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# Regulation of Fc Receptor Expression and Signaling on Murine Mast Cells

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# **Regulation of Fc Receptor Expression and Signaling on Murine Mast Cells**

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of  
Philosophy at Virginia Commonwealth University

By

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## LIST OF ABBREVIATIONS

ATF3	Activating transcription family 3
BFA	Brefeldin A
BMMC	Bone marrow derived mast cell
CTMC	Connective tissue mast cell
DNA	Deoxyribonucleic acid
E4BP4	Adenovirus E4 promoter-binding protein
EAE	Experimental autoimmune encephalitis
ETA-1	Early T-lymphocyte activation-1
FcεRI	High affinity Receptor for the Fc Portion of IgE
FcγRIIb	Low affinity inhibitory Receptor for the Fc Portion of IgG
FcγRIII	Low affinity activation Receptor for the Fc Portion of IgG
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
IFN $\gamma$	Interferon gamma
MC <sub>t</sub>	Tryptase containing mast cell
MC <sub>tc</sub>	Tryptase and chymase containing mast cell
MMC	Mucosal mast cell

NDRG1	N-myc downstream regulated 1
Nurr77	Nuclear receptor subfamily 4, group A, member
Nfil3	Nuclear factor, interleukin 3 regulated
Par1	Protease-activated G protein-coupled receptor
SCF	Stem cell factor
SHIP	SH2-containing inositol-5-phosphatase
Stat6	Signal transducer and activator of transcription 6
TNF $\alpha$	Tumor Necrosis Factor alpha

## ABSTRACT

### REGULATION OF Fc RECEPTOR EXPRESSION AND SIGNALING ON MURINE MAST CELLS

Hey Jin Chong B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2004

Director: John J. Ryan, Ph.D., Associate Professor, Department of Biology

Mast cells have long been appreciated as the primary effector cells in allergy and asthma, and recently have been implicated in other inflammatory diseases. Using high density oligonucleotide probe arrays, we assessed genome-wide transcriptional profiles after FcεRI aggregation for 90 minutes, 5 hours and 24 hours. We describe novel gene regulation in response to FcεRI signaling, including altered expression of CD44, Par1, osteopontin, Nur77, E4BP4, and NDRG1. In addition, the gene encoding FcεRI β was downregulated 5 hours after mast cell activation according to both microarray analysis and RPA, and western blotting confirmed the downregulation of the beta subunit protein. Moreover, this downregulation of beta mRNA correlated with the decreased FcεRI surface expression after mast cell activation. Very little is known about the transcriptional

regulation of the beta subunit of FcεRI. These transcriptome profiling experiments are revealing novel and clinically relevant insight into how FcεRI signaling may be controlled.

Continuing with our focus on how mast cell activation is regulated, we examined the effects of Th2 cytokines on expression of important surface receptors. Murine mast cells co-express the activation receptor FcγRIII and the inhibitory receptor FcγRIIb and can be activated by IgG immune complexes. Using mouse bone marrow-derived mast cells, we report that IL-4 selectively increases FcγRIII expression without altering FcγRIIb. This enhanced expression could be induced by Stat6 activation alone, and appeared to be mediated in part by increased FcγRIIIα protein synthesis without significant changes in transcription. The increase in FcγRIII expression was functionally significant, as it was matched by enhanced FcγR-mediated degranulation and cytokine production. Selective regulation of mast cell FcγR by IL-4 could alter inflammatory IgG responses and subsequently disease severity and progression. Collectively, our studies have used genome-wide screening and reductionist studies to demonstrate mechanisms by which mast cell function is regulated.

## Literature Review

### Mast Cells

In 1877, Paul Ehrlich first observed mast cells in connective tissue and termed them *mastzellen*, meaning “feeding cells”, because he believed that the metachromatic staining granules were a result of overfeeding (Ehrlich, 1878). This reddish purple staining, a key identifying feature of mast cells, is due to the interaction of blue aniline dye with the highly acidic and abundant heparin contained within the secretory granules (Wills-Karp et al. 1998).

Mast cells are the principal cellular effectors of allergic reactions and can be found in tissues that are exposed to the outside environment, such as beneath the epithelial surfaces of the respiratory tract, gastrointestinal tract, and skin. They are distributed throughout connective tissue and concentrated near nerves, blood and lymphatic vessels (Eady et al. 1979; Ryan et al. 2003).

Mast cells originate in the bone marrow from multipotential hematopoietic progenitors which progress to become unipotential mast cell committed progenitors. Although the exact lineage is not clear, it is known that mast cells are of myeloid lineage as determined by the expression of CD34+ on these progenitors. However, unlike other granulocytes, they do not complete their differentiation in the bone marrow. Rather, these committed progenitors travel through the blood stream and it is not until their arrival at the

connective tissues or mucosa that they differentiate and express the proteases and surface antigens indicative of a fully mature mast cell (Huff et al. 1988; Ashman et al. 1991; Rodewald et al. 1996). This differentiation and maturation is under the influence of the microenvironment, and the requirements differ depending on the system. In rodents both stem cell factor (SCF) and interleukin-3 (IL-3) are critical for mast cell maturation, but in human systems, only SCF is critical (Huang et al. 1990; Martin et al. 1990; Matsui et al. 1990; Nocka et al. 1990; Tsai et al. 1991; Irani et al. 1992; Valent et al. 1992; Mitsui et al. 1993; Lantz et al. 1998).

For both humans and rodents, mast cells can be divided into two distinct groups depending mainly on the content of their granules. In humans, mast cells are categorized according to their expression of proteases. Mast cells that contain tryptase alone are designated MC<sub>T</sub>, those that express chymase, carboxypeptidase and cathepsin G as well are designated MC<sub>TC</sub> (Irani et al. 1994). MC<sub>T</sub> can be found in normal lung primarily alveoli, and in the gastrointestinal mucosa, whereas MC<sub>TC</sub> are predominately located in normal skin, blood vessels, synovium and the gastrointestinal submucosa. However, these are generalizations and location is not an absolute determinant of cell type (Weidner et al. 1993).

Rodents also have two distinct categories, connective tissue mast cells (CTMC) and mucosal mast cells (MMC). The names are misleading because like human mast cells, they are classified according to the contents of their granules rather than their location. In general, CTMC can be found in the connective tissue of skin and MMC in the lamina propria. One important distinguishing characteristic is the expression of

different proteoglycans between the two types. CTMC selectively produce heparin proteoglycan whereas MMC produces both heparin proteoglycan as well as chondroitin sulfate proteoglycans. A more precise distinguishing factor is the expression of mast cell specific proteases (MMCPs). Importantly, CTMC's express MMCP-5 whereas MMC selectively express MMCP-1 (Reynolds et al. 1990; McNeil et al. 1991).

Mature mast cells regardless of type and location all express certain receptors. These key defining surface antigens include c-kit, a SCF receptor critical in mast cell proliferation and differentiation, and the high affinity IgE receptor, FcεRI, the most appreciated receptor involved in mast cell activation. FcεRI aggregation on mast cells or basophils, initiates a signaling cascade that results in degranulation and the release of lipid derived mediators, cytokines and chemokines. These events manifest as symptoms associated with Type IV hypersensitivity, such as local edema and swelling (wheal and flare), smooth-muscle contraction, vasodilation, and eventually the recruitment of other inflammatory cells (Schwartz 2002).

Mast cells store pre-formed mediators such as proteoglycans, proteases, tumor necrosis factor alpha (TNFα) and histamine (Metcalfé et al. 1997). One paper recently reported that the cytokine IL-4 is also stored in human mast cells (Wilson et al. 2000). Histamine is a potent biogenic amine that mediates many of the immediate symptoms of allergy including vasodilation, bronchoconstriction, GI smooth muscle contraction and increased mucus production (Schwartz 2002). Shortly after degranulation is the *de novo* synthesis of lipid mediators derived from arachadonic acid, mostly the products of the lipoxygenase and cyclooxygenase pathways. These metabolites, namely leukotrienes

from the former and prostaglandins and thromboxanes from the latter pathway, can stimulate prolonged bronchoconstriction, enhance vascular permeability, promote bronchial mucus secretion, and smooth muscle contraction. Collectively, these granule contents and rapidly synthesized products induce the immediate phase of the Type I response. The late phase is characterized by the synthesis and secretion of various cytokines and chemokines, including IL-3, IL-4, IL-6, IL-8, IL-13, TNF $\alpha$  and MIP1 $\alpha$ . This late phase is clinically discernable by prolonged inflammation, leukocyte recruitment, and edema (Brown et al. 1987; Young et al. 1987; Burd et al. 1989; Gordon et al. 1990; Gordon et al. 1991; Mecheri et al. 1997).

It is the mast cell release of biogenically active mediators, chemokines, proteases and cytokines that allow them to play a central role in a variety of inflammatory processes including asthma, immediate and delayed hypersensitivity reactions, host resistance to bacterial infections, murine models of multiple sclerosis, rheumatoid arthritis, and inflammation in cardiovascular disease (Metcalf et al. 1997; Galli et al. 1999; Gommerman et al. 2000; Kelley et al. 2000; Mekori et al. 2000; Secor et al. 2000; Malaviya et al. 2001; Lee et al. 2002).

### **Fc $\epsilon$ RI**

Fc $\epsilon$ RI, first discovered in 1970, is a multimeric cell surface receptor belonging to the immunoglobulin receptor superfamily (Ishizaka et al. 1970; Blank et al. 1989; Ravetch et al. 1991). In rodents, Fc $\epsilon$ RI is only expressed as a tetramer ( $\alpha\beta\gamma_2$ ) composed of an alpha, beta, and two identical disulfide linked gamma subunits. Humans however



express FcεRI as both a tetramer and a trimer ( $\alpha\gamma_2$ ) with the beta subunit being optional. In rodents, FcεRI is limited to mast cells and basophils, but in humans, FcεRI can also be found on eosinophils, Langerhans cells, monocytes, circulating dendritic cells and platelets albeit only as a trimer, and at significantly lower levels than in basophils (Gounni et al. 1994; Maurer et al. 1994; Maurer et al. 1996; Joseph et al. 1997; Sihra et al. 1997). These quantitative and qualitative differences can be accounted for by understanding the specific function of each receptor subunit.

The alpha subunit is a member of the immunoglobulin superfamily, with a relatively large extracellular domain, a transmembrane domain and a short cytoplasmic tail. It is the Cε3 domain of this subunit that is responsible for binding the Fc portion of IgE, which is the primary function of this chain (Kinet 1999).

The gamma subunit belongs to the  $\gamma/\delta/\eta$  family of receptor subunits and is associated with the Fc receptors for IgG, IgE, and IgA, as well as with non FcR receptors such as paired immunoglobulin-like receptor A and natural killer cell cytotoxicity receptors (Maeda et al. 1998; Kubagawa et al. 1999; Moretta et al. 2001). The gamma subunit is found typically as a disulfide linked homodimer with a short extracellular domain, transmembrane domain and importantly, an intracytoplasmic domain containing a specific sequence called an immunoreceptor tyrosine-based activation motif (ITAM), which is required for signaling. It is the phosphorylation of the ITAMs found on the gamma that allows for the receptor to signal (Kinet 1999).

The beta subunit, like other members of the CD20 family of molecules, has four transmembrane domains with both amino and carboxy termini in the cytoplasm (Kinet et

al. 1988; Ra et al. 1989). In rodent mast cells, the beta subunit associates with both FcεRI and the low affinity Fc IgG receptor, FcγRIII, but is only required for the surface expression of the former (Kurosaki et al. 1992). The beta subunit, like the gamma subunits, contains an ITAM within the intracellular domain, but unlike gamma, the beta subunit does not autonomously signal. Rather, the beta subunit serves to amplify the intensity of gamma signaling by a factor of five to seven, due to the beta subunit association with the src tyrosine kinase, lyn (Dombrowicz et al. 1996; Lin et al. 1996; Dombrowicz et al. 1998; Kinet 1999). In addition to these functions, the beta subunit was also found to enhance surface expression of FcεRI by promoting the processing and export of the alpha chain to the cell surface, as well as enhancing the stability of the receptor complex (Donnadieu et al. 2000). Taking into consideration the augmented signaling and expression, it is estimated that the beta subunit can amplify the host response by a factor of 30 (Donnadieu et al. 2000).

Epidemiologic studies also elucidated another potentially significant role for the beta subunit in atopy. In pursuit of the identification of genes that could be linked to atopy and or bronchial hyperreactivity, an area of chromosome 11 was implicated and later, the region was narrowed down to the gene encoding the Fc receptor beta subunit (Cookson et al. 1989; Sandford et al. 1993). In addition, coding sequence polymorphisms of the beta subunit gene associated with allergic phenotypes have been found to be prevalent in certain populations (Shirakawa et al. 1994; Hill et al. 1995; Hill et al. 1996; Palmer et al. 1997; Cox et al. 1998; Hijazi et al. 1998).

Interestingly, while in search of another beta polymorphism, a short-lived truncated beta subunit was identified as a product of alternative gene splicing. Furthermore, this variant protein was found to negatively regulate surface expression of FcεRI by competing with classical beta and preventing alpha chain maturation (Donnadieu et al. 2003).

### **FcεRI Signaling**

FcεRI signaling is very similar to those of most receptors bearing ITAMs. When the receptor is aggregated by bound IgE capturing antigen, lyn, a src family kinase, becomes activated. Lyn is anchored to the cell membrane or associated with the beta subunit and aggregation allows for lyn to phosphorylate multiple receptors. Lyn will autophosphorylate and subsequently transphosphorylate the ITAM motifs found on FcεRβ and FcRγ. This recruits, amongst other proteins, syk, a tyrosine kinase, by its SH2 domain, which is in turn activated by lyn. This two step activation of lyn and syk initiates a signaling cascade that branches out into other signaling pathways, one involving the phosphorylation of phosphoinositide 3-Kinase (PI3K), which leads to the production of phosphatidylinositol-(3,4,5)-triphosphate (PtdIns(3,4,5)P3). PtdIns(3,4,5)P3 in return recruits Tec kinases and phospholipase C gamma (PLCγ) to the membrane by their pleckstrin-homology (PH) domains. This leads to the production of IP3 and diacylglycerol (DAG), causing a sustained influx of calcium (Daeron 1997; Ravetch et al. 2001). Ultimately this influx of calcium, along with the phosphorylation and activation of various other substrates such as SLP-76 and even sphingosine kinase,

leads to degranulation, transcription of new genes, and cytokine production (Daeron 1997; Kinet 1999). Recent evidence also demonstrates a critical role for the src family kinase Fyn in regulating mast cell degranulation, although the mechanism remains elusive (Parravicini et al. 2002).

## **FcγR**

IgG Fc Receptors can be divided into two distinct classes: activation receptors that contain an ITAM such as FcγRI and FcγRIII, and inhibitory receptors that bear an immunoreceptor tyrosine-based inhibitory motif (ITIM), such as FcγRIIb. Both kinds of receptors are widely expressed throughout the hematopoietic system, and it is the balance of these receptors that dictates the cellular response to the same stimuli. The high affinity FcγRI is found on macrophages, monocytes and neutrophils, and the low affinity FcγRIII on macrophages, activated monocytes, NK cells, pre-B and T cells, as well as murine mast cells (Daeron 1997).

Aggregation of FcγRI or FcγRIII can mediate a variety of inflammatory processes depending on the cell, including degranulation, phagocytosis, antibody-dependent cellular cytotoxicity, cytokine production, and release of inflammatory arachidonic acid metabolites (Daeron 1997; Ravetch et al. 2001). Signal transduction through these receptors is almost identical to that described for FcεRI. Signaling is mediated by ITAMs in the cytoplasmic domains of the Fc receptor γ chain, through recruitment and activation of tyrosine kinases such as lyn and syk. PI3K is activated, eventually leading

to the production of IP<sub>3</sub> and DAG causing degranulation and cytokine production (Ravetch et al. 2001).

In contrast to the activation receptors, FcγRIIb is a single chain low affinity receptor that can be expressed in different isoforms due to differential splicing. While ITAM sequences elicit pro-inflammatory FcγR signaling via ITAM, FcγRIIb possesses ITIM that preferentially interacts with Src homology 2-containing-inositol 5'-phosphatase, or SHIP. When FcγRIIb are crosslinked with an activation receptor such as BCR, TCR, FcεRI, FcγRI, or FcγRIII, SHIP recruitment and activation leads to the hydrolyzation of its primary substrate, PIP<sub>3</sub>, thereby preventing calcium influx and ultimately cell activation (Malbec et al. 1998; Malbec et al. 1999; Ujike et al. 1999).

These two classes of IgG receptors are generally co-expressed on most cell types including monocytes, macrophages, neutrophils, and mast cells (Takizawa et al. 1992; Daeron 1997). In addition, both receptors bind IgG complexes with the same affinity and specificity, thus it is the balance of these receptors on the cell surface that will dictate the biological function triggered by the same stimuli. Studies where one receptor is deleted illustrate the importance of the regulation of the ratio of these receptors in determining the cellular response (Ravetch et al. 1991; Hulett et al. 1994).

FcγRIII is a multimeric low affinity receptor consisting of an FcγRIII alpha subunit responsible for binding IgG complexes, and two gamma subunits responsible for signaling (Ravetch et al. 2001). Thus, mice deficient in the gamma subunit do not express FcγRIII nor FcεRI. In studies using mice deficient in FcR gamma chain, IgG induced passive systemic anaphylaxis was attenuated, and this was the same for FcγRIII-

deficient mice, indicating the capacity of IgG and Fc $\gamma$ RIII to mediate mast cell activation in vivo (Hazenbos et al. 1996; Miyajima et al. 1997; Ujike et al. 1999). Surprisingly, mice deficient in either Fc $\epsilon$ RI or Fc $\gamma$ RIIb had enhanced active anaphylaxis when induced by immunization with antigen and alum, but gamma deficient and Fc $\gamma$ RIII $\alpha$  deficient mice had an attenuated response. This unexpected result was due to the competition between Fc $\epsilon$ RI and Fc $\gamma$ RIII for the gamma subunits. The elimination of the Fc $\epsilon$ RI alpha subunit allowed for greater surface expression of Fc $\gamma$ RIII, tipping the balance of Fc $\gamma$ RIIb/RIII towards activation of the mast cell (Dombrowicz et al. 1997). The balance of these receptors on mast cells also seems to play a critical role in other IgG mediated diseases, as recently demonstrated using a mouse model of multiple sclerosis. Mast cell deficient mice reconstituted with Fc $\gamma$ RIII $^{-/-}$  mast cells had greatly reduced disease severity compared to mice reconstituted with wild type mast cells. In contrast, reconstitution with Fc $\gamma$ RIIb $^{-/-}$  mast cells severely augmented disease pathology (Robbie-Ryan et al. 2003). Mast cells and particularly Fc $\gamma$ RIII expression on these cells is also important in murine model of rheumatoid arthritis. When serum from arthritogenic mice K/BxN was injected into mast cell deficient mice, the severity of rheumatoid arthritis was significantly reduced, and it was theorized that Fc gamma receptors could be playing a role, in an immune complex mediated arthus like reaction (Lee et al. 2002). In addition, mice that lack Fc $\gamma$ RIII, do not develop symptoms of collagen induced arthritis, indicating that IgG complexes are involved in the development of this disease (Diaz de Stahl et al. 2002).

## **Interleukin-4**

IL-4 was initially identified as a T helper cell derived cytokine that stimulated B cell proliferation and growth, as well as enhanced MHC class II expression (Coffman et al. 1986). A member of the four  $\alpha$ -helical cytokine family, IL-4 is produced by a subset of T cells, mostly T helper 2 cells but also NK1.1 and  $\gamma/\delta$  T cells, as well as basophils and mast cells (Brown et al. 1987; Seder et al. 1994; Yoshimoto et al. 1994; Chen et al. 1997).

IL-4 is as a critical player in controlling allergic responses and affects many stages of allergy. Along with its stimulatory effect on B cells, IL-4 is the principal and necessary cytokine that induces B cell isotype switching to IgE and IgG<sub>4</sub> in humans and IgE and IgG<sub>1</sub> in mice (Coffman et al. 1986; Gascan et al. 1991). In fact, in mice that lack either IL-4, its receptor, or the key IL-4 signaling molecule Stat6, IgE production is absent or greatly diminished (Kuhn et al. 1991; Kaplan et al. 1996; Shimoda et al. 1996; Noben-Trauth et al. 1997). Even before the production of IgE, IL-4 promotes the development of T helper 2 cells from antigen stimulated naive T cells while inhibiting the development of T helper 1 cells, thus affecting an even earlier stage in mounting an allergic response (Hsieh et al. 1992; Seder et al. 1992). In accordance with its pro allergic role, IL-4 is a mast cell growth factor and has been shown to induce Fc $\epsilon$ RI expression on in vitro-cultured human mast cells (Mosmann et al. 1986; Toru et al. 1996; Xia et al. 1997).

IL-4 also affects cells other than leukocytes. Along with TNF $\alpha$ , IL-4 induces the expression of vascular cell adhesion molecule-1 (VCAM-1) on vascular endothelial cells

and induces these cells to secrete C-C family chemokines such as monocyte chemoattractant protein-1 (MCP-1) (Thornhill et al. 1991). Recently, IL-4 has also been shown to play a role in skeletal muscle growth by recruiting myoblasts and enhancing fusion (Horsley et al. 2003).

IL-4 can mediate diverse biological functions due to the different signaling pathways utilized by the IL-4 receptor. There are two types of IL-4 receptor, type I and type II. Type I consists of two subunits, a 140 kDa IL-4R $\alpha$  chain which is a member of the hematopoietin receptor superfamily, and the common gamma chain ( $\gamma_c$ ) which is required for the activation of signaling pathways (Keegan et al. 1994). Type II replaces the  $\gamma_c$  chain with the IL-13RI, which has many of the same properties as the former (Callard et al. 1996).

The alpha chain of the IL-4 receptor has 3 different motifs with five highly conserved tyrosine residues critical for signaling. The most membrane proximal tyrosine, Y497 [Y1] is within the Insulin-IL-4 receptor (I4R) motif which generates IL-4 mediated proliferative signals through the recruitment and activation of Insulin receptor substrate 1 and 2 (IRS1/2) (Keegan et al. 1994). Y2-Y4 are within the Stat-6 motif and causes the transcription of various IL-4 inducible genes such as CD23, MHCII, and the IL-4R $\alpha$  through activation of the Jak-Stat pathway (Ryan et al. 1996; Reichel et al. 1997).

In addition to these signaling pathways, the IL-4R $\alpha$  also contains a C-terminal ITIM that allows for interaction with phosphatases such as SH2-containing phosphatase 1 (SHP-1) and SH2-containing inositol-5-phosphatase (SHIP) (Scharenberg et al. 1996; Imani et al. 1997). IL-4 signaling has also been shown to stimulate the phosphorylation



of SHIP, and recent studies have indicated that SHIP could positively regulate proliferation induced by IL-4 (Zamorano et al. 1998; Giallourakis et al. 2000). However, the importance and function of the role of this phosphatase in IL-4 signaling remains to be fully elucidated.

The IL-4 receptor does not have endogenous kinase activity, therefore signaling is dependent on associated kinases that are members of the Janus family of tyrosine kinases (Jak) associated with the cytokine receptor subunits. Jak1 and Jak3 are associated with the IL-4R $\alpha$  chain and  $\gamma$ c chain, respectively. Upon IL-4 binding its receptor, Jak1 and Jak3 phosphorylate the critical tyrosine residues on the alpha chain, creating docking sites for different proteins, namely Stat6 and IRS1/2, by their SH2 domains (O'Shea et al. 2002).

Signal transducer and activator of transcription 6 (Stat6) belongs to a family of molecules activated by each members of the hematopoietin receptor superfamily and receptors for interferon-related molecules (Darnell 1997). After Stat6 docks the IL-4R $\alpha$  via its SH2 domain, Jak1 and Jak3 phosphorylate Stat6. Phosphorylation causes Stat6 to disengage from the receptor and associate with another Stat6 molecule, followed by translocation to the nucleus. Once in the nucleus, Stat6 recognizes a specific palindromic sequence, TTC-N4-GAA, and initiates transcription of IL-4 responsive genes, including MHCII, CD23, germline IgE, IgG1, and IL-4R $\alpha$ . (O'Shea 1997; Nelms et al. 1999). Stat6 is essential for mediating IL-4 lymphocyte responses to IL-4 and is primarily activated only by IL-4 (O'Shea 1997). In studies demonstrating this importance, Stat6 deficient mice are unable to generate a Th2 response, have no IL-4 mediated upregulation

of CD23, MHCII and IL-4, and have decreased proliferation of T cells and B cells. In addition, B cells from Stat6 deficient mice are unable to make IgE, and T cells do not differentiate into Th2 cells in response to IL-4 or IL-13. After nematode infections, Stat6 deficient cells have reduced Th2 cytokines as well as IgE and IgG1 responses (Nelms et al. 1999). Interestingly, a mast cell specific truncated form of Stat6 has been reported. This Stat6 molecule is 65 kDa in size rather than the normal 94 kDa because it lacks the C-terminal transactivation domain. This Stat6 isoform could explain the anti-inflammatory effects caused by IL-4 on mast cells (Sherman et al. 1999).

IL-4 signaling also induces proliferation in cells including mast cells and B cells, and the IRS-1/2 pathway was found to be critical in mediating this function. Analysis of cell lines lacking different Jaks determined that Jak1 is critical for the phosphorylation of IRS 1/2. (Burfoot et al. 1997; Chen et al. 1997; Wang et al. 1997). IRS 1/2 can then associate with proteins that contain SH2 domains, one being the p85 subunit of PI3K. This recruitment activates the p110 catalytic subunit, which phosphorylates membrane lipids, the most important being phosphatidylinositol-(3,4,5)-trisphosphate and PI-3,4-bP. These are produced within seconds of stimulation and are thought to act as second messenger molecules for IL-4 function. These molecules have been shown to be involved in the activation of Akt and PKC which lead to cell growth and survival. Other pathways could be potentially activated by IRS 1/2 such as the Ras/MAPK pathway but the literature is not yet conclusive (Nelms et al. 1999).

## MATERIALS AND METHODS

### *BMMC cultures*

BMMC were maintained as primary, factor-dependent, multi-clonal populations in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 100U/ml penicillin, 100 $\mu$ g/ml streptomycin, 1mM sodium pyruvate, and 1mM HEPES (cRPMI; all materials from Biofluids, Rockville, MD), to which was added 20% volume/volume WEHI-3 cell-conditioned medium (cRPMI/WEHI). BMMC were cultured from bone marrow harvested from femurs of BL6x129 wild type, BL6x129 Fc $\gamma$ RII $\alpha$ -deficient, or FcR $\gamma$ -deficient mice (Taconic farms, Germantown, NY), and maintained in cRPMI/WEHI. After 3-4 weeks in culture, these populations were >99% mast cells, as judged by morphology and flow cytometry staining for expression of Fc $\epsilon$ RI, CD13, Kit, Fc $\gamma$ RII/Fc $\gamma$ RIII and T1/ST2 (data not shown). The resulting populations were generally used between weeks 4-12.

### *Cytokines and reagents*

Saponin, cycloheximide, Brefeldin A, and propidium iodide were purchased from Sigma Immunochemicals (St. Louis, MO). Murine IL-3 and IL-4 were purchased from R&D Systems (Minneapolis, MN). P-Nitropheno- $\beta$ -D-acetamido-2-deoxyglucopyranoside was purchased from Sigma (St. Louis, MO).

### ***Antibodies***

2.4G2 (rat anti-mouse FcγRII/RIII), mouse IgE, FITC-conjugated rat anti-mouse CD13, FITC-conjugated rat anti-mouse Kit, PE-conjugated rat isotype control IgG, and FITC-conjugated mouse-IgG were purchased from BD PharMingen (San Diego, CA). FITC-conjugated goat F(ab'2) anti-rat IgG, and Rat anti-mouse IgE was purchased from Southern Biotechnology Associates (Birmingham, AL). FITC-conjugated rabbit anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). FITC-conjugated rat anti-mouse T1/ST2 was purchased from Morwell Diagnostics (Zurich, Switzerland). K9.361 anti-FcγRIIb was the kind gift of Ulrich Hammerling (Memorial Sloan Kettering Cancer Center, NY, NY).

