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Transformation of human mast cells by interferon-gamma and the potential role of myeloid derived suppressor cells in mastocytosis.

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VCU SOM

Transformation of human mast cells by interferon-gamma and the potential role of myeloid derived suppressor cells in mastocytosis.

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

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Abbreviations

AP-1	activator protein 1
BMMC	Bone marrow derived murine mast cells
BTK	Bruton's tyrosine kinase
c-kit	stem cell factor receptor, CD117
CXCR	receptor for CXC family of chemokines
DAG	diacyl glycerol
DMA	dimethylamiloride
ERK	extracellular signal regulated kinase
FcεRI	high affinity IgE receptor, either tetrameric (α,β, γ, γ) or trimeric (α, γ, γ) form.
Fyn	FYN oncogene related to SRC, FGR, YES
GM-CSF	granulocyte monocyte conolony stimulating factor
Grb-2	Growth factor receptor-bound protein 2
HLA II	human leukocyte antigen II (human version of MHC II)
IFNγ	interferon gamma
IL	interleukin
IP3	inositol trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
LAT	linker for t cell activation
LPS	lipopolysaccharide
Lyn	Lck/Yes novel tyrosine kinase
MAPK	mitogen activated protein kinase
MC_T	mast cell identified by sole expression of tryptase
MC_{TA3}	mast cell identified by co-expression of tryptase and carboxypeptidase, but not chymase
MC_{TC}	mast cell identified by co-expression of both tryptase and chymase
MDSC	myeloid derived suppressor cell
MEK	Mitogen-activated protein kinase kinase
MHC II	major histocompatibility complex II (also called HLA II for humans)
MIP-1α	Macrophage Inflammatory Protein 1α
NFAT	Nuclear factor of activated T-cells
PI3K	Phosphatidylinositol-4,5-bisphophate 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PLCγ1	Phospholipase C, gamma 1 subtype
Raf-1	Rapidly Accelerated Fibrosarcoma-1
SCF	stem cell factor
SH2	Src-homology-2 domain
Slp-76	SH2 domain containing leukocyte protein of 76kDa
Syk	spleen tyrosine kinase
TNFα	tumor necrosis factor α
TSLP	thymic stromal lymphopoietin

Abstract

TRANSFORMATION OF HUMAN MAST CELLS BY INTERFERON-GAMMA AND THE POTENTIAL ROLE OF MYELOID DERIVED SUPPRESSOR CELLS IN MASTOCYTOSIS.

By Sahar Lotfi-Emran, MD, PhD

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

Virginia Commonwealth University, 2014

Director: Dr. Lawrence B. Schwartz, MD, PhD; Charles & Evelyn Thomas Professor of Medicine
Chair, Division of Rheumatology, Allergy & Immunology; Associate Chair for Research
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Mast cells respond to a variety of signals, are associated with both increased inflammation and regulation of the immune response, and are able to interact with a variety of hematopoietic and non-hematopoietic cells. The majority of the work that highlights mast cell pleiotropic abilities has been completed in murine models. Though these models have significantly advanced our understanding of what mast cells *can* do, they cannot inform us as to what mast cells *actually do* in human beings. The goal of this dissertation is to assess fully mature, primary human mast cell function beyond the well-defined type 1 hypersensitivity function and place mature human mast cells in the context of interactions with other immune cells. The first project addresses the ability of IFNγ, a historically Th1 associated cytokine, to dramatically alter mast cell phenotype. In particular, IFNγ stimulation allows mast cells to act as antigen presenting cells to CD4+ T cells. The second project describes and addresses the T cell

suppressive function of myeloid derived suppressor cells in Mastocytosis, a disease of clonal mast cells.

Introduction

Identification and characterization of mast cells

Mast cells were first identified in 1878 by Paul Ehrlich due to reaction to aniline dyes (metachromasia of their granules) as granular cells of the connective tissue. (Figure 1) In his later work, Ehrlich showed an increase in mast cell number in inflamed tissues and tumors; his students would observe an increase in mast cells surrounding, but not within, carcinomas.[1] Ehrlich believed mast cells to be of mesenchymal lineage and the origin of mast cells remained controversial until the late 1970s when Kitamura and colleagues published evidence that mast cells are of hematopoetic origin: first, that bone marrow cell transfer from beige mice (mice with Chediak-Higashi syndrome whose cells can be tracked by particularly large granules) to irradiated normal mice repopulates tissues with beige mast cells; second, that bone marrow transfer from normal mice to mast cell deficient mice (WBB6F1-W/W^{v}) populates tissues with mast cells. [2,3]

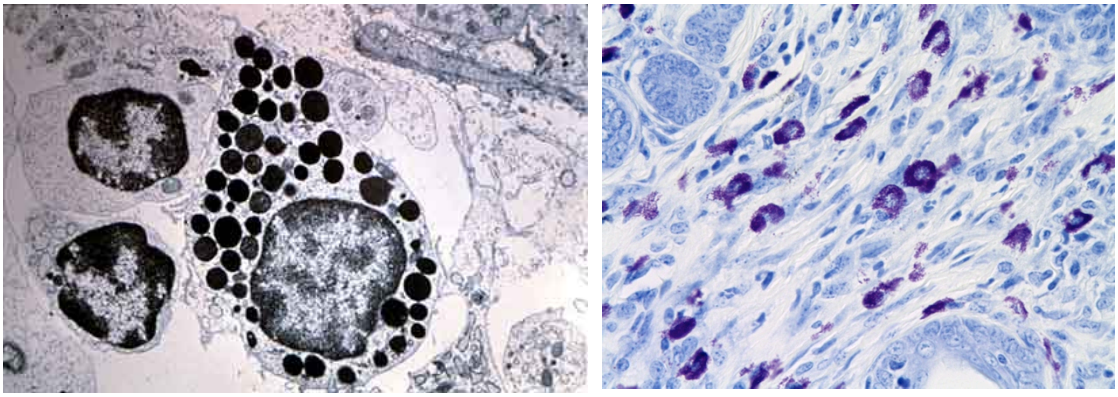

Figure 1. TEM and histochemical identification of human mast cells.

TEM (left) photo displays mast cell (with granules) in close association with lymphocytes in intestinal lamina propria. (from University of South Carolina School of Medicine Medical Microscopy lab at http://millette.med.sc.edu/Lab%206%20pages). Toluidine blue stained mast cells in mouse pinnae demonstrating metachromasia of mast cell granules (right). (from Indiana University Basic histology found at http://www.iupui.edu/~anatd502/Labs.f04/blood%20lab)

With their hematopoietic origin firmly established, contradictory (though circumstantial) evidence accumulated in regards to the nature of the relationship between basophils and mast cells. Like mast cells, basophils are granulocytes of hematopoietic origin, constitutively express high affinity IgE receptor (FcεRI), and are stimulated by IgE:FcεRI crosslinking to exocytose their granules. Basophil granules mainly store histamine, chondroitin sulfate, and proteases**.** [4]Tryptase is stored but at far lower levels than in mast cells.[5-7] In mice, bone marrow cells cultured in media with IL-3 (with or without stem cell factor (SCF)) result in production of both basophils and mast cells. In addition, some mast cell deficient mice also lack basophils.[8,9] As a result, several authors embraced the notion of a common mast cell and basophil progenitor.[10]

In humans the two cell types differ in important ways. Unlike mast cells, mature human basophils are found in peripheral blood, have a short lifespan, have bi-lobed or multi-lobular nuclei, are potent IL-4 producers, do not store chymase or carboxypeptidase, and do not express c-kit (CD117), the receptor for SCF.[4,6] In turn, human mast cells do not express the IL-3 receptor and human CD34+ cells cultured with IL-3 do not differentiate into mast cells.[11] IL-3's known ability to support the expansion of multilineage hematopoietic stem cell progenitors is

likely responsible for the modest increase in mast cell numbers produced from CD34+ human cells cultured in the presence of combined IL-3 and SCF.[12,13]

Recent literature has furthered analyzed mast cells and basophils in comparison to other hematopoietic cells. CD34+CD117+CD13+ cells, the most committed mast cell progenitor population found in humans thus far, give rise to both mast cells and monocytes but not basophils, erythroid precursors, myeloid cells, lymphocytes, or megakaryocytes.[13] Glycomic comparison show striking similarity between freshly isolated basophils and eosinophils, which display part-processed *N*-glycans, whereas mast cells displayed more highly processed N-glycans with sialylated and fucosylated multi-antennary structures, similar to other immune cells.[14] Principle component analysis of primary and cultured human mast cell transcriptomes shows an incredibly unique expression of transcription factors that cluster independently of all other hematopoietic cells, with closest relationships to dendritic cells, Langerhans's cells, and monocytes.[15] Overall, in opposition to data from mice, recent data refutes the concept of a common progenitor of human mast cells and basophils and indicates a very early separation of mast cell progenitors from remaining hematopoietic cells.

An additional layer of complexity can be attributed to the fact that mast cells only fully mature in peripheral organs and fully mature mast cells retain functional plasticity in response to local stimuli. Thus there are many phenotypic and functional differences between murine and human mast cells, between mast cells at different tissue sites and in healthy and inflamed tissues.

Mice have 1/5th to 1/10th of the density of mast cells found in peripheral tissue compared to humans and mice do not naturally develop allergic diseases.[16,17] The localization and phenotype of mast cells also differ: healthy humans almost entirely lack peritoneal mast cells whereas healthy rodents almost entirely lack mast cells in the lung. Rodents express several chymases which are sub-organ specific, whereas humans express a single chymase.[18] Human mast cells produce much higher levels of certain cytokines, such as tumor necrosis factor (TNFα) and IL-5, whereas, in the absence of atopic inflammation, only mouse mast cells have been shown to produce IL-4.[19-21] Immunoglobulin signaling via Fcγ receptors differs between species. Murine mast cells express the inhibitory IgG receptor, FcγRIIB. Heter-crosslinking of IgG bound by FcγRIIB with IgE bound by FcεRI reduces mast cell degranulation.[22] However, mature human mast cells only express the activating FcγRIIA receptor and hetero-crosslinkage increases degranulation.[23] As will be described later, there are also species specific differences in transcription factor activation following FcεRI crosslinkage which has an impact on subsequent mediator production.[24] Some of the difficulty encountered in attempts to translate treatments tested in experimental airway hypersensitivity models in mice to human allergic disease can be attributed to these and other specific differences in mast cell phenotype and function.[25]

The majority of mouse data relies on the use of bone marrow derived mast cells (BMMCs), which cannot fully mature *in vitro*. Neither murine BMMCs, nor human bone marrow, fetal cord blood, or peripheral blood CD34+ cell derived mast cells reflect their tissue counterparts, despite meticulous researcher care in selecting cells that highly express FcεRI and CD117.[26-28]

While many mast cell functions are not dependent on achieving fully mature status, as defined by expression of appropriate proteases, CD117 expression, FcεRI expression, and an inability to further differentiate (terminally differentiated), degree of maturity is particularly important in evaluating HLA II expression. HLA II is upregulated at various stages of myeloid differentiation as well as during periods of rapid cell proliferation as seen in myeloproliferative disorders. Indeed, human CD34+CD117+CD13+ mast cell progenitors express HLA II.[13] They are also hypogranulated and show altered ligand expression: for example, they, like murine cells, express FcγRIIB, which is not expressed on tissue derived mature human skin[23] or lung (unpublished) mast cells.

Fully mature human mast cells were initially characterized by their protease composition as either containing tryptase, chymase, carboxypeptidase A3, and cathepsin G (MC_{TC}) or containing only tryptase (MC_T). These distinct mast cell categories are functionally different: both types degranulate in response to FcεRI crosslinking, but only MC_{TC} cells respond to C5a and compound 48/80.[29] Due to differences in adenosine receptor expression, FcεRI mediated degranulation of MC_T cells from lung, but not MC_{TC} cells from skin, is potentiated by stimulation with low concentrations of adenosine and parallels the ability of IV administered adenosine to produce bronchospasm but not urticaria in asthmatics.[30] There are also differences in mediator production post-degranulation: for example, MC_{TC} cells from skin do not produce the leukotriene LTC_4, whereas both lung MC_{TC} and MC_T do.[29]

The distribution of mast cells, and mast cell subtypes, can change dramatically with disease and associated inflammation. In 2002, Brightling and colleagues compared asthma and eosinophilic bronchitis, both apparently involving IL-4, IL-5, and eosinophils, in an effort to understand the source of airway hyperreactivity seen in asthma but not eosinophilic bronchitis.[21,31] They found that mast cells accumulate in the airway smooth muscles of asthmatic but not eosinophilic bronchitis or healthy patients. The presence of mast cells in the airway smooth muscle, airway mucous glands, and bronchial epithelium and an increase in IL-13 are the main pathological differences with regard to mast cells between the two "Th2" diseases of the lower airway.[32] In severe asthma, the change in lung mast cells number is largely due to increases in the proportion of MC_{TC} cells.[33]

Recently, a third type of mast cell has been described: mast cells that express tryptase and carboxypeptidase A3 but not chymase (MC_{TA3}). MC_{TA3} are uniquely elevated in the airway epithelium of asthmatics, the nasal mucosa of chronic sinusitis, and the esophagus of eosinopilic esophagitis patients.[34,35] Unlike the MC_{TC} cells that accumulate in the airway smooth muscle of asthmatics, the MC_{TA3} cells are located in close association with mucous glands. Aside from correlations with disease, their significance is not known.

Mast cells as Th2 effector cells

Mast cells are best characterized as Type I hypersensitivity effector cells, armed and activated by IgE. Mast cells constitutively express the high affinity IgE receptor, FcεRI, whose surface expression is stabilized by binding to IgE.[36-38] Because tissue resident mast cells are longed lived, as are IgE:FcεRI on mast cell surfaces, mast cells from subjects sensitive are able to

respond to an antigen long after specific IgE production has dropped below detectable levels. A recent metanalysis of allergen sensitivity testing by skin prick versus by serum IgE shows that on average 46% of skin prick test positive individuals are also positive for serum specific IgE for that given allergen. However, 25% of skin prick test positive individuals are not positive for serum specific IgE.[39]

High affinity IgE receptor is made up of an α chain, β chain, and two γ chains ($\alpha\beta\gamma_2$). Human mast cells and basophils constitutively express both the tetrameric ($\alpha\beta\gamma_2$) and trimeric ($\alpha\gamma_2$) forms whereas rodent FcεRI is only expressed as a tetramer. IL-4, the prototypical Th2 cytokine, increases production of α chain mRNA and protein and increases FcεRIα surface expression. α chain binds IgE but lacks any known signaling domains. Though γ chain appears to be required for surface expression of $\alpha\beta\gamma_2$ and $\alpha\gamma_2$ forms, β chain appears to enhance consitutive surface expression of $\alpha\beta\gamma_2$.[40-42]

Both β and γ chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails and, on crosslinking of IgE:FcεRI complex, ITAMs are phosphorylated by receptor associated protein tyrosine kinases Lck/Yes novel tyrosine kinase (Lyn), Fyn, and spleen tyrosine kinase (Syk). Targets of Lyn and Syk include the activation of Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) that produces Phosphatidylinositol (3,4,5)-triphosphate (PIP3), phosphorylates Tec family kinase Bruton's tyrosine kinase (BTK), which is recruited to the membrane by PIP3, and phosphorylates the membrane-localized adapter protein linker for activation of T cells (LAT). LAT provides a docking site for

phospholipase Cγ1 (PLCγ1), which is activated by BTK and, possibly, Syk to generate diacyl-glycerol (DAG) and inositol trisphosphate (IP3). IP3 initiates endoplasmic reticulum calcium release and subsequent opening of plasma membrane calcium channels. Calcium current mobilizes endosomal machinery resulting in degranulation. DAG activates additional protein kinase second messenger systems. Grb-2 and Slp-76 adapters can bind to LAT and are the intermediaries in the initiation of Ras family GTPases Ras, Raf-1, MEK, and ERK, and ensuing mitogen-activated protein kinase (MAP Kinase) activation cascades.[40-42] (Figure 2)

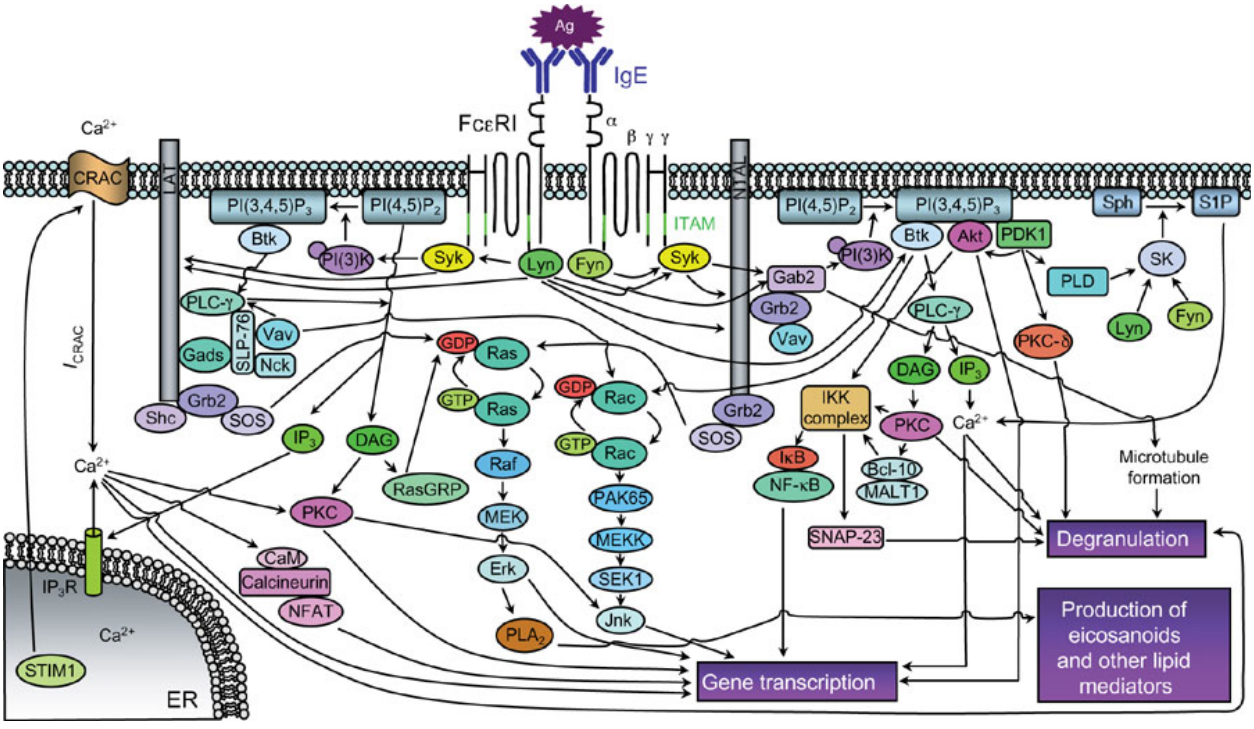

Figure 2. FcεRI signaling cascades in mast cells.

Simplified major pathways activated following crosslinking of of FcεRI on mast cell surface.[43]

Crosslinking of MC_{TC} IgE:FcεRI complexes results in (1) release of granule contents, including heparin, tryptase, histamine, cathepsin G, carboxypeptidase A3, β-hexosaminidase and pre-made TNFα, and (2) the initiation of de novo mediator production. De novo mediator production can be characterized as early production of lipid mediators, including leukotrienes, prostaglandins, and sphingosine-1-phosphate[44,45], and late production of chemokines and cytokines including IL-5, IL-6, IL-8, IL-13, TNFα, MIP-1α, GM-CSF, and more.[38] Production of specific cytokines following FcεRI signaling is controlled by different transcription factors. In mouse mast cells, NF-ƙB is responsible for IL-4 and IL-5 whereas NFAT appears to regulate IL-2 and TNFα.[46-48] In primary human intestinal mast cell, NFAT but also NF-ƙB regulate TNFα, IL-3, and IL-13 production, whereas IL-5 production is regulated by ERK1/2 activation of transcription factor AP-1.[49] Despite species-specific differences, production of some mediators can be uncoupled from Ca^{++} dependent signaling cascades and therefore from degranulation.

Mast cell responses to IgE:FcεRI crosslinking are reflected by the physiological response to allergen in humans characterized as Type 1 Hypersensitivity. The early phase of type 1 hypersensitivity rapidly follows allergen:IgE:FcεRI activation of mast cells and resolves within an hour. In skin, the early phase is characterized by a "wheal" and "flare" response to histamine released by degranulation. In the airways, histamine release produces typical rhinosinusitis symptoms and asthmatic wheezing. Some symptoms of the early phase response are due to rapid secretion of de novo generated lipid mediators. The late phase of type 1 hypersensitivity occurs about 6-8 hours after initial stimulus and lasts up to 24 hours. The late phase is attributed to de novo production of proteins, largely chemokines and cytokines, which takes

longer and results recruitment of other immune cells such as eosinophils, neutrophils, lymphocytes, and monocytes. In the skin, the late phase involves cutaneous erythema, warmth, and induration caused by an inflammatory infiltrate.[50]

Expanding roles in inflammation and immune regulation

Mast cells, like many innate immune cells, express a variety of pattern recognition receptors that induce degranulation and/or mediator production in response to bacteria, viruses, fungi, and parasites.[51,52] Mast cell response to parasites is consistent with their role as Th2 effector cells[53] but their responses to other types of infections are more recently recognized. In addition to infection, mouse mast cells are thought to play a role in regulation of graft versus host disease, transplant tolerance and rejection, autoimmune disease, and response to cancer. However, with the creation of more specific mast cell deficient mice, many of these roles have come into question. In addition, the direction of mast cell regulation, whether inflammation is enhanced or inhibited, is very dependent inflammatory model used.

Mast cell ability to recognize and response to bacteria have been explored in several settings. In one mouse model of AD where skin lesions are induced by repeat stimulation with house dust mite and superantigen, T cells and mast cells, but not B cells or eosinophils, were significantly correlated with lesion onset and severity.[54,55] This finding reflects the association between human mast cells, AD lesions, and the role of bacteria that has already been reviewed. Rodent mast cell innate anti-bacterial response and recruitment of neutrophils is shown to be important in intraperitoneal bacterial infection, either in a caecum ligation and puncture

model[56] or following direct injection of enterobacteria[57]. Aside from circumstantial evidence notes in AD, data for human mast cells involvement in antibacterial response is limited, however, cord blood derived human mast cells and the LAD mast cell line, both of which express HLA II at baseline, can phagocytose and kill Escherichia coli, consequently produce a variety of mediators, and display associated upregulation of CD80.[58] Primary human lung mast cells produce LTC4 in response to and are able to kill Streptococcus pneumonia.[59-62] Overall, in both human and mouse studies, the focus is on innate and immediate antibacterial responses rather than adaptive responses, thus mast cell ability to regulate T cell response to bacterial infection is largely unexplored.

Viral infections and their relationship to asthma led to a focus on mast cell role in antiviral immunity. Mast cell number, histamine concentration, and asthmatic symptoms increase during viral infection of airways[63,64]. In mice, poly(I:C), Newcastle disease virus, and influenza A and B virus stimulation of mast cell induce mediator production, upregulate expression of CD80, and recruitment of CD8+ T cells in WBB6F1-KitW/W-v mice (see description and problems with this model below).[65] Human cord blood derived mast cells and the HMC-1 line recruit NK cells via production of CXCL8 in response to poly(I:C) stimulation or infection with rheovirus.[66] Human CD34+ derived mast cells also produce a robust response to intracellular recognition of poly (I:C) or viral infection including type I interferons, TNFα, and IL-29, largely in the absence of degranulation.[51]

Mast cell role in autoimmune disease has diminished in importance in recent years. Two main mast cell deficient mouse models have been used to address mast cell function in vivo: WBB6F1-Kit$^{W/W-v}$ mice, that have a natural loss of function mutation in the white spotting locus which encodes c-kit, and Kit$^{W-sh/W-sh}$ (w-sash) mice, that have an inversion upstream of the c-kit locus leading to abnormal receptor expression. WBB6F1-KitW/W-v mice have several additional hematopoietic deficiencies including anemia, neutropenia, reduced basophils, and impaired γδ T cell development in the gut. W-sash mice have far fewer hematopoietic defect but are not entirely healthy: they exhibit neutrophilia, thrombocytosis, and reduced levels of IgE.[67-69] Either model can be reconstituted with wild type bone marrow cells to allow for "recovery" of mast cells in tissues.

