either failed clinical trials or have been removed from the market, and most attempts at

antibody-based β_2 integrin antagonism have been abandoned as a result (179, 180). Arguments against using monoclonals to treat inflammation aside (180), our data indicate that previous attempts at controlling β_2 integrin activity have been flawed in approach. As an alternative to antagonism, future therapies should focus instead on β_2 integrin activation in order to reduce myeloid cell-derived inflammation. In this light, Maignel *et al.* have recently reported on the synthesis of small molecule agonistic “leukadherins” that maintain β_2 integrins in their extended, active conformation (181). Leukadherin treatment sustained leukocyte binding to endothelial cells and away from inflamed tissue in numerous disease models (181). However, whether these Leukadherins were also able to diminish cell activation directly – as would be theorized by our conclusions – was untested and remains an enticing possibility.

Our data suggest an important role for cell adhesion events in fine-tuning inflammation. β_2 integrins first encounter their ligands within the luminal side of blood vessels. By finding that β_2 integrins are required for negatively regulating TLR responses, we have highlighted the exciting prospect that cell adhesion events may limit inflammatory cytokine production in the bloodstream and thereby compartmentalize inflammatory cytokine production to the site of inflammation. Beyond this initial β_2 integrin binding, myeloid cells also encounter β_2 integrin ligands within the extracellular matrix while *en route* to their intended targets. Here, these ligands would be modified by local inflammatory mediators (182), suggesting that distinct β_2 integrin ligands may differentially regulate TLR responses in a manner which targets inflammatory cytokine production to the infected tissue and therefore minimizes damage to the host.

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Figures

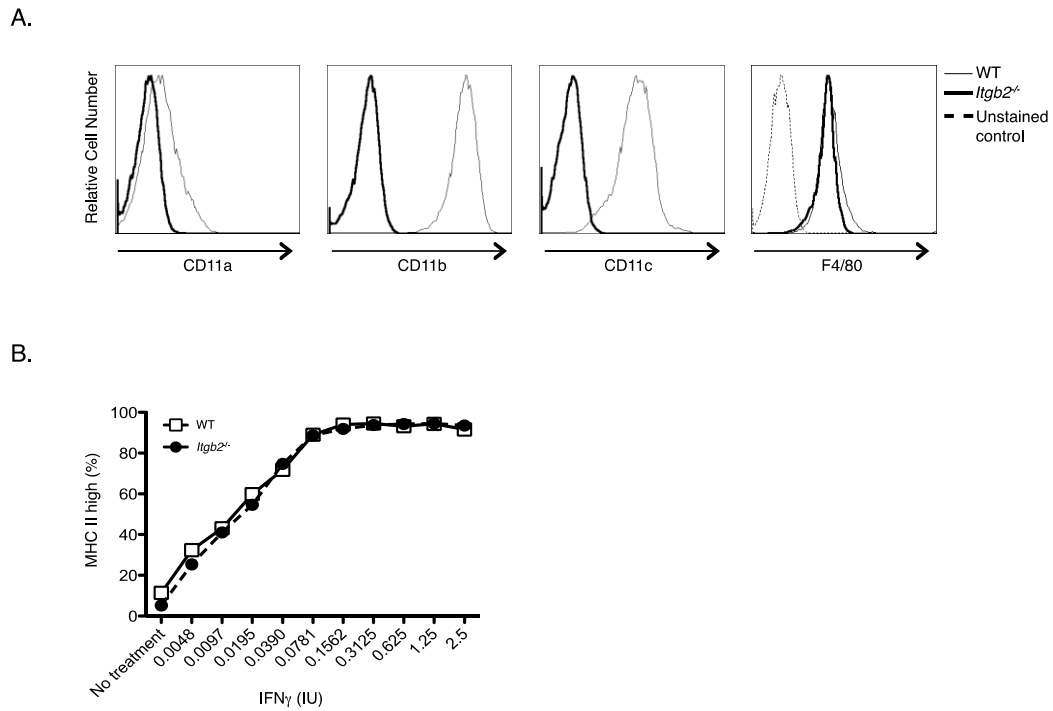
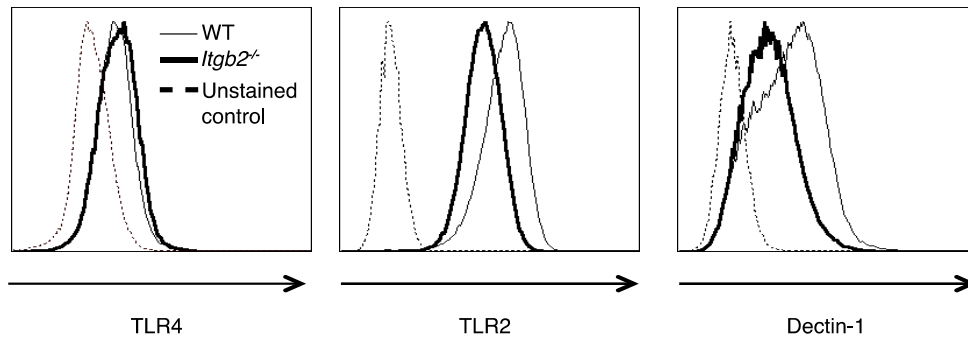


Figure 3.1 Phenotypic characterization of *Itgb2*^{-/-} macrophages. (A) The expression of integrin alpha subunits, CD11a, CD11b, and CD11c, as well as expression of the macrophage marker F4/80 was determined by flow cytometry. (B) Macrophages were stimulated with the indicated concentrations of IFN γ for 48 hours and MHC II expression was determined by flow cytometry.

A.



B.

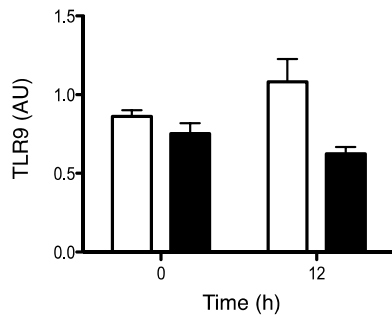


Figure 3.2 TLR and Dectin-1 expression in *Itgb2*^{-/-} macrophages. (A) Surface TLR4, TLR2 and Dectin-1 expression was determined by flow cytometry for WT and *Itgb2*^{-/-} BM-derived macrophages. (B) TLR9 expression was determined by qPCR at baseline and after 12 hours of 1.0 ng/ml LPS stimulation. Data were normalized to GAPDH levels and represent triplicate wells +/- SD.

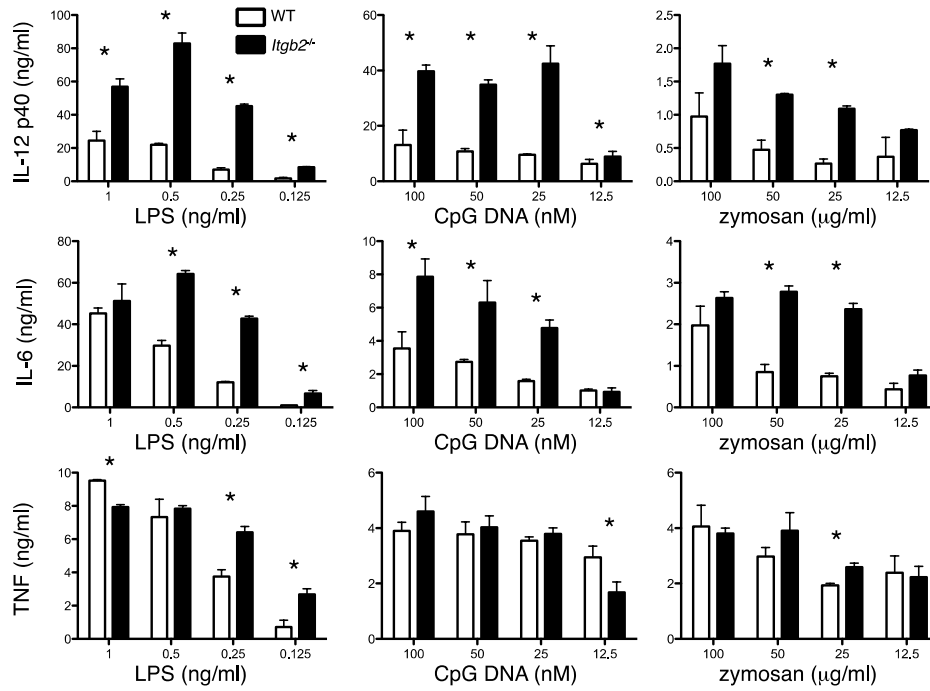


Figure 3.3 *Itgb2*^{-/-} macrophages secrete more inflammatory cytokines after TLR stimulation. Bone marrow-derived macrophages were rested overnight and stimulated with TLR agonists for 24 hours. The concentration of IL-12 p40, IL-6 and TNF in supernatants was measured by ELISA. Results shown are triplicate wells +/- SD. *p,0.05.