### ***Mast Cell Activation***

BMMC were cultured at  $3 \times 10^5$  cells/ml in cRPMI supplemented with IL-3 (5ng/ml) for at least 3 days. Cells were resuspended in cRPMI to a concentration of  $1 \times 10^7$  cells/ml and incubated with IgE (10ug/ml) for 45 minutes at 4 C, washed twice with RPMI and resuspended at the same concentration in cRPMI with rat anti-IgE (5ug/ml). After 40 minutes, pre-warmed cRPMI/IL-3 was added until the cells were at a concentration of  $5 \times 10^5$  cells/ml, and then incubated at 37 C for various time points.

### ***Detection of surface FcεRI***

To detect FcεRI surface expression, BMDC were incubated with unlabelled IgE (10µg/ml) for 30 minutes at 4°C, washed twice with FACS buffer and resuspended in unlabelled rat anti-IgE (5 µg/ml) for 30 minutes at 4°C, washed again and finally resuspended in FITC-goat anti-rat IgG in the aforementioned conditions. For negative staining controls, IgE was omitted from the staining procedure. Cells were then washed twice with FACS buffer and analyzed by FACScan flow cytometry (Becton Dickinson Immunocytometry, Braintree, MA) in the presence of propidium iodide to exclude dead cells.

### ***Immunoprecipitation and western blotting for FcεRIα***

For immunoprecipitation and western blotting of FcεRIα,  $30 \times 10^6$  cells were cultured and activated as described previously, resuspended in cRPMI, pelleted and solubilized in ice-cold lysis buffer (0.5% Nonidet P-40, 10% glycerol; 5M NaCl; 100mM Tris; 100mM MgCl<sub>2</sub>; supplemented with an enhanced inhibitor cocktail consisting of a 20X concentrate of Boehringer Mannheim Complete, with 80mM benzamidine HCL, 50mM ε-caproic acid, 16mM iodoacetamide, 10µg/ml leupeptin, 10µg/ml Pepstatin, and 100µg/ml soybean trypsin inhibitor) then vortexed vigorously for 15 seconds and placed on ice for 40 minutes. The supernatants were clarified at 14,000 x g at 4° C for 30 minutes, aliquoted, snap frozen in liquid nitrogen, and stored at -80° C. The lysates ( $3 \times 10^7$  cells/sample) were assayed (BioRad, Hercules, CA) for protein concentration according to the manufacturer's recommended procedure. 760 µg per sample were

immunoprecipitated using Protein A sepharose beads (50 $\mu$ l) that had been incubated overnight with 25 $\mu$ g of rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories) and either 5  $\mu$ g of clone 5.14 anti-Fc $\epsilon$ RI $\alpha$  monoclonal antibody, or Chrompure mouse IgG whole (Jackson Immunoresearch Laboratories) as a control. After 3 hours of incubation at 4 $^{\circ}$  C, the beads with lysate were centrifuged at 4 $^{\circ}$  C at 14,000 x g for 1 minute, washed 4 times with 1 ml cold 1.0% NP-40 lysis buffer followed by one washing with 0.01% NP-40 lysis buffer. To recover protein, beads were boiled for five minutes with 40  $\mu$ l of loading buffer (20ul BME, 20 ul of 200mM orthovanadate and 960 ul 2X tris-glycine sample buffer) and microfuged at 14,000 x g for 2 minutes. The supernatant was analyzed by SDS-PAGE on a 10% Tris-glycine gel (Invitrogen/Novex, Carlsbad, CA) and transferred to a nitrocellulose membrane. Membranes were blocked and incubated overnight at 4 $^{\circ}$  C with TW anti- Fc $\epsilon$ RI $\alpha$  monoclonal antibody (1  $\mu$ g/ml) washed 4 times for 20 minutes each with 4%BSA/TTBS (10mM Tris pH 7.2 and 150 mM NaCl) followed by Protein A-horse radish peroxidase (Zymed, San Francisco, CA) at a dilution of 1:5000. Size estimates for proteins were obtained using molecular weight standards from BioRad (BioRad, Hercules, CA). The reaction was developed with an ECL Western blotting detection kit (Amersham Life Science, Piscataway, NJ).

### ***Western blot for Fc $\epsilon$ RI $\beta$ and FcR $\gamma$***

Western blotting was performed using 50 $\mu$ g of total cellular protein. Lysates were boiled in 1X sample buffer and separated on either a 10% or 4-20% tris-glycine gel along with a molecular weight marker (BioRad, Hercules, CA). The proteins were then

transferred to a nitrocellulose membrane and blocked for one hour at room temperature in 4%BSA/TTBS. FcεRIβ and γ subunits were detected with mouse anti-FcεRI β (the kind gift of Jean-Pierre Kinet, Boston, MA) at 1:2000 or rabbit anti-FcεRI γ subunit (Upstate Biotechnology, Lake Placid, NY) at a 1:10,000 dilution, overnight at 4° C. Membranes were quick rinsed followed by washing 3 times for 20 minutes each with TTBS (1X TBS with 0.05% Tween-20). The secondary antibodies, rat anti-mouse IgG-horse radish peroxidase (Santa Cruz, Santa Cruz, CA) and by Protein A-horse radish peroxidase (Santa Cruz, Santa Cruz, CA) were added to fresh TTBS at a 1:5000 dilution and incubated at room temperature for 1 hour. The blots were then quick rinsed again and washed as previously described. Immunoblots were then detected by ECL (Amersham Life Science).

### ***Mast Cell Activation for Affymetrix gene chip analysis***

BMMC were cultured at  $3 \times 10^5$  cells/ml in cRPMI supplemented with IL-3 (5ng/ml). After 4 days,  $1 \times 10^7$  cells were counted, washed with RPMI, and resuspended in 1 ml of cRPMI with IgE (10μg/ml) for 45 minutes at 4° C, washed twice with RPMI and resuspended at the same concentration in cRPMI with rat anti-IgE (5ug/ml). After 40 minutes, pre-warmed cRPMI/IL-3 was added until the cells were at a concentration of  $5 \times 10^5$  cells/ml, and then incubated at 37° C for either 90 minutes, 5 hours, or 24 hours, after which time total RNA was harvested using TRIzol Reagent (Invitrogen, Carlsbad, CA). The RNA from these cells were compared to the RNA from a control group of mast cells that were incubated in cRPMI/IL-3 alone for 24 hours.

### *Affymetrix gene chip analysis*

For each sample, total RNA (0.1-10  $\mu\text{g}$ ) was extracted from approximately  $10^7$  cells. RNA was harvested using TRIzol Reagent (Invitrogen, Carlsbad, CA) and further purified using an RNeasy Kit (Qiagen, Valencia, CA). Double stranded cDNA was synthesized using SuperScript Choice system (Life Technologies, Rockville, MD) and a T7-(dT)<sub>24</sub> primer (Amersham Pharmacia Biotech, Buckinghamshire, England). The cDNA was subjected to in vitro transcription in the presence of biotinylated nucleoside triphosphates using a BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Inc., Farmingdale, NY), based on the manufacturer's protocol. The biotinylated cRNA was then purified, fragmented and hybridized to Affymetrix MG-U74A GeneChips (Santa Clara, CA) which contain approximately 6,000 full-length genes and 6,000 expressed sequence tags (ESTs).

Data analysis was performed with GeneChip Analysis Suite 5.0 and Data Mining Tool (Affymetrix), and default settings were used. Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to a constant target intensity for all arrays.

Average fluorescence intensity was used to determine the expression level of an individual mRNA, using the following equation,  $(\Sigma(\text{PM-MM})/(\text{number of probe pairs}))$ , where PM and MM denote perfect-match and mismatch probes. This score is used to assess probe pair saturation and compares it to a pre-defined cut off value in order to determine if the gene will be called Present, Marginal or Absent. If the intensities of mismatched probes were very high, gene expression was judged to be absent.



Cells activated for 90 minutes, 5 hours and 24 hours (target samples) were compared to unstimulated cells cultured in IL-3 alone (reference or baseline sample) and Microarray Suite was used to determine if the target sample is increased, decreased or no change in comparison to the baseline sample. Signal log ratio quantitatively describes the change between the target and reference samples, and is expressed as  $\log_2$  ratio. Genes were sorted so that only those that increased or decreased were included for further analysis. Of these genes, those that were scored absent in the activated sample (for upregulated genes) or absent in the unstimulated sample (for downregulated genes) were excluded.

### ***Hierarchical clustering***

Genes were subjected to hierarchical clustering using Cluster (Eisen, M [<http://rana.lbl.gov/EisenSoftware.htm>]). For hierarchical clustering analysis, similarity was defined based on a modified Pearson's correlation coefficient, by selecting Correlation uncentered in the similarity Metric dialog box. Average linkage clustering was then chosen to cluster the data. The results were graphically displayed using Tree View (Eisen, M [<http://rana.lbl.gov/EisenSoftware.htm>]).

### ***Gene Ontology analysis***

Genes were analyzed using David (Glynn Dennis Jr.) and the GoCharts module which uses designations assigned by the Gene Ontology Consortium (<http://www.geneontology.org>) and displays the distribution of differentially expressed

genes according to the different functional categories. Genes were categorized according to their molecular function with a coverage level of 3 out of 5, one being the least specific and 5 being the most specific.

### ***Tissue culture conditions for regulation of BMMC Fc $\gamma$ R expression***

Cells were washed to remove WEHI-3 CM, and incubated in cRPMI at 37° C for 4-6 hours. Cells were plated at  $3 \times 10^5$  cells/ml, 200 $\mu$ l/well in 96-well flat-bottom plates. IL-3 was added to 5ng/ml, followed by the indicated concentrations of IL-4. Cells were then incubated for the indicated times. Every 4 days half of the media and cytokines were replaced. Fc $\gamma$ RIIb/RIII levels were then determined by flow cytometric analysis.

### ***Detection of Fc $\gamma$ RIIb and Fc $\gamma$ RIII surface expression***

To detect Fc $\gamma$ RIIb expression, wild type BMMC were washed with FACS buffer (phosphate-buffered saline (PBS)/3%FCS/0.1% sodium azide) in 96-well "V" bottom plates, and resuspended in unlabelled K9.361 ascites diluted 1:100, or in FACS buffer alone for 30 minutes at 4° C. Cells were then washed twice with FACS buffer and incubated for 30 minutes at 4° C with 10 $\mu$ g/ml FITC-rabbit anti-mouse IgG, washed twice, and analyzed in the presence of propidium iodide to establish a live cell gate. Flow cytometry was performed using a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA). In some of these experiments, mouse IgG was employed as a control primary antibody in place of FACS buffer alone. To detect Fc $\gamma$ RIII expression, Fc $\gamma$ RIIb-deficient BMMC were washed with FACS buffer and incubated for 30 minutes at 4°C

with 10µg/ml FITC-2.4G2, either in the presence of unlabelled 2.4G2 to serve as a negative control, or without unlabelled 2.4G2. Cells were then washed twice, and analyzed by flow cytometry in the presence of propidium iodide. We also assessed FcγRIIb expression by 2.4G2 staining of BMMC derived from FcR gamma chain-deficient mice, which lack expression of FcγRIII. The percent inhibition of FcγR expression was calculated by comparing mean fluorescent intensities (MFI) of populations cultured with IL-3 alone to those cultured with IL-3 and other stimuli using the following equation:

$$\{[(\text{MFI of IL-3 cultured BMMC}) - (\text{MFI of comparison culture})] / (\text{MFI of IL-3 culture})\} \times 100.$$

To detect expression of CD13, Kit, and T1/ST2, BMMC were incubated with unlabelled 2.4G2 ascites for 10 minutes at 4° C to block non specific binding, followed by FITC-labeled antibodies at 10µg/ml for 30 minutes at 4° C. Cells were then washed twice and analyzed by flow cytometry in the presence of propidium iodide.

### ***2.4G2 Staining of fixed and permeabilized cells***

To detect intracellular FcγRIIIα expression, FcγRIIb deficient BMMC (3x10<sup>5</sup> cells/ml) were washed twice with 1X PBS and fixed with 4% paraformaldehyde/PBS for 20 minutes at room temperature. Cells were washed with and resuspended in FACS buffer, then stored overnight at 4° C. Cells were then resuspended in 100µl of staining buffer (PBS, 0.1% BSA, 0.01M HEPES, and 0.5% Saponin) and incubated at room

temperature for 10 minutes, prior to 2.4G2 staining as described above. Samples were analyzed by flow cytometry using a forward scatter versus side scatter gate.

### ***RNase protection assay (RPA) for FcγRIII alpha and 2.4G2 crosslinkage mediated cytokines***

For each sample,  $5 \times 10^6$  BMDC (cultured at  $5 \times 10^5$  cells/ml) were starved for 4 hours in cRPMI, then stimulated with IL-3 (5ng/ml) alone or IL-3+IL-4 (20ng/ml) for the indicated time points. To detect cytokine mRNA expression after 2.4G2 crosslinkage, cells were incubated for 10 minutes with  $10 \mu\text{g/ml}$  2.4G2 at  $4^\circ \text{C}$ , washed, and incubated with  $2.5 \mu\text{g/ml}$  goat F(ab'2) anti-rat IgG for 90 minutes at  $37^\circ \text{C}$ . Total RNA was harvested using TRIzol Reagent (Invitrogen, Carlsbad, CA) and subjected to RPA analysis using the RiboQuant System (PharMingen, San Diego, CA). Pixel intensity was determined using a Typhoon Phosphorimager 445si System equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

### ***Retroviral Infection***

FcγRIIb deficient BMDC cultures were infected with retrovirus expressing a bicistronic construct consisting of GFP alone or GFP and the constitutively active Stat6 mutant termed Stat6VT as described previously (Daniel et al. 2000; Zhu et al. 2001). FcγRIII surface expression was assessed on the GFP-positive population by flow cytometry analysis using phycoerythrin-coupled 2.4G2 antibody.

### ***β-hexosaminidase assay***

For each sample,  $6 \times 10^4$  BMDC were cultured with cytokines for the indicated times as described above, then stimulated by 2.4G2 crosslinkage in a volume of 50 $\mu$ L as described above for RPA measurements. The supernatant was collected 60 minutes after activation at 37 $^\circ$  C and stored at -20 $^\circ$  C. The pellet was resuspended in PBS/1% NP40, incubated at 0 $^\circ$ C for 30 minutes, vortexed for 5 minutes, and clarified by centrifugation at 10,000 RPM for 20 minutes at 4 $^\circ$  C.  $\beta$ -hexosaminidase activity in cellular supernatants and pellets was determined as described previously (Schwartz et al. 1979). Percent release was calculated by dividing the amount of  $\beta$ -hexosaminidase activity in the supernatant by the sum of  $\beta$ -hexosaminidase activity in the supernatant and pellet. Specific release was determined by subtracting the percent release of unstimulated cells from cells stimulated by 2.4G2 crosslinkage. Non-specific release averages ranged from 7.9 to 15.11 percent on day 4.

### ***Elisa***

For each sample,  $5 \times 10^5$  BMDC were cultured for 4 days with the indicated cytokines in duplicate wells. Equal numbers of BMDC were then stimulated by 2.4G2 crosslinkage in 200 $\mu$ L cRPMI/IL-3 for 24 hours at 37 $^\circ$  C as described above for RPA measurements, with the exception that an extra wash step was included following the incubation with goat anti-rat IgG. Cytokine release was measured using OptEIA ELISA kits (BD Pharmingen, San Diego, CA).

## RESULTS

### ***FcεRI mediated mast cell activation regulates expression of over 400 different genes at three separate time points.***

Mast cells have long been appreciated as the primary effector cells in allergy and asthma. Recently, their role in inflammation has diversified, as mast cells are now implicated in a variety of inflammatory processes including host resistance to bacterial infections, mouse models of multiple sclerosis, rheumatoid arthritis and inflammation in cardiovascular disease (Kelley et al. 2000; Secor et al. 2000; Malaviya et al. 2001; Lee et al. 2002). Mast cell activation results in the synthesis and release of many biologically active mediators and cytokines that can initiate, maintain or contribute to the pathology of the aforementioned conditions (Metcalf et al. 1997). In order to assess changes that occur in gene expression after mast cell activation, bone marrow derived mast cells were activated by FcεRI crosslinkage (IgE + anti-IgE) for 90 minutes, 5 hours and 24 hours and analyzed for gene expression using Affymetrix Murine Genome-U74 GeneChips. The GeneChips were analyzed using Microarray Suite 5.0 followed by further analysis with Data Mining Tool (Affymetrix). Out of over 12,000 different probe sets, 937 were regulated by FcεRI crosslinkage in at least one time point (Figure 1). These probe sets were further filtered to omit expressed sequence tags, creating a list of

487 probe sets for known genes that are regulated by FcεRI crosslinkage in at least one time point. (Appendix 1). To further characterize the data, remaining genes were filtered using Cluster (Eisen, M [<http://rana.lbl.gov/EisenSoftware.htm>]) to include only those that change at least two fold in at least two separate samples. The results were hierarchically clustered and depicted using Tree View (Eisen, M [<http://rana.lbl.gov/EisenSoftware.htm>]). Thus, there remained 259 probe sets representing known genes that either increased or decreased at least two fold after mast cell activation in at least two separate samples (Figure 2).

**Figure 1.** FcεRI mediated mast cell activation regulates the expression of many genes.

Bone marrow derived mast cells were incubated with IgE followed by rat anti mouse IgG for 90 minutes, 5 hours and 24 hours. RNA was harvested from the cell cultures and analyzed by Affymetrix MG-U74 GeneChips. Changes in gene expression levels were determined by comparing activated cells to unstimulated cells, in two separate experiments. Results from both experiments were analyzed simultaneously. Genes were filtered to include only those deemed increased or decreased according to analysis by Microarray Suite 5.0 (Affymetrix). The remaining genes were subjected to hierarchical clustering and displayed graphically using Cluster and Tree View (Eisen). Each row represents a single gene, with each column representing the mRNA levels for all of the genes in single treatment group of cells. Unstimulated samples were normalized to black, with red indicating increased expression and green indicating decreased expression levels. The intensity of the color reflects the degree of change, thus a darker red or green indicates a greater fold change over unstimulated cells.



This image shows a vertical strip of a document page, likely a list or index, with a green margin on the left side. The text is extremely dense and illegible due to the high resolution and small font size. The strip appears to be a scan of a page from a book or a document, with the text arranged in a single column. The green margin is visible on the left edge of the strip.

**Figure 2.** Graphical depiction of genes subjected to hierarchical clustering using Cluster and Tree View. Genes that changed were filtered in Microsoft Excel to exclude expressed sequence tags. The remaining 487 probe sets were further filtered using Cluster to include only those that changed at least two fold in at least two separate time points (M. Eisen; [<http://rana.stanford.edu/software/>]) and were further analyzed by hierarchical clustering and displayed graphical using Tree View (M. Eisen; [<http://rana.stanford.edu/software/>]).



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***FcεRI mediated mast cell gene expression profiles change over time.***

Mast cell activation is not an instantaneous phenomenon, but occurs in a series of events that manifest as symptoms both minutes and even hours later. It is well known that FcεRI aggregation initiates a signaling cascade that results in degranulation of the cell within thirty minutes as well as the transcription of new genes, and that over time this transcriptional profile changes (Schwartz. 2002; Ryan et al. 2003). To understand the changes in gene expression that occur over time, we analyzed the most changed transcripts at 90 minutes, 5 hours and 24 hours. After 90 minutes of activation, 174 probe sets changed at least 2 fold or more compared to unstimulated cells. These genes were categorized using the Database for Annotation, Visualization and Integrated Discovery (DAVID). This program utilizes various databases to annotate and chart different gene lists into chosen specified categories (Glynn Dennis Jr. 2003). In order to get a global overview of the changes occurring after ninety minutes of activation, the genes were categorized according to molecular function using vocabulary from the gene ontology consortium (GO analysis). 21.3% of the 174 genes are involved in DNA binding. When these genes were further categorized according to biological function, 75.7% of these DNA binding genes were involved in transcription or the regulation of transcription with 24.9% of these genes involved in regulation of the cell cycle. 10.9% of the genes transcribed at ninety minutes are involved in purine nucleotide binding, 42.1% of these involved in phosphorylation and 31.6% consisted of genes involved in small GTPase mediated signal transduction. The third and fourth largest category of genes were those involved in cytokine activity and growth factor activity, 9.2% and 5.7%

respectively. These categories consisted of primarily all the inflammatory cytokines (i.e. interleukin-13), growth factors (i.e. GM-CSF) and chemokines such as MIP1 $\alpha$  (Figure 3).

The most upregulated and downregulated transcripts after ninety minutes are presented in table format. Not surprisingly, 6 of the 10 most upregulated genes are chemokines, inflammatory cytokines and growth factors such as Interleukin-13, prostaglandin-endoperoxide synthase 2 (COX-2) and GM-CSF, and the other four are involved in cell signaling. The top three transcripts, chemokine (C-C motif) ligand 7 (MCP-3), CCL-4 (MIP1 beta) and CCL-3 (MIP1 alpha) increased 502, 179, and 169 fold respectively. These results are in agreement with a previously published paper reporting these chemokines as the most upregulated transcripts after mast cell activation. (Nakajima et al. 2002). Interestingly, one of the signaling molecules transcribed, early growth response 2, a transcription factor induced by inflammatory stimuli, was upregulated 72 fold over unactivated cells. The ten most downregulated genes at ninety minutes are involved in cell signaling, cell cycle regulation, or chromatin packaging. One, schlafen 2, has been reported to be involved in the suppression of T cell growth but the presence or role of this protein in mast cells has not been reported (Schwarz et al. 1998). (Table 1).

Five hours after mast cell activation, 202 probe sets are described to change at least two fold compared to unstimulated cells, compared to the 174 genes regulated at ninety minutes. After analysis with DAVID and GOChart, the top three categories were similar, with 16.8% of genes involved in DNA binding, 15.3% in purine nucleotide binding, and 8.4% involved in transferase activity (Figure 4). Of the genes categorized as

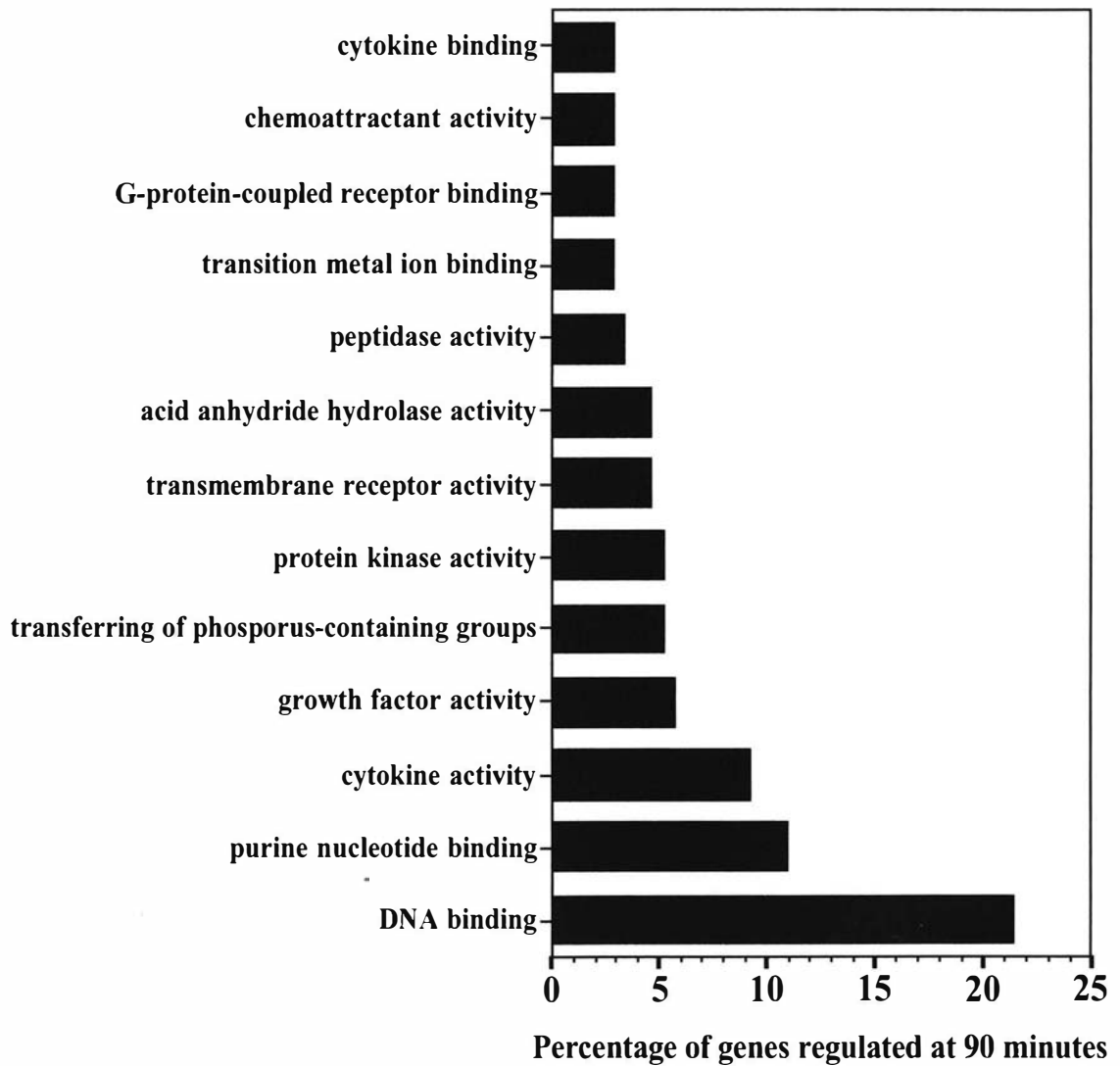
DNA binding, the majority function in the regulation of transcription (73.5%) with cell cycle regulation and DNA replication a far second and third (20.6% and 17.6% respectively). The most changed transcripts at five hours however differed from those seen at 90 minutes. The two most abundantly transcribed genes compared to unstimulated cells were again chemokines, however, the fold change was reduced to 59 fold over baseline, compared to 502 fold at ninety minutes (Table 2). Interestingly, the third most increased transcript at five hours is secreted phosphoprotein 1, better known as osteopontin. Osteopontin contributes to the development of T helper 1 immunity, and is implicated in a wide assortment of inflammatory diseases including experimental autoimmune encephalomyelitis (Jansson et al. 2002). Its role in mast cells has yet to be reported. Further, a known receptor for a breakdown product of osteopontin, CD44 is the ninth most increased transcript five hours after mast cell activation. This surface antigen also binds hyaluronic acid and has been implicated in other diseases including allergic lung inflammation. (Teder et al. 2002; Katoh et al. 2003). Its expression has been reported on human mast cells, but there is little literature suggesting a role for this receptor in mast cell-mediated disease (Kruger-Krasagakes et al. 1996).

Transcriptional activity has significantly slowed down 24 hours after the initial FcεRI mediated activation of the mast cell. Only 69 genes change 2 fold or more, with the top three categories again being DNA binding (13%), purine nucleotide binding (11.6%), and cytokine activity (8.7) (Figure 5). The vast majority of DNA binding genes function in the regulation of transcription, and the majority of purine nucleotide binding genes are important in phosphorylation. The most increased transcript, procollagen, type

XVIII, alpha 1, also known as endostatin, is a potent anti-angiogenic factor (Table 3) (O'Reilly et al. 1997; Dhanabal et al. 1999). This transcript increases a modest 10 fold, compared to the most increased transcript at 90 minutes, CCL7, which increased a tremendous 502 fold compared to unstimulated cells. Another gene regulated at this time point, Nuclear factor, interleukin 3 regulated (E4BP4), is a transcription factor known to be up regulated by IL-3. It has been recently published that in Ba/F3 cells, IL-3 regulates the expression of E4BP4 through GATA1, and that this is important in IL-3 mediated survival and anti-apoptotic signaling (Yu et al. 2002). Although this gene is upregulated both 90 minutes and 5 hours after mast cell activation (7 fold and 3 fold respectively), by 24 hours, the gene coding for E4BP4 is the second most downregulated gene at this time point, at about 4 fold below unstimulated cells (Table 3).

