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Itk is a Dual Action Regulator of Immunoreceptor Signaling in the Innate and Adaptive Immune System: A Dissertation

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Itk is a dual action regulator of immunoreceptor signaling in the innate and adaptive immune system

A dissertation presented by

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This thesis is dedicated to my grandparents.

Grace E. Evans
John W. Evans, Sr
Dorothy E. Iannino
Joseph R. Iannino

Your influence and inspiration has shaped the person I am today.
And although you are not here, your spirits live on through me.

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ABSTRACT

The cells and molecules that comprise the immune system are essential for mounting an effective response against microbes. A successful immune response limits pathology within the host while simultaneously eliminating the pathogen. The key to this delicate balance is the correct recognition of the pathogen and the appropriate response of immune cells. Cellular activation originates through receptors that relay information about the state of the microenvironment to different compartments within the cell. The rapid relay of information is called signal transduction and employs a network of signaling mediators such as kinases, phosphatases, adaptor molecules, and transcription factors. IL-2 inducible T cell kinase (Itk) is a non-receptor tyrosine kinase that is an integral component of signal transduction downstream of many immunoreceptors. This dissertation describes two distinct pathways that utilize Itk in both phases of the immune response.

T cells use the TCR to sense a multitude of peptide-based ligands and to transmit signals inside the cell to activate cellular function. In this regard, the diversity of ligands the T cells encounter can be portrayed as analog inputs. Once a critical threshold is met, signaling events transpire in close proximity to the plasma membrane to activate major downstream pathways in the cell. The majority of these pathways are digital in nature resulting in the on or off activation of T cells. We find, however, that altering the TCR signal strength that a T cell receives can result in an analog-based response. Here, the graded expression of a transcription factor, IRF4, is modulated through the activity of Itk. We link

this graded response to an NFAT-mediated pathway in which the digital vs. analog nature has been previously uncharacterized. Finally, we demonstrate that the repercussions of an analog signaling pathway is the altered expression of a second transcription factor, Eomes, which is important in the differentiation and function of T cells. These results suggest that Itk is crucial in the modulation of TCR signal strength.

Mast cells primarily rely on the IgE-bound FcεR1 for pathogen recognition. Crosslinking this receptor activates mast cells and results in degranulation and cytokine production via an expansive signaling cascade. Upon stimulation, Itk is recruited to the plasma membrane and phosphorylated. Little else is known about how Itk operates inside of mast cells. We find that mast cells lacking Itk are hyperresponsive to FcεR1-mediated activation. This is most apparent in the amount of IL-4 and IL-13 produced in comparison to wild-type mast cells. Increased cytokine production was accompanied by elevated and sustained signaling downstream of the FcεR1. Finally, biochemical evidence demonstrates that Itk is part of an inhibitory complex containing the phosphatase SHIP-1. These results indicate a novel function for Itk as a negative regulator in FcεR1-mediated mast cell activation.

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Chapter 1: Introduction

Opening remarks

One of nature's crowning achievements, the human body is an intricate combination of complex organ systems working in concert to sustain and foster life. It is because of this biological accomplishment we continue to thrive and flourish in our environment. However, we do not subsist alone, nor could we. There are millions and millions of different organisms we must co-exist with in order to survive. The vast majority of these co-inhabitants are microorganisms. Of the known microbes, most are innocuous to humans, even beneficial, providing a source of energy and nutrition. A small fraction of microbes, however, are potentially infectious and may cause disease in humans; we call these microbes - pathogens. Fortunately, evolution has imparted us with an immune system to prevent and circumvent the adverse effects of an infection.

Once a pathogen has breached primary barriers (e.g. skin, saliva, etc.) and established an infection by colonizing a niche within a host, the immune system is charged with recognition and elimination of the microbe. The collection of tissues, cells, and molecules that constitute the immune system is essential to mounting a successful response against foreign invaders. Typically, activation of the immune system by a pathogen results in a two-phased attack initiated by innate immune cells and subsequently followed by an adaptive immune response. The goal of this tandem effort is life-long, sterilizing immunity for the host against that particular pathogen. Occasionally, however, the modus operandi breaks down and the host is left vulnerable and potential life-

threatening ailments ensue. Frequently, these ailments result from such disorders as immunodeficiency, autoimmunity, and hypersensitivity (allergy). In order to prevent these conditions or to treat existing immunological diseases, it is imperative to understand the mechanisms at play in regulating a proper immune response. This dissertation explores the mechanisms that are involved in the proper activation of two specific cell types, T cells and mast cells, which are important in an immune response. The remainder of this chapter is divided into three major sections: T cells, Tec kinases, and mast cells. Following a review of the literature, the goals for this dissertation are stated systematically.

Hematopoiesis, T cell development, and the concept of signal strength

T lymphocytes develop in the thymus where they undergo a step-wise maturation into functionally competent naïve cells. The cells residing in the thymus cannot self-renew and therefore require the constant replenishment of progenitor cells from the bone marrow. In the bone marrow, hematopoietic stem cells (HSCs) are that constant source of self-renewal that feed the immune system's cellular component. The derivation of a multipotent progenitor (MPP) from HSCs begins the necessary commitment steps for cells seeding the thymus. Differentiation of cells into MPPs results in the loss of an ability to self-renew but allows for the classic bifurcation of hematopoiesis that is depicted in Figure 1.1 (1-5). This is demarcated by the generation of the common

Figure 1.1

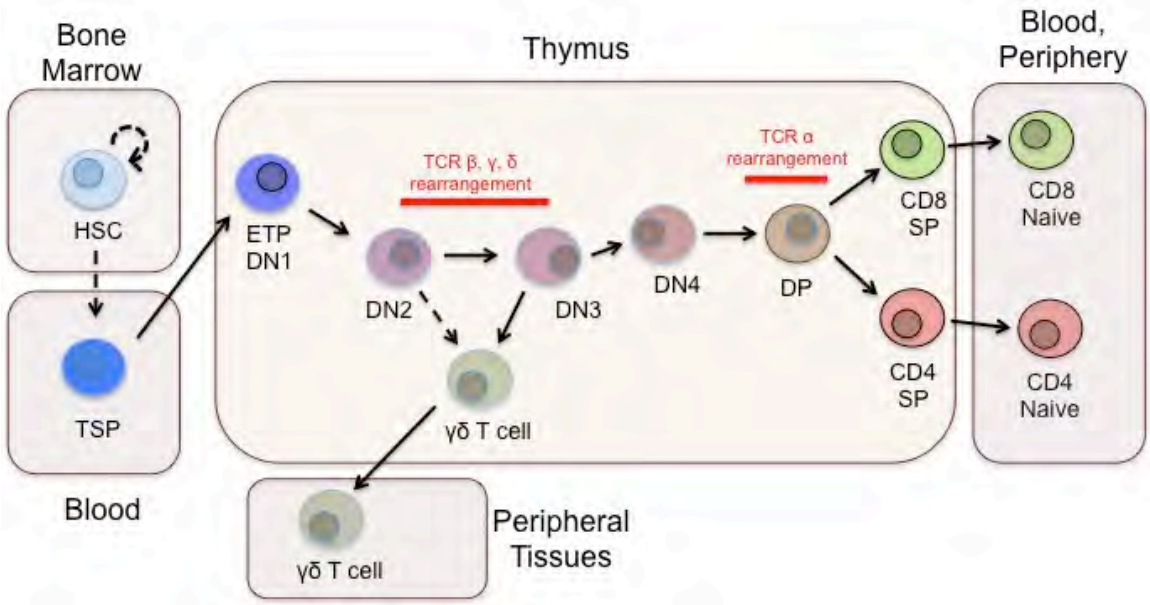

Figure 1.1 T cell development

Derived from the hematopoietic stem cell (HSC), the thymic seeding progenitor (TSP) migrates from the bone marrow to seed the thymus. Notch signals promote the differentiation to the early thymic progenitor (ETP). The rearrangement of TCRβ, γ, and δ begins at the double negative 2 (DN2) stage. DN3 cells undergo β-selection, become γδ T cells, or die. Rapid proliferation into DN4 occurs and TCRα rearrangement occurs at the double positive (DP) stage. Positive and negative selection regulates TCR specificity at the DP and single positive (SP) stages, respectively. Carefully scrutinized SP thymocytes mature and emigrate from the thymus into the periphery as naïve T cells.

myeloid progenitor (CMP) and common lymphoid progenitor (CLP) from the MPP[a]. In 1997, it was discovered that the CLP was a committed progenitor that could give rise to natural killer (NK) cells, B lymphocytes, and T lymphocytes (6, 7). It is thought that the CLP seeds the thymus for T cell development, however, variations in experimental settings have challenged this notion (8, 9). Irrespective of which cell actually seeds the thymus, interaction with the thymic stroma results in the differentiation of the most immature T cell progenitors. These cells are called early thymic progenitors (ETPs) and are phenotypically identified by the expression of c-Kit (CD117) and the IL-7 receptor (CD127), the lack of expression of lineage markers (CD8, CD3, TCRβ, TCRγδ, NK1.1, CD11c, Mac1, Gr1, B220, CD19, Ter119), and are CD25 negative (10, 11).

At this point the ETP remains uncommitted to the T cell lineage, though the ability to form other lymphoid populations, like B cells, is greatly diminished. A key determinant in T cell commitment is the expression of Notch ligands on thymic epithelial cells and Notch receptors on the ETP. The lack of the receptor, Notch1, results in the complete loss of T cells (12, 13). Once, Notch signaling has occurred the ETP can proceed into the textbook differentiation program of T cell development. The cells that enter this developmental phase are said to be double negative (DN) for CD4 and CD8 expression yet can be phenotypically marked by the expression pattern of CD44 and CD25 (14-16). Progression from the DN1 stage ($CD44^{hi}$ $CD25^{-}$), where ETPs reside, into the DN2 stage ($CD44^{hi}$ $CD25^{+}$) is highlighted by the accessibility of genes that will form the TCR and the

[a] The multipotent progenitor is also thought to give rise to a mast cell precursor. This will be discussed later in this chapter.

initial expression of recombination activating genes (RAG) (17, 18). Because developing thymocytes are not synchronous, monitoring the DN2 to DN3 stage ($CD44^{lo}$ $CD25^{+}$) is more like looking at a continuum rather than the stop and go of single defined events. This is not meant to imply that the process is not tightly controlled. In fact, it's just the opposite. The proceeding paragraphs will explain the regulatory steps in becoming a T cell.

The continuous expression of RAG1 and RAG2 during the DN2 to DN3 transitional phase provides the thymocyte with the ability to rearrange the *TCRγ*, *TCRδ*, and *TCRβ* loci (19-21). In addition to gene rearrangement, this period is also a critical point in the commitment of thymocytes to become αβ or γδ T cells. A central factor in determining this is the IL-7 receptor. Signaling through the IL-7 receptor is thought to be important for opening the *TCRγ* locus (22-24). Furthermore, thymocytes with the highest amount of IL-7 receptor preferentially become γδ T cells, whereas those with the lowest amount become αβ T cells (10, 25). The ultimate fate determination between these two T cells is the productive rearrangement of TCR genes in conjunction with functions of Id3 (γδ T cells) and Notch signaling (αβ T cells) (26-31). The full commitment to become an αβ T cell throughout the DN3 stage begins during β-selection. This process gives the cell several chances to successfully rearrange the genes that will comprise the TCR. The first attempt comes from the *TCRβ* gene that becomes expressed along with a surrogate α-chain (pre-TCRα) and the CD3 signaling chains (32-34). If the *TCRβ* gene does not undergo successful rearrangement, an attempt on the second allele will occur. A functional TCR complex prompts

the cell to cease rearrangement on the *TCRβ* gene, a process known as allelic exclusion. The DN3 cells that survive β-selection differentiate to the DN4 stage ($CD44^{lo}$ $CD25^{-}$). During the DN3 to DN4 transition, RAG proteins are down regulated and a period of rapid proliferation ensues that will feed the double positive (DP) compartment. DP ($CD4^{+}CD8^{+}$) cells acquire the surface expression of CD4 and CD8 and re-express the RAG proteins to begin working on the *TCRα* gene until a functional protein is generated (11, 35, 36). It is up to the thymus to scrutinize the reactivity and specificity of the productive T cell receptor; and thus begins the concept of TCR signal strength.

The concept of signal strength

When the functionality of a T cell receptor has been verified, the specificity of the T cell must next be controlled (37, 38). This is where the original model of TCR signal strength originates (Figure 1.2). DP cells that demonstrate intermediate levels of specificity for host MHC alleles receive signals through the TCR to promote their survival and further differentiation. This is known operationally as positive selection. It is clear that a critical threshold for strength of TCR signaling exists because T cells that bind MHC proteins too weakly will not receive the necessary anti-apoptotic cues and die by neglect. Furthermore, signals from the TCR also direct the fate of cells that will become CD4 single positive (SP) or CD8 SP. This is accomplished through the actions of a complex

Figure 1.2

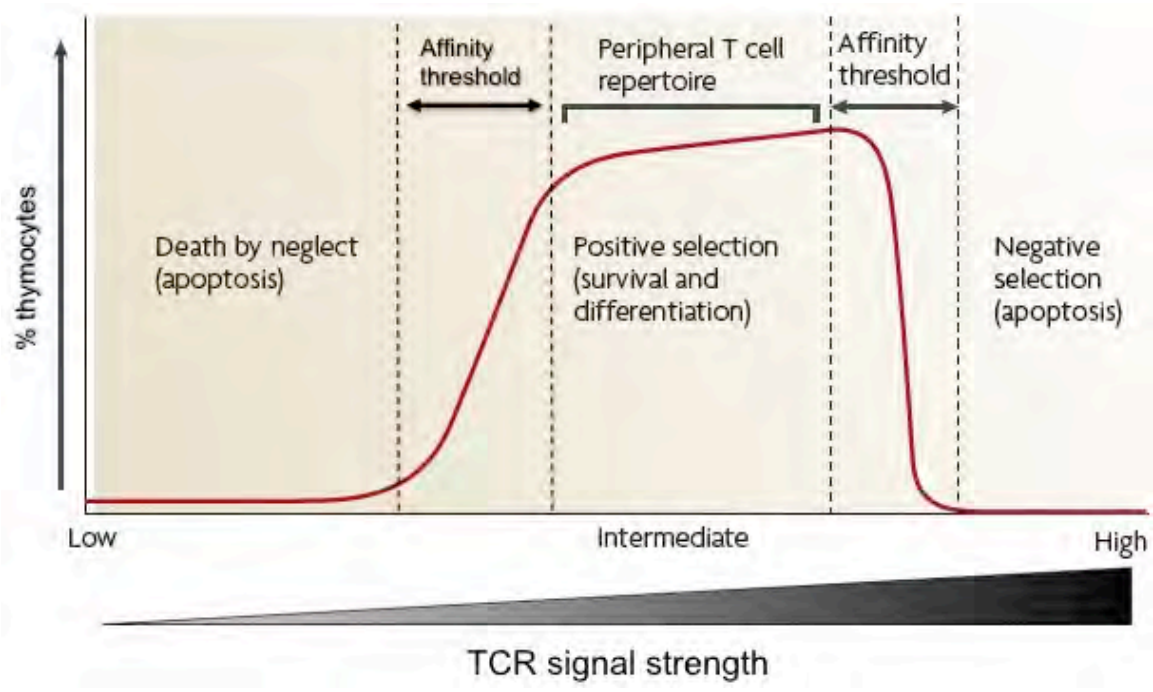

Figure 1.2 TCR signal strength in the thymus

The classic model of TCR signal strength in the thymus adapted from Nature Reviews Immunology, 2009, vol. 9, pp. 833-844. Positive selection ensures that intermediate amounts of TCR affinity towards self-pMHC will recognize antigen presented in the periphery. Thymocytes with very little TCR affinity generate low TCR signal strength and die by neglect. Thymocytes receiving strong TCR signal strength recognize self-pMHC too well and undergo negative selection.

transcription factor network, including proteins like Th-POK, Runx, and GATA-3 (11, 39, 40). TCR signal strength is thought to be important in this process as well. For example, the differential expression of GATA-3 links TCR signal strength to the lineage commitment of CD4 SP or CD8 SP (41, 42). Strong TCR signals result in high levels of GATA-3 expression, which promotes the development of CD4 SP cells, whereas weak TCR signals generate less GATA-3 and drive the lineage toward CD8 SP cells.

Once DP thymocytes become single positive cells, they are ready to undergo a second round of scrutiny, called negative selection. In this process, the presentation of self-peptide embedded within MHC molecules determines whether a T cell recognizes that antigen or not. This is where TCR signal strength takes center stage again and dictates that a cell receiving too strong of a stimulus will die by apoptosis; whereas a cell that receives an intermediate level of stimulation will survive through positive selection. Another critical threshold point for strength of TCR signaling delineates the very fine transition between positive and negative selection. For example, it was elegantly demonstrated that small incremental changes (analog) in ligand potency result in dramatic changes (digital) in the sensitivity of TCR signal transduction, as shown in the Ras – MAPK pathway (43, 44). High affinity ligands that promoted negative selection showed a cellular redistribution of signaling proteins at the plasma membrane. On the other hand, cells stimulated with a ligand 1.5 times less potent displayed a more sparse distribution pattern of signaling proteins and survived via positive selection. Importantly, this was not due to a lack of signal transduction because

all cells expressed CD69. These experiments demonstrated that small changes in ligand quality result in the yes/no event that determines survival or death.

The collective actions of positive and negative selection, as mediated by TCR signal strength, provide the primary means of preventing autoimmunity in peripheral tissues. This is referred to as central tolerance. Survival of these regulatory processes in the thymus renders a mature CD4 or CD8 single positive cell functionally competent to serve in the immune system. It also prompts CD4 and CD8 T cells to emigrate from the thymus and populate the rest of the body as naïve T cells, where they are free to engage peptide-MHC complexes.

T cell activation

The identification of a receptor

Prior to the 1980's it was unknown exactly how T cells were activated. Many years of research, mostly on allogeneic rejection, helped solidify the notion that the lymphoid compartment of the blood was responsible for cytoxicity and could provide help to antibody secreting B cells. Around the turn of 1960's, a series of *in vitro* studies elegantly demonstrated that the cytotoxicity of T cells was specific and likely due to clonal receptors on the cell surface (45-48). This was shown by the incubation of lymphoid cells, obtained from an immunized animal, on top of a monolayer of cells containing the immunogen. Several hours following incubation, the monolayer began dying. Cells that were gently washed

off the monolayer, and therefore non-adsorbed, could not replicate the cytoxicity when placed on a fresh monolayer. The adsorbed cells, however, when eluted and incubated on a fresh monolayer, were cytotoxic. It would take another decade to actually discover the determinant of this specificity – the T cell receptor.

If the flurry of activity in the literature during the 1980's showcased anything, it was that this decade was a pioneering time for the T cell. The discovery that MHC molecules presented peptides to T cells, the structure of the TCR, and the identity of components for the entire T cell antigen receptor complex were but a few of the highlights. As expected then, many groups partook in the identification of the T cell receptor, which was spearheaded by the use of monoclonal antibodies on a number of human and murine T cell tumor lines (37, 49-53). These studies opened the door to the biochemical, structural, and genetic nature of the TCR. Two groups led, by Tak Mak and Mark Davis, independently cloned the *TCRβ* locus from human and mouse T cells (54-57). The presence of variable and constant regions revealed remarkable similarity to the BCR and served as the basis for TCR diversity. Shortly thereafter, Don Wiley's group resolved the crystal structure of MHC Class I and proposed that it presented peptides to T cells via a deep, electron dense pocket (58-60). These initial studies laid the groundwork for the molecular mechanism by which T cells recognize antigen. There is, however, one missing component linking this with the molecular circuitry inside the cell.

Significant pieces of information pointed towards CD3 as that missing link (61, 62). Monoclonal antibodies directed towards CD3 promoted T cell activation. The presence of long intracellular domains suggested that CD3 was an integral part of TCR signal transduction. A key study using Jurkat mutant T cells demonstrated the requirement of CD3 to be co-expressed with the TCR on the cell surface of a T cell (63, 64). Thus all the necessary components for examining TCR signaling have been assembled (Figure 1.3).

Proximal TCR signaling

At present, we understand many of the molecular details involved in TCR signal transduction. As described above, the T cell antigen receptor complex consists of two disulfide-linked chains (e.g. α and β), associated in the membrane with CD3 signaling chains (i.e. γ , δ, ε, ζ). These signaling chains contain immunoreceptor tyrosine based activation motifs (ITAMs) in the cytosol. Crosslinking or ligating the T cell receptor results in the induction of a membrane proximal kinase cascade involving three distinct families of non-receptor protein tyrosine kinases (PTKs): the Src family, the Syk family, and the Tec family (65, 66). The initialization of this cascade is mediated by the recruitment of Src family kinases, such as Lck and Fyn, to the intracellular portion the TCR complex. For example, Lck is associated with the intracellular domains of the co-receptors CD4 and CD8. CD8 and CD4 bind conserved regions on MHC Class I or MHC Class II, respectively, and therefore usher Lck in close proximity to the ITAMs.

Figure 1.3

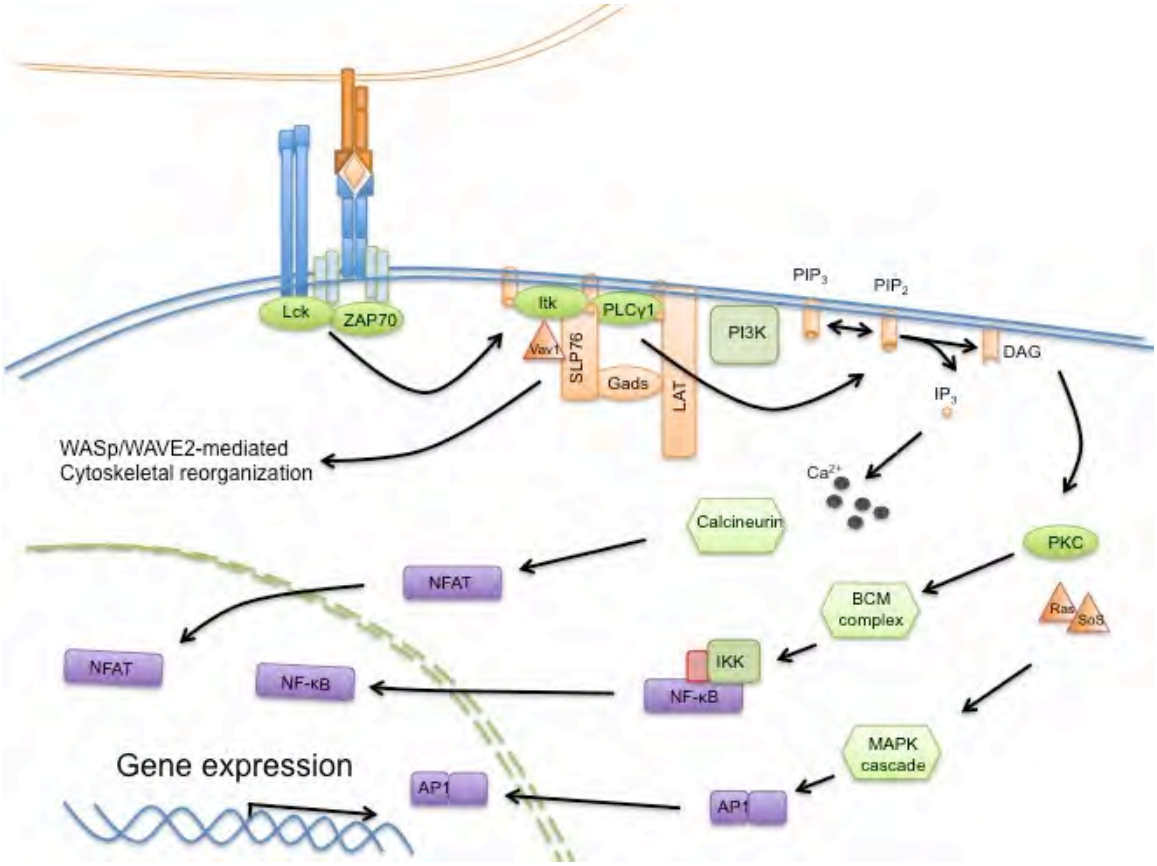

Figure 1.3 T cell receptor signal transduction

The schematic above highlights the important players and pathways in TCR signaling. A more detailed description is in the text. Briefly, ligation of the TCR by pMHC recruits PTKs from three families (Src, Syk, Tec). Activation of these PTKs promotes the assembly of a membrane proximal signaling complex. This complex regulates the generation of secondary messengers, IP_3 and DAG, which activate major downstream pathways: Ca^{2+} mobilization, NF-κB, and MAPK cascade. The culmination is transcription factor activation and gene expression.

The dephosphorylation of Src family kinases by the phosphatase, CD45, facilitates their activation. ITAMs in the intracellular domains of the TCR complex are then phosphorylated by Src family kinases. The phosphotyrosines in the ITAMs provide a docking site for the Syk family kinase, ZAP-70, which in turn, gets phosphorylated by Lck.

The recruitment and activation of Zap-70 is a crucial signaling event that determines whether a T cell will be switched on or remain off; there is no intermediary at this point. Once activated, ZAP-70 potentiates the nucleation of a large macromolecular complex by phosphorylating the adaptor proteins, LAT and SLP-76 (37, 67, 68). These two adaptor proteins form the scaffold that will provide functional access for a number of signaling molecules, including enzymes (e.g. PLCγ1, Tec kinases, and PI3K) and adaptors (e.g. Grb2, Gads, Nck and Vav) (69, 70). The activity of PI3K promotes the membrane accumulation of PIP_3. This increases the probability that PH domain-containing proteins, like Itk and PLCγ1, will join the complex. Multiple interaction sites amongst the proteins within this complex act as a molecular glue to maintain stability and propagate the signal. In doing so, Tec family kinases, such as Itk, phosphorylate PLCγ1, which in turn cleaves PIP_2 into DAG and IP_3 (71, 72). The generation of the second messengers, DAG and IP_3, mediates the induction of three pathways required for the expression of effector genes in T cells. DAG is responsible for evoking two pathways, NF-κB and MAPK, whereas IP_3 regulates Ca^{2+} mobilization.

The major downstream pathways

DAG is the membrane bound remnant of PIP_2 hydrolysis from PLCγ1 activity. The cleavage of PIP_2 reveals a docking site for the PKC family member, PKCθ, which associates with DAG via a lipid-binding domain (73, 74). At the membrane, PKCθ can phosphorylate CARMA1 to promote the assembly of a complex containing Bcl10 and MALT1 (75, 76). MALT1 is then thought to activate the E3 ligase, TRAF6, which polyubiquitinates the regulatory subunits of IKK. Once the regulatory subunits of IKK have been degraded, the catalytic portion of IKK then phosphorylates IκB promoting its degradation and the subsequent release of NF-κB. NF-κB can then translocate to the nucleus to promote the expression of target genes important for T cell function.

The DAG – PKCθ tandem is also important in the activation of the guanine nucleotide-binding protein (G-protein), Ras. Ras activation occurs when GTP is loaded by guanine nucleotide exchange factors (GEFs), such as son of sevenless (SOS) and Ras guanyl nucleotide-releasing protein (RasGRP). SOS is in the same complex as LAT but RasGRP is recruited to the membrane via DAG where it becomes phosphorylated by PKCθ. Both of these GEFs promote Ras activation. However, RasGRP-dependent GTP production catalyzes SOS activity thereby promoting a positive feedback loop for Ras activation (69, 77, 78). Active Ras is required for the initiation of the first serine/threonine kinase in the MAPK cascade, Raf-1 (79, 80). The MAPK cascade culminates with the activation and formation of transcriptional activators, such as AP-1, that induce

target gene expression.

In contrast to DAG, the cleavage of PIP_2 by PLCγ1 releases IP_3 from the membrane into the cytosol, where it can liberate intracellular Ca^{2+} stores from the endoplasmic reticulum (ER). Sensors in the ER membrane detect the depletion of intracellular Ca^{2+}, via domains called EF hands, and promote the opening of Ca^{2+} release-activated Ca^{2+} (CRAC) channels in the plasma membrane, a process known as store-operated Ca^{2+} entry (SOCE) (81-84). SOCE results in the activation of a number of Ca^{2+} sensitive signaling molecules, including calmodulin (CaM). CaM is another protein that is able to detect high concentrations of intracellular Ca^{2+}, via EF hands. The conformational change that occurs in CaM allows it to interact with and promote the enzymatic activity of the phosphatase calcineurin. Calcineurin is then able to dephosphorylate the transcription factor NFAT. Dephosphorylated NFAT is then free to translocate to the nucleus and regulate the expression of TCR-dependent genes.

Other pathways

In addition to the major signaling pathways downstream of the TCR, there are several other pathways that are known to be important in the activation of T cells. Stimulation through the TCR promotes cytoskeletal reorganization that aids in the polarization of signaling molecules towards the TCR complex (85, 86). The most well studied mediators of this pathway are the GEF Vav1 and its GTPase effectors Rac1 and Cdc42. Phosphorylation of Vav1 promotes the

activity of Rac1 and Cdc42, which in turn induce F-actin polymerization via WASp and WAVE2. The rearrangement of cytoskeletal actin is important for the adhesion of T cells to target cells and migration throughout the body.

Another critical moderator of T cell activation is costimulation. The costimulatory molecule, CD28, is a staple in proper T cell activation. Ligated by B7 molecules on APCs, costimulation of T cells via CD28 is the epitomized second signal required for T cell activation. PI3K is one of the major players downstream of CD28 signaling. As mentioned elsewhere, PI3K promotes the accumulation of PIP_3 in the plasma membrane. In the context of costimulation, however, the activation of PI3K promotes the activity of phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates its target Akt (62). Akt is a major regulator of T cell activation and is involved in such processes as differentiation and survival.

TCR signal strength as a function of activation

The initial concept of TCR signal strength has been introduced superficially as a way to convey the physiological importance (i.e. T cell development) of this phenomenon. A few more words are in order to describe some of the parameters that are important in understanding the role of TCR signal strength in T cell activation.

Qualitative and quantitative differences in TCR – pMHC interactions influence the T cell response. Collectively these interactions are referred to as

TCR signal strength. There are a number of ways in which TCR signal strength can be modulated experimentally and physiologically. Many studies utilize monoclonal antibodies against CD3 as a surrogate means to activate T cells. Even prior to the TCR being cloned and arduously analyzed, experiments using anti-CD3 revealed much about the nature of T cell activation. Crosslinking the TCR via increasing amounts of anti-CD3 caused corresponding increases in the uptake of tritiated thymidine (87). More recent studies using anti-CD3 have confirmed the idea that T cell activation is either on or off, which is commonly referred to as digital signaling. This was shown by examination of multiple aspects of the NF-κB pathway, which revealed that it acts in a digital manner in response to TCR ligation (88). Experimentally, cells analyzed for the activation of NF-κB showed that only the proportion of cells responding to TCR stimulation changed in response to altered signal strength. The magnitude of NF-κB activation, however, did not vary, indicating the presence of a robust 'all-or-none' response. Interestingly, these observations were also made when numerous components upstream of NF-κB were examined. This included PKCΘ membrane localization, formation of the BMC complex, and activation of IκK. However, the strength at which anti-CD3 elicits its action on the TCR is orders of magnitude greater than what a T cell sees under physiological circumstances (89).

Another approach to investigating TCR signal strength is by using pMHC complexes known to induce T cell responses *in vivo*. In using these TCR ligands, the binding parameters of TCR – pMHC can be analyzed in the context

of signal transduction. For example, measurement of TCR – pMHC interaction kinetics determined that ligands with slower off-rates induced more extensive phosphorylation of the CD3ζ chains (90). In similar studies, it has been revealed that measuring early activation events can sometimes not construe the entire story. In response to weak TCR ligands, the amount of Ca^{2+} inside the cell takes considerably longer to elevate to levels equivalent to strong ligands (91). The limitations of these types of assays aside, they have quantitatively provided us with the ability to distinguish the boundaries of T cell activation.

In addition to studying the potency of TCR – pMHC interactions, a number of other factors influence TCR signal strength. This includes, but is not limited to, the density of pMHC presented to a T cell, duration of TCR – pMHC interaction, biophysical constraints imposed on the T cell - APC conjugate, and the binding of co-receptors and adhesion molecules (92). Moreover, a T cell's ability to distinguish between different pMHC ligands likely involves the regulatory activities of signaling molecules like kinases, which can further modify TCR signal strength.

Numerous examples of T cells utilizing signal strength can be found in the literature. The foremost example, thymic development, has already been described. The strength of TCR signaling also has an important role in regulating the *in vivo* response of CD4 T cells as well as directing the lineage fate of helper T cells. Analysis of tetramer binding approximates the average affinity of TCR – pMHC in an antigen-specific CD4 T cell population responding to primary and secondary immunizations, and therefore the entire T cell repertoire can be

examined against a given immunogen (93). Here, it was observed that T cells responding to a secondary challenge displayed a greater intensity of tetramer binding than T cells from the primary challenge. This narrowing of the repertoire indicates that T cells with a greater affinity towards antigen displayed a selective advantage over T cells with a lower affinity.

One of the central themes in T cell biology is the differentiation of CD4 helper T cells into effector cells such as T_H1, T_H2, and T_H17 cells. Differentiation of CD4 T cells into these effector subsets is controlled by many factors, most notably the cytokine microenvironment (94). CD4 T cell differentiation occurs in the context of TCR stimulation and it is here that TCR signal strength has an influence on which lineage the cell will become. *In vitro* studies of CD4 T cell differentiation suggest that weaker TCR-pMHC interactions preferentially promote the generation of IL-4-producing T cells, and thus the generation of T_H2 cells (95). Conversely, CD4 T cells exposed to strong TCR stimulation become T_H1 cells producing an abundance of IFN-γ (96). More recently, T follicular helper cells (T_{FH}) appear to also be influenced by TCR signal strength. In a polyclonal population of CD4 T cells, differentiation into the T_{FH} subset is more efficient when the cells have a higher affinity towards cognate tetramer than non-T_{FH} cells (97). Therefore, a precedent is set whereby the strength of TCR stimulation can drastically alter the differentiation fate of CD4 T cells.