Using these two models, mast cells were reported to regulate inflammation in the MOG_{35-55} experimental autoimmune encephalomyelitis (EAE)[70] model of multiple sclerosis and anti-glucose-6-phosphatase antibody model of autoimmune arthritis through antibody mediated activation.[70] More recently, mouse models have been developed that are not dependent on c-kit loss of function and instead utilize targeted cre-lox systems to eliminate most mast cells or particular subsets of mast cells without effecting hematopoiesis.[71] Use of newer models refutes previous findings showing equal susceptibility of mast cell deficient and wild type mice in EAE and arthritis.[72] Unlike EAE and arthritis, systemic lupus erythematosis provides a unique role for mast cells and basophils as autoreactive IgE is associated with disease severity.[73]

In humans, the association of mast cells with autoimmune disease predates most murine findings. Mast cells are almost absent in normal synovium but elevated mast cell numbers have been associated with both juvenile and adult rheumatoid arthritis; increasing cell numbers correlate with disease severity.[74-76] More recently, it is recognized that elevated mast cell number in the joint are predominantly MC_T type, that they express FcγRI and FcγRII and can be activated by IgG to degranulate, and that they colocalize with synovial IL-17.[77] Tryptase can be detected in cerebral spinal fluid of patients with multiple sclerosis (MS) but not healthy controls[78-80], mast cells numbers are elevated in MS brain and surround plaques, particularly "chronic" or large lesions, and mast cell numbers are elevated in women with MS in comparison to men with MS.[81].

More surprising than additional inflammatory roles, mast cells are now associated with inhibition of inflammation. The mechanism of UVB associated inhibition of contact sensitivity in mouse skin is mast cell dependent and requires the migration of skin mast cells to the draining lymph node. (Hart 1998)[82] In a series of elegant studies, Dr. Randolph Noelle's group has shown that T_{reg} mediated skin transplant tolerance is associated with increased mast cell numbers in the skin graft, drawn by T_{reg}. Rejection of grafts correlates with absence of mast cells and w-sash mast cell deficient mice fail to achieve tolerance. [83]His later studies (not in w-sash mice but in normal, mast cell competent mice) would show that mast cells enter into a reciprocal relationship with T_{regs} which promotes graft tolerance. Mast cell degranulation breaks that relationship and consequently the skin graft is rejected.[84] Unlike previous findings with mouse AD, a different model of allergic dermatitis implicates mast cell-T_{reg} interactions in suppression

of inflammation. Mast cells migrated from the AD lesion to the spleen and where their production of IL-2 caused an increase in T_{reg} cells.[85]

Dissertation Goals

The nature of the exploration of mast cells in tolerance is qualitatively different than that of mast cells in inflammation. In models of tolerance mast cells are not assessed as innate effector cells only but as a bridge between innate and adaptive immunity, in particular they are attributed with direct and indirect ability to regulate T cell responses. It is a placement of mast cells normally reserved for dendritic cells and an approach that has been relegated to the sidelines in studies of inflammation. In mouse models, as described below, it is incresaingly clear that mast cells are not primary initiators of T cell activation, that mast cell effect on T cells are mediated through dendritic cells, and that mast cells are unlikely initiators of naïve T cell activation and certainly do not skew naïve T cells towards a Th2 phenotype.

Prior to examining the body of work that relates to mast cell-T cell interactions, a key point must be highlighted: the majority of disease models in mice must initiate the disease de novo and run a fast course. There are multiple reasons for this pattern that range from the cost of mouse facilities to inability of mice to naturally replicate human disease. Yet sick people come to attention because, and not before, they are sick. The majority of human diseases are experienced as chronic diseases and experienced over a lifetime that, even when reduced, is

much longer than the life of a mouse, certainly longer than we, as researchers, allow a mouse to experience disease states.

The problem for humans is not only what initiates inflammation and tolerance, but what sustains it. When a viral infection can run its course, why does asthma last a lifetime? When a bacterial infection is cleared following antibiotics, why doesn't eczema, diabetes, or multiple sclerosis disappear following treatment? We have assumed that the answer lies in the continual availability of antigen and consequently the continual, if low grade, stimulation of T cell and overall immune cell response. Recent and robust data shows that low-grade antigen exposure leads to tolerance, not inflammation, that natural regulatory mechanisms (e.g. the upregulation of CTLA-4 on T cells following activation, the widespread expression of PD-L1) are plentiful, and that T effector (T_{eff}) responses are frequently suppressed by infectious organisms, cancer, and even simply the aging process.

Both projects of this dissertation are concerned with primary human mast cells as a bridge between innate and adaptive immune response, interacting with and regulating other immune cells, and promoting inflammation or tolerance through direct and indirect mechanisms. The first project presents evidence that, with the appropriate signal, primary human mast cells act as antigen presenting cells at peripheral sites. Extending beyond the realm of available evidence, it can be imagined that mast cells sustain human T memory cells and T_{eff} cells, a role that is important for vigorous immune response to infection and one that may be hijacked to sustain chronic, peripheral inflammation. The second project presents evidence that

dysregulated mast cell proliferation, as exemplified by Mastocytosis, is associated with an increase in myeloid derived suppressor cells (MDSCs), which are capable of suppressing both T cell and mast cell activation. In addition, although mouse models have significantly advanced our understanding of what mast cells *can* do, they cannot inform us as to what mast cells *actually do* in human beings. Thus this dissertation examines roles of primary human mast cells in the context of human disease.

Part I. Transformation of Human Mast Cells by IFNγ

Introduction

Mast cell interactions with T cells

Some of the earliest acknowledgement that a relationship exists between mast cells and T cells lies in the literature on mast cell subset differentiation. Mouse T cell production of IL-3 was the unknown factor in WEHI-concavalin A conditioned media that differentiates BMMCs from bone marrow. Studies of T cell deficient mice showed an absence of mucosal type mast cells.[86] Eventually, it would be recognized that human mast cells do not require IL-3 for differentiation or survival and do not express the IL-3 receptor. Regardless, the notion that T cells regulated mast cell differentiation or recruitment was pursued.

Individuals with T cell deficiency, whether acquired or congenital, had markedly decreased numbers of MC_T cells in their GI mucosa but increased mast cell numbers overall due to increased MC_{TC} density. Significant changes in mast cell subset distribution or overall burden were not see when comparing individuals with inflammatory bowel disease to healthy controls.[87] In contrast, skin biopsies from patients with scleroderma, a T cell mediated autoimmune disease, showed elevated MC_T expression when healthy skin contains almost entirely MC_{TC} cells.[87]

By the late 1990s, cellular contact between mast cells and T cells, cognate interactions, had been observed in a variety of inflammatory human diseases and responses including delayed type hypersensitivity, sarcoidosis, inflammatory bowel disease, psoriasis, atopic dermatitis, and rheumatoid arthritis.[88-95] Besides visualization in tissue sections, serum tryptase is used as a surrogate marker of mast cell burden and degranulation. In patients with chronic urticaria, tryptase and IL-2, most likely produced by T cells, are significantly elevated compared to healthy controls and correlate best in known autoimmune mediated disease.[96] Mast cells have been identified as the major source of IL-10 induced inhibitory HLA-G expression in chronic hepatitis C virus infection associated liver fibrosis.[97]

With the development of W/W-v and w-sash mast cell deficient models, examination of the effects of T cell – mast cell interactions could be conducted *in vivo*. Murine mast cells can regulate T cell inflammatory response directly or indirectly in the defense against virus infection. BMMCs express major histocompatibility Complex I (MHC I), produce IL-2, and can activate CD8+ T cell responses in an antigen specific manner in vitro and in EAE.[98] Rodent and human mast cells degranulate and produce chemokines in response to infection with Dengue virus, which, in mice, recruits natural killer cells and NK T cells into infected skin. The mast cell mediated rapid containment of Dengue virus improved recovery response in mast cell reconstituted versus deficient mice.[99] Similarly, human cord blood derived mast cells produce chemokines in response to infection with rheovirus that induced the chemotaxis of IFNγ producing CD56+ cytotoxic T cells in vitro.[49] Indirectly, murine mast cells promote dendritic cell

and Langerhan cell maturation, migration to draining lymph node, and consequently promote Th1 and Th17 cytokine production by CD4+ T cells.[100-103]

Consistent with their role in T_{reg} mediated transplant tolerance[83], BMMCs are a source of TGFβ which is required for generation of T_{reg} cells in mice and T_{regs}, in turn, are a source of TGFβ which dampens mast cell cytokine production.[104,105] Murine mast cell-T cell cross-communication is very reliant on OX40-OX40L interactions through which T_{regs} inhibit mast cell degranulation and mast cells enhance T effector (T_{eff}) proliferation.[106-108] However, in the setting of graft versus host disease, murine mast cells are able to inhibit T cell responses independently of T_{regs} and through a mechanism involving IL-10 secretion. [109]

Unfortunately, some findings from c-kit deficient mice cannot be replicated in mast cell specific Cre-lox deletion systems. For example, Mcpt5-Cre/diphtheria-toxin-lox mice do not have any deficiencies or dysregulation in skin wound healing and bleomycin induced fibrosis as is seen in c-kit deficient mice [110] Many of the noted mechanisms of mast cell-T cell interaction have yet to be assessed in mast cell deficient mice. Although neither c-kit deficient mouse strain has noted T cell compartment defects, T cell response to viral infection has not been assessed and baseline Th/T_{eff}/T_{reg}/CTL distribution comparisons are unavailable. Nevertheless, although the terms are not fully defined, it is clear that a relationship, likely a reciprocal relationship exists between mast cells and T cells.

Mast cells as antigen presenting cells

Mast cell regulation of the T cell response is widely accepted, but direct antigen presentation by mast cells to CD4+ T cells has been more controversial. Early work with *in vitro* rodent models identified major histocompatibility complex II (MHC II) expression on mast cells[111,112], but did not exclude the possibility of contaminating antigen presenting cells. Careful exclusion of such cells showed that *in vitro* derived murine mast cells do not express surface MHC II at baseline and require a combination of IFNγ and LPS or IFNγ and IL-4 to upregulate expression.[113,114] Although most data implies a necessary, if insufficient, role for IFNγ, other groups have found that MHC II expression on mast cells can be induced by GM-CSF during differentiation of BMMCs in culture and is temporally restricted to early phases of mast cell differentiation.[115] Notch ligand delta-like-1 is reported to increase BMMC and peritoneal mast cell expression of MHC II and OX40L.

Despite the phenotypic and functional differences between murine and human mast cells, several human mast cell models have corroborated murine findings *in vitro*. As some of the earliest investigators of mast cells antigen presenting capabilities, Poncet *et al* first used HMC-1 cells, a leukemic cell line with some properties of immature mast cells, and then used cord blood derived mast cells, to show that human leukocyte antigen II (HLA II) is expressed at baseline, upregulated by IFNγ, IL-4, or GM-CSF, and is able to activate CD4+ T cells via superantigen. Guadenzio *et al* showed that human mast cells derived from CD133+ peripheral blood cells in vitro express little baseline HLA DR but increase expression in response to IFNγ alone—they did not require costimulation with IL-4.[114] CD133+ IFNγ primed MCs could

stimulate CD4+ memory T cells via superantigen and such stimulation shifted cytokine production of T cells towards simultaneous production of IFNγ and IL-22, a T cell phenotype that the authors indicate are enriched in psoriatic lesions.

Though superantigen mediated activation of CD4+ T cells may be a mechanism of inflammation in some diseases, it bypasses the requirement for protein uptake, processing, HLA II loading, and peptide presentation. Utilizing peripheral blood CD34+ in vitro derived mast cells, Suurmond *et al* provide the best evidence to date that mast cells are true antigen presenting cells: CD34+ derived mast cells from HLA –DRB1*03:01 positive individuals primed with peptide from adenoviral hexon protein II or whole hexon II protein were able to activate the M2.11 T cell clone that is specific for hexon II peptide in the context of HLA-DRB1*03:01. Suurmond *et al* attempted to show that primary human CD4+ T cells, expanded pre-mast cell culture by stimulation of PBMCs with bulk antigen, could also respond to antigen primed CD34+ derived mast cells, but lacked controls showing an absent response of primary CD4+ T cells from an individual without exposure to the bulk antigens or from CD4+ T cells not previously stimulated with those antigens.[116] The bulk antigen preparation used in this setting, a combination of tetanus toxoid, tuberculin, and candida albicans, likely harbors endotoxin. Therefore, it is difficult to conclude that the CD4+ T cell response to bulk antigen was antigen specific.

Indeed, the controversy surrounding recently described basophil antigen presenting function highlights the importance of fulfilling all criteria of antigen presentation. In 2009, several murine studies indicated that basophils played a direct role in activation of Th2 CD4+ T cells.[117-

[119] Shortly thereafter those findings were questioned and attributed to the antibody mediated depletion methods used.[120,121] A transgenic basophil deficient mouse was developed and showed that basophils can amplify Th2 responses, but are dispensable for Th2 initiation and cannot take up and process antigens.[122-124] Most recently, in 2013, human basophils were shown to require a combination of IL-3, IFNγ, and GM-CSF to express low levels of HLA II but could not induce antigen specific T cell activation of proliferation due to an inability to efficiently take up and process antigen.[125,126] Thus, the question, "Can mast cells act as antigen presenting cells?" requires a robust and thorough answer.

Rational and Hypothesis

Given the evidence of mast cell sensitivity to microenvironmental signals, their strategic location at host/environment interfaces, and their hematopoietic similarity, though in a limited manner, to monocytic cells as compared to other myelocytes, this project tests the hypothesis that mast cells can act as antigen presenting cells. In particular, our data will demontrate that IFNγ is the major determinant of primary human mast cell antigen presenting function by assessing (1) mast cell HLA II, CD80, CD40, and other costimulatory molecule expression, (2) mast cell activation of T cells and the determinants of that activation, (3) mast cell ability to take up and process antigen and the mechanism(s) by which that occurs, and (4) mast cell integration of uptake, processing, and presentation of CMV antigen to CD4+ T cells.

Methods

Primary human cells

This study is conducted in accordance with the amended Declaration of Helsinki. The Human Studies Internal Review Board (IRB) at Virginia Commonwealth University (FWA 00005287) approved the protocol. Most human skin tissue is collected in a de-identified manner. For some skin tissue, and all blood collection, written informed consent is obtained from patients as per IRB approved protocols #413 and #HM14456.

Antibodies and reagents

Recombinant human stem cell factor (SCF, Swedish Orphan Biovitrum, Stockholm, Sweden), the non-competing mouse IgG1 anti-FcεRIα mAb, 22E7 (provided by J. P. Kochan, Hoffman-LaRoche, Nutley, NJ), 5D12 anti-CD40 antibody (provided by L. Boon, Bioceros, Ultrecht, Netherlands), and G28.5 anti-CD40 antibody (provided by D. Conrad, Virginia Commonwelath University, Richmond, VA) were gifts. IgE anti-NP (AbD Serotec, Raleigh, NC); 4-hydroxy-3-nitrophenylacety (NP)-BSA (Biosearch Technologies, Novato, CA); RosetteSep CD4+ T cell negative selection kit (Stemcell Technologies, Vancouver, BC, Canada) X-vivo 15 media (Lonza, Walkersville, MD); serum-free freezing media (ATCC, Manassas, VA); recombinant human IL-2, IFNγ, IL-4, IL-17, and IL-6 (Peptrotech; Rocky Hill, NJ); Brefeldin A (BfA); phorbol 12-myristate 13-acetate (PMA); Ionomycin (I); FcBlock (Miltenyi Biotech); antibodies against IFNγ-PE, IL-4-APC, IL-17-PerCP, CD3-PeCy7, CD3-PE, CD69-APC, CD25-PerCP, CD117 biotin, and PeCy7-streptavidin (Biolegend, San Diego, CA); carboxyfluorescein diacetate succinimidyl ester (CFSE) and Far-red Live Dead Kits (Life technologies, Grand Island, NY); FoxP3 intracellular staining kit

(eBiosciences, San Diego, CA); and inactivated human cytomegalovirus virus (CMV) lysate and control lysate (Advanced Biotechnologies, Columbia, MD) were obtained and used as described.

Mast Cell Isolation and Purification

Fresh samples of human post-surgical skin are obtained from the Department of Pathology at Virginia Commonwealth University, Cooperative Human Tissue Network of the National Cancer Institute, or Richmond Plastic Surgeons. The tissues are digested at 37°C with collagenase type 2 (1.5 g/L, Worthington, Lakewood, NJ), hyaluronidase (0.7 g/L), and DNase type 1 (0.18 g/L, Sigma, St. Louis, MO, USA) for three consecutive 1 hour incubations in modified Hank's Balanced Salt Solution (HBSS) buffer (HBSS with 0.04% $NaHCO_3$, 1% fetal bovine serum, 1% HEPES, 0.1% $CaCl_2$) containing amphotericin B (0.5%) and antibiotic/antimycotic solution (1%, Sigma). After digestion, the samples are filtered through 70- then 40-µm nylon cell strainers, washed, and separated on a Percoll gradient. Cells at the buffer/Percoll interface are collected, washed, and re-suspended at 5×10^5 cells/ml in X-VIVO 15 media (Lonza, Walkersville, MD) containing SCF (100 ng/ml) and cultured in 24-well plates with weekly medium changes.

Mast cell degranulation and degranulation assays

Mast cells can be induced to degranulate via IgE receptors through several methods.[30,127-129] Use of a particular method is selected according to downstream applications. 22E7 induced degranulation is not possible when when labeling with an anti-mouse IgG1 secondary flourescent antibody. If such labeling was necessary, mast cells were incubated overnight with IgE anti-NP (1 µg/mL), excess/unbound IgE was washed away, and degranulation was induced with NP-BSA (10 ng/mL).[128] Because IgE anti-NP is a chimera of mouse variable regions linked to

human IgE constant regions, anti-mouse Fcγ specific secondary flourescent antibodies must be used.

Similarly, measurement of degree of degranulation is selected according to upstream applications. When mast cells alone were degranulated, a standard β hexoseaminidase assay was used to determine % degranulation as previously described. However, as T cells also produce β hexoseaminidase, assessment of degranulation in co-culture supernatants required the use of G4 total tryptase ELISA as previously described.[130]

T cell isolation and storage

Jurkat cells are a gift from Dr. David Straus (VCU). Peripheral blood primary CD4+ T cells are isolated by RossetteSep purification according to manufacturer's instructions. In brief, blood is incubated with RosetteSep CD4+ T cell enrichment cocktail (Tetrameric Antibody Complexes recognizing CD8, CD16, CD19, CD36, CD56, CD66b, TCRγ/δ and glycophorin A) for 20 min at room temperature then dilute two fold with 2% Fetal Bovine Serum in Phosphate Buffered Saline. Dilute blood is layered over RosetteSep DM-L density medium and centrifuged. Enriched cells (>95% CD4+, data not shown) are removed from DM-L/plasma interface and washed. T cells are cryopreserved in serum-free freezing media then thawed and rested overnight in X-VIVO 15 containing recombinant human IL-2 (20 U/mL) prior to use. For HLA II matched cocultures, blood and skin tissues are collected from the same individual.

Superantigen co-cultures

Mast cells are stimulated for 2 days with IFNγ (10 ng/mL) in the presence of Soy Bean Trypsin Inhibitor (100 µg/mL, SBTI) or medium alone with SBTI, washed extensively, then put into coculture with Jurkat cells or CD4+ T cells. Mast cell-Jurkat cocultures are stimulated with varying concentrations of superantigens Toxic Shock Syndrome Toxin-1 (TSST) or Stapylococcus Enterotoxin E (SEE) for 2 days.

To assess effects of endogenous IFNγ production by T cells, CD4+ T cells are sorted according to CXCR3 expression into CXCR3+ and CXCR3- populations then cocultured with mast cells with or without TSST (10 ng/mL) stimulation for 4 days. In addition, non-sorted, CD4+ primary T cells were labelled with CFSE (2.5 µM) according to manufacturer's instructions then cultured for 4 days with mast cells in the presence of TSST (10 ng/mL) alone, in the presence of IFNγ neutralizing antibody (100 µg/mL), IFNγR blocking antibody (100 µg/mL), both, or isotype control MOPC (100-200 µg/mL as appropriate).

CMV co-cultures

Inactivated lysates of cytomegalovirus (CMV) infected cells and control cells are added to mast cells for 3 days, the last 2 of which are with SBTI and with or without IFNγ (10 ng/mL). The four groups are designated Cont'MC_{TC}, CMV'MC_{TC}, IFNγ'Cont'MC_{TC}, and IFNγ'CMV'MC_{TC}. As experimental controls, CMV lysate is added to T cells alone (designated $CD4^+$ T + CMV) and mast cells are cocultured with T cells in the presence of TSST (designated MC_{TC} + TSST).

For experiments assessing effects of degranulation, mast cells are incubated with CMV lysate and IFNγ as above, then stimulated for 30 min with 22E7, an antibody that crosslinks FceRI, or isotype control mIgG1. Degranulated and control IFNγ'CMV'MC_{TC}s are washed extensively and returned to culture with fresh SBTI for 2 hours as a precaution against residual protease release.

As inhibitors of antigen uptake can cause cell death over extensive periods of time, a different protocol was used to assess their ability to alter subsequent T cell proliferation. Cell viability was examined after 4, 6, 8, and 12 hours of incubation with uptake inhibitors 5-(N,N-Dimethyl)amiloride hydrochloride (DMA) and Dynasore alone and in combination. Initial slight decrease in viability was detected at 8 hours and higher cell loss was detected at 12 hours by trypan blue exclusion. Thus, mast cells were incubated for 6 hours with CMV lysate, with or without the presence of uptake inhibitors, then washed extensively and incubated with or without IFNγ, in the presence of SBTI, for 2 days.

Following any of the above priming protocols, mast cells are washed extensively and placed into coculture with HLA matched CFSE labelled CD4+ T cells at a 1:2 mast cell:CD4+ T cell ratio. For assessment of inhibitor effects, T cell proliferation was assessed after 6 days. For all other cocultures, T cell proliferation and cytokine production were assessed after 7 days.

Statistics

All data assessed by Shapiro-Wilks test for normal distribution. Thereafter the appropriate parametric or non-parametric statistical test was used as noted. If the same mast cell source was assessed in multiple conditions, paired t test or paired ANOVA testing was used. All data was analyzed with SigmaPlot software (Systat). Unless otherwise notes, $\alpha = 0.5$.

Results

MC_{TC} cells do not express HLA DR at baseline but upregulate its expression in response to IFNγ

Mast cells express IFNγ receptor (Figure 3) and increase FcγRI surface expression in response to IFNγ stimulation.[131] IFNγ drives transcription of transcription factor CIITA, a master regulator of HLA II and associated genes, and is responsible for upregulation of MHC II on murine mast cells, human mast cell lines HMC-1 and LAD-1/2, and cord blood derived human mast cells. Initially, incubation of MC_{TC} cells with varying concentrations of IFNγ did not produce robust upregulation of HLA DR. However, the addition of SBTI increased response to IFNγ ten fold indicating IFNγ susceptibility to mast cell produced cathepsin G or chymase. (Figure 4) This is likely an artifact of culture as *in vivo*, chymase and cathepsin G are inhibited by an excess of natural protease inhibitors, diffuse away when not restrained by plastic walls and are released mainly via degranulation.[44] Therefore, all experiments hereafter are conducted in the presence of SBTI (100 ng/mL).