**Figure 3.** Probe sets that increased or decreased at least two fold after ninety minutes of FcεRI crosslinkage were analyzed using the Database for Annotation, Visualization and Integrated Discover (David). The 174 probe sets were categorized according to their functional categories using the vocabulary given by the Gene Ontology Consortium (GO) and displayed in graphical format. Ninety-eight of the 174 probe sets listed (43.7%) were unclassified and not represented graphically.

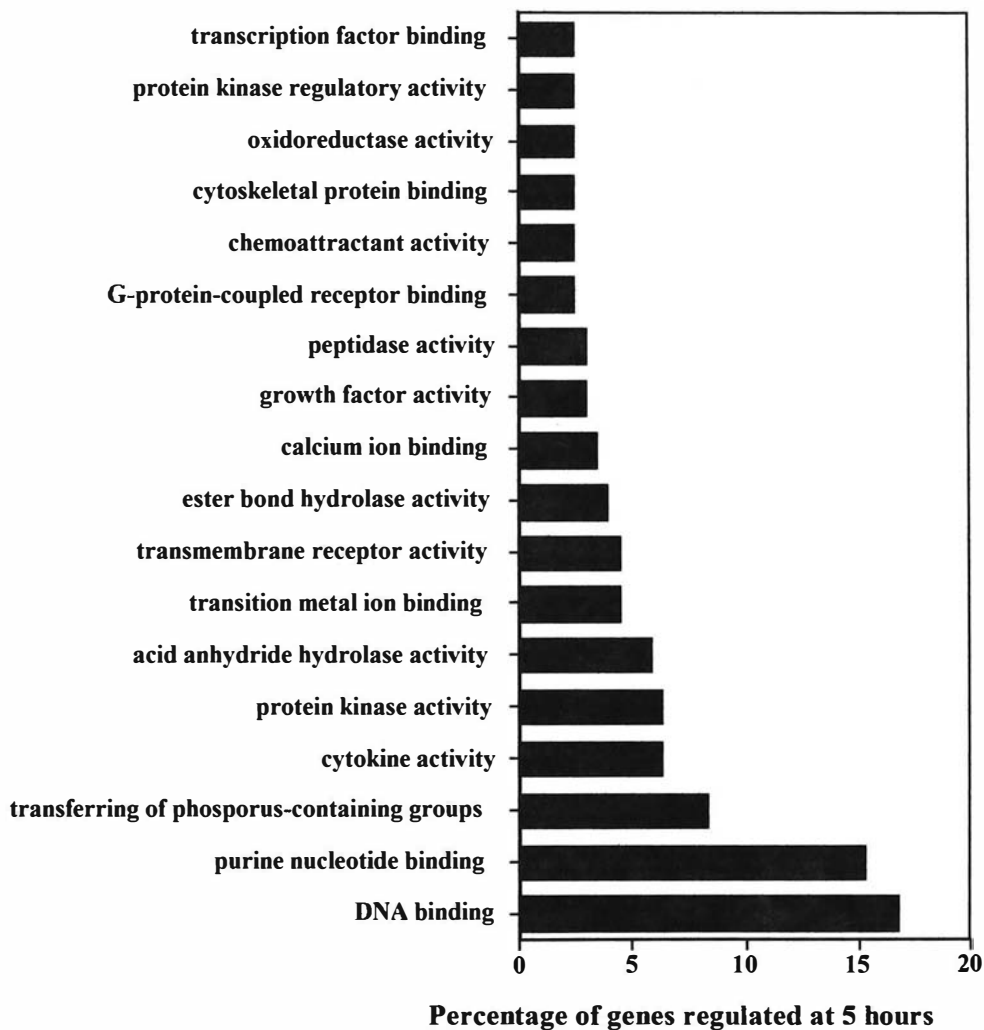




**Table 1. Top 20 most changed genes 90 minutes after FcεRI aggregation.**

Probe ID	Gene Name	Gene Symbol	Average Fold Change		
			90 mins	5 hrs	24 hrs
94761_at	chemokine (C-C motif) ligand 7 (MCP-3)	Ccl7	502.70	55.98	4.80
94146_at	chemokine (C-C motif) ligand 4 (MIP1 beta)	Ccl4	179.45	11.28	1.13
102424_at	chemokine (C-C motif) ligand 3 (MIP1 alpha)	Ccl3	168.87	59.04	3.08
94168_at	interleukin 13	Il13	95.52	18.27	-0.05
104647_at	prostaglandin-endoperoxide synthase 2 (COX-2)	Ptgs2	93.16	1.80	-1.12
102661_at	early growth response 2	Egr2	72.53	1.81	-1.65
92948_at	colony stimulating factor 2 (granulocyte-macrophage)	Csf2	68.07	0.08	-0.10
102780_at	neoplastic progression 3	Npn3	62.79	11.95	0.63
104155_f_at	activating transcription factor 3	Atf3	46.15	1.51	-1.30
92356_at	protein tyrosine phosphatase, non-receptor type 8	Ptpn8	40.28	8.13	-1.06
102318_at	sialyltransferase 8 (alpha-2, 8-sialyltransferase) D	Siat8d	-9.91	-8.09	-0.25
98010_at	nuclear factor, erythroid derived 2	Nfe2	-7.34	-6.98	-0.02
102397_at	core-binding factor, runt domain, alpha subunit 2: translocated to, 3 homolog (human)	Cbfa2t3h	-7.25	-4.72	1.05
92472_f_at	schlafen 2	Slfm2	-6.02	-1.75	1.40
92409_at	zinc finger protein 260	Zfp260	-4.18	-4.59	-0.72
103342_at	embryonic ectoderm development	Eed	-4.12	-1.24	-1.09
93414_at	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	Abcb1b	-3.97	-5.49	1.08
93697_at	chromobox homolog 4 (Drosophila Pc class)	Cbx4	-3.50	-2.64	-1.55
92471_i_at	schlafen 2	Slfm2	-3.34	-1.79	1.56
103346_at	CDC-like kinase 2	Clk2	-3.02	-1.58	-1.08

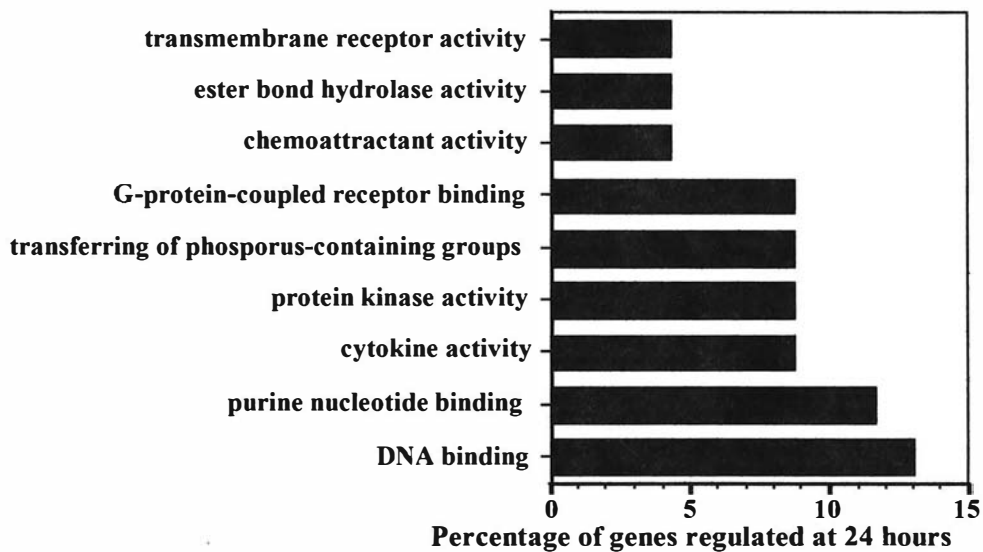
**Figure 4.** Probe sets that increased or decreased at least two fold after 5 hours of FcεRI crosslinkage were analyzed using the Database for Annotation, Visualization and Integrated Discover (DAVID). The 202 probe sets were categorized according to their functional categories using the vocabulary given by the Gene Ontology Consortium (GO) and displayed in graphical format. One hundred twenty five of the 202 probe sets listed (38.1%) were unclassified and not represented graphically.



**Table 2. Top 20 most changed genes 5 hours after FcεRI aggregation.**

Probe ID	Gene Name	Gene Symbol	Average Fold Change		
			90 mins	5 hrs	24 hrs
102424_at	chemokine (C-C motif) ligand 3 (MIP1 alpha)	Ccl3	168.87	59.04	3.08
94761_at	chemokine (C-C motif) ligand 7 (MCP-3)	Ccl7	502.70	55.98	4.80
97519_at	secreted phosphoprotein 1 (osteopontin)	Spp1	11.95	29.72	2.52
93328_at	histidine decarboxylase	Hdc	21.56	29.13	2.48
101881_g_at	procollagen, type XVIII, alpha 1 (endostatin)	Col18a1	-1.25	25.28	10.55
100127_at	cellular retinoic acid binding protein II	Crabp2	7.71	23.95	-4.61
93930_at	LIM and SH3 protein 1	Laspl	11.67	22.05	6.61
94168_at	interleukin 13	Il13	95.52	18.27	-0.05
AFFX-TransRecMur/X57349_M_at	transferrin receptor	Trfr	7.97	15.08	-0.12
103005_s_at	CD44 antigen	Cd44	15.06	14.69	2.02
102806_g_at	CEA-related cell adhesion molecule 1	Ceacam1	5.87	12.47	3.27
95364_at	guanine nucleotide binding protein, alpha 14	Gna14	-2.17	-13.93	0.11
99457_at	antigen identified by monoclonal antibody Ki 67	Mki67	-2.04	-12.57	-3.11
103468_at	meiosis-specific nuclear structural protein 1	Mns1	-2.12	-9.39	-1.29
104333_at	G7e protein	G7e-pending	-2.05	-8.53	-2.19
102337_s_at	Fc receptor, IgG, low affinity IIb	Fcgr2b	-1.65	-8.35	-0.51
102318_at	sialyltransferase 8 (alpha-2, 8-sialyltransferase) D	Siat8d	-9.91	-8.09	-0.25
97421_at	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	Smc2ll	-1.41	-7.89	-1.96
98010_at	nuclear factor, erythroid derived 2	Nfe2	-7.34	-6.98	-0.02

**Figure 5.** Probe sets that increased or decreased at least two fold after 24 hours of FcεRI crosslinkage were analyzed using the Database for Annotation, Visualization and Integrated Discover (David). The 69 probe sets were categorized according to their functional categories using the vocabulary given by the Gene Ontology Consortium (GO) and displayed in graphical format. Twenty-nine of the 69 probe sets listed (58.0%) were unclassified and not represented graphically.



**Table 3. Top 20 most changed genes 24 hours after FcεRI aggregation.**

Gene Name	Gene Symbol	Average Fold Change		
		90 mins	5 hrs	24 hrs
procollagen, type XVIII, alpha 1 (Endostatin)	Col18a1	-1.25	25.28	10.55
LIM and SH3 protein 1	Lasp1	11.67	22.05	6.61
acidic epididymal glycoprotein 1	Aeg1	0.11	2.36	6.17
fatty acid-Coenzyme A ligase, long chain 4	Facl4	8.72	10.83	6.06
chemokine (C-C motif) ligand 7 (MCP-3)	Ccl7	502.70	55.98	4.80
RAB4A, member RAS oncogene family	Rab4a	6.56	8.42	4.47
CD68 antigen	Cd68	-0.09	2.09	4.25
tumor necrosis factor, alpha-induced protein 3	Tnfaip3	1.91	7.76	3.95
homodomain interacting protein kinase 1	Hipk1	35.53	10.46	3.95
paired-Ig-like receptor A3	Pira3	1.44	0.80	3.74
cytoplasmic tyrosine kinase, Dscr28C related	Tec	5.78	6.04	3.70
cellular retinoic acid binding protein II	Crabp2	7.71	23.95	-4.61
nuclear factor, interleukin 3, regulated (E4bp4)	Nfil3	6.90	2.58	-3.87
CDC28 protein kinase 1	Cks1	-1.14	-1.99	-3.39
serine/threonine kinase 6	Stk6	-1.92	-5.38	-3.38
antigen identified by monoclonal antibody Ki 67	Mki67	-2.04	-12.57	-3.11
MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)	Mad2l1	-1.55	-2.37	-3.10
phosphoglycerate kinase 1	Pgk1	0.24	0.33	-2.92
proviral integration site 2	Pim2	-1.36	-3.68	-2.77
kinesin family member 11	Kif11	-1.76	-2.90	-2.74
solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	Slc7a5	2.74	2.63	-2.72



***Mast Cell Activation regulates the expression of genes involved in cell signaling.***

Probe sets of interest were selected and categorized into four groups, genes involved in signaling, cell cycle and/or apoptosis, immune response, and surface antigens. Thirty-two cell signaling genes were listed in table format, analyzed by hierarchical clustering, and also graphically represented using Tree View (Figure 6 and Table 4). Of the 32 genes listed, only nine genes consistently decrease over the three time points, including GATA2, a transcription factor that is required for the early differentiation of mast cells from progenitors (Tsai et al. 1997).

As would be expected, many genes involved in early signaling events are upregulated at 90 minutes. Almost immediately after FcεRI aggregation, the gene for activating transcription factor 3 (ATF3) is upregulated 40 fold. This gene is a member of the basic region leucine zipper family of transcription factors and is known to be upregulated in many tissues as an immediate response gene to stress and damage (Hai et al. 2001). Fyn, another member of this family, recently found to be critical for mast cell degranulation, is regulated by mast cell activation (Parravicini et al. 2002). Transcription factors are also regulated, such as FosB and JunD, members of the AP-1 transcription family, which are upregulated at 90 minutes (Table 4). Moreover, genes thought to inhibit signaling are also upregulated 90 minutes after mast cell activation, such as SOCS3 and CIS. One noteworthy gene that is upregulated after mast cell activation is the nuclear receptor subfamily 4, group A, member 1 (Nurr77). This protein is a member of the orphan nuclear receptor family, and the message for it increased an average of 38 fold 90 minutes after mast cell activation. Nurr77 protein has been shown to play a role in

TCR mediated cell death and thymocyte negative selection, as well as death in macrophages (He 2002; Kim et al. 2003). Its induction after mast cell activation has not been reported and could warrant further study.

**Figure 6.** Graphical depiction of selected genes important in cell signaling and regulated by FcεRI crosslinkage. Selected genes were analyzed and subjected to hierarchical clustering using Cluster and displayed graphically using Tree View. Both independent experiments are depicted in the same graph.

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 6nA no xL  
 10nA 90min  
 6nA 90min  
 10nA 5hr  
 6nA 5hr  
 10nA 24hr  
 6nA 24hr



Myb  
 serine/threonine kinase 10  
 CDC-like kinase 2  
 Chromobox homolog 4  
 GATA2  
 nuclear factor, erythroid derived 2  
 Ptn2  
 PIM2  
 Ezh2 (mEnx-1)  
 Hivk2  
 ATF4  
 ATF3  
 Nfya-A binding protein 2  
 E4bp4  
 SOCS3  
 JunD1  
 rhoB  
 Nr4a2 (Nurr1)  
 Cebpbeta  
 N-myc  
 regulator of G-protein signaling 2  
 Madh7 (Smad7)  
 Teios1 (mGIF)  
 Nr4a2 (Nurr1)  
 FosB  
 Map2K3 (MEK3)  
 DUB2  
 CIS  
 dual specificity phosphatase 1  
 Nr4a1 (Nur77)  
 Tec Kinase  
 F2r (Par1)

**Table 4. Cell Signaling Genes regulated by FcεRI aggregation.**

Name/Description	Identifier	Fold change		
		90 mins	5 hrs	24 hrs
ATF3	104156_r_at	39.09	0.54	0.34
ATF4	100599_at	2.47	0.1	-1.71
Cebp beta	92925_at	6.59	1.91	-1.18
CIS	100022_at	3	1.99	0.2
dual specificity phosphatase 1	104598_at	26.76	2.63	1.63
DUB2	99905_at	15.36	5.22	3.16
E4bp4	102955_at	6.9	2.58	-3.87
F2r (Par1)	92356_at	40.28	8.13	-1.06
FosB	103990_at	12.96	-0.1	-1.34
Fyn proto-oncogene	100133_at	1.87	4.09	1.15
Hipk2	103833_at	-1.03	8.29	-0.03
JunD1	102364_at	2.97	1.3	1.11
Madh7 (Smad7)	92216_at	5.53	0.85	1.21
Map2K3 (MEK3)	93314_g_at	2.4	2.39	-0.89
Ngfi-A binding protein 2	100962_at	12.49	2.54	-1.21
N-myc	103048_at	4.12	1.22	1.4
Nr4a1 (Nur77)	102371_at	37.89	-1.78	2.54
Nr4a2 (Nurr1)	92248_at	31.69	2.22	1.13
Nr4a2 (Nurr1)	92249_g_at	15.58	2.22	1.59
regulator of G-protein signaling 2	97844_at	12.87	2.62	1.12
RhoB	101030_at	6.12	2.39	1.34
SOCS3	92232_at	12.37	0.12	3.7
Tec Kinase	103539_at	5.78	6.04	1.08
Tiegl (mGIF)	99603_g_at	5.96	3.25	1.20
CDC-like kinase 2	103346_at	-3.02	-1.58	-1.08
chromobox homolog 4	93697_at	-3.5	-2.64	-1.55
Ezh2 (mEnx-1)	99917_at	-1.48	-2.62	-1.76
GATA2	102789_at	-2.7	-1.31	-1.11
Myb	92644_s_at	-2.81	-0.07	1.05
nuclear factor, erythroid derived 2	98010_at	-7.34	-6.98	-0.02
PIM2	101926_at	-1.36	-3.68	-2.77
Ptpn2	101996_at	-1.86	-4.99	-1.8
serine/threonine kinase 10	93680_at	-2.51	-1.74	-0.03

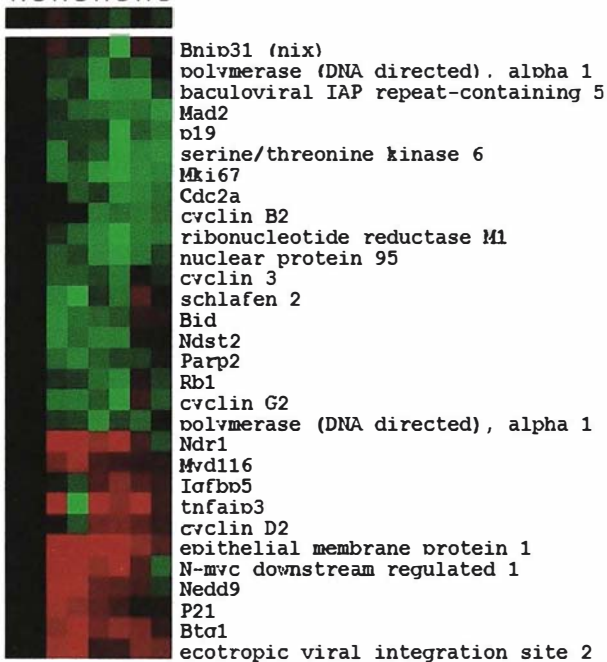
***Mast cell activation regulates the expression of cell cycle and apoptosis involved genes.***

Cell cycle and apoptosis regulation are important in the ability of the mast cell to maintain an inflammatory response. In order to gain insight into the role of mast cell activation in cell cycle regulation, we selected genes known to be involved in cell cycle regulation and apoptosis for further study. Cyclin 3, cyclin B2, cyclin G2, and retinoblastoma gene are all downregulated at 90 minutes, 5 hours and 24 hours, between one fold and three fold. Cyclin D2 however, is modestly upregulated at 5 hours and 24 hours. Concomitantly, p21, a cyclin dependent kinase inhibitor, is upregulated almost 5 fold at 90 minutes. However, in contrast, p19, cyclin dependent kinase inhibitor of CDK4, is downregulated almost 7 fold at 5 hours.

One interesting gene involved in cell cycling, N-myc downstream regulated 1, or NDRG1, is a serine protease recently characterized to be highly expressed in mast cells cultured in stem cell factor. This protein has been theorized to be responsible for mast cell maturation, allowing for degranulation to stimuli (Taketomi et al. 2003). Our microarray analysis revealed that 90 minutes after mast cell activation, this gene is upregulated almost 13 fold and is back to baseline by 24 hours. (Figure 7, Table 5).

**Figure 7.** Graphical depiction of selected genes important in cell cycling and apoptosis and regulated by FcεRI crosslinkage. Selected regulated genes were analyzed and subjected to hierarchical clustering using Cluster and displayed graphically using Tree View. Both independent experiments are depicted in one graph.

10MA no xL  
 6MA no xL  
 10MA 90min  
 6MA 90min  
 10MA 5hr  
 6MA 5hr  
 10MA 24hr  
 6MA 24hr





**Table 5. Selected genes important in cell cycle and survival.**

Name/Description	Identifier	Fold change		
		90 mins	5 hrs	24 hrs
Btg1	93104_at	4.53	2.89	1.90
cyclin D2	97504_at	-0.25	2.36	1.60
ecotropic viral integration site 2	98025_at	4.98	1.41	2.38
epithelial membrane protein 1	97426_at	23.55	8.57	2.53
Igfbp5	100566_at	-1.32	2.33	1.57
Myd116	160463_at	3.37	1.50	1.25
Ndr1	96596_at	12.56	4.94	0.04
Nedd9	101469_at	5.92	2.01	0.14
N-myc downstream regulated 1	160464_s_at	12.81	4.67	0.05
p21	98067_at	4.23	1.71	0.04
tnfaip3	99392_at	1.91	7.76	3.95
Bid	98433_at	-2.84	-1.75	1.22
Bnip31 (nix)	96255_at	0.20	-6.40	0.17
Cdc2a	100128_at	-1.38	-2.50	-2.47
cyclin 3	160545_at	-1.75	-2.02	-0.02
cyclin B2	94294_at	-1.02	-2.39	-2.20
cyclin G2	98478_at	-2.49	-3.25	-0.08
Mad2	99632_at	-1.55	-2.37	-3.10
Mki67	99457_at	-2.04	-12.57	-3.11
Ndst2	92400_at	-2.62	-2.80	1.09
nuclear protein 95	99564_at	-1.576	-4.20	-1.87
p19	94521_at	-1.37	-6.70	-2.12
Parp2	100903_at	-2.51	-1.58	-1.22
polymerase (DNA directed), alpha 1	103207_at	-1.39	-2.53	-0.43
Rb1	97948_at	-1.298	-2.68	-0.10
ribonucleotide reductase M1	100612_at	-1.64	-5.05	-2.42
schlafen 2	92472_f_at	-6.02	-1.75	1.40
serine/threonine kinase 6	92639_at	-1.92	-5.38	-3.38

***Mast cell activation strongly regulates genes involved in the inflammatory response.***

Activation of the mast cell through FcεRI results in the new synthesis of cytokines, chemokines, and growth factors. This process has been highly studied, especially in the context of mast cell mediated disease. (Plaut et al. 1989; Galli et al. 1993; Bradding et al. 1996; Lane et al. 1996; Metcalfe et al. 1997). As would be expected, we also found by microarray analysis a rapid and strong induction of pro-inflammatory molecules after 90 minutes of FcεRI aggregation, including chemokines, inflammatory cytokines and growth factors. As previously mentioned, the top 3 most increased transcripts at ninety minutes are chemokines CCL7, CCL4, and CCL3. It is also not surprising that the 4<sup>th</sup> most increased transcript at ninety minutes is the inflammatory cytokine Interleukin-13, which has many overlapping functions with Interleukin-4 and plays a significant role in the progression and severity of asthma (Wills-Karp et al. 1998). Mast cell activation also upregulates certain enzymes involved in the process of manufacturing more inflammatory mediators such as histidine decarboxylase and COX-2. Another pro-inflammatory protein, Activin A, was transcriptionally upregulated 4 fold at 90 minutes in our analysis. This confirms a previous report demonstrating the induction of activin βA transcription in mast cells after activation with PMA and the calcium ionophore A23187, and that this protein promoted the proliferation of human airway smooth muscle cells (Cho et al. 2003) (Figure 8, Table 6).

**Figure 8.** Graphical depiction of selected immune response genes and regulated by FcεRI aggregation. Selected genes were analyzed and subjected to hierarchical clustering using Cluster and displayed graphically using Tree View. Both independent experiments are depicted in one graph.

10nA no xL  
 6nA no xL  
 10nA 90min  
 6nA 90min  
 10nA 5hr  
 6nA 5hr  
 10nA 24hr  
 6nA 24hr



CD8 beta  
 Siat8d  
 PDGF alpha  
 secreted phosphoprotein 1  
 TGF beta 1  
 CCL9 (MRP-2)  
 GM-CSF  
 COX-2  
 TNFL superfamily 9 (4IBB ligand)  
 IL-4  
 INF beta  
 diphtheria toxin receptor (HB-EGF)  
 CCL2 (MCP1)  
 inhibin beta-A (activin A)  
 CCL7 (MCP-3)  
 TNF alpha  
 TNFL superfamily 8 (CD153)  
 IL-6  
 IL-13  
 CCL4 (MIP1 beta)  
 CCL3 (MIP1 alpha)  
 MCSF  
 histidine decarboxylase  
 IL-1 beta  
 CCL1  
 VEGF  
 SAM domain and ED domain, 1

**Table 6. Table of selected immune response genes regulated by FcεRI aggregation.**

Name/Description	Identifier	Fold Change		
		90 mins	5 hrs	24 hrs
CCL1	94166_g_at	4.98	4.34	-1.62
CCL2 (MCP1)	102736_at	10.44	0.17	0.13
CCL3 (MIP1 alpha)	102424_at	168.87	59.04	3.08
CCL4 (MIP1 beta)	94146_at	179.45	11.28	1.13
CCL7 (MCP-3)	94761_at	502.70	55.98	4.80
CCL9 (MRP-2)	104388_at	1.71	2.60	2.32
COX-2	104647_at	93.16	1.80	-1.12
Diphtheria toxin receptor (HB-EGF)	92730_at	31.80	3.97	0.80
GMCSF	92948_at	68.07	0.08	-0.10
histidine decarboxylase	93328_at	21.56	29.13	2.48
IL-1 beta	103486_at	11.89	7.08	1.68
IL-13	94168_at	95.52	18.27	-0.05
IL-4	92283_s_at	15.23	1.74	1.04
IL-6	102218_at	14.27	3.26	0.08
INF beta	160092_at	10.44	1.51	0.50
inhibin beta-A (activin A)	100277_at	3.80	0.22	0.19
MCSF	101450_at	19.05	4.82	2.51
PDGF alpha	94932_at	2.98	7.12	2.88
SAM domain and HD domain, I	103080_at	3.10	0.12	1.75
secreted phosphoprotein I	97519_at	11.95	29.72	2.52
TGF beta 1	101918_at	1.56	2.31	1.19
TNF	102629_at	16.99	2.99	0.80
TNFL superfamily 8 (CD153)	101136_at	5.51	1.34	0.46
TNFL superfamily 9 (41BB ligand)	92415_at	3.44	-0.01	1.36
VEGFa	103520_at	4.00	1.42	-0.83
CD8 beta	94000_at	-2.17	-4.98	-0.32
Siat8d	102318_at	-9.91	-8.09	-0.25

***Mast cell activation modulates the transcription of cell surface antigen genes.***

It is reasonable to assume that mast cell activation would initiate a cascade that would ultimately alter the ability of the cell to undergo further stimulation, and one method could be through the regulation of its surface antigens. The most strongly induced surface receptor is the hyaluronic acid receptor CD44. Recent studies have implicated its role in both mediating inflammation as well as resolving it (Teder et al. 2002; Katoh et al. 2003). However, its role in mast cell function has not been fully elucidated and could be worth further study. The most downregulated surface receptor after mast cell activation is the inhibitory low affinity IgG receptor, FcγRIIb. (Figure 9, Table 7). Dysregulation of FcγRIIb expression or signaling can lead to the susceptibility of many autoimmune diseases including SLE, EAE and rheumatoid arthritis (Clynes et al. 1998; Johansson et al. 2001; Robbie-Ryan et al. 2003). The regulation of FcγRIIb by mast cell activation could be of importance in the role of the mast cell in these diseases (Ravetch et al. 2001).