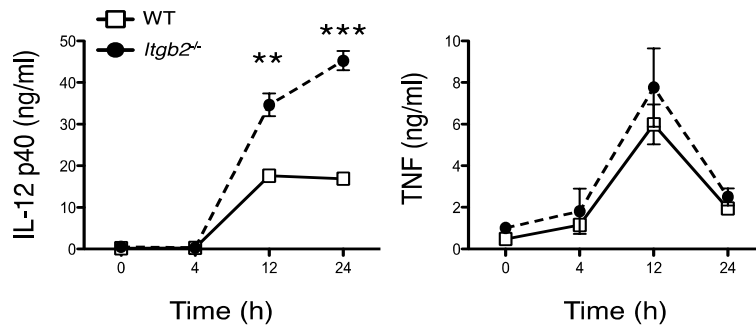
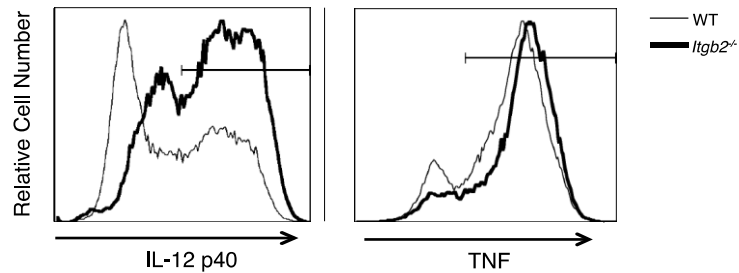


Figure 3.4 β_2 integrin deficiency enhances inflammatory cytokine secretion following TLR stimulation. Bone marrow-derived macrophages were stimulated with 1 ng/ml LPS for the indicate times and concentrations of cytokines in the supernatants was measured by ELISA. The data are shown as +/- SD of triplicate wells. ** $p < 0.01$ and *** $p < 0.001$.

A.



B.

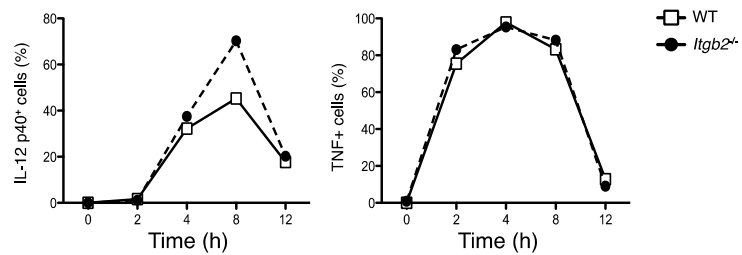


Figure 3.5 Increased inflammatory cytokine synthesis by macrophages in the absence of β_2 integrin signals. Macrophages were stimulated with 1 ng/ml LPS for indicated times and Brefeldin A was added for the last 2 hours of stimulation. TNF and IL-12 p40 production was measured by intracellular cytokine staining and flow cytometry. (A) Gating strategy for IL-12 p40⁺ and TNF⁺ cells 8 hours after stimulation. (B) Percent of macrophages staining positive for IL-12 p40 and TNF at each time point.

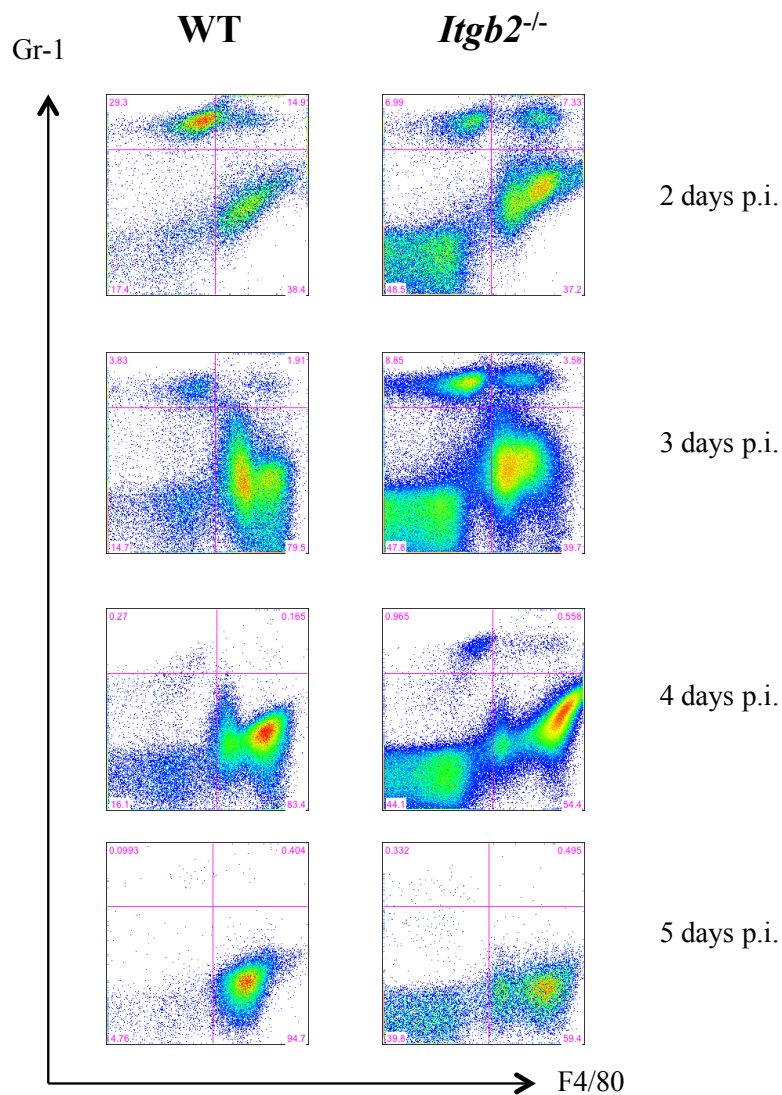


Figure 3.6 Emigration of neutrophils and macrophages into the peritoneal cavity of WT and *Itgb2*^{-/-} mice following thioglycollate injection. Mice were injected with 1 ml of 3% thioglycollate broth i.p. and cells were isolated by lavage. Surface staining for F4/80⁺Gr-1^{+/neg} macrophages and F4/80^{neg}Gr-1⁺ neutrophils was assessed by flow cytometry up to 5 days post-injection (p.i.).