HLA DR expression is significantly elevated with IFNγ concentrations as low as 100 pg/mL (Figure 4). Considering that serum IFNγ concentration is roughly 50 pg/mL[132] in healthy

individuals, that mast cells are frequently located near blood vessels[133], and that a subset of perivascular mast cells sample blood vessel contents, it is likely that mast cells are exposed to IFNγ *in vivo*. Indeed, freshly isolated mast cells express low levels of HLA DR (Figure 5A) though neither effects of the isolation procedure nor aberrant c-kit expression on other myeloid cells can be ruled out. For almost all mast cell sources tested after 1.5 to 2 months in culture, HLA DR detection is nearly equivalent to that of isotype control but is considerably increased after 3 day stimulation with 10 ng/mL IFNγ (Figure 5B). In addition, Dr. Brant Ward could not detect intracellular HLA DR expression by immunohistochemistry in the absence of IFNγ stimulation. HLA DR, once expressed, appears to be very stable, and though levels decrease over time they remain significantly elevated compared to unstimulated MC_{TC} cells up to 3 days after IFNγ is washed away. (Figure 6)

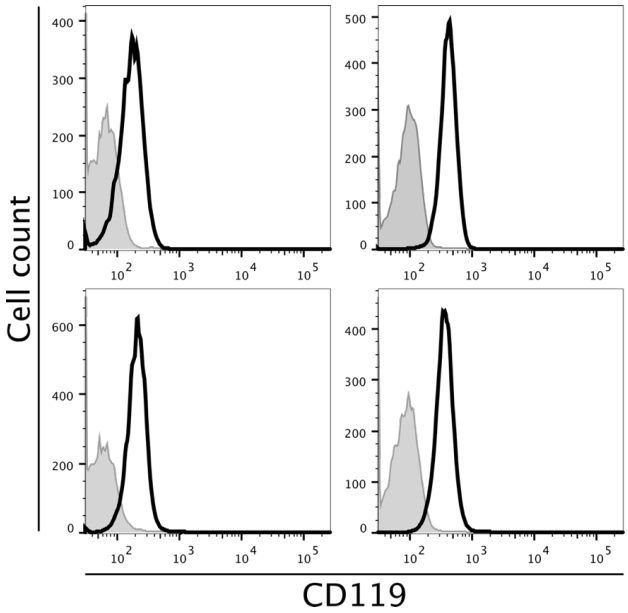

Figure 3. MC_{TC} cells express IFNγ Receptor.

MC_{TC} cells were labeled with anti-CD119 (IFNγRα chain) antibody (black line) or isotype control (shaded gray), followed by biotinylated goat anti-mouse IgG, then PE-streptavidin. Each histogram represents a different source of mast cells. Geometric mean fluorescence intensity (MFI) was calculated for isotype control and subtracted from that of CD119 labelled cells to determine net MFI for each sample. On average, net MFI of MC_{TC} expressed CD119 was 229 with 95% confidence interval between 58 and 400.

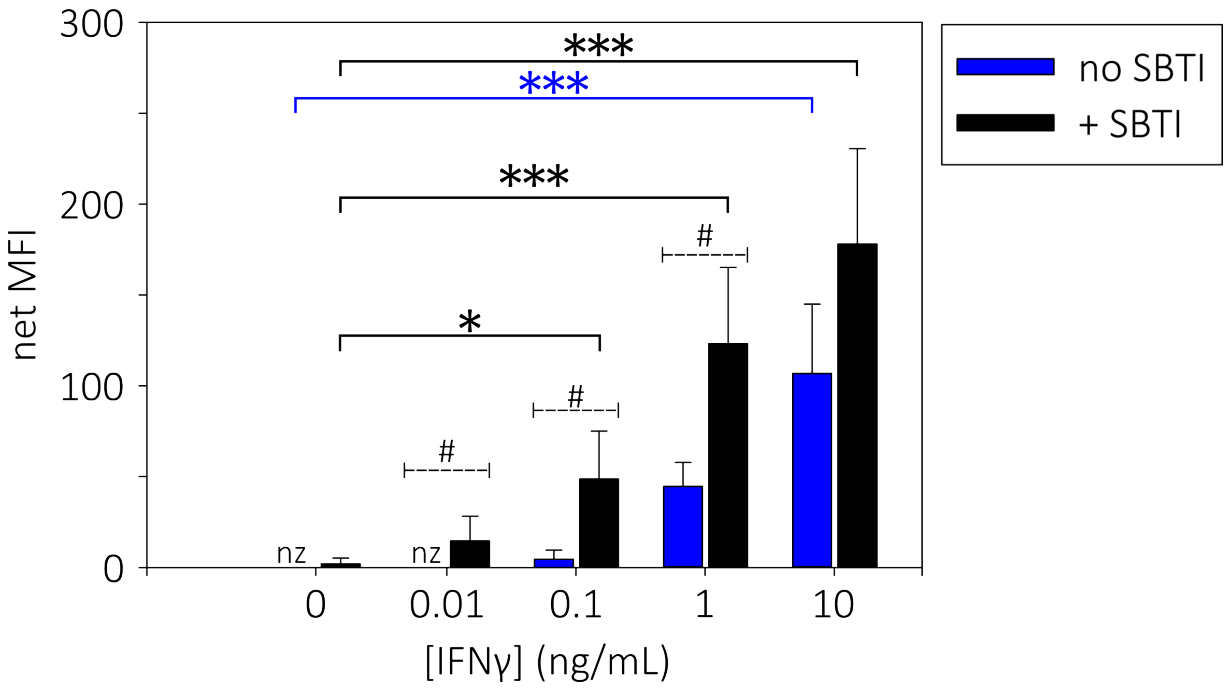

Figure 4. IFNγ induced HLA DR expression is augmented in the presence SBTI.

MC_{TC} cells stimulated for 3 days with IFNγ at concentrations above ± soy bean trypsin inhibitor (SBTI) (100 ng/mL), labeled with anti-HLA DR antibody or isotype control, and assessed by flow cytometry. Geometric mean fluorescence intensity (MFI) was calculated for each sample. Net MFI was calculated by subtracting isotype control MFI from that of labelled cells for each condition. Mean ± one standard deviation is shown. Net zero (nz) indicates net MFI ≤zero. *#,*

p<0.05, two tailed Students' t test comparison of a given [IFNγ] ± SBTI. For +SBTI (black bars/ stars) or –SBTI (blue bars/stars), mean HLA DR at each [IFNγ] was assessed by repeated measures ANOVA followed by Bonferroni's comparison to 0 ng/mL IFNγ for each. **,p<0.05, ***,p<0.001*

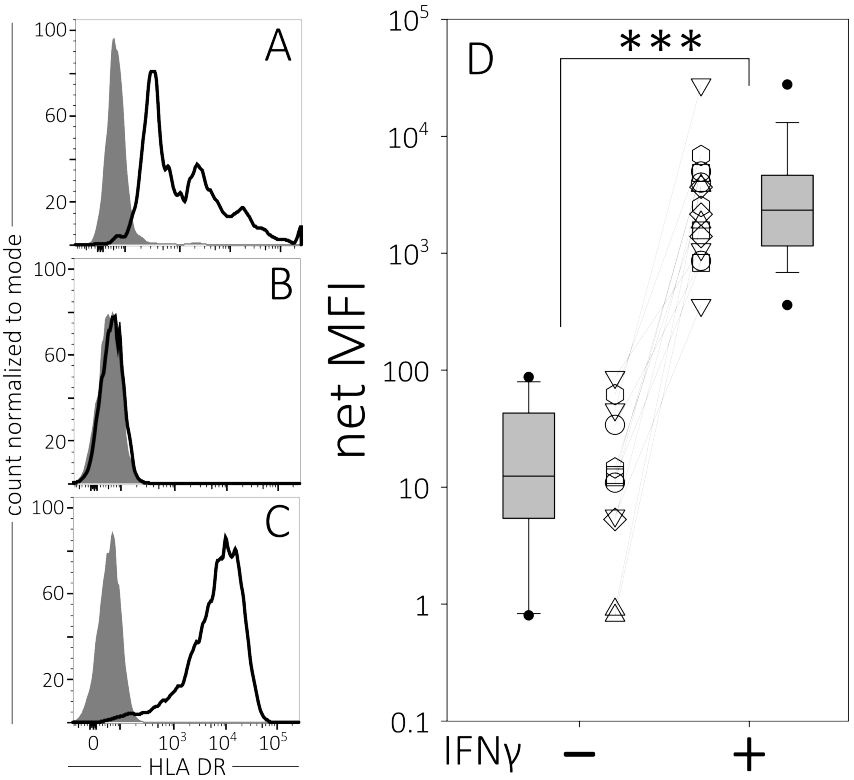

Figure 5. MC_{TC} HLA DR expression is lost during culture but recovers with IFNγ stimulation.

HLA DR (black line) expression of compared to isotype control (shaded gray) for *A*, freshly isolated CD117+ gated skin cells, *B*, after 6 weeks in culture, and *C*, following 3 day stimulation with IFNγ (10 ng/mL). MC_{TC} cells were labeled with anti-HLA DR antibody or isotype control (mIgG2b) followed by isotype specific secondary antibody. For freshly isolated skin cells, HLA DR

staining was combined with anti-CD117 biotin antibody (mIgG1) followed by streptavidin secondary. *D*, MC_{TC} cells were stimulated ±IFNγ stimulation, as *B* and *C*, for 3 days. Data was not normally distributed. *n=16, ***, p<0.001, Wilcoxon Signed Rank test*

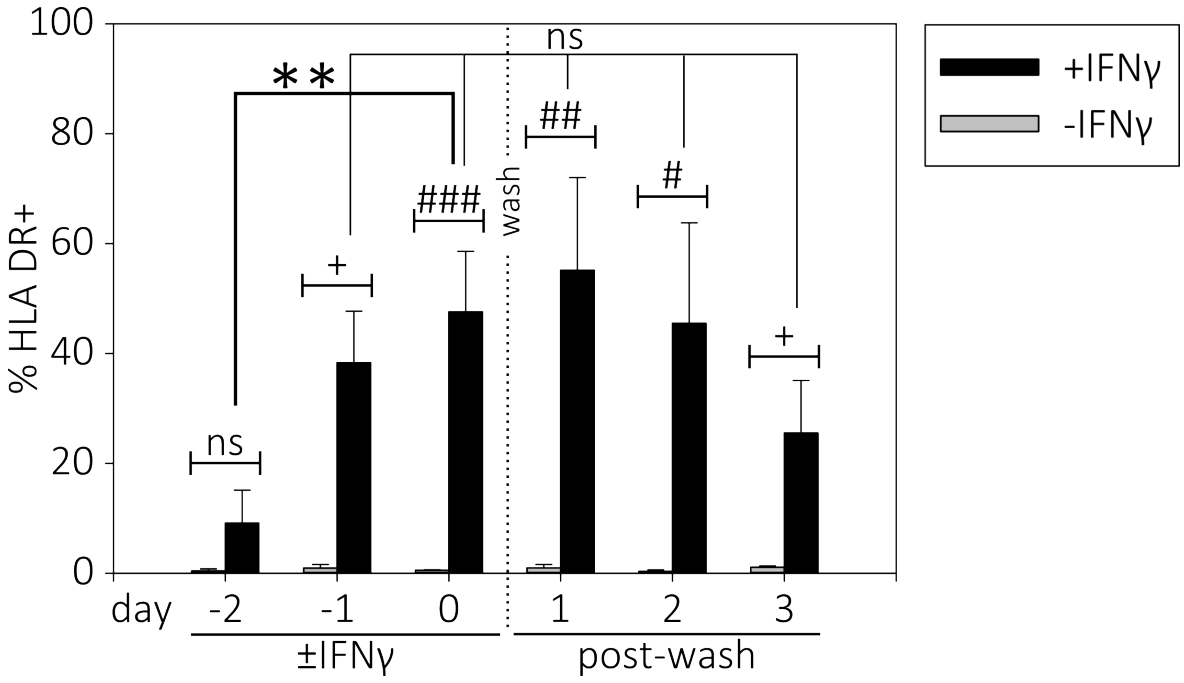

Figure 6. MC_{TC} HLA DR expression is sustained after IFNγ is removed.

MC_{TC} cells were incubated ±IFNγ (10 ng/mL) for 3 days, then thoroughly washed and returned to culture without IFNγ (post wash). Percent HLA DR positive cells, of gated mast cells, was assessed each day. Data shown is mean ± one standard deviation. +IFNγ and –IFNγ means are compared for each day. Paired t test was used for days -2, -1, and 3 as all data came from the same 3 sources and both +IFNγ and –IFNγ groups had an *n=3*. On days 0, 1, and 2, *n=6* for +IFNγ and *n=3* for –IFNγ. *+, p<0.05* with paired Students' t test. *#, p<0.05, ##, p<0.01, ###, p<0.001* with Students' t test. %DR+ means for the +IFNγ group was compared across all days with one

way ANOVA followed with Bonferroni's post-test comparison to day 0. Mean %DR+ was not significantly different from day 0 on any day but day -2 (***, p<0.01).*

Although baseline HLA DR expression on cultured MC_{TC} cells is undetectable by flow cytometry, it is possible that low amounts of HLA DR are expressed below our detection limit. To address this possibility, mast cells, primed (IFNγ'MC_{TC}) or un-primed (MC_{TC}), are cultured with the Jurkat T cell line in the presence of superantigens Staphylococcus Enterotoxin E (SEE) or Toxic Shock Syndrome Toxin (TSST). Superantigens link HLA II molecules to variable region of the β chain (Vβ) of T cell receptors (TCRs). By binding outside of the HLA peptide-binding groove, superantigens induce polyclonal activation of up to 30% of all T cells. Superantigen specificity is determined by binding affinity for the Vβ region of the TCR; thus, SEE binds TCRs with Vβ 5.1, 8, 16, 18, 21.3 whereas TSST binds Vβ 2[134]. Unlike primary T cells, clonal T cell lines by definition have the same Vβ region. In the case of Jurkat cells, their TCR, which has a Vβ 8, can be bound by SEE but not TSST[135]. This characteristic makes Jurkat cells a ready avenue by which any HLA DR expression can be amplified and made measureable. Only IFNγ'MC_{TC} cells are able to activate Jurkat cells, as measured by CD69 expression, via SEE. (Figure 7) Thus, MC_{TC} cell HLA DR is not only undetectable by flow cytometry in the absence of IFNγ stimulation, it is functionally absent as well.

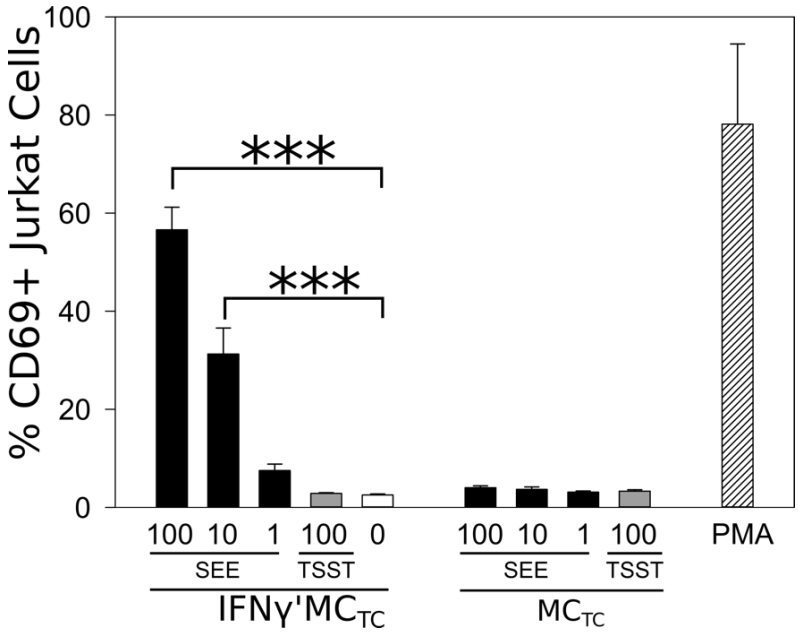

Figure 7.Only IFNγ'MC$_{TC}$ cells, not un-primed MC$_{TC}$, can activate Jurkat cells via Staphylococcus Enterotoxin E (SEE).

MC$_{TC}$ cells were incubated for 3 days ±IFNγ (IFNγ'MC$_{TC}$ or MC$_{TC}$), then thoroughly washed and put into coculture with Jurkat cells in the presence of SEE or toxic shock syndrome toxin (TSST) at various concentrations. Jurkat cells were stimulated with phorbol 12-myristate 13-acetate (PMA) to serve as a positive control for Jurkat activation. Cells were assessed after 2 days of coculture by flow cytometry for activation as defined by percent of CD3+ cells that were CD69^{high}. ****, p<0.001, repeated measures ANOVA followed with Bonferroni's post test multiple comparison to IFNγ'MC$_{TC}$ with Jurkat cells in the absence (0) of superantigen. n=3, mean ±one SE.*

As reviewed earlier, other mediators have been reported to increase HLA DR expression on various mast cell models. To address these possibilities, MC_{TC} cells were incubated with a variety of stimulants, with or without IFNγ, and HLA DR expressed was assessed. Neither IL-4, IL-13 (not shown), IL-6 (not shown), IL-17 (not shown), thymic stromal lymphopoietin (TSLP), lipopolysaccharide (LPS), nor CpG stimulation upregulated surface HLA DR expression alone. IL-4, TSLP, LPS, and CpG also did not augment IFNγ mediated increases in HLA DR. (Figure 8) IFNγ co-stimulation with TLR2 agonist Heat Killed *Listeria Monocytogenes* (HKLM) did enhance MC_{TC} HLA DR expression compared to IFNγ stimulation alone but did not induce HLA DR expression alone. (Figure 9A) Finally, as HLA DR colocalized with granule proteases shortly after IFNγ stimulation, prior to significant surface expression, degranulation modestly increased HLA DR surface expression. (Figure 9B)

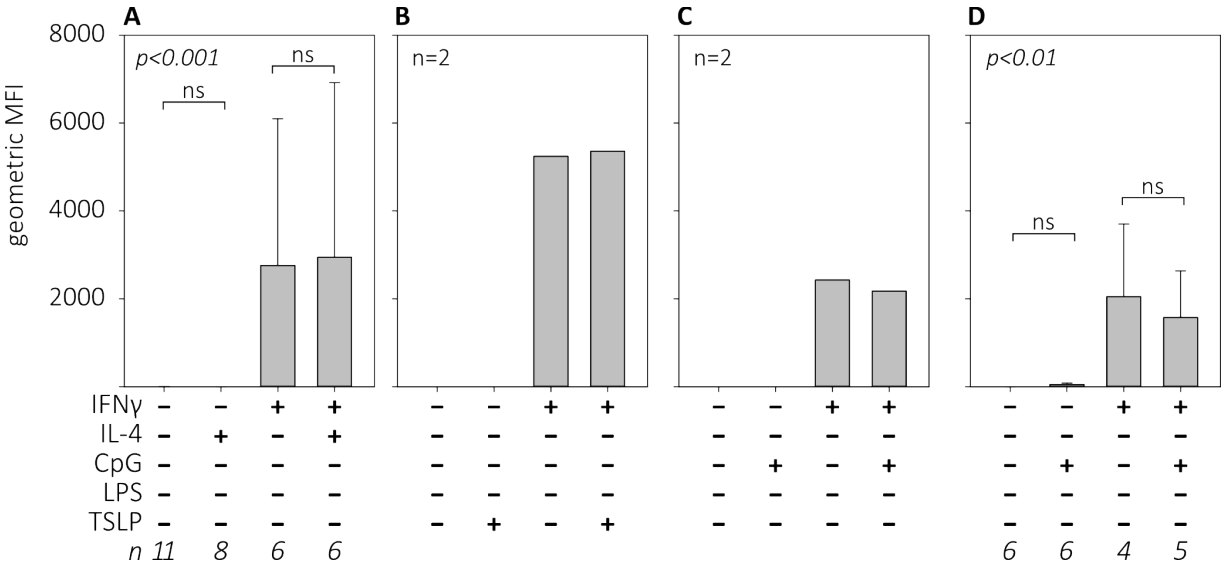

Figure 8. IFNγ induced HLA DR expression on MC_{TC} cells is not altered by co-stimulation with IL-4, TSLP, CpG, or LPS.

MC_{TC} cells were incubated for 3 days ±IFNγ (10 ng/mL) and ***A,*** ±IL-4 (100 U/mL), ***B,*** ±TSLP (100 ng/mL), **C,** with CpG or control oligo (10 µg/mL), and ***D,*** ± LPS (50 ng/mL). Cells were labelled for HLA DR expression and assessed by flow cytometry. Bar graphs show mean ± one standard deviation. For ***A*** and ***D***, one-way analysis of variance was conducted to evaluate the relationship between stimulation conditions and HLA DR expression (*p* and *n* values shown above) followed by Dunn's multiple comparison post-test. In both ***A*** and ***D***, all pairwise comparisons were significantly different *except* for those marked "ns", or not significant.

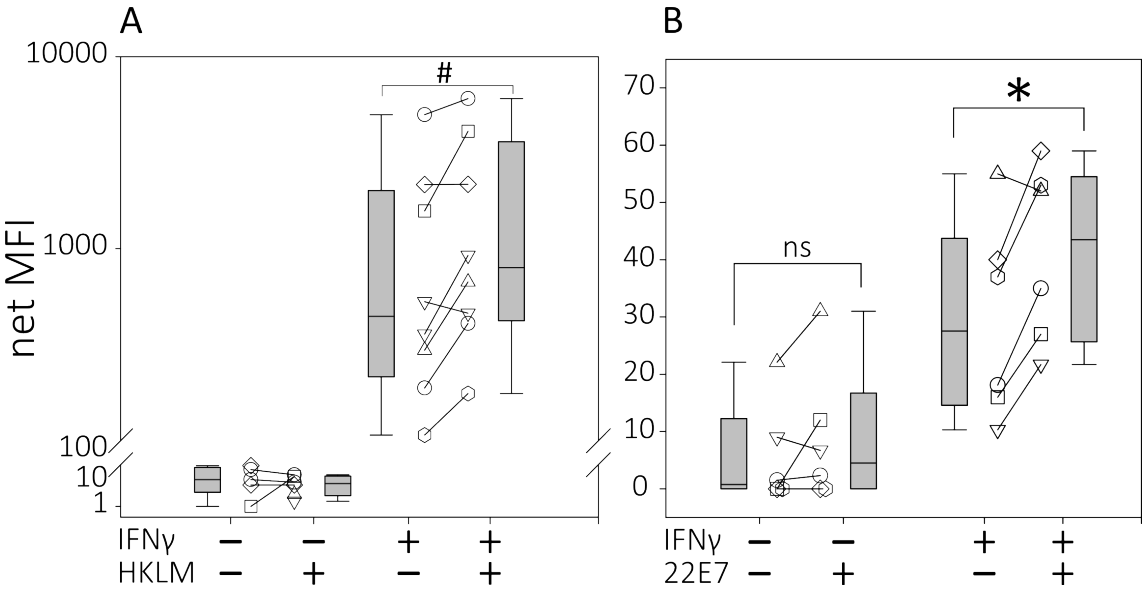

Figure 9. IFNγ induced HLA DR expression on MC_{TC} cells is augmented by HKLM or FcεRI-mediated degranulation.

A, MC_{TC} cells were incubated for 3 days ±IFNγ (10 ng/mL) ±HKLM (10^8 equivalents/mL) then assessed for net MFI of surface HLA DR expression as described. Data was not normally distributed and Wilcoxin signed rank test was used to assess for difference in mean net MFI between IFNγ only and IFNγ+HKLM groups. *n=8, #, p<0.05 B*, MC_{TC} cells were incubated for ±IFNγ (10 ng/mL) and then stimulated with 22E7, an antibody that crosslinks FcεRI and causes degranulation, or with an isotype control. Data was normally distributed and Students' paired t test was used to assess for difference in mean net MFI between IFNγ only and IFNγ+22E7 groups. *n=6, *, p<0.05*

IFNγ regulates surface ligands involved in antigen presenting cell-T cell interactions

Naïve T cells require 2 signals for activation; an HLA II mediated signal through the T cell receptor and a B7 family (CD80/86) costimulatory signal through CD28. Primarily recognized for their required presence during activation of naïve T cells, signal 2 ligands are increasingly recognized for their role in sustaining the activity of effector and memory T cells and preventing their apoptosis or senescence.[136-138] CD80 and CD86 are thus considered hallmark markers of antigen presenting cells. MC_{TC} cells express low levels of CD80 at baseline and increase its expression when stimulated with IFNγ. (Figure 10) However, MC_{TC} cells neither express baseline CD86, as is noted with monocytes, nor upregulate it in response to IFNγ, as is observed with monocytes and dendritic cells.[139]

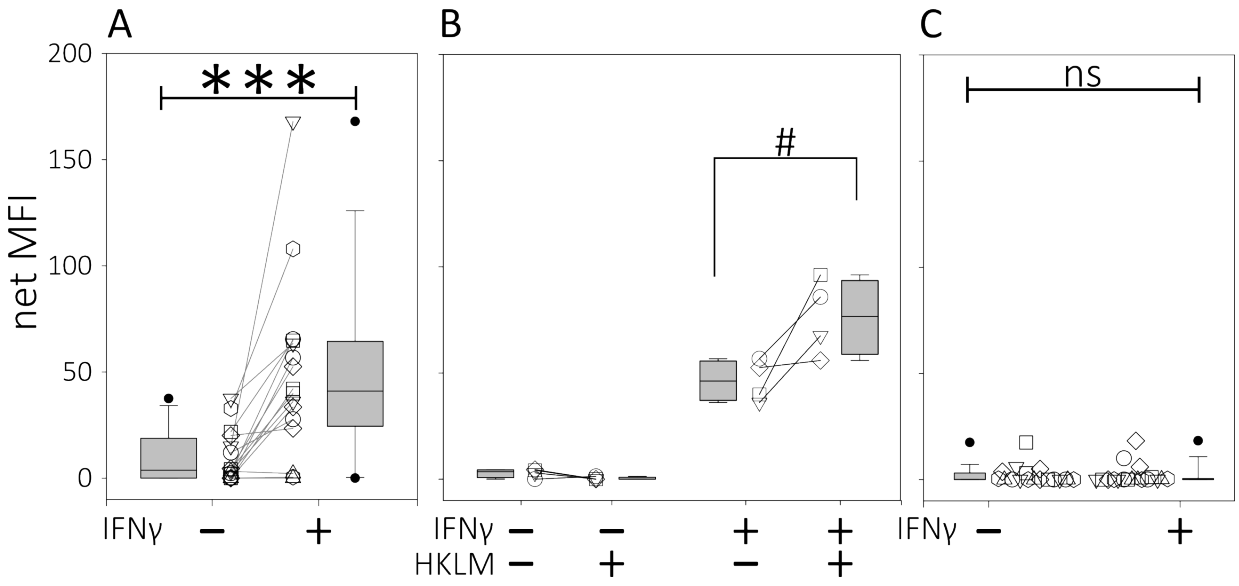

Figure 10. MC_{TC} cells express CD80, not CD86.