Also of interest is coagulation factor II (thrombin) receptor, or F2r, or Par1. It has previously been reported that thrombin can activate mast cells causing degranulation, cytokine release and mast cell adherence to fibronectin (Vliagoftis 2002). We found by microarray analysis that the transcription of Par1 increased 2.8 fold, 4.62 fold and 3.14 fold over 90 minutes, 5 hours and 24 hours after activation.

**Figure 9.** Graphical depiction of surface antigen genes regulated by FcεRI crosslinkage. Selected regulated genes were analyzed and subjected to hierarchical clustering using Cluster and displayed graphically using Tree View. Both independent experiments are depicted in one graph.

10MA no xL  
 6MA no xL  
 10MA 90min  
 6MA 90min  
 10MA 5hr  
 6MA 5hr  
 10MA 24hr  
 6MA 24hr



FcεRI alpha  
 IL-18R1  
 CD48  
 c-kit  
 IL-4R alpha  
 FcεRI beta  
 FcαRIIb  
 IL-17R  
 CCR1  
 PIRB  
 IL-3Rb (GM-CSFR2b1)  
 PIRA3  
 IL-3Rb (GM-CSFR2b1)  
 CD68  
 Lasp1  
 Itcb2 (LFA-1)  
 C5aR  
 F2R (PAR1)  
 CD9  
 CD53  
 CD44



**Table 7. Table of selected surface antigen genes.**

Name/Description	Identifier	Fold Change		
		90 mins	5 hrs	24 hrs
CCR1	99413_at	3.98	1.52	0.24
CD44	103005_s_at	15.06	14.69	2.02
CD53	94939_at	2.05	3.14	1.62
CD68	103016_s_at	-0.09	2.09	4.25
CD9	95661_at	1.83	2.47	1.67
F2R (PAR1)	92267_at	2.797	4.62	3.14
IL-17R	99991_at	2.34	-0.24	1.29
IL-3Rb (GM-CSFR2b1)	94748_g_at	-0.11	1.74	2.11
IL-3Rb (GM-CSFR2b1)	94747_at	-1.37	1.44	1.74
Laspl	93930_at	11.67	22.05	6.61
PIRA3	100328_s_at	1.44	0.80	3.74
PIRB	98003_at	0.00	1.16	2.41
CD48	103089_at	-1.57	-2.34	-1.14
c-kit	99956_at	-1.47	-1.41	1.36
Fc gamma RIIb	102337_s_at	-1.65	-8.35	-0.51
FceRI alpha	101209_at	-1.15	-1.27	1.15
FceRI beta	96509_at	-0.28	-4.29	1.80
IL-18R1	101144_at	-2.29	-2.62	0.37
IL-4R alpha	102021_at	-2.22	-1.41	0.09

***Mast cell activation regulates the transcription of the FcεRI beta subunit.***

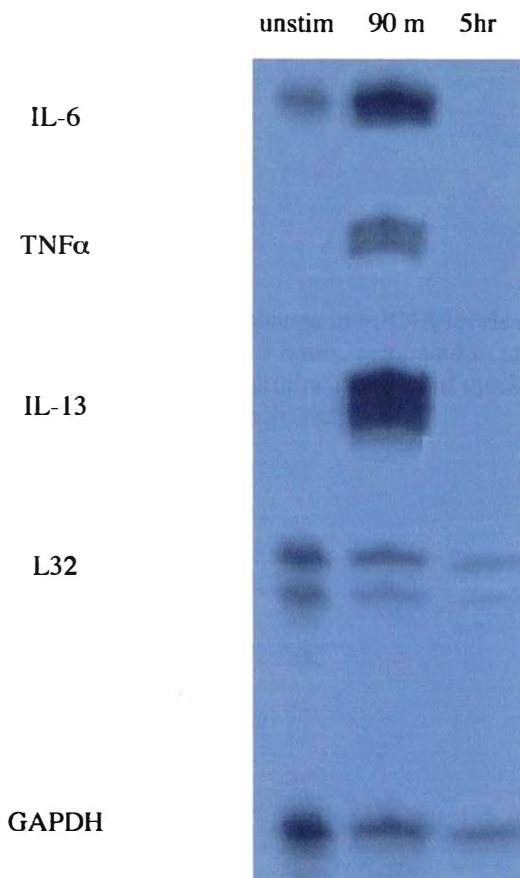
We found that levels of FcεRI alpha and gamma transcripts were not significantly altered after mast cell activation. However, the level of the beta subunit, shared by both FcεRI and FcγRIII, was reduced 4 fold after five hours of mast cell activation compared to unstimulated cells. The beta subunit is critical for FcεRI expression in rodents, and although not required for human FcεRI expression, can augment both its signaling and expression (Dombrowicz et al. 1998; Donnadieu et al. 2000). Besides the recent finding that GATA-1 binds the beta chain promoter, little else is known about events that regulate its transcription and expression (Maeda et al. 2003). Thus our finding by GeneChip analysis that mast cell activation could potentially regulate the transcription of the beta chain was of great interest.

***Ribonuclease Protection Assay confirms GeneChip results.***

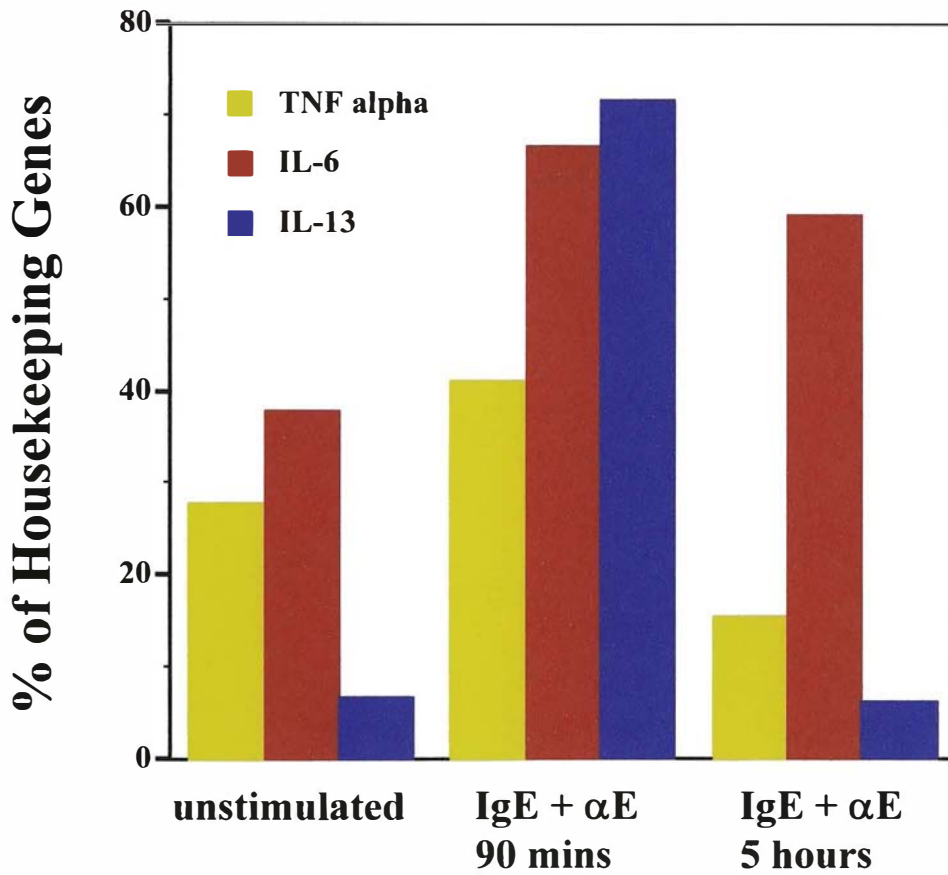
To confirm our microarray results, we analyzed mRNA levels of various cytokines and Fc receptor subunits by ribonuclease protection assay. BMMC were incubated in IL-3 and activated by Fc $\epsilon$ RI crosslinkage (IgE+anti-IgE) for ninety minutes, 5 hours and 24 hours, after which RNA was harvested and subjected to RPA analysis. In accordance with the microarray analysis, there was an upregulation of IL-13, IL-6 and TNF $\alpha$  message levels ninety minutes after activation (Figure 10 and Figure 11).

The mRNA levels of Fc $\epsilon$ RI subunits were also assessed by RPA using IgE crosslinkage or pharmacological activation with the calcium ionophore, ionomycin (Figure 12). In agreement with the results from GeneChip analysis, 5 hours after activation, the Fc $\epsilon$ RI alpha subunit does not significantly change after Fc $\epsilon$ RI crosslinkage (Figure 13). The gamma subunit on the other hand is significantly reduced after 5 hours of crosslinkage (Figure 14). This experiment has been repeated and because of inconsistent results needs to be further studied. Importantly, Fc $\epsilon$ RI $\beta$  message levels are reduced by almost 80 percent compared to unstimulated cells 5 hours after mast cell activation, and this result has remained consistent throughout 5 separate experiments (Figure 15).

**Figure 10.** RPA analysis confirming upregulation of cytokines after mast cell activation. Wild type BMMC were cultured in IgE followed by rat anti-mouse IgG for 90 minutes and 5 hours, or were left unstimulated.



**Figure 11.** Summary of percent change in mRNA levels of TNF $\alpha$ , IL-6, and IL-13 in cells activated for 90 minutes and 5 hours, compared to unstimulated cells. Phosphorimaging was used to determine the ratio of cytokine expression to the expression of L32+GAPDH for each sample.



**Figure 12.** RPA demonstrates the different levels of FcεRI α, β and γ subunits after FcεRI mediated mast cell activation. Wild type BMNC were cultured in either IgE followed by rat anti-mouse IgG, or ionomycin for 5 hours, or were left unstimulated.



unstimulated  
IgE + anti-IgE  
Ionomycin



alpha

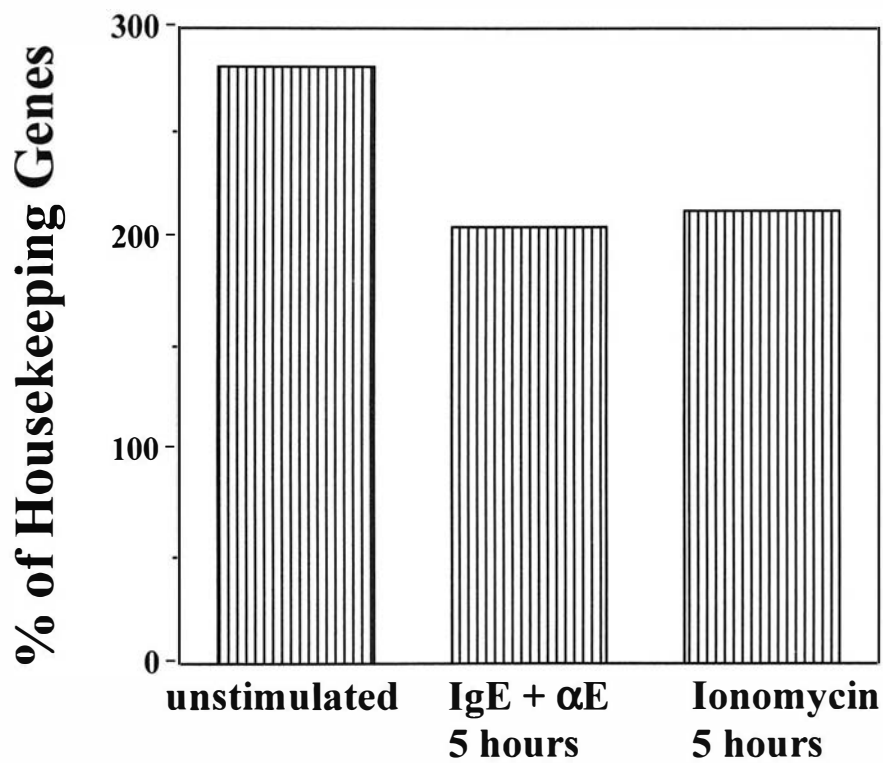
beta

gamma

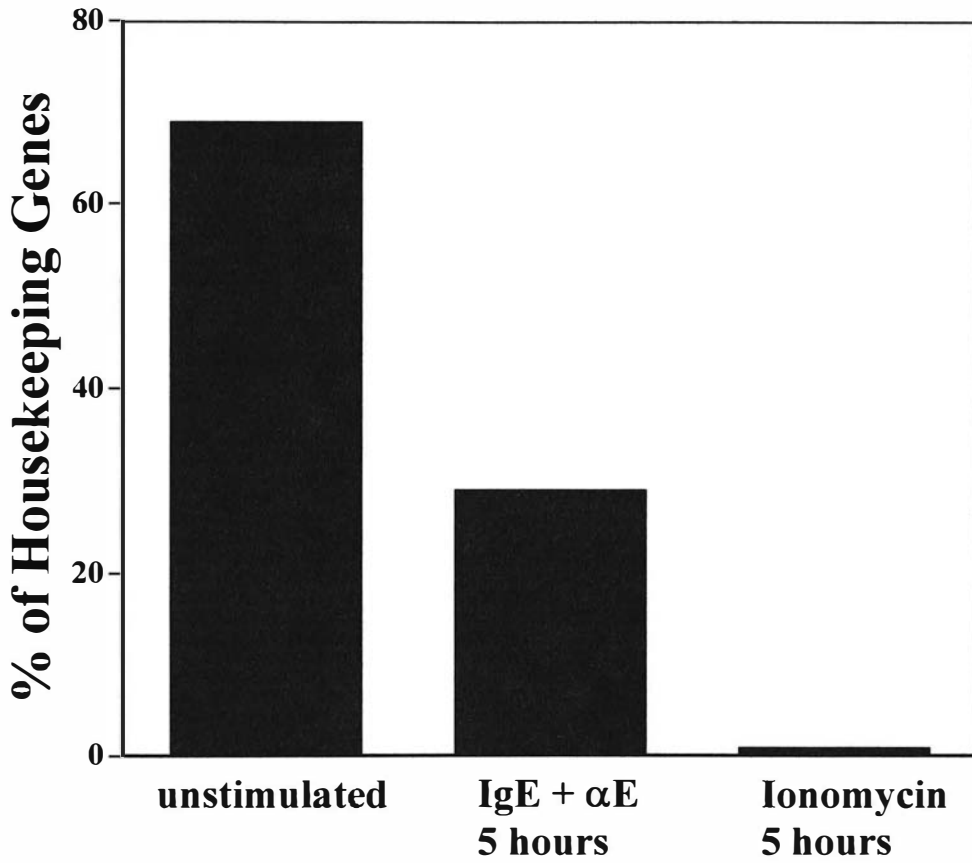
L32

GAPDH

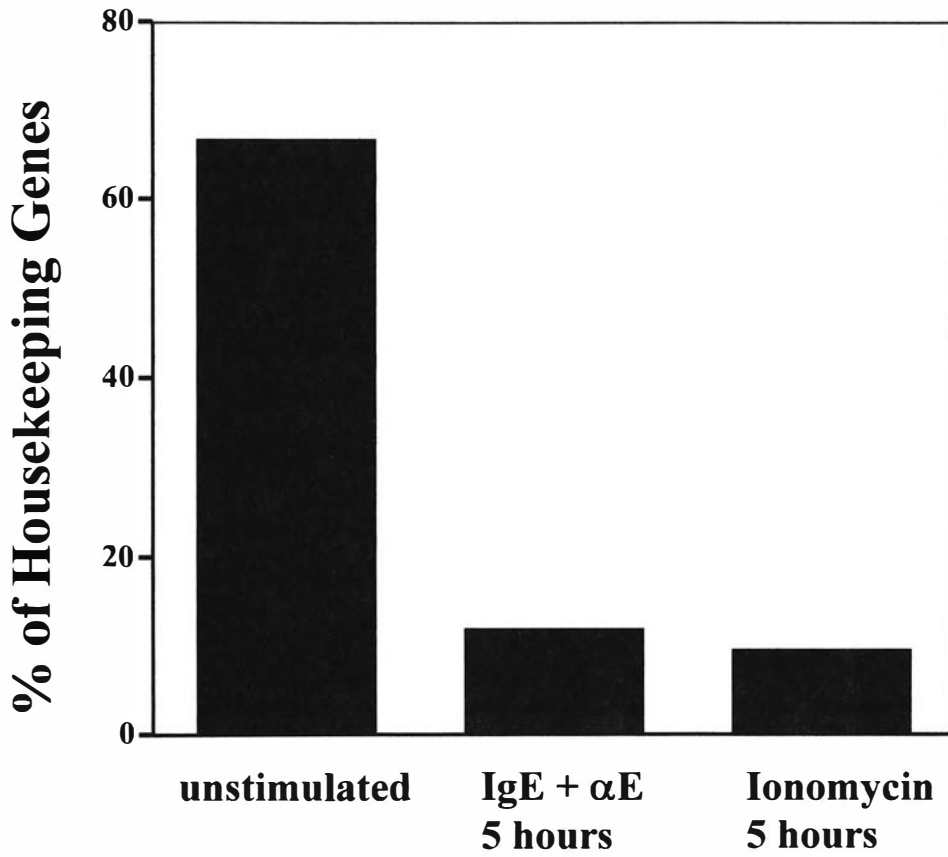
**Figure 13.** Percent change of FcεRI α mRNA in cells activated by FcεRI aggregation or ionomycin for 5 hours and compared to unstimulated cells. Phosphorimaging was used to determine the ratio of FcεRI α expression to expression of L32+GAPDH for each sample.



**Figure 14.** Percent change of FcεRI γ mRNA in cells activated by FcεRI aggregation or ionomycin for 5 hours and compared to unstimulated cells. Phosphorimaging was used to determine the ratio of FcεRI α expression to expression of L32+GAPDH for each sample.



**Figure 15.** Percent change of FcεRI β mRNA in cells activated by FcεRI aggregation or ionomycin for 5 hours and compared to unstimulated cells. Phosphorimaging was used to determine the ratio of FcεRI α expression to expression of L32+GAPDH for each sample.



***FcεRI beta chain protein levels are reduced after mast cell activation.***

In order to determine if changes in message levels corresponded to changes in protein levels, we assessed protein levels of the different subunits of FcεRI after mast cell activation. FcεRI alpha protein did not significantly change after mast cell activation (Figure 16 and Figure 17). However, there was a slight decrease in protein levels of FcεRI beta and gamma chains after mast cell 90 minutes and 5 hours of mast cell activation. (Figure 17).



**Figure 16.** FcεRI α protein levels after FcεRI aggregation. BMDC were activated with IgE + anti-IgE for 90 minutes, 5 hours and 24 hours, or left unstimulated. Whole cell lysates were immunoprecipitated for FcεRI α and immunoblotted for FcεRI α.

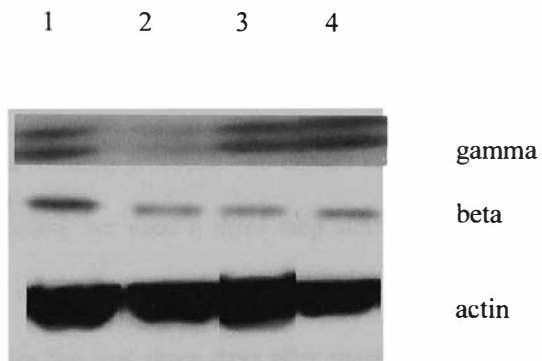
IP: alpha  
WB: alpha

1 2 3 4 5



1. unstimulated cells
2. IgE + anti-IgE for 90 minutes
3. IgE + anti-IgE for 5 hours
4. IgE + anti-IgE for 24 hours
5. IgG control

**Figure 17.** FcεRI γ and β protein levels after mast cell activation. BMNC were activated with IgE and anti-IgE for 90 minutes, 5 hours and 24 hours, or left unstimulated. Whole cell lysates were separated on a 4-20% gradient gel, transferred and probed for FcεRI β or γ. Nitrocellulose was stripped and reprobed for actin.

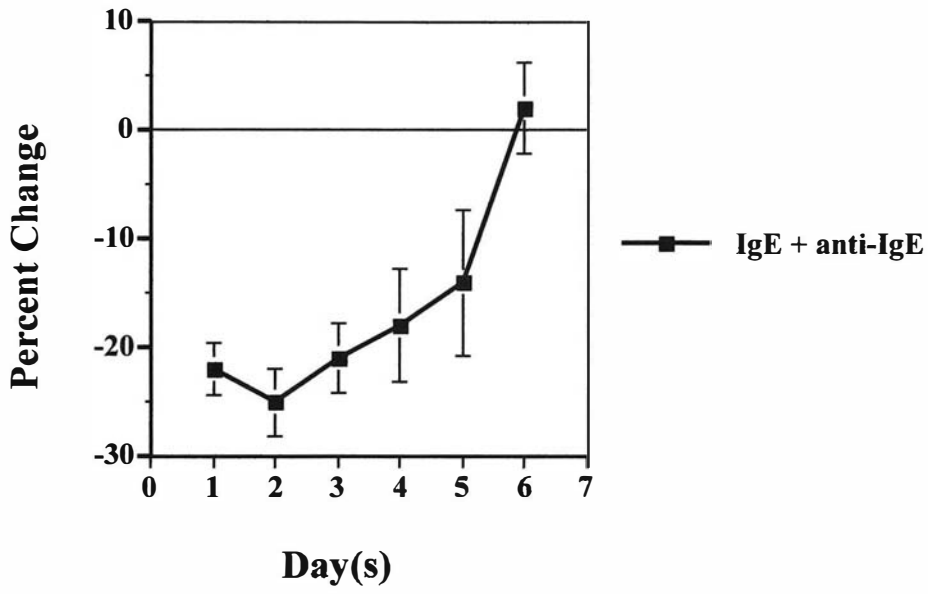


1. unstimulated cells
2. IgE + anti-IgE for 90 minutes
3. IgE + anti-IgE for 5 hours
4. IgE + anti-IgE for 24 hours

***Mast cell activation correlates with downregulation of surface FcεRI.***

In rodents, the surface expression of FcεRI requires the association of alpha with both the beta and gamma chains (Kinet 1999). Thus it is reasonable to presume that regulation of the beta subunit could correlate with a change in FcεRI surface expression. We activated BMDC for various time points and checked for surface FcεRI by flow cytometry using a modified staining protocol. To ensure complete staining of FcεRI on the cell surface after activation, we used a three step staining consisting of unlabelled IgE, unlabelled anti IgE and FITC coupled anti-IgG. Surface FcεRI is decreased approximately 25 percent for the first three days after mast cell activation, compared to unstimulated cells and returns to baseline by day 7 (Figure 18). The mechanism by which mast cell activation regulates surface expression of FcεRI has not been fully elucidated and needs to be further studied. It would be of great interest if a causal relationship could be established between mast cell activation, beta chain regulation and FcεRI surface expression.

**Figure 18.** Mast cell activation alters surface expression of FcεRI. BMMC were incubated in IgE followed by anti-IgE and cultured for the days indicated. Surface FcεRI was measured by flow cytometry.



***Part II. IL-4 selectively enhances Fc $\gamma$ RIII expression.***

Pro-inflammatory signaling followed by a feedback homeostatic effect on the cell is a theme constantly explored by our lab. We have previously reported that Interleukin-4, which is thought of predominantly as a pro-inflammatory cytokine, downregulated the surface expression of Fc $\epsilon$ RI and c-kit after 3 days of culture. (Ryan et al. 1998; Mirmonsef et al. 1999). In further support of the homeostatic effects of IL-4, we have reported that IL-4 in addition with IL-10 can induce mast cell apoptosis 6 days after culture (Yeatman et al. 2000). This led us to explore the possibility that IL-4 could also regulate other surface receptors.

Mast cells express both the inhibitory and activation low affinity IgG receptors, Fc $\gamma$ RIIb and and Fc $\gamma$ RIII, respectively (Daeron 1997). These two receptors have opposite functions, Fc $\gamma$ RIII aggregation activates the cell whereas Fc $\gamma$ RIIb co-aggregation with an activating receptor will terminate the activation signal. Despite their opposing functions, the receptors have virtually identical extracellular domains and bind IgG complexes with the same affinity (Ravetch et al. 2001). Recent studies have indicated the importance of balancing these receptors in mast cell-mediated inflammation observed in multiple sclerosis and rheumatoid arthritis (Lee et al. 2002; Robbie-Ryan et al. 2003). The differential regulation of these two receptors was our next area of focus.

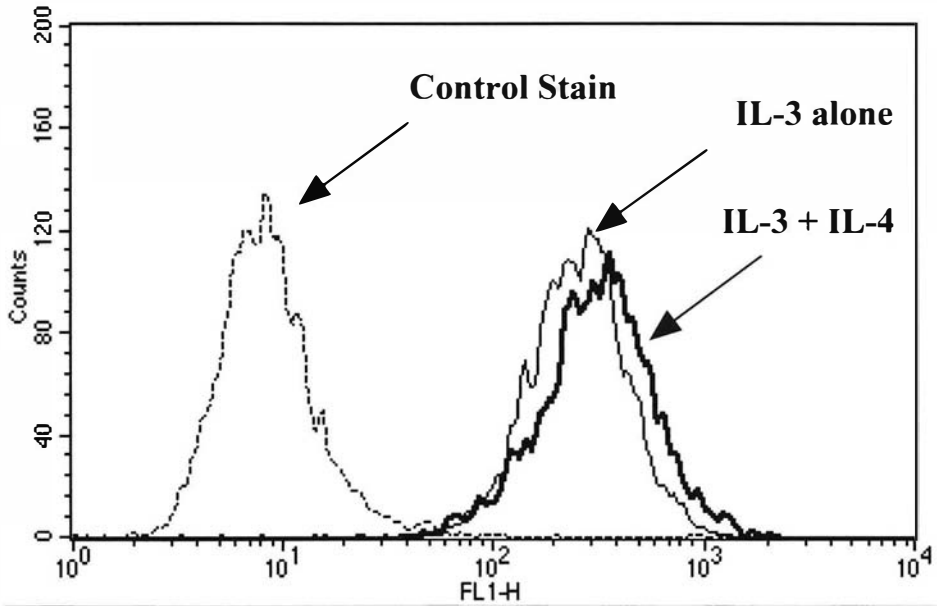
To assess the effects of IL-4 on Fc $\gamma$ RIIb and Fc $\gamma$ RIII expression, we cultured BMDC in IL-3 alone or IL-3 + IL-4. After three days of culture, there was a slight increase in Fc $\gamma$ R expression as assessed by flow cytometry with FITC-2.4G2, an antibody that recognizes both Fc $\gamma$ RIIb and Fc $\gamma$ RIII (Figure 19). To determine if this increase in



Fc $\gamma$ R expression was due to an increase in Fc $\gamma$ RIIb, these BMMC were stained with K9.361, an antibody specific for Fc $\gamma$ RIIb. No change in Fc $\gamma$ RIIb expression was detected after the addition of IL-4 in culture for three days as compared to cells cultured in IL-3 alone (Figure 20). This led us to explore the possibility that IL-4 upregulated Fc $\gamma$ RIII surface expression without altering Fc $\gamma$ RIIb expression. In order to test this directly, we harvested BMMC from FcR $\gamma$ <sup>-/-</sup> mice that lack Fc $\gamma$ RIII but express Fc $\gamma$ RIIb, and from Fc $\gamma$ RIIb<sup>-/-</sup> mice that lack Fc $\gamma$ RIIb but express Fc $\gamma$ RIII (Takai et al. 1994; Takai et al. 1996). In concordance with K9.361 staining, the addition of IL-4 did not significantly alter Fc $\gamma$ RIIb surface expression. In contrast, culture in IL-3+IL-4 consistently increased Fc $\gamma$ RIII surface expression more than 2-fold over BMMC cultured in IL-3 alone (Figure 21). Because BMMC basal surface levels of Fc $\gamma$ RIIb is greater than Fc $\gamma$ RIII expression (Lobell et al. 1993), an IL-4-mediated increase in Fc $\gamma$ RIII could be expected to yield only a slight increase in staining total Fc $\gamma$ R expression, consistent with the data shown in Figure 19.