Consequences of TCR signaling

Controlling CD8 T cell activation and differentiation

The overall goal of TCR signaling is the functional activation of T cells. T cell function manifests as proliferation, differentiation, cytokine secretion, cytoxicity, migration, etc. In order to bridge the gap between the environmental input and the functional output of T cells, TCR signaling must initiate a number of intracellular processes. These processes include such modifications as cytoskeletal rearrangements, induction of cell cycle, and the mobilization of transcription factors that act on specific genes. Several transcription factors have been described above (e.g. NFAT, NF-kB, AP-1, etc.) and these represent the initial group of cellular activators responsible for gene expression. Some of the genes that are regulated by this initial group include a second wave of transcription factors that are important in facilitating the effector phase of T cell activation.

The T-box transcription factors, T-bet and Eomesodermin (Eomes), are two of the most well-characterized regulators from this second tier of transcription factors. They have roles in both CD4 and CD8 T cell activation and differentiation. For example, T-bet is highly expressed in effector CD8 T cells that are activated in the context of a pro-inflammatory environment. This was demonstrated in *Tbx21*-deficient mice that fail to develop short-lived effector cells (SLECs), defined by reduced IL-7 receptor (CD127) expression and increased

levels of surface KLRG1, in response to an LCMV infection (98). SLECs are the predominant effector cell generated during the acute phase of a CD8 T cell response to infection. The other major effector cells generated are the memory precursor effector cells (MPECs). It is thought that this cell type populates the pool of memory CD8 T cells that forms upon infection resolution. As opposed to T-bet and SLECs, Eomes is typically associated with CD8 T cells that become MPECs (99). Two important lines of evidence support this notion. First, Eomes was found to preferentially segregate in daughter cells that form MPECs (100, 101). Secondly, Eomes expression is suppressed in response to high levels of the pro-inflammatory cytokine IL-12, which can induce high levels of T-bet (102). Like, T-bet, Eomes expression is controlled by a complex signaling pathway that is initiated by the TCR and utilizes inputs from a variety of other pathways (e.g. cytokine signaling and costimulation).

More recently, additional transcription factors, that are regulated at various time points following TCR signaling, seem to be important in mediating the differentiation of CD8 T cells (103). Examples include Blimp-1, Bcl6, and TCF-1. Similar to T-bet and Eomes, Bcl6 and Blimp-1 share a reciprocal role in the differentiation of CD8 T cells. Blimp-1, like T-bet, appears to control the generation of SLECs in numerous models of infection (104-106). Direct regulatory control over Blimp-1 can occur with binding of Bcl6 to the *Prdm1* (Blimp-1) promoter, and vice versa (107). As such, Bcl6 mediates the expansion of memory CD8 T cells. In models of infection, mice that lack Bcl6 activity exhibit a reduction in memory CD8 T cells; whereas transgenic mice that overexpress

Bcl6 have elevated numbers of memory CD8 T cells (108). Similarly, TCF-1 appears to regulate the formation of MPECs over SLECs. Mice that are deficient in TCF-1 show increases in the SLEC population with concomitant decreases in MPECs suggesting that the lack of TCF-1 prohibits the production of MPECs (109). It is believed that TCF-1 acts upstream of Eomes to promote the formation of MPECs following infection. Several experiments support this conclusion. First, the loss of TCF-1 prevents severely diminishes the expression of Eomes. Second, TCF-1 is capable of direct regulation of Eomes by binding upstream regulatory elements within the Eomes promoter. Finally, the lack of MPEC formation that occurs in TCF-1 deficiency can be rescued by retroviral transduction of Eomes, which restores memory CD8 T cell differentiation. It is currently unclear exactly how these transcription factors control CD8 T cell differentiation. For example, despite the overwhelming amount of evidence to suggest that Eomes is responsible for the formation of memory CD8T cells, it was recently shown that Eomes deficient T cells could still form a functional memory population even after a pathogen had been cleared (110). Intriguingly, however, these cells were not able to efficiently compete with WT T cells in mixed bone marrow chimera experiments when challenged with a pathogen.

Tec kinases

In the late 1940's, a young boy was admitted to the Walter Reed Army Hospital in Washington, D.C. with shaking chills, high fever, arthritic-like pain,

and nausea. He was given a thorough physical examination that yielded few additional symptoms. Blood work showed white blood cell counts that were within normal range and a 10-day culture that turned up negative. Treatment with penicillin alleviated the existing symptoms and he was consequently discharged. A couple of weeks later, the boy was readmitted with similar symptoms. Again, he was treated with penicillin and released. This course of hospitalization and release would continue for many months until an attending physician, Col. Ogden Bruton, analyzed the serum content by protein electrophoresis. It was discovered that the boy lacked circulating IgG antibodies; he was then diagnosed with agammaglobulinemia, a rare x-linked genetic disorder [b] (112). Many years later, the genetic basis for x-linked agammaglobulinemia (XLA) was identified as Btk, a member of the Tec kinase family (113-115).

The Tec family is one of the most prominent protein tyrosine kinase (PTK) families known and the second largest of the non-receptor variety. As listed in Table 1.1, Tec family kinases are predominantly expressed in hematopoietic cells. Cellular expression is usually overlapping with two or more Tec kinases in a given cell (Figure 1.4). In mice, the five members include: Tec, Btk, Itk, Rlk, and Bmx. They are primarily involved in the signal transduction of several receptors in the immune system, most notably the multi-chain immune recognition receptor family, or immunoreceptors. Tec kinases have roles in cellular development and activation of immune responses. This is supported by

[b] The boy was eventually treated with adoptive immunotherapy via introduction of exogenous IgG, and his clinical manifestations improved (111).

Table 1.1

Member	Full Name	Expression Pattern
Tec	tyrosine expressed in hepatocellular carcinoma	T cells B cells Mast cells Hepatocytes
Btk	Bruton's tyrosine kinase	B cells Macrophages Mast cells Dendritic cells
Itk	IL2-inducible T-cell kinase	T cells Mast cells NK cells
Rlk	resting lymphocyte kinase	T cells Mast cells NK cells
Bmx	bone marrow tyrosine kinase gene on chromosome X	Granulocytes Epithelial cells

Table 1.1 Tec family kinases

The table above lists the 5 Tec family kinases found in mice. Several Tec family kinases are also known by other names (e.g. Itk was also called Tsk and Emt in the early 1990's). The full name commonly used in the literature is listed in the second column. The known cellular expression of each family member is listed in the third column.

Figure 1.4

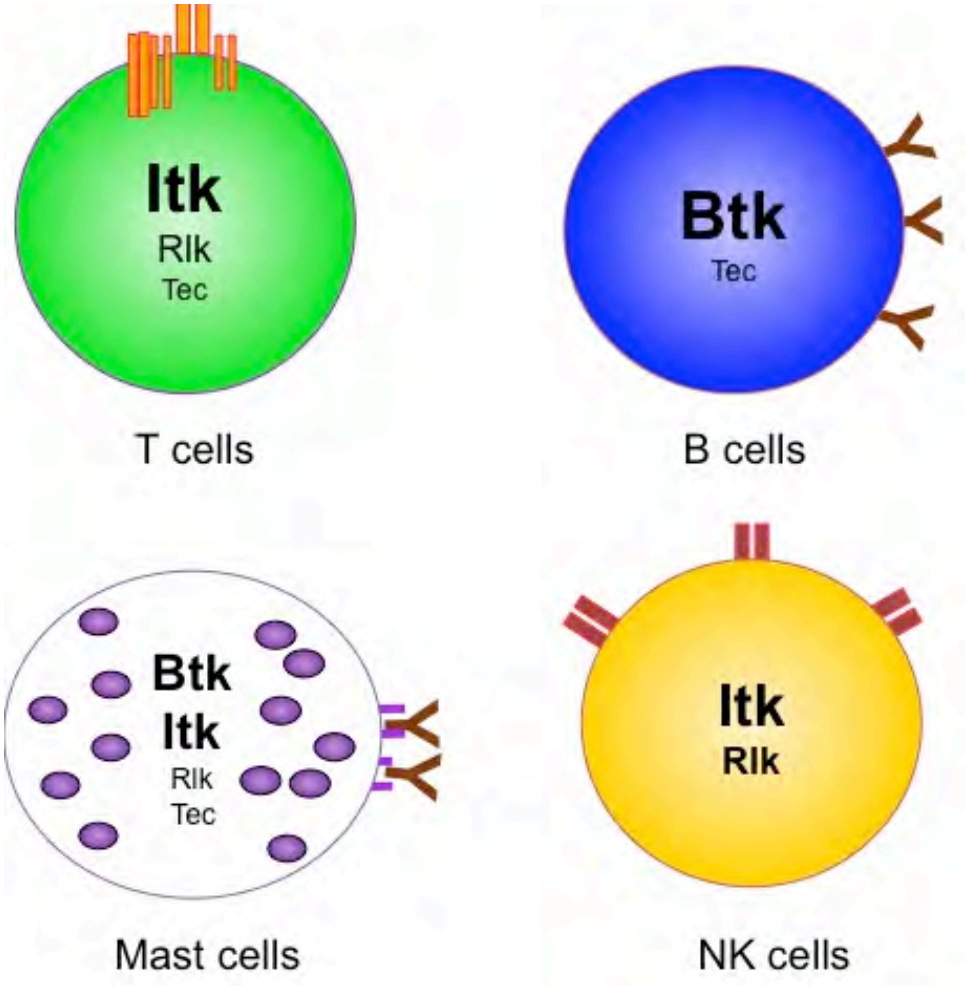

Figure 1.4 A representative expression pattern of Tec kinases in the immune system

The expression pattern of Tec family kinases frequently overlaps in many cells of the immune system. The relative amount of expression of each Tec family member is represented by the font size. For example, Itk is the dominant Tec family member in T cells, which contain less Rlk and even less Tec. Mast cells express four out of five members with Btk and Itk being co-dominant.

in vitro and *in vivo* experimentation using mouse models (72). Humans with genetic deficiencies in Tec kinases are highly susceptible to recurrent infections and increased mortality (116, 117).

Structure of Tec kinases

Close to 20 years ago, molecular cloning identified a multitude of new intracellular proteins from immune cells. These proteins were characterized by a catalytic tyrosine kinase region as well as molecular interaction domains like the Src-homology (SH) domain and the Pleckstrin-homology (PH) domain (118). It was unclear as to whether or not these proteins constituted a discrete PTK family (119). The cause for such confusion was apparent from the organizational structure of modular domains and the high degree of sequence homology with Src family kinases (120). For example, Tec kinases (as shown in Figure 1.5) have a catalytic kinase domain and SH domains (i.e. SH2 and SH3) that reside in the carboxy-terminus (C-terminus). Closer inspection of the amino-terminal (N-terminal) portion determined that these proteins comprise their own family of non-receptor tyrosine kinases (120, 121). In the N-terminal region, Tec kinases are distinguished from other PTK families by the presence of a PH domain and a Tec homology (TH) domain. The TH domain contains a Zn^{2+}- binding Btk homology motif and proline-rich regions (PRR). Noticeably absent from Tec kinases are the N-terminal myristoylation sites and C-terminal regulatory tyrosine residues found in Src family kinases (122).

Figure 1.5

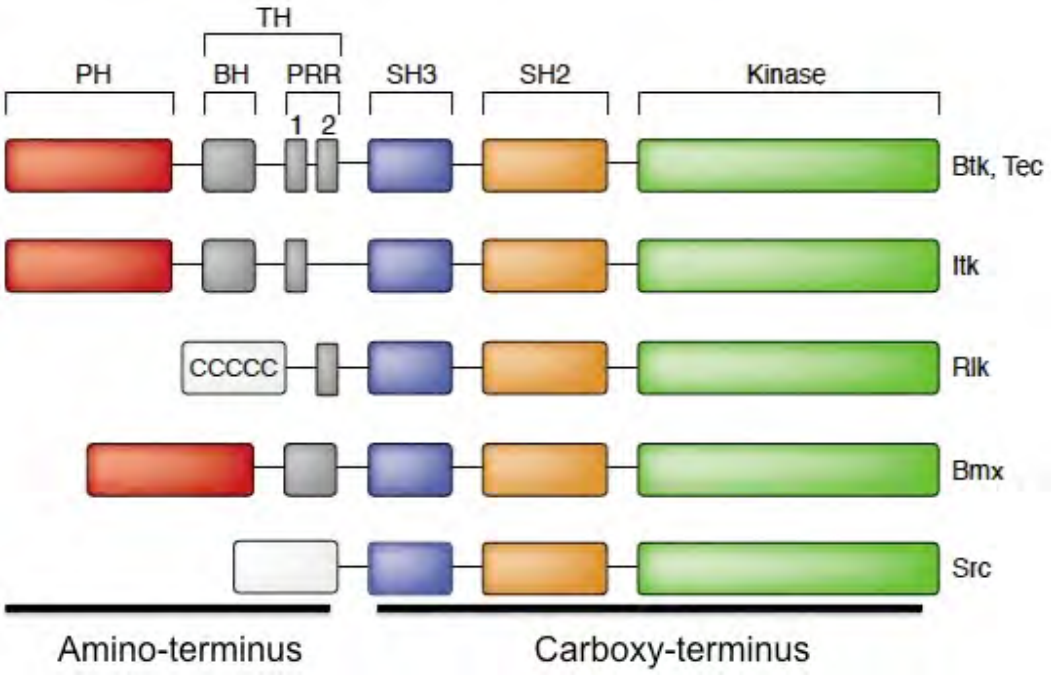

Figure 1.5 Structural domains of Tec family kinases

Adapted from Current Opinions in Immunology, 2002, vol. 14, pp. 331-340. Five mammalian kinases of the Tec family are compared with Src kinase, of the Src family. The C-terminal portion of the Tec family contains a high degree of homology (kinase, SH2, and SH3 domains) with the Src family. The N-terminal portion of the Tec family houses the Tec homology domains, which contains a Btk homology motif and proline-rich regions. Four of the five members are capped with a Pleckstrin homology domain. Rlk/Txk contains a cysteine string that is post-translationally modified. In comparison, Src, has an N-terminal membrane localization motif and a C-terminal regulatory tail.

Described above is the general structure of Tec family kinases. Some members have slight variations in the number or position of these regions. The exception to this organizational structure is Rlk/Txk. Rlk/Txk is a unique member that typically contains a cysteine-string motif, which allows for post- translational palmitoylation, in place of the PH domain. Overall, the structural features of the N-terminal region appear to be limited to the Tec family tyrosine kinases and, therefore, provide a unique mode of regulation in PTKs.

Regulation of Tec kinases

The intramolecular structure of Tec kinases places restrictions on their regulation and function. Well characterized regulatory mechanisms occur through components of the N-terminal portion of Tec family kinases (72). For instance, the PH domain plays a critical role in the subcellular localization of the Tec kinase Itk. Here, the PH domain of Itk allows it to become tethered to the plasma membrane upon generation of phosphatidylinositol (3,4,5) trisphosphate (PIP_3) (123, 124). This means that in a cellular resting state, when the levels of PIP_3 are low, PH domain-containing Itk is largely cytosolic. Conversely, when levels of PIP_3 are high, Itk aggregates at the membrane where kinase activity can ensue. Regulation of these events can be controlled by a number of proteins. Upon immunoreceptor ligation (e.g. TCR), phospoinositide 3-kinase (PI3K) becomes active and generates PIP_3 from the phosphorylation of phosphatidylinositol (4,5) bisphosphate (PIP_2). In contrast, phosphatase and

tensin homolog (PTEN) can directly counteract PI3K activity by dephosphorylating PIP_3 under inhibitory or homeostatic conditions (125). This action is instrumental in regulating Itk because PTEN-deficient T cells constitutively localize Itk at the plasma membrane.

This is not the sole means of Tec family kinase regulation via the PH domain. The association of the Tec PH domain with PIP_3 at the plasma membrane can be influenced by the activity of SH2-containing inositol-5-phosphatase (SHIP), which also diminishes the amount of PIP_3 in the membrane (126). Moreover, this mechanism appears to be mediated through a direct interaction between the SH3 domain of Tec and SHIP proteins.

Another mechanism for the regulation of Tec family kinases through PH domains includes a positive feedback loop promoted by Btk in B cells (127). The association of Btk with PI5K, upon BCR activation, promotes co-localization and recruitment to the membrane. Once there, PI5K can generate PIP_2 by phosphorylating phosphatidylinositol 4-phosphate (PIP). PI3K can then convert this product into PIP_3, which in turn promotes the recruitment of more PH domain-containing proteins to the membrane. This feedback loop is negatively regulated by SHIP, as ablation of SHIP promotes Btk-mediated BCR activation (128, 129).

Recruitment of Rlk/Txk to the plasma membrane is regulated in a completely different manner. The lack of a PH domain suggests a different mode of membrane localization. The existence of a cysteine-string motif permits the constitutive association of Rlk/Txk to the plasma membrane upon post-

translational palmitoylation. It has been further shown that association with the plasma membrane localizes Rlk/Txk to lipid raft microstructures, which cluster together upon immunoreceptor crosslinking (130).

In addition to the regulation that occurs at the plasma membrane, Tec kinases use their other domains to form protein-protein interactions. Ligation of immunoreceptors causes a recruitment of Tec family kinases to the receptor-signaling complex so that they may relay signals to the rest of the cell. This type of recruitment involves the nucleation of a large macromolecular complex. In T cells, this complex is scaffolded by the adaptor proteins linker for activation of T cells (LAT) and SH2-domain-containing leukocyte protein of 76 kDa (SLP-76). These two molecules are critically important for Itk to impose its function. The regulation of this event occurs through the specific phosphorylation of tyrosine residues on LAT and SLP-76 by the Syk family kinase zeta-chain-associated protein kinase 70 (ZAP-70). The molecular recognition of Itk by SLP-76 and LAT is regulated both directly and indirectly. Indirectly, Itk can associate with LAT through the adaptor protein growth factor receptor-bound protein 2 (Grb-2) via proline-rich regions in the TH domain (124). Direct regulation of Itk can occur through the SH2 domain. The importance of the Itk SH2 domain is highlighted in a SLP-76 mutant that prevents docking of Itk at Tyr 145, resulting in diminished downstream signal transduction (131). Because the PH domain of Itk is still present, the membrane localization of Itk is not ablated, only its kinase activity.

Kinase activity of the Tec family is initiated by tyrosine phosphorylation on a conserved activation loop within the catalytic domain (121). This results in a

conformational change that allows access to substrates (e.g. PLCγ). Though auto-phosphorylation of Tec kinases does occur, the activation loop is actually trans-phosphorylated by Src family kinases. This feature further distinguishes the Tec family from the Src family, in which auto-phosphorylation of the activation loop does happen. The auto-activation event that transpires in Tec kinases takes place on conserved residues in the SH3 domain and is best documented in Itk and Btk (132-134). The significance of this auto-phosphorylation event is unclear, though, as signal transduction is not impaired in B cells, but is diminished in T cells. It is likely that this event is more important in mediating protein-protein interactions than direct kinase activity (121). What is certain is that tyrosine phosphorylation of Tec kinases and subsequent conformational changes are crucial to proper activation of downstream targets.

One final major mode of regulation that occurs in Tec family kinases is inter- and intramolecular interactions. This is a mechanism at play during times of cellular homeostasis, ensuring that inadvertent kinase activity is not initiated. This type of phenomenon is well-documented in the Src family, where C-terminal negative regulatory residues stabilize an intramolecular interaction with its own SH2 domain (135). The Tec family, however, fails to possess these autoinhibitory sequences and must rely on other means to self-regulate. *In vitro* experiments have found that the SH3 domain of Tec, Btk, and Itk can interact with its adjacent PRR, essentially folding up on itself, to limit binding with other proteins (136-139). It appears likely, however, that intermolecular dimers preferentially form. A model set forth proposes that, with Itk for example, the

intermolecular interaction between SH3 and SH2 domains, and resulting homodimer, supersedes that of the intramolecular associations (140).

Taken together, the information detailed above demonstrates the existence of several layers of regulation for Tec family kinases. This is necessary because the Tec family is positioned in such a way as to relay the propagation of membrane proximal signaling events to major downstream pathways required for cellular activation. Dis-regulation of Tec kinases leads to severe signaling defects in B cells and T cells.

Itk

Several groups cloned Itk in the early 1990's using degenerate PCR to screen for novel tyrosine kinases in hematopoietic cells (141-145). Most of these reports found this newly discovered gene was expressed in T cells and was inducible in response to IL-2, hence the name IL-2-inducible T cell kinase. One report found further expression of this gene in mast cells and called it Emt (expressed in mast cells and T cells) (143). What's known about Itk in mast cells is limited and will be discussed at the end of this chapter. Much of what is known about Itk has been derived through *in vitro* and *in vivo* studies on murine T cells. Itk has been implicated in signaling downstream of the CD2, IL-2 (CD25), CD28, and chemokine receptors; its most renowned role, though, is downstream of the TCR (146-149).

Actions of Itk

The primary substrate of Itk in T cells is PLCγ1. As described previously, the activation of PLCγ is essential for integrating membrane proximal events with major downstream signaling pathways. Accumulation of PIP_3 in the plasma membrane promotes the aggregation of Itk to the cell surface via the PH domain. The SH2, SH3, and TH domains act as a molecular adhesive when they interact with adaptor proteins such as LAT, SLP-76, and Grb-2. This forms a large macromolecular complex, sometimes referred to as a signalosome (150). Itk can then be activated by the tyrosine phosphorylation of Src family kinases. The now accessible catalytic domain of Itk is then able to specifically phosphorylate PLCγ1 (151-153). This step regulates the production of secondary messengers like IP_3 and DAG, promoting further signal transduction and gene expression.

Itk is also involved in other aspects of TCR signaling such as cytoskeletal reorganization. A requirement for multiple domains, including the PH and SH2 domains, is necessary to induce TCR-mediated actin polymerization (154, 155). Subsequently, it was found that Itk could directly interact with Vav, a regulator of actin machinery, via its SH2 domain (156). Interestingly, this event does not require the kinase activity of Itk, indicating a novel function for Itk as an adaptor. It also indicates that the other modular domains of Itk are not simply required for its regulation, but rather are a functional unit in TCR signaling.

Itk deficiency

The clinical relevance of Itk was not fully appreciated until fairly recently when two young sisters developed a fatal lymphoproliferative disorder (117). These girls suffered from recurrent infections and a general inability to control EBV-induced B cell proliferation. This severe immune dis-regulation was genetically mapped to a candidate gene that encoded *Itk*. The result of a homozygous missense mutation in the SH2 domain of Itk led to unstable protein expression in these girls. Since this initial case, additional carriers of *Itk* mutant alleles have been found and, like human Btk deficiency, the mutations in Itk span across the protein (e.g. PH, SH2, and kinase domains) (157). Unlike Btk, however, the mortality associated with Itk deficiency is much higher, and treatment requires total replacement of the immune system by hematopoietic stem cell transplant (HSCT).

The initial body of research on the characterization of Itk carried out *in vitro* has yielded much information about the implicit nature of Itk. Much more was learned about Itk when Itk-deficient mice were made. The first report of Itk-deficient mice provided a physiological role for Itk in T cell development, showing a partially intact but severely altered T cell repertoire (158). Phenotypically this was demonstrated by an observation in the thymus of fewer total mature thymocytes, decreased CD4 T cells, and a skewed CD4:CD8 T cell ratio. Itk-deficient T cells had diminished proliferative capacity in response to a mixed lymphocyte reaction, mitogen stimulation, and TCR crosslinking. This defect in T

cells lacking Itk was rescued by using PMA and ionomycin, which bypasses membrane proximal signaling events. Moreover, supplementation with exogenous IL-2 also restored proliferation. This genetic ablation of Itk corroborated the TCR signaling defect from previous *in vitro* reports and also provided the field with a means to study Itk *in vivo*.

The second major report[c] using Itk-deficient mice made substantial headway into the biochemical mechanism by which Itk exerted its function (159). It was here that a significant link between Itk, Ca^{2+} mobilization, and IL-2 production was firmly established. Although intracellular stores of Ca^{2+} could be released, the potentiation of this major pathway by extracellular Ca^{2+} could not be sustained in the absence of Itk. This result correlated with diminished PLCγ1 phosphorylation and subsequent IP_3 generation. Importantly, defects in ZAP-70 activation or activity were not observed, placing Itk in line with or parallel to the initial transduction events mediated by ZAP-70. These two studies were milestones in the understanding of Itk and its role in T cell biology.

Since these studies, more information has been gleaned about Itk deficiency in mice. Itk-deficient mice have trouble controlling a number of infections as well as differential responses to acute hypersensitivities like atopic dermatitis and airway hyperresponsiveness (AHR). The role of Itk in allergies will be discussed later. In infectious models of parasitic challenge, $Itk^{-/-}$ mice cannot mount the protective T_H2-response required to control *N. brasilienis* or *S.*

[c] It should be noted that two different lines of Itk-deficient mice were created. The first mouse generated by Dan Littman and Charlene Liao targeted a region within the PH domain of Itk for deletion (158). Karen Liu created the second mouse in Leslie Berg's lab and excised a portion of the kinase domain of Itk (159). Both lines of mice do not express Itk protein.

mansoni (160, 161). This defect is accompanied by a decrease in the production of T_H2 cytokines such as IL-4, IL-5, and IL-13. Itk-deficient mice are also extremely susceptible to infection by the obligate intracellular parasite *T. gondii*, having a mean survival time of just over 2 months (162). Interestingly, Itk deficient mice were resistant to a challenge with another parasite, *L. major* (160). In this case, the T_H2 response normally present in wild-type mice was replaced by a protective T_H1 response, marked by IFNγ production, in Itk-deficient mice. These infection models indicated that Itk-deficient mice cannot mount effective T_H2 immunity and are also heavily skewed in their response towards a T_H1 type response. This may suggest that Itk-deficient mice respond appropriately to viral infections, which are classically associated with type-1 immunity and CD8 T cells. To the contrary, Itk is fundamental to mounting an optimal CD8 T cell-mediated response against viral pathogens such as lymphocytic choriomeningitis virus (LCMV) and vaccinia virus (VV) (163, 164). The defect in the CD8 T cell response is likely due to the altered developmental profile attributed to these cells in Itk-deficient mice (165).

One other major phenotype associated with Itk-deficiency is an inherent T_H2-like environment under homeostatic conditions. This condition develops normally in unmanipulated mice lacking Itk and is marked by extremely elevated levels of IgE in the serum. Because the cytokines IL-4 and IL-13 are required for the production of IgE, this sets up an apparent paradox in which mice that cannot mount effective T_H2 responses have excessive amounts of antibodies found in T_H2 responses. This issue is partially resolved when Itk-deficient mice are

crossed to mice lacking γδ T cells (166). Consequently, γδ T cells are, in part, responsible for the elevated levels of IgE in the serum in Itk-deficient mice and display an altered cytokine profile (i.e. elevated IL-4 and decrease IFNγ) in response to TCR ligation.

Overall the data on Itk deficiency indicates that Itk is pivotal in a number of immunological responses. Itk deficiency represents global defects in countless aspects of T cell biology and afflicts many different cell types (e.g. CD4 T cells, CD8 T cells, and γδ T cells).

The relationship of Itk and mast cells

The role of Itk in T cells is firmly established with decades of scientific experimentation as documented above. Many of the major functional characteristics of Itk in T cells have been described and are currently being expanded. Further, many of the *in vivo* phenotypes associated with Itk deficiency have been attributed to defects in T cell receptor signaling. By comparison, the role of Itk in mast cells is notably less characterized. Perhaps an analogous situation may be likened to the relationship of mast cells and immunology. Due to the contribution of mast cells to the pathology of many conditions, many early scientists viewed these cells with skepticism and beguilement (167). The following section will now delve into the important aspects of mast cell biology concluding with their rapport with Tec kinases.

The colorful history of mast cells

The discovery of mast cells, in the late 1870's, has more to do with the emergence of hematology than it does with immunology. While studying the chemical properties of aniline dyes (e.g. toluidine blue) and their utility in histological staining, Paul Ehrlich observed a unique cell type that stained metachromatically within connective tissues (168) (Figure 1.6). The reddish, violet granules in the cytoplasm were vastly abundant and gave the impression that the cell ingested them. For this reason, Ehrlich called the cells *mastzellen*, which roughly translates to 'overfed cells.' An important histological observation made by Ehrlich was that mast cells seemed to be excluded from the vasculature and confined to peripheral tissues. Furthermore, they were distinct from a previously described group of heterogeneous cells that Wilhelm von Waldeyer called *plasmazellen*[d].

The convergence of mast cells and immunology began during the quest to uncover the pathogenesis of allergies, and in particular anaphylactic shock. It was known by the 1920's that allergy, or hypersensitivity, was an immunological phenomenon, likely mediated by antibodies (170, 171). This was most evident in smallpox vaccinations where some patients would develop severe reactions to a booster. In the 1960's, it was learned that the reaginic antibodies responsible for

[d] Waldeyer's *plasmazellen* do not refer to the commonly recognized antibody-secreting cell of the B cell lineage, called plasma blasts or plasma cells. Classically, early hematologists and cell biologists, including German pathologist Rudolf Virchow, used the term plasma cell as almost a catchall term to encompass a cell type of unknown origin or even a space within connective tissues. In fact, Waldeyer's initial description of plasma cells was so ambiguous that it virtually included all cells containing granular protoplasm within the connective tissues (169).

Figure 1.6

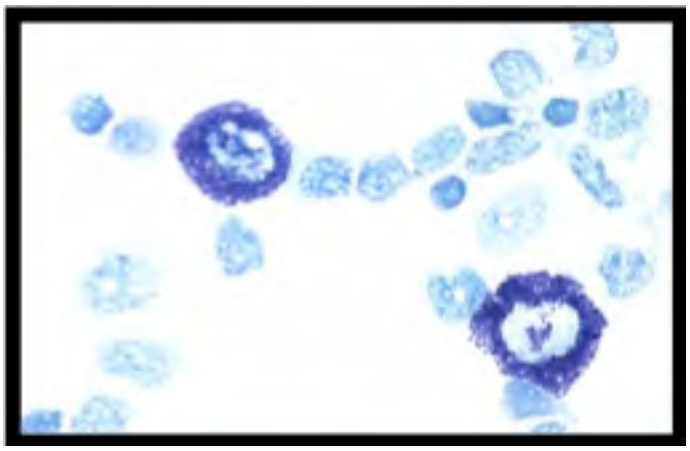

Figure 1.6 Mast cells from the peritoneal cavity

Cells from the peritoneum of wild-type mice were stained with Toluidine blue. Metachromatic staining of granules within two mast cells can be clearly seen. Image was courtesy of Yoko Kosaka.

these types of hypersensitivities were of the IgE isotype (63, 172). Concomitantly, independent experiments were conducted to investigate the pharmacological activity of histamine and heparin in canines. Once heparin was discovered to be the chromotrope that had famously reacted with Ehrlich's dyes, it was linked to mast cells in the liver. Around the same time, subcutaneous injections of histamine were discovered to elicit the classic wheal-and-flare reactions (hives) frequently associated with anaphylaxis (75). Finally, in the 1950's, Riley and West performed the key studies linking together histamine, heparin, mast cells, and anaphylactic reactions (173-176). In a stroke of ingenuity, it was realized that a chemical liberator of histamine, stilbamidine, would fluoresce under ultraviolet light and could be tracked to the source of histamine. Injection of this molecule into rats induced histamine release, as expected, and accumulated in the perivascular regions of connective tissues. As lines of fluorescent spots appeared along the walls of blood vessels, the localization of the dye and mast cells was indisputable. In fact, it was noted that some mast cells were less fluorescent than others, which corresponded with a loss of histamine. Riley and West bore witness to what is now known as mast cell degranulation. The studies outlined above helped forge the link between mast cells and allergies but perhaps more importantly, they set the stage for more in depth analyses of mast cell function and development.

Mast cell development, differentiation, and heterogeneity

Ehrlich's initial description of mast cells suggested that they might be derived from fibroblasts within the connective tissue. In 1977, it was reported, however, that mast cells could be generated in a classic bone marrow chimera experiment (177). Taking advantage of the unusually large granules found in the cells of beige mice (C57BL-Bg^J/Bg^J), Kitamura and colleagues engrafted the bone marrow from these mice into irradiated wild-type mice. Several months following reconstitution, it was observed that the vast majority of mast cells within a variety of peripheral tissues were donor-derived. Thus, like all cells of the immune system, the mast cell lineage can be traced to bone marrow and the hematopoietic stem cell (HSC) (Figure 1.7). Unlike the majority of hematopoietically-derived cells, mast cells complete maturation within their final destination (178). This indicates the existence of an intermediary between the bone marrow and peripheral sites. Due to the heterogeneity and plasticity of various progenitor cells that can give rise to mast cells, a committed precursor remained elusive. In the 1990's a mast cell precursor, defined by the expression of CD34, CD13, and c-Kit (CD117) and lack of FcεR1, was found in the blood of humans and mice (179-181) (182). This progenitor cell retained some plasticity, as it could develop into the blood basophil, but it was a monumental step in the right direction. More recently, a committed mast cell precursor was further characterized to express β7 and T1/ST2 (IL-33 receptor) in addition to CD34, CD13, and c-Kit (183). Additional delineation of mast cell poiesis has proven

Figure 1.7

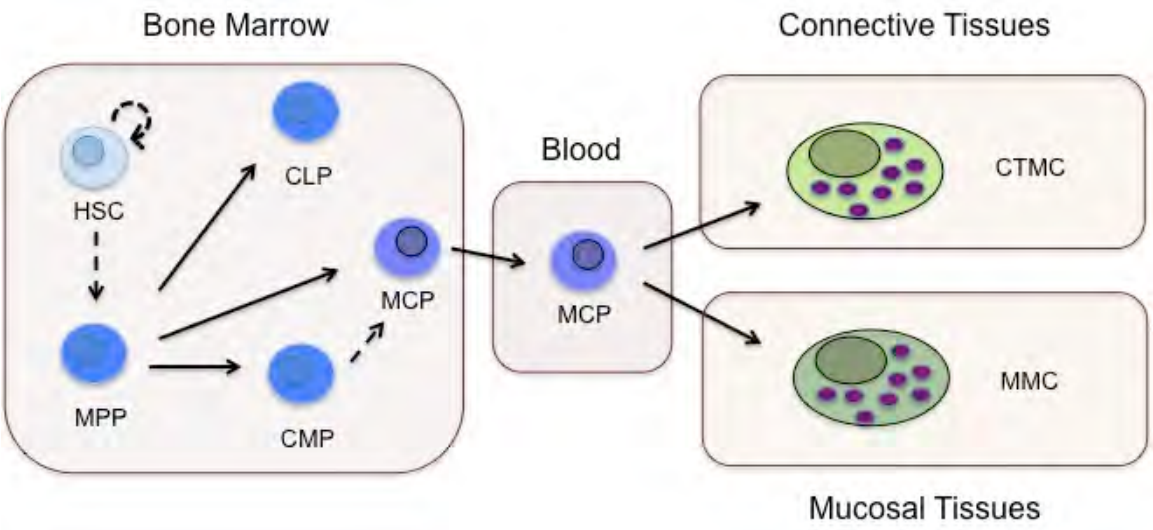

Figure 1.7 Mast cell development

Derived from the hematopoietic stem cell (HSC), the multipotent progenitor (MPP) can give rise to the common lymphoid progenitor (CLP), the common myeloid progenitor (CMP), and the mast cell precursor (MCP). The MCP can be found in the blood and is thought to migrate to peripheral tissues via chemokine receptors and integrins. Upon extravasation into peripheral tissues, the MCP can terminally differentiate into mature mast cells. Two major types of mast cells can be identified based on granule content, the connective tissue-associated mast cell (CTMC) and the mucosal associated mast cell (MMC).

difficult as the very subtle changes in gene expression between pluripotent cells and committed progenitors have yet to be resolved (63, 184).