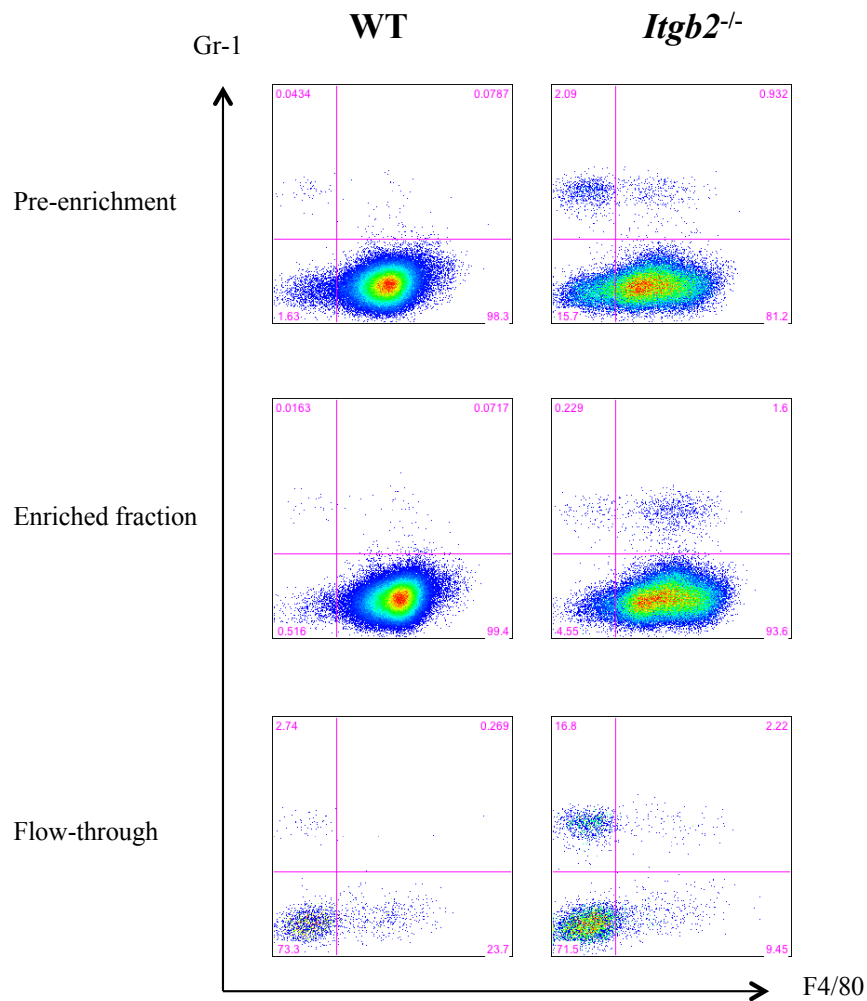


Figure 3.7 Magnetic bead isolation for F4/80-expressing macrophages. Peritoneal cells were isolated by lavage 5 days after injection with 3% thioglycollate broth. Cells were stained with F4/80-biotin Ab followed by α -biotin magnetic beads and enriched by MACS sorting. Staining for F4/80 and Gr-1 was conducted by flow cytometry for pre-sort, enriched and flow-through fractions.

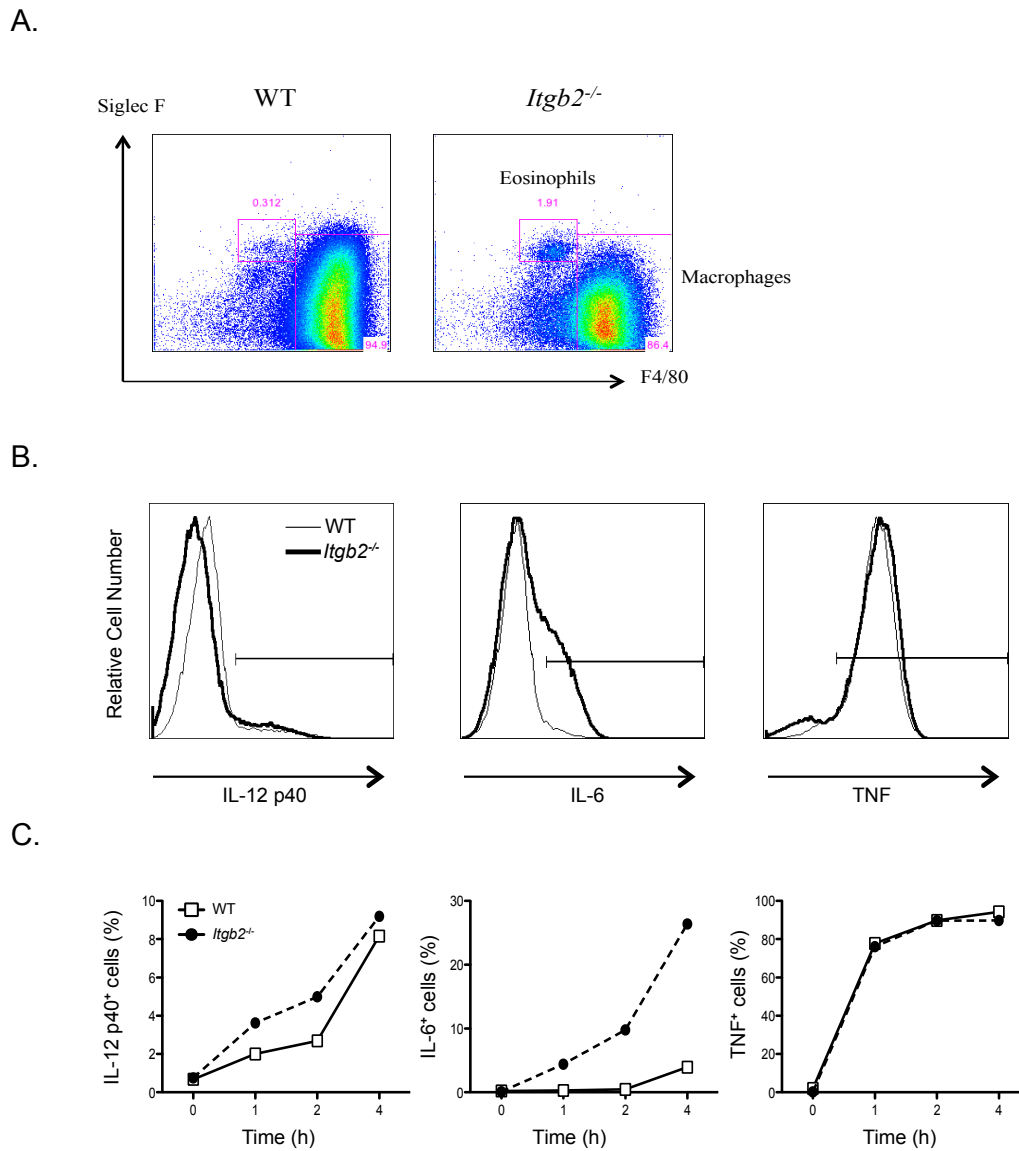


Figure 3.8 β_2 integrins inhibit TLR activity in inflammatory macrophages. D5 thioglycollate-elicited cells were enriched for F4/80⁺ cells, rested overnight before treatment with 1 ng/ml LPS with Brefeldin A for the last 2 hours of stimulation, and cytokine levels were determined by flow cytometry. (A) Gating strategy for F4/80^{high} macrophages. (B) Representative histograms of IL-12 p40, IL-6 and TNF after 4 hours of treatment. (C) The percent of cytokine-producing macrophages as gated in (B).

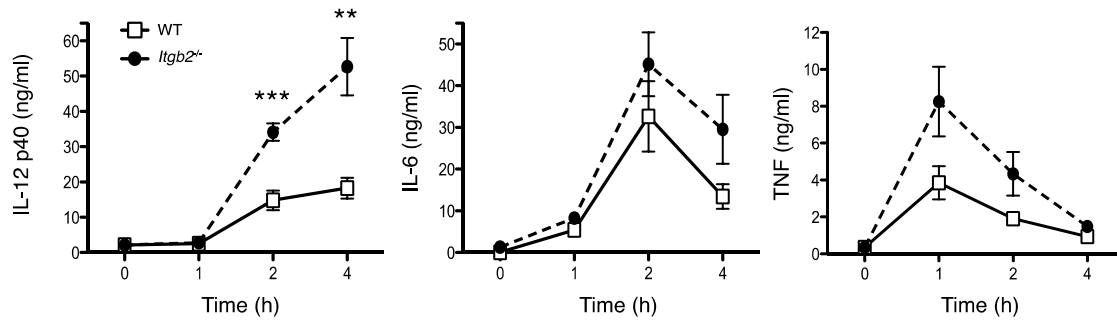
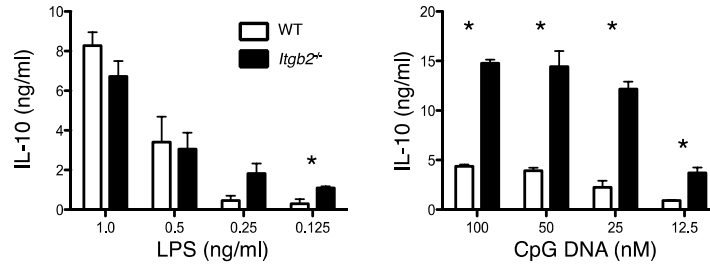


Figure 3.9 β_2 integrins dampen TLR responses in vivo. WT (n=5) and *Itgb2*^{-/-} (n=5) mice were injected i.p. with 1 mg/kg LPS. Blood was collected at the indicated time points and serum cytokine amounts were measured by ELISA. These data are displayed as mean \pm SEM of biological replicates. **p<0.01 and ***p<0.001.