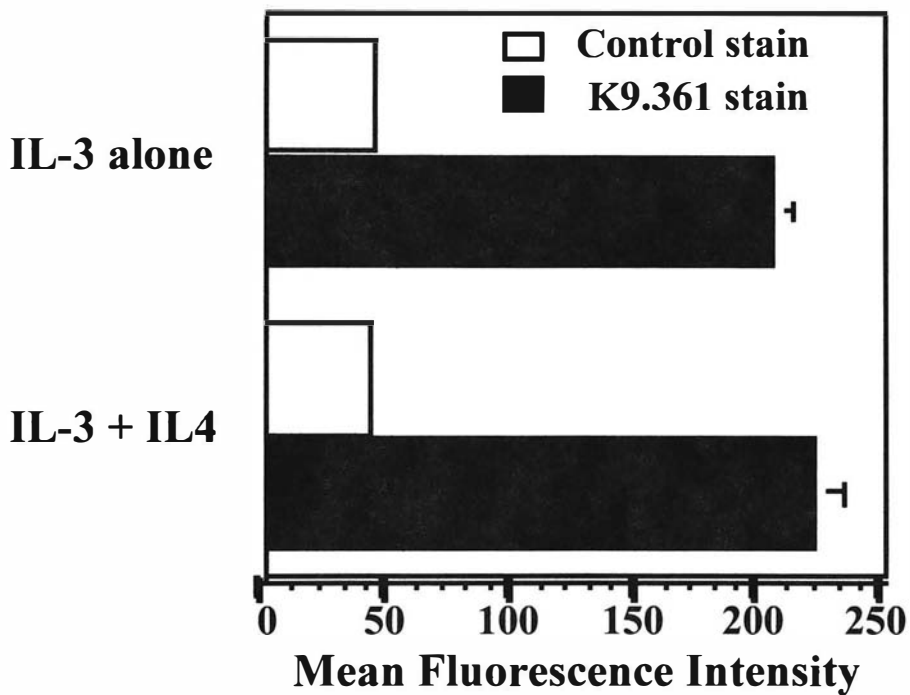
IL-4 elevated Fc $\gamma$ RIII expression from days 2 through at least day 14 of culture. This effect required a minimum of 1 ng/ml of IL-4, with maximal effects at 10 ng/ml. Fc $\gamma$ RIIb expression showed little or no change during this culture period (Figures 22 and 23). Thus, IL-4 selectively enhanced mast cell Fc $\gamma$ RIII surface expression without significantly altering the expression of Fc $\gamma$ RIIb for up to 14 days.

**Figure 19.** IL-4 regulates Fc $\gamma$ R expression. BMDC derived from wild mice were cultured in IL-3 or IL-3 + IL-4 for three days and assessed for Fc $\gamma$ R expression by flow cytometry with anti-Fc $\gamma$ RII/Fc $\gamma$ RIII antibody (2.4G2). Data shown are from at least 7 independent experiments using 12 different cell populations. (\*) =  $p < 0.05$  as determined by analysis of variance (ANOVA) and least significant difference (LSD) analysis.

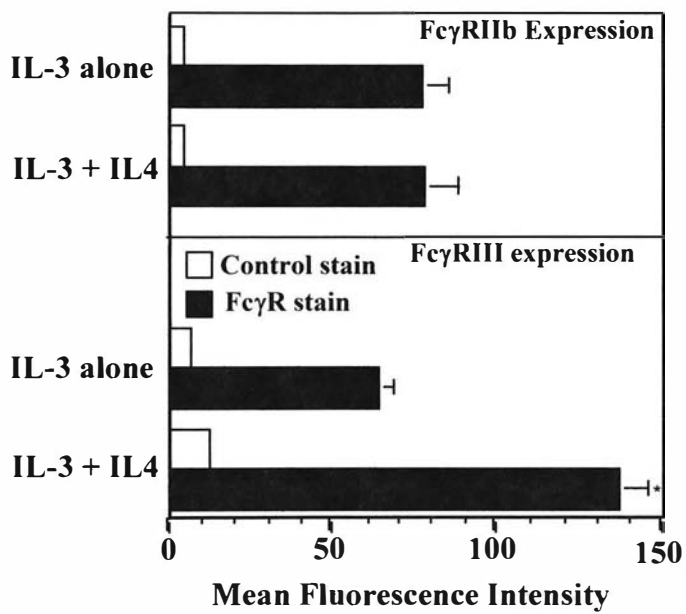


Mean Fluorescence Intensity

**Figure 20.** IL-4 does not regulate the surface expression of Fc $\gamma$ RIIb. BMMC derived from wild mice were cultured in IL-3 or IL-3 + IL-4 for three days and assessed for Fc $\gamma$ RIIb expression by flow cytometry with K9.361. Data shown are from at least 7 independent experiments using 12 different cell populations. (\*) =  $p < 0.05$  as determined by analysis of variance (ANOVA) and least significant difference (LSD) analysis.

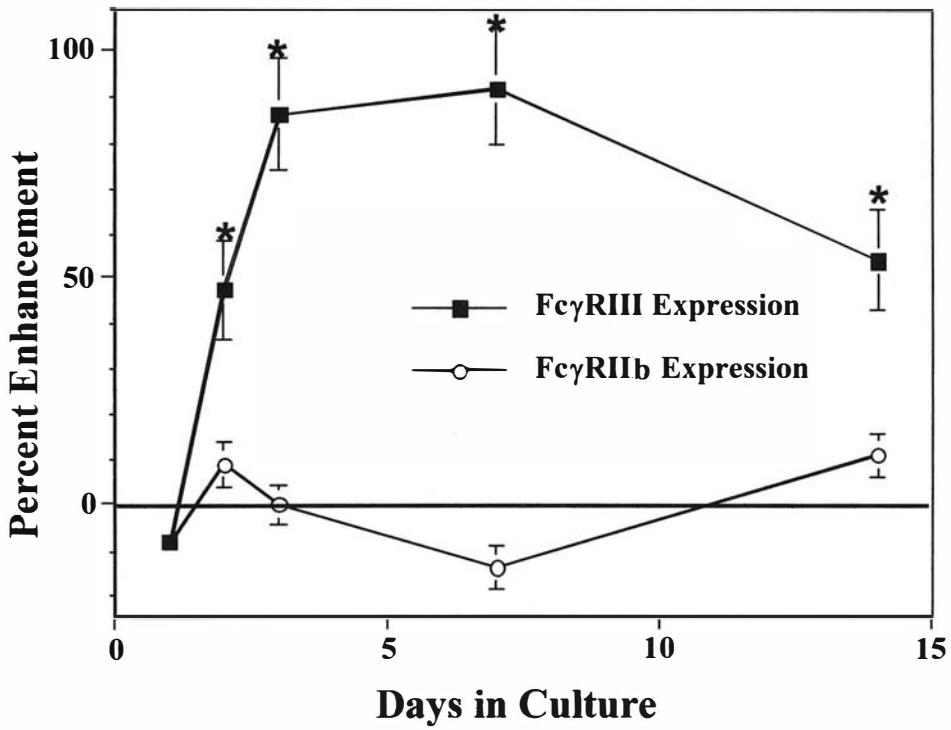


**Figure 21.** Fc $\gamma$ R surface staining of FcR $\gamma$ <sup>-/-</sup> or Fc $\gamma$ RIIb<sup>-/-</sup> mast cells. BMMC derived from FcR $\gamma$ <sup>-/-</sup> or Fc $\gamma$ RIIb<sup>-/-</sup> mice were cultured in IL-3 or IL-3 + IL-4 for three days and assessed for Fc $\gamma$ R expression by flow cytometry using FITC-2.4G2. Data shown are from at least 7 independent experiments using 12 different cell populations. (\*) =  $p < 0.05$  as determined by analysis of variance (ANOVA) and least significant difference (LSD) analysis.

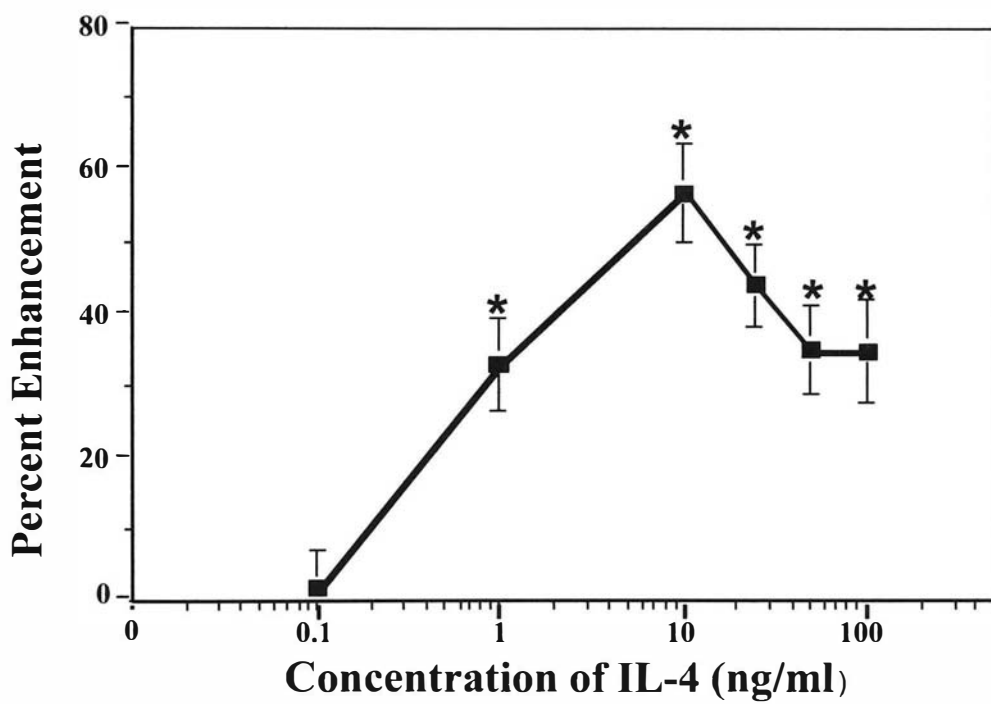


**Figure 22.** BMDC derived from  $Fc\gamma R^{-/-}$  or  $Fc\gamma RIIB^{-/-}$  mice were cultured in IL-3 or IL-3 + IL-4 and Fc $\gamma$ R expression was assessed by flow cytometry on the indicated days by FITC-2.4G2 staining. Percent enhancement and standard error measurements were calculated by comparing mean fluorescence intensities of IL-3+IL-4 treated BMDC to the same cells cultured in IL-3. Data shown are from 9 independent BMDC populations analyzed in at least 6 separate experiments. (\*) =  $p < 0.05$  as determined by ANOVA and LSD analysis.





**Figure 23.** Dose Response to IL-4. BMMC derived from  $Fc\gamma RIIB^{-/-}$  mice were cultured in IL-3 or IL-3 with increasing concentrations of IL-4. Percent enhancement was calculated by comparing the mean fluorescence of cells cultured in IL-3+IL-4 to cells cultured in IL-3. (\*) =  $p < 0.05$  as determined by ANOVA and LSD analysis.



***IL-4 modestly alters FcγRIIIα mRNA expression and upregulates protein expression.***

We investigated the mechanism of IL-4-mediated FcγRIII regulation by first assessing FcγRIIIα mRNA changes through RNase Protection Assay (RPA) analysis. The addition of IL-4 to BMDC cultured in IL-3 for 30 minutes to 72 hours did not significantly alter FcγRIIIα mRNA expression, though a moderate increase was noted after 96 hours of culture (Figure 24). This finding corroborates a related study in which we found that IL-4 downregulates FcεRI surface expression without significantly altering mRNA expression of FcεRI subunits including the FcεRIγ chain (Gillespie et al. 2003).

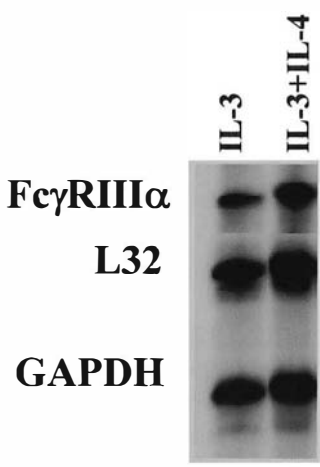
There are several post-transcriptional mechanisms by which IL-4 could increase FcγRIIb surface expression, including increased protein synthesis, increased protein stability, and enhanced protein trafficking from preformed intracellular pools. To determine if the changes in FcγRIII surface expression were matched by an increase in total FcγRIIIα protein levels, we employed 2.4G2 staining of fixed and permeabilized FcγRIIb-deficient BMDC. This technique, modeled after intracellular staining of cytokines, allowed detection of both surface and intracellular FcγRIIIα, since unlabelled 2.4G2 antibody added prior to fixation could block surface but not intracellular FITC-2.4G2 staining (data not shown). These experiments demonstrated an IL-4-mediated increase in total FcγRIIIα protein expression that resembled the enhanced surface expression (Figure 25).

To determine if the increase in FcγRIIIα protein expression was related to changes in protein stability, we employed the translational inhibitor cycloheximide.

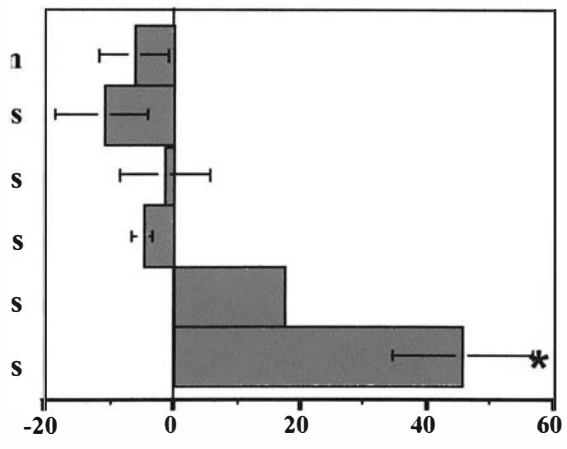
Fc $\gamma$ RIIb-deficient BMMC were stimulated for three days with IL-3 alone or with IL-3+IL-4, after which cycloheximide was added to the cultures. Intracellular 2.4G2 staining during the proceeding 24 hours showed that the rate of total Fc $\gamma$ RIII $\alpha$  degradation was unaltered by IL-4 treatment, indicating that IL-4 stimulation had no effect on Fc $\gamma$ RIII $\alpha$  stability (Figure 26).

There remained the possibility that IL-4 facilitated recycling of endocytosed Fc $\gamma$ RIIb, resulting in increased surface expression. Brefeldin A (BFA) prevents surface expression of newly synthesized proteins by inhibiting transport from the endoplasmic reticulum to the golgi, and blocks surface protein recycling by preventing endosomes from fusing with the trans-golgi (Lippincott-Schwartz et al. 1991; Wood et al. 1991). In order to determine if IL-4 upregulated Fc $\gamma$ RIII surface expression by altering protein transport or recycling, we assessed changes in surface levels of Fc $\gamma$ RIII after the addition of BFA to the culture. Fc $\gamma$ RIIb-deficient BMMC were cultured in IL-3 alone or IL-3+IL-4 for 3 days prior to the addition of BFA. Surface Fc $\gamma$ RIII expression was measured 20 hours later, the latest time point possible without affecting cell viability. Cells cultured in IL-3+IL-4 had virtually the same sensitivity to BFA as cells cultured in IL-3 alone, as both groups demonstrated a 25% reduction in Fc $\gamma$ RIII surface expression compared to cells treated with vehicle alone (Figure 27).

**Figure 24.** IL-4 has minimal effects on transcription. Wild type BMMC were stimulated for 4 days with the indicated cytokines, and total RNA was subjected to RPA analysis as described in Materials and Methods. Data shown are a representative BMMC population from 3-11 populations assessed in 5 independent experiments spanning days 1-4. (B) Summary of percent change in Fc $\gamma$ RIII $\alpha$  mRNA in cells cultured in IL-3+IL-4, as compared to cells cultured in IL-3 alone. Phosphorimaging was used to determine the ratio of Fc $\gamma$ RIII $\alpha$  expression to expression of L32+GAPDH for each sample. This ratio was used to compare Fc $\gamma$ RIII $\alpha$  expression in BMMC treated with IL-3+IL-4 to those cultured in IL-3 alone. Data are means and standard errors from 3-11 samples from 5 independent experiments.

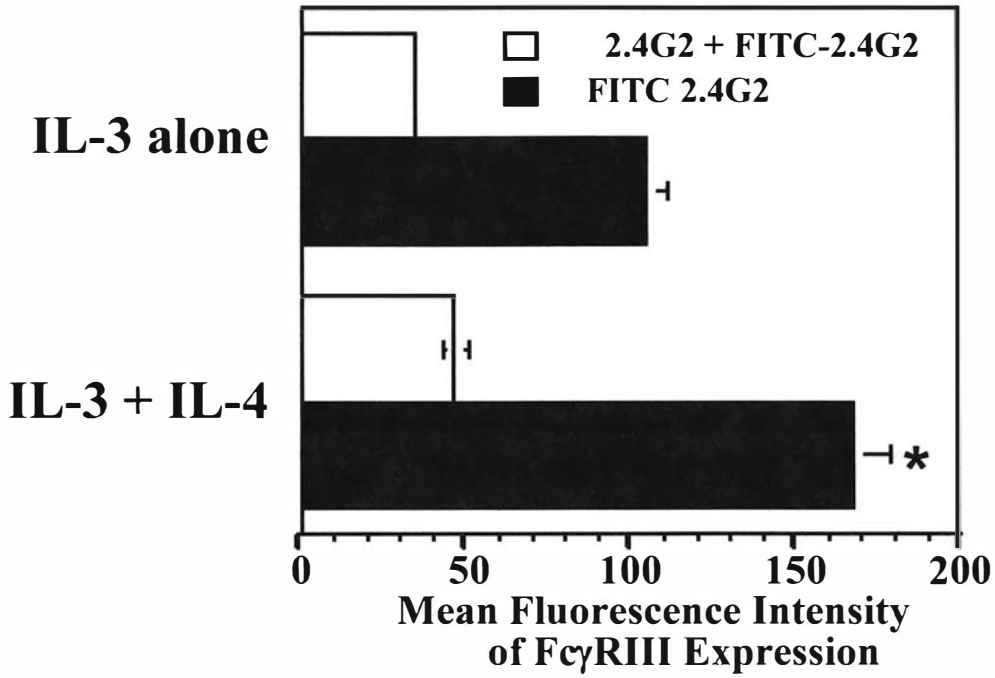


# B

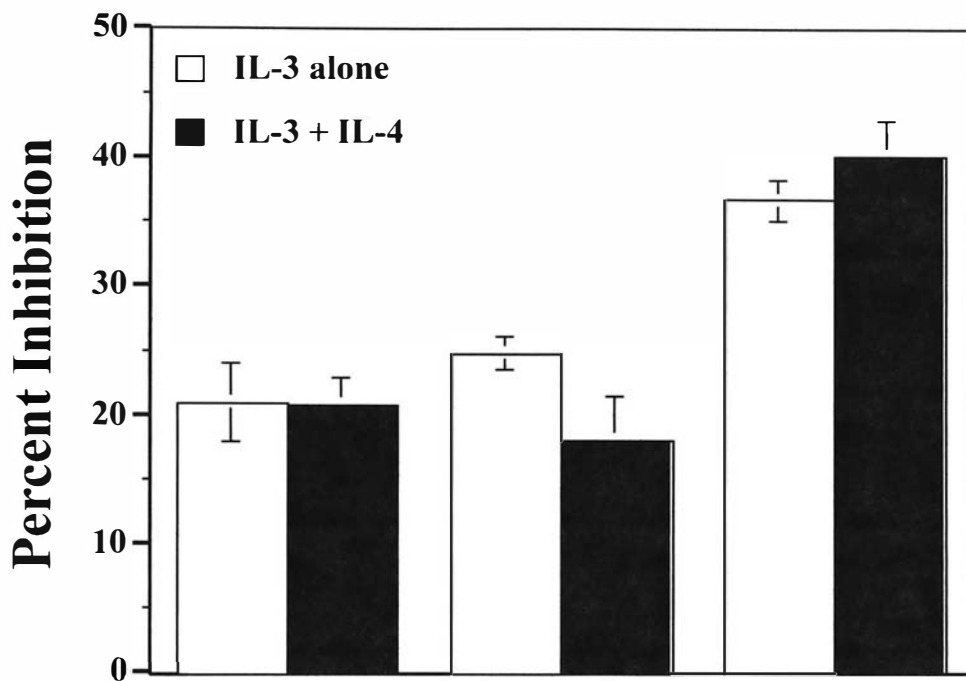


**Figure 25.** Intracellular staining of FcγRIIIα. BMMC derived from FcγRIIbα<sup>-/-</sup> mice were cultured in the indicated cytokines for three days, and assessed for total FcγRIIIα expression by intracellular staining with FITC-2.4G2, as described in Materials and Methods. Statistical significance was evaluated as described in figure 1. (\*) = p < 0.05 as determined by ANOVA and LSD analysis.

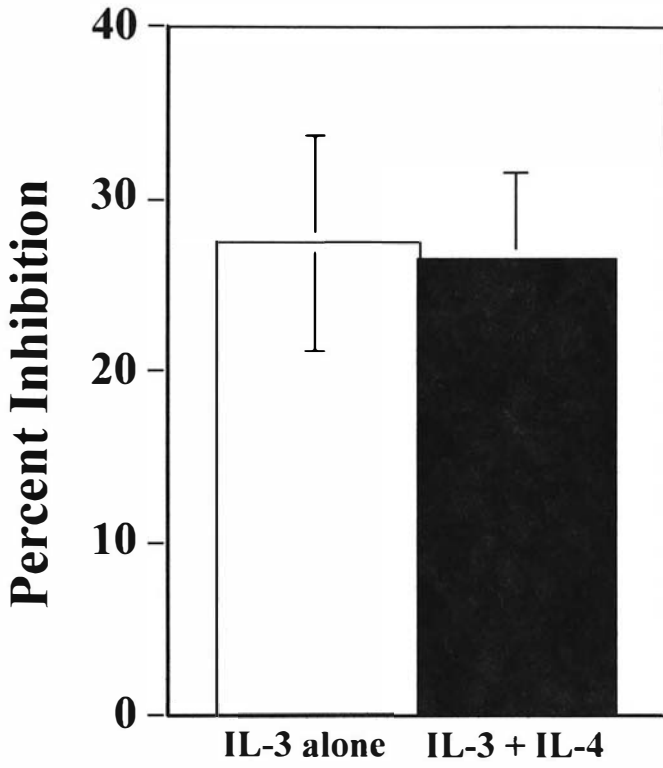




**Figure 26.** IL-4 enhances FcγRIII expression without altering protein stability. BMDC derived from FcγRIIbα<sup>-/-</sup> mice were cultured for three days in IL-3 alone or in IL-3+IL-4 prior to the addition of cycloheximide (4μg/ml final) for the times indicated. Intracellular staining with 2.4G2 was used to assess total FcγRIIIα expression. Percent change in expression was determined by comparing mean fluorescence intensities of cells treated with cycloheximide to those in DMSO. Data shown are means and standard errors of 9 different BMDC populations from three representative experiments.



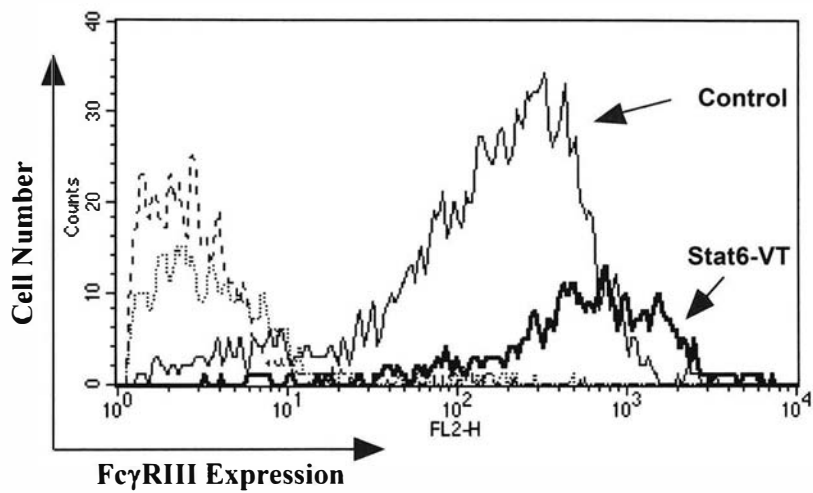
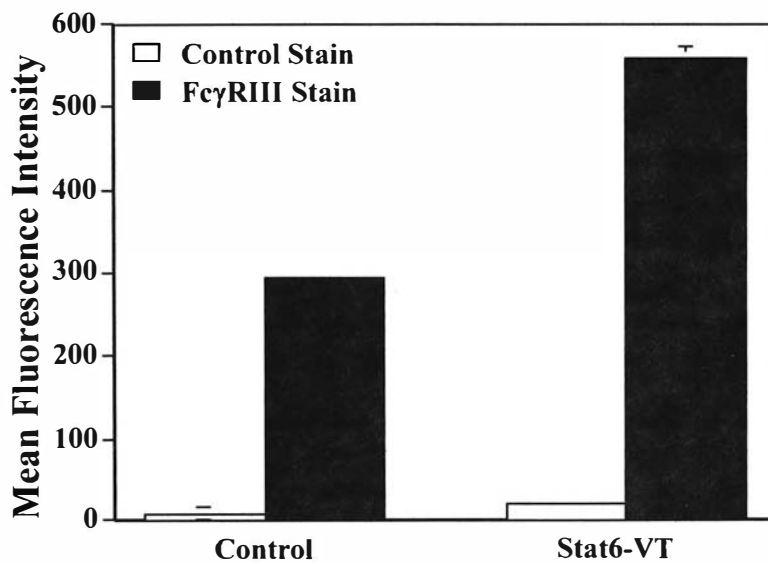
**Figure 27.** IL-4 does not affect Fc $\gamma$ RIII recycling. BMMC derived from Fc $\gamma$ RIIb $\alpha^{-/-}$  mice were cultured for three days in IL-3 alone or in IL-3+IL-4 prior to the addition of Brefeldin A (3 $\mu$ g/ml) for 20 hours. Staining with 2.4G2 was used to assess surface Fc $\gamma$ RIII expression by flow cytometry. Percent inhibition was determined by comparing mean fluorescence intensities of cells treated with BFA to those treated with media alone. Data shown are means and standard errors of 6 different cell populations in three independent experiments.



***The role of Stat6 in IL-4-mediated Fc $\gamma$ RIII expression.***

Because of the importance of Stat6 in IL-4 signaling, we explored its role in IL-4 mediated Fc $\gamma$ RIII upregulation. In order to determine if Stat6 was sufficient to upregulate Fc $\gamma$ RIII, we infected Fc $\gamma$ RIIb deficient BMDC with a bicistronic retroviral expression vector encoding green fluorescence protein (GFP) alone or GFP and a constitutively active mutant of Stat6, termed Stat6VT (Daniel et al. 2000; Zhu et al. 2001). These cells were maintained in IL-3 alone and assessed for Fc $\gamma$ RIII expression 5 days post-infection. As shown in Figure 28, Stat6VT increased Fc $\gamma$ RIII approximately surface expression 2-fold, closely matching IL-4-mediated upregulation (Figure 21).

**Figure 28.** Stat6 activation is sufficient to enhance derived from FcγRIII expression. (A) BMDC derived from FcγRIIb<sup>-/-</sup> mice were transfected with bi-cistronic retrovirus expressing GFP alone (lower left), or GFP and constitutively active Stat6VT (lower right) as described in Materials and Methods. The cells were cultured in IL-3 for three to five days and stained with PE-2.4G2 to determine surface FcγRIIIA expression. The upper dot plot is a negative control stained with unlabelled 2.4G2 before the addition of PE-2.4G2. (B). Average mean fluorescence intensity and standard errors of 6 samples.