A, MC_{TC} cells were stimulated ± IFNγ (10 ng/mL) for 3 days then assessed for surface expression of CD80 by flow cytometry. Net MFI is calculated as described. *n=16, ***, p<0.001, Wilcoxon*

signed rank test. B, MC_{TC} cells were stimulated ± IFNγ (10 ng/mL) ± HKLM (10^8 equivalents/mL) for 3 days and assessed for surface CD80 expression. Mean net MFI of IFNγ versus IFNγ + HKLM stimulation groups is compared by one tailed paired Students' t test. *n=4, *, p<0.05 C,* MC_{TC} cells were stimulated as in *A* then assessed for CD86 surface expression. *n=18*, mean net MFI of the two groups are not significantly (ns) different, *Wilcoxon signed rank test*

The B7 family are involved in T cell activation, proliferation, and ultimately function. Timing is key as B7 binding partners are differentially upregulated on the T cell surface following activation: for example CD28 is present prior to activation and Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4 or CD152), a higher affinity binding partner for CD80 and CD86, is expressed after activation. CD80 and CD86 binding to CTLA-4 induce an inhibitory signal that provides a natural damper on T cell responses. Activated T cells also upregulate Programmed Death 1 (PD-1 or CD279) which can be bound by B7 family member Programmed Death –Ligand 1 (PD-L1 or CD274), providing another inhibitory signal. PD-L1 signaling appears to be more nuanced as inhibitors of PD-1/PD-L1 interactions used in treatment of cancer patients have a very different, and largely better tolerated, safety profile than CTLA-4 inhibitors.[140]

Although CTLA-4 and PD-1 signaling is largely characterized as inhibitory, certain subsets of T cells constitutively express these ligands and require signaling through these ligands for proper function. T follicular helper (T_{FH}) cells constitutively express PD-1 and require PD-1 signal for adequate IL-21 production during induction of plasma cells. Similarly, T regulatory (T_{reg}) cells constitutively express CTLA-4 and CTLA-4 signaling does not inhibit their proliferation.[141]

The function of B7 family ligands is context specific and often controversial, but given their significant role in regulating T cell responses, their expression on MC_{TC} cells was assessed. Primary MC_{TC} cell expression of ICOS-L varies widely by source, but is largely absent on most mast cells, with or without IFNγ stimulation. As previously reported on murine bone marrow derived mast cells, MC_{TC} cells express PD-L1 and PD-L2. Both PD-1 ligands are ubiquitously expressed but there are some subtle differences. For example, in contrast to MC_{TC} cells, human blood monocytes do not express PD-L2 without IFNγ stimulation.[142] In mice, PD-L1 and PD-L2 are linked to differential regulation of Th1 or Th2 CD4+ T cells though no such evidence has been reported in humans. Thus, although PD-1 ligands are intriguing, their role in MC_{TC} –CD4+ T cell interactions is left largely un-explored. Finally, B7-H3, a newly recognized B7 family protein, is expressed at baseline on MC_{TC} cells and its expression is not altered by IFNγ stimulation. (Figure 11)

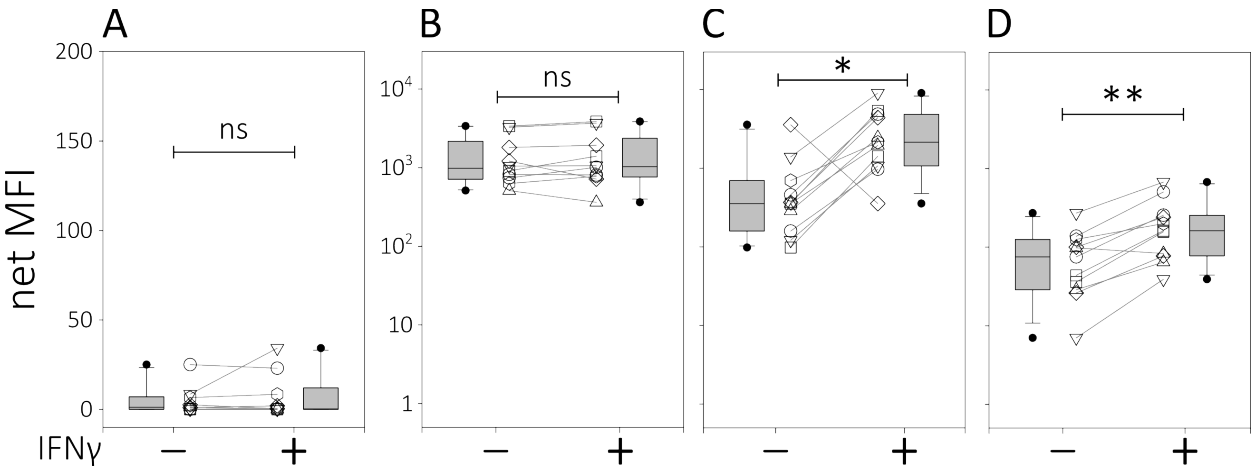

Figure 11. MC_{TC} cells express B7 family ligands including PD-L1, PD-L2, and B7-H3 but not ICOS-L and increase PD-L1 and PD-L2 expression with IFNγ stimulation

MC_{TC} cells were incubated for 3 days ±IFNγ (10 ng/mL) then assessed for *A*, ICOS-L, *B*, B7-H3, *C*, PD-L1, and *D*, PD-L2 surface expression. Data is not normally distributed, **, p<0.05, **, p<0.01, Wilcoxon signed rank test.*

Antigen presenting cell interactions with T cells are multifaceted, bidirectional, and involve many more ligand-ligand interactions than those of the B7 family alone. Two TNF/TNFR family proteins are of particular interest: CD40/CD40L and OX40/OX40L. CD40/40L interactions are well characterized and integral to antigen presenting cell-T cell interactions. CD40L is upregulated on T cells following initial activation by APCs. Activation of CD40 further matures the APC[143], increasing HLA II, CD80, and CD86 expression, increases APC innate recognition receptor expression such as TLRs[144], and enhancing APC cytokine production, particularly production of IL-12[145]. IL-2 is not only a survival and growth factor for Th1 T cells, it also increases T cell CD40L expression, completing the feedback circuit between APC and T cell that

is necessary for proper amplification of T cell responses.[146] CD40/40L interactions are also integral to CD4+ regulation and activation of CD8+ CTLs[147,148], to Th17 differentiation of naïve CD4+ cells[144], to T_{reg} homeostatis[149], and to CD4+ T cell proliferation and cytokine production in general.[150,151]

CD40 expression has not been reported on human mast cell lines or murine mast cells. In fact, CD40 *Ligand* expression has been reported on human mast cell lines, but not bone marrow derived mast cells, and thought to be important for induction of IgE producing plasma cells.[152,153] On the other hand, OX40-ligand, but not OX40, is reported on human cord blood derived mast cells[154], cultured primary human tonsillar mast cells[106], and bone marrow derived murine mast cells[107]. OX40-ligand is responsible for tonsillar and bone marrow derived mast cell induced T cell proliferation post mast cell degranulation.[106,107]

In contrast to findings reported in the literature, primary MC_{TC} cells do not express OX40L or OX40 pre- or post- FcεRI-triggered degranulation, but instead express CD40 when stimulated by IFNγ. (Figure 12) With the intent of blocking CD40/CD40L interactions during coculture, two CD40 specific antibodies were used to further characterized CD40 function on MC_{TC} cells. G28.2 is an activating antibody of mouse mIgG1 subclass. Activating antibodies are thought to function by crosslinking cell surface CD40 though it is not clear if CD40L itself functions through the same mechanism. 5D12, mIgG2b subclass, on the other hand, is one of the few available CD40/CD40L interaction blocking antibodies with little intrinsic CD40 activating properties.[155]

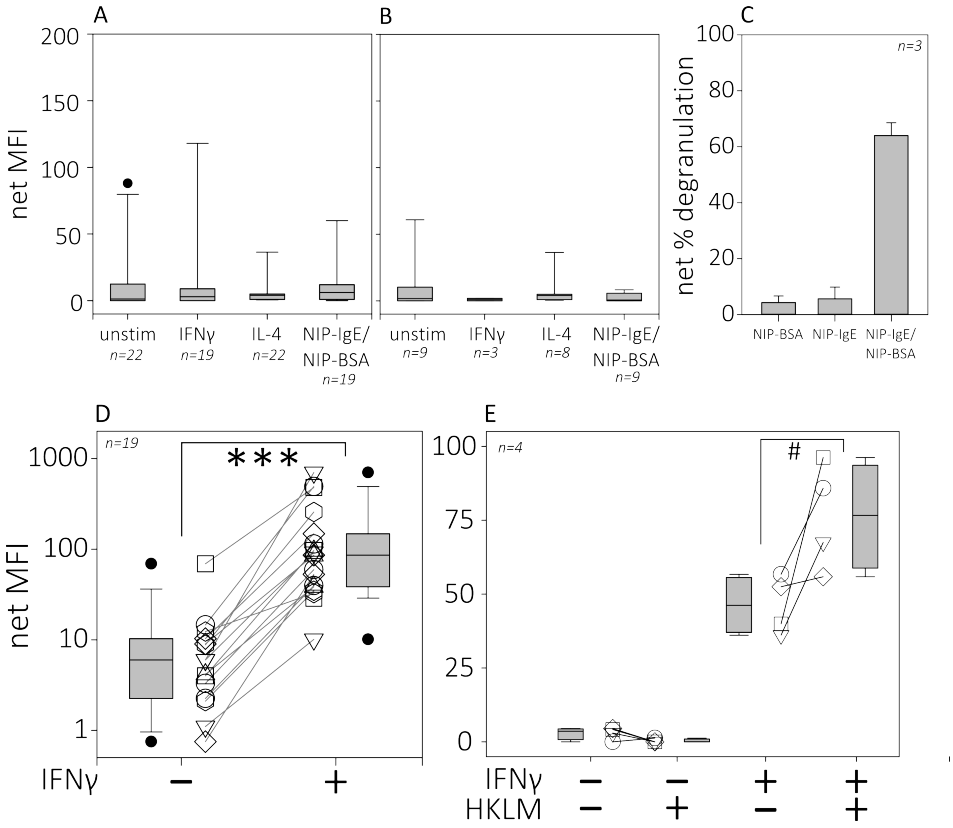

Figure 12. MC_{TC} cells do not express detectable OX40, OX40L, or CD40 but upregulate CD40 when stimulated.

MC_{TC} cells were incubated ±IFNγ (10 ng/mL) or ± IL-4 (10U/mL) for 3 days or incubated with anti-(4-hydroxy-3-iodo-5-nitrophenyl)acetyl IgE (NIP-IgE, 1 µg/10^6 cells/mL) overnight then stimulated with 4-Hydroxy-3-iodo-5-nitrophenylacetyl hapten is conjugated to bovine serum albumin (NIP-BSA, 100 ng/mL) for 30 min. Surface expression of OX40 (*A*) and OX40 (*B*) was assessed by flow cytometry. Net MFI was calculated relative to source and condition specific isotype control fluorescence. *n* for each condition is noted at x-axis. Mean net MFI for each condition was compared by *Kruskal-Wallis One Way Analysis of Variance on Ranks* and showed no significant differences between groups. *C*, Net % degranulation was calculated for a subset

of cells used in *A* and *B* by βhexoseaminidase activity assay to confirm . *D*, MC_{TC} cells were incubated ±IFNγ (10 ng/mL) for 3 days. Surface CD40 expression was assessed by flow cytometry as described. ****, p<0.001, Wilcoxon Signed Rank Test, n=19.E,* A subset of MC_{TC} cells from *D* were stimulated ±HKLM (10^8 equivalents/mL) and ±HKLM±IFNγ during the 3 day incubation. *#, p<0.05, one tailed paired Student's t test. n=4.*

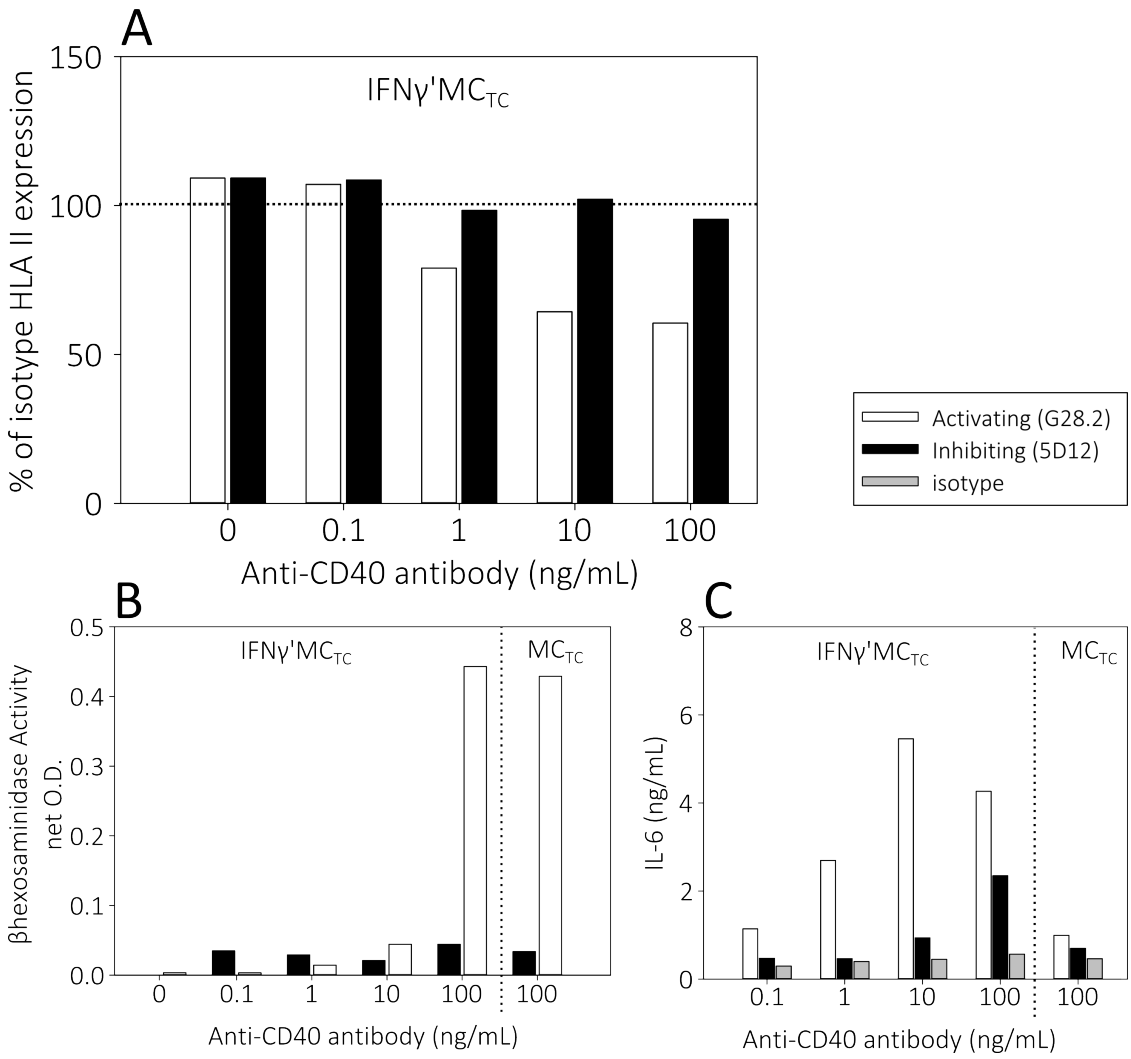

Figure 13. CD40 activation on MC_{TC} cells induces IL-6 production but decreases HLA II expression.

MC_{TC} cells were incubated ±IFNγ (10 ng/mL) ±G28.2 or 5D12 or isotype control antibodies for 3 days. G28.2 is a potent CD40 activating antibody and 5D12 has been shown to be an effective inhibitor of CD40-Cd40L interactions. However, 5D12 is documented to cause cell activation, via CD40 or other mechanisms, at concentrations ≥100 ng/mL. Both cells and supernatants were collected. *A*, MC_{TC} cells were assessed for HLA II expression by flow cytometry, *n=1. B*, Supernatants were assessed for β-hexosaminidase (βhex) activity. Net activity was calculated by subtracting isotype control activity at each antibody concentration. βhex activity correlated with cell source and not antibody concentration or whether isotype control used was IgG1 or IgG2b. n=2 and mean (CI) isotype βhex activity for each MC_{TC} source was 0.06 (0.006) and 0.14 (0.003).

Surprisingly, addition of G28.2 in the presence of IFNγ decreased HLA II expression relative to respective isotype control. (Figure 13A) HLA II was not detectable in MC_{TC} cells not stimulated with IFNγ. (data not shown). Interpretation of this finding is complicated. As mentioned earlier, it is not clear that CD40/CD40L interactions are truly replicated by any one activating antibody. CD40L interacts with CD40 at several epitope sites, which are differentially recognized by different activating antibodies, and each epitope site appears to modulate CD40 function in a different manner; for example, some activating antibodies can prevent B cell apoptosis but do not enhance immunoglobulin production whereas others, such as G28.2, are potent elicitors of antibody production.

In addition, as CD40/40L interactions are *trans* interactions, CD40 endocytosis following activation is unlikely. Finally, as with many members of the TNF Receptor family, CD40 signaling is mediated by TNF Receptor Associated Factor (TRAF) proteins and can include downstream activation of canonical and non-canonical NFκB-signaling, MAPKs, PI3K, and PLCγ pathways.[156] TRAF6 is reported to be downstream of FcεRI signaling and TRAF6 deficient bone marrow murine mast cells degranulate normally to IgE mediated activation yet produce significantly less IL-6 and IL-13.[157] However, because CD40 activation can utilize multiple TRAF proteins, it is possible that CD40 activation produces some degree of degranulation, releasing an excess of proteases that overwhelmed SBTI, allowing degradation of IFNγ and thus decreasing HLA II expression over the 3 days of culture. Use of recombinant CD40L stimulation, instead of CD40 specific antibody, both immobilized and in solution, would clarify these findings.

Supernatants from MC_{TC} cells incubated ± IFNγ (10 ng/mL) ± anti-CD40 antibody did exhibit β hexosaminidase (βhex) activity, though it was particularly high only with 100 ng/mL of 5D12. (Figure 13B) However, equal βhex activity was detected in 100 ng/mL 5D12 cultures without IFNγ (Figure 13B), suggesting that 5D12 mediated βhex release is not due to CD40 crosslinking but perhaps to FcγRIIA crosslinking: 5D12 may agglutinate at higher concentrations, causing aggregation of FcγRIIA. Indeed, mouse IgG2b isotype antibodies have relatively higher affinity for human FcγRII receptors.[158] Separation of IFNγ stimulation and CD40 stimulation and use of $f(ab')_2$ antibody fragments and recombinant CD40L are required to clarify these findings.

Despite the technical issues that must be resolved, G28.2, the CD40 activating antibody, produced a dose dependent increase in IL-6 production in IFNγ'MC_{TC} cells. 5D12 increased IL-6 production only at the highest concentration (100 ng/mL) and activation of CD40 by 5D12 at high concentrations is well documented[159]. (Figure 13C) G28.2 did induce IL-6 production from MC_{TC} cells in the absence of concurrent IFNγ stimulation, though less than half that seen with IFNγ'MC_{TC}, suggesting the presence of functional CD40 on MC_{TC} cells below detection by flow cytometry. (Figure 13C) In contrast, IL-12 was not detected in any supernatant (data not shown). Importantly, some cytokines, such as IL-4, are particularly susceptible to MC_{TC} proteases and require both the presence of SBTI and a protective antibody to prevent degradation.[44] IL-12 susceptibility to mast cell proteases has not been assessed.

CD40/CD40L interactions are vital to both CD8+ and CD4+ T cell interaction with antigen presenting cells. CD40 signaling enhances dendritic cell expression of costimulatory molecules CD80 and CD86, prevents dendritic cell death in response to HLA II crosslinking, and increases dendritic cell cytokine production. [143,160,161]The inflammatory feedback loop mediated by CD40/CD40L interactions is important for efficient amplification of both T cell and antigen presenting cell functions. For example, both CD40/CD40L and B7/CD28 signaling must be abrogated to induce tolerance during *in vitro* mixed lymphocyte reaction[162] and blockade of CD40/CD40L interactions improves symptoms in murine models of inflammatory bowel disease[163]. Although our data provide evidence that MC_{TC} cells express functional CD40, it is difficult to interpret the influence of CD40 function in the context of mast cell- T cell interactions due to the technical issues described above. Additional experiments, particularly

those that utilize CD40L and non-activating antibodies or antibody fragments, are required to determine if CD40/CD40L interactions promote inflammation or dampen inflammation in the mast cell context.

CD4+ T cell production of IFNγ is sufficient for upregulation of HLA II on MC_{TC} cells

Jurkat T cells do not produce IFNγ or IL-2 in their 'resting' state.[164] Therefore, MC_{TC} cells in Jurkat cocultures require priming to express HLA II and be able to activate Jurkat cells via Superantigen. This is not the case with primary human CD4+ cells. As seen in figure 14, in the presence of 100 ng/mL TSST, MC_{TC} and IFNγ'MC_{TC} cocultures display nearly equal percentages of activated CD4+ T cells as measured by activation marker CD25, 61.4% and 74.9% respectively. However, at lower concentrations of TSST, the difference is marked: 34.5% versus 75.9% at 1 ng/mL, 4.95% versus 34.7% at 0.01 ng/mL. This finding implies that robust T cell activation can overcome differences in MC_{TC} priming.