Upon entry to peripheral sites, the mast cell precursor begins to populate the tissue and undergo terminal differentiation. Several factors may influence this process, including chemokine receptors (e.g. CCR3), adhesion molecules (e.g. β7), and signaling by cytokines (e.g. IL-3 & SCF) (185-187). The most significant determinant in mast cell differentiation is the ligand for c-Kit, stem cell factor (SCF) (188, 189). Several lines of evidence demonstrate the importance of the c-Kit – SCF signaling axis for mast cell development. First, the mast cell is the only known hematopoietically derived cell to express c-Kit for the duration of its life (189). Secondly, SCF can prevent apoptosis in mast cells starved for IL-3 (188). Further, IL-3-deficient mice have a slight decrease in total numbers of mast cells but are otherwise developmentally fine (190). Finally, mast cells are noticeably absent in several mice (e.g. $Kit^{W/Wv}$, $Kit^{W\text{-}sh/W\text{-}sh}$, and $KitL^{Sl/Sl\text{-}d}$) with mutations in c-Kit or SCF (191).

Terminally differentiated mast cells are not homogeneous. Several subsets of mast cells exist with distinguishing features such as granule content, morphological phenotype, and anatomical location (i.e. connective tissue-associated mast cells & mucosal-associated mast cells). Mast cell heterogeneity is not a new concept. In the 1960's and 1970's, mast cells were shown to exhibit differential histochemical staining patterns and reacted dissimilarly to compound 48/80, a known histamine liberator (192, 193). Histochemical staining was suitable until the discovery of countless mast cell proteases (MCP) (e.g.

esterases, metalloproteases, etc.) showed considerably more heterogeneity amongst mast cells (194). The gold standard in distinguishing mast cell subsets is through examination of the protease content within granules. In mice, two major classes of mast cells exist: connective tissue mast cells and mucosal mast cells (75). Mast cells in the airways and gastrointestinal tract express the chymases MCP-1 and MCP-2, whereas mast cells in skin contain chymases MCP-4 and -5 and the tryptase MCP-6. The evolutionary divergence between proteases in mice and humans has led to a slightly different nomenclature in humans (194). Mucosal-associated mast cells are tryptase positive (MC-T) while skin-associated mast cells are tryptase/chymase positive (MC-TC). The variety within the mast cell population may make mast cells appear abound and easily accessible. On the contrary, much of what is known about mast cells has only been uncovered within the last couple of decades due to advances in cell culture techniques.

Studying mast cells

The bone marrow-derived mast cell culture system

So, how can mast cells be studied in a highly controlled manner? Ideally, primary mast cells are purified from an animal model or from human samples. However, since mast cells are confined to tissues, isolation may be tedious, and the number of mast cells obtained may be too low to use (63). An alternative

approach must be used. As aforementioned, the derivation of pure mast cell cultures is *in vitro*. The determination of researchers to culture mast cells in this way led to the identification of several factors important for mast cell development *in vivo*. As previously described, the key experiments that opened the door to an *in vitro* culture system were the 1977 bone chimeras described previously (177). Building off their studies in beige mice, Kitamura and colleagues used a mast cell-deficient mouse strain ($Kit^{W/Wv}$), with a naturally occurring mutation in the white (*W*) locus, to demonstrate natural reconstitution of the mast cell population with congenic wild-type bone marrow (195). Within a few years, the culture of bone marrow-derived mast cells (BMMCs) was firmly established by several groups (196-200). The major growth factor required for long-term maintenance of these cells *in vitro* was found to be IL-3 (201-203). To date, the BMMC remains the go to culture system for immunologists studying all aspects of mast cell biology (Figure 1.8).

Mast cell-deficient mice

The use of mast cell-deficient mice has been a powerful tool in deciphering the *in vivo* functionality of mast cells for a variety of medically relevant maladies. Naturally occurring mutations in the dominant white spotting (*W*) and semi-dominant Steel (*Sl*) loci were known to eliminate mast cells in peripheral tissues (195, 204). Anemia, impaired pigmentation, and sterility were all phenotypes that characterized mice containing mutations at the *W* or *Sl* loci.

Figure 1.8

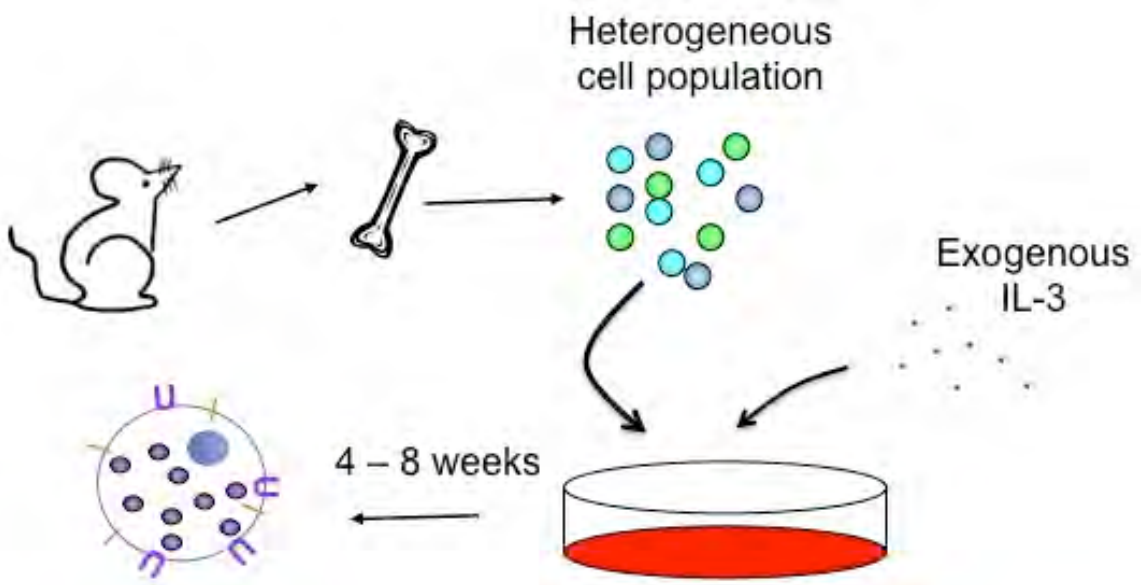

Figure 1.8 Bone marrow-derived mast cell culture system

Bone marrow from the femurs and tibias of mice are flushed and washed to obtain a heterogeneous mixture of cells. Culturing this mixture in the presence of exogenous IL-3 for 4 to 8 weeks generates a highly pure population (>95%) of bone marrow-derived mast cells.

Moreover, a gene dosing effect exists because the severity of afflictions varies depending on which alleles are present. For example, KitL$^{Sl/+}$ mice have a less severe form of anemia than KitL$^{Sl/Sl\text{-}d}$ mice (205). KitL$^{Sl/Sl}$ mice are embryonic lethal due to defects in erythropoiesis. A similar phenomenon occurs at the *W* locus as well. The differences between the two loci, from the mast cell's perspective, were not fully appreciated until Kitamura and colleagues performed their bone marrow chimera experiments in these mice. As explained above, Kit$^{W/Wv}$ mice could overcome their mast cell-deficiency with bone marrow transplantation from a wild-type donor; this was not true in KitL$^{Sl/Sl\text{-}d}$ mice, however, and suggested that the abnormality in the *Sl* locus was intrinsic to the host and not the mast cell (204). It took nearly ten years for researchers to figure out that the *W* locus encoded the transmembrane receptor, c-Kit, and the *Sl* locus encoded the cytokine SCF (205-207). By this time, several lines of mice containing c-Kit or SCF mutations had been used as a source of mast cell-deficiency. Currently, the two most prominent models are the Kit$^{W/Wv}$ mice and Kit$^{W\text{-}sh/W\text{-}sh}$ mice (208).

The Kit$^{W/Wv}$ mice that Yukihiko Kitamura used are sterile and must be derived from a WB/ReJ x C57BL/6 cross. Closer inspection of this cross and numerous other *W* mutations revealed that allelic variation in mice resulted in graded phenotypic outcomes dependent on the expression and functionality of the c-Kit receptor (209). The original *W* allele is classified as a severe lethal mutation and generates a truncated form of c-Kit lacking the transmembrane region. Although the protein is still expressed, it does not localize to the plasma

membrane. The W^v allele, on the other hand, exhibits a much weaker phenotype than the *W* allele. A single amino acid substitution (T → M) at position 660 results in stable c-Kit expression, on the cell surface, but decreased kinase activity. Crossing these alleles results in viable $Kit^{W/Wv}$ litters. However, the reduced signaling of c-Kit results in macrocytic anemia, sterility, and pigmentation defects due to diminished melanocytes (210). Further, impairments in the gastrointestinal tract show a complete lack of interstitial cells of Cajal as well as a reduction in intraepithelial γδ T cells (191). Nonetheless, $Kit^{W/Wv}$ mice have been used extensively for mast cell studies and despite the extraneous phenotypes associated with this mouse strain much has been learned.

Given the tremendous 'off-target' defects present in $Kit^{W/Wv}$ mice, researchers have found the $Kit^{W\text{-}sh/W\text{-}sh}$ mice to be a more suitable alternative in studying mast cells *in vivo*. Although the W^{sh} mutation was discovered almost 30 years ago, mice carrying this allele have only recently found their way into mainstream immunology research (191, 211). Mice carrying the homozygous W^{sh}/W^{sh} mutation arose spontaneously from a C3H/HeH x 101/H mouse cross and are called *W-sash* mice. Unlike many other *W* alleles, the W^{sh} mutation is not located in the coding region of the gene; instead an inversion in upstream regulatory elements results in altered transcriptional activity of the c-Kit gene (212). This is significant because the more severe ailments afflicting the other *W* mutant mice seem to appreciably absent in the *W-sash* mice (191). Notably, *W-sash* mice are fertile and lack an anemic phenotype, yet still retain mast cell deficiency (211, 213). These mice, however, are not devoid of all phenotypes

associated with c-Kit mutations. The most noticeable alteration in these mice is the lack of pigmentation on their fur coat, which can be used for genotyping. An exhaustive comparison between the phenotypes of $Kit^{W\text{-}sh/W\text{-}sh}$ and $Kit^{W/Wv}$ mice is ongoing, but thus far the *W-sash* mice seem to have become the gold standard in assessing mast cell function *in vivo*.

More recently, several new lines of mice that are genetically deficient in mast cells have been developed (208). Importantly, these mice retain the c-Kit – SCF signaling axis vital for mast cell development and differentiation. Many of these new mice make use of Cre-recombinase insertions to alter the genomic DNA of a targeted allele. The usefulness of each type of mouse has not yet been fully established and all but one mouse retains some peripheral mast cells. The Cre-master mice, on the contrary, lack terminally differentiated mast cells in the peripheral tissues but retain a pool of MCPs in the circulation (214). The insertion of the Cre-recombinase gene in the locus that encodes carboxypeptidase A3 (Cpa3) induces p53-mediated apoptosis suggesting that this mutation is genotoxic. Interestingly, mast cell numbers in $Cpa3^{Cre/+}$ mice crossed to $Trp53^{-/-}$ mice (p53-deficient) are only partially restored. This observation suggests that Cpa3 is potentially significant in the terminal differentiation of mast cells. A possible alternative to this hypothesis is that the Cpa3 protease is important in creating a niche for mast cells within the tissues and without it they can no longer gain a foothold. The full meaning of these findings remains to be seen.

Mast cell function and products

Mast cells play a role in an assortment of physiological processes. Immunologically, they can respond to a variety of microbial pathogens, mediate hypersensitivity, and infiltrate tumors for immunosurveillance (208, 215). More recently, mast cells have been linked to cardiovascular diseases such as atherosclerosis as well as metabolic disorders like diabetes and obesity (216). Functionally, mast cells orchestrate these processes primarily through the release of mediators at the site of activation. Activation of mast cells, in these aspects, is a multiphase process that exerts effector function in waves beginning with degranulation and culminating in *de novo* synthesis of mast cell products. The list of mast cell products shown in Table 1.2 is immense and only depicts a small sampling. The multitude of products permits mast cells to respond in a variety of manners tailored towards the stimuli. As such, the types of mediators generated by mast cells can largely categorize activation into two classes: preformed and newly synthesized.

Degranulation and preformed mediators

The hallmark of mast cell activation is degranulation and the release of preformed mediators, such as biogenic amines and proteases, into the microenvironment. Degranulation was one of the earliest recognized functions of mast cells, as noted above, and remains one of the quickest responses elicited

Table 1.2

Class of Product	Examples	Function
Preformed Mediators		
Biogenic amines	Histamine, Serotonin, Dopamine, Polyamine	Vasodilation, Angiogenesis, Mitogenesis, Vasoconstriction, Pain, Neurotransmitter
Proteases	Tryptase, Chymase, Granzyme, Cathepsins	Tissue damage, Inflammation, Pain, Activation of PAR, Angiotensin II synthesis
Proteoglycans	Heparin, Hyaluronic acid, Chondroitin sulfate	Angiogenesis, Connective tissue component, Cartilage synthesis, Anti-inflammatory
Cytokines	TNF-α, IL-4, IL-15	Inflammation, Leukocyte regulation
Newly Synthesized Mediators		
Lipid mediators	Leukotriene, Prostaglandins	Leukocyte chemotaxis, Vasoconstriction, Pain, Bronchoconstriction
Cytokines	TNF-α, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-18, IL-25, IL-31, IFN-α, IFN-β, IFN-γ	Inflammation, Leukocyte migration, Pain
Chemokines	CCL2/MCP-1, CCL3, CCL4, CCL5/RANTES, CCL7, CCL11, CCL13, CCL20, CXCL1, CXCL2, CXCL8/IL-8, CXCL9, CXCL10, CXCL11, MIP-1α, MIP-1β	Chemoattraction, Tissue infiltration of leukocytes

Table 1.2 Mast cell products

The table above is a partial list of the mast cell products. It is divided into two major classes: preformed mediators and newly synthesized mediators. Examples are shown in the second column with a brief description of function in the third column.

by the immune system. The physiological consequences of mast cell degranulation are quite diverse and range from local inflammation at the site of activation to systemic regulation of the blood vessels and airways (10).

Most of the immediate effects of mast cell degranulation can be attributed to the actions of readily soluble biogenic amines (217). The synthesis of these molecules comes from the catalysis of available amino acid substrates by their corresponding enzymes within the cell. Histamine is the most well known biogenic amine and has been shown to regulate the vasodilation, capillary permeability, and bronchoconstriction frequently implicated in allergic reactions such as anaphylaxis. Histamine is generated by the decarboxylation of histidine, which is catalyzed by histidine decarboxylase.

Another well-studied biogenic amine in mast cells is the neurotransmitter serotonin. Serotonin is synthesized in the one-step hydroxylation of tryptophan by tryptophan hydroxylase and has been linked to immunomodulation, mast cell adhesion and chemotaxis, and tissue regeneration (218-220). Other biogenic amines such as polyamine and dopamine are also suspected of residing within mast cell granules but their role in immune responses is less clear (10). Once generated, biogenic amines are thought to be packaged in granules by proteoglycans and upon release are poised to act immediately within the local environment as well as in distal peripheral tissues (221).

Surprisingly, the effects of some preformed mediators are not immediately elicited. These mediators, instead, take time to solubilize in the microenvironment and disseminate systemically (37). It is thought that the

charged residues of proteins and proteoglycans within the granule stabilize an interaction such that particulate dissociation takes place slowly following secretion (222). One example of this is the interaction between glycosylated heparin and mast cell proteases; the highly negative charge of heparin sequesters positively charged proteases. Interestingly, this mechanism is likely responsible for the distribution of preformed inflammatory cytokines (e.g. TNF) to the draining lymph nodes during an immune response (223).

The two most common proteoglycans found within mast cell granules are the serglycins heparin and chondroitin. These molecules consist of a peptide core that is glycosylated with sulfated glycosaminoglycans (GAGs). The glycosylation pattern displayed by serglycins varies greatly depending on the cell type in which they are present. For example, heparin GAGs are frequently found in connective tissue-associated mast cells, while chondroitin GAGs are prominent in mucosal-associated mast cells (1, 3). Contrary to the well-known roles as an anti-coagulant (i.e. heparin) and in cartilage integrity (i.e. chondroitin), it appears that the major function for serglycins within mast cells is to mediate packaging and secretion of granules (6).

Accounting for greater than 25% of the total cellular proteome, the most abundant proteins within mast cells are proteases (8). Typically, proteases are stored as prozymes that are activated by cleavage of a peptide leader upon secretion. Surprisingly, the majority of mast cell proteases are stored as fully active enzymes (10). This potentially destructive situation is subverted by stabilization with proteoglycans thereby limiting substrate exposure. As noted

above, the variety of proteases and their distribution are major components of mast cell heterogeneity. Multiple types of proteases are secreted during degranulation, including metalloproteases (e.g. carboxypeptidase A) and cathepsins (e.g. cathepsin G) (12). The major protease classes in mast cells, though, are the trypsin-like (i.e. tryptase) and chymotrypsin-like (i.e. chymase) peptidases. Mast cell tryptases selectively target protein substrates for cleavage at residues proceeding lysine and arginine. They are involved in elements of pruritogenic activity and nociception via the neuronally expressed protease-activated receptor (PAR-2) (14, 16). Chymases have a propensity to cleave target proteins at aromatic residues (e.g. phenylalanine and tyrosine) and have been directly implicated in cardiovascular regulation through the formation of angiotensin II (17). On top of the non-overlapping functions just mentioned, tryptases and chymases promote general inflammation by degrading the extracellular matrix and facilitating leukocyte infiltration in response to pathogens (19).

Mast cells are capable of delivering a rapid infusion of pro-inflammatory and anti-inflammatory cytokines into the microenvironment during an immune response, which in part, is accomplished by the release of preformed stores. This was first demonstrated in 1990 when it was found that mast cells represented a viable source of preformed TNF (22). It is unclear whether the major effects of TNF are due to preformed stores or *de novo* expression (10). It is very clear, though, that mast cell-derived TNF is pleiotropic in its actions. In this regard, TNF can promote the migration of dendritic cells, induce neutrophil

recruitment, and enhance cytokine production in T cells (26, 28, 30, 31). Moreover, mast cell-derived TNF can have a systemic influence in the outcome, both beneficial (e.g. *Escherichia coli*) and detrimental (e.g. *Salmonella typhimurium*), of bacterial infections (32, 34). Other preformed cytokines that can be released by mast cells include IL-4 and TGFβ, but again, the roles of the preformed versions of these cytokines is uncertain (35, 36).

By virtue of densely compact granules, mast cells are able to act swiftly in the face of stimulation through release of preformed mediators. The location within peripheral tissues that mast cells reside indicates that they act as sentinels and surveyors of the immune system. These two facts have prompted researchers to label mast cells as 'first responders' to environmental insults such as microbes and allergens (37).

De novo synthesis of mediators

The second major component of mast cell activation takes place following degranulation and consists of *de novo* synthesis of mast cell products. Most major stimuli of mast cells induce signal transduction, transcriptional activity, and *de novo* synthesis of gene targets like cytokines and chemokines. In addition, the natural cellular metabolism associated with mast cell activation generates metabolites, such as eicosanoids, that play a role in many allergic reactions. These two categories of *de novo* mediators that mast cells manufacture can be classified into protein products or lipid metabolites.

Of the protein products that mast cells express, cytokines are by far the most expansive (Table 1.2). Mast cells were first found to synthesize and release cytokines in 1987 (39, 40). As discussed above, mast cells have a prominent role in both local and systemic inflammation, which can be partially attributed to the actions of TNF. In the same vein, mast cells can promote immunity and survival to peritonitis through the production of the T_H1-type cytokine IL-12 (41). Mast cells are probably most noted for their ability to mediate type-2 immunity; they are a potent reservoir for T_H2-associated cytokines like IL-4, IL-5, and IL-13 (43). The importance of mast cell derived-type-2 cytokines can be seen in the response to a variety of extracellular bacteria and multicellular parasites. This is aptly exemplified in the immune response to parasitic helminthes where IL-4 and IL-13 promote the formation of granulomas around the pathogen sequestering it from host tissues (45). Mast cells can also promote immunity through the production of chemokines, which serve to recruit other immune cells to sites of infection by extravasation and chemotaxis (37). For instance, mast cell-derived CXCL-8 (i.e. IL-8) causes NK cell migration when stimulated with nucleic acids frequently associated with viral replication (54). The massive collection of cytokines and chemokines allows mast cells to modulate the actions of responding leukocytes, placing them at the center of an immune response to a number of microbial infections.

Activation of mast cells results in the rapid synthesis of a class of lipid metabolites known as eicosanoids. These inflammatory lipids are generated from the cleavage of arachidonic acid from membrane bound phospholipids by

the signaling enzyme phospholipase A_2 (58). Multiple catalytic events using a variety of enzymes, notably cyclooxygenase and lipoxygenase, ultimately derive prostaglandins, leukotrienes, and thromboxanes (61). These mast cell-derived lipid mediators are known to cause acute allergic reactions including nasal discharge, bronchospasms (sneezing & coughing), and urticaria (wheal-and-flare reactions) (63).

The culmination of signal transduction in mast cells is the expression and synthesis of lipid-derived and protein products. The vast range of newly synthesized mediators allows mast cells to orchestrate the assault on microbial infections for the duration of an immune response. The tailored response of mast cells is the basis for their flexibility in regulating cells in the immediate vicinity as well as systemically. The immensity of their response, unfortunately, is also central in the pathogenesis of allergic reactions. Taken together, the mediators that mast cells produce contribute to the broad functions ascribed to these cells.

Mast cell Activation

Observed almost a century ago, the classic depiction of mast cell activation is degranulation, which is the exocytosis of preformed mediators contained within secretory granules. Not until the last 25 years or so, was it appreciated that mast cells undergo a second wave of activation epitomized by the generation of cytokines (65). Complete mast cell activation includes both

degranulation and *de novo* synthesis, though the latter is capable of occurring independent of degranulation in response to some types of stimuli. Nonetheless, mast cell activation can be triggered through a variety of receptors expressed within the cell and on the plasma membrane. Mast cells employ these receptors to facilitate immunity, regulate the pathogenesis of allergies, and participate in physiological disorders. In the context of an immune response, mast cells can be alerted to the presence of microbes directly by virtue of Toll-like receptors (TLR) or indirectly through ligation of complement receptors, for example.

IgE-independent activation

Increasing evidence has suggested that mast cells are crucial to the innate immune response against pathogens (37). This is accomplished primarily through the action of surface TLRs, such as TLR-2 and -4, and intracellular TLRs, such as TLR-7 and -9 (69). Mast cell responses to TLR stimulation can vary tremendously and are highly dependent on the microbial product present (71). LPS stimulation of TLR-4, for example, can induce cytokine production from mast cells independent of degranulation. On the other hand, a complete mast cell response is observed when TLR-2 is engaged by peptidoglycans. Moreover, mast cell activation through these two TLRs induces differential cytokine expression; TLR-4 promotes IL-1β whereas TLR-2 induces IL-4 and IL-5. TLR signaling in mast cells, like other cells, is initiated by the recruitment of a single adaptor molecule or a specific combination of adaptors, which includes

MyD88, TRIF, TRAM, or TIRAP (73). The signal transduction cascade that ensues culminates in the activation and mobilization of transcription factors, like NF-κB and IRF3. TLR stimulation of mast cells primarily leads to the expression of inflammatory products (e.g. cytokines and chemokines) in a degranulation-independent manner (75).

The complement system can indirectly and directly alert mast cells to the presence of a pathogen through ligation of complement receptors on the cell surface. The proteolytic products of C3 convertase (i.e. C3a and C3b) have differential effects on mast cells. Ligation of C3a to its receptor C3aR promotes the migration of mast cells to sites of inflammation, whereas C3b forms a covalent bond with bacterial surfaces priming them for opsonization via the receptor C3R (CD11b-CD18) on mast cells (69, 77). Furthermore, C3a can potently induce mast cell degranulation in several types of immune responses (79).

IgE-dependent activation

By far, the most well characterized receptor on mast cells is the high-affinity IgE receptor, FcεR1. The discovery of receptors for IgE was facilitated by a series of studies, primarily from Henry Metzger and colleagues in the mid 1970's (81, 83, 84). These experiments used rat immunocytomas that secreted copious amounts of IgE to show specific binding at the surface of rat basophilic leukemia (RBL) cells, a frequently used mast cell line. Subsequent analysis of

the IgE receptor revealed abundant expression on mast cells and an affinity for IgE on the order of K_D 10^{-11} M (85). It was further shown that crosslinking the receptor with IgE dimers was sufficient to induce degranulation. In 1989, FcεR1 was finally cloned and discovered to comprise a tetrameric complex assembled from four subunits: the IgE-binding α, a transmembrane β subunit, and two γ chains (2, 4, 5, 224).

The foremost activation pathway responsible for the majority of mast cell-mediated immune responses is transduced through the FcεR1. IgE-dependent activation occurs when IgE-coated FcεR1 molecules on mast cells encounter antigens or allergens specific for that antibody in the periphery. As shown in MC-Figure 1.9, crosslinking of the FcεR1 initiates an impressive signal transduction cascade invoking classical intracellular pathways, such as MAPK activation and Ca^{2+} mobilization. The primary signaling events occur when immunoreceptor tyrosine-based activation motifs (ITAMs), within the β and γ signaling chains, become phosphorylated through the kinase activity of Src family kinases such as Lyn (7, 225). This event is thought to be associated with membrane microstructures called lipid rafts (9, 226). In turn, the recruitment and activation of Syk kinases leads to the nucleation of a macromolecular complex consisting of adaptor proteins (e.g. LAT, SLP-76, Gads, Grb2, and Vav) and enzymes (e.g. PLCγ, PI3K, Btk). Correct assembly of this complex is of the utmost importance as mast cell activation, both degranulation and gene expression, is severely diminished when many of these molecules are missing (11, 227-229). Interestingly, a complementary pathway can be induced at this point by the Src

Figure 1.9

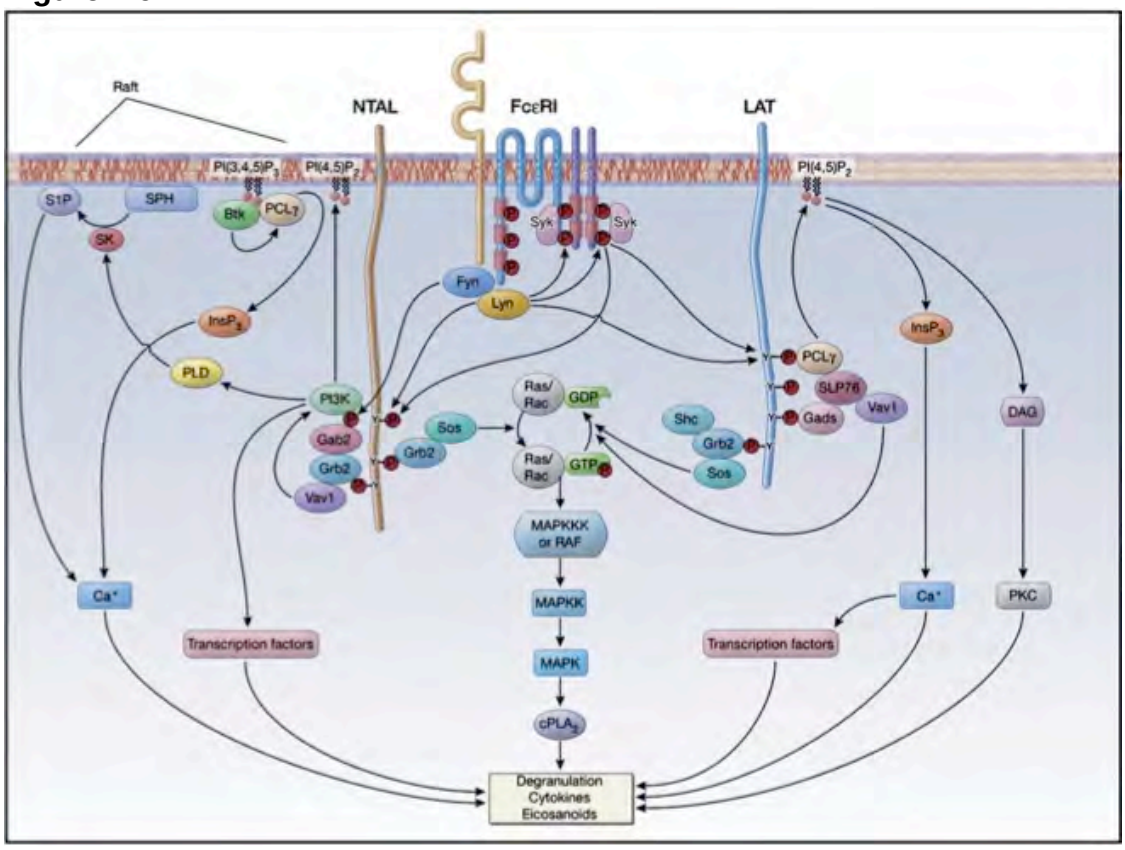

Figure 1.9 FcεR1 signal transduction

The schematic above was adapted from the Journal of Allergy and Clinical Immunology, 2006, vol. 117, pp. 1214-1225. It depicts the important players and pathways in FcεR1-mediated signaling. A more detailed description is in the text. Briefly, ligation of the IgE-bound FcεR1 by antigen recruits Src, Syk, and Tec family kinases. Activation of these PTKs promotes the assembly of two membrane proximal signaling complexes scaffolded by LAT and LAT2. These complexes regulate the generation of secondary messengers, IP_3 and DAG, which activate major downstream pathways including Ca^{2+} mobilization and the MAPK cascade. The culmination is degranulation, production of lipid mediators, and gene expression.

family kinase Fyn, which promotes the formation of another scaffold primarily held together by LAT2 (NTAL) (13, 230). The significance of these two branches is not completely understood but it is clear that substantial 'cross-talk' exists amongst these pathways. What is known is that the LAT pathway provides the principal activation of PLCγ, which can be regulated by Tec family kinases, whereas the LAT2 pathway appears to provide a means for PI3K activity via the adaptor Gab2 (15, 231). It should be noted that PI3K is also recruited and activated in the LAT complex as well, highlighting the amount of 'cross-talk' that can occur.

With this in mind, phosphorylation of PLCγ by Tec kinases (e.g. Btk) can initiate the production of important second messengers like DAG and IP_3 from the hydrolysis of PIP_2. This is a critical step linking membrane proximal events to the two major downstream signaling pathways in mast cells (i.e. Ca^{2+} and MAPK) (18, 232). Specifically, IP_3 induces the release of intracellular Ca^{2+} stores primarily held in the endoplasmic reticulum, which in turn leads to opening of CRAC channels thereby flooding the cells with extracellular Ca^{2+}. The transient increase in cytosolic Ca^{2+} allows for the direct regulation of the phosphatase calcineurin, a gatekeeper for NFAT activity. Dephosphorylation of NFAT allows for nuclear translocation where target genes can be expressed. DAG primarily regulates the second major pathway elicited by FcεR1 signaling. Unlike IP_3, DAG remains membrane bound where it can interact with and activate PKC family members (e.g. δ, ε, and θ) in a Ca^{2+}-independent manner. PKC activity potently induces several MAPK molecules including Erk1/2, Jnk, and p38. The

MAPK pathways promote the activation of major transcription factors, like AP-1 and NF-κB, which regulate the transcription of many mast cell products.

Tec kinases in mast cells

Mast cells express four of the five Tec family kinases: Btk, Itk, Rlk, and Tec. Very little is known about the role of Itk, Rlk, and Tec in mast cells; Btk, on the other hand, is very well characterized (20, 21, 72). Kawakami and colleagues spearheaded a number of studies investigating the role of Btk in FcεR1-mediated mast cell activation (23, 24, 233). They first showed that Btk was phosphorylated in response to FcεR1 crosslinking (25, 234). Degranulation and production of lipid mediators (i.e. leukotrienes) were diminished in mast cells lacking Btk (27, 29, 235, 236). The nature of signaling defects became apparent when it was shown that Btk-deficient mast cells made less IL-2, TNF, IL-6, and GM-CSF (33, 237). The general phenotype associated with mast cells lacking Btk is strikingly similar to Btk-deficient B cells, which is to say that signal transduction is severely impaired (11, 235, 238). The one major distinction between these two cells types regarding Btk is the developmental requirement. B cell development is severely impaired in two different mice with mutations in Btk, whereas mast cells develop normally (38, 237, 239). Furthermore, the human immunodeficiency, x-linked agammaglobulinemia, demonstrates the clinical importance of Btk in B cells (11, 113, 114). In general, the overall role of Btk in FcεR1-mediated mast cell activation is one of positive regulation.