**A****B**



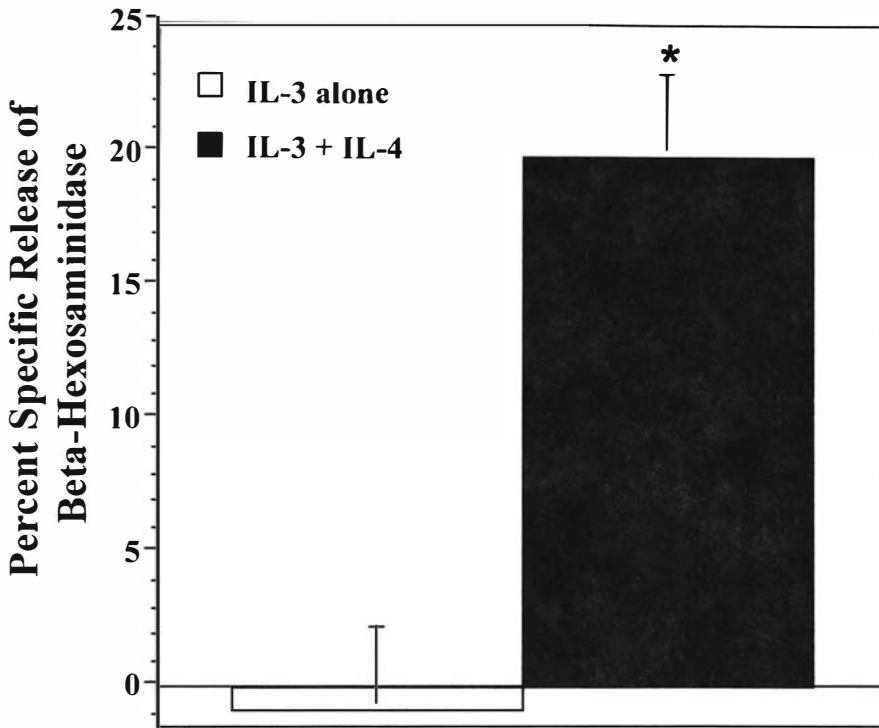
***IL-4 increases FcγR-mediated inflammatory function on wild type mast cells.***

We assessed the effects of IL-4 stimulation on the immediate phase of mast cell activation by measuring FcγR-induced release of β-hexosaminidase, a marker of mast cell degranulation (Schwartz et al. 1979). Wild type BMMC expressing both FcγRIIb and FcγRIII were cultured in IL-3 alone or in IL-3+IL-4 for three days, followed by crosslinkage with anti-FcγRIIb/FcγRIII mAb (2.4G2) and goat anti-rat IgG for 60 minutes. Release of the granule component β-hexosaminidase into culture supernatants was then assessed by enzyme assay. Degranulation was measured as percent specific release of stimulated cells over unstimulated cells. BMMC cultured in IL-3 alone showed no significant β-hexosaminidase release after 2.4G2 crosslinkage, consistent with the high level of FcγRIIb expression on these cells (Lobell et al. 1993). However, BMMC cultured in IL-3+IL-4 demonstrated enhanced β-hexosaminidase release after activation (Figure 29).

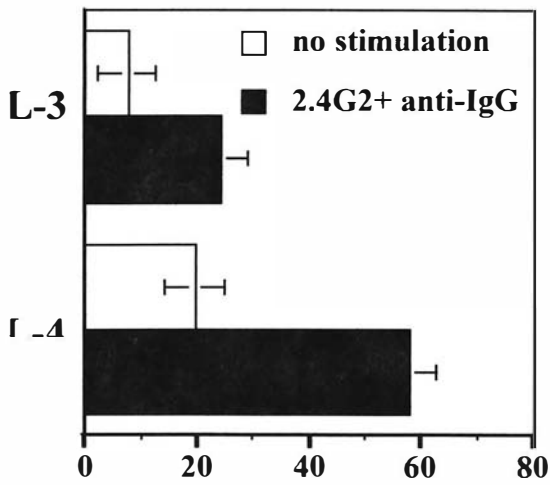
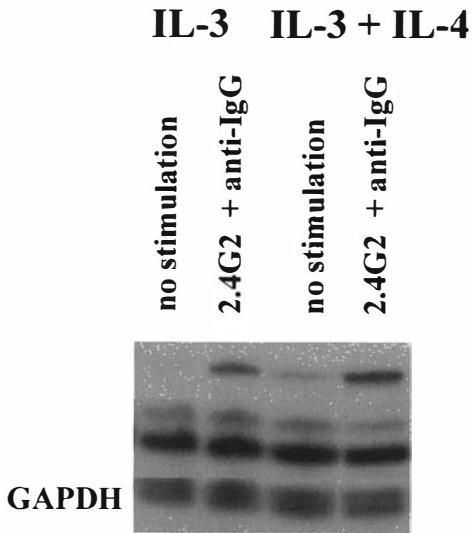
While mast cell degranulation is part of the early phase of mast cell activation, cytokine production is an indicator of the late phase response, and is a critical component of inflammatory infiltration and tissue damage (Wills-Karp 1999). To determine if IL-4 altered FcγR-mediated cytokine production, wild type BMMC were cultured for three days in IL-3 alone or IL-3 +IL-4, followed by activation with FcγR crosslinkage. TNFα mRNA and protein levels were measured by RPA and ELISA, respectively. These experiments showed that IL-4 enhanced both FcγR-mediated TNFα mRNA levels and protein secretion (Figure 30 and Figure 31), though this increase was modest in

comparison to the effects on beta hexosaminidase release. Together with the degranulation studies, these results demonstrate that IL-4 stimulation enhances the early and late phases of FcγR-mediated pro-inflammatory mediator secretion in mast cells.

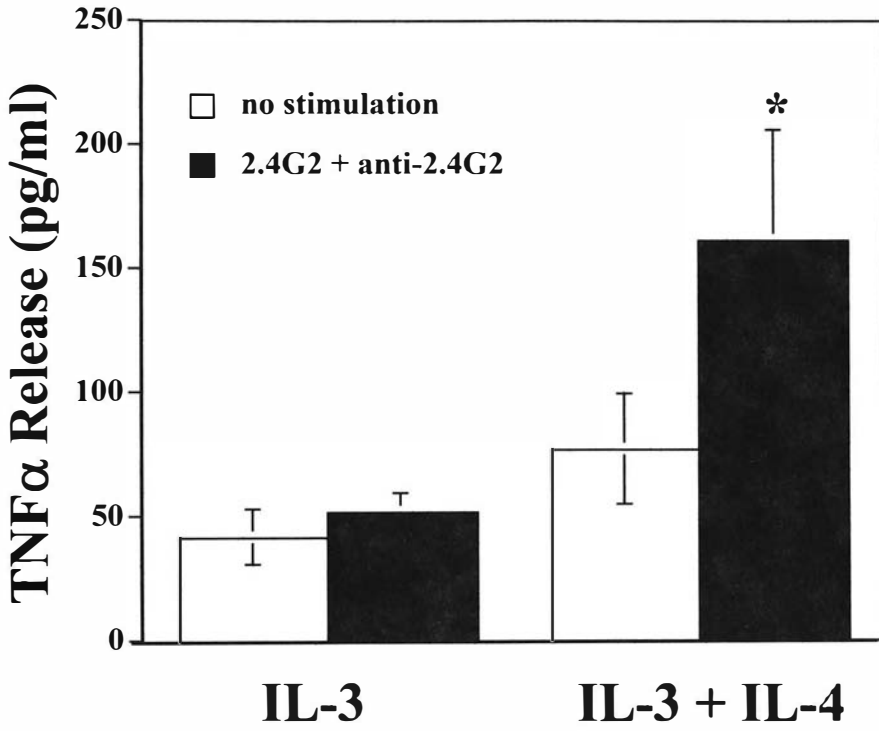
**Figure 29.** IL-4 enhances Fc $\gamma$ R-mediated  $\beta$ -hexosaminidase release on wild type BMMC. Wild type BMMC were cultured in IL-3 alone or in IL-3 plus IL-4 for the number of days indicated. Cells were then stimulated with 2.4G2 and goat anti-rat IgG (Fc $\gamma$ R XL) or were left unstimulated, and assessed for  $\beta$ -hexosaminidase release as described in Materials and Methods. Percent specific release was calculated by subtracting  $\beta$ -hexosaminidase release in non-activated cells from that of Fc $\gamma$ R XL-stimulated cells as described in Materials and Methods. Data shown are means and standard errors from a minimum of 6 BMMC populations assessed in a minimum of 5 independent experiments. (\*) =  $p < 0.05$  when comparing to IL-3 alone, as determined by ANOVA followed by LSD analysis.



**Figure 30.** IL-4 alters Fc $\gamma$ R-mediated cytokine mRNA synthesis. Wild type BMMC were cultured for 3 days with the IL-3 or IL-3+IL-4, followed by Fc $\gamma$ R XL as described previously. Total RNA was subjected to RPA analysis. Data shown are one representative RPA from 5 independent experiments with similar results using 3-11 independent BMMC populations. (B) Summary of changes in TNF $\alpha$  mRNA expression. Pixel intensities from (A) were obtained by phosphorimager analysis, and the ratio of cytokine gene expression to the sum of the L32 and GAPDH housekeeping gene expression was determined. Data are means and standard errors from 3-11 samples from 3-5 independent experiments.

**A**

**Figure 31.** IL-4 effects on TNF alpha release. Wild type BMMC were cultured in the indicated cytokines for 3 days, then stimulated with FcγR XL for 24 hours. TNFα release was measured using ELISA as described in Materials and Methods. Data shown are means and standard errors from 3 different BMMC populations assessed in 3 independent experiments. Each sample was done in triplicate. The lower limit of detection was 15.4 pg/ml. for TNFα. (\*) =  $p < 0.05$  when compared to cells cultured in IL-3 alone, using ANOVA and LSD analysis.





## DISCUSSION

FcεRI aggregation initiates a signaling cascade that results in degranulation, the immediate production of lipid-derived mediators, and the later release of newly transcribed chemokines and cytokines. With the advanced technology of cDNA and oligonucleotide arrays, it is now possible to monitor the genome wide transcriptional profile of cells and tissues (Brown et al. 1999). The MG-U74 GeneChip contains the probe set for over 6000 full length genes and 6000 expressed sequence tags (Affymetrix). Using these probe arrays we were able to assess the global transcriptional changes that occurred after mast cell activation for 90 minutes, 5 hours and 24 hours, and compare these results to cells that remained unstimulated. These time points correspond to the early, late and resolution phases of mast cell activation. Analysis with Microarray Suite 5.0 (Affymetrix) revealed that 937 of these probes were regulated by FcεRI crosslinkage in at least one time point, and that approximately half of these probes were for ESTs (Figure 1). Further analysis revealed that 259 probes for full length genes were regulated at least two fold by FcεRI crosslinkage (Appendix 1).

Gene changes over time revealed that the transcriptional profiles were similar at 90 minutes, 5 hours and 24 hours after activation. The most regulated category at each time point was DNA binding genes, mostly those involved in transcriptional regulation and cell cycling. There were however general differences. Ninety minutes after

activation, the three most abundantly transcribed genes compared to unstimulated cells were the chemokines CCL7 (MCP-3), CCL4 (MIP1 $\beta$ ) and CCL-3 (MIP1 $\alpha$ ) which were upregulated an astonishing 502, 179 and 168 fold, respectively (Table 1). In comparison, the most abundantly transcribed genes after 5 hours were CCL-3 (MIP1 $\alpha$ ), CCL7 (MCP-3), and secreted phosphoprotein 1 (osteopontin), which increased a modest 59, 55, and 30 fold respectively. Twenty-four hours after activation, the most abundantly transcribed gene, endostatin, is induced only 10.55 fold over unstimulated cells (Table 3). Looking at the total number of genes that were regulated, there were a total of 174 genes that were regulated at least two fold after ninety minutes, 202 after 5 hours, and a meager 69 genes were regulated after 24 hours. Upon analysis with DAVID, which integrates information from a variety of sources including the National Library of Medicine and the Gene Ontology consortium, of the 20 most changed transcripts at 24 hours after activation, 12 of them remained unclassified according to GO analysis (Glynn Dennis Jr.). It can be reasonably inferred that most of these gene changes are secondary to Fc $\epsilon$ RI aggregation, and are most likely due to an autocrine effect from the mast cell release of preformed mediators and/or cytokines. Therefore it is difficult to interpret these results without knowing the microenvironment of the mast cell at the time.

Our analysis revealed genes previously unreported to be regulated in mast cells. Some of these will be discussed in detail.

*Osteopontin*. This gene is also known as secreted phosphoprotein 1, and early T lymphocyte activation 1 (ETA-1). Osteopontin is expressed by T cells and is a regulator of T helper 1 immunity (Patarca et al. 1989). It was previously reported that ETA-1 sustained the autoimmune responses associated with EAE, the murine model of multiple sclerosis. Mice deficient in ETA-1 had lower clinical scores, faster recovery, and fewer spontaneous relapses as well as decreased inflammatory infiltration and demyelination of the spinal cord. T cells from these mice also produced less TNF $\alpha$  and IFN $\gamma$ . (Jansson et al. 2002). Mast cells have recently been reported to play an essential role in the pathology of EAE. Mice deficient in mast cells had significantly reduced disease incidence as well as decreased clinical scores compared to wild type littermates, and reconstitution with mast cells restored disease symptoms (Secor et al. 2000). It has also been reported that the expression of Fc receptors is critical in the regulation of this disease, but the link between mast cell activation and disease pathology remains to be elucidated (Brown et al. 2002; Robbie-Ryan et al. 2002; Robbie-Ryan et al. 2003). We found that osteopontin is the third most upregulated gene five hours after mast cell activation, just after CCL3 and CCL7 (Table 2). If it was shown that mast cells produce osteopontin after activation, it would be an interesting functional link between mast cells and EAE worthy of further exploration.

Osteopontin is also known to be important in cell attachment and motility, by engaging the surface antigen CD44. Interaction between osteopontin and CD44 can regulate macrophage migration (Weber et al. 1996; Weber et al. 2002). If mast cells are able to make osteopontin, it is possible that they can recruit other inflammatory cells

such as macrophages via their CD44 expression. This could be an important component in mast cell involvement in T helper 1 immune responses and diseases.

*CD44*. In addition to binding osteopontin, CD44 is the major cell surface receptor for the nonsulfated glycosaminoglycan hyaluronan (HA). CD44 is a transmembrane adhesion receptor that can be expressed as a standard protein or as a variant containing spliced exon sequences. Although these different variants have been demonstrated to have slightly different functions, CD44 generally is important in cell migration, including lymphocyte trafficking, limb development, axon growth, wound healing, tumorigenesis, metastasis formation, and autoimmune diseases. Anti-CD44 treatment has demonstrated its role in collagen induced arthritis, experimentally induced colitis and delayed type hypersensitivity reactions (Mikecz et al. 1995; Ponta et al. 1998; Nedvetzki et al. 1999). One recent study reported that administration of anti-CD44 monoclonal antibodies prevented lymphocyte and eosinophil accumulation in the lung after challenge with helminth and mite antigens. Treatment with this antibody blocked the elevation of T helper 2 cytokines, chemokines, and leukotrienes in bronchoalveolar lavage fluid, as well as airway hyperresponsiveness (Kato et al. 2003). Thus CD44 could be important in airway allergic inflammation. Previous studies have reported the expression of CD44 on human mast cells (Nedvetzki et al. 1999). Our GeneChip analysis identified CD44 as the ninth most increased transcript five hours post mast cell activation (Table 2). Considering the importance of mast cells in allergic lung inflammation, the regulation of CD44 surface expression after mast cell activation could be important in the pathology of

this disease. CD44, as well as HA ligand has also been found in considerable amounts in the joint synovium of rheumatoid arthritis patients. In addition, anti-CD44 monoclonal antibody can markedly reduce inflammation in murine models of RA (Naor et al. 2003). It is possible that mast cell expression of CD44 regulates its role in these conditions and would be interesting to pursue further.

*Protease-activated G protein-coupled receptor.* Par1, also known as F2R or coagulation factor II (thrombin) receptor, is a G protein-coupled receptor that mediates cellular responses to thrombin. Thrombin is a serine protease usually generated in response to tissue damage or inflammation, and acts to form fibrin matrices and activate platelets and endothelial cells during injury (Kannan 2002). It has been reported that mast cells express Par1 and signaling could induce adherence to fibronectin. We report that mast cell activation induces the transcription of Par1 around 3-5 fold during the 90 minutes through 24 hour time points measured (Figure 9, Table 7). Therefore, Fc $\alpha$ RI aggregation could upregulate the surface expression of Par1, allowing for further activation of mast cells near tissue injured sites where thrombin is prevalent, resulting in further mast cell activation by other receptors.

*Nuclear receptor subfamily 4, group A, member (Nurr77).* Nurr77, also known as TR3 or NGFI-B, is an immediate early response gene that is rapidly induced during apoptosis of immature thymocytes, T cell hybridomas and other various cancer cells. Inhibition of Nurr77 activity inhibits apoptosis in T cells and its overexpression leads to

massive apoptosis. This protein has also been shown to play a role in TCR mediated cell death, thymocyte negative selection, as well as caspase independent activation induced cell death in macrophages (He 2002; Kim et al. 2003). Nurr77 is a steroid receptor and a member of the orphan nuclear receptor family, because its ligand is unknown. We found that Nurr77 gene expression peaked at ninety minutes post FcεRI aggregation, increasing 38 fold over unstimulated cells, and levels returned to baseline by 5 hours (Figure 6, Table 4). Nurr77 expression in mast cells has not been reported. The early upregulation of this gene could be indicative of an apoptotic phenotype induced by FcεRI aggregation.

*Schlafen 2.* Schlafen 2 belongs to a family of genes identified because of their upregulation during positive selection of T lymphocytes. Schlafen is German for “sleep”, because induction of this gene causes cell cycle arrest (Schwarz et al. 1998). We found that schlafen2 expression was downregulated between 3-6 fold ninety minutes after mast cell activation, and that transcript levels return to baseline by 24 hours. (Figure 7, Table 5). To our knowledge, there are no reports of schlafen2 expression in mast cells. However, if it could be demonstrated that schlafen2 protein is expressed and regulated in mast cells by activation, it would be interesting if this affects cell survival after stimulation.

*Nuclear factor, interleukin 3 regulated (NFIL3 aka E4BP4).* E4BP4 was originally identified by its ability to recognize and repress the adenovirus E4 promoter, hence its name adenovirus E4 promoter-binding protein. Identified as NF-IL3A in T

cells, E4BP4 is a basic region/leucine zipper transcription factor known to be upregulated by IL-3 and capable of binding the human IL-3 promoter (Cowell 2002). E4BP4 expression was induced in a pro-B cell line Ba/F3 after stimulation with IL-3, and forced expression delayed apoptosis caused by IL-3 deprivation, while over expression of a dominant negative E4BP4 attenuated the survival response of IL-3 (Ikushima et al. 1997). We found by microarray analysis that FcεRI aggregation regulated the expression of this transcript at all three time points assessed. Transcript levels are increased both 90 minutes and 5 hours after mast cell activation (7 fold and 3 fold respectively), but by 24 hours, E4BP4 is the second most downregulated gene, 4 fold below unstimulated cells (Figure 6, Table 3, Table 4). It can be inferred that initially mast cell activation enhances mast cell survival, but due to an unknown regulatory mechanism, E4BP4 expression is repressed by 24 hours. This implies a greater sensitivity to apoptotic signals during the resolution phase of mast cell activation.

*N-myc downstream regulated 1 (NDRG1)*. NDRG1 is a serine protease that is strongly induced in several cell types during cellular differentiation or stress. In most reports, the expression of this gene has been found to be growth inhibitory, but when transfected into RBL cells, proliferation remained unchanged but degranulation after FcεRI aggregation was enhanced (Kurdistani et al. 1998; Guan et al. 2000). Recently, a study found that mast cells co-cultured with fibroblasts in the presence of stem cell factor had high levels of NDRG1, and that the levels correlated with enhanced degranulation (Taketomi et al. 2003). Our microarray analysis revealed that 90 minutes after mast cell activation, this

gene is upregulated almost 13 fold and back to baseline 24 hours later. It is possible that upregulation of this gene could allow for the mast cell to mature after activation, thus enhancing its response to other stimuli.

Microarray analysis reveals genome wide gene expression patterns after mast cell activation, therefore rather than looking at one gene and its effect on a biological process, it is possible to look at temporal expression patterns of many related genes and predict a potential concerted effect on biological function. To illustrate this point, the regulation of mast cell survival cell after FcεRI activation was analyzed. Genes known to be involved in cell cycle regulation were analyzed, and their expression patterns in context of other gene changes were summarized in a table (Table 32). Collectively, the gene expression profiles are inconclusive. Ninety minutes after mast cell activation, Nurr77, a pro apoptotic gene is highly upregulated, and p21 is also elevated compared to unstimulated cells. However, schlafen2 which induces cell cycle arrest, is downregulated at this time point, and E4BP4 is upregulated, arguing for cell cycle progression and survival.

The literature on FcεRI aggregation and mast cell survival are mixed as well. Some studies argued that FcεRI aggregation enhanced survival and proliferation, prevented apoptosis due to factor withdrawal (Plaut et al. 1989; Matsuda et al. 1999). One study using BMDCs found that FcεRI aggregation enhanced survival through the upregulation of A1, a member of the Bcl-2-family (Xiang et al. 2001). Others indicated the opposite, stating that FcεRI aggregation significantly reduced proliferation in response to growth factors (Tsai et al. 1993). The difficulty interpreting the literature lies in the fact that all of the studies use different cell types and culture conditions (Kawakami



et al. 2002). Further studies are needed to determine if these genes are even expressed as proteins, and if they play a significant role in mast cell survival and proliferation.

**Table 8. Summary of Genes involved in cell cycle regulation, apoptosis and survival.**

Gene	Role	90 minutes	5 hours	24 hours
Cyclin D2	cell cycle progression	—	↑	—
Cyclin D3	cell cycle progression	—	↓	—
Cyclin B2	cell cycle progression	—	↓	↓
p19	cell cycle arrest	—	↓↓	↓
p21	cell cycle arrest	↑	—	—
schlafen2	cell cycle arrest	↓↓	—	—
Rb	cell cycle arrest	—	↓	—
Nurr77	apoptosis	↑↑↑	—	—
E4BP4	anti apoptotic	↑	↑	↓

***Regulation of the beta subunit by FcεRI aggregation.***

Microarray analysis revealed that FcεRI aggregation resulted in a four fold decrease in beta chain mRNA after five hours, without significantly altering alpha or gamma transcripts (Figure 9, Table 7). RPA analysis partially confirmed the array, demonstrating that 5 hours after activation, the message levels of the alpha chain remain unchanged, whereas beta chain levels decrease significantly (Figure 12, Figure 13, Figure 15). RPA analysis of the gamma subunits however gave mixed results and further studies are needed to clarify if gamma is regulated by FcεRI aggregation (Figure 12, Figure 14).

Little is known about the transcriptional regulation of the individual subunits of FcεRI. Previous studies have demonstrated that IL-4 can upregulate the transcription of FcεRIα in human mast cells, and that IFNγ can increase the rate of FcRγ chain transcription (Brini et al. 1993; Toru et al. 1996; Xia et al. 1997). However, there is to our knowledge no literature on known factors that specifically regulate transcription of the beta subunit. Recently, Maeda et. al. published that GATA-1 can bind the beta subunit promoter at four different sites, and that these four sites are required for full transcriptional activation by GATA-1 (Maeda et al. 2003). GATA-1 is highly expressed in mast cells and could be the primary transcription factor responsible for its expression (Weiss et al. 1995). Our microarray analysis revealed that GATA-1 transcript levels were slightly downregulated after mast cell activation (Appendix 1). It is possible that this moderate decrease in GATA-1 transcript results in less total GATA-1 protein, leading to decreased transcription of the beta subunit. It is also plausible that FcεRI aggregation regulates the expression of other transcripts that have an effect on the

stability of GATA-1 transcript and/or protein, or affect the ability of GATA-1 to bind the promoter of the beta subunit. It is also possible that a different yet unidentified transcription factor is involved in beta chain regulation.

FcεRI could also regulate beta transcript by decreasing its stability or increasing degradation. Ribonuclease Protection Assay (RPA) can only assess steady state levels of mRNA. Repeating the experiments using Actinomycin D, a transcriptional inhibitor, could give insight into FcεRI activation and beta chain regulation. If the half-life of the β subunit mRNA is decreased by FcεRI crosslinkage, this could provide evidence that FcεRI activation is either actively mediating an increase in the degradation of mRNA or is alleviating some protection from degradation. Further studies are needed to elucidate the potentially complex steps that connect FcεRI aggregation with transcriptional control of the beta subunit.

We found that downregulation of the beta transcript correlated with downregulation of the beta protein, with no change in alpha protein levels (Figure 16 and Figure 17). Again, gamma protein levels seemed reduced 90 minutes after activation but this needs to be repeated in order to confirm. Western blotting can only indicate steady state levels of protein, thus studies utilizing treatment with the translational inhibitor cycloheximide can determine the effects of FcεRI aggregation on protein stability.

Finally, we found that FcεRI aggregation correlated with a decreased surface expression of FcεRI (Figure 18). This corroborates the reduced expression of FcεRI β, which is required for FcεRI surface expression. Further studies are needed to directly

connect mast cell activation to beta chain regulation and regulation of the surface expression of FcεRI.

A potential factor that could regulate the surface expression of FcεRI is through the mechanism of a newly described isoform of the beta subunit,  $\beta_T$ . It has recently been discovered that alternative splicing of the beta subunit gene results in the expression of a truncated variant,  $\beta_T$ , that can compete functionally with its normal counterpart. This isoform pairs with immature FcεRI $\alpha$  and inhibits its maturation, thus preventing surface expression of FcεRI.  $\beta$  and  $\beta_T$  are expressed in mast cells so the balance between the two isoforms could alter the mast cell response. The mechanism that controls the splicing of the beta chain is still unknown (Donnadieu et al. 2003). It is possible that mast cell activation initiates a signal cascade resulting in the upregulation of the  $\beta_T$  while downregulating or even maintaining  $\beta$  levels. The probe used for the Ribonuclease Protection Assay binds both isoforms of the beta gene. Therefore we could not determine if the downregulation of beta message seen 5 hours after activation is due to decreased  $\beta$ ,  $\beta_T$  or both.  $\beta_T$  is a slightly smaller protein than  $\beta$ , but due to its low level of expression and instability, it is difficult to detect without proteasome inhibitors. We would need to repeat our studies using a probe that could bind only  $\beta_T$  or  $\beta$ , and repeat the western blots using protease inhibitors.

Another experiment that would more directly link beta chain downregulation with inhibition of FcεRI surface expression is needed. We propose the transfection of BMDC with a vector containing the beta subunit with a constitutively active promoter. These

cells could be activated with IgE+anti-IgE, and surface expression could be analyzed by flow cytometry. If the FcεRI expression remains stable, this could indicate that downregulation of the beta gene is required for downregulation of surface FcεRI.

If decreased beta chain resulted in downregulation of FcεRI surface expression, this could lead to decreased activation of the mast cell. Human mast cells do not require the beta subunit for FcεRI expression, however the trimer does not signal nearly as well as its tetrameric counterpart. Increased expression of this form after mast cell activation could be homeostatic, preventing further activation to dampen the inflammatory response.

## **Part II. Interleukin-4 regulation of FcγRIII expression and signaling.**

BMMC cultured in the presence of IL-4 for three days had a twofold increase in surface FcγRIII, with no significant change in surface levels of FcγRIIb. This upregulation of surface FcγRIII could be seen through day 14 of culture with as little as 1 ng/ml of IL-4. In an attempt to determine the level of regulation by IL-4, we looked at total mRNA and intracellular protein levels of FcγRIIIα. We found that total intracellular levels of FcγRIIIα were elevated by the second day of culture, mimicking the surface expression of FcγRIII. However, there was only a nominal increase in mRNA levels. Thus it did not appear that the upregulation of surface FcγRIII was a result of IL-4 enhanced transcription of FcγRIIIα.