A.



B.

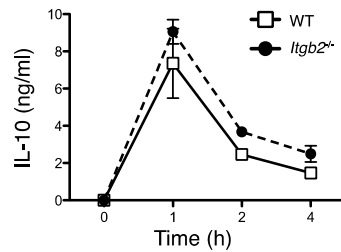


Figure 3.10 β_2 integrins do not block TLRs via IL-10 synthesis. (A) Bone marrow-derived macrophages were stimulated with either LPS or CpG DNA for 24 hours and IL-10 secretion was measured by ELISA. (B) WT (n=3) and *Itgb2*^{-/-} (n=3) mice were injected i.p. with 1 mg/kg LPS and blood was collected at indicated time points. Serum IL-10 concentration was determined by ELISA.

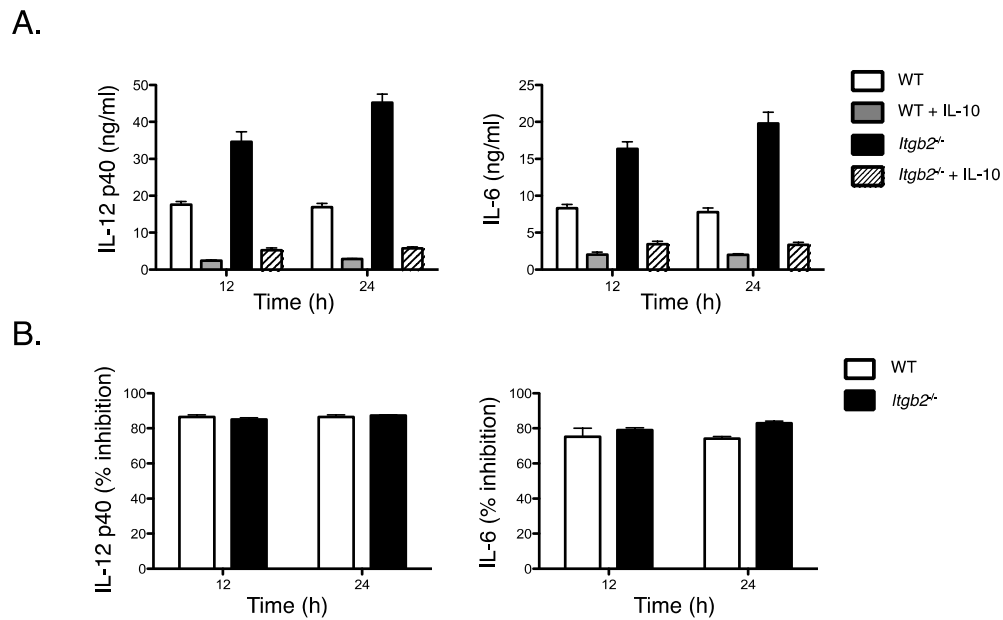


Figure 3.11 β_2 integrin deficiency does not influence IL-10 sensitivity. Macrophages were treated with 10 ng/ml IL-10 for 30 minutes prior to stimulation with 1 ng/ml LPS. (C) Cytokine production was assessed by ELISA. (D) The percent decrease in cytokine production was determined by normalizing to TLR stimulation without exogenous IL-10 treatment. The data are shown as mean \pm SD of triplicate wells.

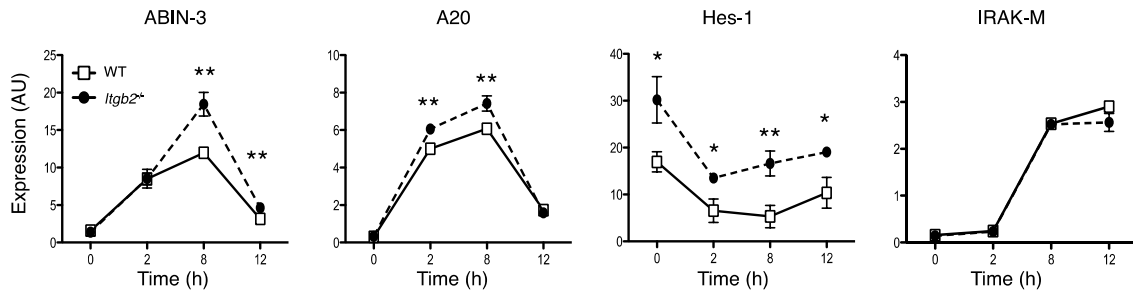


Figure 3.12 *Itgb2*^{-/-} macrophage hypersensitivity is not due to a defect in intracellular TLR inhibitor expression. ABIN-3, A20, Hes-1 and IRAK-M mRNA levels were determined by qPCR after stimulation of macrophages with 1 ng/ml LPS. Expression levels for each inhibitor are normalized to GAPDH and the results are displayed as mean +/- SD of triplicate wells.

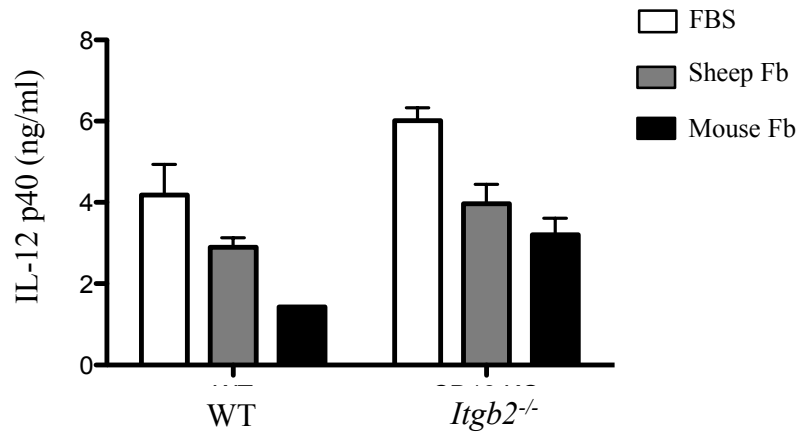


Figure 3.13 Fibrinogen inhibits cytokine secretion in a β_2 integrin-independent manner. WT and *Itgb2*^{-/-} macrophages were cultured on plates coated with 10 μ g/ml fibrinogen (Fb) derived from sheep and mouse plasma for 1 hour, followed by stimulation with 0.5 ng/ml LPS overnight. IL-12 p40 synthesis was determined by ELISA. Results are shown as mean \pm SD of triplicate wells.