Despite being cloned from mast cells, for about 10 years there was very little known about Itk and this cell type (42, 143). During this initial period, it was found that Itk was phosphorylated and recruited to the plasma membrane upon FcεR1 stimulation (44, 240). Subsequently, studies done by two groups have been performed examining the role of Itk in mast cells *in vivo* (46-48, 241-244). In these studies, a model of allergic airway responsiveness was used to examine airway hypersensitivity in Itk-deficient mice or in $Kit^{W/Wv}$ mice reconstituted with BMMC lacking Itk (49-53, 241, 244). To much dismay, the results of each report are at odds with each other. In Itk-deficient mice, mast cell degranulation was severely impaired despite wild-type levels of reaginic antibodies. Moreover, leukocyte infiltration and type-2 cytokines were absent when these mice were challenged with allergen. These results suggest that Itk is required to facilitate mast cell responses, *in vivo* (55-57, 241).

A second report used a reconstitution model and found normal airway responsiveness in mice containing Itk-deficient BMMC (59, 60, 244). They concluded that the amount of natural IgE in mice lacking Itk potentially confounded the results of the previous study by demonstrating that degranulation remained intact in mast cells lacking Itk. These results correlated with *in vitro* observations that degranulation was normal in Itk-deficient BMMC. Oddly, increased cytokine production over wild-type BMMC occurred in these cells when stimulated through FcεR1. This was unexpected because the intracellular defect that was observed actually showed diminished signal transduction, when quite the opposite would be predicted (62, 244). Thus, the limited information about

the role of Itk in mast cells is incomplete, conflicting, and perhaps even more turbid. More work is required to appreciate the role of Itk in mast cells.

Thesis Objectives

Itk is a major player in the signal transduction of T cells. The lack of Itk leads to a severe reduction, but not a complete loss, of TCR signaling even in the face of very weak signals. In contrast, the role of Itk in the signal transduction of mast cells is less clear. The goal of this thesis is to explore the role of Itk in modulating the activation of T cells and mast cells. The major objectives of this dissertation are stated below.

1. To examine the influence of TCR signal strength on the activation of CD8 T cells (Chapter II).
    a. Determine the contribution of TCR – pMHC interactions on the expression of TCR responsive genes in CD8 T cells.
    b. Assess the involvement of the Tec kinase Itk in altering TCR signal strength.
    c. Examine the major downstream pathways involved in the regulation of TCR signal strength.
2. To investigate the role of Itk in the activation of mast cells (Chapter III).
    a. Examine the immediate and late phase responses of mast cell activation in the context of Itk deficiency.

b. Identify signaling abnormalities associated with Itk deficiency.

c. Mechanistically characterize Itk's role in the involvement of mast cell activation.

Chapter 2:

Modulation of TCR signal strength reveals an analog response in T cells that correlates with Itk activity

Chapter II Attributions

The material in chapter II has been submitted for publication. The research contained within this chapter is my own work [JE].

Introduction

The T cell antigen receptor (TCR) is the gateway to T cell activation. It is charged with engaging peptide-MHC (pMHC) at the interface of an immunological synapse and transducing information inside the cell. Potentiation of this information is achieved through numerous signaling cascades utilizing molecules such as kinases, phosphatases, and adaptors (62, 64). The culmination of these signaling events results in the mobilization of a transcription factor network that ultimately directs the myriad biological functions that a T cell employs during an immune response.

How a T cell sees antigen in the context of pMHC can have dire consequences for that cell (38, 66). During thymopoiesis, for example, signals emanating from the TCR that are too strong lead to negative selection, whereas signals that are too weak are neglectful. The end result of both of these signaling events is apoptosis. Only those cells receiving signals of intermediate strength survive by positive selection. A precedent, therefore, is set by the TCR to recognize pMHC in such a way as to induce just the right amount of signal to respond appropriately (67, 68, 92). Demonstration of the importance of TCR signal strength is not hard to come by, nor is it limited to the thymus. Indeed, signal strength has been implicated in such cellular processes as CD8 T cell differentiation (70, 245) and cell division (72, 246). It also has far reaching systemic effects on the way one fights infection (74, 247) or copes with

autoimmunity (76, 248). Yet, the precise molecular mechanisms that control TCR signal strength remain to be completely elucidated.

To aid in the understanding of signal strength, TCR signaling can be classified as digital or analog. Digital signaling can be characterized by switch-like events that trigger on or off effects by T cells. There is no intermediary. On the other hand, analog signaling occurs when the signal input is proportional to the response elicited by the T cell. A range of intermediates exist between the on or off responses seen in digital signaling.

Several examples of digital signaling cascades have been reported in T cells. The majority of the components necessary to activate NF-κB are digital in response to stimulation with anti-CD3 antibodies (78, 88). This includes the formation of the Bcl-10, Carma1, and Malt1 (BCM) complex, IκB degradation, and NF-κB nuclear translocation. In addition to the NF-κB pathway, the MAPK pathway has been shown to propagate digital signals in response to TCR stimulation. An analog to digital switch is thought to occur at the level of Ras activation (80, 249). Here, a positive feedback loop facilitated by the guanine nucleotide exchange factors, RasGRP and son of sevenless (SOS), promotes the GTPase activity of Ras, sending digital signals down the rest of the pathway (78, 82). Another group proposed that digitization of the MAPK pathway occurs through an analog-based negative feedback of SH2 domain containing tyrosine phosphatase (SHP-1) (86, 250). Here, weakly potent TCR ligands induce the negative regulation of SHP-1 preventing activation of the MAPK cascade. Only

TCR ligands that reach a certain signaling threshold can overcome the negative feedback loop and induce MAPK activity (62, 251).

An interesting observation in these types of studies is that titration of PMA in T cells induces analog signaling within the NF-κB and MAPK pathways. This suggests that digitization of major signaling pathways occurs at the level of membrane proximal signaling events. A quick look at the major components of this part of TCR signaling reveals three major protein tyrosine kinase (PTK) families, Src, Syk, and Tec (66, 87). Of these PTKs, Src and Syk family members are absolutely required for signal transduction. For example, T cells lacking Zap-70 or Lck result in the complete absence of TCR signaling (88, 252, 253). Therefore, both Lck and Zap-70 are capable of transducing digital signals downstream of the TCR. On the other hand, stimulation of T cells deficient in Tec family kinases results in reduced TCR signaling from the point of PLCγ1 activation through the major downstream pathways (89, 121).

Itk is the predominant Tec family kinase in T cells. Itk is recruited to the membrane via interactions with the LAT scaffolding complex and PIP_3 upon TCR stimulation. Here, Itk can phosphorylate its target substrate PLCγ1. One of the major pathways that is affected by Itk is the Ca^{2+} - NFAT pathway (90, 159). As opposed to the NF-κB and MAPK pathways, limited data exists on the nature of the Ca^{2+} - NFAT pathway, but it too has characteristics of both digital and analog behavior (91, 254-256). For example, the release of intracellular Ca^{2+} is thought to provide analog control of CRAC channels until store-operated calcium entry (SOCE) can occur (92, 256). SOCE is one of the major defects in stimulated T

cells lacking Itk (111, 159). We therefore postulate that Itk modulates TCR signal strength by controlling analog to digital signal conversion and that limiting Itk activity will result in a graded T cell response.

In this study, we show that changing TCR signal strength using altered peptide ligands generates both digital and analog responses. The analog response produces a skewed transcription factor profile in CD8 T cells. This is highlighted by the exquisite control of IRF4 expression in response to a wide range of APLs with varying affinity toward the TCR. We further show that the graded expression of IRF4 is controlled by the amount of Itk activity within CD8 T cells. Finally, we link NFAT activity with graded IRF4 expression and an altered distribution of transcription factors in CD8 T cells. Taken together, the implications of these results suggest that modulation of TCR signal strength through Itk and NFAT can alter the manner in which CD8 T cells respond to pMHC.

Results

CD8 T cells behave like a switch in response to TCR – pMHC ligation

In order to assess the responsiveness of T cells to varying concentrations of cognate pMHC, we examined the expression levels of several proteins by flow cytometry. Ova peptide (pOva) was titrated against OT-I CD8 T cells resulting in the bi-modal distribution of CD69 expression (Fig 2.1A & B). At a critical

Figure 2.1

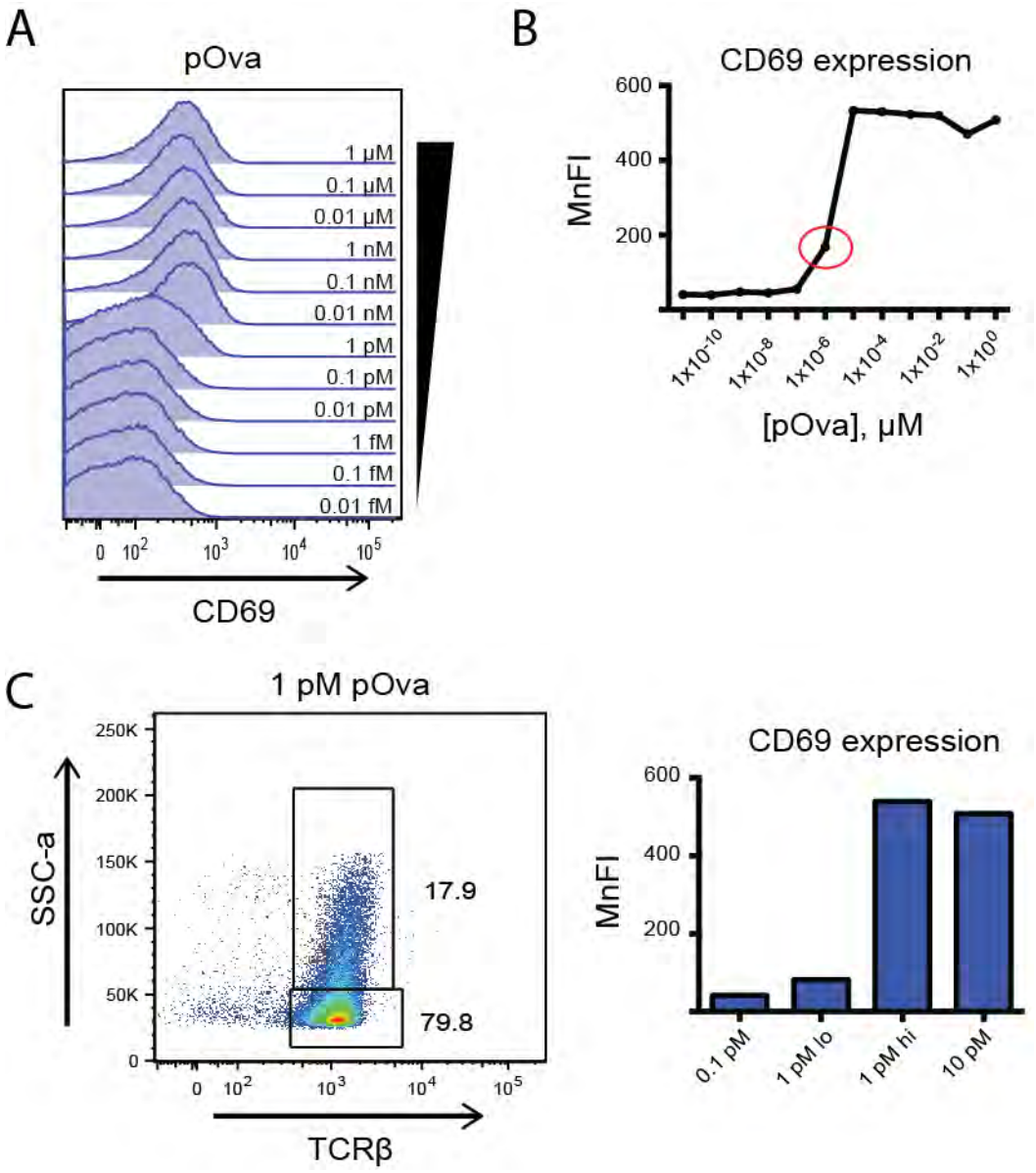
A
pOva
1 μM
0.1 μM
0.01 μM
1 nM
0.1 nM
0.01 nM
1 pM
0.1 pM
0.01 pM
1 fM
0.1 fM
0.01 fM
0
10^2
10^3
10^4
10^5
CD69
B
CD69 expression
MnFI
600
400
200
1x10^-10
1x10^-8
1x10^-6
1x10^-4
1x10^-2
1x10^0
[pOva], μM
C
1 pM pOva
SSC-a
250K
200K
150K
100K
50K
0
17.9
79.8
TCRβ
CD69 expression
MnFI
600
400
200
0.1 pM
1 pM lo
1 pM hi
10 pM

Figure 2.1 Digital expression of CD69 in CD8 T cells stimulated with pOva

(A) OT-I CD8 T cells were stimulated for 24 hours in the presence of pOva. CD69 expression was determined by flow cytometry. The triangle to the right of histograms represents the amount of pOva that was used as indicated at the right of each histogram.

(B) Graphical representation of the data from (A). Mean fluorescent intensity of CD69 is shown. The red circle at 1 pM is further examined in (C).

(C) Cells stimulated with 1 pM of pOva were differentiated by side scatter area (SSC-a), and then examined for CD69 expression. The adjacent concentrations, 0.1 and 10 pM, are shown in the bar graph for comparison.

The data represent 1 independent experiment.

threshold of 1pM, stimulation with pOva results in approximately 20% of T cells expressing CD69 and 80% of cells not expressing CD69 (Fig 2.1C). At concentrations above 1pM, all cells expressed CD69, whereas no CD69 expression was observed at concentrations below 1pM. Moreover, the mean fluorescent intensity (MnFI) of all cells expressing CD69 was consistently comparable (Fig 2.1B). Therefore, CD8 T cells display a digital (on or off) pattern of activation in response to pOva.

Altering TCR – pMHC potency results in a differential T cell response and a skewed transcription factor profile

In order to determine if this effect on CD8 T cells was unique to pOva, we stimulated OT-I cells with varying concentrations of an altered peptide ligand (APL) called G4. This peptide has an amino acid substitution at position 4 (N→G) of the pOva 8-mer and results in a decreased affinity for the OT-I TCR while retaining the same stability with MHC class I as pOva (44, 112, 257, 258). Titration of G4 on OT-I CD8 T cells appeared to result in a similar pattern of CD69 expression compared to pOva (Fig 2.2A). As expected, the critical threshold for the activation of OT-I T cells with G4, which was 1uM (Fig 2.2B), was much higher than pOva. Cells that were stimulated with concentrations of G4 higher than 1uM displayed a similar MnFI, whereas those cells stimulated at concentrations lower than 1uM did not express CD69. Closer inspection of OT-I cells stimulated with 1uM of G4 revealed a graded expression pattern of CD69

Figure 2.2

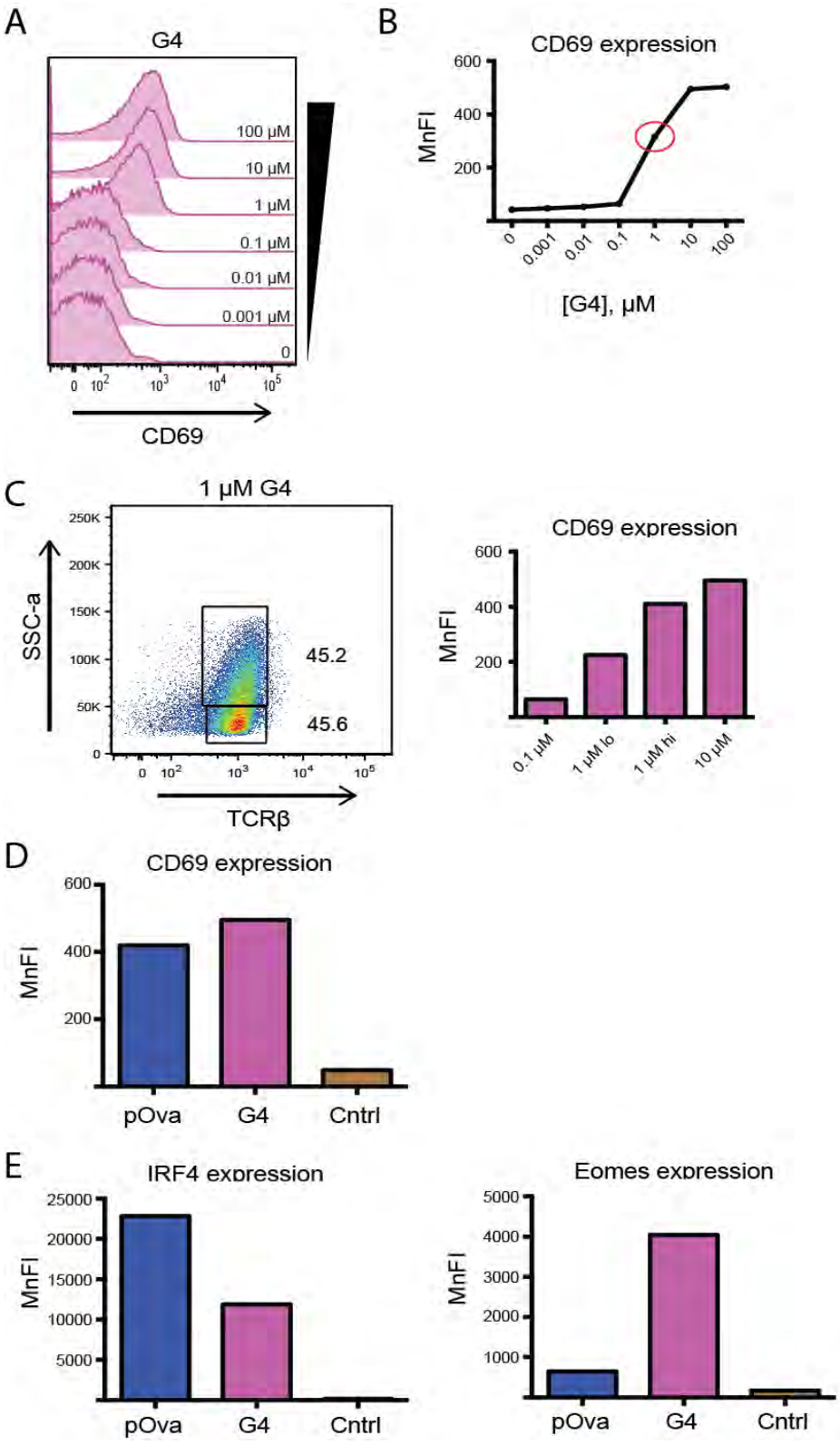
A
G4
100 μM
10 μM
1 μM
0.1 μM
0.01 μM
0.001 μM
0
CD69
B
CD69 expression
MnFI
[G4], μM
C
1 μM G4
SSC-a
45.2
45.6
TCRβ
CD69 expression
MnFI
0.1 μM
1 μM lo
1 μM hi
10 μM
D
CD69 expression
MnFI
pOva
G4
Cntrl
E
IRF4 expression
MnFI
pOva
G4
Cntrl
Eomes expression
MnFI
pOva
G4
Cntrl

Figure 2.2 Weak TCR signal strength leads to skewed transcription factor profile

(A) OT-I CD8 T cells were stimulated for 24 hours in the presence of G4 peptide. CD69 expression was determined by flow cytometry. The triangle to the right of histograms represents the amount of G4 peptide that was used as indicated in next to each histogram.

(B) Graphical representation of the data from (A). Mean fluorescent intensity of CD69 is shown. The red circle at 1 uM is further examined in (C).

(C) Cells stimulated with 1 uM of G4 peptide were differentiated by side scatter area (SSC-a), and then examined for CD69 expression. The adjacent concentrations, 0.1 and 10 uM are shown in the bar graph for comparison.

(D) CD8 T cells were stimulated for 24 hours with 10 uM pOva, G4 peptide, or a control peptide. CD69 expression was quantified by flow cytometry.

(E) CD8 T cells were stimulated as in (D). Cells were fixed, permeabilized and, stained for IRF4 (left) and Eomes (right) expression. MnFI was quantified by flow cytometry.

The data represent 1 independent experiment.

(Fig 2.2C). This implied that altering the interaction potency between TCR – pMHC impacted the ability of CD8 T cells to respond in a switch-like manner.

The density at which pMHC is presented to CD8 T cells can influence the amount of signal that a T cell receives and thereby alter the response. To address this, we stimulated OT-I CD8 T cells with equivalent concentrations of pOva, G4, and a control peptide. In response to TCR – pMHC ligation with either pOva or G4, CD8 T cells expressed similar amounts of CD69 and CD44 on the cell surface (Fig 2.2D & not shown). This suggested that the cell surface phenotype of OT-I CD8 T cells does not appreciably change in response to stimulation with its cognate ligand or an APL. When the transcription factor profile of these two populations was analyzed, we observed a dramatic difference in the expression of interferon regulatory factor 4 (IRF4) and Eomesodermin (Eomes) (Fig 2.2E). OT-I CD8 T cells stimulated with pOva displayed increased levels of IRF4 compared to cells stimulated with G4. This consistently resulted in about a two-fold difference. Conversely, Eomes expression was drastically reduced in T cells stimulated with pOva. Subsequent experiments revealed this difference was consistently between two- and four-fold. These data suggest that TCR signal strength is vital to the expression pattern of transcription factors in CD8 T cells.

Stimulation of CD8 T cells with APLs results in the graded expression of IRF4

To assess the responsiveness of CD8 T cells to varying amounts of TCR signal strength, we utilized a panel of APLs with a range of affinities towards the OT-I TCR (44, 113-115). All of these peptides have a one-amino acid substitution with the parent peptide, pOva. Importantly, the binding kinetics to MHC class I is not changed in the peptide panel. Stimulation of OT-I CD8 T cells with 5 out of 6 peptides from the panel resulted in equivalent expression levels of CD69 (Fig 2.3A). A peptide, E1, with very low affinity towards the OT-I TCR did not stimulate the expression of CD69 and was comparable to unstimulated cells, an isotype control (not shown), and a control peptide. These data suggest that altering the interaction affinity between TCR and pMHC does not regulate the expression of CD69 and results in its digital expression.

A different conclusion was reached when the expression pattern of IRF4 and Eomes was observed. When we examined the amount of IRF4, activated OT-I CD8 T cells displayed an expression pattern that was graded (Fig 2.3B). This stepwise pattern was dependent on the strength of the TCR – pMHC interaction. T cells receiving the strongest amount of signal expressed the most amount of IRF4; whereas those cells receiving decreased signal strength expressed the least amount of IRF4. Importantly, the E1 peptide did not induce expression of IRF4. This result indicates the presence of a critical threshold between the signal strength generated by G4 and E1. Above this threshold, an analog signal occurs in response to increasing amounts of TCR signal strength.

Figure 2.3

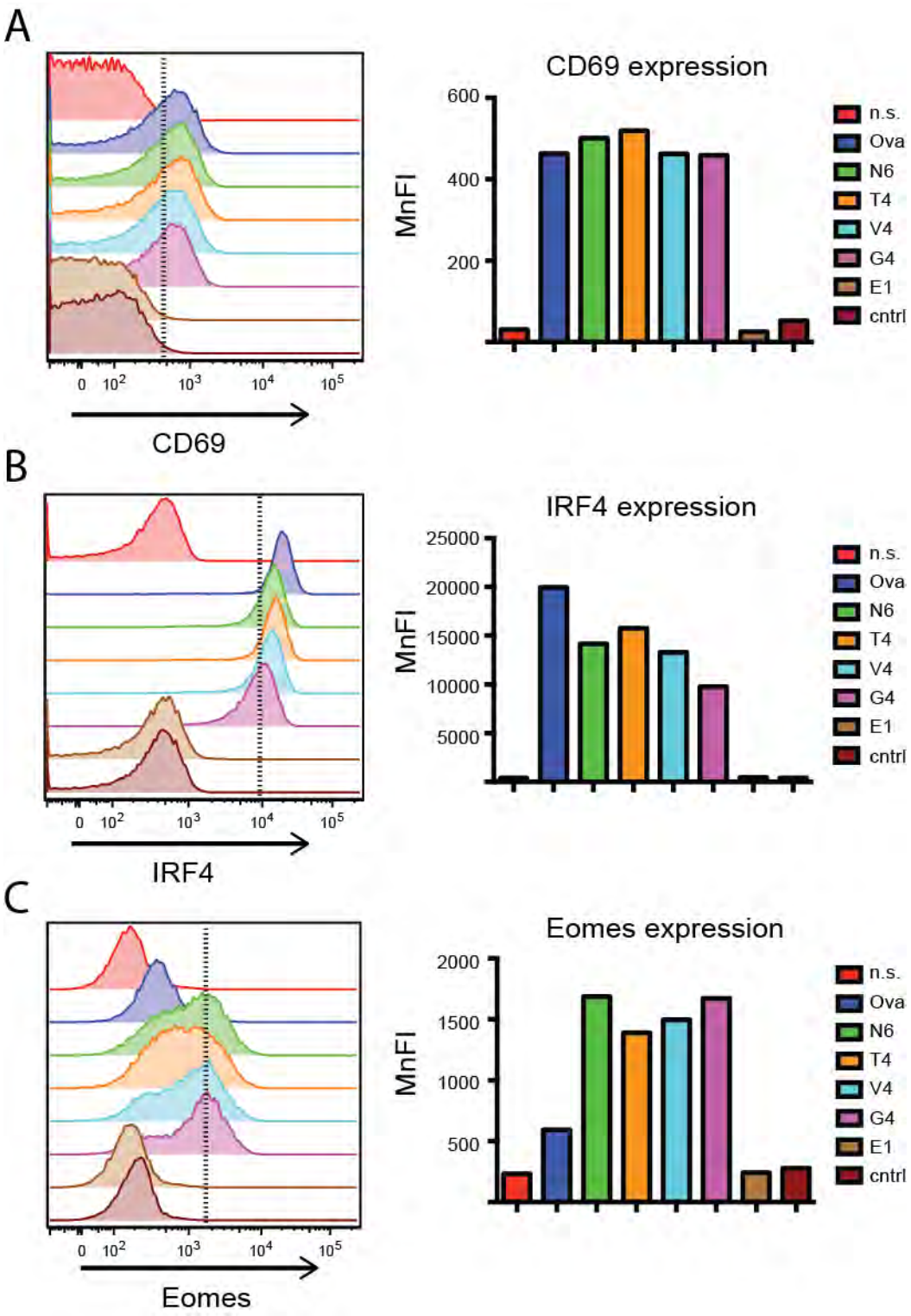
A
CD69 expression
MnFI
600
400
200
n.s.
Ova
N6
T4
V4
G4
E1
cntrl
0 10^2 10^3 10^4 10^5
CD69
B
IRF4 expression
MnFI
25000
20000
15000
10000
5000
n.s.
Ova
N6
T4
V4
G4
E1
cntrl
0 10^2 10^3 10^4 10^5
IRF4
C
Eomes expression
MnFI
2000
1500
1000
500
n.s.
Ova
N6
T4
V4
G4
E1
cntrl
0 10^2 10^3 10^4 10^5
Eomes

Figure 2.3 Altered peptide ligands generated graded IRF4 expression

(A - C) OT-I CD8 T cells were stimulated for 24 hours in the presence of the indicated peptides. Cells were stained for cell surface expression of CD69 (A) then fixed, permeabilized, and stained for intracellular expression of IRF4 (B) and Eomes (C). The dotted line in the histograms is the mean fluorescent intensity of cells stimulated with the G4 peptide. MnFI is quantified in the bar graphs on the right.

Data are representative of 3 independent experiments.

The data in Figure 2.2E suggested that Eomes expression is altered in response to varying TCR signal strength and is not dependent on the expression of CD69. Indeed, 5 out of 6 peptides stimulated OT-I CD8 T cells and induced the expression of Eomes (Fig 2.3C). Stimulation with the E1 peptide resulted in the same level of expression as unstimulated cells and a control peptide. CD8 T cells stimulated with a very potent ligand, pOva, induced the least amount of Eomes expression. In contrast, cells stimulated with lower affinity (N6, T4, V4, and G4) ligands induced robust expression of Eomes. Interestingly, the expression pattern of Eomes was not uniform amongst the lower affinity APLs (Fig 2.3C, histograms). The majority of the cells stimulated with G4 and V4 peptides expressed high amounts of Eomes. Cells stimulated with peptides of slightly greater affinity for the TCR (T4 and N6) contained a more uniform distribution of Eomes. These data suggest that Eomes expression is highly sensitive to TCR signal strength and inversely correlates with IRF4 expression. Consistent with previously published data from our lab, it is likely that IRF4 negatively regulates Eomes expression in T cells (72, 259).

Graded expression of IRF4 is not due to altered kinetics

We hypothesized that weak TCR signal strength results in the delayed expression of IRF4 and that it simply takes longer to catch up to the levels exhibited by strong TCR signal strength. As early as 3 hours post stimulation, OT-I CD8 T cells began expressing substantial amounts of IRF4 in response to 4

out of 6 of the peptides in the APL panel (Fig 2.4A). CD8 T cells stimulated in the presence of G4 peptide expressed IRF4 at levels very close to the limit of detection and was comparable to unstimulated cells, E1 peptide, and a control peptide. By 18 hours post stimulation 5 out of 6 peptides from the panel drastically increased IRF4 expression. Unexpectedly, at both of these time points, a graded pattern of IRF4 expression was observed in response to stimulation with 5 out of 6 peptides.

We were curious if other time points exhibited the same trend. A full time course experiment was performed choosing additional periods between 3 and 24 hours post stimulation. As demonstrated in Figure 2.4B, at no time point examined did we observe any of the APLs expressing similar amounts of IRF4 to pOva. In fact, the major difference that we detected was an increase in the magnitude of expression. This is most aptly exhibited between pOva and G4 peptides, where the difference in IRF4 expression between the two peptides only increased as time progressed. Following 24 hours of stimulation, the cells in culture begin to divide and a direct comparison of protein expression between the populations becomes unattainable (data not shown).

Given the sensitivity of Eomes expression to TCR stimulation, we wanted to assess the kinetics for this transcription factor. As opposed to the very early expression of IRF4, we could not detect Eomes expression in any condition up to 6 hours post stimulation (Fig 2.4C). By 12 hours post stimulation, 5 out of the 6 peptides had expressed Eomes above background (unstimulated cells, E1 peptide, and control peptide). Whereas lower affinity peptides (N6, T4, V4, and

Figure 2.4

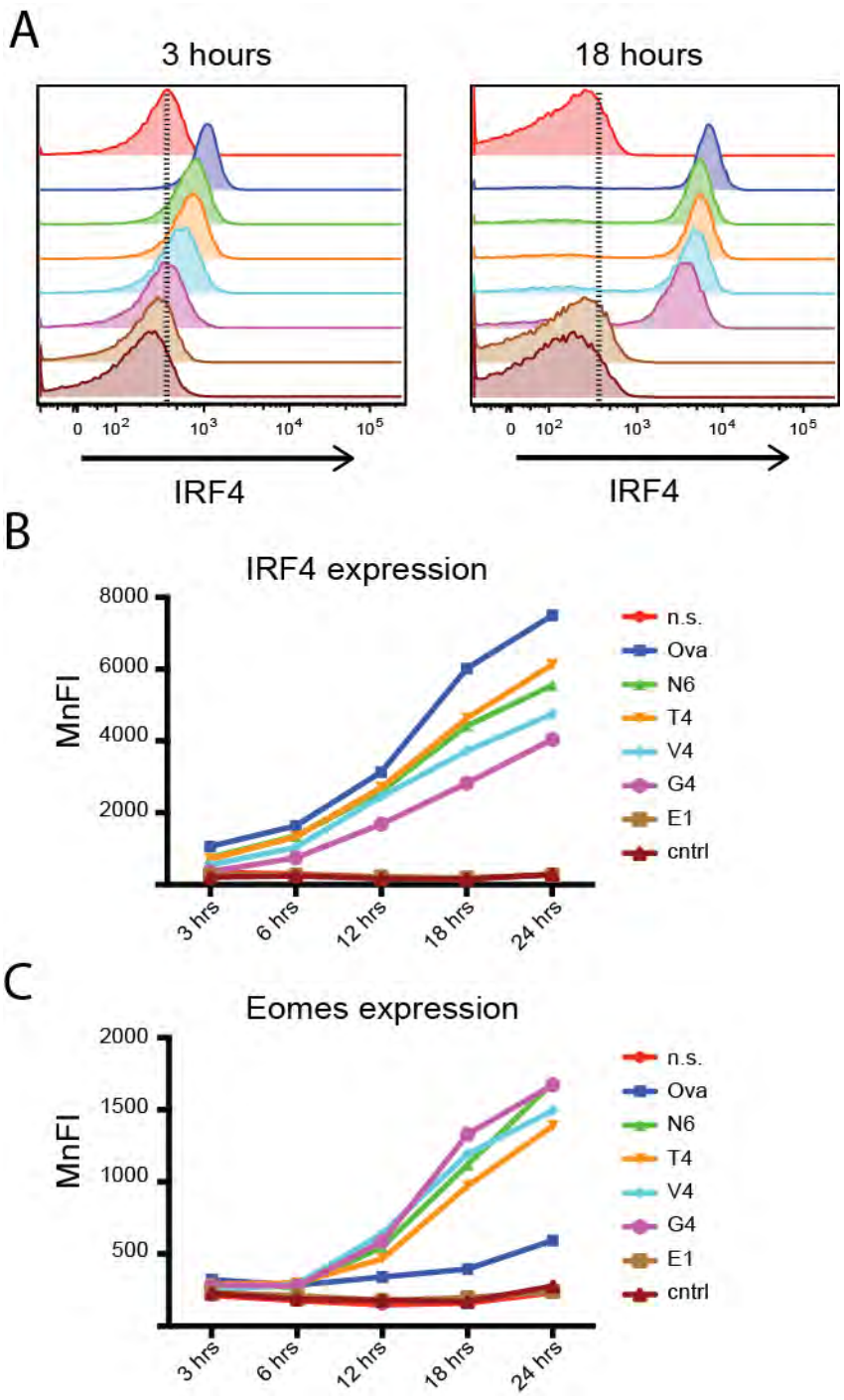
A
3 hours
18 hours
0
10^2
10^3
10^4
10^5
IRF4
B
IRF4 expression
MnFI
8000
6000
4000
2000
3 hrs
6 hrs
12 hrs
18 hrs
24 hrs
n.s.
Ova
N6
T4
V4
G4
E1
cntrl
C
Eomes expression
2000
1500
1000
500

Figure 2.4 Altered transcription factor profile is not due to delayed kinetics

(A - C) OT-I CD8 T cells were stimulated in the presence of the indicated peptides and assayed at the indicated time points for IRF4 expression (A,B) and Eomes expression (C). The dotted line in the histograms (A) is the mean fluorescent intensity of cells stimulated with the G4 peptide at 3 hours. MnFI is quantified for IRF4 and Eomes in the line graphs in B and C, respectively.