To assess the possibility that IL-4 regulation occurs post transcriptionally, we employed the translational inhibitor cycloheximide to determine if IL-4 was enhancing FcγRIIIα protein stability. After 3 days of culture in either IL-3 alone or IL-3 + IL-4, cycloheximide was added to the cell culture. We found that cells cultured in the presence of IL-4 had the same rate of degradation of FcγRIIIα as cells cultured in IL-3 alone, indicating that IL-4 did not affect protein stability (Figure 26).

There remained the possibility that IL-4 facilitated recycling of endocytosed FcγRIII, resulting in increased surface expression. Brefeldin A (BFA) prevents surface expression of newly synthesized proteins by inhibiting transport from the endoplasmic reticulum to the golgi, and blocks surface protein recycling by preventing endosomes from fusing with the trans-golgi (Lippincott-Schwartz et al. 1991; Wood et al. 1991)

Cells cultured in IL-3+IL-4 had virtually the same sensitivity to BFA as cells cultured in IL-3 alone (Figure 27). These data agree with an earlier study by Kubo and co-workers showing that surface Fc $\gamma$ RIII is very stable and undergoes little receptor recycling (Kubo et al. 2001). Collectively these data argue that IL-4 enhances Fc $\gamma$ RIII $\alpha$  protein expression by increasing protein synthesis, without altering Fc $\gamma$ RIII $\alpha$  mRNA levels, protein stability or surface protein recycling. One possible mechanism by which this could occur is through enhancing translational efficiency. IL-4 signaling may regulate mRNA binding proteins, leading to increased translation without altering mRNA levels (Al-Maghrebi et al. 2002).

The ability of IL-4 to enhance Fc $\gamma$ RIII expression is an interesting contrast to our previous finding of IL-4-mediated downregulation of Fc $\epsilon$ RI. Although these events may have opposing effects on mast cell function, reduced Fc $\epsilon$ RI expression may be related to the increase in Fc $\gamma$ RIII. Our recent study demonstrated that IL-4 diminishes Fc $\epsilon$ RI expression by decreasing Fc $\epsilon$ RI $\beta$  protein expression without affecting Fc $\epsilon$ RI $\alpha$  or FcR $\gamma$  (Gillespie et al. 2003). Since Fc $\gamma$ RIII expression requires pairing with Fc $\epsilon$ RI $\gamma$  but not Fc $\epsilon$ RI $\beta$ , it is possible that loss of Fc $\epsilon$ RI $\beta$  results in a *de facto* increase in Fc $\gamma$ RIII expression due to reduced competition for the obligatory gamma chain. This theory is supported by the demonstration that Fc $\epsilon$ RI $\alpha$ -deficient mast cells exhibit increased Fc $\gamma$ RIII expression (Dombrowicz et al. 1998). Further, these changes would likely be mast cell-specific, explaining the differences between our study and those of Te Velde et al., and Pricop et al. (te Velde et al. 1990; Pricop et al. 2001) who found that IL-4 inhibits



Fc $\gamma$ RIII expression on monocytes. Therefore IL-4 may be enhancing surface Fc $\gamma$ RIII expression both by increasing Fc $\gamma$ RIII $\alpha$  protein synthesis and limiting competition for its expression partner, Fc $\epsilon$ RI $\gamma$ .

Because of the importance of Stat6 in IL-4 signaling, we explored the role of Stat6 in IL-4 mediated Fc $\gamma$ RIII upregulation. We found that Stat6 activation alone is sufficient to increase Fc $\gamma$ RIII expression, data in keeping with IL-4 mediated regulation of Fc $\epsilon$ RI (Ryan et al. 1998; Gillespie et al. 2003). Since Fc $\gamma$ RIII expression is enhanced without altering Fc $\gamma$ RIII $\alpha$  transcription, it would appear that Stat6-mediated effects are indirect, perhaps by regulating expression of RNA binding proteins as previously suggested.

Increasing expression of pro-inflammatory Fc $\gamma$ RIII might enhance mast cell responses to IgG-mediated stimuli, however co-expression of inhibitory Fc $\gamma$ RIIb receptors could block these signals, preventing mast cell activation. With recent data emphasizing the role of mast cell Fc $\gamma$ RIII-mediated signaling in inflammatory disease (Oettgen et al. 1994; Takai et al. 1994; Hazenbos et al. 1996; Dombrowicz et al. 1997; Miyajima et al. 1997), the effect of IL-4 on IgG-mediated activation of wild type mast cells has clinical importance. BMDC cultured in IL-3 alone showed no significant  $\beta$ -hexosaminidase release after Fc $\gamma$ R crosslinkage, consistent with the high level of Fc $\gamma$ RIIb expression on these cells (Lobell et al. 1993). However, BMDC cultured in IL-3+IL-4 demonstrated enhanced  $\beta$ -hexosaminidase release after activation (Figure 6). These data indicate that selective upregulation of Fc $\gamma$ RIII by IL-4 is functionally

significant, leading to increased IgG-mediated mast cell degranulation. BMDC cultured in IL-4 also had enhanced Fc $\gamma$ R-mediated TNF $\alpha$  mRNA levels and protein secretion, although this increase was modest in comparison to the effects on beta hexosaminidase release. Together with the degranulation studies, these results demonstrate that IL-4 stimulation enhances the early and late phases of Fc $\gamma$ R-mediated pro-inflammatory mediator secretion in mast cells.

The ability of IL-4 to alter Fc $\gamma$ R signaling in the context of both pro- and anti-inflammatory IgG receptors could be due solely to the selective upregulation of Fc $\gamma$ RIII. However, Fc $\gamma$ RIIb expression is still considerable after IL-4 stimulation. It remains possible that enhanced Fc $\gamma$ R-mediated mast cell activation is due partly to enhanced Fc $\gamma$ RIII expression and partly to inhibition of Fc $\gamma$ RIIb signaling.

Fc $\gamma$ RIIb mediated inhibitory signaling requires an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) that preferentially interacts with Src homology 2-containing-inositol 5'-phosphatase, or SHIP-1. Upon Fc $\gamma$ RIIb aggregation with an activation receptor such as the BCR, TCR, Fc $\epsilon$ RI or Fc $\gamma$ RIII, lyn phosphorylates the critical tyrosine within the ITIM motif, recruiting SHIP-1 by its SH2 domain. This recruitment results in tyrosine phosphorylation of SHIP-1. SHIP-1 subsequently dephosphorylates the major PI3K product, PIP3, which abolishes the recruitment of PH domain containing molecules, abrogating activation receptors signaling (Rauh et al. 2003). Normally, mast cells express more Fc $\gamma$ RIIb than Fc $\gamma$ RIII and so the activation signal is abrogated, preventing degranulation (Lobell et al. 1993). However, it is possible

that in the presence of IL-4, this inhibition is relieved. This could occur through inhibition of the phosphorylation of ITIM or decreased recruitment or activation of SHIP. Either scenario could reduce FcγRIIb signaling, allowing FcγRIII to activate the cell. Thus the effects of IL-4 on SHIP-1 expression and activation warrant further study.

The current study demonstrates that IL-4 upregulates FcγRIII surface expression without significantly altering expression of FcγRIIb. IL-4 conveys these changes via alterations in FcγRIIIα protein expression without affecting mRNA synthesis, protein stability, or receptor recycling. Stat6 activation alone induces FcγRIII upregulation, emphasizing the importance of this transcription factor in IL-4-initiated signal transduction. Importantly, modifying the surface ratio of FcγRIIb to FcγRIII is functionally significant, allowing for degranulation and cytokine production after FcγR crosslinkage. Understanding FcγR regulation could be significant to inflammatory disease, as recently demonstrated in a rodent model of multiple sclerosis, where selective FcγRIII expression on mast cells altered disease severity (Robbie-Ryan et al. 2003). Similarly, there is recent evidence that FcγR play an important role in a mouse model of rheumatoid arthritis (Lee et al. 2002). Manipulation of the FcγRIIb/FcγRIII ratio by cytokines could therefore be important for controlling and treating inflammatory disease.

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## APPENDIX

Probe ID	Gene Name	Gene Symbol	Average Fold Change		
			90m	5h	24h
96713_at	3-phosphoadenosine 5-phosphosulfate synthase 2	Papss2	-1.41	-4.00	-1.14
96989_at	7-dehydrocholesterol reductase	Dhcr7	-1.63	-2.96	1.05
103024_at	a disintegrin and metalloprotease domain 8	Adam8	3.09	3.25	1.68
92581_at	acetyl-Coenzyme A dehydrogenase, medium chain	Acadm	-1.65	-0.13	1.22
93122_at	acidic epididymal glycoprotein 1	Aeg1	0.11	2.36	6.17
104156_r_at	activating transcription factor 3	Atf3	39.09	0.54	0.34
104155_f_at	activating transcription factor 3	Atf3	46.15	1.51	-1.30
100599_at	activating transcription factor 4	Atf4	2.47	0.10	-1.71
103392_at	adenylate cyclase 7	Adcy7	-1.10	2.18	2.21
95148_at	adenylate kinase 2	Ak2	-0.02	2.24	-1.47
99039_g_at	adenylosuccinate synthetase 2, non muscle	Adss2	1.62	1.74	1.49
160082_s_at	ADP-ribosylation factor 4	Arf4	1.54	0.12	-1.09
100903_at	ADP-ribosyltransferase (NAD <sup>+</sup> : poly(ADP-ribose) polymerase)-like 2	Adprt12	-2.51	-1.58	-1.22
102249_at	adwillin	Avil	1.25	2.02	1.29
93904_f_at	AF059706: clone N1.1.b immunoglobulin heavy chain VDJ region gene		-2.38	-19.10	-2.20
99993_at	alarlyl (membrane) aminopeptidase	Anpep	-1.22	1.44	1.78
160215_at	amino-terminal enhancer of split	Aes	0.00	-1.34	1.13
102710_at	amyloid beta (A4) precursor protein-binding, family B, member 1 interacting	Appb1ip	-2.83	1.52	-1.17
93496_s_at	amyloid beta (A4) precursor-like protein 2	Aplp2	1.75	1.93	-0.04
102815_at	annexin A11	Anxa11	-1.06	1.41	1.36
93083_at	annexin A5	Anxa5	1.50	1.96	1.43
99457_at	antigen identified by monoclonal antibody Ki 67	Mki67	-2.04	-12.57	-3.11
103796_at	apoptotic protease activating factor 1	Apaf1	-1.97	-1.09	1.06
102200_at	aquaporin 8	Aqp8	1.75	4.30	0.42
95600_at	ariadne homolog 2 (Drosophila)	Arih2	8.02	3.35	1.97
160495_at	aryl-hydrocarbon receptor	Ahr	1.50	1.82	1.62
93984_at	ATPase inhibitor	Atpi	-1.42	-1.45	0.00
103275_at	ATPase, H <sup>+</sup> transporting, lysosomal V0 subunit a isoform 1	Atp6v0a1	0.46	2.98	1.54
99579_at	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, beta 3 polypeptide	Atp1b3	-1.20	-1.27	1.05
93414_at	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	Abcb1b	-3.97	-5.49	1.08
160612_at	ATP-binding cassette, sub-family G (WHITE), member 1	Abcg1	-2.00	0.07	1.82
101521_at	baculoviral IAP repeat-containing 5	Birc5	-1.50	-3.85	-2.54
93527_at	basic transcription element binding protein 1	Bteb1	8.91	3.36	0.06
98868_at	B-cell leukemia/lymphoma 2	Bcl2	1.85	-1.88	-0.11
93104_at	B-cell translocation gene 1, anti-proliferative	Btg1	4.53	2.89	1.90
96146_at	B-cell translocation gene 3	Btg3	4.89	2.32	0.17
96255_at	BCL2/adenovirus E1B 19kDa-interacting protein 3-like	Bnip3l	0.20	-6.40	0.17
93088_at	beta-2 microglobulin	B2m	0.03	1.13	1.40
98433_at	BH3 interacting domain death agonist	Bid	-2.84	-1.75	1.22
98052_at	blocked early in transport 1 homotog (S. cerevisiae)-like	Bet1l	0.48	2.16	1.26
92220_s_at	bridging integrator 1	Bin1	-1.14	1.57	1.08
99668_at	bridging integrator 1	Bin1	-1.30	1.36	-1.15
98066_r_at	bromodomain containing 2	Brd2	2.48	1.09	-0.19
101961_at	budding uninhibited by benzimidazoles 3 homolog (S. cerevisiae)	Bub3	-1.33	-0.01	-1.37
96522_at	calmodulin 1	Calm1	1.20	1.60	-1.13
93293_at	calmodulin 2	Calm2	1.11	-1.37	-1.17
101040_at	calpain 2	Capn2	0.51	3.11	1.67
100610_at	calpain, small subunit 1	Capns1	-0.06	0.09	1.40
94004_at	calponin 2	Cnn2	-1.81	-5.25	1.17

95142_s_at	capping protein (actin filament) muscle Z-line, beta	Capzb	-1.24	-1.35	1.09
160106_at	capping protein (actin filament), gelsolin-like	Capg	1.18	1.24	1.65
92642_at	carbonic anhydrase 2	Car2	2.32	6.08	1.83
98535_at	catechol-O-methyltransferase	Comt	-1.45	-0.03	1.39
160430_at	catenin beta	Catnb	-0.07	2.08	0.04
94831_at	cathepsin B	Ctsb	1.13	1.70	1.64
101019_at	cathepsin C	Clsc	1.74	1.50	0.03
101973_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal do	Cited2	-1.27	-2.08	-1.04
92925_at	CCAAT/enhancer binding protein (C/EBP), beta	Cebpb	6.59	1.91	-1.18
98980_at	CD37 antigen	Cd37	-1.17	-1.32	1.50
103005_s_at	CD44 antigen	Cd44	15.06	14.69	2.02
103611_at	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	Cd47	-1.61	-1.70	-1.13
103089_at	CD48 antigen	Cd48	-1.57	-2.34	-1.14
94939_at	CD53 antigen	Cd53	2.05	3.14	1.62
103016_s_at	CD68 antigen	Cd68	-0.09	2.09	4.25
94000_at	CD8 antigen, beta chain	Cd8b	-2.17	-4.98	-0.32
95661_at	CD9 antigen	Cd9	1.83	2.47	1.67
97468_at	CDC28 protein kinase 1	Cks1	-1.14	-1.99	-3.39
103346_at	CDC-like kinase 2	Clk2	-3.02	-1.58	-1.08
102806_g_at	CEA-related cell adhesion molecule 1	Ceacam1	5.87	12.47	3.27
100128_at	cell division cycle 2 homolog A (S. pombe)	Cdc2a	-1.38	-2.50	-2.47
101088_f_at	cellular nucleic acid binding protein	Cnbp	1.14	1.85	-0.17
100127_at	cellular retinoic acid binding protein II	Crabp2	7.71	23.95	-4.61
93595_at	ceroid-lipofuscinosis, neuronal 2	Cln2	-0.08	-0.08	1.62
160671_at	ceroid-lipofuscinosis, neuronal 8	Cln8	13.74	5.74	3.00
94166_g_at	chemokine (C-C motif) ligand 1	Ccl1	4.98	4.34	-1.62
102736_at	chemokine (C-C motif) ligand 2	Ccl2	10.44	0.17	0.13
102424_at	chemokine (C-C motif) ligand 3	Ccl3	168.87	59.04	3.08
94146_at	chemokine (C-C motif) ligand 4	Ccl4	179.45	11.28	1.13
94761_at	chemokine (C-C motif) ligand 7	Ccl7	502.70	55.98	4.80
104388_at	chemokine (C-C motif) ligand 9	Ccl9	1.71	2.60	2.32
99413_at	chemokine (C-C motif) receptor 1	Ccr1	3.98	1.52	0.24
93697_at	chromobox homolog 4 (Drosophila Pc class)	Cbx4	-3.50	-2.64	-1.55
100946_at	Cluster Incl AF109906 MHC class III region RD gene		10.16	7.68	-2.69
92267_at	coagulation factor II (thrombin) receptor	F2r	2.80	4.62	3.14
101450_at	colony stimulating factor 1 (macrophage)	Csf1	19.05	4.82	2.51
92948_at	colony stimulating factor 2 (granulocyte-macrophage)	Csf2	68.07	0.08	-0.10
94748_g_at	colony stimulating factor 2 receptor, beta 1, low-affinity (granulocyte-macro	Csf2rb1	-0.11	1.74	2.11
94747_at	colony stimulating factor 2 receptor, beta 1, low-affinity (granulocyte-macro	Csf2rb1	-1.37	1.44	1.74
93454_at	complement component 1, q subcomponent, receptor 1	C1qr1	2.24	1.56	1.23
101728_at	complement component 5, receptor 1	C5r1	2.21	7.28	1.38
95460_at	COP9 (constitutive photomorphogenic) homolog, subunit 5 (Arabidopsis th	Cops5	1.28	1.96	1.17
93547_at	core binding factor beta	Cbfb	-1.30	-1.30	-1.16
102397_at	core-binding factor, runt domain, alpha subunit 2, translocated to, 3 homok	Cbfa2t3h	-7.25	-4.72	1.05
94046_at	c-src tyrosine kinase	Csk	-1.03	-3.42	1.06
98073_at	cut-like 1 (Drosophila)	Cutl1	-1.00	-0.10	1.34
94294_at	cyclin B2	Ccnb2	-1.02	-2.39	-2.20
97504_at	cyclin D2	Ccnd2	-0.25	2.36	1.60
160545_at	cyclin D3	Ccnd3	-1.75	-2.02	-0.02
160127_at	cyclin G1	Ccng1	-1.51	-1.82	1.32
98478_at	cyclin G2	Ccng2	-2.49	-3.25	-0.08
98067_at	cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	4.23	1.71	0.04
94521_at	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	Cdkn2d	-1.37	-6.70	-2.12
100581_at	cystatin B	Cstb	1.46	2.21	2.31
92608_at	cysteine and glycine-rich protein 1	Csrp1	0.01	-2.40	-1.32
100022_at	cytokine inducible SH2-containing protein	Cish	3.00	1.99	0.20
103539_at	cytoplasmic tyrosine kinase, Dscr28C related	Tec	5.78	6.04	3.70
103518_at	cytotoxic T lymphocyte-associated protein 2 beta	Ctla2b	2.14	2.71	1.68
93493_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	Ddx5	-1.15	-1.79	0.13
103250_at	deafness, autosomal dominant 5 homolog (human)	Dfna5h	1.06	2.45	0.76

103617_at	decay accelerating factor 1	Daf1	1.23	-1.66	-0.22
99543_s_at	deoxyguanosine kinase	Dguok	-1.35	-1.90	1.20
99905_at	deubiquitinating enzyme 2	Dub2	15.36	5.22	3.16
92887_at	dimethylarginine dimethylaminohydrolase 2	Ddah2	0.11	-2.13	-0.13
92730_at	diphtheria toxin receptor	Dtr	31.80	3.97	0.80
96254_at	DnaJ (Hsp40) homolog, subfamily B, member 1	Dnajb1	12.71	-1.22	-1.29
104598_at	dual specificity phosphatase 1	Dusp1	26.76	2.63	1.63
92758_at	dual specificity phosphatase 2	Dusp2	3.53	-0.18	1.06
94522_at	dynactin 3	Dctn3	1.44	1.40	1.05
98579_at	early growth response 1	Egr1	13.86	-0.10	-2.02
102661_at	early growth response 2	Egr2	72.53	1.81	-1.65
98025_at	ecotropic viral integration site 2	Evi2	4.98	1.41	2.38
98026_q_at	ecotropic viral integration site 2	Evi2	3.80	-0.01	2.06
103342_at	embryonic ectoderm development	Eed	-4.12	-1.24	-1.09
100472_at	enabled homolog (Drosophila)	Enah	-1.24	-1.77	-0.06
99917_at	enhancer of zeste homolog 2 (Drosophila)	Ezh2	-1.48	-2.62	-1.76
160857_at	ephrin B2	Efnb2	4.35	3.37	0.02
97426_at	epithelial membrane protein 1	Emp1	23.55	8.57	2.53
98525_f_at	erythroid differentiation regulator	edr	-1.31	0.79	-1.85
92553_at	esterase 10	Es10	1.23	4.89	1.65
93058_at	eukaryotic translation initiation factor 1A	Eif1a	1.95	1.61	0.17
95046_s_at	eukaryotic translation initiation factor 2B, subunit 4 delta	Eif2b4	-1.21	2.12	0.44
96883_at	eukaryotic translation initiation factor 3, subunit 4 (delta)	Eif3s4	-0.02	1.52	-0.12
93089_at	eukaryotic translation initiation factor 4A2	Eif4a2	-1.54	-0.07	-0.14
98121_at	farnesyltransferase, CAAX box, alpha	Fnta	-1.89	-1.43	-1.01
98575_at	fatty acid synthase	Fasn	-1.53	-1.44	0.12
104017_at	fatty acid-Coenzyme A ligase, long chain 4	Facl4	8.72	10.83	6.06
160901_at	FBJ osteosarcoma oncogene	Fos	7.79	-4.84	-1.86
103990_at	FBJ osteosarcoma oncogene B	Fosb	12.96	-0.10	-1.34
101209_at	Fc receptor, IgE, high affinity I, alpha polypeptide	Fcer1a	-1.15	-1.27	1.15
102337_s_at	Fc receptor, IgG, low affinity IIb	Fcgr2b	-1.65	-8.35	-0.51
92188_s_at	feline sarcoma oncogene	Fes	-1.11	-2.04	-1.33
92587_at	ferredoxin 1	Fdx1	2.29	-2.11	-1.20
99872_s_at	ferritin light chain 1	Ftl1	1.40	1.57	1.22
97949_at	fibrinogen-like protein 2	Fgl2	1.20	2.98	2.03
99546_at	FK506 binding protein 2	Fkbp2	-1.16	-1.35	-1.45
92809_r_at	FK506 binding protein 4	Fkbp4	-1.75	-2.01	-1.59
97327_at	flap structure specific endonuclease 1	Fen1	-2.94	-2.35	-1.97
100515_at	furin (paired basic amino acid cleaving enzyme)	Furin	2.05	1.30	1.25
100133_at	Fyn proto-oncogene	Fyn	1.87	4.09	1.20
104333_at	G7e protein	G7e-pending	-2.05	-8.53	-2.19
94192_at	ganglioside-induced differentiation-associated-protein 10	Gdap10	-1.61	1.58	0.13
102654_at	GATA binding protein 1	Gata1	-1.54	-1.50	-1.09
102789_at	GATA binding protein 2	Gata2	-2.70	-1.31	-1.11
93750_at	geisolin	Gsn	1.75	2.30	1.80
94295_at	general transcription factor II I	Gtf2i	-1.57	-1.33	-1.05
92655_at	glucosaminyl (N-acetyl) transferase 1, core 2	Gcnt1	1.78	-0.07	0.27
100573_f_at	glucose phosphate isomerase 1	Gpi1	0.12	-1.45	-1.52
99649_at	glutamate-cysteine ligase, catalytic subunit	Gclc	1.90	1.39	-1.35
94897_at	glutathione peroxidase 4	Gpx4	1.03	-1.41	1.20
99583_at	glutathione S-transferase, pi 2	Gstp2	-1.21	-1.43	-1.27
97525_at	glycerol kinase	Gyk	1.62	1.80	0.31
98984_f_at	glycerol phosphate dehydrogenase 2, mitochondrial	Gpd2	1.75	0.04	-0.06
101620_at	glycoprotein 1b, alpha polypeptide	Gp1ba	-1.22	-2.96	-1.65
92611_at	GPI-anchored membrane protein 1	Gpiap1	-1.57	-1.10	-0.13
102877_at	granzyme B	Gzmb	1.42	0.13	1.16
102313_at	GTP cyclohydrolase 1	Gch	5.99	2.52	0.12
100514_at	guanine nucleotide binding protein, alpha 13	Gna13	2.08	1.21	1.13
95364_at	guanine nucleotide binding protein, alpha 14	Gna14	-2.17	-13.93	0.11
160114_at	guanosine diphosphate (GDP) dissociation inhibitor 3	Gdi3	-1.26	-1.32	0.00



95419_at	H1 histone family, member 0	H1f0	0.54	-2.12	-1.60
93019_at	H2A histone family, member X	H2afx	0.41	-1.43	-1.75
101954_at	H2A histone family, member Z	H2afx	1.05	0.00	-1.43
100708_at	H3 histone, family 3B	H3f3b	2.87	-0.02	-1.33
95282_at	heat shock protein 1, alpha	Hspca	-1.09	-0.06	-1.68
98111_at	heat shock protein 105	Hsp105	-1.37	2.36	-1.68
93875_at	heat shock protein 1A	Hspa1a	9.16	8.78	1.79
93276_at	hematological and neurological expressed sequence 1	Hn1	-1.23	-2.03	-1.29
160101_at	heme oxygenase (decycling) 1	Hmox1	4.12	2.23	-0.24
102410_at	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	Hs3st1	12.22	10.55	2.19
92629_f_at	hepatoma derived growth factor	Hdgf	-1.28	-1.36	-1.21
93117_at	heterogeneous nuclear ribonucleoprotein A2/B1	Hnrpa2b1	-1.34	-1.23	-0.23
93095_at	high mobility group box 1	Hmgb1	-0.29	-1.55	-1.46
93250_r_at	high mobility group box 2	Hmgb2	-1.14	-3.70	-1.97
98038_at	high mobility group box 3	Hmgb3	-1.26	-1.81	-2.07
101589_at	high mobility group nucleosomal binding domain 2	Hmgnd	-1.52	-1.93	-1.77
93328_at	histidine decarboxylase	Hdc	21.56	29.13	2.48
99581_at	histidine triad nucleotide binding protein	Hint	-1.25	-1.23	-0.07
101886_f_at	histocompatibility 2, D region locus 1	H2-D1	-0.02	1.10	1.50
97540_f_at	histocompatibility 2, D region locus 1	H2-D1	0.01	-1.02	1.25
99379_f_at	histocompatibility 2, K region	H2-K	-0.02	1.13	1.41
99378_f_at	histocompatibility 2, Q region locus 1	H2-Q1	0.21	1.35	1.78
101876_s_at	histocompatibility 2, T region locus 17	H2-T17	-1.39	1.24	1.65
94288_at	histone 1, H1c	Hist1h1c	11.30	0.99	0.93
93833_s_at	histone 1, H2bc	Hist1h2bc	10.00	5.04	2.45
160176_at	histone cell cycle regulation defective interacting protein 5	Hirp5	-1.44	-1.65	1.05
96416_f_at	histone1, H3d	Hist1h3d	2.32	1.47	-0.39
97809_at	HLA-B associated transcript 8	Bat8	-1.25	-1.46	1.23
160384_at	HLA-B-associated transcript 1A	Bat1a	-1.46	-1.22	-1.40
103262_at	homeodomain interacting protein kinase 1	Hipk1	35.53	10.46	3.95
103833_at	homeodomain interacting protein kinase 2	Hipk2	-1.03	8.29	1.15
101096_s_at	HS1 binding protein	Hs1bp1	1.26	1.69	-2.64
160451_at	hypothetical protein MGC18745	MGC18745	2.12	1.86	0.00
160107_at	hypoxanthine guanine phosphoribosyl transferase	Hprt	-1.10	-1.50	-1.33
104500_at	iduronidase, alpha-L-	Idua	-1.77	-2.15	-0.14
99109_at	immediate early response 2	Ier2	1.96	-0.64	-2.38
94384_at	immediate early response 3	Ier3	1.71	0.23	0.49
100360_f_at	immunoglobulin heavy chain 4 (serum IgG1)	Igh-4	-1.72	-6.39	-2.01
100682_f_at	immunoglobulin kappa chain variable 8 (V8)	Igk-V8	-2.00	-4.91	-1.55
100277_at	inhibin beta-A	Inhba	3.80	0.22	0.19
93013_at	inhibitor of DNA binding 2	Ildb2	2.03	1.65	1.47
100578_at	inosine 5-phosphate dehydrogenase 2	Impdh2	-1.43	-1.56	-1.56
101441_i_at	inositol 1,4,5-triphosphate receptor 5	Itpr5	-1.21	-1.40	-1.08
94398_s_at	inositol polyphosphate 5-phosphatase B	Inpp5b	-1.70	-1.53	1.23
102884_at	inositol polyphosphate 5-phosphatase D	Inpp5d	-1.24	-1.70	0.09
100566_at	insulin-like growth factor binding protein 5	Igfbp5	-1.32	2.33	1.57
101123_at	integral membrane protein 2B	Itm2b	-1.21	-1.40	1.11
102353_at	integrin beta 2	Itgb2	0.35	3.50	1.66
100906_at	integrin beta 7	Itgb7	-1.24	-1.47	0.05
160092_at	interferon-related developmental regulator 1	Ifrd1	10.44	1.51	0.50
103486_at	interleukin 1 beta	Il1b	11.89	7.08	1.68
98500_at	interleukin 1 receptor-like 1	Il1rl1	-1.09	-1.23	1.18
98501_at	interleukin 1 receptor-like 1	Il1rl1	5.04	4.10	0.19
94168_at	interleukin 13	Il13	95.52	18.27	-0.05
99991_at	interleukin 17 receptor	Il17r	2.34	-0.24	1.29
101144_at	interleukin 18 receptor 1	Il18r1	-2.29	-2.62	0.37
92283_s_at	interleukin 4	Il4	15.23	1.74	1.04
102021_at	interleukin 4 receptor, alpha	Il4ra	-2.22	-1.41	0.09
102218_at	interleukin 6	Il6	14.27	3.26	0.08
93029_at	isocitrate dehydrogenase 3 (NAD+), gamma	Idh3g	-0.18	-1.36	1.15
102364_at	Jun proto-oncogene related gene d1	JunD	2.97	1.30	-0.03
102362_i_at	Jun-B oncogene	Junb	22.14	2.41	1.19
102363_f_at	Jun-B oncogene	Junb	4.50	0.46	0.03
99541_at	kinesin family member 11	Kif11	-1.76	-2.90	-2.74
97991_at	Kirsten rat sarcoma oncogene 2, expressed	Kras2	4.09	-1.44	-0.01
99956_at	kit oncogene	Kit	-1.47	-1.41	1.36
98059_s_at	lamin A	Lmna	2.01	1.35	-1.15
96065_at	lactexin	Lxn	1.13	1.46	1.69
95706_at	lectin, galactose binding, soluble 3	Lgals3	2.16	5.27	2.78