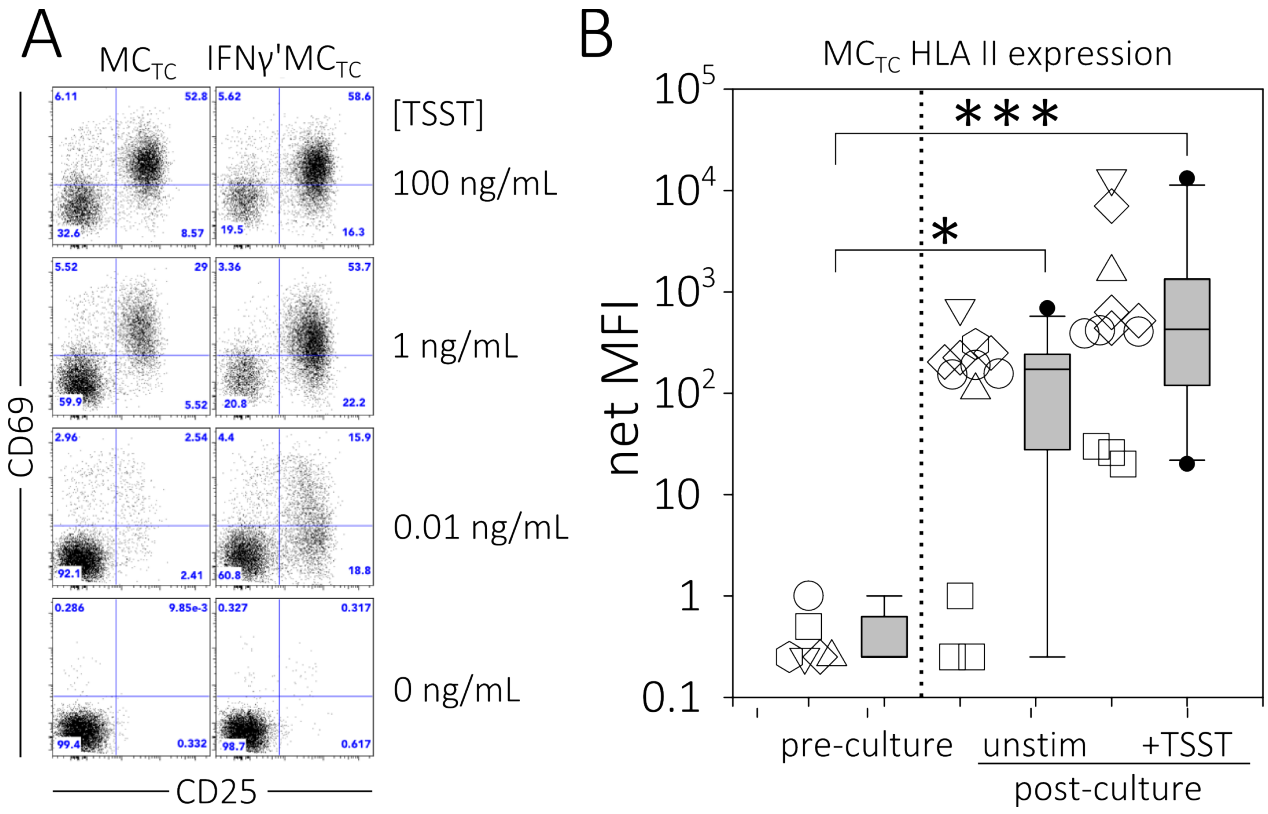

Figure 14. CD4+ T cell activation by MC_{TC} cells is not dependent on IFNγ priming as MC_{TC} cells upregulate HLA II when cocultured with T cells.

A, MC_{TC} cells are primed for 2 days ± IFNγ (10 ng/mL), then washed thoroughly and cocultured for 4 days with primary human CD4+ T cells in the presence of various concentrations of superantigen TSST. Cells from coculture are then labeled for CD3, CD117, CD25, and CD69 expression. CD3+CD117- cells (T cells) are gated and CD25 and CD69 expression is shown above. *B*, 6 different MC_{TC} (CD117+CD3-) sources were assessed for HLA II expression prior to and after 4 day coculture with 6 different sources of primary CD4+ T cells ± TSST (10 ng/mL) generating 12 unique pairings. In this figure, each mast cell source is represented by a unique symbol shape regardless of the CD4+ T cell source it was paired with. *Pre-culture n=6, post-culture n=12. *, p<0.05, ***, p<0.001, t-test comparison.*

To better understand the loss of IFNγ priming effect when primary T cells are used, CD4+ T cell cytokine production was assessed prior to coculture and resulting CD4+ T cell proliferation was assessed after MC_{TC} and IFNγ'MC_{TC} cocultures. To compensate for the natural variability that is integral to use of primary human cells, an internally normalized proliferation ratio calculation was created:

$$P_{ratio} = \frac{P_{IFN\gamma' MC+TSST} - P_{IFN\gamma' MC}}{P_{MC+TSST} - P_{MC}}$$

where *P* is the %proliferated CD4+ T cells defined as $CD117^{neg}CD3^{+}CFSE^{low}CD25^{+}$

P_{ratio} is, therefore, the ratio of CD4+ T cell proliferation between paired cocultures wherein the only difference is the IFNγ primed state of MC_{TC} cells. For cocultures for which IFNγ priming is irrelevant, the ratio goes to 1. P_{ratio} was found to significantly correlate with the percentage of IFNγ producing CD4+ T cells (Th1 cells) as measured prior to coculture and P_{ratio} approached unity when >20% of initial T cells were positive for IFNγ production. P_{ratio} does not correlate with the percentage of IL-17 (Th17) or IL-4 (Th2) producing cells. (Figure 15) This finding implies that robust IFNγ production, either by unstimulated T cells or due to superantigen stimulation, overcomes differences in MC_{TC} priming during MC_{TC}-T cell coculture.

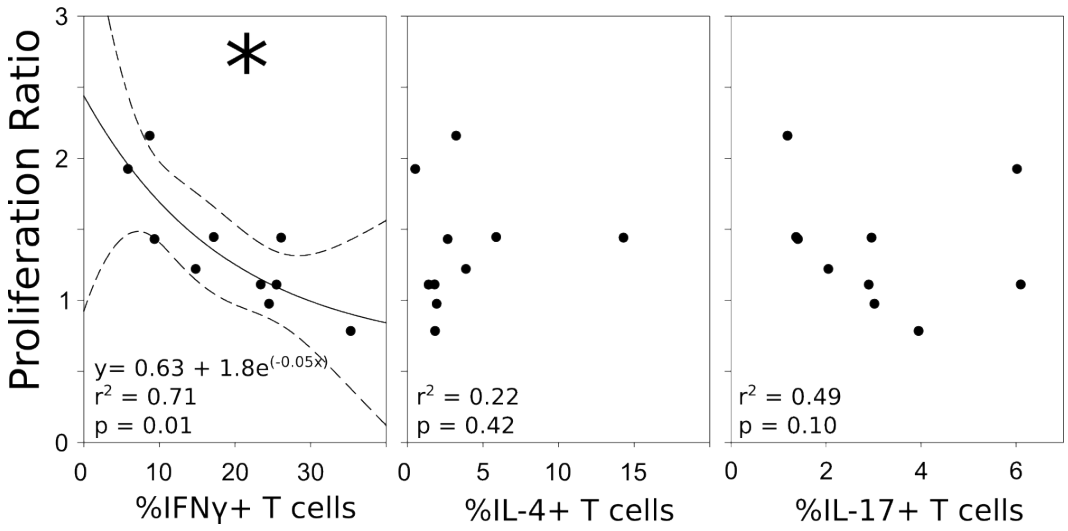

Figure 15. The proportion of IFNγ, but not IL-4 or IL17, producing CD4+ T cells prior to coculture correlates with the proliferation ratio across 10 separate cocultures.

As indicated in Figure 15, primary human CD4+ T cell harbor a subset of Th1 cells that, unlike Jurkat cells, are already producing IFNγ and can therefore initiate an activation feedback loop between mast cells and T cells: Th1 cells secrete IFNγ that induces HLA II upregulation on mast cells, mast cells are then able to activate Th1 cells via TSST which, in turn, make IFNγ and upregulate HLA II on more mast cells. Indeed, coculture of MC_{TC} cells with primary CD4+ T cells results in increased HLA II expression that is further augmented by stimulation with TSST. (Figure 14B) To validate this notion, the Th1 portion of CD4+ T cells was altered by sorting primary CD4+ cells according to CXCR3 expression. CXCR3 is a chemokine receptor that is highly expressed on Th1 CD4+ T cells. Not all CXCR3+ CD4+ T cells actively produce IFNγ at any given time; nonetheless, CXCR3+ T cells harbor a much higher proportion of IFNγ producing cells.

(Figure 16A) In the absence of stimulation, CXCR3+ T cell efficiently upregulate HLA II expression on MC_{TC} cells but CXCR3- T cells do not. This upregulation is dependent on CXCR3+ cell number and is enhanced by stimulation with TSST. Surprisingly, CXCR3- T cells are poor inducers of MC_{TC} HLA II expression even in the presence of superantigen TSST. (Figure 16B)

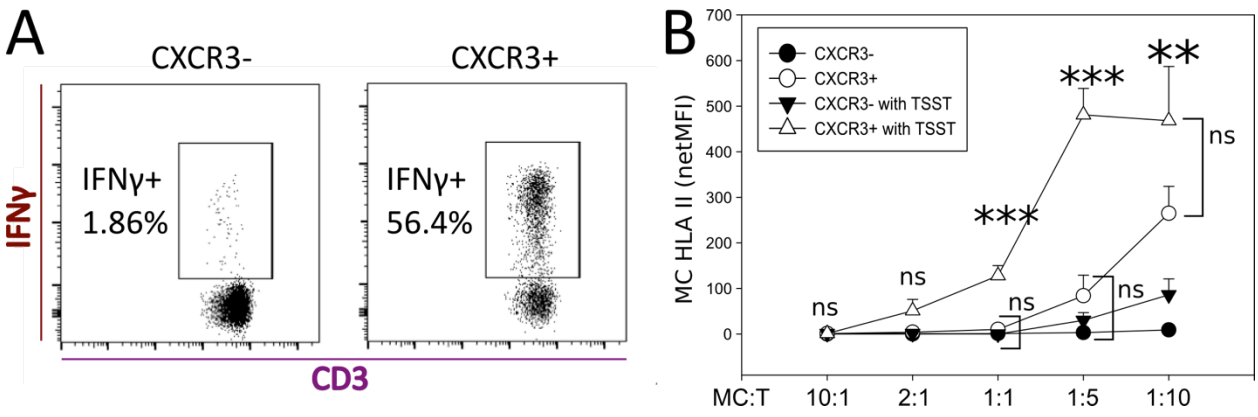

Figure 16. The IFNγ producing Th1 subset of CD4+ T cells are efficient inducers of MC_{TC} HLA II expression.

A, CD4+ T cells are sorted into CXCR3+ and CXCR3- populations then assessed for intracellular cytokine production following a 4 hour incubation with PMA, Ionomycin, and Brefeldin A. *B,* MC_{TC} cells are cocultured for 4 days with increasing numbers of CXCR3+ or CXCR3- CD4+ T cells +/- TSST stimulation. HLA II expression is assessed on CD117+CD3- gated cells. MC_{TC} HLA II expression across 3 cocultures at each MC:T ratio by ANOVA and multiple comparison. MC:T ratios that are significantly different are indicated and multiple comparisons that were not significantly (ns) different are highlighted. ***, p<0.01, ***, p<0.001*

The role of IFNγ production during MC_{TC} cocultures with CD4+ T cells is more directly addressed by the use of IFNγ/IFNγR inhibition. Neutralizing IFNγ antibody, clone NIB42[165], added at the beginning of coculture, and competitive IFNγ receptor inhibitor antibody GIR-208[166], added 2 hours prior to addition of CD4+ T cells, reduce subsequent TSST stimulated proliferation of CD4+ T cells in cocultures with un-primed MC_{TC} but not IFNγ'MC_{TC} cells. (Figure 17) The improved efficacy of direct IFNγR inhibition (as indicated by multiple comparison testing) in comparison to IFNγ neutralization suggests that IFNγRs are susceptible to inhibition prior to synapse formation whereas IFNγ secreted into the synaptic cleft is less susceptible to neutralization. Murine CTLs have been shown to release high concentrations of IFNγ at synaptic clefts that subsequently and quickly diffuses away. However, the ability of anti-IFNγ antibody ability to inhibit IFNγ at the synaptic cleft has not been directly evaluated.[167]

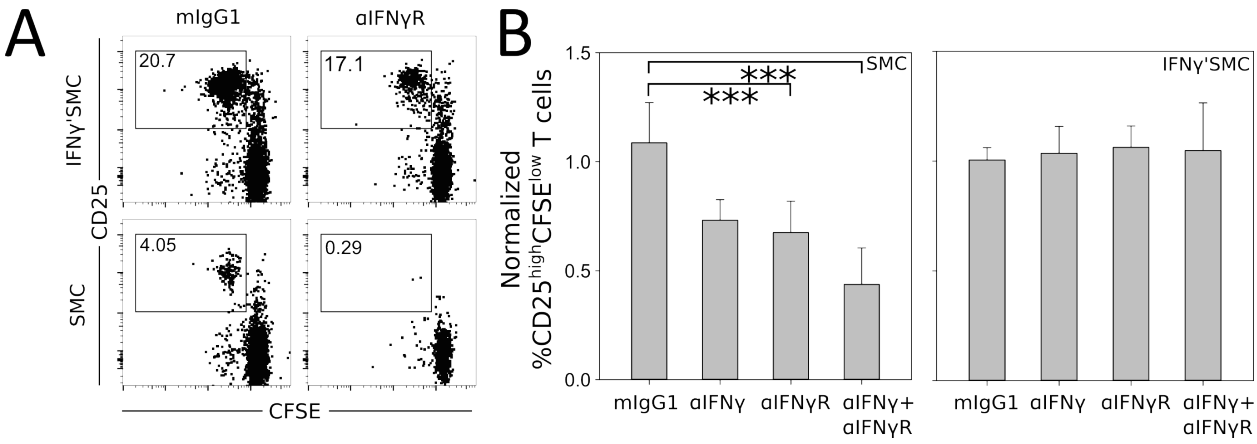

Figure 17. MC_{TC}, but not IFNγ'MC_{TC}, activation of CD4+ T cells is inhibited in the presence of IFNγ and IFNγR antibodies.

Mast cells are primed with IFNγ for 2 days then washed and returned to culture. 100 ng/mL of GIR-208, a competitive inhibitor of IFNγ at the IFNγR, or isotype control, is added to MC_{TC} and

IFNγ'MC_{TC} for 2 hours prior to addition of CFSE labelled CD4+ T cells. IFNγ neutralizing antibody NIB42 or isotype control is added just prior to addition of CD4+ T cells. Cocultures are incubated for 4 days in the presence of TSST stimulant at 10 ng/mL then evaluated for CD4+ T cell proliferation as shown in A. *A*, Live cells are gated on CD3+CD117- T cells then evaluated for CFSE dilution and CD25 expression as shown. *B*, CD4+ proliferation for each coculture with the presence of antibodies was normalized to mast cell-CD4+ cocultures stimulated with TSST in the absence of antibodies. *Data was evaluated by ANOVA followed by multiple comparison analysis. ***, p<0.001*

These data show that IFNγ, whether added to mast cells prior to coculture or provided to mast cells by Th1 cells, is the major determinant of MC_{TC} HLA II expression and subsequent superantigen mediated T cell activation. Though TSST-1 is notorious for its role in toxic shock syndrome, the incidence of toxic shock due to any superantigen is low, most recently measured at less than 1 in 100,000, and with decreasing symptom severity due to early treatment and increasing immunity.[168] Superantigens have a putative role in pathology of atopic dermatitis (AD). Since the 1970s, it is noted that *Staphylococcus aureus* colonize lesional skin of atopic dermatitis patients at higher rates than non-lesional skin or mucosa, that colonization correlates with disease severity, and that treatment with anti *Staphyloccocus* antibiotics improves symptoms.[169-171] Lesional skin is also associated with increased mast cell density and increased mast cell degranulation; mouse models of AD show improvement with protease inhibitors; and polymorphisms in the mast cell expressed FcεRIβ subunit are associated with increased risk for development of AD.[169,172,173] It has been suggested that mast cells drive AD

associated inflammation in part through *S. aureus* superantigen HLA II mediated activation of T cells. However, this concept remains controversial because individuals with AD have measureable IgE against a variety of superantigens and a newly identified *S. aureus* toxin, delta toxin, has been shown to directly activate mast cell degranulation in an FcεRI independent manner.[171,174]

High concentrations of IFNγ are able to induce HLA II expression on a variety of hematopoietic and non-hematopoietic cells. Superantigens bypass mechanisms required by professional antigen presenting cell ability to activate CD4+ T cells. Antigen presenting cells must take up and process extracellular antigen to peptides and load those peptides onto HLA II molecules to elicit CD4+ T cell response. As endocytosis of antigens is historically associated with mast cell progenitors, our model utilizes fully mature, primary MC_{TC} cells and evaluates for their ability to take up extracellular antigen.

Fully mature, primary human MC_{TC} cells are capable endocytic cells

Dr. Brant Ward has evaluated MC_{TC} cells for endocytic ability and mechanisms and his data is summarized below:

- MC_{TC} cells are able to internalize glycosylated (ovalbumin) and non-glycosylated (bovine serum albumin) proteins, as well as complex, branched carbohydrates (dextran).
- Uptake of ovalbumin and dextran by MC_{TC} is sensitive to inhibition of either macropinocytosis/phagocytosis by dimethyl amiloride (DMA) or dynamin-dependent, receptor-mediated endocytosis by Dynasore

- Uptake of bovine serum albumin is less sensitive to inhibition, and decreased uptake was only seen with inhibition of both endocytic pathways.
- Gene expression microarrays reveal that unlike dendritic cells and other antigen presenting cells, MC_{TC} cells do not express RNA for a wide variety of endocytic receptors at baseline. Flow cytometry confirms negligible detectable expression of multiple endocytic receptors on the surface of MC_{TC} cells.
- However, DEC-205, a receptor expressed on antigen presenting cells and known to effectively target antigens for presentation in HLA II, is significantly expressed on MC_{TC} cells.
- DEC-205 on MC_{TC} is quickly internalized after antibody ligation, indicating that it is a functional receptor.

Cytomegalovirus primed MC_{TC} cells activate CD4+ T cells of CMV seropositive, but not seronegative, individuals

To fully evaluate MC_{TC} antigen presenting function, a model antigen system is necessary. Cytomegalovirus (CMV) commonly causes an asymptomatic to mild primary infection in immunocompetent hosts, and establishes lifelong latency with periodic, non-symptomatic reactivation. Prevalence in the United States is about 50% and increases with age, from 38% in 6-11 year olds to 58% in 40-49 year olds, and with even greater prevalence, upward of 70%, in non-white ethnic groups. With high prevalence at the population level and high and recurrent antigenic exposure at the individual level, CMV protein is a robust model antigen. In addition,

CMV membrane protein gB activates TLR2, which might enhance HLA II expression on mast cells analogous to the TLR2 ligand HKLM, as shown earlier.[175]

MC_{TC} were incubated for 3 days with inactivated CMV lysate and control lysate. On the second day of lysate incubation, SBTI (100 µg/mL) and IFNγ (10 ng/mL) were added to some MC_{TC}. On day three, MC_{TC} were thoroughly washed and replated with CFSE labelled CD4+ T cells from the same individual and cocultured for 7 days. At the end of coculture, T cells were assessed for activation ($CD25^{high}$) and proliferation ($CFSE^{low}$) as well intracellular cytokine production. Serum from tissue donors was used to assess seroreactivity, allowing separation of cocultures into CMV seropositive and CMV seronegative groups.

In the absence of IFNγ priming, CMV seropositive, but not seronegative, cocultures exhibit activation and proliferation of CD4+ T cells (Fig 18A, first two columns). Seropositive CD4+ T cell proliferation was enhanced in cocultures with IFNγ primed MCs (IFNγ'CMV'MC_{TC}); seronegative cocultures did not show any proliferation despite IFNγ priming (IFNγ'Cntrl'MC_{TC}). (Figure 18A, third and fourth columns) All sources, regardless of serology status, were able to show CD4+ proliferation in the presence of TSST (Figure 18A, column five) and addition of CMV lysate to T cells alone, without washing, did not show any proliferation, indicating an absence of contaminating dendritic cells. (Figure 18A, last column) In seropositive individuals, both CMV'MC_{TC} and IFNγ'CMV'MC_{TC} cocultures were significantly elevated above control lysate primed cocultures. (Figure 18B, top panel) As CMV serology status does not correlate directly with the *degree* of CD4+ T cell response, there is evident variation in the degree of T cell

proliferation by subject. Based on our studies with mast cell- T cell cocultures in the context of TSST, IFNγ production by CMV responsive T cells further augments mast cell HLA II expression and ability to activate T cells, particularly for those mast cells that present CMV peptides in their HLA II; therefore, individual differences in number of CMV responsive Cd4+ T cells and activatibility of those T cells would be greatly amplified. The ability of CMV'MC_{TC} cells to activate CD4+ T cells in some individuals, albeit to a lesser extent than IFNγ'CMV'MC_{TC} cells, indicates that mast cells are capable of storing antigen for at least 1 day prior to peptide presentation: CMV antigen is washed away prior to CMV'MC_{TC} cell exposure to CD4+ T cell produced IFNγ. Nonetheless, none of the seronegative cocultures display any T cell proliferation. (Figure 18B) Proliferating CD4+ T cells from seropositive cocultures produce mainly IFNγ, low IL-4, and almost no IL-17, consistent with the known T cell response to CMV (Figure 18C).[176-180]

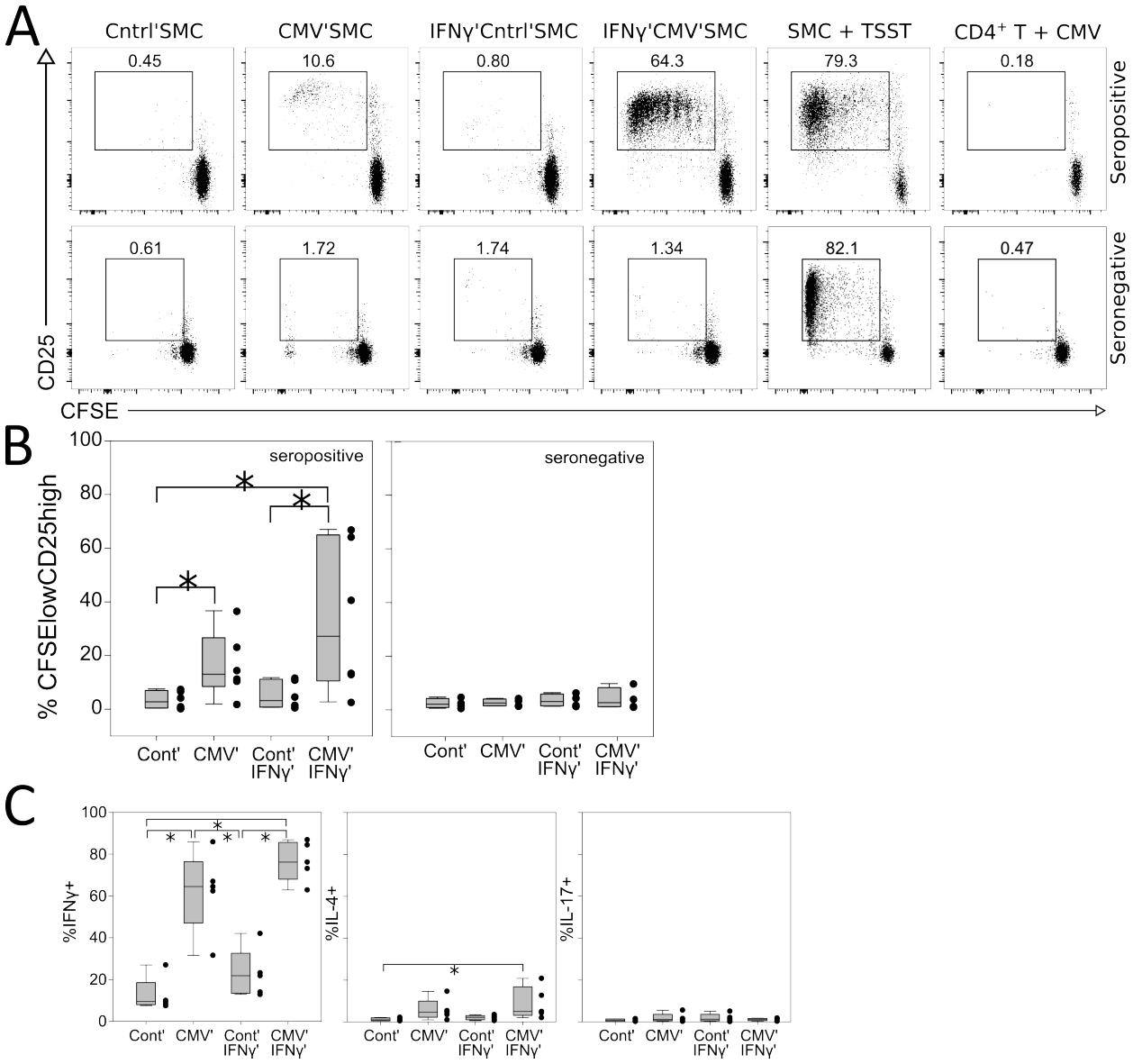

Figure 18 CMV primed MC_{TC} induce proliferation of CD4+ T cells from CMV seropositive, but not seronegative, individuals.