Data are representative of 2 independent experiments.

G4) rapidly induced high levels of Eomes expression, CD8 T cells stimulated with pOva remained low for the duration of the time course. Interestingly, we were unable to discern graded expression of Eomes within the APLs N6, T4, V4, or G4. Taken together, these data indicate that the expression of IRF4 and Eomes is not simply delayed in response to lower TCR signal strength.

Itk activity modulates the graded expression of IRF4

The data thus far has indicated the presence of at least two distinct signals emanating from the TCR upon stimulation. The first signal is digital and controls the expression of TCR responsive genes like CD69. The second signal is analog and controls the expression of IRF4. We wanted to examine the signaling mediators responsible for controlling the graded expression of IRF4. In order to do this we utilized an Itk-specific pharmacological inhibitor called 10N (116, 117, 259). We performed a titration of this inhibitor on cells stimulated with pOva. To limit the toxicological effects of this inhibitor, we gated on cells that were CD69+ and then examined the expression of IRF4 and Eomes. OT-I CD8 T cells stimulated with pOva in the presence of increasing amounts of 10N displayed a graded pattern of IRF4 expression (Fig 2.5A, left histograms). To ensure that this effect was not isolated to the strong agonist pOva, we performed the inhibitor titration on the lower affinity peptide T4. OT-I CD8 T cells stimulated in the presence of increasing amounts of 10N also displayed a graded expression pattern for IRF4 (Fig 2.5A, right histograms). These data suggest

Figure 2.5

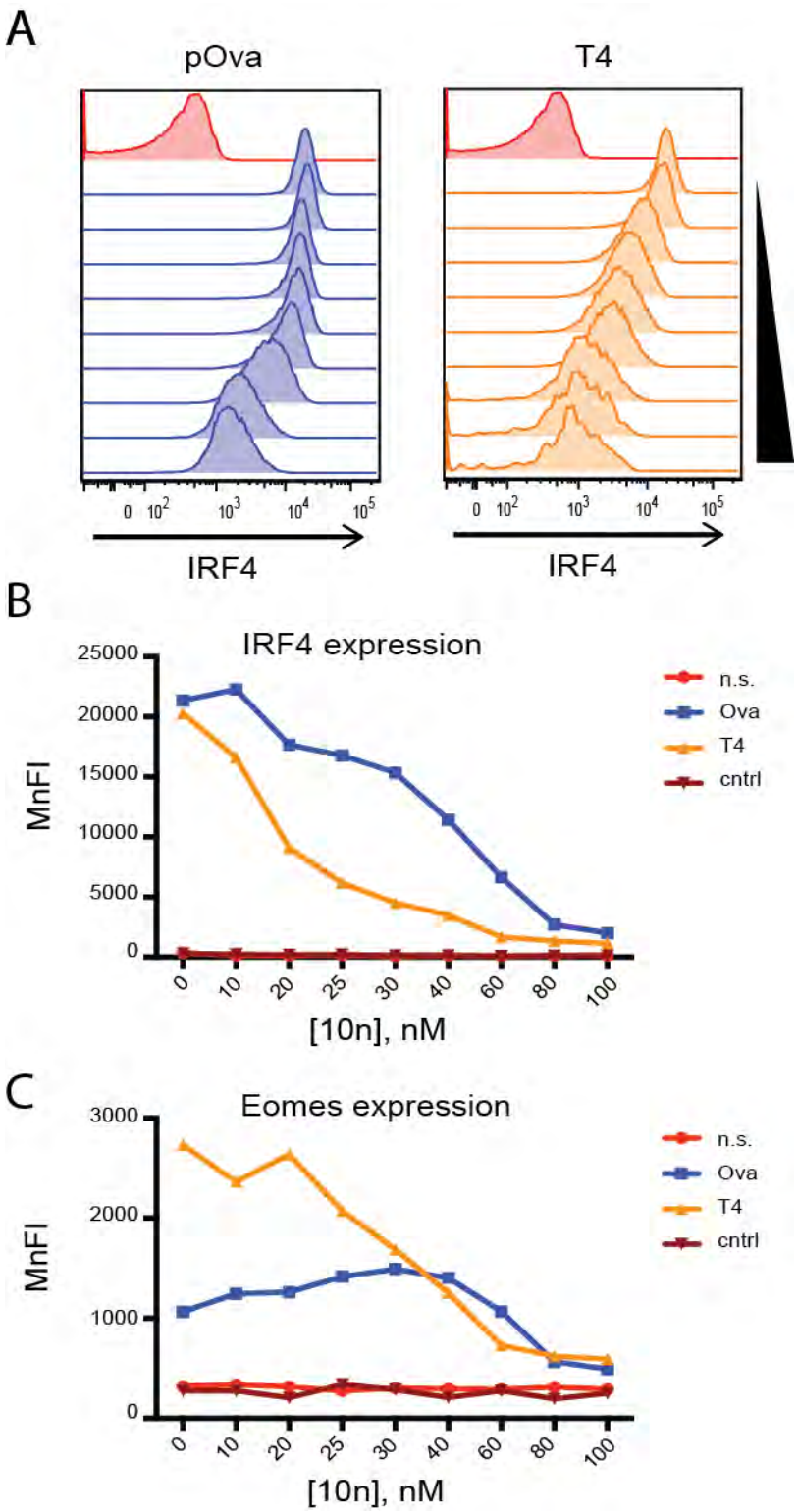
A
pOva
T4
0 10^2 10^3 10^4 10^5
IRF4
IRF4
B
IRF4 expression
MnFI
25000
20000
15000
10000
5000
0
0 10 20 25 30 40 60 80 100
[10n], nM
n.s.
Ova
T4
cntrl
C
Eomes expression
MnFI
3000
2000
1000
0
0 10 20 25 30 40 60 80 100
[10n], nM
n.s.
Ova
T4
cntrl

Figure 2.5 The transcription factor profile in CD8 T cells is sensitive to Itk activity

(A) OT-I CD8 T cells were stimulated with of pOva (left histograms) and T4 (right histograms) in the presence of an Itk inhibitor. The inhibitor concentrations used are indicated in B and C. Cells were harvested at 24 hours and subjected to an intracellular stain for IRF4 and Eomes. CD69+ cells were used for analysis.

(B and C) The data from A is depicted as a dose response curve for IRF4 (B) and Eomes (C). The data is compared against unstimulated cells and a control peptide.

Data are representative of 3 independent experiments.

that Itk activity can tune the signal that the TCR transmits in response to varying inputs.

We generated a dose-response curve to directly compare stimulation with the pOva and T4 peptides. In addition to the graded expression of IRF4 that is observed in response to peptide stimulation, the sensitivity of CD8 T cells to the 10N inhibitor can also be seen (Fig 2.5B). The expression of IRF4 is more sensitive to lower concentrations of 10N inhibitor in OT-I CD8 T cells stimulated with the APL T4 than pOva. Cells stimulated with T4 peptide have lost more than half their expression of IRF4 at 20nM 10N, whereas it takes about 50nM of 10N to reach 50% IRF4 expression in response to pOva. This indicates that the strong TCR signal generated by pOva can compensate for or withstand a greater loss in Itk activity than the TCR signal generated by T4 peptide.

Given that altering TCR signal strength by using APLs results in differential Eomes expression, we formulated a dose-response curve for this transcription factor. Similar to IRF4 expression, the sensitivity of OT-I CD8 T cells stimulated with T4 peptide is greater than that of pOva (Fig 2.5C). Only at concentrations greater than 60nM 10N was Eomes expression affected in cells stimulated with pOva. On the other hand, at concentrations between 20 and 25nM 10N, cells stimulated with T4 peptide began losing Eomes expression. Interestingly, the amount of fluorescence increased by about 40% in cells stimulated with pOva in the presence of 30nM 10N compared with those cells stimulated in the absence of 10N (MnFI values of 1062 no inhibitor vs 1492 30nM). This suggests that decreasing Itk activity can dampen the strong signals

produced by pOva. The result of this is an increased expression of Eomes, strengthening the argument that Eomes expression in CD8 T cells is inversely proportional to TCR signal strength.

TCR signal strength can be altered by NFAT activity resulting in the graded expression of IRF4

Itk is a critical regulator downstream of TCR signaling in T cells, linking membrane proximal events to major downstream pathways by activating PLCγ1. The generation of secondary messengers, IP_3 and DAG, which are produced by PLCγ1, induces the MAPK cascade, NF-κB activation, and Ca^{2+} mobilization. One of the major defects in $Itk^{-/-}$ T cells is an uncoupling of TCR signaling with NFAT activity (118, 159). Due to the graded IRF4 expression seen during Itk inhibition, we wanted to assess the ability of NFAT to transduce analog signals in stimulated CD8 T cells. In order to examine NFAT activity on the expression of IRF4, we used the calcineurin inhibitor FK506. OT-I CD8 T cells stimulated with pOva in the presence of increasing amounts of FK506 displayed a graded distribution of IRF4 (Fig 2.6A, left histograms). For lower concentrations of FK506 (0.075, 0.15 and 0.3 nM), the expression of IRF4 changed very little in cells stimulated with pOva compared to cells stimulated in the absence of FK506. Even at the highest concentration of FK506, pOva-induced expression of IRF4 remained above the levels of unstimulated cells. This suggests that it takes substantial amounts of NFAT inhibition to alter the expression of IRF4. Similarly,

Figure 2.6

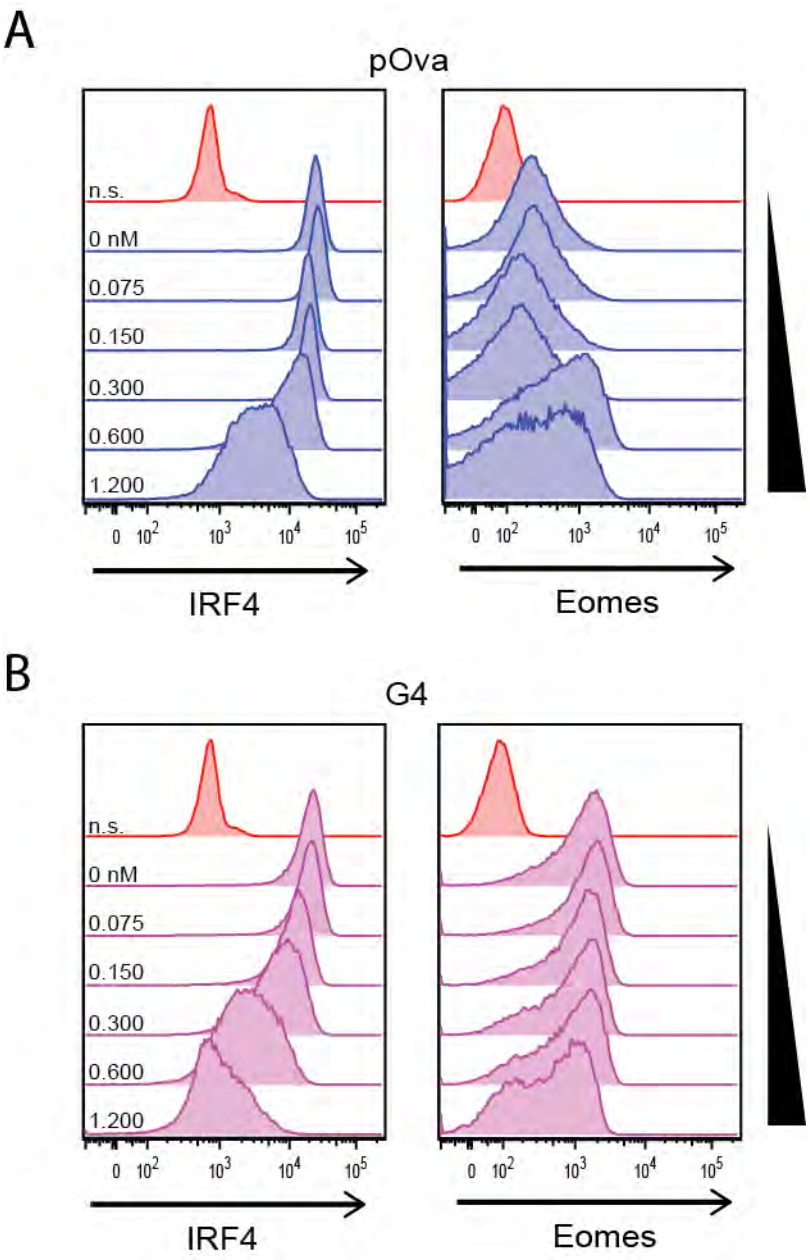
A
pOva
n.s.
0 nM
0.075
0.150
0.300
0.600
1.200
0 10^2 10^3 10^4 10^5
IRF4
0 10^2 10^3 10^4 10^5
Eomes
B
G4
n.s.
0 nM
0.075
0.150
0.300
0.600
1.200
0 10^2 10^3 10^4 10^5
IRF4
0 10^2 10^3 10^4 10^5
Eomes

Figure 2.6 TCR signal strength is altered by NFAT activity

(A) OT-I CD8 T cells were stimulated with of pOva in the presence of an NFAT inhibitor. The inhibitor concentrations, in nanomolar, used are indicated in the left panel of histograms. Cells were harvested at 24 hours and subjected to an intracellular stain for IRF4 (left histograms) and Eomes (right histograms).

(B) OT-I CD8 T cells were stimulated with of G4 peptide in the presence of an NFAT inhibitor. The inhibitor concentrations, in nanomolar, used are indicated in the left panel of histograms. Cells were harvested at 24 hours and subjected to an intracellular stain for IRF4 (left histograms) and Eomes (right histograms).

For comparative purposes, the red histograms in each panel are unstimulated cells. The triangle at the right indicates the concentration of NFAT inhibitor.

Data are representative of 3 independent experiments.

Eomes expression in CD8 T cells stimulated with pOva in the presence of low concentrations of FK506 (0.075, 0.150. 0.3nM) was not considerably altered but remained detectable above background levels (Fig 2.6A, right histograms). Intriguingly, the same concentrations of FK506 (0.6 and 1.2nM) that caused a decrease in IRF4 expression in cells stimulated with pOva resulted in an increase in Eomes expression. This suggests that NFAT activity is required for full expression of IRF4, and further provides evidence that IRF4 regulates Eomes expression.

The analog response observed when CD8 T cells are stimulated with pOva in the presence of FK506 was not as impressive as we had expected. We reasoned that a lower affinity peptide would allow us to more readily resolve the effect that NFAT activity had on TCR signal strength. Therefore, we repeated these experiments using the G4 peptide. When OT-I CD8 T cells were stimulated with G4 peptide in the presence of FK506, we observed a much more pronounced analog response as demonstrated by IRF4 expression (Fig 2.6B, left histograms). The influence of FK506 on IRF4 expression was seen at concentrations as low as 0.15nM and continued to the highest non-toxic dose (1.2nM). Interestingly, at 1.2nM FK506 an almost complete loss of IRF4 expression was observed, suggesting that NFAT activity is required for IRF4 expression when TCR signal strength is low. We were unable to detect any appreciable change in Eomes expression at low concentrations of FK506 (Fig 2.6B, right histograms). At 0.6 and 1.2nM FK506, we noticed the levels of Eomes starting to decrease despite the reduction in IRF4 expression. This was

somewhat surprising because we are unaware of any direct regulation of Eomes induction by NFAT. Nonetheless, the data from these experiments demonstrate the ability of NFAT activity to transmit an analog signal from the TCR in response to both strong and weak signal strength.

Discussion

The information that a T cell receives to induce activation can most aptly be conveyed as one of signal strength. The notion of TCR signal strength is frequently used to describe the development of T cells in the thymus, whereby 'too strong' signals lead to negative selection, 'just right' signals lead to positive selection, and no signal leads to death by neglect. In this context, a suitable T cell population can arise that will provide adequate protection to the host. Altering the strength of TCR signaling can completely change the developmental dynamics required for an appropriate T cell repertoire (119, 165, 260). Once T cell development is complete, TCR signal strength influences the fate of CD4 T cell differentiation into T_H1 vs T_H2 cells and controls T_H17 cell responses (120, 261, 262). The important determinants of TCR signal strength include, but are not limited to: the potency of TCR – pMHC interactions, the duration of TCR – pMHC interactions, the density of pMHC presentation, and the activity of signaling mediators.

In our studies, we employ two experimental models of TCR signal strength, potency of TCR – pMHC interactions and activity of signaling

mediators, to show that a CD8 T cell integrates digital and analog signals to promote activation. Several lines of evidence support such a conclusion. CD8 T cells stimulated with decreasing concentrations of a given peptide display a uniformly similar pattern of expression for TCR responsive genes, such as IRF4 and CD69. T cell activation persists until a critical concentration threshold is reached; at this point T cells cease to respond. This implies that T cells can be activated in an on/off manner, similar to a light switch, and is consistent with current models that propose T cell responses occur in a digital manner (120, 121, 263). Indeed, a preponderance of research indicates that several pathways downstream of the TCR, notably the NF-κB and MAPK pathways, can mediate digital activation of T cells (88, 122, 249).

On the other hand, if CD8 T cells are exposed to altered peptide ligands (APLs) of varying potency, and therefore varying signal strength, the response changes from all digital to partially digital and partially analog. For example, the cell surface markers CD69 and CD44 display a digital pattern of expression. This makes sense because expression of CD69 and CD44 require the actions of AP-1 or NF-κB and AP-1, respectively (72, 264, 265). Conversely, the transcription factor network established during T cell activation is skewed in response to varying TCR signal strength. This is best documented by the changes in the expression of IRF4, where strong TCR signals lead to more IRF4 and weak TCR signals generate less IRF4. It is not completely clear which transcription factors regulate IRF4 expression but it is clear that TCR signal strength is important.

The identification of digital and analog signals simultaneously emanating from the TCR immediately prompts the question – how is this regulated? To examine this, we conducted experiments that involved modulating the activity of signaling mediators. The selection of potential targets that can mediate an analog response is limited, largely because of the essential requirements for so many components of the TCR signaling cascade. For instance, both Src and Syk family kinases have major developmental blocks in thymopoiesis, which is likely due to the complete absence of TCR signaling (123, 124, 266, 267). This is demonstrated in T cells that genetically lack Zap-70 (125, 268). Crosslinking the TCR in these T cells results in the complete ablation of downstream signaling events, as evidenced by lack of phosphorylated PLCγ1, nonexistent Ca^{2+} mobilization, and no IL-2 production. By comparison, the defect in Itk-deficient T cells is relatively mild. In the absence of Itk, T cells display diminished TCR signaling with mild defects in PLCγ1 phosphorylation, decreased NFAT activity, and reduced IL-2 production. Because of the inherent developmental defects associated with Itk deficiency, we sought to tune Itk activity with a pharmacological inhibitor. In order to eliminate toxicological bias of the inhibitor, only cells that expressed CD69 were included for analysis. Despite the finite range of useful concentrations, IRF4 expression was graded in CD8 T cells stimulated with given peptides in the presence of increasing concentrations of inhibitor. There are several important implications of this finding. First, it demonstrates that IRF4 expression is sensitive to Itk activity. Second, it demonstrates that Itk activity is capable of propagating analog signals from the

TCR. Third, despite the reduction in IRF4 expression, CD69 expression remained intact on all cells. Fourth, it phenocopies the results obtained through the stimulation of T cells with altered peptide ligands of varying potency. Finally, the data is in line with previously published work describing the ability of Itk to act as a rheostat (126, 269).

The relationship between Itk and NFAT has been firmly established. Itk functions upstream of the primary signaling pathways in T cells. The majority of these pathways have been shown to transmit digital signals in response to TCR stimulation (88, 127, 249). Consistent with this idea, we find that CD69 is expressed in a digital manner. Very little data exists on whether the NFAT pathway responds in a digital or analog manner. Using a non-competitive inhibitor of NFAT, the activity of this transcription factor can be modulated in response to various concentrations of the inhibitor. CD8 T cells exposed to strong TCR signals in the presence of increasing NFAT inhibition exhibit an altered transcription factor profile exemplified by analog control of IRF4 expression. This is especially true at higher amounts of inhibition. Moreover, the result is more pronounced in cells that were stimulated with weak TCR ligands. This suggests that high TCR signal strength can overcome NFAT inhibition to a greater extent than low strength TCR signals. Overall, our experiments suggest that NFAT is capable of transmitting analog signals that result in the graded expression of IRF4.

With this understanding, we constructed a model to describe the manner in which T cells are activated. The model in Figure 2.7 depicts the pulse curve of

T cell activation as a function of signal strength. It compares the two types of T cell responses that pass through digital and analog mediators. Digital and analog responses occur once a critical activation threshold is overcome. Digital signals use on - off mediators such as ZAP-70 to culminate in bi-modal responses like CD69 expression. These responses are independent of TCR signal strength. Oppositely, analog responses are dependent on TCR signal strength. Analog signals use mediators that do not function as on - off switches like Itk. They are tunable dials. As TCR signal strength increases so too does the T cell response, as evident by graded IRF4 expression.

Figure 2.7

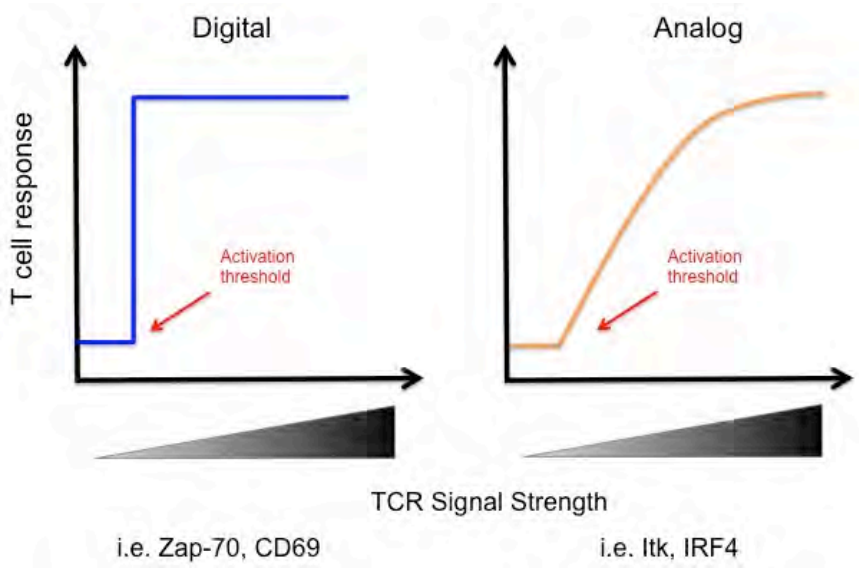

Figure 2.7 Model of T cell activation in response to TCR signal strength

Upon reaching a critical threshold, signals pass through ZAP-70 to generate digital expression of CD69, whereas Itk acts as a digital to analog converter producing the graded expression of IRF4.

Materials and Methods

Mice

Mice were bred and housed under specific pathogen-free conditions at the University of Massachusetts Medical School (UMMS) in accordance with institutional animal care and use committee guidelines. OT-I TCR transgenic Rag1$^{-/-}$ mice were purchased from Taconic. Mice were euthanized between 8 and 12 weeks of age.

Cell Culture Conditions

Spleens from OT-I Rag1$^{-/-}$ mice were harvested and processed using aseptic technique in a laminar flow hood. Red blood cells were lysed with ammonium chloride for 5 minutes at room temperature. Pure splenocytes were filtered and counted using a Cellometer Auto T4 cell counter (Nexcelom Bioscience). Splenocytes with greater than 85% viability were seeded at 1.25x10^6 cells/mL in 96-well round bottom tissue culture plates. Complete media consisted of RPMI-1640 base, 10% heat-inactivated FBS, 100 U/mL penicillin, 100 ug/mL streptomycin, 100 mM L-glutamine, 100 µM non-essential amino acids, 50 µM 2-ME, and 10 µM HEPES buffer. Cells were incubated for the indicated time points at 37°C, 5% CO_2 in a water-jacketed incubator.

Peptides

The peptides used are listed in Table 2.1 and were synthesized to greater than 90% purity by 21st Century Biochemicals. Peptides that activated OT-I CD8 T cells were used at concentrations sufficient to induce maximal CD69 expression, unless otherwise indicated. Control peptides or peptides that do not sufficiently induce CD69 expression were used at 10uM.

Antibodies and staining

CD8-PE- Texas Red (PETR) and Live/Dead Violet were purchased from Invitrogen. CD44-v500 and CD69-PE Cy7 were purchased from BD Biosciences. Eomes-FITC, Eomes-PerCP eFluor710, IRF4-Allophycocyanin (APC), IRF4-phycoerythrin (PE), and TCR-β–APC eFluor780 were purchased from eBioscience. Cells were harvested at the indicated time points and washed with 2% FBS/PBS. Cells were incubated with the indicated surface stains for 20 minutes at 4°C. Cells were washed with 2% FBS/PBS, fixed and permeabilized using the FoxP3 fixation and permeabilization kit (eBiosciences) according to the manufacturer's protocol. Flow cytometry was performed using a BD LSRII (BD Biosciences). Analysis was done using FlowJo (TreeStar) and Prism (GraphPad) software. In all experiments, cells were gated using doublet discrimination, Live Dead staining, and fluorescence minus one (FMO) controls.

Reagents

The Itk inhibitor 10N was synthesized at the National Institutes of Health and was kindly provided by Jian-kang Jiang and Craig Thomas (Chemical Genomics Center, NIH). The NFAT inhibitor FK506 was purchased from Calbiochem. The concentrations of inhibitors used are indicated in the text and figure legends.

Table 2.1 Peptide variants presented by MHC Class I

The table above depicts the peptides used to ligate the TCR on OT-I CD8 T cells. The abbreviation is listed in the first column with the corresponding amino acid sequence in the second column. The third column quantifies the potency relative to pOva of each peptide's ability to stimulate CD69 expression on OT-I CD8 T cells (44, 128, 129, 257, 258). The fourth column is the concentration of peptide used in experiments unless otherwise indicated in the figure legend. P815 and m97 are control peptides that do not stimulate OT-I CD8 T cells.

Table 2.1

Peptide Name	Sequence	Potency	Conc. (uM)
Ova	SIINFEKL	1	0.01
N6	SIINFNKL	1/5(aprx)	0.1
T4	SIITFEKL	1/71-122	1
V4	SIIVFEKL	1/680	1
G4	SIIGFEKL	1/7515	10
E1	EIINFEKL	1/56,524	10
P815	HIYEFPQL	-	10
m97	IISPFPGL	-	10

Chapter 3:

Itk is a negative regulator of mast cell activation

Chapter III Attributions

The material in chapter III has been submitted for publication and consists of a collaboration between Yoko Kosaka [YK], Markus Falk [MF], Stefan Schattgen [SS], and myself [JE]. The specific contributions for each figure are as follows: Figure 3.1 [YK], Figure 3.2 [YK and JE], Figure 3.3 [YK and JE], Figure 3.4 [YK and MF], Figure 3.5 [MF and SS], and Figure 3.6 [MF and JE].

Introduction

Upon activation, mast cells secrete an extensive variety of effector molecules, including biogenic amines, enzymes, lipid metabolites, and cytokines. Many of these mediators are preformed and stored in granules, while others are newly synthesized. Historically, mast cell-derived products are responsible for an assortment of allergic responses ranging from localized wheal-and-flare reactions to systemic anaphylaxis (75, 130). More recently, mast cells have been linked to atherosclerosis, obesity, and cancer (124, 215, 216). In addition, the diversity of mediators that are released when mast cells are stimulated puts them in a prime position to regulate the adaptive immune response to pathogens (37, 131). In light of such observations, the biochemical signaling events involved in generating effector responses in mast cells is of significant interest.

Mast cells can be activated through the signaling of many cell surface receptors, including the high affinity Fc receptor for IgE (FcεRI). Aggregation of the FcεRI triggers a cascade of events that is highly reminiscent of the signals elicited by other multi-chain immune recognition receptors, such as the antigen receptors on T cells and B cells (the TCR and BCR, respectively) (121, 231, 232). Upon FcεRI crosslinking, two functionally complementary pathways are activated by the Src family kinases, Lyn and Fyn. Lyn acts by phosphorylating the β and γ chain immunoreceptor tyrosine-based activation motifs (ITAMs) of FcεRI, resulting in the recruitment and activation of Syk. Activation of Lyn and Syk lead to the assembly of a membrane-associated signaling complex that is

nucleated by LAT and promotes the activation of PLCγ. As in lymphocytes, PLCγ activation is a key event, leading to the production of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), thereby linking receptor-proximal events to major downstream pathways that regulate gene expression.

Like Lyn, Fyn also associates with FcεRI and is activated as a result of FcεRI crosslinking (132-134, 230). Fyn phosphorylates the adaptor molecule Gab2, thereby recruiting the p85 subunit of PI3K to the membrane. PI3K activity then produces membrane phosphatidylinositol-3,4,5-trisphosphate (PIP_3), leading to the recruitment of PH domain-containing proteins, such as PLCγ and Tec family kinases. Despite the distinct biochemical nature of the Lyn-and Fyn-dependent signals, it is clear that crosstalk exists between these two pathways and that both are essential for optimal FcεRI signaling. The activation of mitogen-activated protein kinases (MAPK), NFAT, and NF-κB has all been shown to require the activity of both branches of the FcεRI signaling network (121, 270).

Previous studies in T cells, B cells, and mast cells have demonstrated that Tec family kinases are integral components of antigen receptor signaling pathways (72, 121, 135). The predominant function of Tec family kinases is the phosphorylation and activation of PLCγ. In T cells, Itk localizes to the membrane by associating with the LAT complex and by binding to PIP_3. This brings Itk into proximity with its target substrate, PLCγ1. Thus, T cells lacking Itk exhibit reduced PLCγ1 phosphorylation, leading to impaired IP_3 production, diminished Ca^{2+} mobilization, and decreased NFAT activation. The reduction in PLCγ1

phosphorylation also decreases activity in the MAPK cascade. The consequence of these signaling defects is severely impaired T cell effector function, which is highlighted by the susceptibility of $Itk^{-/-}$ mice to various pathogenic infections (136-139, 160, 161). Based on these data, it is well accepted that Itk has a positive regulatory role in TCR-mediated signaling pathways.

In mast cells, as in B cells, the Tec kinase Btk has a similar positive signaling role in the activation of PLCγ in response to antigen receptor stimulation. For instance, Btk-deficient mast cells exhibit multiple defects following FcεRI activation, including impairments in degranulation, production of leukotrienes, and synthesis of cytokines (140, 233, 235-237). Biochemical studies show that PLCγ2 activation, IP_3 generation, and Ca^{2+} mobilization are all decreased in mast cells lacking Btk.

In addition to Btk, mast cells express other Tec family members: Itk, Rlk, and Tec (141-145, 234, 271) (and our unpublished observations). To date, the biochemical function(s) of these additional Tec kinases in mast cell signaling pathways has not been well addressed. Initial data suggested the likelihood that Itk participates in mast cell signaling based on the observation that Itk is recruited to the membrane and phosphorylated in response to FcεRI stimulation (143, 240). Additional, studies have examined the function of Itk *in vivo*, using models of allergic airway responsiveness. Forssell and colleagues indirectly investigated Itk-deficient mast cells by challenging $Itk^{-/-}$ mice with an airway allergen and looking for signs of inflammation (146-149, 241). In this report, $Itk^{-/-}$ mice

exhibited significant impairments in lung mast cell degranulation following antigen challenge in the airways. Interestingly, a direct comparison of $Itk^{-/-}$ mice with $Btk^{-/-}$ mice in this system indicated that the lack of Itk led to a more severe defect than that seen in the absence of Btk. In contrast, a more recent study by Iyer and colleagues used an *in vivo* reconstitution model where wild-type or $Itk^{-/-}$ bone marrow-derived mast cells (BMMC) were adoptively transferred into mice lacking mast cells (150, 244). Here, mice reconstituted with $Itk^{-/-}$ BMMC demonstrated equivalent levels of airway responsiveness to mice reconstituted with wild-type BMMC indicating that Itk was not required for mast cell degranulation. Interestingly, they also showed that $Itk^{-/-}$ mast cells secreted elevated levels of cytokines following FcεRI stimulation, however no apparent signaling defects were observed. Therefore the role of Itk in mast cell activation remains incompletely defined.

In our studies, we examine mast cell responses *in vitro* to determine whether the absence of Itk has an intrinsic effect on mast cell function. We show that freshly isolated peritoneal mast cells (PMCs) from Itk-deficient mice have dramatically increased levels of IgE on their surface. This corresponds to an increase in cytokine secretion following IgE crosslinking. Strikingly, we also find that Itk-deficient BMMCs are functionally hyperresponsive, exhibiting enhanced cytokine production in response to FcεRI stimulation. Analyses of biochemical events downstream of FcεRI show increased levels of PLCγ2, Erk1 and -2 phosphorylation, Ca^{2+} mobilization, and NFATc1 activation in $Itk^{-/-}$ versus wild-type mast cells. To account for this phenotype, we demonstrated an inducible

interaction between Itk and SHIP-1 following FcεRI stimulation. Furthermore, we showed that Itk could phosphorylate Dok-1, Dok-2, and SHIP-1 *in vitro* as well as co-localize with a multi-molecular negative signaling complex upon FcεRI stimulation. Taken together, these observations support the conclusion that Itk is important for the formation of an inhibitory signaling pathway that represses cytokine production following mast cell activation.