103335_at	lectin, galactose binding, soluble 9	Lgals9	0.01	-2.03	0.07
93600_at	leptin receptor gene-related protein	Obrgrp	-1.40	-1.81	1.31
100540_at	leukotriene A4 hydrolase	Lta4h	-1.14	-1.37	-1.16
93930_at	LIM and SH3 protein 1	Lasp1	11.67	22.05	6.61
98122_at	LIM domain only 4	Lmo4	-1.35	-3.91	-1.76
93078_at	lymphocyte antigen 6 complex, locus A	Ly6a	-1.19	-1.69	-1.31
101487_f_at	lymphocyte antigen 6 complex, locus E	Ly6e	1.15	-0.03	-2.01
98000_at	lymphocyte antigen 64	Ly64	-0.29	-1.76	-1.30
102957_at	lymphocyte cytosolic protein 2	Lcp2	1.69	-1.12	0.01
160089_at	lysosomal membrane glycoprotein 1	Lamp1	1.02	-1.11	-1.32
AFFX-MurIL4_at	M25892 Mus musculus interleukin 4 (Il-4) mRNA		4.12	0.14	0.06
94805_f_at	M33988 histone H2A.1 gene		-1.40	-2.38	-2.21
97719_at	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	Mst1r	9.66	-0.53	-1.99
92216_at	MAD homolog 7 (Drosophila)	Madh7	5.53	0.85	1.11
99632_at	MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)	Mad2l1	-1.55	-2.37	-3.10
101082_at	maic enzyme, supematant	Mod1	-1.34	5.89	1.10
160580_at	mannosidase 1, alpha	Man1a	3.71	2.59	1.81
97203_at	MARCKS-like protein	Mlp	19.94	2.21	-0.09
99024_at	Max dimerization protein 4	Mad4	-1.34	-0.10	1.59
103468_at	meiosis-specific nuclear structural protein 1	Mns1	-2.12	-9.39	-1.29
96767_at	membrane bound C2 domain containing protein	Mbc2	-1.17	-1.80	-1.17
96509_at	membrane-spanning 4-domains, subfamily A, member 2	Ms4a2	-0.28	-4.29	1.80
93573_at	metallothionein 1	Mt1	2.76	-0.10	0.03
104340_at	methyl-CpG binding domain protein 1	Mbd1	-2.55	-0.06	-1.15
102161_f_at	MHC (Qa) Q2-k gene for class I antigen	X5860C	0.04	1.21	1.62
100062_at	minichromosome maintenance deficient 3 (S. cerevisiae)	Mcm3	-1.43	-2.79	-1.70
93041_at	minichromosome maintenance deficient 4 homolog	Mcm4	-1.66	-2.14	-1.93
100156_at	minichromosome maintenance deficient 5, cell division cycle 46	Mcm5	-1.69	-2.49	-1.86
93356_at	minichromosome maintenance deficient 7 (S. cerevisiae)	Mcm7	-1.68	-3.45	-1.97
93314_g_at	mitogen activated protein kinase kinase 3	Map2k3	2.40	2.39	1.21
96632_at	mortality factor 4 like 2	Morf4l2	1.73	1.57	-1.18
92644_s_at	myeloblastosis oncogene	Myb	-2.81	-0.07	1.05
160463_at	myeloid differentiation primary response gene 116	Myd116	3.37	1.50	1.25
96285_at	myeloid-associated differentiation marker	Myadm	-1.14	-2.07	-1.11
92400_at	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 2	Ndst2	-2.62	-2.80	1.09
102780_at	neoplastic progression 3	Npn3	62.79	11.95	0.63
101469_at	neural precursor cell expressed, developmentally down-regulated gene 9	Nedd9	5.92	2.01	0.14
93101_s_at	neural precursor cell expressed, developmentally down-regulated gene 4	Nedd4	-0.12	-2.04	-0.02
103048_at	neuroblastoma myc-related oncogene 1	Nmyc1	4.12	1.22	-1.21
103662_at	neutrophil cytosolic factor 4	Ncf4	-1.31	-1.88	1.67
100962_at	Ngfi-A binding protein 2	Nab2	12.49	2.54	-0.89
103805_at	nibrin	Nbn	-1.54	-5.91	-1.55
98114_at	Niemann Pick type C1	Npc1	1.43	1.11	1.16
160464_s_at	N-myc downstream regulated 1	Ndr1	12.81	4.67	-0.05
96596_at	N-myc downstream regulated-like	Ndr1	12.56	4.94	0.04
95380_at	non MHC restricted killing associated	Nmrk	2.23	1.95	1.59
102209_at	nuclear factor of activated T-cells, cytoplasmic 1	Nfatc1	7.55	2.54	1.41
98427_s_at	nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	Nfkb1	1.56	-1.47	-0.01
98010_at	nuclear factor, erythroid derived 2	Nfe2	-7.34	-6.98	-0.02
102955_at	nuclear factor, interleukin 3, regulated	Nfil3	6.90	2.58	-3.87
99564_at	nuclear protein 95	Np95	-1.58	-4.20	-1.87
103288_at	nuclear receptor interacting protein 1	Nrip1	2.17	1.75	1.71
102371_at	nuclear receptor subfamily 4, group A, member 1	Nr4a1	37.89	-1.78	1.40
92248_at	nuclear receptor subfamily 4, group A, member 2	Nr4a2	31.69	2.22	2.54
92249_g_at	nuclear receptor subfamily 4, group A, member 2	Nr4a2	15.58	2.22	1.13
102197_at	nucleobindin 2	Nucb2	-1.38	2.32	1.49
100144_at	nucleolin	Ncl	-1.25	0.07	-1.59
94372_at	nudix (nucleoside diphosphate linked moiety X)-type motif 1	Nudt1	-1.54	-2.28	-1.33
99019_at	P450 (cytochrome) oxidoreductase	Por	0.08	1.79	1.23
100328_s_at	paired-Ig-like receptor A3	Pira3	1.44	0.80	3.74
98003_at	paired-Ig-like receptor B	Pirb	0.00	1.16	2.41
95491_at	Parkinson disease (autosomal recessive, early onset) 7	Park7	-1.12	-1.53	-1.94
96128_at	PCTAIRE-motif protein kinase 3	Pctk3	2.43	2.17	1.26
101700_at	per-hexamer repeat gene 4	Phxr4	6.59	1.61	1.36
97758_at	peroxiredoxin 1	Prdx1	1.70	3.70	-1.13
96256_at	peroxiredoxin 3	Prdx3	-1.19	-1.39	-0.03
93495_at	peroxiredoxin 4	Prdx4	1.11	0.02	-1.72
92637_at	phosphofructokinase, liver, B-type	Pfkl	0.04	-2.44	-1.84
93346_at	phosphoglycerate kinase 1	Pgk1	0.24	0.33	-2.92

100607_at	phospholipase D3	Pld3	-1.13	1.62	2.08
102839_at	phospholipid scramblase 1	Plscr1	5.03	2.25	1.46
101027_s_at	pituitary tumor-transforming 1	Pttg1	-1.11	-1.49	-0.27
94932_at	platelet derived growth factor, alpha	Pdgfa	2.98	7.12	2.88
160829_at	pleckstrin homology-like domain, family A, member 1	Phlda1	7.99	1.46	-1.35
100720_at	poly A binding protein, cytoplasmic 1	Pabpc1	0.17	1.54	1.47
103207_at	polymerase (DNA directed), alpha 1	Pola1	-1.39	-2.53	-0.43
101461_f_at	praja1, RING-H2 motif containing	Pja1	-1.46	-1.19	0.01
100606_at	pnin protein	Pnp	-0.46	-3.58	1.11
101881_g_at	procollagen, type XVIII, alpha 1	Col18a1	-1.25	25.28	10.55
101585_at	progesterone receptor membrane component 1	Pgrmc1	-0.13	-1.31	-1.37
103029_at	programmed cell death 4	Pdcd4	1.30	-1.97	1.27
96858_at	programmed cell death 8	Pdcd8	-1.31	-1.34	-1.25
101065_at	proliferating cell nuclear antigen	Pcna	1.67	-1.34	-1.67
101468_at	properdin factor, complement	Pfc	1.24	1.02	-1.78
96588_at	prostaglandin E receptor 3 (subtype EP3)	Ptger3	-1.51	-2.33	-0.12
104647_at	prostaglandin-endoperoxide synthase 2	Ptgs2	93.16	1.80	-1.12
100733_at	proteasome (prosome, macropain) subunit, alpha type 2	Pasma2	1.00	1.40	-0.05
92544_f_at	proteasome (prosome, macropain) subunit, alpha type 3	Pasma3	-1.01	1.54	-0.01
101558_s_at	proteasome (prosome, macropain) subunit, beta type 5	Psmb5	-1.19	1.75	0.28
94263_f_at	proteasome (prosome, macropain) subunit, beta type 7	Psmb7	-1.00	1.52	-1.57
99510_at	protein kinase C, beta	Prkcb	-1.24	-1.37	1.19
104531_at	protein kinase C, delta	Prkcd	-1.51	-1.50	1.19
160698_s_at	protein kinase C, delta	Prkcd	-1.57	-1.41	1.11
103559_at	protein kinase, cAMP dependent, catalytic, alpha	Prkaca	-1.03	-1.37	0.03
93116_at	protein kinase, cAMP dependent, catalytic, beta	Prkacb	-1.34	-1.08	1.09
101482_at	protein phosphatase 1, catalytic subunit, gamma isoform	Ppp1cc	-1.97	-1.35	-1.25
94929_at	protein tyrosine phosphatase, non-receptor type 1	Ptpn1	-1.13	1.60	0.17
101996_at	protein tyrosine phosphatase, non-receptor type 2	Ptpn2	-1.86	-4.99	-1.80
92356_at	protein tyrosine phosphatase, non-receptor type 8	Ptpn8	40.28	8.13	-1.06
100976_at	protein tyrosine phosphatase, non-receptor type 9	Ptpn9	-1.43	-1.71	0.04
101298_g_at	protein tyrosine phosphatase, receptor type, C	Ptprc	1.73	2.12	2.34
99478_at	protein tyrosine phosphatase, receptor type, C polypeptide-associated prot	Ptprcap	1.06	1.53	1.59
93323_at	proteolipid protein 2	Plp2	0.13	1.37	1.36
102791_at	proteasome (prosome, macropain) subunit, beta type 8 (large multifunction	Psmb8	-1.20	-1.77	-0.06
100718_at	prothymosin alpha	Ptma	0.03	-1.12	-1.54
101926_at	proviral integration site 2	Pim2	-1.36	-3.68	-2.77
95586_at	purinergic receptor P2X, ligand-gated ion channel 4	P2rx4	-1.88	-3.41	1.28
97415_at	RAB3D, member RAS oncogene family	Rab3d	-1.33	-5.71	-0.14
101921_at	RAB4A, member RAS oncogene family	Rab4a	6.56	8.42	4.47
104108_at	Rab6 interacting protein 1	Rab6ip1	-0.26	-2.28	0.16
95785_s_at	RAB7, member RAS oncogene family	Rab7	3.93	3.23	2.77
95516_at	RAB9, member RAS oncogene family	Rab9	-1.64	-1.60	0.11
94768_at	RAD21 homolog (S. pombe)	Rad21	-1.15	-2.31	-1.41
98573_r_at	RAN binding protein 1	Ranbp1	-1.51	-1.29	-1.61
101254_at	RAN, member RAS oncogene family	Ran	-1.25	0.03	-1.47
102028_at	Ras association (RalGDS/AF-6) domain family 5	Rassf5	2.59	1.82	1.30
101030_at	ras homolog gene family, member AB	Arhb	6.12	2.39	1.12
93319_at	RAS p21 protein activator 3	Rasa3	-1.67	-1.75	1.15
101584_at	Ras suppressor protein 1	Rsu1	0.00	-1.49	1.16
103642_at	Ras-GTPase-activating protein SH3-domain binding protein	G3bp-pending	-1.14	0.05	-1.43
102821_s_at	RAS-like, family 2, locus 9	Rasl2-9	-1.21	0.05	-1.64
97319_at	Ras-related associated with diabetes	Rrad	7.27	1.47	1.17
98950_at	Ras-related GTP binding C	Rragc	-1.13	-1.29	1.11
102300_at	receptor-associated protein of the synapse	Rapsn	1.25	-2.03	-2.25
94378_at	regulator of G-protein signaling 16	Rgs16	6.06	3.09	1.36
97844_at	regulator of G-protein signaling 2	Rgs2	12.87	2.62	1.59
97948_at	retinoblastoma 1	Rb1	-1.30	-2.68	-0.10
92647_at	retinoblastoma binding protein 4	Rbbp4	-1.38	-1.67	-1.34
93081_at	retinoblastoma binding protein 7	Rbbp7	-1.39	0.06	-1.87
98434_at	Rho guanine nucleotide exchange factor (GEF7)	Arhgef7	-1.28	2.17	1.56
100612_at	ribonucleotide reductase M1	Rrm1	-1.64	-5.05	-2.42
98007_at	ribosomal protein S6 kinase, polypeptide 2	Rps6ka2	2.81	6.15	1.67
96785_at	RIKEN cDNA 0610013D04 gene	0610013D04Rik	-8.56	-4.76	0.08
160205_f_at	ring finger protein 11	Rnf11	1.36	1.99	1.21
101965_at	ring finger protein 13	Rnf13	-0.22	-2.91	0.12
92539_at	S100 calcium binding protein A10 (calpactin)	S100a10	1.30	1.54	1.72
98600_at	S100 calcium binding protein A11 (calizzarin)	S100a11	1.32	2.44	2.82
100960_g_at	S100 calcium binding protein A13	S100a13	-1.10	1.02	1.42

100959_at	S100 calcium binding protein A13	S100a13	-1.29	1.04	1.30
92770_at	S100 calcium binding protein A6 (calcyclin)	S100a6	-1.18	0.05	1.59
96024_at	S-adenosylthionocysteine hydrolase	Ahcy	-1.24	-2.09	-1.57
103080_at	SAM domain and HD domain, 1	Samhd1	3.10	0.12	1.75
92471_i_at	schlafen 2	Slfn2	-3.34	-1.79	1.56
92472_f_at	schlafen 2	Slfn2	-6.02	-1.75	1.40
92636_f_at	SEC61, gamma subunit (S. cerevisiae)	Sec61g	0.03	1.10	-1.24
97519_at	secreted phosphoprotein 1	Spp1	11.95	29.72	2.52
103468_at	selectin, platelet (p-selectin) ligand	Selp1	-1.01	-1.44	0.02
160836_at	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) ;	Sema4d	-1.11	-1.51	1.03
101995_at	sequestosome 1	Sqstm1	1.99	1.49	1.34
102860_at	serine (or cysteine) proteinase inhibitor, clade A, member 3G	Serpina3g	1.43	-2.27	-1.40
92978_s_at	serine (or cysteine) proteinase inhibitor, clade B, member 2	Serpnb2	2.66	1.62	0.21
96060_at	serine (or cysteine) proteinase inhibitor, clade B, member 6a	Serpnb6a	-1.13	1.46	1.93
94147_at	serine (or cysteine) proteinase inhibitor, clade E, member 1	Serpine1	10.71	2.43	0.21
93680_at	serine/threonine kinase 10	Stk10	-2.51	-1.74	-0.03
93272_at	serine/threonine kinase 16	Stk16	-1.92	-1.22	0.06
92639_at	serine/threonine kinase 6	Stk6	-1.92	-5.38	-3.38
99847_at	sialyltransferase 4A (beta-D-galactoside alpha-2,3-sialyltransferase)	Siat4a	3.16	3.86	-0.07
102318_at	sialyltransferase 8 (alpha-2, 8-sialyltransferase) D	Siat8d	-9.91	-8.09	-0.25
101519_at	signal recognition particle 14	Srp14	0.00	1.48	1.08
102994_at	signal transducer and activator of transcription 4	Stat4	-1.23	-2.21	1.11
97421_at	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	Smc2l1	-1.41	-7.89	-1.96
92582_at	solute carrier family 1, member 7	Slc1a7	1.65	2.43	-0.28
99133_at	solute carrier family 3 (activators of dibasic and neutral amino acid transp	Slc3a2	1.51	0.11	-1.35
104221_at	solute carrier family 7 (cationic amino acid transporter, y+ system), membe	Slc7a5	2.74	2.63	-2.72
97243_at	solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 1	Slc9a3r1	-1.09	-1.44	1.15
160543_at	sorting nexin 3	Snx3	-1.19	-1.66	-1.16
92540_f_at	spermidine synthase	Smm	-1.43	0.19	-1.41
94017_s_at	splicing factor, arginine/serine-rich 2 (SC-35)	Sfrs2	-1.15	-0.01	-1.61
99876_at	src-like adaptor	Sla	15.77	1.24	0.17
94057_g_at	stearoyl-Coenzyme A desaturase 1	Scd1	1.56	2.48	1.86
94056_at	stearoyl-Coenzyme A desaturase 1	Scd1	1.27	2.20	1.61
95758_at	stearoyl-Coenzyme A desaturase 2	Scd2	-1.49	-0.05	1.19
160471_at	stem-loop binding protein	Sibp	-2.64	-2.65	-0.28
103421_at	stromal cell derived factor receptor 2	Sdfr2	-1.06	2.67	1.41
160428_at	succinate-Coenzyme A ligase, GDP-forming, beta subunit	Succlg2	-1.15	-2.00	-1.49
92232_at	suppressor of cytokine signaling 3	Socs3	12.37	0.12	1.34
92855_at	suppressor of initiator codon mutations, related sequence 1 (S. cerevisiae)	Sui1-rs1	2.36	1.59	0.03
100499_at	syntaxin 3	Stx3	-1.77	-2.14	-0.04
101398_at	syntaxin binding protein 2	Stxbp2	-1.32	-1.85	-0.01
99448_at	tain	Tln	0.07	0.19	1.28
92339_at	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, A	Taf1a	5.84	3.19	1.35
97973_at	T-cell acute lymphocytic leukemia 1	Tal1	-1.24	1.14	1.13
102744_at	T-cell receptor gamma, variable 2	Tcrg-V2	-2.12	-77.03	-3.01
102745_at	T-cell receptor gamma, variable 4	Tcrg-V4	-1.54	-22.55	-1.69
101551_s_at	testis derived transcript	Tes	1.16	1.84	1.42
101502_at	TG interacting factor	Tgif	3.92	-1.82	1.63
99602_at	TGFB inducible early growth response 1	Tieg1	3.16	1.35	1.14
99603_g_at	TGFB inducible early growth response 1	Tieg1	5.96	3.25	1.08
92807_at	thioredoxin 1	Txn1	-0.01	1.43	-1.56
99985_at	thioredoxin reductase 1	Txnrd1	2.00	3.35	-0.16
95694_at	topoisomerase (DNA) 1	Top1	1.26	-1.92	-1.28
101959_f_at	transcription factor Dp 1	Tfdp1	-1.97	-2.12	-1.39
93789_s_at	transcriptional regulator, SIN3B (yeast)	Sin3b	1.62	3.22	1.11
104623_at	transducin-like enhancer of split 3, homolog of Drosophila E(spl)	Tle3	-0.04	2.38	1.07
AFFX-TransRecMurr/X57349_M_at	transferrin receptor	Trfr	7.97	15.08	-0.12
101918_at	transforming growth factor, beta 1	Tgfb1	1.56	2.31	1.19
103035_at	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	Tap1	-1.42	-2.53	1.20
102873_at	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	Tap2	-1.40	-1.82	1.13
99566_at	triosephosphate isomerase	Tpi	0.33	-1.89	-2.61
93071_at	trypartite motif protein 28	Trim28	-1.65	-1.38	-1.40
160532_at	tropomyosin 1, alpha	Tpm1	1.70	1.85	0.07
99972_at	tryptophan hydroxylase 1	Tph1	-0.22	-2.20	1.03
102308_at	tubby-like protein 3	Tulp3	-1.43	-1.70	-0.07
100342_i_at	tubulin, alpha 1	Tuba1	-1.09	-1.67	0.00
100343_f_at	tubulin, alpha 1	Tuba1	-1.02	-1.59	-1.27
98759_f_at	tubulin, alpha 2	Tuba2	1.11	-1.57	-1.42
94835_f_at	tubulin, beta 2	Tubb2	-1.33	-1.41	1.10

94788_f_at	tubulin, beta 5	Tubb5	-1.36	-1.96	-1.24
94789_r_at	tubulin, beta 5	Tubb5	-1.17	-1.89	-1.26
100151_at	tumor differentially expressed 1	Tde1	-1.11	-2.27	1.08
102629_at	tumor necrosis factor	Tnf	16.99	2.99	0.80
101136_at	tumor necrosis factor (ligand) superfamily, member 8	Tnfsf8	5.51	1.34	0.46
92415_at	tumor necrosis factor (ligand) superfamily, member 9	Tnfsf9	3.44	-0.01	1.36
94928_at	tumor necrosis factor receptor superfamily, member 1b	Tnfrsf1b	1.66	2.10	-0.45
99392_at	tumor necrosis factor, alpha induced protein 3	Tnfaip3	1.91	7.76	3.95
160499_at	tumor rejection antigen gp96	Tra1	-1.26	-1.44	-1.42
104679_at	TXK tyrosine kinase	Txk	1.34	-1.71	1.64
93908_f_at	type IIB intracisternal A-particle (IAP) element encoding integrase	X16670	2.00	1.74	1.84
AFFX-MURINE_b1_at	U01310 Mus musculus C57/Black6 BC1 scRNA		-1.52	-1.16	-1.46
92217_s_at	U05265 Glycoprotein 49 B		2.20	1.39	1.34
101255_at	ubiquitin B	Ubb	1.58	1.13	1.08
95215_f_at	ubiquitin C	Ubc	1.95	1.59	1.28
96695_at	ubiquitin-conjugating enzyme E2A, RAD6 homolog (S. cerevisiae)	Ube2a	1.68	-1.28	1.16
95703_at	ubiquitin-like 1 (sentrin) activating enzyme E1A	Uble1a	-1.55	-1.86	-1.24
103002_at	UDP-Gal betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	B4galt1	1.01	1.66	1.40
97934_at	UDP-N-acetyl-alpha-D-galactosamine polypeptide N-acetyl-galactosaminylb	Galnt1	-1.38	-1.83	-1.09
95555_at	unc119 homolog (C. elegans)	Unc119h	-1.23	-4.17	0.00
94381_at	uridine monophosphate kinase	Umpk	-2.23	-1.65	0.10
103520_at	vascular endothelial growth factor A	Vegfa	4.00	1.42	-0.83
93305_f_at	vesicle-associated membrane protein 8	Vamp8	-1.47	-1.61	1.02
100302_at	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian);	Maff	6.89	-0.10	2.92
102919_at	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein K (avian);	Mafk	2.88	2.14	0.17
101458_at	wee 1 homolog (S. pombe)	Wee1	1.64	0.14	0.10
92262_at	wild-type p53-induced gene 1	Wig1	-1.79	-0.16	1.40
100522_s_at	WW domain binding protein 5	Wbp5	0.06	-1.61	-1.42
AFFX-18SRNAMurrX00686_5_at	X00686: gene for 18S rRNA		-1.19	-1.56	-1.83
93909_f_at	X04120 Intracisternal A particles, Thbd linked		2.24	2.08	0.89
96657_at	X58609: Mouse MHC (Qa) Q2-k gene for class I antigen	Sat	4.45	1.87	1.83
160321_at	zinc finger protein 216	Zfp216	3.03	1.57	-0.01
92409_at	zinc finger protein 260	Zfp260	-4.18	-4.59	-0.72
99001_at	zinc finger protein 292	Zfp292	-2.48	-1.08	-1.30
93324_at	zinc finger protein 36, C3H type-like 1	Zfp36l1	4.33	-1.27	1.29
102293_at	zinc finger protein, subfamily 1A, 1 (Ikaros)	Znfn1a1	2.25	3.91	2.55
100046_at		Mthrd2	-1.01	-0.82	-1.89

94788_f_at	tubulin, beta 5	Tubb5	-1.36	-1.96	-1.24
94789_r_at	tubulin, beta 5	Tubb5	-1.17	-1.89	-1.26
100151_at	tumor differentially expressed 1	Tde1	-1.11	-2.27	1.08
102629_at	tumor necrosis factor	Tnf	16.99	2.99	0.80
101136_at	tumor necrosis factor (ligand) superfamily, member 8	Tnfsf8	5.51	1.34	0.46
92415_at	tumor necrosis factor (ligand) superfamily, member 9	Tnfsf9	3.44	-0.01	1.36
94928_at	tumor necrosis factor receptor superfamily, member 1b	Tnfrsf1b	1.66	2.10	-0.45
99392_at	tumor necrosis factor, alpha-induced protein 3	Tnfaip3	1.91	7.76	3.95
160499_at	tumor rejection antigen gp96	Tra1	-1.26	-1.44	-1.42
104679_at	TXK tyrosine kinase	Txk	1.34	-1.71	1.64
93908_f_at	type IIB intracisternal A-particle (IAP) element encoding integrase	X16670	2.00	1.74	1.64
AFFX-MURINE_b1_at	U01310 Mus musculus C57/Black6 BC1 scRNA		-1.52	-1.16	-1.46
92217_s_at	U05265 Glycoprotein 49 B		2.20	1.39	1.34
101255_at	ubiquitin B	Ubb	1.58	1.13	1.08
95215_f_at	ubiquitin C	Ubc	1.95	1.59	1.28
96695_at	ubiquitin-conjugating enzyme E2A, RAD6 homolog (S. cerevisiae)	Ube2a	1.68	-1.28	1.16
95703_at	ubiquitin-like 1 (sentinel) activating enzyme E1a	Ubl1a	-1.55	-1.86	-1.24

## VITA