A, Representative day 7 dot plot comparison of one seropositive and one seronegative individual for cocultures with control lysate primed (Cntrl'MC_{TC}), CMV lysate primed (CMV'MC_{TC}), control lysate and IFNγ primed (IFNγ'Cntrl'MC_{TC}), CMV lysate and IFNγ primed (IFNγ'CMV'MC_{TC}) MC_{TC} as well as experimental controls T cells alone with CMV lysate (T+CMV)

and un-primed MC_{TC} with superantigen TSST (MC_{TC} + TSST). B, Summary of day 7 proliferation and activation data for CMV seropositive and seronegative cocultures. C, $CFSE^{low}CD25^{+}$ cells from seropositive individuals production of cytokines IFNγ, IL-4, and IL-17.

MC_{TC} uptake of CMV protein utilizes dynamin dependent, receptor-mediated endocytosis.

Dr. Ward showed inhibition of ovalbumin and dextran uptake by MC_{TC} in the presence of either macropinocytosis and phagocytosis inhibitor dimethyl amiloride (DMA) or dynamin-dependent, receptor-mediated endocytosis inhibitor Dynasore[181]. To determine the mechanisms of CMV antigen uptake, CMV priming of MC_{TC} was altered to include either or both inhibitors. MC_{TC} cells were incubated for 6 hours with CMV lysate alone, in combination with each uptake inhibitor, in combination with both uptake inhibitors, and with Dynasore's vehicle control DMSO. CMV'MC_{TC} cells were then washed thoroughly and returned to culture with 100 ng/mL SBTI and 10 ng/mL IFNγ. After two days, CMV'IFNγ'MC_{TC} cells were washed again and put into coculture with matched CD4+ T cells for six days. Cocultures were assessed for proliferation ($CFSE^{low}$) and activation ($CD25^{high}$) of CD4+ T cells (CD3+CD117- gated) by flow cytometry. T cell proliferation was inhibited when CMV'IFNγ'MC_{TC} were exposed to CMV lysate in the presence Dynasore, less so in the presence of DMA, but only significantly so in the presence of both inhibitors, indicating that CMV protein uptake involves multiple pathways. (Figure 19)

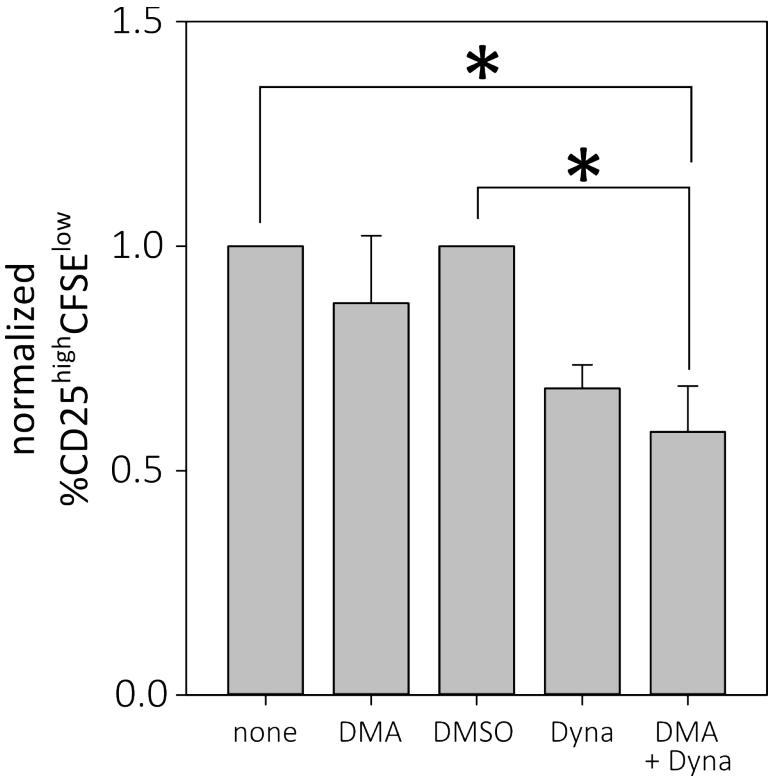

Figure 19. Uptake of CMV lysate proteins involves multiple pathways.

MC_{TC} cells are incubated with CMV lysate alone, in the presence of DMA, DMSO (vehicle control for Dynasore), Dynasore, or both DMA and Dynasore for 6 hours, washed thoroughly, then stimulated with IFNγ (10 ng/mL) for 2 days. IFNγ'MC_{TC} cells were then washed and put into coculture with source matched CD4+ T cells for 6 days, then assessed for CD4+ T cell activation ($CD25^{high}$) and proliferation ($CFSE^{low}$). *Repeated Measures ANOVA with Tukey's Multiple comparison. *, p<0.05, n=3*

Conclusions

- IFNγ'MC_{TC} cells are capable antigen presenting cells, able to take up and store protein antigen, process proteins to peptides, and load those peptides into HLA II when

provided with an IFNγ stimulus. HLA II:peptide complex is then surface expressed allowing recognition by HLA matched T cell receptors on CD4+ T cells in an antigen specific manner.

- Mast cell function, in turn, may be altered by interaction with T cells as is indicated by the effects of T cell produced IFNγ and by the ability of mast cells to produce IL-6 in response to CD40 crosslinking.
- Given the increase in mast cell density noted at most sites of inflammation and the lack of evidence thus far in regards to mast cell ability to travel to lymph nodes in humans, we postulate that mast cell antigen presenting function may sustain peripheral inflammation and chronic inflammatory diseases. CD40 expression on mast cells indicates that they can also mediate CD4+ T cell regulation of other immune cell responses.
- Finally, although indirectly related to Project 2 of this dissertation, it is interesting to note that CD40 crosslinking is documented to cause the upregulation of CD25 expression on human CD34-derived Langerhan cell and plasmacytoid dendritic subsets during interactions with CD4+ T cells. Clonal mast cell in mastocytosis frequently and aberrantly express CD25.[143,182] It is possible to evaluate changes in CD25 expression on mast cells with the data already collected for mast cell- CD4+ cocultures in the context of CMV and superantigen.

Future Studies

- Though the work presented in this project utilizes fully mature, primary human cells, all of it has been done *in vitro*. Future studies will determine if IFNγ or a Th1 response to PPD can induce *in vivo* mast cell HLA II expression.
- The modulation of IFNγ induced HLA II expression by TLR2 signaling (HKLM) and degranulation need to be addressed. In particular, do quiescent versus degranulated mast cells preferentially sustain a particular type of inflammatory environment as was noted by some authors. Also, it is important to determine if IgE mediated antigen uptake enhances mast cell APC function.
- The reciprocal effects of T cells on mast cells require evaluation, particularly as low levels of CD40 activation induced profound changes in IL-6 production in the absence of appreciable degranulation.
- Finally, though human mast cell ability to chemotax to lymphoid organs has not been described, mast cells are known to be present in lymph nodes and tonsils. Although LN and tonsilar mast cells could have matured in those tissues, it is also possible that those mast cells originated in other peripheral tissues (as has been seen with mouse models).[81,183] The CMV cocultures indicate that MC_{TC} cells can store antigenic protein for at least 1 day prior to HLA mediated presentation and future work can test the limit of antigen storage prior to presentation. In addition, our work indicates that mast cells load, if not also process, peptides onto HLA II in the same granules as those which store tryptase. Murine mast cell degranulation releases less-soluble, and therefore long lived, complexes of heparin encapsulated proteins, such as TNFα, that reach draining lymph

nodes intact.[184] It is possible, regardless of migration to lymph nodes, that MC_{TC} cells can package antigenic proteins or peptides in a similar manner. The formation of such theoretical complexes is readily testable *in vitro*.

Part II. Myeloid Derived Suppressor Cells in Mastocytosis

Introduction

Myeloid Derived Suppressor Cells

Tumor associated granulocytosis, associated lymphopenia, accumulation of lineage negative Null Cells or Natural Suppressor cells, and concurrent loss of T cell function have been recognized since the late 1970s[185]. The advent of improved phenotypic markers in the late 1990s in conjunction with functional assays improved research into these, now named, Myeloid Derived Suppressor Cells (MDSCs). MDSCs are heterogeneous tumor or inflammation induced early myeloid cells that lack most lineage surface markers and are potent inhibitors of T cell activation and proliferation. To validate cells as MDSCs, suppressive activity must be demonstrated as bone marrow cells from naïve mice can display the same surface ligand distribution but are not immunosuppressive.[185,186] Human MDSC progenitor potential is highlighted by reported in vitro differentiate into osteoclasts, monocytes, granulocytes, endothelial cells, or dendritic cells. In the context of cancer, MDSCs are recruited by tumor and microenvironment produced mediators such as CSF-1, G-CSF, GM-CSF, VEGF, IL-6, IL-10, IL-4, IL-13, PGE2, and Histamine.[185,187] The variety and tumor-specificity of recruitment factors contributes to the wide heterogeneity of this population of cells.

MDSCs regulate tumors directly by (1) enabling tumor invasion via secretion of proteolytic enzymes, particularly matrix metalloprotease-9; (2) regulating tumor cell arrest, niche

formation, and formation of tumor permissive pre-metastatic sites; and (3) increasing angiogenesis and vasculogenesis. Unfortunately, direct MDSC effects in the tumor microenvironment are difficult to dissect from the remaining environment milieu and the strengths and weaknesses of findings in this regard are reviewed elsewhere.[185]

It is MDSC capacity to inhibit T cell responses that defines the population and has been most extensively studied. MDSCs inhibit T cell activation and proliferation by (1) production of reactive oxygen species, (2) depletion of amino acids specifically required by T cells, (3) production of inhibitory mediators such as TGFβ and IL-10, and (4) enhanced production of T regulatory cells. MDSCs express high levels of arginase-1 (ARG-1), which metabolized L-arginine to L-ornithine in the urea production pathway. Arginine is particularly required for T cell proliferation and cytokine production as its absence reduces CD3ζ chain recycling at cell surface as well as de novo CD3ζ production and selectively impairs the expression of cyclin D3 and cyclin dependent kinase 4, blocking their downstream signaling, and trapping T cells in the G_0 – G_1 phase of their cell cycle.[188-191]

L-Arginine is also a substrate for nitric oxide synthase (NOS) whose product, nitric oxide (NO), interferes with the signaling cascade downstream of the IL-2 receptor, reducing IL-2 mediated survival and proliferation of T cells.[192] NO effects are mediated by dispersal into adjacent cells, followed by S-nitrosylation of cysteine residues or by activation of soluble guanylate cyclase and cGMP dependent protein kinases.

20.[193-195] When ARG-1 and NOS are expressed in the same cell, as they are in MDSCs, arginine becomes rate limiting and NOS begins to produce superoxide instead of NO. Superoxide is highly reactive and quickly reacts with other molecules to produce reactive intermediates such as peroxynitrite and hydrogen peroxide.[196] Granulocytic MDSCs are potent sources of ROS and peroxynitrite. Granulocytic MDSCs from patients with pancreatic cancer induced the loss of CD3ζ chain in naïve T cells, resembling murine MDSC ability to down regulate CD3ζ chain from CD8+ T cells in a mouse tumor model.[197-199]

T cells lack cystathionase and do not have a cystine transporter. They are dependent on uptake of cysteine produced and exported by other cells. Most antigen presenting cells can take up cystine and reduce it to cysteine. Excess cysteine is excreted through Alanine-Serine-Cysteine (ASC) transporters. MDSCs can also take up cystine and reduce it, but do not express an ASC transporter and thus act as a cystine trap, reducing overall cysteine availability in their microenvironment. [200,201]

MDSCs are reported to produce inhibitory cytokine TGFβ and IL-10 and are implicated in cancer tolerant T regulatory cell production.[202] The details of these suppressive mechanisms are currently being ironed out thanks to improved murine models and recent consensus in regards to MDSC surface marker identification. Murine MDSCs are identified as glutathione reductase 1 (Gr1 aka Ly-6), CD11b (integrin-αM) double positive cells that can be further subcategorized according to specific Gr1 subtype expression.[203] Ly-6G$^{low/neg}$Ly-6C^{high} MDSCs have monocyte-like morphology, preferentially express iNOS, and have increased per-cell T cell suppressive

capacity. Ly-6G+Ly-6C^{low} MDSCs have a granulocytic-like morphology, frequently displaying a bilobed or multilobed nucleus, express high levels of arginase 1, and are most consistently elevated in mouse tumor models.

Until recently, human MDSCs have not been as clearly defined as their mouse counterparts. Although various forms of immature myeloid cells have been identified in several tumors, a consistent surface expression profile has been elusive. However, a consensus is building on the identification of human MDSCs as HLA DR$^{neg/low}$, CD11b+, and CD33+ cells which can be differentiated to CD15+ granulocytic type and CD14^{high} monocytic type.[204-206] Early work with human MDSCs utilized Lineage cocktails that included either CD15 or CD14 or both, thus peripheral blood MDSC frequency has been largely underestimated in both healthy controls and individuals with various cancers.

Human MDSCs do not show as clear delineation as their mouse counterparts in regards to subset specific mechanism of ROS production. In addition, it is not clear if granulocytic MDSCs are precursors of granulocytes or granulocytes that have been previously aberrantly activated, in peripheral blood. In many studies they display nuclear morphology that resembles granulocytic precursors, express neutrophil progenitor surface ligand profiles, and are found in the monocytic layer following Ficoll PBMC prep.[207] Choi et al argue that neutrophils are activated by a soluble serum factor in tumor bearing hosts, exocytose their granules, and downregulate CD16 expression while retaining surface CD15. By definition, such neutrophils become hypogranular and thus able to contaminate Ficoll PBMC prep.[208,209] However, the two

concepts are not mutually exclusive and peripheral blood granulocytic MDSCs can harbor both distinct immature and degranulated populations or represent a single population that is immature *and* degranulated, all in a tumor specific manner. No matter their "true" origin, granulocytic MDSCs, like their monocytic counterparts, are highly suppressive of T cell responses.

Unlike T regulatory cells, MDSC suppression function is not HLA II restricted. However, some specificity is conferred by MDSC requirement for cell-cell contact.[205] As MDSC mechanism of action largely revolves around the production of short lived and highly reactive oxygen species such as hydrogen peroxide or pereoxytine or the depletion of local levels of arginine and cysteine, it is easy to imagine that suppression requires, at minimum, proximity if not direct cell-cell contact. Indeed, though monocytic MDSCs do not express HLA II, they do express HLA I as well as many cell surface ligands that promote contact with T cells, such as CD80 and CD83. *In vivo*, MDSCs are drawn by inflammatory cytokine and chemokines to peripheral sites where they inhibit local T cell responses. Thus, MDSC depletion from PBMCs restores normal T cell function: *in vitro*, MDSCs suppress T cell activation and proliferation in a wide range of antigen and antigen-independent models, likely an artifact of culture induced proximity, though an accurate measure of MDSC suppression capacity.[204]

Mouse tumor models clearly show a profound association between MDSCs and an increase in tumor progression, both in regard to tumor size and number of metastasis. Human data in this regard is quickly growing. Various forms of MDSCs are reported to be elevated in malignant

melanoma[205,210], non-Hodgkin's lymphoma[211], head and neck carcinomas, urological cancers of bladder and ureter, and lung cancers.[207] MDSCs are shown to have prognostic significance in NHL, breast, pancreatic, esophageal, and gastric cancers where increased MDSCs correlate with decreased overall survival. MDSC burden predicts patient response to IL-2 therapy for kidney cancer or melanoma with increasing MDSCs associated with a better response, theoretically due to IL-2's ability to recover the T cell response.

In patients with myeloid dysplasia, MDSCs appear to be a separate, non-clonal population that suppress hematopoiesis, promotes T cell tolerance, and serves as a key source of myelosuppressive and inflammatory molecules such as IL-10, TGF-β, NO, and arginase.[212] Recent human studies show age related increases in serum levels of inflammatory cytokines (e.g., TNF-α, IL-6, and IL-1β), accompanied by rising in blood MDSC concentration, and that indicate senescence-dependent changes drive MDSC expansion and play a role in the observed age-dependent predisposition to Myelodysplastic sydrome.[213]

Down-modulation of MDSCs in mouse models inhibits tumor progress. Several major strategies are used to reduce MDSC activity: (1) Sildenafil and other NOS inhibitors reduce MDSC NO production, (2) VEGFR inhibitors, soluble VEGF receptor, and antihistamines reduce MDSC recruitment, (3) all-trans-retinoic-acid (ATRA) and high dose Vitamin D force MDSCs to mature and thereby lose their suppressive activities while concurrently increasing the monocytes and dendritic cells they mature into, and (4) gemcitabine and doxitaxil, to which MDSCs are particularly vulnerable, kill these cells.[214] Attempts to modulate MDSC burden in human beings,

few though they are, have had varying success. VEGF[215] targeting in humans has failed to alter MDSC burden and highlights the heterogeneity of MDSCs in a heterogeneous population. On the other hand, ATRA improves *in vitro* T cell response to tetanus toxoid function in patients with renal cell carcinoma and improves T cell responsiveness to cancer vaccine therapy in small cell lung carcinoma.[216] Likely, ATRA's success can be attributed to its relatively low toxicity and ability to not only decrease MDSC burden but also diminish the elevated immature-to-mature myeloid ratio.

Mast Cells mediators

Mast cells produce many of the mediators documented to recruit MDSCs and increase their survival. Mast cells complete maturation in peripheral tissues and there display characteristics that are subset and tissue specific. All mast cells store histamine, heparin, and proteases, largely tryptase, in granules whose contents are released with the appropriate stimulus. As reviewed in Section I, human mast cells are categorized according to their protease composition. The distinct mast cell subsets are functionally different and display heterogeneity in production of leukotrienes, prostaglandins, cytokines, and chemokines.

Dr. Daniel Conrad and Dr. John Ryan's labs describe the importance of mast cell-MDSC interactions in regulating inflammation and tolerance. Effects of MDSCs on tumor progression or clearance of parasite infection are absent in $Kit^{Wsh/Wsh}$ mice and $Cpa3^{cre}/Mcl^{fl/fl}$ mice and return with mast cell reconstitution[187,217] MDSCs increase mast cell cytokine production following IgE receptor activation *in vitro* and *in vivo* increase airway hyper-responsiveness to allergen challenge[218] Most recently, MDSC-mast cell interactions have been observed to

depend on histamine production. MDSCs chemotax and proliferate to histamine, an effect which can be blocked by cetirizine or cimetidine.[187]

Indeed, humans experiencing allergic symptoms have significantly elevated blood MDSCs compared to healthy, non-symptomatic controls.[187] Is mast cell produced histamine responsible for the elevated MDSC levels in atopic individuals and if so, does it indeed function through H1 and H2 receptors as in the mouse?

Histamine Pharmacology

Histamine was discovered in 1910 by Dale and Laidlaw. It is synthesized by histidine decarboxylase in a variety of cells, stored by mast cells and basophils only, and degraded by diamine oxidase extracellularly and by N-methyltransferase intracellularlly.[219] It has a short blood half-life of about 4 minutes in human plasma and normal plasma concentrations in healthy individuals are <1 ng/mL (<9 nM). [220-222]The biological effects of histamine are mediated through four G-protein coupled receptors, H1 through H4, which share surprisingly low sequence homology, 16-35%, at the protein level.[223] There are important species differences throughout the histamine/histamine receptor systems. For example healthy mice exhibit much higher plasma concentrations of histamine (~320 nM).[224]

H1 receptors are the most ubiquitously expressed receptors and are known to mediate vasodilation, bronchoconstriction, and alter sleep-wake cycles. H2 receptors are also widely expressed and most famously noted for their role in control of stomach acid release. As a presynaptic autoreceptor, H3 receptors inhibit the release of histamine and other

neurotransmitters from the central and peripheral nervous system. Indeed, whereas H1R antagonists cause a feeling of sleepiness, H3R antagonists cause a feeling of alertness. [225] H3 receptors may also be involved in blood-brain barrier regulation.

In the early 1990s, when only H1 and H2 histamine receptors had been identified, inconsistencies in eosinophil responses to pharmacological agonists and inhibitors implicated the existence of additional histamine receptors.[226] The discovery of the H3 receptor[227] quickly led to the identification the fourth histamine receptor[228-231]. Compared to other histamine receptors, the H4 receptor has a restricted expression profile and is mainly found on cells of hematopoietic origin.[230,232]. Four alternative splice variants of the H4 receptor gene (HRH4) have been identified, two of which are truncated. Homodimers of truncated forms and heterodimers of truncated and full-length forms are retained intracellularly.[233,234]

The H4 receptor has a unique pharmacologic profile that shares the strong histamine binding affinity and constitutive baseline activity of the H3 receptor. Most H1 and H2 receptor antagonists show no inhibition of histamine at H4Rs. However, the H3R *antagonist* clobenpropit acts as a high affinity partial *agonist* at the H4R.[232] Such dramatic differences are also seen across species: Johnson & Johnson's specific H4R antagonist JNJ 7777120, developed for human H4R, behaves as a partial agonist on mouse, rat, and dog H4Rs, complicating interpretation of animal findings.[235]

Histamine receptors coupled to different G proteins and trigger varying signaling cascades. H1Rs signal through G_q proteins and activate phospholipase C (PLC), initiating inositol phosphate (IP3) production, calcium mobilization, and phosphoinositide-3-kinase (PI3K) activation. H2Rs couple to G_s, increase cAMP formation, and activate protein kinase A (PKA). H3 and H4 receptors both couple to pertussis toxin sensitive $G_{i/o}$ protein but their signaling pathways differ. H3 receptors primarily inhibit cAMP formation, whereas H4 receptors primarily activate PLC and MAPK pathways.[223,236] (Table 1)

H4R signaling pathways remains controversial. Initially, H4R was reported to weakly inhibit forskalin mediated increases in cAMP in a pertussis toxin sensitive manner. [228,230,237]However, one study showed that histamine could not alter cAMP levels in human H4 receptor-transfected cells, and instead increased calcium mobilization if the cells were cotransfected with $G\alpha_{q/i1/2}$, $G\alpha_{q/i3}$, or $G\alpha_{16}$ proteins. The same study showed histamine induced MAPK phosphorylation, which was inhibited by pertussis toxin.[238] Evaluation of native histamine receptor activity in murine mast cells confirmed the association of H4R and calcium flux. Histamine produced an H2R mediated increase in cAMP and an H4R activation of PLC with subsequent calcium mobilization in murine bone marrow derived mast cells.[239] What's more, recent work links measureable H4R activity to the recruitment of β arrestin to H4R in a non-pertussis toxin sensitive manner.[240] Although native H4R activity has not been thoroughly evaluated in any human cells, it is interesting that murine H1 and H4 receptors utilize the same major pathways as they are also reported to produce similar and somewhat redundant effects. (Table 1)

Table 1. Histamine Receptors[a]				
	H1	H2	H3	H4
Histamine K_i (nM)	14,249	25,609	10	11
Constitutive activity	low	low	high	High
G protein	$G\alpha_q$	$G\alpha_s$	$G\alpha_{i/o}$	$G\alpha_{i/o}$
Signaling mechanism **major** **minor**	**PLC/DAG/IP3/PI 3K**	**↑cAMP/PK A**	**↓cAMP** MAPK/ERK/JNK/A P1	**PLC/DAG/IP3/PI3 K** ↓cAMP MAPK/ERK/JNK/A P1 β-arrestin
Antihistamine K_i (nM)				
• **Diphenhydramin e**	12	?	>10,000	>10,000
• **Cetirizine**	6	?	?	>10,000
• **Fexofenadine**	47	?	?	>10,000
• **Ranitidine**	>10,000	133	>10,000	>10,000
• **Cimetidine**	>10,000	1,504	>10,000	>10,000
Antidepressants/ Antipsychotics K_i (nM)				
• **Doxepin**	0.6	162	>10,000	>10,000
• **Amitriptyline**	0.7	66.1	>10,000	>10,000

[a]See Appendix I. Human histamine receptor specificities for extended table and references.