Results

Augmented levels of IgE on the surface of peritoneal mast cells from Itk$^{-/-}$ mice

We first assessed the phenotype of mast cells isolated from Itk$^{-/-}$ mice *ex vivo*. Total peritoneal exudate cells (PECs) were collected and stained with anti-c-kit and anti-IgE antibodies (Fig. 3.1A). c-kit^{+} PECs from Itk$^{-/-}$ mice consistently showed higher levels of anti-IgE staining compared to WT PECs. Given that Itk$^{-/-}$ mice exhibit spontaneously high levels of IgE in the serum and that IgE alone induces upregulation of FcεRI on mast cells, this finding was not surprising (151-153, 161, 272).

We then assessed the functional responsiveness of PMCs from Itk$^{-/-}$ and WT mice by crosslinking the FcεRI. To do this, c-kit^{+} cells were isolated from total peritoneal exudate by magnetic bead selection. Toluidine blue staining of the resultant cells confirmed a highly pure population of mast cells (data not

Figure 3.1

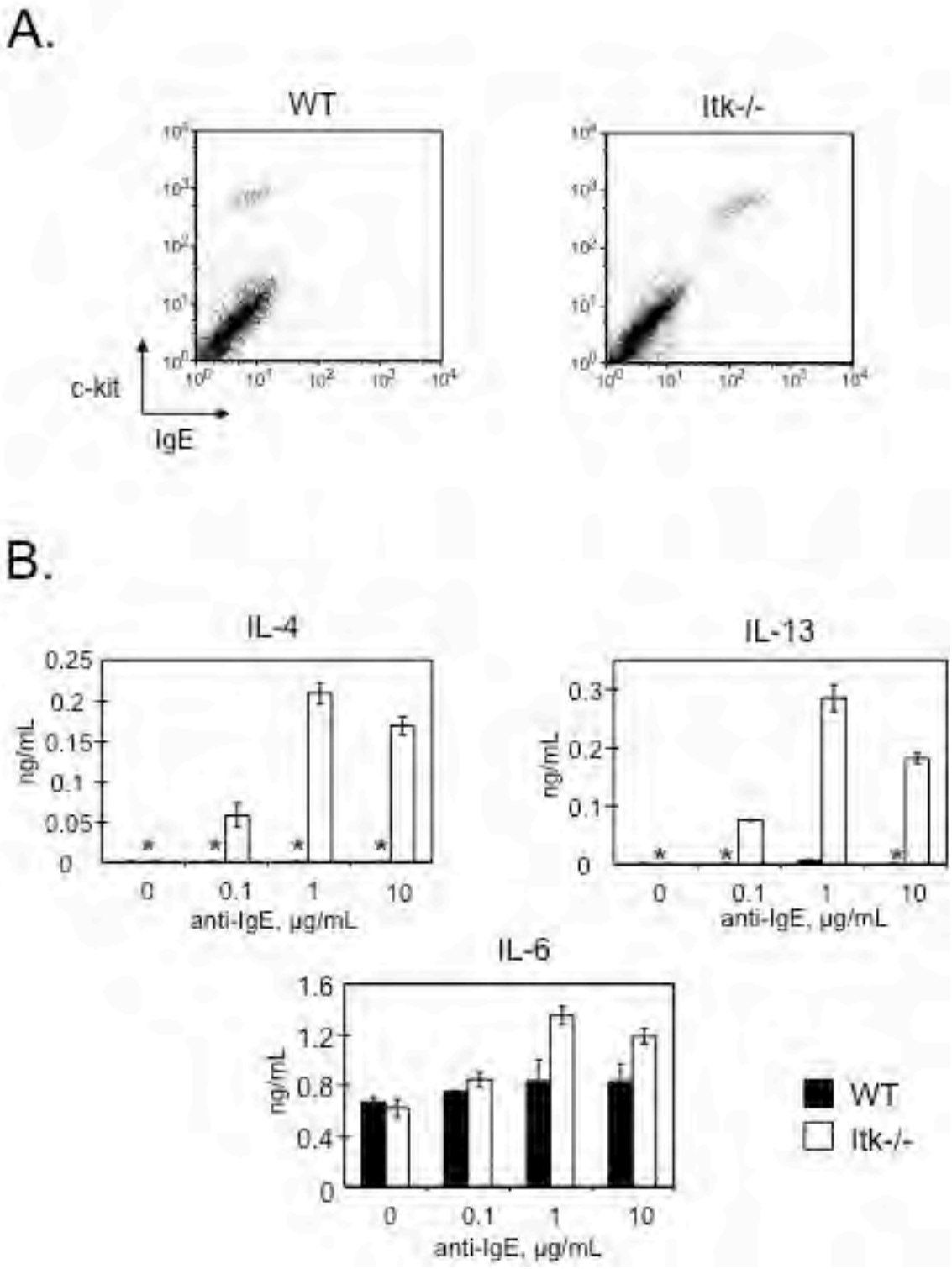
A.
WT
Itk-/-
c-kit
IgE
B.
IL-4
IL-13
IL-6
ng/mL
anti-IgE, µg/mL
0
0.1
1
10
WT
Itk-/-

Figure 3.1 Peritoneal mast cells from Itk$^{-/-}$ mice show elevated surface IgE and enhanced responsiveness to FcεRI stimulation

(A) PECs were stained with anti-IgE-FITC and anti-c-kit-PE and analyzed by flow cytometry. Plots shown are gated on live SSChi cells.

(B) WT (black bars) or Itk$^{-/-}$ (white bars) c-kit+ PECs were isolated by positive selection using magnetic beads. After overnight incubation, cells were stimulated with plate-bound anti-IgE at the indicated concentrations. Cytokines were detected by ELISA 20 hrs post IgE crosslinking. (*below limit of detection: IL-4, 0.03 ng/mL; IL-6, 0.016 ng/mL; IL-13, 0.02 ng/mL). Data shown are representative of 4 independent experiments.

shown). When Itk$^{-/-}$ PMCs were stimulated with plate-bound anti-IgE antibody, IL-4 and IL- 13 secretion was readily observed. In contrast, WT PMCs produced amounts of cytokine that were generally below the level of detection (Fig. 3.1B). Unlike IL-4 and IL-13, IL-6 secretion by both types of PMCs was detectable. However, Itk$^{-/-}$ PMCs showed a slight increase in IL-6 release upon IgE crosslinking. The elevated cytokine response exhibited by Itk$^{-/-}$ mast cells is likely due to enhanced mast cell stimulation generated by the high levels of IgE/FcεRI complex on the surface of these cells.

Itk$^{-/-}$ BMMCs appear developmentally normal and produce a normal degranulation response

To examine mast cell responses in the absence of the environmental influences present in Itk$^{-/-}$ mice, we generated mast cell cultures from bone marrow progenitors (BMMCs). As expected, Itk is expressed in WT BMMCs and is absent from Itk$^{-/-}$ BMMCs (Fig. 3.2A). We also found no detectable compensatory changes in Btk expression in Itk$^{-/-}$ BMMCs. Importantly, and in contrast to the *ex vivo* data, Itk$^{-/-}$ BMMCs showed no consistent difference in the expression levels of FcεRI compared to WT BMMCs. This enabled a functional comparison of cells with similar levels of surface FcεRI (Fig. 3.2B).

To address whether the lack of Itk in mast cells affected degranulation, BMMCs were first sensitized with anti-DNP IgE then incubated with varying amounts of DNP-HSA to crosslink the receptor. Degranulation, as assessed by

Figure 3.2

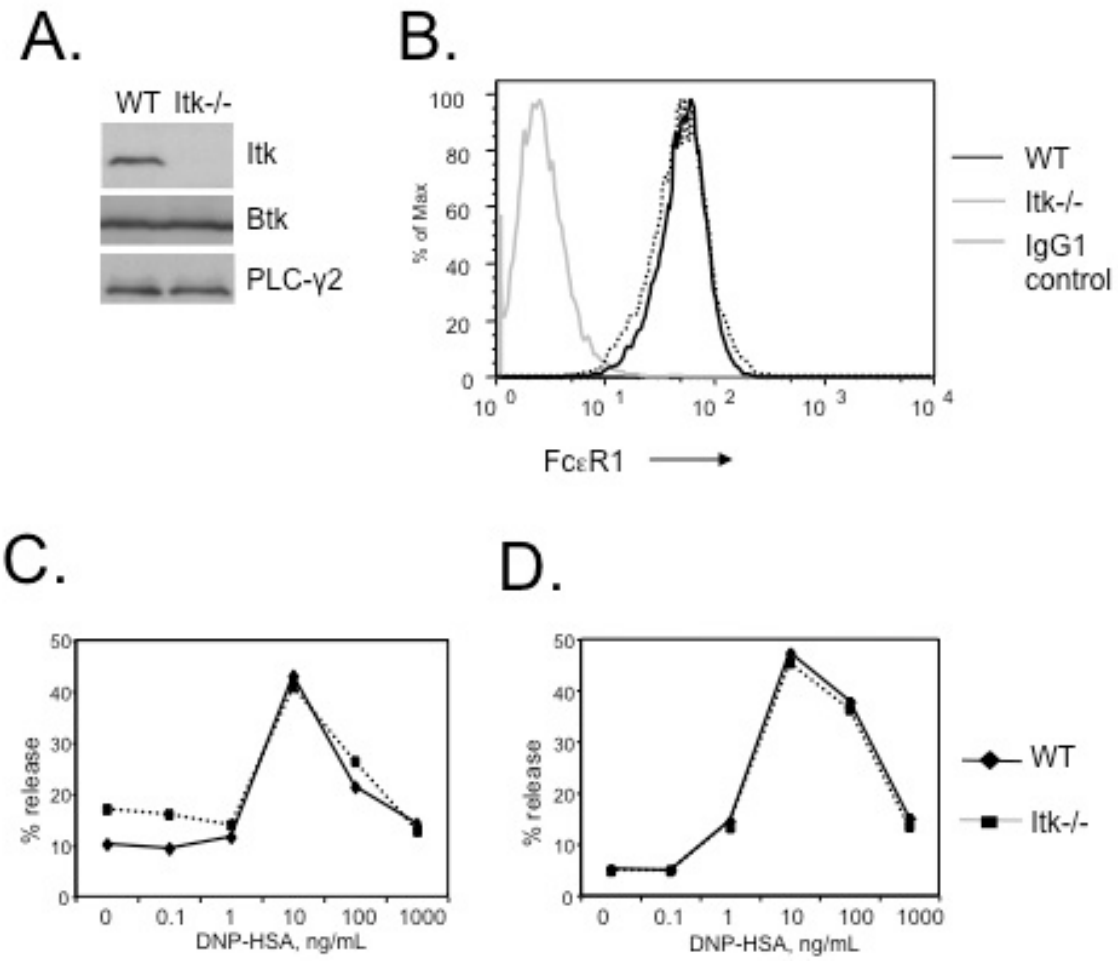
A.
WT Itk-/-
Itk
Btk
PLC-γ2
B.
% of Max
FcεR1
WT
Itk-/-
IgG1 control
C.
% release
DNP-HSA, ng/mL
D.
% release
DNP-HSA, ng/mL
WT
Itk-/-

Figure 3.2 Degranulation responses of WT and Itk$^{-/-}$ BMMCs are similar

(A) Itk protein in WT and Itk$^{-/-}$ BMMC lysates was assessed by immunoblotting with anti-Itk antibody. The membrane was reprobed with anti-Btk and anti-PLC-γ2 antibodies.

(B) WT (solid line) or Itk$^{-/-}$ (dotted line) BMMCs were blocked with 2.4G2 for 5-10 min, then stained with anti- FcεRI or control IgG (thick grey line) for 20 min, and analyzed by flow cytometry.

(C) BMMCs were sensitized with IgE (SPE-7) for 4 hrs, followed by incubation with the indicated doses of DNP-HSA. After 30 min, β-hexosaminidase release was assessed (WT, solid line; Itk$^{-/-}$, dotted line). Data shown are representative of 4 independent experiments.

(D) Assay was performed as in (C) but with H1-DNP-α-26 IgE for sensitization. Data shown are representative of 2 independent experiments.

β-hexosaminidase release, was similar between WT and Itk$^{-/-}$ mast cells (Fig. 3.2C). However, we consistently observed slightly higher 'background' degranulation (no antigen) in Itk$^{-/-}$ BMMCs compared to WT BMMCs. We interpreted this as an indication that Itk$^{-/-}$ mast cells were hypersensitive to the low amount of signal generated by the binding of the SPE-7 clone (anti-DNP IgE antibody) to the FcεRI. A review of the literature confirmed our suspicions, as this IgE clone has been suggested to induce some receptor signaling even in the absence of antigen-mediated crosslinking (154, 155, 273). Accordingly, when these experiments were repeated using the anti-DNP IgE clone H1-DNP-α-26 the augmented 'background' degranulation disappeared (Fig. 3.2D) (156, 274). Therefore, maximal FcεRI-mediated degranulation is similar in magnitude and dose response to antigen in Itk$^{-/-}$ and WT BMMCs. However, Itk$^{-/-}$ mast cells have a heightened sensitivity to IgE binding alone following SPE-7 incubation.

Increased cytokine production by Itk$^{-/-}$ BMMCs upon FcεRI stimulation

In T cells, a deficiency in Itk leads to impaired production of cytokines following TCR stimulation. With this in mind, we assessed the ability of Itk$^{-/-}$ mast cells to produce cytokines upon FcεRI stimulation. To our surprise, we consistently found that the secretion of cytokines (IL-4, IL-6, and IL-13) by Itk$^{-/-}$ BMMCs was higher than that produced by WT BMMCss (Fig. 3.3A). Furthermore, incubation of BMMC with IgE (SPE-7), in the absence of antigen,

Figure 3.3

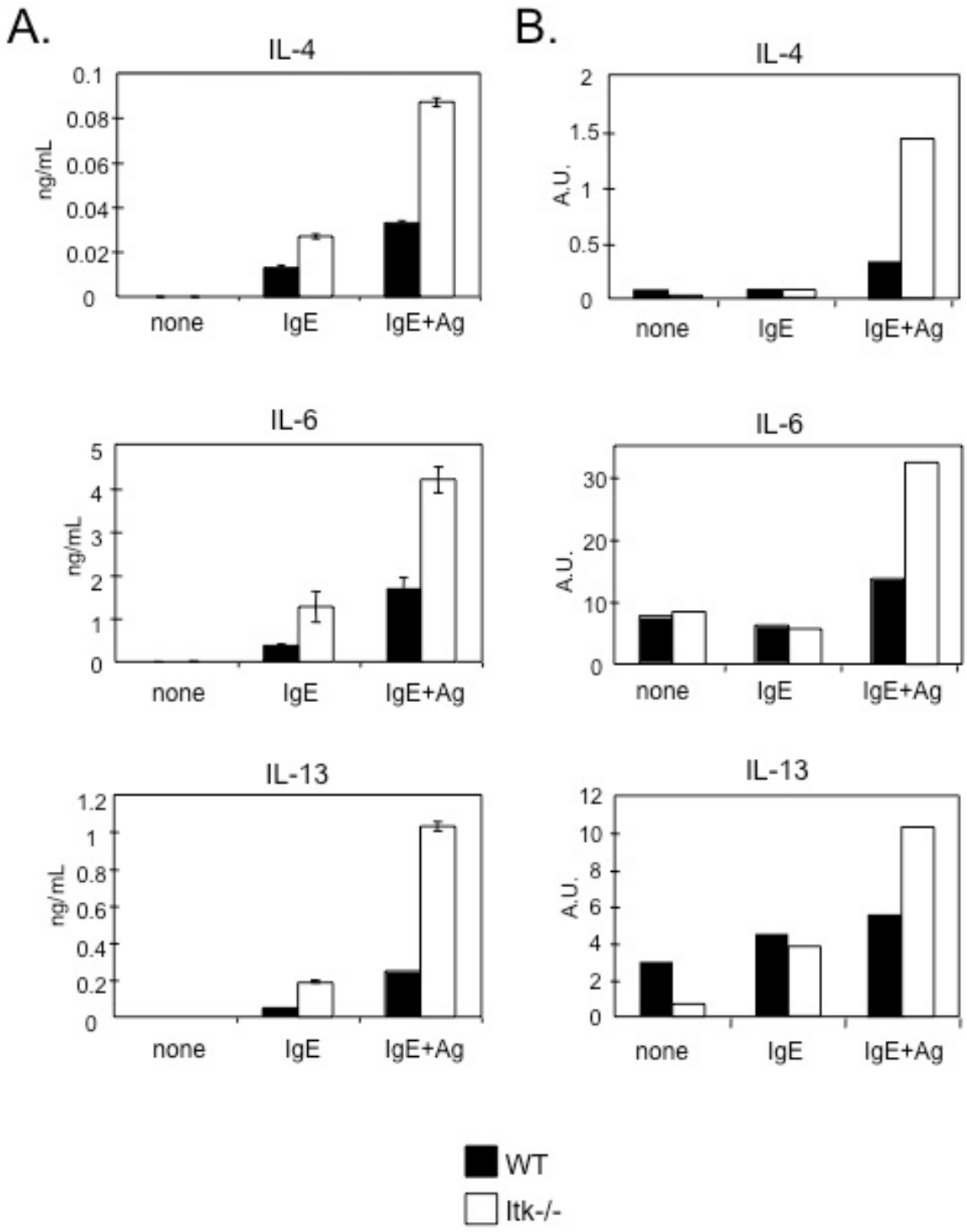
A.
IL-4
ng/mL
0.1
0.08
0.06
0.04
0.02
0
none
IgE
IgE+Ag
B.
IL-4
A.U.
2
1.5
1
0.5
0
none
IgE
IgE+Ag
IL-6
ng/mL
5
4
3
2
1
0
none
IgE
IgE+Ag
IL-6
A.U.
30
20
10
0
none
IgE
IgE+Ag
IL-13
ng/mL
1.2
1
0.8
0.6
0.4
0.2
0
none
IgE
IgE+Ag
IL-13
A.U.
12
10
8
6
4
2
0
none
IgE
IgE+Ag
WT
Itk-/-

Figure 3.3 Increased cytokine production by Itk$^{-/-}$ BMMC upon FcεRI stimulation

(A) WT (black bars) or Itk$^{-/-}$ (white bars) BMMC were incubated alone (none), or with 1 μg/mL IgE for 4 hrs with (IgE+Ag) or without (IgE) 30 ng/mL DNP-HSA (Ag) for 20 hrs. Supernatants were analyzed for cytokines by ELISA; limits of detection: IL-4, 0.002 ng/mL; IL-6, 0.008 ng/mL; IL-13, 0.02 ng/mL. Data shown are representative of 4 independent experiments.

(B) WT (black bars) or Itk$^{-/-}$ (white bars) BMMC were incubated as in (A), but cells were harvested 4 hrs after addition of Ag. Amounts of cytokine mRNA were determined by quantitative RT-PCR, and are expressed as arbitrary units (A.U.) relative to β2-microglobulin mRNA. Data shown are representative of 2 independent experiments.

stimulated higher levels of cytokine production from Itk$^{-/-}$ mast cells compared to WT mast cells. This observation correlated with the degranulation response.

Cytokines secreted from mast cells can be stored as preformed mediators or be newly synthesized in response to signal transduction. The release of cytokines in both situations can be mediated through FcεRI stimulation. To distinguish between the two events, we assessed the levels of cytokine mRNA in WT and Itk$^{-/-}$ mast cells by quantitative real-time PCR. As shown in Figure 3.3B, the pattern of cytokine transcript levels mirrored the pattern of cytokine secretion. Itk$^{-/-}$ BMMCs consistently transcribed more mRNA for IL-4, IL-6, and IL-13 than WT BMMCs in response to FcεRI stimulation. Thus, the augmented cytokine secretion observed in the absence of Itk is due to increases in the *de novo* synthesis of these cytokines. These data also rule out the possibility that the increased cytokine production by Itk$^{-/-}$ BMMCs is an indirect effect of protease-mediated cytokine degradation. These results indicated the existence of a signaling defect in Itk$^{-/-}$ mast cells.

Enhanced FcεRI-induced signaling in Itk$^{-/-}$ BMMCs

A major function of Itk in T cells is to phosphorylate and activate PLCγ1. Consequently, in Itk$^{-/-}$ T cells, phosphorylation of PLCγ1 following TCR stimulation is reduced. To determine whether PLCγ phosphorylation is affected by the loss of Itk in mast cells, BMMCs were stimulated by FcεRI crosslinking and lysates were immunoblotted with phospho-specific antibodies against PLCγ1

Figure 3.4

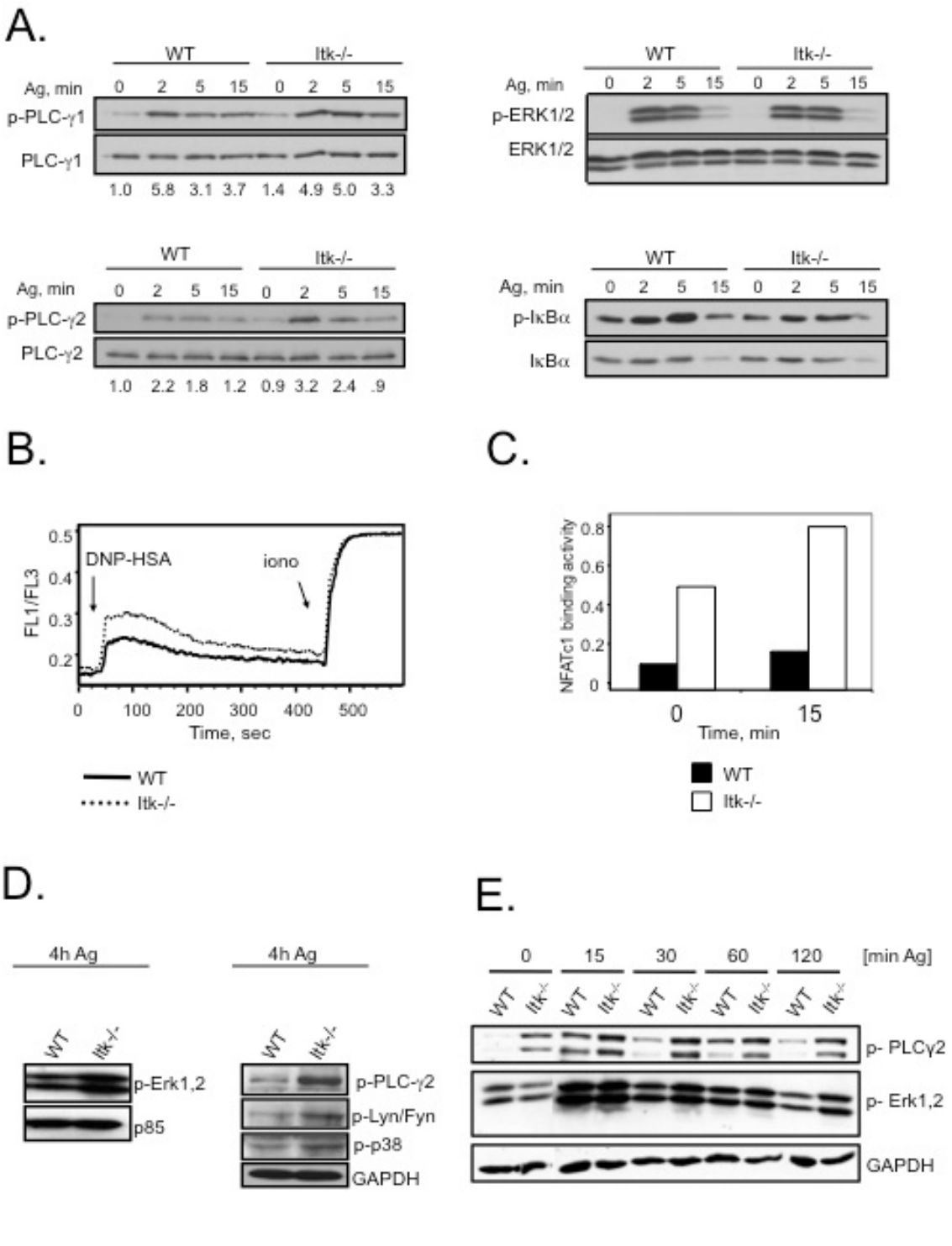
A.
WT
Itk-/-
Ag, min
0 2 5 15 0 2 5 15
p-PLC-γ1
PLC-γ1
1.0 5.8 3.1 3.7 1.4 4.9 5.0 3.3
p-PLC-γ2
PLC-γ2
1.0 2.2 1.8 1.2 0.9 3.2 2.4 .9
p-ERK1/2
ERK1/2
p-IκBα
IκBα
B.
DNP-HSA
iono
FL1/FL3
Time, sec
WT
Itk-/-
C.
NFATc1 binding activity
Time, min
WT
Itk-/-
D.
4h Ag
WT
Itk-/-
p-Erk1,2
p85
p-PLC-γ2
p-Lyn/Fyn
p-p38
GAPDH
E.
0 15 30 60 120 [min Ag]
p- PLCγ2
p- Erk1,2
GAPDH

Figure 3.4 Enhanced FcεRI-induced activation of signaling pathways in Itk$^{-/-}$ BMMCs

(A) BMMCs were sensitized with 0.5 μg/mL IgE and incubated with 100 ng/mL DNP-HSA for the indicated times. Lysates were immunoblotted with anti-phospho-PLC-γ1 (upper left), anti-phospho-PLC-γ2 (lower left), anti-phospho-ERK1/2 (upper right), or anti-phospho-IκBα (lower right). Membranes were then stripped and reprobed with antibodies to detect total protein levels. Numbers under left panels represent densitometric values of phospho-PLC-γ bands normalized to total PLC-γ (loading), relative to the value for WT cells at the 0 time point. Data shown are representative of 3 independent experiments.

(B) WT (solid line) or Itk$^{-/-}$ (dotted line) BMMCs were sensitized with IgE and loaded with Fluo-3 and Fura Red. DNP-HSA (100 ng/mL), at 30 sec, and ionomycin (2 mM), at 8 min, were added and fluorescence was assessed by flow cytometry. Data shown are representative of 4 independent experiments.

(C) WT (black bars) or Itk$^{-/-}$ (white bars) BMMCs were incubated for 4 hrs with 0.5 μg/mL IgE and stimulated for 0 or 15 min with 100 ng/mL DNP-HSA. Nuclear extracts were isolated and subjected to NFATc1 ELISA assay. Data shown are representative of 3 independent experiments.

(D) WT or Itk$^{-/-}$ BMMCs were sensitized with 1 μg/mL IgE for 4 hrs and stimulated for 4 hrs with DNP-HSA. Lysates were probed for phospho-Erk1/2 or PI3-kinase p85 as a control (left panel), or phospho-PLCγ2, phospho-p38, phospho-Lyn/Fyn, and GAPDH as a control (right panel). Data shown are representative of 2 independent experiments.

(E) WT or Itk$^{-/-}$ BMMCs were stimulated as described above for the indicated time. Lysates were probed for phospho-PLCγ2, phospho-Erk1/2 and GAPDH as a control. Data shown are representative of 2 independent experiments.

and PLCγ2 (Fig. 3.4A). Although in some experiments Itk$^{-/-}$ mast cells showed a slight enhancement in PLCγ1 phosphorylation compared to WT mast cells, this effect was not consistently observed. In contrast, PLCγ2 phosphorylation was reproducibly increased in Itk$^{-/-}$ versus WT BMMCs following stimulation.

Since Ca^{2+} mobilization is a response that occurs downstream of PLCγ–induced IP_3 generation, we assessed whether intracellular Ca^{2+} levels were affected by the increased PLCγ2 phosphorylation observed in Itk$^{-/-}$ BMMCs. As shown in Fig. 3.4B, Itk$^{-/-}$ BMMCs exhibit an elevated rise in intracellular Ca^{2+} following FcεRI stimulation compared to WT BMMCs. This increased Ca^{2+} response led to enhanced activation of NFAT in stimulated Itk$^{-/-}$ BMMCs, indicating that the observed increases in PLCγ phosphorylation and Ca^{2+} mobilization lead to functionally significant changes in transcription factor activity (Fig. 3.4C). The effects on downstream signaling pathways at these time points were selective, however. The activation of ERK and NF-κB pathways was not detectably altered in Itk$^{-/-}$ versus WT BMMCs at periods up to 15 minutes following activation (Fig. 3.4A).

To reconcile the apparently modest effects on signaling pathways with the more robust effects on cytokine production in Itk$^{-/-}$ BMMC, we considered whether alterations in signaling might occur at time points later than those initially assessed. Therefore, we stimulated WT and Itk$^{-/-}$ BMMC for 4 hours and then prepared lysates for biochemical analysis. These experiments showed that Itk$^{-/-}$ BMMCs sustain elevated levels of PLCγ2 phosphorylation, Lyn/Fyn

phosphorylation, and phosphorylation of ERK1/2 compared to WT BMMCs for several hours following activation (Fig. 3.4D-E).

Itk binding to SHIP-1 is mediated by the Itk SH2 and SH3 domains

Given the aberrant regulation of cytokine production and the presence of a signaling defect in $Itk^{-/-}$ mast cells, we postulated that Itk could participate in an inhibitory pathway. To identify a mechanism for the inhibitory role of Itk on mast cell signaling, we considered the SHIP (SH2-containing inositol phosphatase) family of regulators. SHIP-1 and SHIP-2 have been implicated in the negative regulation of various signaling pathways, including FcεRI signaling in mast cells(117, 275-278). Further, mast cells lacking SHIP-1 produce elevated levels of cytokines following activation(157, 279, 280). These data, together with previous studies documenting an interaction between SHIP-1 and the Tec family kinase, Tec, led us to investigate a potential interaction with Itk and subsequent regulation of the SHIP pathway (126, 158). To test this, we first co-expressed Itk and SHIP-1 in 293T cells. When SHIP-1 was immunoprecipitated from lysates of pervanadate-treated cells, we observed that Itk was co-precipitated (Fig. 3.5A).

To identify the Itk protein domains involved in this interaction, pull-down experiments were performed using lysates of RBL-2H3 mast cells and GST-fusion proteins containing individual Itk domains. These experiments demonstrated that the SHIP-1 present in the RBL-2H3 cells bound to both the Itk SH2 and SH3 domains. However, binding to the Itk SH2 domain was only

Figure 3.5

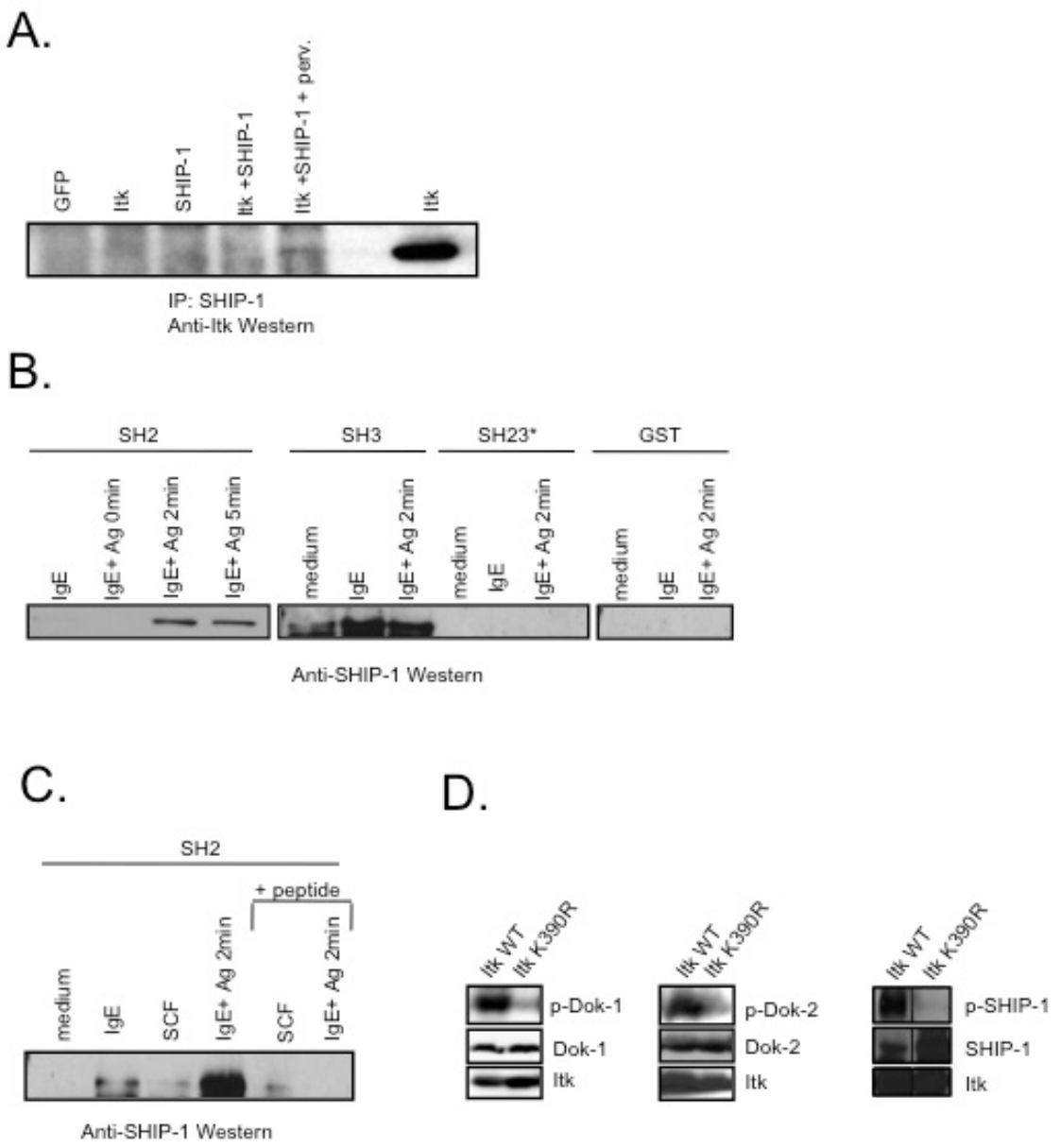
A.
GFP
Itk
SHIP-1
Itk +SHIP-1
Itk +SHIP-1 + perv.
Itk
IP: SHIP-1
Anti-Itk Western
B.
SH2
SH3
SH23*
GST
IgE
IgE+ Ag 0min
IgE+ Ag 2min
IgE+ Ag 5min
medium
IgE
IgE+ Ag 2min
medium
IgE
IgE+ Ag 2min
medium
IgE
IgE+ Ag 2min
Anti-SHIP-1 Western
C.
SH2
+ peptide
medium
IgE
SCF
IgE+ Ag 2min
SCF
IgE+ Ag 2min
Anti-SHIP-1 Western
D.
Itk WT
Itk K390R
p-Dok-1
Dok-1
Itk
Itk WT
Itk K390R
p-Dok-2
Dok-2
Itk
Itk WT
Itk K390R
p-SHIP-1
SHIP-1
Itk

Figure 3.5 Itk binds to and can phosphorylate SHIP-1

(A) 293T cells were transfected with expression vectors encoding GFP, Itk, SHIP-1, or both Itk and SHIP-1. After 24 hrs cells were left untreated or were stimulated with pervanadate as indicated. SHIP-1 was immunoprecipitated and filters were probed with antibodies to Itk. Lane at far right indicates Itk levels in the whole cell lysate.