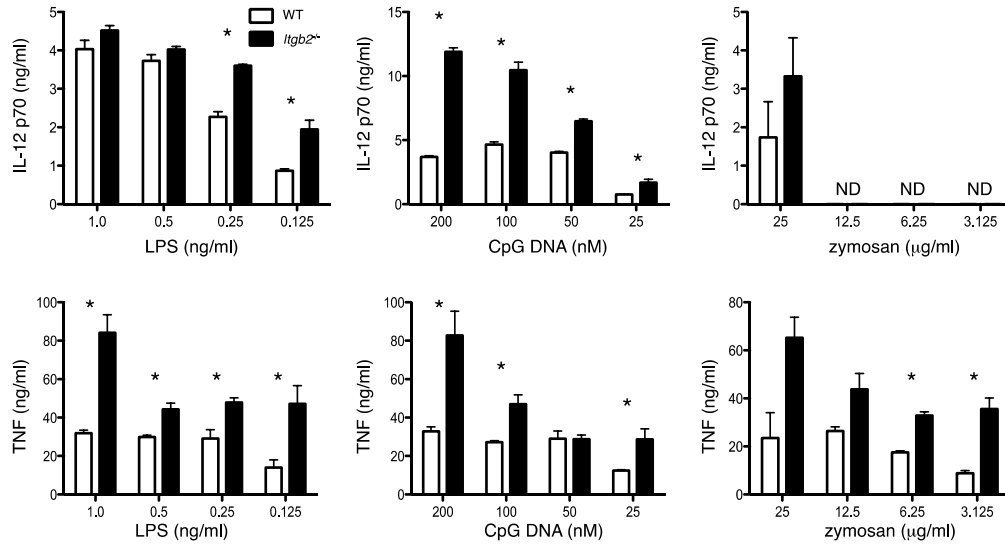


Figure 3.14 *Itgb2*^{-/-} DCs are hypersensitive to TLR stimulation. Bone marrow-derived DCs were isolated by magnetic bead separation for MHC II⁺ cells after GM-CSF culture. DCs were stimulated with TLR agonists overnight and cytokine concentrations in the supernatant were determined by ELISA. Results are shown as mean +/- SD of triplicate wells. *p<0.05.

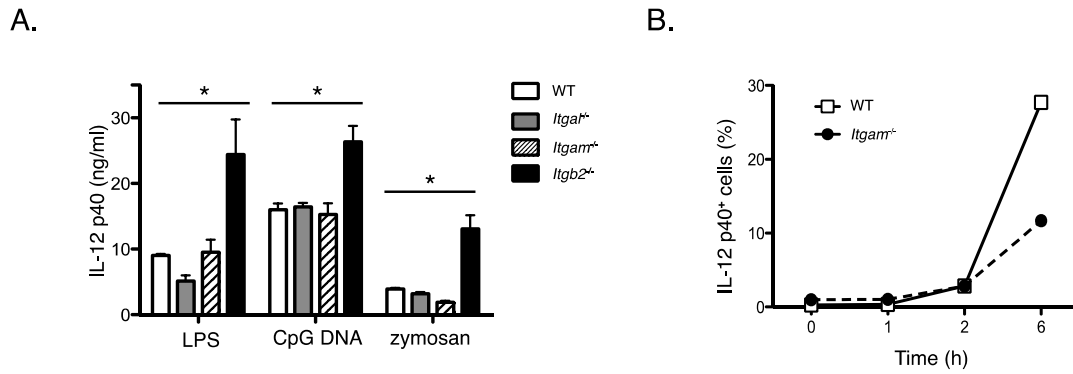


Figure 3.15 LFA-1 and Mac-1 are dispensable for TLR inhibition. (A) Macrophages from WT, *Itgal*^{-/-} (CD11a-deficient) and *Itgam*^{-/-} (CD11b-deficient) macrophages were treated with 1 ng/ml LPS, 100 nM CpG or 100 μg/ml zymosan particles for 24 hours. IL-12 p40 concentrations in the supernatants were determined by ELISA and results are shown as mean +/- SD of triplicate wells. F4/80+ thioglycollate-elicited peritoneal macrophages were treated with 1 ng/ml LPS and IL-12 p40 production was determined by flow cytometry as in Figure 3.8.

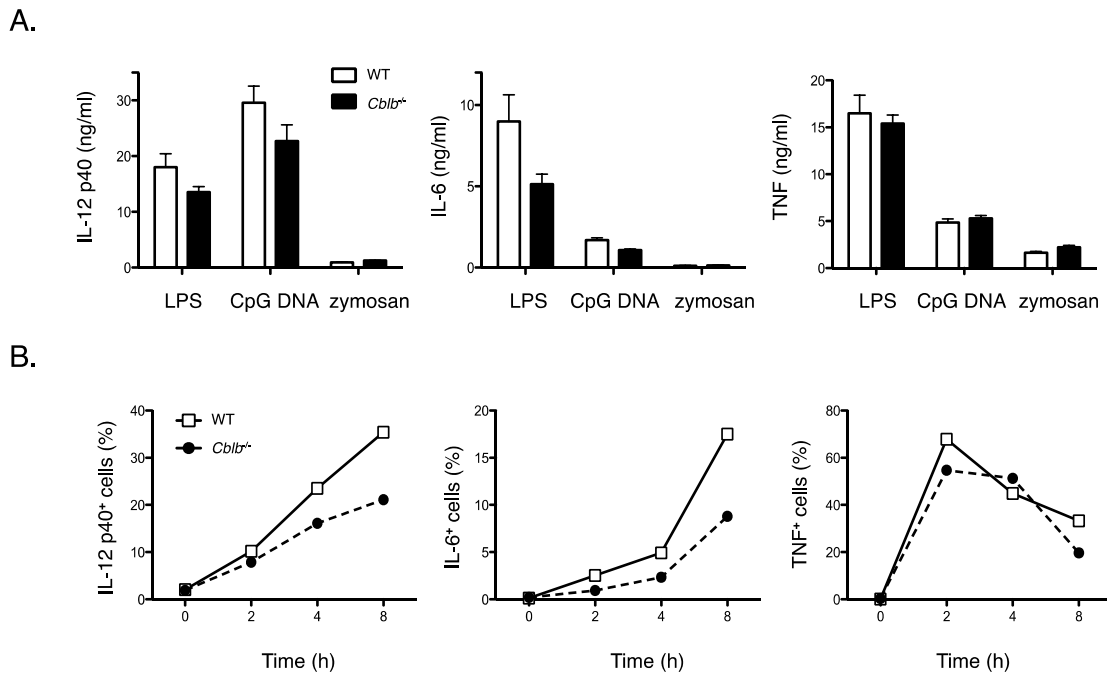


Figure 3.16 *Cbl-b* is dispensable for β_2 integrin-mediated inhibition. (A) Bone marrow-derived WT and *Cblb*^{-/-} macrophages were stimulated with 1 ng/ml LPS, 100 nM CpG or 100 μ g/ml zymosan particles overnight and inflammatory cytokine concentrations were measured by ELISA. Results are shown as mean \pm triplicate wells. (B) F4/80⁺ thioglycollate-elicited peritoneal macrophages were stimulated with 1 ng/ml LPS in the presence of Brefeldin A for the last two hours of stimulation and cytokine responses were measured by intracellular cytokine staining for up to 8 hours.

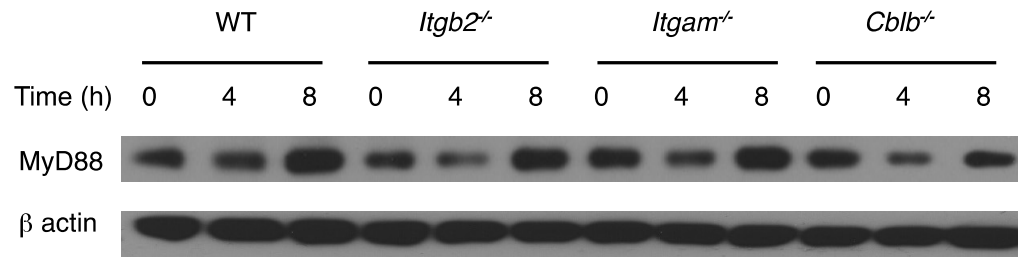


Figure 3.17 MyD88 degradation is unaffected in *Itgb2*^{-/-}, *Itgam*^{-/-}, and *Cblb*^{-/-} macrophages after LPS stimulation. Macrophages were stimulated with 10 ng/ml LPS and cytoplasmic lysates were assessed for MyD88 expression by Western blot, with β actin used as a loading control.