Histamine and Hematopoiesis

In 1977, Byron published the ability of 4-methylhistamine to trigger CFU-S bone marrow stem cells (today called CMPs or common myeloid progenitors) from mice to exit the G_0 phase and enter the S phase of their cell cycle, as indicated by susceptibility to hydroxyurea. The effect of 4-methylhistamine was inhibited by cimetidine.[241] Byron's work was affirmed in 1988 when Shounan and Xu showed that histamine itself triggered murine CFU-S to cell cycle in an H2R

dependent manner.[242] In the same year, Nakaya and Tasaka noted the ability of histamine to increase proliferation and differentiation of CFU-C stem cells (now CFU-GM or the common granulocyte/monocyte progenitor) in a cimetidine and ranitidine inhibited manner. Histamine response of granulocytic progenitors, at the myeloblast and promyelocyte stage, was not H1 or H2 mediated. [243]Finally, Michael Dy's group showed that the IL-3 mediated transition of CFU-S cells into S phase is partly mediated by IL-3 induced histamine production and that this effect could be inhibited by H2, but not H1, receptor antagonists.[244] H3 and H4 receptors were discovered a decade after the work outlined above. It is interesting to note that 4-methylhistamine is a far better H4R agonist than for any other histamine receptors.

The hematopoietic specific expression of H4R inspired Dy's group to return to the study of histamine in hematopoiesis.[245] H4R, but not H3R, is expressed on c-kit+ bone marrow cells with myeloid and lymphoid potential. Clobenpropit, an H3/H4 receptor agonist, stimulation reduced cAMP in c-kit+ cells, an effect that was prevented by an H4R antagonist. Consequently, myeloid, erythroid, and lymphoid colony formation in response to respective growth factors is inhibited by H4R activation. Hematopoietic progenitors become trapped in G_1 phase.[245] Such contradictory findings of histamine effects on hematopoietic progenitors highlight the importance of a need for thorough pharmacological and physiological data on human and mouse histamine receptors and thorough assessment of histamine receptor expression throughout hematopoietic differentiation.

Histamine and Immune regulation

Histamine's role in immune regulation is more nuanced with the discovery of H4R. According to current literature, H4 receptors are implicated in chemotaxis, cytokine production, and cell cycle regulation. In 1975, Clark and colleagues published their finding that histamine induced chemotaxis of human eosinophils.[246] Twenty seven years later, O'Reilly and colleagues would show that eosinophil chemotaxis to histamine is, in fact, H4R mediated.[247] Histamine chemotactic effects were absent when eosinophils were stimulated with H1 and H2 receptor agonists and could only be blocked by H3/H4 receptor antagonists. Because eosinophils do not express H3R, it became clear that H4R was responsible for actin polymerization and upregulation of adhesion ligands CD11b/CD18 and CD54.[222,247-249] Histamine is a relatively weak chemotactic factor when compared to eotaxin (CCL11) and it has a short blood half-life. Although it is easy to assume that it represents a redundant chemotactic pathway for eosinophils, histamine is, in fact, a better mediator for establishing short distance chemotaxis gradients *thanks* to its short half-life. Thus, eosinophils may follow the eotaxin gradient from bone marrow to peripheral tissue then follow histamine gradients to tissue specific sites.

Histamine and H4Rs have also been shown to regulate dendritic cell chemotaxis and function. Histamine induces migration of murine and guinea pig dendritic cells in both *in vitro* (transwell) and *in vivo* (ear skin) systems in both an H4R (JNJ 7777120 inhibited) and H1R (diphenhydramine inhibited) manner. However, JNJ 7777120 has become very controversial: initially characterized as a neutral antagonist, it has since been shown to be a partial inverse agonist in steady state GTPase assays of human H4R and a partial agonist on mouse, rat, and

dog H4Rs.[235,240] Nonetheless, JNJ 7777120 also inhibited H4R agonist clobenpropit induced dendritic cell migration.[250]

H4R mRNA and protein is expressed in monocytes, monocyte derived dendritic cells, 6-sulfo LacNac expressing dendritic cells, plasmacytoid dendritic cells, and Langerhan's cells.[251] Its expression on monocytes and some dendritic cells is upregulated by stimulation with IFNγ.[252,253] H4R mediates human myeloid cell chemotaxis and suppresses TLR induced production of CCL2, CXCL8 (IL8), IL-27, IL-12, TNFα, and Interferon α.[234,251,253-255]

As with other myeloid cells, H4R agonists induce mast cell migration *in vitro* and in mouse models.[239] Mast cell precursor chemotaxis towards CXCL12, for which the receptor is CXCR4, is enhanced by histamine costimulation. That costimulation is lost with siRNA knockdown of H4R and in the presence of JNJ 7777120.[256] H4R drives PI3Kγ and ERK mediated IL-6 production from murine bone marrow derived mast cells alone and enhances LPS induced IL-6 production.[236]

Histamine, MDSCs, Mastocytosis

In addition to histamine specific mouse data and association between atopy and MDSC burden[187], the ability of histamine to regulate both hematopoiesis in general, growth factor dependent hematopoietic differentiation, particularly of the myeloid lineage, to induce chemotaxis of myeloid cells and upregulation of CD11b, makes histamine a strong candidate for regulation of human MDSCs. In humans, histamine is rarely systemically elevated due to its short half-life. However, patients with mastocytosis have consistently elevated plasma and urinary histamine levels.[257]

Mastocytosis

Mastocytosis is a heterogeneous disease characterized by the excessive accumulation of clonal mast cells in one or more tissues.[258] Unlike other clonal tumors or myelodysplasias, aside from mastocytoma, mastocytosis mast cells generally accumulate in one or more peripheral tissue. Symptoms and prognosis vary according to disease subtype, degree of mast cell hyperplasia, the organ(s) involved and the degree of organ impairment, mast cell activatbility, and non-mast cell hematopoietic changes. In most but not all patients, mastocytosis, or clonal mast cell disease, increases the risk of mast cell activation events, and mastocytosis does not preclude co-existence of allergy. A conglomeration of symptoms, and their otherwise idiopathic presentation, is of great importance in raising the suspicion of mastocytosis as each symptom or sign by itself is non-specific and overlaps with other diseases.

Many cases of mastocytosis are first identified by skin findings.[258] A classic and fairly unique finding is Urticaria Pigmentosa (UP), a darkly colored, maculopapular rash that frequently displays Darier's sign: scratching a papule activates mast cells within and causes itching, flushing, and swelling over the lesion (an urticarial response) due largely to released histamine. UP is an excellent example of two sources of pathology in mastocytosis: symptoms due to the disruptive presence of increased mast cell number in an organ (maculopapular rash itself) and symptoms due to mast cell activation (Darier's sign and itching). Some cases of mastocytosis present with recurrent idiopathic anaphylaxis and/or secondary anaphylaxis. Individuals with increased mast cell burden are at higher risk of anaphylaxis than individuals without mastocytosis for a given mast cell stimulant. A history of recurrent and severe anaphylaxis to

insect sting, regardless of the presence of venom specific IgE, should raise suspicion of mastocytosis.[259,260] Table 2 lists additional mastocytosis symptoms according to mechanism(s) involved and mast cell mediators thought to be responsible.[261] Parallel signs and symptoms in two or more organ systems should raise the suspicion of systemic mastocytosis.

In recent years, several international consensus proposals have been published to define World Health Organization criteria for diagnosis of mastocytosis and its subsets, to place mastocytosis in the context of mast cell disorders as a whole, and to better define aggressive forms of mast cell diseases.[258,261,262]

The 2007 based WHO criteria identifies “mastocytosis in the skin” (MIS) as a pre-diagnostic checkpoint and utilizes that checkpoint as a screening tool. MIS criteria are the finding of typical skin lesions (UP, diffuse cutaneous mastocytosis, or mastocytoma) with one or two of the following minor criteria on skin biopsy: (1) mast cell aggregates or increased mast cell density and (2) detection of *KIT* mutation. If adults fulfill MIS criteria, they can be assessed by criteria that define Systemic Mastocytosis (SM). SM is diagnosed by the fulfillment of 1 major and 1 minor criterion or at least three minor criteria as listed in Box 1. If SM criteria are not met, the final diagnosis is cutaneous mastocytosis (CM).

Mastocytosis presents in a bimodal age pattern: (a) pediatric onset which presents with MIS, particularly UP and Mastocytoma, is largely diagnosed before 2 years of age, and (b) adult onset. Pediatric cases are generally self-limited: most cases do not fulfill the SM criteria, are

diagnosed as CM, and the disease regresses by puberty. In the largest published retrospective pediatric review, pediatric mastocytoma totally resolved in 74% of cases and partially resolved in 18.5% and pediatric UP totally resolved in 54.2% of cases and partially resolved in 24.2%.[263] Across several studies it is calculated that about 84% of pediatric cases spontaneously resolve and the D816V point mutation, responsible for the majority of adult forms of mastocytosis, is difficult to detect in children, CM was considered by some to be a reactive disease: an accumulation of non-clonal mast cells due to mediator production by other cells.[264]

Recently, higher sensitivity assays have identified the D816V and other point mutations and/or aberrant CD25 or CD2 expression (suggesting clonality) on bone marrow mast cells in pediatric cases (25-83%).[265-269] In a multicenter study by Bodemer et al, 42% of 50 pediatric cases had the D816V mutation and 42% had a mutation elsewhere in the *KIT* gene.[270] However, presence of D816V mutation versus other mutations did not correlate with phenotypic presentation of disease and neither the detection of D816V nor the extent of cutaneous disease were predictive of persistent disease.[271]

Because pediatric mastocytosis has such a good prognosis, children with MIS are not assessed for SM as would be done for adults. Fulfillment of the major criteria, which generally requires a bone marrow or skin biopsy, is delayed until after puberty and conducted only if symptoms persist.[272] However, baseline serum tryptase levels are measured and used to monitor mast cell burden. A bone marrow biopsy is conducted in cases with baseline tryptase >100 ng/mL,

indicative of a high mast cell burden, or with progressive diseases (e.g. if organomegaly develops).[258,272]

The Spanish Network for Mastocytosis conducted 27 bone marrow biopsies of children to determine the rate of systemic involvement in childhood mastocytosis. Flow cytometric analysis of bone marrow aspirate detected 7 children (26%) with CD25+CD117+ mast cells, 6 (22%) of which had detectable bone marrow c-kit mutation.[268] Although the cohort is small, the percentage of children with clonal bone marrow involvement, 26%, is consistent with a resolution rate of 84%. [263] It is possible that children whose disease appears to extend into adulthood as SM always had the "adult" form of the disease in regards to bone marrow involvement. Nonetheless, as treatment of either CM or SM consists of symptom control, routine bone marrow biopsy is not justified in young children.

Table 2. Mastocytosis Symptoms			
	Infiltration	Activation	
		Location	Mediator(s)
Skin			
Urticaria Pigmentosa	MC infiltrates cause hyper-pigmented maculopapular rash	Pressure sensitive activation produces Darier's sign	Histamine
Mastocytoma	MC infiltrates produce large hyperpigmented cytoma		
Telangiectasia macularis eruptive perstans	MC infilterates produce redish hyperpigmented macules		
Pruritis		MC activation in skin	Histamine, PAF
Flushing			PGD_2
Urticaria			Histamine, PAF, LTC_4
Blistering		MC activation in the skin	IL-6, tryptase, PGD_2, PAF
Gastrointestinal			
Increased gastric acid		MC activation in stomach mucosa	Histamine
Ulcerations			
Intestinal cramping		MC activation in small and large intestines	Histamine, PAF, LTC_4, PDG_2
Diarrhea and/or vomiting			PDG_2
Bleeding		MC activation in colon	
Malabsorption	MC infiltrates disturb normal mucosal absorption	MC activation in GI tract can contribute to malabsorption	
Lungs			
Bronchoconstriction		MC activation in bronchial smooth muscle	Histamine, PGD_2, PAF, LTs, endothelin
Mucus and edema		MC activation in bronchial mucosa	Histamine, PGD_2, PAF, LTs, proteases
Skeletal			
Pain w/o osteopenia/ osteoporosis		MC activation in bone marrow, near bones, and in bone vasculature	
Osteosclerosis			
Osteopenia/Osteoporosis			Heparin, tryptase
Osteolysis	MC infiltrates	MC activation can contribute	
Constitutional/Systemic			
Headache		MC systemic activation or strong activation in one or more peripheral organs	Histamine, PDG_2
Poor concentration/ memory loss/ fogginess			PDG_2
Fatigue, weight loss, cachexia	Weight loss due to malabsorption caused by MC infiltrates		TNFα, IL-1β, IL-6
Hypotension and swelling			Histamine, PAF, PGD_2, LTs, endothelin
Bone marrow dysplasia/ myeloproliferation		MC activation in bone marrow or in distant organ	IL-5 (eosinophilia), Histamine & GM-CSF, (myeloproliferation)
Bone marrow dysfunction/ cytopenia	MC infiltrates overrun marrow		
Fibrosis		MC activation in any specific site	TGFβ
Inhibition of localized clotting			Heparin
Lymphadenopathy		MC systemic activation or within lymphoid organs	IL-6, lymphotaxin
Organomegaly and dysfunction	MC infiltrates can cause splenomegaly, hepatomegaly, ascites, and/or portal hypertension		

Table 3. World Health Organization Systemic Mastocytosis Criteria	
Fulfillment of 1 major and 1 minor *or* 3 minor criteria are required for diagnosis of SM	
Major	• Multifocal dense infiltrate of mast cells (>= 15 mast cells in an aggregate) detected in bone marrow biopsy or other extracutaneous organ(s) and confirmed by tryptase staining (or other special stains).
Minor	• In biopsy of bone marrow or other extracutaneous organ(s), >25% of MCs in the infiltrates are spindle-shaped or have atypical morphology; or, of all MCs in bone marrow aspirate smears, >25% are immature or atypical mast cells. • Detection of Kit point mutation at codon 816 in extracutaneous organ(s) known as D816V • Mast cells in blood or extracutaneous organ(s) that co-express CD117 with CD2 or CD25. • Baseline serum tryptase persistently greater than 20 ng/mL in the absence of associated myeloid disorder.

Not all adult cases initially present with cutaneous symptoms. Isolated bone marrow mastocytosis (a form of ISM, or indolent systemic mastocytosis), may instead be brought to medical attention by unexplained anaphylaxis, osteopathy, neurologic or constitutional symptoms, gastroduodenal disease, or chronic diarrhea. Aggressive systemic mastocytosis (ASM) and mast cell leukemia (MCL) may lack MIS but present with more severe symptoms.

In order to better address such cases and in an effort to create a unifying framework for understanding mast cell activation disorders, a second consensus proposal was published in 2012. In place of MIS, the 2012 framework designates systemic mast cell activation (MCA) as the pre-diagnostic checkpoint. Systemic MCA is defined by (1) typical signs and symptoms of mast cell activation (see table 1), (2) substantial and transient increase in measured mast cell mediators in body fluids (tryptase, histamine, PGD_2), and (3) objective response of clinical symptoms to agents that decrease production or inhibit signaling of mediators. In some cases,

clinical response to drugs may not be clear. In such cases, MCA may still be applicable if the first two conditions are met and a primary underlying mast cell disease or IgE mediated disease is known. From the MCA checkpoint, patients are evaluated to determine if they exhibit a clonal mast cell disease (primary MCA syndrome or MCAS), allergy or other underlying disease (secondary MCA), or idiopathic MCA. As touched upon earlier, an individual with primary MCA may also suffer from an allergy that produces mast cell activation and thus exhibit both primary and secondary MCA. Similarly, an individual may exhibit both a secondary MCA such as a known allergy and idiopathic MCA when a trigger cannot be identified.

Patients with primary MCAS are then assessed to determine if they fit WHO criteria for SM. IF SM criteria are not met but MIS criteria are met, as outlined above, the patient can be diagnosed with CM. In some cases individuals that have evidence of monoclonal MCAS do not fulfill the SM criteria and do not exhibit skin symptoms that fulfill the MIS criteria. Such patients are categorized as "monoclonal MCAS" and may represent a precursor or limited form of SM, particularly if they are shown to harbor the D816V mutation in *KIT*.

Rationale and hypothesis

The role of each mediator and the degree of its production is variable in SM and may depend on tissue and microenvironment regulation of clonal mast cell, clonal mast cell subtype, involvement of non-mast cell lineages, and the sensitivity of clonal mast cells to activation. Integration of surface *KIT* receptor signaling and FcεRIα crosslinking has been shown to

markedly increase mast cell sensitivity to IgE mediated degranulation suggesting that, in SM, though the majority of mutant *KIT* is intracellular, mast cells are relatively hyper-activatable.[273]

Nevertheless, a large portion of mastocytosis symptoms can be attributed to histamine release in particular (Table 2). Indeed, many, but not all, symptoms of mastocytosis are inhibited by combination therapy with high dose H1R and H2R antagonists. Given the high plasma histamine concentrations found in SM, there is undoubtedly some local, and possibly systemic, histamine receptor "escape". The recent discovery of high affinity H3 and H4 histamine receptors means that a portion of histamine mediated events is entirely un-inhibited.

Therefore, this project addresses the hypothesis that MDSC levels are elevated in patients with mastocytosis, likely due to histamine mediated recruitment and survival of MDSCs despite high doses of H1 and H2 receptor antagonists taken by mastocytosis patients. Other mast cell mediators may be involved. Because pediatric mastocytosis frequently regresses after onset of puberty, this project proposed that mastocytosis MDSCs inhibit a natural anti-mastocytosis immune response and can predict disease regression of pediatric mastocytosis. (Figure 20)

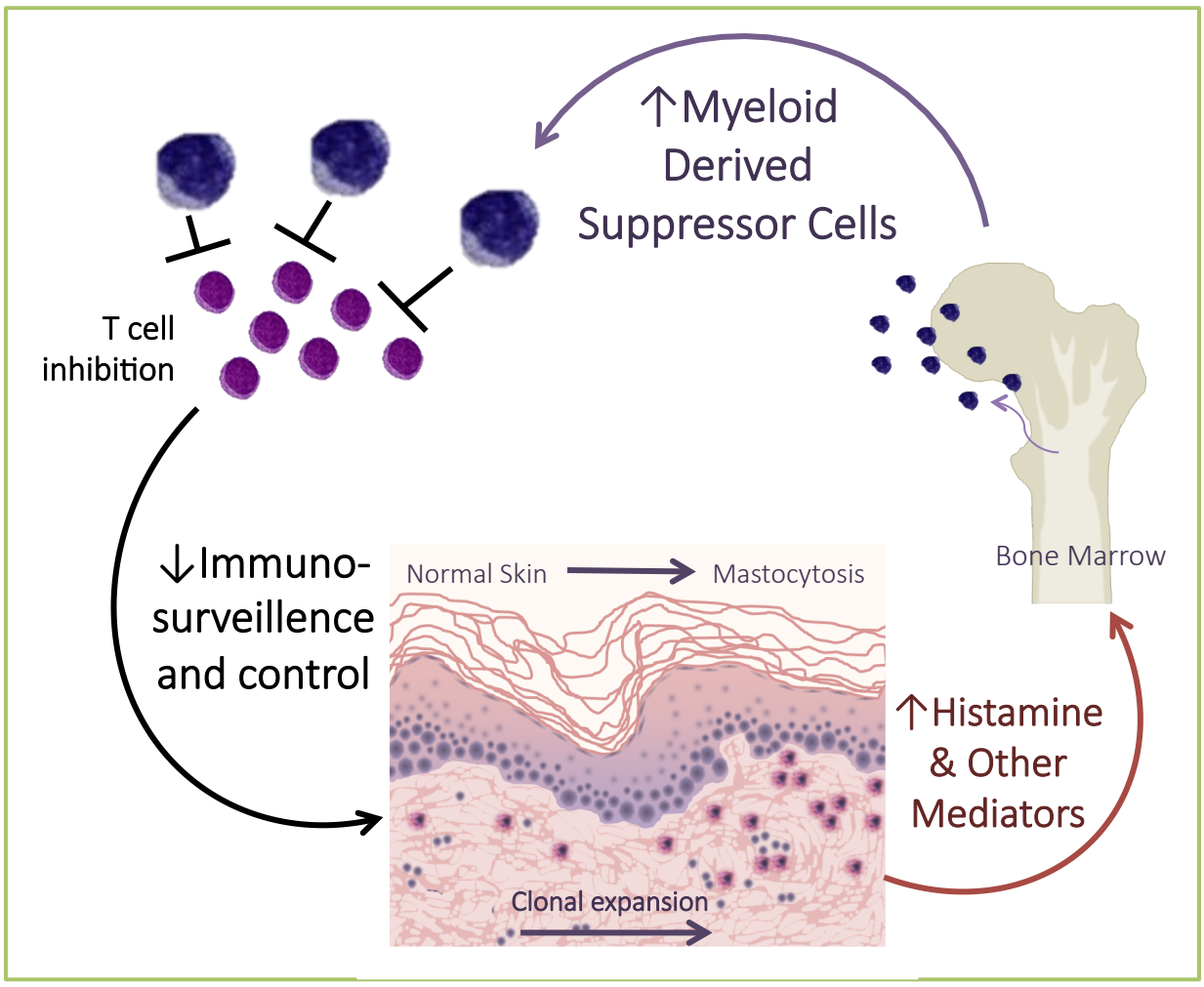

Figure 20. Project Hypothesis

T cells, via immune surveillance, normally eliminate clonal mast cells or clonal mast cell progenitors. As clonal mast cells escape immune surveillance and accumulate, plasma concentrations of histamine and other mast cell mediators increase, causing MDSC development and/or recruitment. MDSCs inhibit the T cell anti-clonal mast cell effect and allow further accumulation of mast cells. In adults, clonal mast cells are likely well established and MDSC burden elevated. In

children, an elevated MDSC level may predict whether their mastocytosis will persist into adulthood.

Methods

Reagents

Recombinant human SCF was a gift (Swedish Orphan Biovitrum, Stockholm, Sweden). X-vivo 15 (Lonza, Walkersville, MD); ficoll-paque PLUS (GE Healthcare, Piscataway, NJ); Fc Block (Miltenyi Biotech); antibodies targeting HLA DR-Alexa 488 (LN3 clone), CD33-PE, CD11b-APC, CD14-PerCP-Cy5.5, CD15-PECy7, CD20 PerCp-Cy5.5, CD3-PECy7, CD4-PE, and CD8-PerCpCy5.5 (Biolegend, San Diego, CA); and CFSE stain and Near-IR Live Dead stain kits (Life technologies, Grand Island, NY) were obtained and used as described. 22E7 and tryptase antibodies B12 and G4, used for the G4 ELISA assay, are produced, and biotinylated if necessary, in-house.

Primary Human Mast cells

As described, primary human mast cells are extracted from de-identified, post-surgical human skin and cultured in XVIVO-15 medium with SCF (100 ng/mL).

Patient recruitment

This study is conducted in accordance with the amended Declaration of Helsinki. The Human Studies Internal Review Board (IRB) at Virginia Commonwealth University (FWA 00005287) approved the protocol. Written informed consent is obtained from all participants as per IRB approved protocols #HM20000022. Symptoms and medications of patients with mastocytosis

or chronic urticaria are recorded at time of consent. Patients with mastocytosis are entered into the Mastocytosis MDSC registry for 10 year follow up to evaluate disease course.

Blood collection, Tryptase testing, and MDSC enumeration

3 cc (children) or 10 cc (adult) of blood is collected by venous puncture following consent. 0.5 cc or more of blood is sent to VCU Pathology Outreach to run a complete blood count with differential (CBC w/diff). 0.5 cc of blood or more is spun down at room temperature to collect plasma. Plasma is stored frozen and sent to the VCU Tryptase Diagnostic Lab for quantification of total and mature tryptase concentrations. Remaining blood is dilute 4 fold with 2% fetal bovine serum (FBS) in PBS, layered over ficoll-paque PLUS and spun down. PBMCs are removed from the ficoll/plasma interface, remaining red blood cells (RBCs) are lysed by ACK lysing buffer (containing 8 g/L NH_4CL, 1 g/L $KHCO_3$, 3.7 g/L $EDTA \bullet Na_2 \cdot 2H_2O$). Cells are washed, labelled with near IR Live/Dead, Fc Blocked (Miltenyi), and subsequently labeled with antibodies for flow cytometric enumeration of MDSCs.

MDSC, Myeloid cell, and T cell sorting

For some adults with mastocytosis, 50 cc of blood is collected. A small portion is treated as above for CBC w/diff, tryptase level, and MDSC enumeration. Remaining blood is diluted 2 fold with 2% FBS in PBS and PBMCs are purified as above. If possible, ACK buffer RBC lysis is avoided as it could potentially activate granulocytic cells in the PBMC prep. PBMCs are then labeled with antibodies for flow cytometry assisted cell sorting (FACS) into purified MDSCs (HLA DR-CD33+CD11b+), myeloid cells (HLA DR+CD33+CD11b+), and T cells (lymphocyte gated, CD20-) for use in functional assays.