(B) RBL-2H3 mast cells were left untreated, or were sensitized with 1 μg/mL IgE for 4 hrs and then stimulated with 100 ng/mL DNP-HSA for the indicated times. Lysates were incubated with beads containing GST-fusion proteins of the Itk SH2 domain (SH2), the Itk SH3 domain (SH3), the mutated Itk SH2+SH3 domains (SH23*), or GST alone (GST). Bound SHIP-1 was detected by immunoblotting with anti-SHIP-1 antibodies. Data shown are representative of 3 independent experiments.

(C) WT BMMCs were left untreated, stimulated with IgE alone, stimulated with 20 ng/mL SCF for 4 hrs, or sensitized with 1 μg/mL IgE (4 hrs) and stimulated with 100 ng/mL DNP-HSA (IgE+Ag) for the indicated time points. Lysates were incubated with the Itk GST-SH2 domain fusion protein, and bound SHIP-1 was detected with anti-SHIP-1 antibodies. Where indicated, the Itk GST-SH2 fusion protein was pre-incubated with 3 mM of a competitive phospho-peptide (AC-ADpYEPP-NH2) prior to incubation with cell lysates.

(D) Sf9 insect cells were infected with baculoviruses expressing WT Itk (Itk WT) or a kinase-inactive Itk mutant (Itk K390R) together with viruses expressing Dok-1 (left panel), Dok-2 (middle panel) or SHIP-1 (right panel). Sf9 cells were

harvested after 3 days. Cell lysates were probed with anti-phosphotyrosine antibody (top panels) or antibodies to Itk, Dok-1, Dok-2, or SHIP-1 (bottom panels).

detected when the RBL cells were activated prior to lysis (Fig. 3.5B). Single amino acid substitutions in the ligand-binding pockets of the Itk SH2 and SH3 domains (SH23*) prevented binding to SHIP-1 (Fig. 3.5B). In addition, binding of SHIP-1 to the Itk SH2 domain was blocked by prior incubation of the SH2 domain with a phospho-peptide ligand, AC-ADpYEPP-NH2 (Fig. 3.5C). This peptide binds to the canonical phospho-tyrosine binding pocket of the Itk SH2 domain (158, 281).

SHIP-1 binds to LAT and recruits 'downstream of kinase' molecules, Dok-1 and Dok-2, to form an inhibitory complex in T cells (159, 282). We hypothesized that Itk could potentially directly phosphorylate SHIP-1, Dok-1, or Dok-2, thereby contributing to the activity of this complex. For these experiments, Itk was co-expressed with SHIP-1, Dok-1, or Dok-2 in insect cells using baculovirus constructs. As a negative control, each potential substrate was also co-expressed with a kinase-inactive form of Itk. As shown in Figure 3.5D, wild type Itk, but not kinase-inactive Itk, was able to phosphorylate SHIP-1, Dok-1, and Dok-2.

Itk co-localizes with the SHIP-1 complex after mast cell activation

To examine whether Itk subcellular localization is regulated by mast cell activation, we performed confocal microscopy on wild type BMMCs. In unstimulated BMMCs, Itk is diffusely distributed in the cytosol, whereas LAT is membrane-localized, as expected (Fig 3.6A,C). Following activation by FcεRI

Figure 3.6

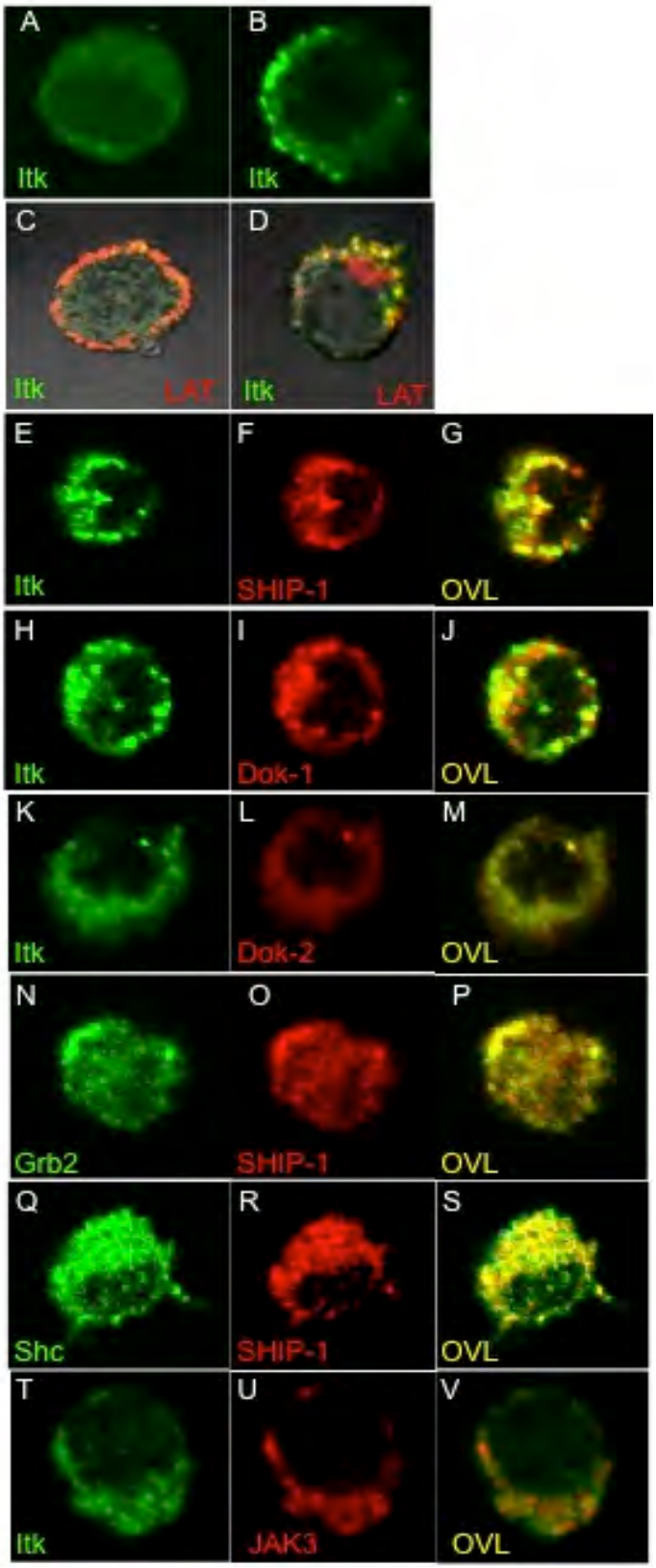
A
Itk
B
Itk
C
Itk
LAT
D
Itk
LAT
E
Itk
F
SHIP-1
G
OVL
H
Itk
I
Dok-1
J
OVL
K
Itk
L
Dok-2
M
OVL
N
Grb2
O
SHIP-1
P
OVL
Q
Shc
R
SHIP-1
S
OVL
T
Itk
U
JAK3
V
OVL

Figure 3.6 Itk co-localizes with components of the SHIP-1 complex following FcεRI stimulation

WT BMMCs were left untreated (A,C) or stimulated with IgE/DNP-HSA for 5 min (B, D, E-V). Cells were fixed, permeabilized and stained with primary antibodies against Itk, LAT, SHIP-1, Dok-1, Dok-2, Shc, and Grb2 as indicated. Coverslips were incubated with secondary antibodies AlexaFluor 488 and AlexaFluor 568 and analyzed by confocal microscopy. Right panel represent superimposed images (D,G,J,M,P,S,V). Yellow color of overlay indicates co-localization of proteins. Data are representative of at least 2 independent experiments with at least 10 cells analyzed per antibody stain.

stimulation for 5 min, Itk is recruited to the cell membrane where it co-localizes with a substantial proportion of LAT molecules (Fig. 3.6B,D). Furthermore, we observed Itk co-localization with components of the previously described inhibitory complex that includes SHIP-1, Dok-1, and Dok-2 (Figure 3.6E-M). In addition, Grb2 and Shc were found to co-localize with SHIP-1 in activated BMMCs (Fig. 3.6N-S), supporting interactions between these proteins, as has been previously reported (159, 282, 283). In contrast, a cytoplasmic kinase that is not involved in FcεRI signaling, Jak3, does not co-localize with Itk after mast cell activation (Fig 3.6T-V). These results indicate that Itk is part of an inhibitory complex in mast cells stimulated through the FcεRI.

Discussion

In contrast to T and B lymphocytes, mast cells express high levels of Itk and Btk as well as low levels of Rlk and Tec. It is well established that Itk deficiency in T cells drastically impairs TCR signaling, which is consistent with a positive regulatory function. In this report, we describe a novel function for Itk as a negative regulator in mast cell activation. This conclusion is based on the initial observation that Itk-deficient mast cells produce higher amounts of IL-4, IL-6, and IL-13 than WT mast cells following FcεRI stimulation. We demonstrate that the elevated cytokine levels are due to aberrant *de novo* synthesis of cytokine transcript, an indication of a genuine signaling defect. Consistent with these

findings, our biochemical studies indicate that PLCγ, ERK1/2, and LAT phosphorylation are elevated in activated mast cells lacking Itk. This enhanced signaling, in turn, leads to enhanced Ca^{2+} mobilization and increased NFATc1 activation.

Interestingly, the most striking differences in phosphorylation of signaling proteins between WT and $Itk^{-/-}$ mast cells occur hours after FcεRI stimulation, rather than minutes. These data support a role for Itk in a late-acting negative feedback pathway that dampens FcεRI-mediated activation. Moreover, $Itk^{-/-}$ BMMCs share several striking similarities with BMMCs lacking the inositol phosphatase, SHIP-1. For example, both $SHIP\text{-}1^{-/-}$ and $Itk^{-/-}$ BMMCs show an enhanced Ca^{2+} flux in response to FcεRI crosslinking. Even more interesting, both types of cells respond to stimulation with anti-DNP IgE (SPE-7) in the absence of antigen (160, 161, 279). These findings suggest that Itk may function in a SHIP-1-dependent inhibitory pathway downstream of the FcεRI.

SHIP-1 signaling is mediated by the recruitment of adapter proteins, Dok-1 and Dok-2 (162, 284). Dok proteins have been identified as leukemia suppressors, and are substrates of the oncogene p210bcr-abl. These adaptor proteins suppress activation signals by inhibiting the Ras-Erk pathway (160, 285). Similar to $Itk^{-/-}$ and $SHIP\text{-}1^{-/-}$ BMMCs, mast cells that lack Dok-1 also produce elevated levels of cytokine when stimulated with IgE alone (163, 164, 286). In addition, Dok-1 over-expression leads to reduced phosphorylation of Erk-1/2, as well as reduced TNFα production in RBL-2H3 cells stimulated by FcεRI crosslinking (165, 284).

These striking similarities between mast cells lacking Itk, SHIP-1, and Dok-1 suggest that Itk may function in this inhibitory signaling pathway. Supporting this, we find that Itk is recruited to the cell membrane following FcεRI stimulation, where it co-localizes with SHIP-1, Grb2, Dok-1, and Dok-2. Our biochemical studies suggest that, once recruited to the cell membrane, Itk associates with SHIP-1 via the Itk SH2 and SH3 domains. In T cells, the SHIP-1 complex is formed with the adaptor protein LAT. Therefore, a similar mechanism may exist in mast cells, as we detect substantial co-localization of Itk and LAT upon FcεRI engagement. SHIP-1 has also been reported to bind to phosphorylated ITAMs of the FcεRI beta subunit, indicating an additional pathway for recruitment of SHIP-1 to the activated FcεRI (166, 287).

Biochemical interactions between components of a SHIP-1 signaling complex have been described in several systems. For instance, tyrosine phosphorylation of Dok-1 is associated with increased binding of Grb2 (167, 286). In addition, LAT and SHIP-1 are required for TCR-induced Dok-2 tyrosine phosphorylation in T cells, with SHIP-1 acting as an adapter protein coupling Dok-2 to LAT (168, 282). Finally the Tec kinase, Tec, forms a tri-molecular complex with SHIP-1 and Lyn in cells stimulated through cKit, and in T cells the Tec SH3 domain interacts with SHIP-1 and SHIP-2 (126, 169, 288). Taken together, these data support the hypothesis that SHIP-1 signaling complexes can contain a Tec family tyrosine kinase, in addition to Lyn, Dok-1, Dok-2, and other adapter proteins.

The function of Itk following its interaction with SHIP-1 in activated mast cells remains to be determined. At the FcεRI, SHIP-1 is activated by the Lyn kinase, however it is currently unclear whether Lyn is directly or indirectly mediating this effect (170, 171, 289). One possibility is that Itk acts as an intermediary between Lyn and SHIP-1 and may directly phosphorylate SHIP-1. Along these lines, Itk phosphorylation of SHIP-1 could provide additional docking sites for the SH2 domains of other signaling molecules recruited to the SHIP-1 complex. Alternatively, Itk may not phosphorylate SHIP-1 in intact mast cells, but instead may bind to SHIP-1 and thereby be brought into proximity with other potential substrates, such as Dok-1 or Dok-2. Thus far, our preliminary experiments have not demonstrated reduced phosphorylation of SHIP-1, at least at Y1020, in mast cells lacking Itk. This may indicate that SHIP-1 is not a substrate of Itk in mast cells or that Itk phosphorylates SHIP-1 at a different site.

Our data has also demonstrated that mast cells isolated from the peritoneum of $Itk^{-/-}$ mice have high levels of surface IgE. Consequently, cytokine production by $Itk^{-/-}$ PMCs in response to IgE crosslinking was dramatically increased compared to WT PMCs. This reflected, at least in part, an elevated signal generated through the high numbers of FcεRI on the surface of these cells. Importantly, IL-4 and IL-13 secretion by $Itk^{-/-}$ PMCs were undetectable in the absence of IgE crosslinking, indicating that these cells are not spontaneously secreting cytokine. Based on these findings, we speculate that enhanced cytokine production by $Itk^{-/-}$ mast cells *in vivo* may contribute to the spontaneous T_H2-biased phenotype of the $Itk^{-/-}$ mouse, as indicated by elevated levels of IgE in

the serum and germinal center hyperplasia (63, 161, 166, 172). Studies using a variety of experimental systems have shown that, *in vivo*, mast cells can influence the responses of B cells, T cells, and dendritic cells. Further, mast cell-derived cytokines have been shown to induce T_H2 cytokine production by CD4 *T cells in vitro*.

The studies described here reveal a regulatory role for Itk in mast cell signaling that is in direct contrast to its function in T cells. Our findings are also confirmed by a report by Iyer, et al. showing enhanced production of cytokines by $Itk^{-/-}$ mast cells stimulated through the FcεRI (75, 244). This study, however, was unable to detect any biochemical defect in FcεRI-mediated signaling from mast cells lacking Itk. Taken together these data provide strong support for a negative regulatory role for Itk in mast cells. As Itk inhibitors are being developed for therapeutic purposes in dampening T cell responses, particularly in the context of atopic diseases, these studies underline the importance of considering contributions from other cells that express Itk.

Materials and Methods

Mice

C57BL/10 (B10) and Itk$^{-/-}$ mice (159, 173-176) (backcrossed to B10 for 13 generations) were housed and bred in the pathogen-free animal facility at University of Massachusetts Medical School. All mice were used in accordance with the Institutional Animal Care and Use Committee.

Mast cells

BMMCs were generated by culturing total bone marrow isolated from B10 or Itk$^{-/-}$ mice in RPMI-1640 supplemented with 10% FCS, 10% WEHI-3 conditioned supernatant, 100 U/mL penicillin, 100 ug/mL streptomycin, 100 mM L-glutamine, 100 µM non-essential amino acids, 50 µM 2-ME, 10 µM HEPES buffer and 5-20 ng/mL mouse rIL-3 (R&D Systems). BMMCs were cultured at 1x10^6 cells/mL for 4 weeks before maturation was determined by staining with anti-FcεRI FITC (eBioscience) and anti-c-Kit PE (BD Biosciences). Flow cytometry was performed on a FACS Calibur or LSRII (both from BD Biosciences) and data was analyzed by FlowJo software (Tree Star). BMMCs that were >95% FcεRI+ c-Kit+ were used for biochemical assays between 4-6 weeks in culture. Additionally, primary mast cells from peritoneal exudate were isolated with anti-c-kit magnetic beads on an AutoMACS (Miltenyi Biotec) and analyzed as indicated. The RBL-

2H3 cell line were purchased from ATCC and cultured according to the supplier's recommendations.

Cell transfection

293T cells were transfected with indicated plasmids using Lipofectamine Reagent (Invitrogen) according to the manufacturer's instructions.

Cytokine measurements

Peritoneal mast cells were stimulated with plate-bound anti-IgE (Southern Biotechnology) for 20 hrs. BMMCs were sensitized with anti-DNP IgE, either SPE-7 (Sigma) or H1-DNP-α-26 (purified H1-DNP-α-26 was a generous gift from Dr. Juan Rivera, the hybridoma cell line was kindly provided by Dr. F. T. Liu), in IL-3-free media for 4-6 hrs. Cells were washed twice and incubated with DNP-HSA (Sigma) for 20 hrs. Cytokine release was detected by ELISA (IL-4, IL-6: BD Biosciences; IL-13: R&D Biosystems) or by Cytometric Bead Array (BD Pharmingen). Cytometric bead array sample data were analyzed on an LSRII using the BD CBA Analysis Software 1 (BD Pharmingen).

To assess cytokine mRNA, BMMCs were stimulated as described for cytokine release, but incubated with DNP-HSA for 4 hrs. RNA isolation (Qiagen) and cDNA synthesis (Invitrogen) were performed according to manufacturer's

instructions. Primer sequences for RT-PCR were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/). The following primer pairs (IDT), designated by PrimerBank ID, were used: IL-4: 10946584a1, IL-6: 13624311a1, IL-13: 6680403a2, β2 -microglobulin: 31981890a1. Quantitative real-time-PCR was performed using conditions as indicated by PrimerBank, with FastStart SYBR Green Master (Roche) on an iCycler (Bio-Rad).

Degranulation

β-hexosaminidase release was performed essentially as described previously (177, 229). Briefly, BMMCs were sensitized with 1 μg/mL IgE for 4-6 hrs then incubated with indicated doses of DNP-HSA for 30 min. Supernatants and cell lysates were incubated with 1 mM p-NAG (Sigma) for 1 hr. Na2CO3/NaHCO3 buffer (0.1 M) was added and absorbance read at 405nm. Percent degranulation was calculated as O.D. values of sup/(sup+pellet) X 100.

Immunoblotting

BMMCs were sensitized with 0.5-1 μg/mL IgE for 4-6 hrs in IL-3-free media, incubated with 100 ng/mL DNP-HSA as indicated, and lysed. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose (Schleicher & Schuell), and blotted with antibodies against Itk (178, 290), Btk, PLC-γ2, IκBα (Santa Cruz Biotechnology), p-PLC-γ1 (BioSource), PLC-γ1 (Upstate Biotech), p-PLC-γ2, p-

ERK1/2, ERK1/2, p-IκBα, p-Src, p-p38, GAPDH, and PI3-kinase p85 (Cell Signaling Technology). HRP-conjugated secondary antibodies (Sigma) and chemiluminescence reagents (Pierce) were used for detection. Densitometric analysis was performed with NIH ImageJ software.

Ca^{2+} flux

BMMCs were sensitized with 1 μg/mL IgE for 4 hrs, washed, and incubated in RPMI-3% FBS containing 16 μM Fura Red and 16 μM Fluo-3 (Molecular Probes) for 45 min at 37˚C. Cells washed in indicator-free media, resuspended in Tyrode's-BSA, and further incubated for 30 min at room temperature. Cells were analyzed by flow cytometry, with addition of DNP-HSA at 30 sec and ionomycin (Calbiochem) at 8 min. Ca^{2+} mobilization was assessed as a ratio of Fluo-3/Fura Red fluorescence.

NFAT assay

NFATc1 ELISA (Active Motif) was performed according to manufacturer's instructions.

Confocal Microscopy

BMMCs from B10 WT or Itk$^{-/-}$ mice were seeded on Fibronectin (Sigma-Aldrich, 1:50) coated coverslips, fixed in 2% paraformaldehyde, and permeabilized with 0.025% saponin. Cells were stained with primary antibodies against Itk (179-181, 290), Shc, Dok-1, Dok-2, Grb-2, LAT, or JAK3 (Santa Cruz Biotechnology) for 16 hours at 4°C. For co-staining, cells were incubated sequentially with two primary antibodies. Cells were washed twice for 5 min and incubated with secondary antibodies (rabbit anti-mouse AlexaFluor 488, donkey anti-goat AlexaFluor 568) for 1 hr at 4°C. Coverslips were mounted on microscope slides in 20 µL ProLong antifade mounting medium (Molecular Probes) and examined by confocal microscopy using a Leica SP1 laser scanning confocal microscope (Leica Microsystems).

GST-pulldown

GST fusion proteins were prepared as described previously (136, 182, 291). The Itk GST-SH23* construct contains the tandem Itk SH2 and SH3 domains, each of which carries a single amino acid substitution that abolishes ligand binding to the canonical binding pockets (124, 183, 291). Lysates from untreated or stimulated cells were incubated with the washed GST fusion matrices. For the competition assay, an Itk SH2 domain specific phosphopeptide (AC-ADpYEPP-NH2) was pre-incubated for 30 min with the matrix prior to the pulldown (63, 184, 281). For Western blots, membranes were probed with antibodies to SHIP-1 (P1C1, Santa Cruz Biotechnology).

Co-immunoprecipitation

Transfected 293T cells were left untreated or were treated for 15 min with pervanadate (0.4 mM Na3Vo4, 0.04% H2O2) just prior to harvesting at 24 hrs post-transfection. Immunoprecipitations were performed for 18 hrs with 2 µg anti-SHIP-1 antibody (clone P1C1, Santa Cruz Biotechnology) followed by Protein Agarose A+G (Santa Cruz Biotechnology) beads for 1 hr. Immunoprecipitates were washed in lysis buffer, boiled in sample buffer, and separated by 7.5% SDS polyacrylamide gel electrophoresis. For Western blots, proteins were transferred onto nitrocellulose membrane and probed with anti-Itk antibodies (185-187, 290).

Baculoviral expression

Sf9 insect cells were obtained from ATCC. Baculoviral stocks were generated using the Bac-to-Bac system (Invitrogen). Sf9 cells were infected with viruses encoding wild-type Itk or the kinase-inactive Itk (K390R). These cells were then co-infected with viruses expressing Dok-1, Dok-2, or SHIP-1 and incubated 72-96 hrs prior to harvesting. Membranes were incubated with antibodies against p-Tyr (4G10 Platinum, Upstate Biotechnology), Dok-1 (Santa Cruz Biotechnology), Dok-2 (Santa Cruz Biotechnology), or Itk (188, 189, 290).

Chapter 4: Discussion

Synopsis

The theory of signal transduction describes a series of sequential steps in which an extracellular ligand induces chemical changes inside a cell. This multistage process is initiated by ligation of a receptor embedded within a cell membrane and is potentiated, in part, by intracellular signaling kinases. Itk is a non-receptor tyrosine kinase that facilitates signal transduction downstream of several multi-chain immune recognition receptors. Since its discovery in the early 1990's, hundreds of scientific reports have elucidated the biochemical, cellular, and physiological nature of Itk. Biochemically, we understand how Itk is regulated and have identified several binding partners. We know the hematopoietic expression of Itk and its primary function in signal transduction. In a physiological sense, we are beginning to learn the role of Itk in several immunological processes. Despite this plethora of information, there is still much that remains unknown about Itk. This dissertation explores the behavior of Itk in the signal transduction of two cell types: T cells and mast cells.

Chapter II expands upon the body of work that describes Itk as a positive regulator of T cell activation. The data demonstrates that two types of signals, digital and analog, emanate from the TCR upon stimulation. Digital signals are readily observed when T cells are stimulated with very potent TCR ligands, a concept that is easily found in the literature. Less is known about the analog signals that a T cell generates. When T cells see antigens that are less potent but able to overcome tonic signaling, an analog T cell response is revealed.

Altering the activity of signaling mediators such as Itk and NFAT can modulate the signals generated when a T cell encounters a strong ligand. The significance of characterizing these two signals is underscored by the expression pattern of IRF4 and Eomes, two transcription factors important for the activation of CD8 T cells. As such, we propose that Itk is responsible for the graded response of T cell activation.

In contrast to the positive regulatory role that Itk plays in T cells, the role of Itk in mast cells is less understood. The data in chapter III describes a unique function for Itk as a negative regulator of mast cell activation. Mast cells lacking Itk, synthesize elevated levels of cytokines when stimulated through the FcεR1 compared to their wild-type counterparts. This hyperactivation of Itk-deficient mast cells correlates with enhanced and sustained signaling downstream of the FcεR1. Interestingly, SHIP-1 is identified as a novel binding partner for Itk. In this regard, we postulate that Itk participates in an inhibitory pathway that is responsible for dampening the *de novo* synthesis of cytokines.

The two data chapters contain an individual discussion section that considers the direct implications of the data presented with respect to signal transduction. This chapter attempts to expand upon those implications and further speculates about the physiological role that Itk has in both T cells and mast cells. This chapter is divided into two sections, each dealing with the signaling characteristics and physiological aspects of Itk in T cells and mast cells.

T cells

As described earlier, Itk has been shown to regulate TCR-mediated signals through the phosphorylation of PLCγ1 (151-153, 189). In turn, the second messengers IP_3 and DAG invoke conserved signaling pathways required for gene expression and optimal cellular activation. Precluding Itk function, either through genetic manipulation or pharmacological inhibition, severely disrupts the linkage between membrane proximal signaling events and major downstream pathways (159, 188). Intriguingly though, TCR signal transduction is not completely ablated in the absence of Itk. PLCγ1 still gets phosphorylated and gene transcription remains partially intact, albeit at a fraction of wild-type levels. Several questions immediately arise. Why, then, is Itk needed at all? In what manner does Itk modulate TCR signaling? Does Itk favor one major pathway over another? What transcriptional targets does Itk regulate? What other factors are implicated in this regulatory process? And finally, what are the cellular fates that Itk determines?

The data contained in chapter II directly addresses several of these questions. For example, one of the primary roles that Itk has is the modulation of TCR signal strength. It does so by converting digital signals, presumably derived from Src and Syk family kinases, into analog responses. A model that depicts the function of Itk in this capacity is described at the end of chapter II, Figure 2.7. Rather than reiterate the digital and analog manner in which Itk behaves downstream of the TCR, a more speculative examination of Itk in TCR signaling

and T cell responses will be discussed. In accordance with this, two more models are proposed that illustrate the interrelated aspects that Itk plays in:

1. TCR signal transduction.
2. The physiological response of T cells.

1. TCR signaling pathways

As mentioned previously, IRF4 expression is sensitive to the effects of Itk activity. Currently, it is not clear how IRF4 expression is regulated in CD8 T cells. Although we have implicated an NFAT-mediated pathway in graded IRF4 expression, it's uncertain as to whether other signaling pathways could be responsible for transducing analog signals. It is currently accepted that the NF-κB pathway transduces digital signals from the TCR to induce gene expression of targets like TNF and CD44 (190, 265, 292). The MAPK pathway possesses features of analog and digital activation but it is believed that the analog components of this pathway exist to switch the signal to a digital one (191, 249). By and large the contention in the field is that the MAPK pathway, notably Erk1/2, are partially responsible for digital activation of T cells (192, 193, 263, 293, 294).

An intriguing proposition then remains that the Ca^{2+} pathway could display analog characteristics. One of the more profound defects associated with Itk deficiency lies in its relationship with Ca^{2+} mobilization and NFAT activity (159, 194). Specifically, Itk-deficient T cells stimulated through the TCR display an intermediate level of Ca^{2+} mobilization compared to wild-type T cells and

unstimulated T cells. This suggests the existence of an implicit sensitivity of this pathway to Itk activity, and by extension to TCR signal strength. Hence, it came as no surprise to us that the NFAT inhibitor experiments essentially phenocopied the Itk inhibitor experiments in chapter II. These initial experiments conducted in chapter II have revealed that limiting the activity of NFAT by titration of the inhibitor yields graded IRF4 expression when CD8 T cells are stimulated with various peptides. This suggests that an NFAT-mediated pathway is capable of transmitting an analog signal from the TCR to the nucleus where induction of genes like *Irf4* will occur. At this time we are unsure whether graded Ca^{2+} mobilization is involved in our system. James Conley, in our lab, has planned two types of complementary experiments to address this issue. The first experiment involves the titration of the calcium ionophore ionomycin. Here, CD8 T cells will be stimulated with or without APLs in the presence of decreasing amounts of ionomycin. If Ca^{2+} mobilization can transduce analog signals then cells stimulated without additional TCR signals (ionomycin alone) should display graded IRF4 expression. Alternatively, if we see bimodal IRF4 expression it would suggest that other signals are required to cooperate with the Ca^{2+} pathway to generate intermediate levels of IRF4. These other signals would likely be resolved in the presence of TCR stimulation. A second type of experiment will use a calcium chelator, EGTA, to limit the availability of free calcium in the media. This will effectively change the extracellular calcium concentration and the amount of store operated calcium entry (SOCE) in stimulated cells can be controlled. This approach brings the added advantage of not bypassing

components of the TCR signaling cascade. Calcium chelation using increasing amounts of EGTA is expected to result in the graded expression of IRF4 when CD8 T cells are stimulated through the TCR. If neither of these experiments reveals an analog response, then another possibility is that NFAT activity can be induced in a Ca^{2+}-independent manner. Ca^{2+}-independent NFAT activation has been demonstrated in cardiomyocytes in response to leptin-mediated signaling (75, 295). Therefore it remains a possibility that CD8 T cells use similar signaling machinery to induce Ca^{2+}-independent NFAT activation.

Our data do not distinguish between the possibilities of direct or indirect regulation of IRF4 by NFAT. Preliminary analysis of the IRF4 proximal promoter has identified several putative binding sites for the transcription factor NFAT. Future chromatin immunoprecipitation experiments have been planned to investigate the direct regulation of IRF4 by NFAT. However, given what is known about the regulation of IRF4 expression in other cell types, it is unlikely that NFAT participates in this process alone. In B cells for example, IRF4 expression is induced by a combination of BCR stimulation and IL-4 signaling, which in turn activate the transcription factors NF-κB and STAT6, respectively (194, 296, 297). This is thought to lead to the graded expression of IRF4 in B cells (63, 298). Indeed, our analysis also identified STAT6 binding sites in the promoter as a potential candidate in the regulation of IRF4. However, both published and unpublished data from our lab indicates that when CD8 T cells are stimulated through the TCR in the presence of IL-4, IRF4 expression levels do not change (177, 259). It remains to be seen whether other cytokine signaling pathways can

synergize with TCR signaling to induce IRF4. It is a possibility that multiple pathways downstream of the TCR cooperate for full IRF4 expression. One report has suggested that the NF-κB pathway can induce transcriptional changes of *Irf4* in lymphocytes (195, 297). The potential for NF-κB to cooperate with NFAT is not novel, as they have both been linked to IL-2 transcription (196-200, 299). Future experiments in our lab will explore the cooperativity of multiple TCR-induced signaling pathways in the regulation of digital and analog responses. Nonetheless, we propose a model, illustrated in Figure 4.1, for T cell signaling that incorporates currently published theories described above with our own data.

2. The physiological response of T cells

One other aspect of our results on TCR signal strength deserves attention. Our experiments fail to directly test the immunological influence that TCR signal strength may pose. However, the literature is replete with examples of physiological processes using the exact cells and epitopes we have used. All of these processes are regulated by currently unexplained mechanisms; we feel that our data provides such a mechanism. Two specific examples will be discussed with respect to our data.