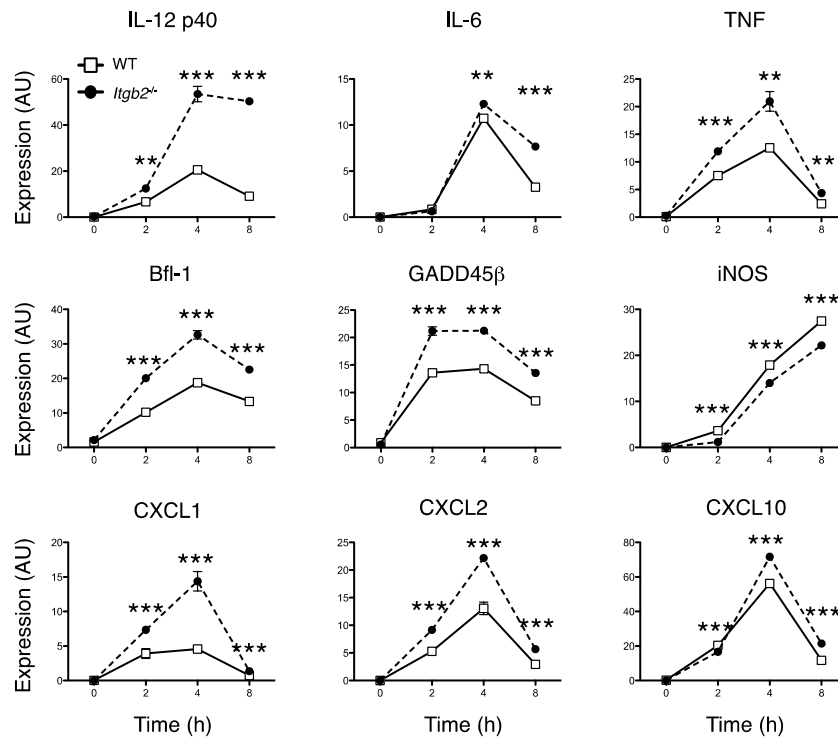


Figure 3.18 β_2 integrin ablation enhances NF- κ B-dependent gene expression in *Itgb2*^{-/-} macrophages. Cells were stimulated with 1 ng/ml LPS. Expression levels of the indicated genes were determined by qPCR with results normalized to GAPDH expression. Results are displayed as mean \pm SD.

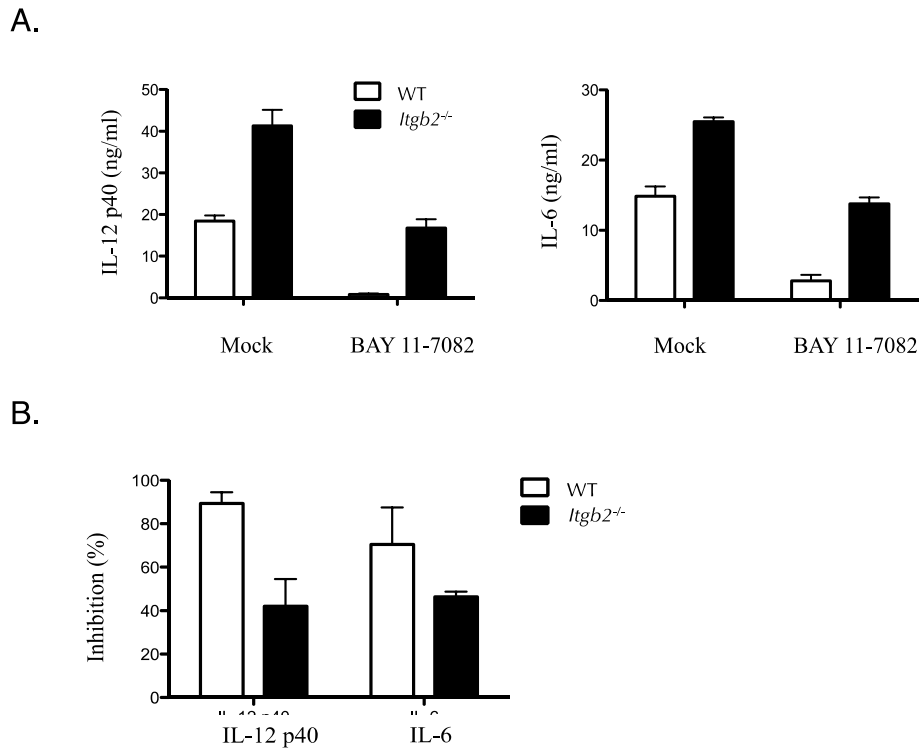


Figure 3.19 *Itgb2*^{-/-} macrophages are less sensitive to NF- κ B pathway inhibition. (A) WT and *Itgb2*^{-/-} macrophages were treated with 10 μ M IKK α / β inhibitor BAY 11-7082 or DMSO for 30 minutes prior to stimulation with 0.5 ng/ml LPS overnight. Supernatants concentrations of IL-12 p40 and IL-6 were determined by ELISA. (B) The percent inhibition was determined by normalizing cytokine levels from BAY 11-7082 treatment to mock levels found for mock-treated macrophages. Results are shown as mean \pm SEM of 3 independent experiments.

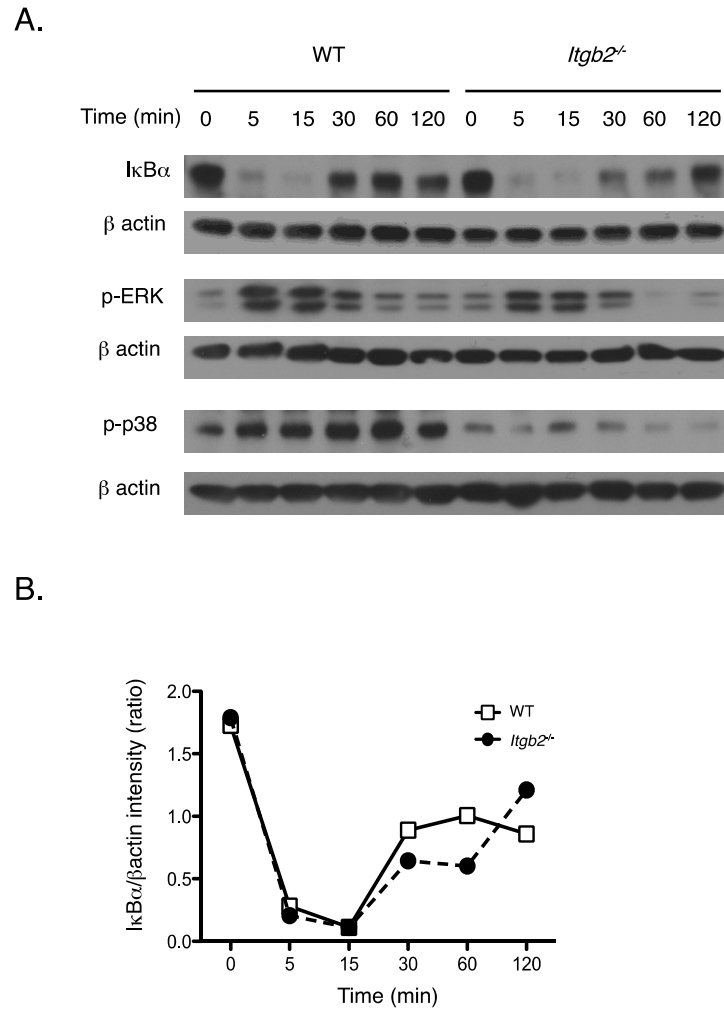


Figure 3.20 *Itgb2*^{-/-} macrophages display enhanced early-phase NF- κ B activation. (A) Bone marrow-derived macrophages were stimulated with 10 ng/ml LPS for the indicated times. Cytoplasmic lysates were assayed for I κ B α , phospho-ERK, and phospho-p38 by Western blot with β actin used as a loading control. (B) Densitometry analysis for the I κ B α protein levels found in (A).

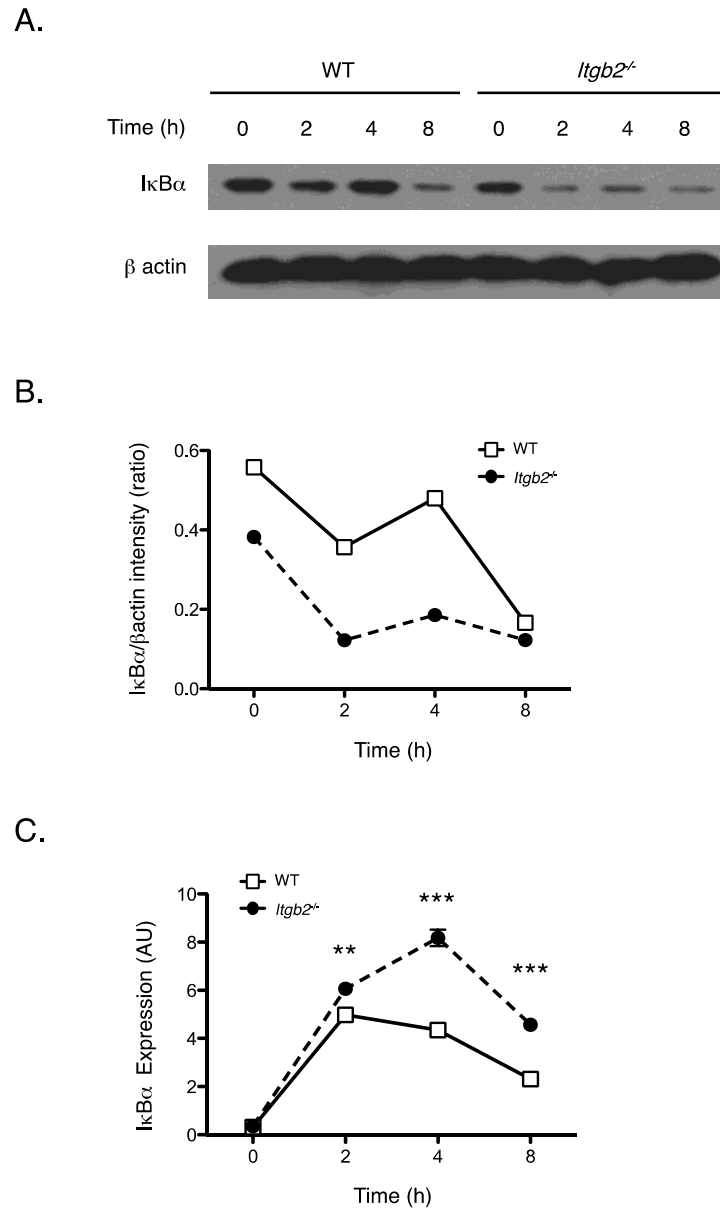


Figure 3.21 *Itgb2*^{-/-} macrophages display enhanced late-phase NF- κ B activation. (A) Bone marrow-derived macrophages were stimulated with 1 ng/ml LPS for the indicated times. Cytoplasmic lysates were assayed for I κ B α with β actin used as a loading control. (B) Densitometry analysis for the I κ B α protein levels found in (A). (C) mRNA expression of I κ B α following 1 ng/ml LPS stimulation. Data are normalized to GAPDH.

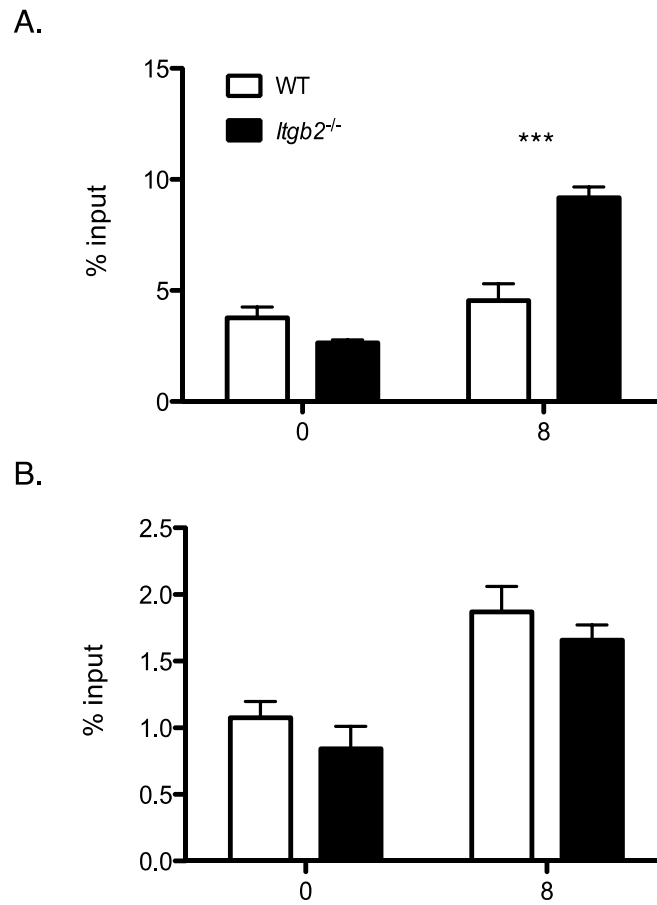
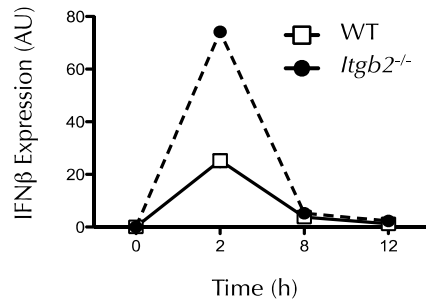


Figure 3.22 β_2 integrin deficiency enhances p65/RelA binding to the *IL12b* promoter. Macrophages were stimulated for 8 hours with 1 ng/ml LPS and ChIP was performed on nuclear extracts using anti-p65/RelA (A) and anti-c-Rel (B) antibodies. Recruitment to the IL-12 p40 (*IL12b*) promoter was determined by qPCR and normalized to total (“input”) chromatin. Results are shown as mean +/- SD of triplicate wells. ***p<0.001

A.



B.

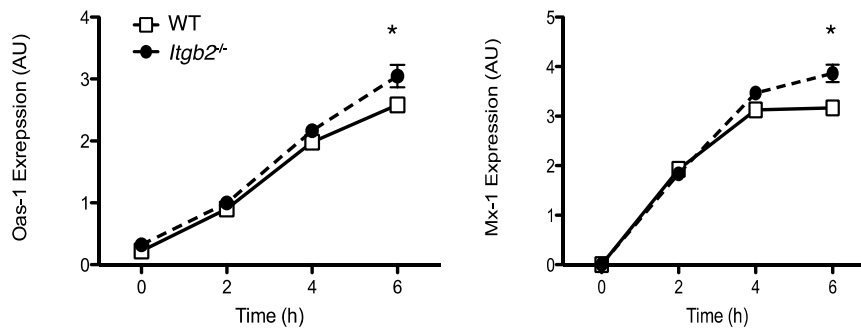


Figure 3.23 *Itgb2*^{-/-} macrophages produce more type I IFNs in response to TLR stimulation and are hypersensitive to IFN β treatment. (A) WT and *Itgb2*^{-/-} macrophages were stimulated with 1 ng/ml LPS and IFN β expression was determined by qPCR. (B) Macrophages were stimulated with 100 U/ml IFN β and ISG expression was determined by qPCR. Results in both (A) and (B) are normalized to GAPDH expression. The data in (B) is mean \pm SEM of 3 independent experiments.