In a report published in *Nature*, Zehn and colleagues utilize an infection model that changes epitopes specific for CD8 T cells in order to study the immune response to *Listeria monocytogenes* (201-203, 247). Although all T cells respond

Figure 4.1

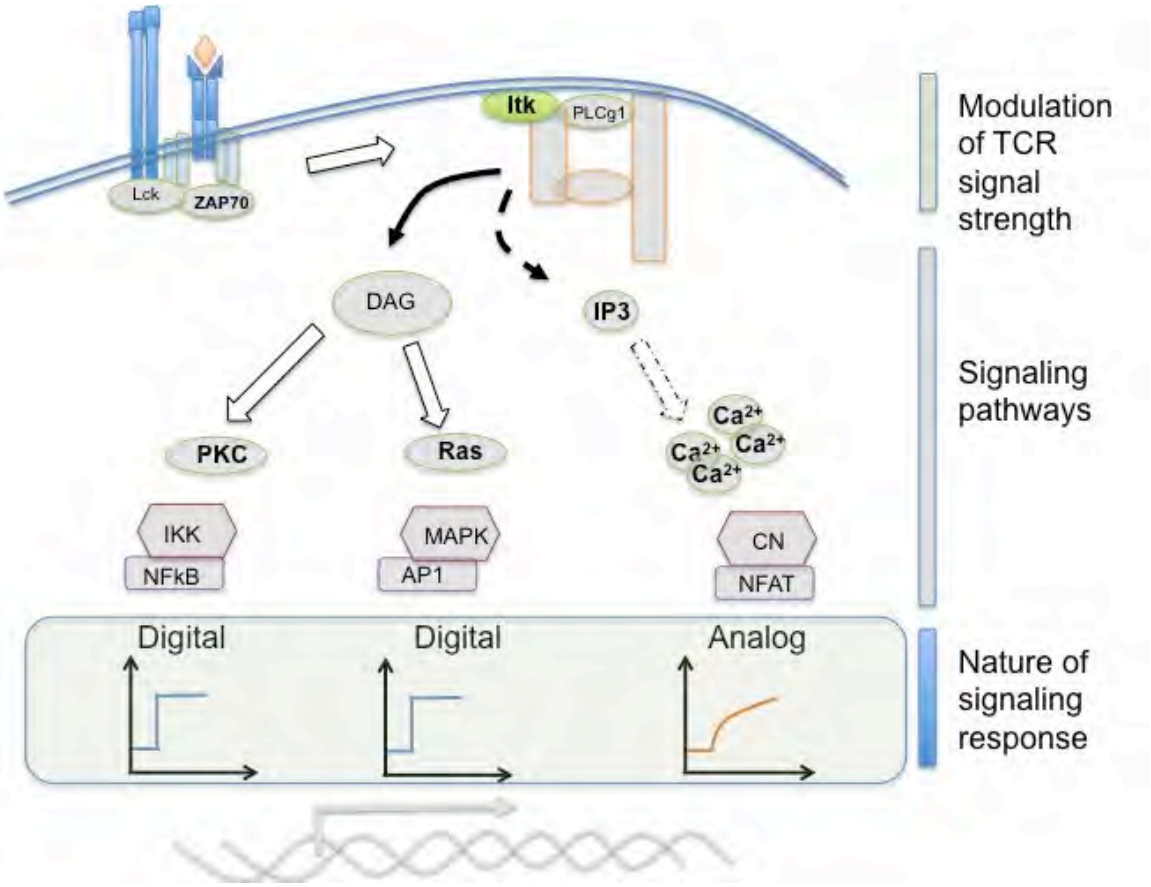

Figure 4.1 Model for T cell receptor signaling

TCR ligation (an analog input) leads to the recruitment and activation of PTKs at the plasma membrane. Digital conversion of the signal occurs by PTKs like Lck and ZAP-70. DAG-mediated pathways display digital activation and induce digital responses (88, 195, 204, 249). Concurrently, Itk can modulate the digital signal, which is dependent on the strength of the initial input, like a rheostat, thereby generating an analog signal. The analog signal then emanates through NFAT producing the graded expression of target genes.

to the initial challenge, they do so with dissimilar kinetics and magnitude. The initial rate of proliferation of CD8 T cells responding to high and low potency ligands is similar amongst the two cell populations. However the expansion of cells primed in response to low potency epitopes peaks two days earlier, at day 5.5, than those cells exposed to higher potency epitopes. Moreover, cells exposed to low potency ligands leave the draining lymph nodes prematurely and undergo contraction much earlier than cells exposed to strong TCR ligands. This implies that TCR signal strength is not important for the initial proliferative burst associated with CD8 T cell clonal expansion, but rather for the total amount of expansion, lymph node retention, and onset of contraction. Data from our lab indicates that CD8 T cells stimulated with APLs undergo similar proliferation out to 4 days in culture. This is consistent with what the authors observed *in vivo*. The findings in this report also demonstrate that the cells exposed to low potency ligands display a similar cell surface phenotype compared to those exposed to high potency ligands. This is the exact result that we see *in vitro*. The authors did not examine the transcription factor profile and therefore were unable to properly decipher the differentiation pathways that CD8 T cells undergo in response to a pathogen. We suspect, though, that more IRF4 would be seen in cells stimulated in the presence of strong TCR ligands, whereas those cells stimulated with weak TCR ligands would display low levels of IRF4. Here is where an interesting dichotomy occurs with our data. In contrast to the phenotype associated with IRF4 expression levels, we see an inverse relationship with Eomesodermin (Eomes). That is, weak signals from the TCR

induce more Eomes expression than strong signals. Our lab has recently published data suggesting that IRF4 can negatively regulate the expression of Eomes in CD8 T cells (205, 259). Given the importance of Eomes expression levels in determining the fate of CD8 T cell differentiation in response to infection, it is tempting to speculate that this might be regulated, in part, by IRF4. Ribhu Nayar and Beth Schutten, in our lab, are currently performing experiments to address the role of IRF4 in the response of CD8 T cells to LCMV. Thus far, these studies seem to indicate an important role for IRF4 in the generation of short-lived effector cells (SLEC), as mice infected with LCMV in the absence of IRF4 have about one third as many responding cells compared to wild-type. Taken together, we postulate that the strength of TCR stimulation received by a CD8 T cell has a direct influence on the outcome of differentiated T cells. We further propose that this outcome is regulated by the analog signals, mediated by the activity of molecules such as Itk and NFAT, which originate from the TCR.

In a second example recently published in *Immunity*, King and colleagues demonstrate the importance of TCR signal strength in mediating cell division of CD8 T cells (204, 246). Cells receiving stimulation from highly potent ligands undergo asymmetric cell division. These cells sustain long-lasting T cell - APC conjugates and undergo substantial proliferation. Asymmetry is demarcated by CD8 expression, where proximal daughter cells receive the most amount of CD8, while distal daughter cells receive less CD8. Interestingly the proximal daughter cells exhibit a phenotype resembling SLECs. In contrast to cells stimulated with strong TCR signals, cells receiving stimulation from less potent ligands undergo

symmetric division, do not have as long a contact time with an APC, and don't proliferate as much. The proliferation results are somewhat at odds with Zehn and colleagues but this is likely due to variation in the design and time points used in the *in vivo* system. *In vitro,* it is clear that cell division is influenced by TCR signal strength. Furthermore, it has previously been shown that asymmetric division is a key determinant in the differentiation of CD8 T cells *in vivo* (101, 205-207). Here, progeny of CD8 T cells that have divided asymmetrically are differentially marked by Eomes and T-Bet segregation. The authors in the current study fail to examine the transcription factor profile of daughter cells. Based on our data, however we would predict that cells receiving strong signal strength would express the least amount of Eomes and the most amount of IRF4. This is consistent with the idea that SLECs express less Eomes than their counterparts, memory precursor effector cells (MPECs).

In conjunction with our data, the two physiological examples described help us construct a more complete model for CD8 T cell differentiation. This model is depicted in Figure 4.2. We propose that cells receiving strong TCR signals will exhibit high levels of Itk activity, induce more IRF4, and promote the formation of SLECs. On the other hand, cells that receive weak TCR signals exhibit lower levels of Itk activity, induce more Eomes, and promote the formation of MPECs.

Figure 4.2

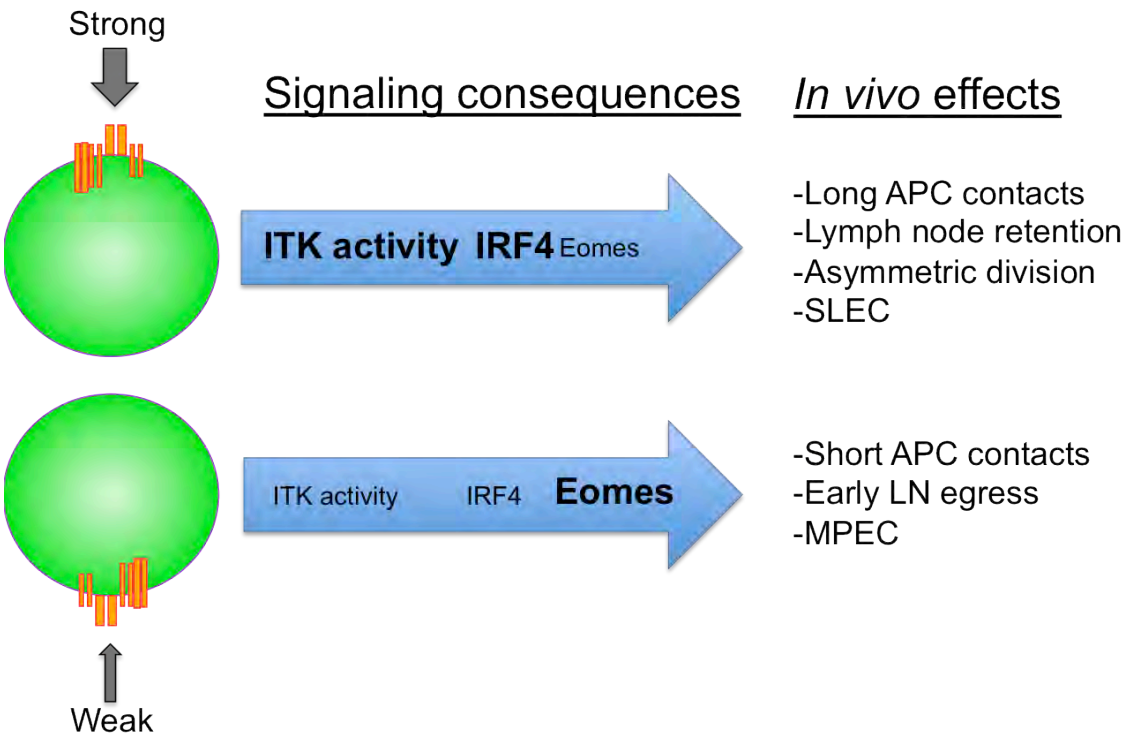

Figure 4.2 Physiological implications of altering TCR signal strength

T cell stimulation with ligands that induce strong signals result in high Itk activity, which generates copious amounts of IRF4. IRF4 represses Eomes expression. The immunological outcome is prolonged T cell:APC interactions and retention in the LN, asymmetric division, and differentiation of short lived effector cells (208, 300). T cells stimulated with weaker ligands do not impose as much Itk activity and therefore do not express high levels of IRF4. Low levels of IRF4 allow for high amounts of Eomes. This results in short T cell:APC contacts and early exit from LNs. Unpublished data from our lab suggests these cells become memory precursor effector cells.

Mast cells

In contrast to T cells, far less information is known about the role of Itk in mast cells. Although Itk is activated and recruited to the membrane upon crosslinking of the FcεR1, substrates for Itk have not been identified inside mast cells. A similar Tec family kinase, Btk, is also expressed in mast cells. The role of Btk in mast cells has been extensively studied. Btk-deficient mast cells have defects in all aspects of mast cell activation, including reduced cytokine expression and reduced degranulation. This phenotype is accompanied by a reduction in the activation of key signaling molecules, such as PLCγ, a major substrate for Btk. These defects highlight the importance of Btk in transducing FcεR1-mediated signals. The conclusion is that Btk is a positive regulator of mast cell activation.

The data in chapter III demonstrates that mast cells lacking Itk are hyperresponsive when activated through the FcεR1. This is shown by the increased levels of cytokines and enhanced signaling downstream of the FcεR1. We have proposed that Itk is involved in an inhibitory pathway that is regulated by the phosphatase SHIP-1. Another interesting observation in chapter III is the *ex vivo* phenotype of mast cells from Itk-deficient mice. Here, mast cells show elevated expression of FcεR1 on the cell surface. When these cells are stimulated by crosslinking the FcεR1, they produce more IL-4 and IL-13. The remainder of this chapter focuses on two major themes. First, the results from two major studies investigating the contribution of Itk-deficient mice in allergic

airway hyperresponsiveness are reconciled. These results are then linked to our results to form a model for the role Itk and Btk in mast cell activation. Finally, a speculative role for mast cells is attributed to one of the major unresolved phenotypes in Itk-deficient mice.

1. Itk limits the responsiveness of mast cells in the airway through an inhibitory pathway

The few studies of Itk in mast cells have been less fruitful and have generated contradicting conclusions. An initial report from Forssell and colleagues, concluded that mast cells in Itk-deficient mice have an important role in mediating immediate and late-phase allergic responses *in vivo* (209, 241). Compared with Btk-deficient mice, Itk-deficient mice displayed greater protection to an airway allergen challenge as demonstrated by a lack of plasma leakage into blood vessels. Examination of mast cells in the lungs of Itk-deficient mice revealed that this result was due to a severe impairment in the ability of cells to degranulate in response to the aerosolized allergen. These findings suggest that Itk strongly promotes mast cell degranulation in immediate airway hypersensitivity. The authors also looked for signs of inflammation in the airways by examining leukocyte infiltration. This is a hallmark of late-phase hypersensitivity, which occurs many hours after allergen challenge. In comparison to wild-type and Btk-deficient mice, Itk-deficient mice had very few cellular infiltrates in the lung tissue or bronchoaveolar lavage (BAL) fluid. The

authors concluded that Itk-deficient mast cells failed to promote inflammation because of an inability to generate the T_H2-cytokines required for chemotaxis and extravasation. These results further implicate Itk as an important mediator of airway hypersensitivity.

This paper was important for two reasons. First, it recapitulated many of the results seen in Btk-deficient mice and second, it ascribed a function for Itk in mast cells. One of the major faults with this study, though, is that Itk-deficient mice have an inherent inflation of circulating IgE within them (166, 210), and mast cells are sensitive to the amounts of IgE in the microenvironment. Mast cells acquire unbound IgE in the periphery by extending cellular projections through the walls of the blood vessels (191, 301). Upon sampling the environment, IgE is then bound by FcεR1 until a specific allergen or antigen can crosslink the receptors. Although the specificity of IgE antibodies in Itk-deficient mice is unknown, it is unlikely that they recognize the sensitizing allergen in this case. Moreover, the amount of available IgE has been shown to regulate mast cell proliferation, survival, and functionality (191, 211, 302). Consistent with this notion, our data has shown that the amount of IgE in the serum also influences the level of IgE receptor on the cell surface of mast cells. This is unsettling because the corresponding wild-type mast cells have fewer FcεR1 molecules per cell, giving Itk-deficient mast cells an unfair advantage in signal transduction. The other major caveat in this report is the model of hypersensitivity used. The authors chose to inject a mixture of ovalbumin and aluminum hydroxide as a way to sensitize and challenge mast cells. However, the cellular reactivity to this type

of allergen is not limited to mast cells (212, 303). Both antibody responses and T cell reactivity can play an important role in the immunological response to allergens in this hypersensitivity model. Itk-deficient mice, as far as we know, do not have any major developmental or functional defect in the B cell compartment. As documented several times in this dissertation, the T cell compartment is heavily skewed in these mice and functionality is impaired. For the reasons presented, the complete significance of Itk in the function of mast cells in this system is unclear.

A second group chose a different approach to examine the role of Itk in acute airway responsiveness (191, 244). Itk-deficient mice were sensitized with an anti-hapten IgE antibody. Aerosolized exposure to the hapten resulted in diminished airway responsiveness compared to wild-type mice when challenged with methacholine. This was attributed to defective release of histamine and is consistent with Forssell and colleagues. However, like our data shows, Iyer and colleagues also demonstrate that mast cells in Itk-deficient mice have elevated levels of IgE receptor on the cell surface. To bypass this caveat, they chose to incorporate an *in vivo* reconstitution model where wild-type or Itk-deficient BMMCs were adoptively transferred into mice lacking mast cells. When airway responsiveness was examined, mice reconstituted with Itk-deficient BMMCs responded the same way as wild-type controls. This indicates that *in vivo*, Itk is not necessary for mast cell degranulation. This correlates with our observation that Itk-deficient BMMCs do not have any noticeable degranulation defect *in vitro*. When Iyer and colleagues examined cytokine production in BMMC lacking Itk,

they observed increases in IL-2, IL-4, IL-13, TNF, and GM-CSF compared to wild-type BMMCs. The data in this dissertation support this observation and take it several steps further. Since mast cells can potently release preformed mediators, including cytokines like IL-4 and TNF, we wanted to determine whether this defect was from *de novo* synthesis (35, 65, 211, 213). We show that, in accordance with elevated protein levels, transcripts for the indicated cytokines were also increased in Itk-deficient mast cells. Our data implies that Itk-deficient mast cells do indeed have a signaling abnormality. Admittedly, when we look at early time points proceeding FcεR1 stimulation in Itk-deficient mast cells, we note only a mild signaling impairment. This is highlighted in the activation of PLCγ and is accompanied by a slight increase in Ca^{2+} mobilization and increased NFAT activity. Similarly, Iyer and colleagues also detect increased localization of NFAT in the nucleus of Itk-deficient mast cells. This is perplexing because they do not observe a single signaling defect in these cells. In fact, in some of their experiments they noticed some signaling components were diminished when compared to wild-type BMMCs. Nonetheless, the substantial elevation in cytokines we have both observed do not seem to fit with the signaling data. We rationalized that because the major defects observed in Itk-deficient mast cells were during the second wave of activation, perhaps we were not looking at the correct time points. We attempted to unravel this disconnect by inspecting the status of signaling molecules at much later time points following FcεR1 stimulation. Much to our surprise, Itk-deficient BMMCs not only displayed elevated levels of activated signaling molecules but they were

also sustained several hours following stimulation. Our data substantiates a bona fide signaling defect in response to FcεR1-mediated stimulation and suggests that Itk is a negative regulator of mast cell activation.

Negative regulation of FcεR1-mediated mast cell activation is facilitated by several signaling molecules including kinases (e.g. Lyn), phosphatases (e.g. SHIP), and adaptor proteins (e.g. Dok). One of the major inhibitory pathways that mast cells use to shutdown FcεR1-induced signaling is regulated by SHIP-1. This pathway is initiated by Lyn phosphorylation of SHIP-1, which upon activation, translocates to the membrane where phosphatase activity can catalyze the conversion of PIP_3 to PIP_2 (208, 304). Activation of SHIP-1 triggers the assembly of a larger complex containing adaptor proteins such as Dok1, Shc, and Grb2 that bind to the C-terminal domain of SHIP-1 (214, 305). Interestingly, mast cells lacking SHIP-1 or Dok1 exhibit a strikingly similar phenotype to Itk-deficient mast cells with one notable exception (208, 215, 279, 306). The amount of degranulation is about 4-fold higher in mast cells lacking SHIP-1 compared to wild-type controls. The other enticing piece of information was that the Tec family kinase, Tec, has been reported to associate with SHIP-1 via the SH3 domain in T cells (126, 216). A number of our biochemical experiments demonstrated a specific interaction between Itk and SHIP-1, mediated by the SH2 and SH3 domains of Itk. This was somewhat surprising because it had previously been reported that the SH3 domain of Itk did not bind to SHIP-1 in T cells (10, 126). Given the promiscuity attributed to SH3 domains in general, a likely explanation for this discrepancy is that other proteins not expressed in mast

cells occupied the SH3 domain of Itk. Nonetheless, the interaction we observed by the SH2 domain of Itk appeared to be specific because competitive inhibition or mutation of this domain ablated SHIP-1 association. Using confocal microscopy, we were also able to demonstrate co-localization of Itk with several members of the negative regulatory complex that SHIP-1 nucleates. The significance of SHIP-1 phosphorylation by Itk is unclear at the moment. Due to technical limitations, we have only been able to demonstrate phosphorylation in insect cells. Taken together, the biochemical data and microscopy suggest that Itk forms a novel interaction with a known negative regulatory complex in mast cells.

Given the data presented in this dissertation, in conjunction with the available information in the literature, we propose the model in Figure 4.3. Upon FcεR1-mediated activation of mast cells, Tec family kinases translocate to the plasma membrane to regulate signal transduction. Btk is responsible for the phosphorylation of PLCγ, which propagates the activating pathway in mast cells. Concomitantly, Itk associates with a macromolecular complex containing SHIP-1 to participate in the inhibition or dampening of FcεR1 signal transduction. The presence of both of these kinases is required for optimal mast cell activation.

2. Mast cells contribute to the T_H2 phenotype in Itk-deficient mice

One of the initial phenotypes described in Itk-deficient mice was the existence of a spontaneous T_H2-like environment (161, 217). This environment

Figure 4.3

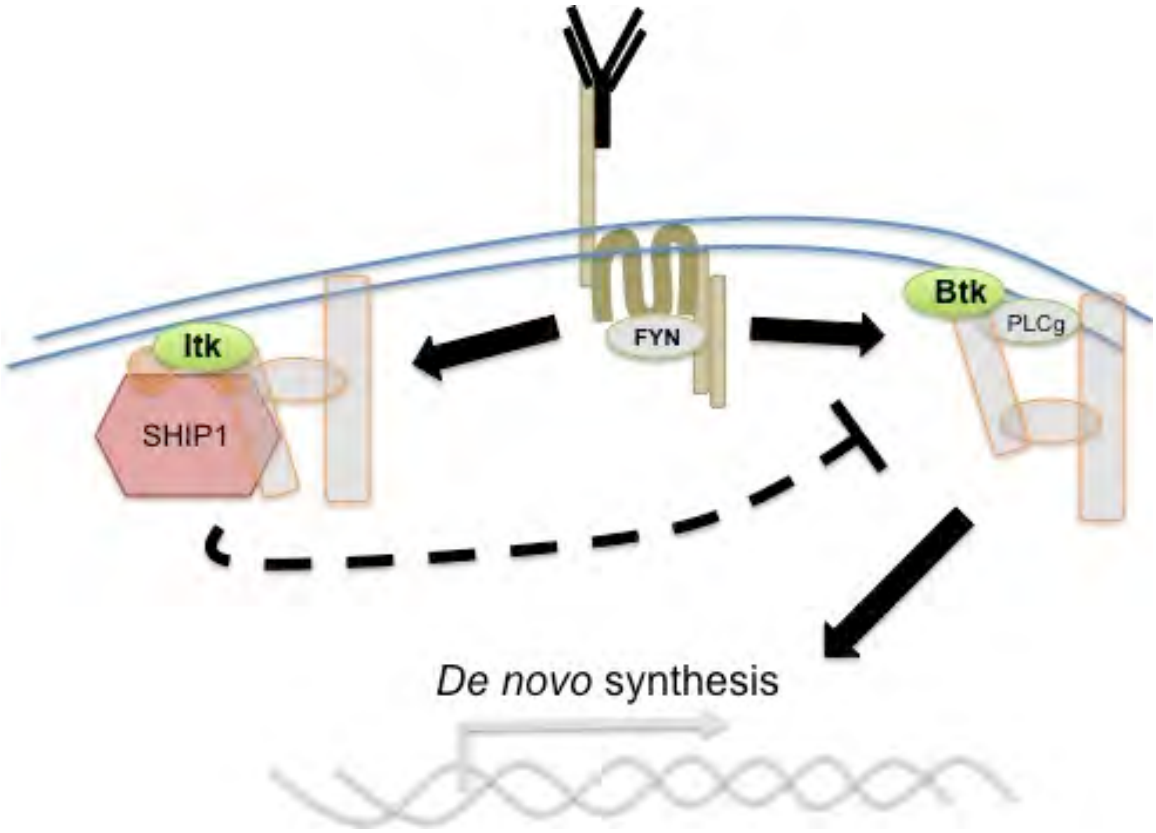

Figure 4.3 Itk negatively regulates mast cell activation

Crosslinking the FcεR1 results in the induction of gene transcription through activating pathways regulated by Btk. Concurrently, inhibitory pathways, mediated by Itk, turn down or dampen the *de novo* synthesis of mast cell products like cytokines.

displays extremely high levels of T_H2-associated antibodies (IgG1 and IgE) in the serum and germinal center B cell hyperplasia. This phenotype is greatly exacerbated in mice doubly deficient for Itk and Rlk. In order for B cells to class-switch and secrete IgE they must be exposed to IL-4 and IL-13 (218-220, 307). It was found by Catherine Yin, a former graduate student in our lab, that γδ T cells from Itk-deficient mice have a skewed cytokine profile compared with wild-type γδ T cells. A specific subset of γδ T cells normally associated with their ability to make T_H1-associated cytokines, like IFNγ, begins making copious amounts of IL-4 upon TCR stimulation. She further found a vast increase in the total numbers of this γδ T cell subset in Itk-deficient mice. These two observations were attributed to a developmental abnormality in the γδ T cell subset caused by a lack of Itk (10, 308).

Interestingly, when Itk-deficient mice are crossed to mice lacking γδ T cells, the progeny have reduced levels of IgE in the serum and are largely rescued from the T_H2-like phenotype (166, 221). However, a complete rescue is not observed as a significant amount IgE (about 5-fold higher than wild-type) remains in the circulation. Furthermore, B cell germinal center hyperplasia is not completely restored to wild-type levels. Intriguingly, the T_H2 phenotype in the Itk/Rlk double knockout mice is not rescued by a cross to mice lacking γδ T cells (our unpublished observations). This indicates that γδ T cells are not the only cell type that contributes to the T_H2-like environment. Because of the enhanced cytokine production, specifically IL-4 and IL-13, associated with mast cells lacking Itk, it remains plausible that this cell type is partially responsible for this

phenotype. The other interesting facet of the data in chapter III, is that Itk-deficient mast cells that are exposed to IgE alone are hyperresponsive and secrete more cytokines than wild-type mast cells. This suggests that even in the absence of antigen, mast cells lacking Itk can secrete IL-4 and IL-13 into the local environment. These cytokine can then instruct B cells to class switch to IgE and generate the extremely high levels seen in Itk-deficient mice. The access IgE can in turn reactivate cytokine production in mast cells, thereby forming a positive feedback loop. It is therefore postulated that mast cells contribute to the spontaneous T_H2-like environment in Itk-deficient mice by promoting a continuous IL-4/IL-13 - IgE cycle (Figure 4.4).

Concluding remarks and significance

One major question that remains is - why does Itk behave so differently in T cells compared to mast cells? The answer may lie in the examination of the major substrate of Itk that is available in each cell type and the presence of other Tec kinase family members. In T cells, three Tec family kinases exist: Itk, Rlk, and Tec. Ablation of Itk in T cells leads to a diminished TCR signaling. Similarly, Rlk-deficient T cells also have decreased T cells signaling, albeit not as severe as $Itk^{-/-}$ T cells. Tec-deficient T cells have the mildest impairments in signaling, and Tec-deficient mice are phenotypically normal. T cells that are doubly deficient in Itk and Rlk have an extremely severe impairment in TCR signaling, and exacerbated defects in T cell development and function. These facts

Figure 4.4

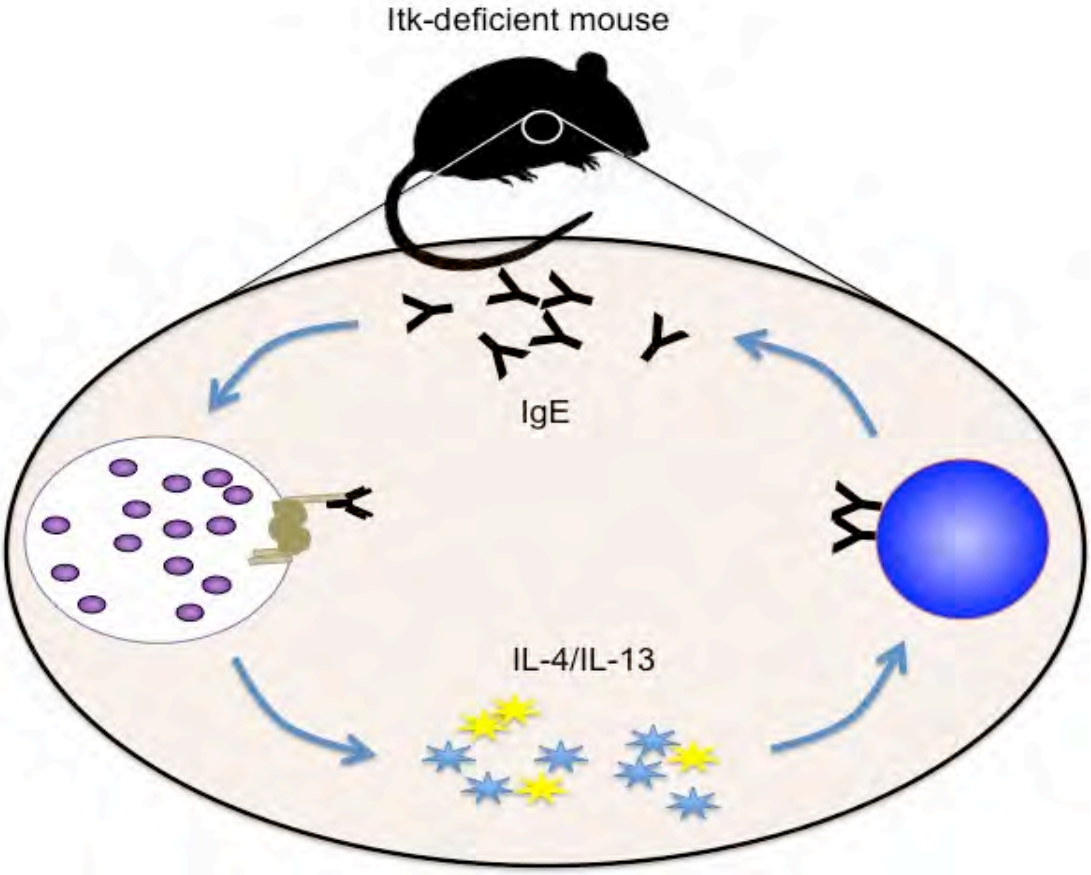

Figure 4.4 Mast cells contribute to the T_H2 phenotype in Itk-deficient mice

Itk-deficient mice have an inherent T_H2-like environment within them. This phenotype occurs spontaneously without any intervention or manipulation. The phenotype is marked by elevated levels of IgE in the serum. Itk-deficient mast cells produce enhanced amounts of IL-4 and IL-13 in response to FcεR1 crosslinking. A positive feedback loop is therefore in place in which mast cells produce IL-4 and IL-13 that induces B cells to secrete IgE that bind to and activate more cytokine production from mast cells.

indicate the potential for functional redundancy amongst Tec family kinases, such that in the absence of one Tec family kinase another can lessen the defect by acting on the substrate. The major substrate of Itk in T cells is PLC-γ1; a closely related molecule, PLC-γ2, is not expressed in T cells. The expression level of Itk is many fold higher than Rlk or Tec, and this likely gives it a competitive advantage by simple stoichiometry for PLC-γ1 over the other Tec family kinases. This indicates that Itk is free to participate in the nucleation of the LAT complex and phosphorylate PLC-γ1 upon TCR ligation.

Interestingly, a synonymous situation occurs in B cells. B cells express Btk at very high levels and Tec at very low levels. In B cells, Btk has been shown to be the primary regulator of PLC-γ2; PLC-γ1 is not expressed in B cells. Similar to T cells, B cells deficient in Tec or Btk have defects in BCR signaling, with Btk deficiency exhibiting a more severe phenotype than $Tec^{-/-}$ B cells. Double deficient cells experience an even greater defect in BCR signaling, again suggesting a compensatory role for Tec family kinases in immunoreceptor signaling.

Mast cells, on the other hand, have components of both B cell signaling and T cell signaling. That's to say that many of the proteins that are exclusively found in B cells or T cells are found together in mast cells. For example, mast cells express both Itk and Btk at very similar levels. Furthermore, their substrates PLC-γ1 and PLC-γ2 are also co-expressed. This is where the notion of functional redundancy breaks down, however. As shown in chapter three, mast cells that lack Itk have the opposite phenotype as mast cells lacking Btk.

This is exemplified by the enhanced signaling that occurs downstream of the FcεR1 in Itk-deficient mast cells; Btk-deficient mast cells, on the other hand, have a severe reduction in the amount of signaling that occurs in response to FcεR1 stimulation. Perplexingly, PLC-γ1 phosphorylation is notably enhanced in the absence of Itk. It is thought that Btk is capable of phosphorylating both PLC-γ1 and PLC-γ2 in order to propagate signal transduction. Since, Itk and Btk are expressed at similar levels, it is unlikely that the enzyme – substrate stoichiometry is a factor. The likely explanation for this conundrum lies in the catalytic activity of each kinase. Btk has been shown to have enhanced enzymatic activity for substrate when directly compared to Itk (309). Furthermore, it is also possible that minor variations in peptide sequence within the kinase domains of Btk and Itk allow Btk to bind substrate with a greater propensity than Itk. This is not to say that Itk is inferior. In fact, the defects associated with Itk deficiency have been aptly described and it's function should be further investigated.

Given the many immunological processes that Itk can partake in, it is an attractive target for therapeutic intervention. The development of pharmaceutical inhibitors targeting Itk has found traction in several private companies for the treatment of human disorders mediated by the immune system (37, 310). Selective inhibition of Itk has been proposed as novel means for the treatment of HIV and skin inflammation disorders like atopic dermatitis (222, 311, 312). A large majority of the research on Itk has been done in T cells and its function has been well characterized. Our own studies have determined that Itk subtly

modulates T cell receptor signaling by tuning signal strength. In light of this, we agree that targeting Itk could potentially work for a variety of diseases. However, the data and commentary in this dissertation expand the functional role of Itk in the signal transduction of immunoreceptors. The novel phenotype ascribed to mast cells lacking Itk cannot be ignored, especially when thinking about skin disorders. We caution those interested in exploring Itk inhibition in the clinic. The novelty of Itk must also be considered in natural killer cells, where Itk is the predominant Tec family kinase expressed. We have virtually zero knowledge on the role of Itk in this cell type. Finally, the importance in understanding Itk has become even more pressing in recent years now that a cohort of people with genetic mutations in Itk has been found (157, 223). Unfortunately, these people typically do not fair very well when Itk function is ablated and succumb to EBV-linked infections. Further inquiry into the pathogenesis of Itk deficiency must be performed. I am confident that future investigation of Itk, in our lab and others, will address the outstanding issues surrounding Itk.

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