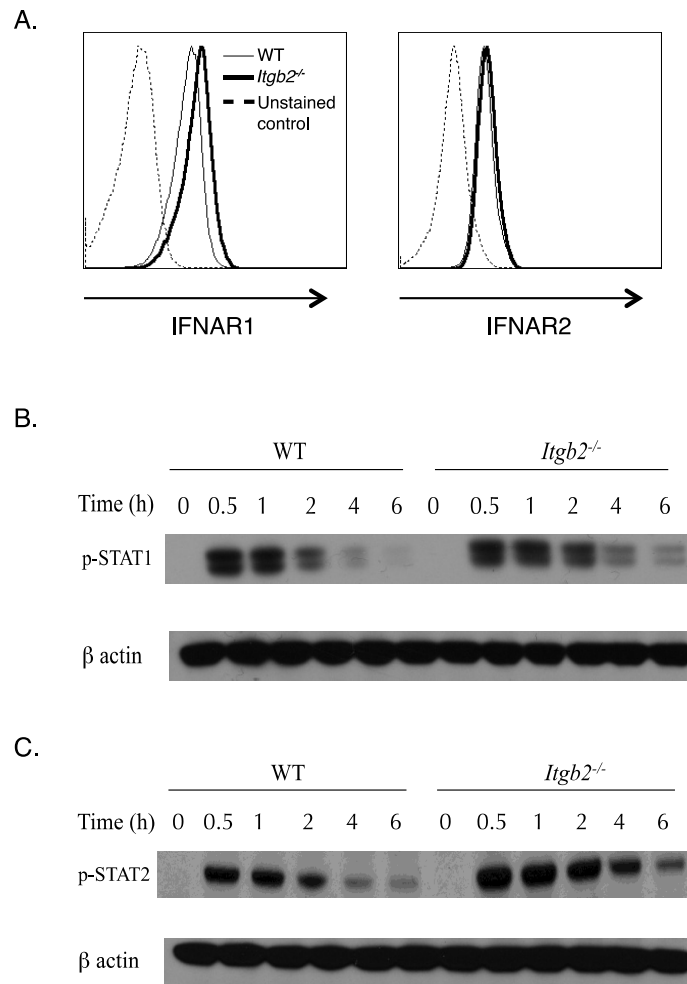
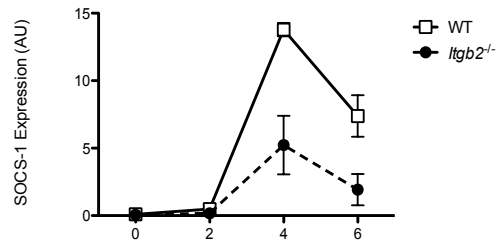
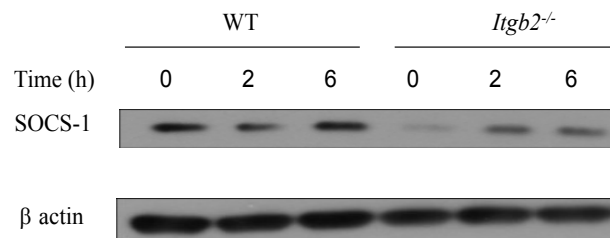


Figure 3.24 β_2 integrins control IFNAR1 expression and STAT1/2 activation. (A) WT and *Itgb2^{-/-}* macrophages were assessed for IFNAR1 and IFNAR2 subunit expression by flow cytometry prior to stimulation. (B and C) Macrophages were stimulated with 100 U/ml IFN β and cytoplasmic lysates were examined for phospho-STAT1 (B) and phospho-STAT2 (C) by Western blot, with β actin used as loading controls.

A.



B.



C.

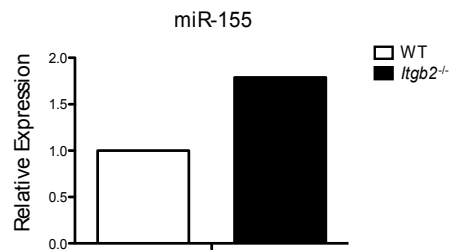


Figure 3.25 SOCS-1 is diminished in *Itgb2*^{-/-} macrophages. (A) SOCS-1 mRNA levels were determined by qPCR after stimulation with 1 ng/ml LPS for up to 6 hours with data normalized to GAPDH. Results are shown as mean \pm SD of triplicate wells. (B) Cytoplasmic lysates from 1 ng/ml LPS stimulated macrophages were assessed for SOCS-1 by Western blot at the indicated time points, with β actin used as a loading control. (C) Macrophages were stimulated with 1 ng/ml LPS for 12 hours and miR-155 expression was determined by qPCR with data normalized to U6 endogenous control.

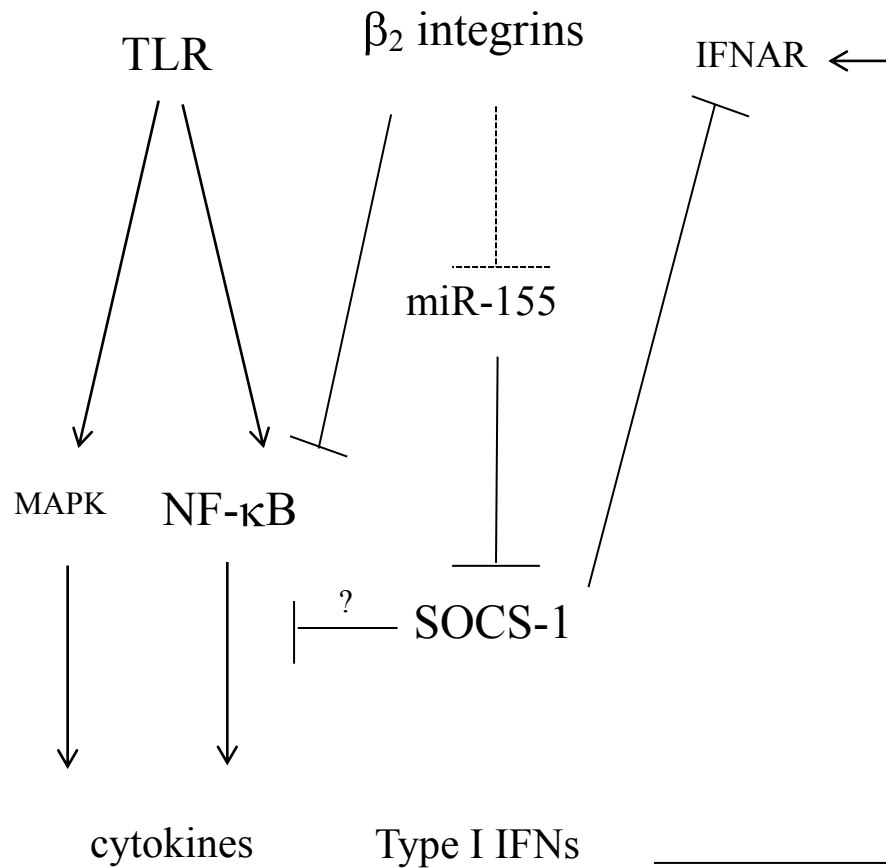


Figure 3.26 Proposed model of β_2 integrin-mediated TLR and type I IFN inhibition. β_2 integrin signaling inhibits early and late-phase NF- κ B activation to diminish inflammatory cytokine production after TLR stimulation. This may occur by control of miR-155, which in turn blocks SOCS-1 expression. In addition to limiting cytokine production by this mechanism, SOCS-1 modulation also controls “secondary” type I IFN signaling after TLR stimulation.

Curriculum Vitae
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Education

- 2000-2004 B.S., Biochemistry; University of Washington
- 2000-2004 B.S., Microbiology; University of Washington
- 2005-2012 Ph.D., Immunology; University of Washington

Research and Professional Experience

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- 2004- 2005 Research Scientist I; University of Washington
- 2001-2004 Undergraduate Research Assistant; University of Washington

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- 2010 Sandra L. Clark Foreign Travel Scholarship
- 2010 International Congress of Immunology Travel Scholarship

Teaching Experience

- 2006 Teaching Assistant; University of Washington Dept. of
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- 2004 Teaching Assistant; University of Washington Dept. of
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- 2003-2004 Tutor; University of Washington CLUE Program