T cell suppression assay

Following cell sorting, T cells are labelled with CFSE and activated with CD3/CD28 human T cell activator beads (Life Technologies) in the presence of increasing numbers of either MDSCs or other myeloid cells. Following a 3 day incubation, cocultures are collected, Fc Blocked, and labeled with antibodies for flow cytometry.

Mast cell suppression assay

Following cell sorting, MDSCs or other myeloid cells are cultured overnight with primary MC_{TC} cells. The following day, cocultures are washed and resuspended in Tyrode's Buffer. 22E7 is added at varying concentrations for 20 min at 37 degrees. Activation is stopped with ice cold Tyrode's buffer, cells are spun down, supernatant is separated, and pellets are resuspend in a volume equal to that of the total supernatant. Triton-X 100 is added to 1% to both supernatant and pellet suspensions and pipetted repeatedly to promote cell lysis. Supernatants and pellets are stored frozen at -20°C for up to 1 month. Samples are thawed and total tryptase concentrations of supernatant and pellet are measured by the G4 total tryptase ELISA as described.[130]

Statistics

Unless otherwise noted, data is analyzed by ANOVA followed by a Mann Whitney Rank Sum Test for multiple comparisons. Correlations are calculated with Spearman's correlation test. $p<0.05$ is considered significantly different than the null hypothesis. All statistics presented in this project are preliminary and under-powered.

Results

Blood concentration of total MDSCs is significantly elevated in adults with mastocytosis compared to adults with chronic urticaria

Peripheral blood is collected from all study participants and medications and disease relevant symptoms are recorded for participants with mastocytosis and chronic urticaria. All patients with mastocytosis are entered into a ten year registry for followup. As mastocytosis is a rare disorder, initial efforts have been concentrated on recruitment for the mastocytosis group (M). Indeed, delay in control recruitment, particularly healthy controls, allow for age, gender, and ethnicity matching as best as possible. After consent, blood is collected by venous puncture and a portion is used for complete blood count testing by VCU pathology.

Remaining blood is processed within 4 hours of collection. Plasma is collected for tryptase testing and peripheral blood mononuclear cells (PBMCs) are prepped. Total Myeloid Derived Suppressor Cell (tMDSC) percentage of total, live, PBMCs is calculated by flow cytometry. (Figure 21A) tMDSCs are defined as HLA-DR-CD33+CD11b+ cells. Single, live lymphocytic cells are gated according to forward scatter and light scatter properties, as would be done during automated complete blood count, and used to calculate tMDSC concentration in blood ($[tMDSC]_{blood}$) as described in methods.

A total of 3 adult healthy controls (H), 9 adults with chronic urticaria (CU, antihistamine control), and 10 adults with mastocytosis (M) have been recruited at this time. The majority of group M is diagnosed with indolent systemic mastocytosis (ISM) though 3 of the 10, indicated

by blue circles, can be categorized as smoldering indolent mastocytosis (SmSM). (Figure 21B) SmSM is sometimes associated with myelodysplasia, and an association with particularly high tMDSC concentration was anticipated. However, as can be seen in Figure 21B, that is not the case: several patients with ISM have higher $[tMDSC]_{blood}$ than two SmSM patients.

As for SM, CU patients are treated with high dose H1 and H2 receptor antagonists, sometimes leukotriene inhibitor Montelukast and/or Doxepin, and rarely with steroids. The mechanism of action of most of the treatment medications is well described. Prostaglandins and not leukotrienes are reported to recruit and activate MDSCs in mouse models. Therefore, we did not anticipate, nor have we observed, a correlation between Monteleukast treatment and $[tMDSC]_{blood}$. Steroids, noted for their ability to reduce eosinophil and neutrophil numbers, seemed likely to produce medication associated changes in $[tMDSC]_{blood}$. Therefore, study eligibility is dependent on one month or more without the use of systemic steroids or regular use of topical steroids. Doxepin, on the other hand, was first designed as a tricyclic antidepressant but has non-specific activity at many sites including strong antagonism at H1 receptors and either antagonist or agonist behavior at catecholamine and cholinergic receptors. Doxepin use does not exclude an individual from this study. However, as with all other medications, doxepin dose used is noted. As is apparent in Figure 21B, M individuals taking 25-75 mg per day of Doxepin have significantly lower $[tMDSC]_{blood}$ compared to their doxepin-free M counterparts.

Taken together, doxepin users are categorized separately from non-users. Although recruitment numbers are still limited, M patients show a trend towards elevated $[tMDSC]_{blood}$ compared to CU patients when excluding both M+doxepin and CU+doxepin patients. (Figure 21B)

Only 2 healthy and 6 mastocytosis pediatric cases have been recruited thus far. Pediatric M $[tMDSC]_{blood}$ is not significantly different than adult CU controls. All pediatric M cases recruited thus far are diagnosed as, and display classic symptoms of, cutaneous mastocytosis (CM) or mastocytosis in the skin (MIS). Though there are no reports of $[tMDSC]_{blood}$ for children as young as 6 months old, as is recruited in this study, MDSCs have been noted to slightly increase with age in healthy adults. Therefore, though healthy children are extremely difficult to recruit, age matched controls are vital for proper evaluation of this cohort. Given that 80-90% pediatric mastocytosis cases spontaneously regress after puberty, all children with mastocytosis in this study will be part of a ten year registry that will allow evaluation of a relationship, if present, between $[tMDSC]_{blood}$ and disease outcome. Interestingly and despite that lack of age matched control, of the 6 pediatric M cases, only 1 (33%), shows elevated $[tMDSC]_{blood}$ thus far. (Figure 21B)

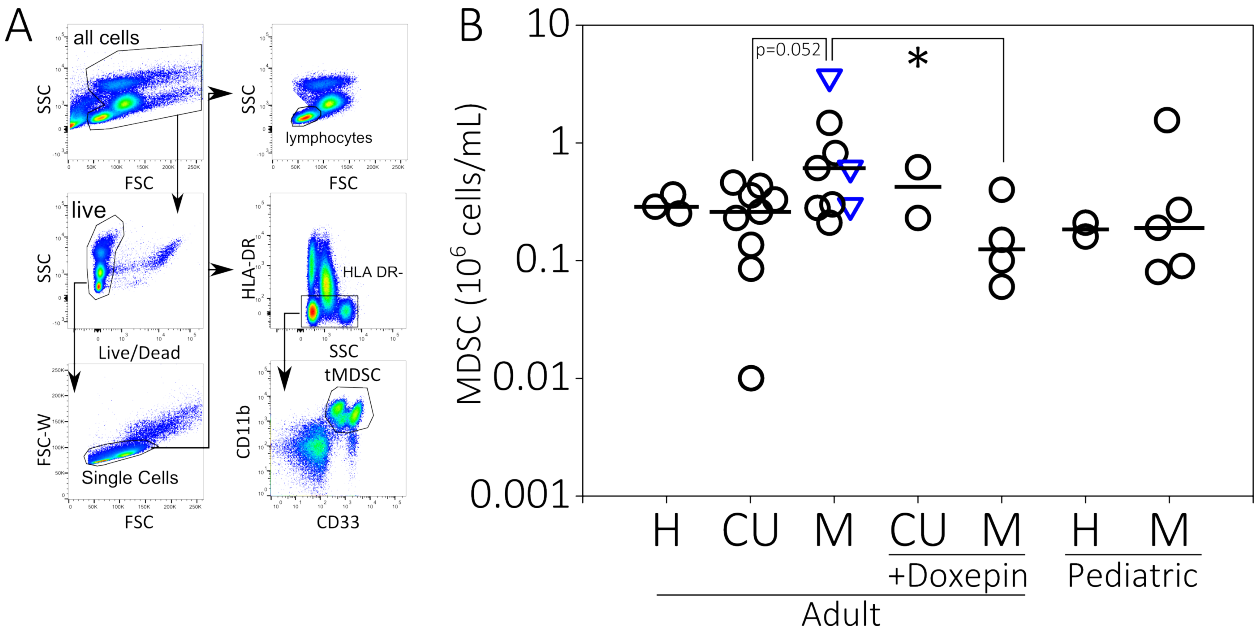

Figure 21. [tMDSC] is elevated in patients with systemic mastocytosis.

A, Necrotic cells and doublets are removed. Lymphocytes are selected by light scatter properties and used to calculate [tMDSC] as described. Live, single, HLA DR-CD33+CD11b+ MDSCs are selected as shown. B, [tMDSCs] for pediatric and adult healthy controls (H), chronic urticaria (CU), and mastocytosis (M). All adults are diagnosed with SM, all children with cutaneous mastocytosis (CM). As for SM, CU patients are treated with high dose H1 and H2 receptor antagonists, sometimes Montelukast, and rarely with steroids. Blue triangles (▽) indicate smoldering SM. **,p<0.05, Mann-Whitney Rank Sum test.*

Significantly elevated MDSC concentration in adults with mastocytosis is due to elevation in granulocytic, and not monocytic, subset of MDSCs

tMDSCs were further divided into $CD14^{bright}$ monocytic subset (monoMDSC) and $CD15^{bright}$ granulocytic subset (granMDSC). (Figure 22A) As with tMDSCs, each subset was binned

according to disease and doxepin use. Once again, ISM and SmSM mastocytosis patients did not segregate according to MDSC subset concentration. Adults with mastocytosis have significantly elevated $[granMDSC]_{blood}$ when compared to CU patients. M patients also trend higher than M+doxepin. (Figure 22B) Whereas granulocytic MDSCs differ between M and CU groups, no significant difference is detected between monocytic MDSCs of adults with mastocytosis versus chronic urticaria or mastocytosis patients taking doxepin. (Figure 22C)

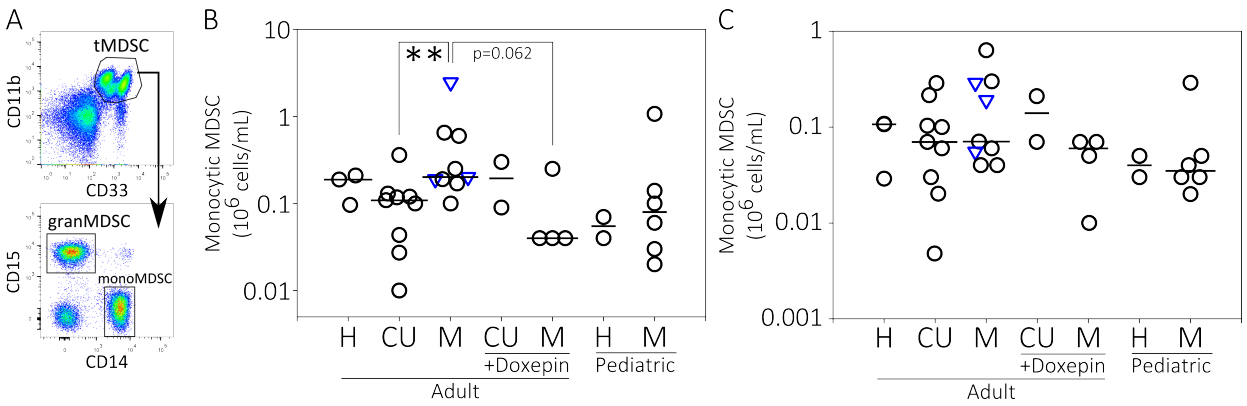

Figure 22. [granMDSC] is elevated in patients with systemic mastocytosis.

A, tMDSCs are separated into granulocytic (CD15+) and monocytic (CD14+) subsets. Blood concentrations are calculated relative to lymphocyte count as described. B, [granMDSC] for adult and pediatric H, CU, and SM groups. C, [monoMDSC] for adult and pediatric H, CU, and SM groups. Blue triangles (▽) indicate smoldering SM. ***,p<0.01, Mann-Whitney Rank Sum test.*

Mastocytosis associated tMDSCs are able to suppress CD3/CD28 bead stimulated T cell activation and proliferation *in vitro*

MDSCs are named for their suppressive function and evidence of suppressive function must be provided as gold standard for MDSC identification. Therefore, some adult mastocytosis study participants donated 50 cc of blood such that their tMDSCs can be sorted and tested for suppressive activity. After PBMC purification, cells are labeled for HLA DR, CD33, CD11b, and CD20 and sorted into 3 groups: (1) tMDSCs: HLA-DR-CD33+CD11b+, (2) non MDSC myeloid cells: HLA-DR+CD33+CD11b+, and (3) T cells: lymphocyte gated, CD20-. After sort, cells in the T cell group are evaluated for CD3, CD4, and CD8 expression and are >90% CD3+. (data not shown).

T cells are CFSE labelled and put into culture alone or with increasing numbers of MDSCs or non MDSC myeloid cells then stimulated with CD3/CD28 T cell activation beads for 3 days. On the final day, relative proliferation of T cells ($CFSE^{low}$) is calculated for each coculture ratio (Figure 21) and normalized to proliferation of T cells with CD3/CD28 beads alone. Whereas increasing non MDSC myeloid cells slightly increases the degree of T cell proliferation, increasing MDSCs suppress T cell proliferation. T cell proliferation is significantly lower at a 2:1 MDSC to T cell ratio compared with 10:1. In addition, MDSC-T and non MDSC myeloid-T cocultures are significantly different at 2:1 and 1:1 ratios. (Figure 23)

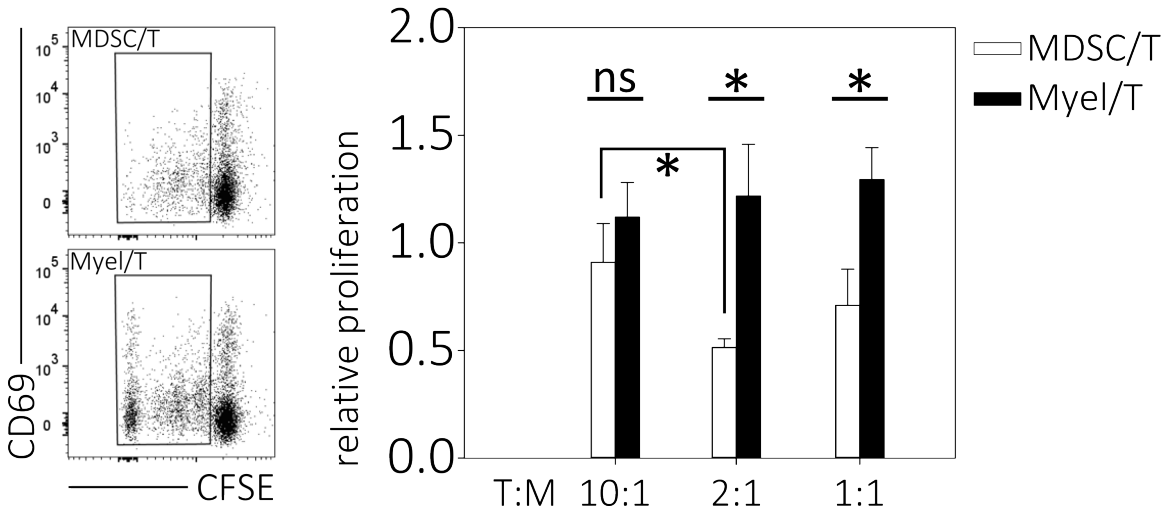

Figure 23. Mastocytosis MDSCs, but not myeloid cells, inhibit primary T cell proliferative response to CD3/CD28 activation beads.

A, Cells are cultured and labeled as described. Proliferation is assessed as reduced CFSE fluorescence as shown, and is then normalized to proliferation of T cells to beads alone. *B*, Summary of three experiments comparing proliferation of T cells cocultured with MDSCs or other myeloid cells. *n=3. *,p<0.05, one-tailed t test*

Mastocytosis MDSC concentration does not correlate with total tryptase

Plasma or serum total tryptase was measured by an ImmunoCAP commercial assay for each study participant. Total tryptase is elevated in adults with mastocytosis compared to healthy controls, CU patients, and CU patients taking doxepin. (Figure 24A) Although not yet significant, the M+doxepin group total tryptase trends much higher than that of H, CU, or CU+doxepin groups. Many, though not all, children with mastocytosis have elevated total tryptase as well.

As has been reported, children with mastocytosis tend to have lower levels of serum tryptase in comparison with their adult counterparts.

In vitro, whereas *immature* tryptase is constitutively released by mast cells, *mature* tryptase is mainly released from activated mast cells during degranulation. Therefore, *in vivo*, immature tryptase concentration likely correlates with mast cell burden whereas mature tryptase correlates with mast cell activation. [274,275] Baseline total tryptase, which is almost entirely immature tryptase, for healthy individuals (in the absence of mast cell activation, anaphylaxis, mastocytosis, or other clonal proliferative disease) is weakly determined by haplotype distribution of tryptase subtypes, gender, age, severe chronic renal failure, extensive alcohal consumption, and possibly obesity. [276-279] Unknown, additive genetic effects are responsible for as much as 82% of baseline serum tryptase variation as estimated by twin study comparison.[280]

Phadia's total tryptase assay, developed by Dr. Schwartz, measures both immature and mature forms of tryptase and, in the absence of mast cell activation, serum total tryptase levels reflect immature tryptase. Unique to our laboratory is the separate ELISA assay that specifically measures mature tryptase. The immature tryptase level can be estimated from the mature concentration and allows mast cell burden to be assessed even in the face of mast cell activation.

Despite the clear segregation of tryptase levels with disease groups (Figure 22A), neither total, mature, nor immature tryptase levels correlate with tMDSC or granMDSC concentrations in

patients with mastocytosis. (Figure 22B and Table 4) Intriguingly, in chronic urticaria subjects, total and immature tryptase levels do show significant correlation with granulocytic MDSC concentration. (Table 4) As all chronic urticaria participants are adults, the mastocytosis group was restricted to adults only, assessed again, without any observed significant correlations to MDSC burden found. Coupled with the observation that MDSC concentrations of CU patients treated with doxepin, though only 2 thus far, do not appear lower than those of CU doxepin-free patients, initial observations indicate that the mechanism of MDSC recruitment, or perhaps baseline CU MDSC levels, is different than the mechanism of MDSC recruitment in patients with Mastocytosis. Currently, in the absence of suppression assays, we lack key evidence that the low concentrations of HLA DR-CD11b+CD33+ identified in healthy and chronic urticaria controls are indeed MDSCs. Increasing recruitment and targeted experiments may clarify this point.

Table 4. Spearman's analysis of [MDSC] and [tryptase] correlations. r^2 (p)

	Total tryptase	Immature tryptase	Mature tryptase
Mastocytosis, all			
n	15	14	15
[tMDSC]	0.02 (0.94)	0.04 (0.89)	0.16 (0.57)
[granMDSC]	0.07 (0.79)	0.11 (0.70)	0.17 (0.54)
Mastocytosis, adult only			
n	9	8	9
[tMDSC]	-0.15 (0.68)	-0.14 (0.71)	-0.02 (0.95)
[granMDSC]	-0.13 (0.71)	-0.10 (0.79)	0.03 (0.91)
Chronic Urticaria			
n	8	8	8
[tMDSC]	0.51 (0.10)	0.56 (0.12)	0.25 (0.54)
[granMDSC]	***0.71 (0.04)***	***0.71 (0.04)***	0.25 (0.54)

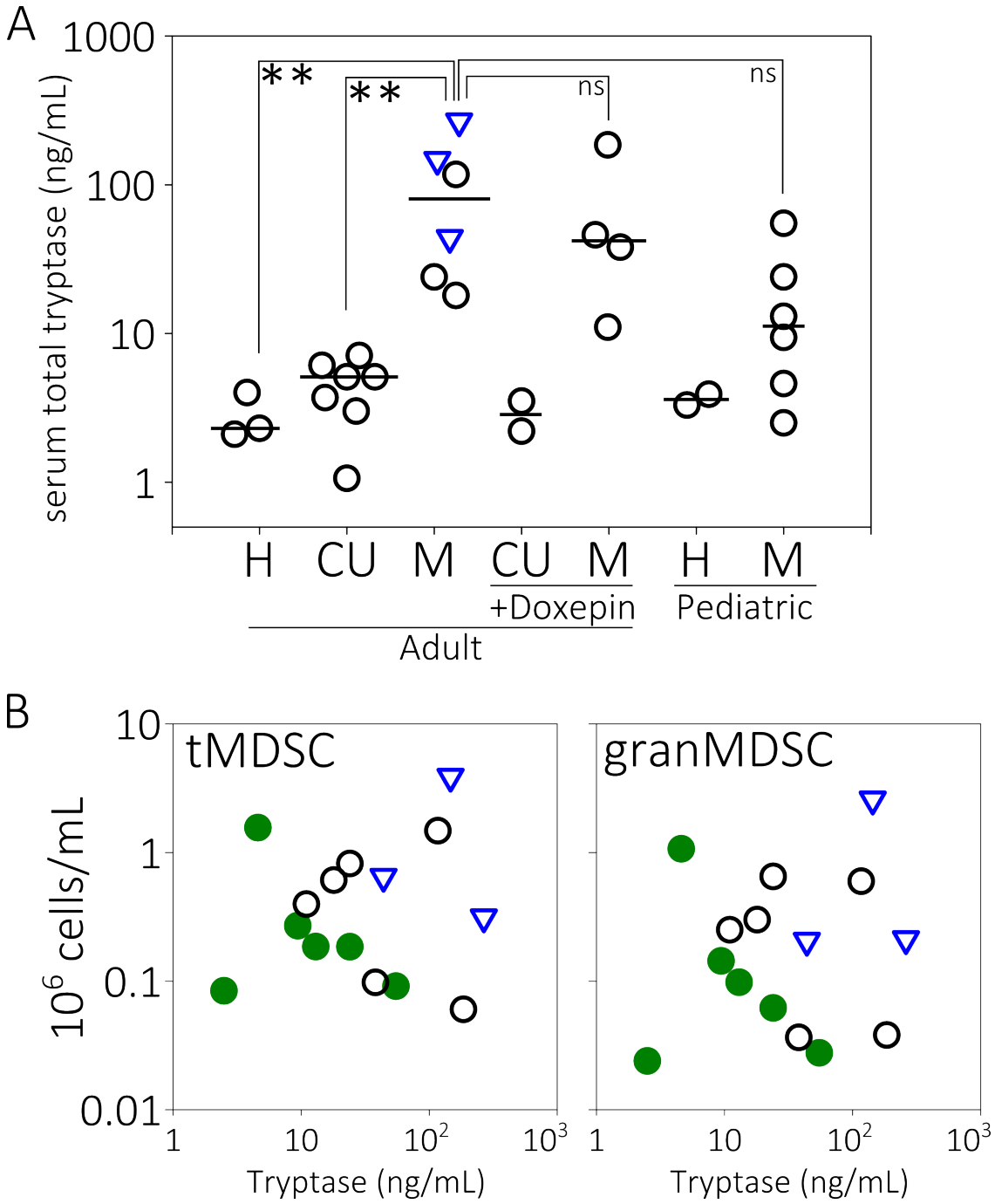

Figure 24. Total tryptase does not correlate with $[tMDSC]_{blood}$ or $[granMDSC]_{blood}$.

A, [Tryptase] for adult and pediatric H, CU, and SM groups. ***, p<0.001, ANOVA followed by Dunn's multiple comparison (if n<3, category was not included in ANOVA) B,* [Tryptase] versus

[tMDSC] or [granMDSC] for all patients with mastocytosis. Triangles (▽) are smoldering SM. Filled circles (●) are pediatric cases. Spearman's correlation coefficient (r^2) and p value are listed in Table 4. Spearman's correlation coefficient was also assessed post-hoc for various subgroups including doxepin-free only, indolent SM only, and by age/disease grouping as in *A.* [Tryptase] did not show correlation with [tMDSC] or [granMDSC] for any subgroup.

Mastocytosis associated tMDSCs are able to suppress IgE receptor stimulated mast cell activation *in vitro.*

As shown in Table 4, mature tryptase, an indicator of mast cell activation, does not correlate with [tMDSC] or [granMDSC] for patients with mastocytosis. As discussed, MDSC suppressive mechanisms are locally specific, not antigen specific, and largely mediated through production of NO and reactive oxygen species ROS. Macrophage produced NO, and NO from chemical donors, has been shown to inhibit IgE mediated degranulation, LTC4 secretion, IL-6 and TNFα production by rodent mast cells through modulation of PLCγ 1 and transcription factors AP1, Fos, and Jun. [281-286]Human CD34-derived mast cell IgE mediated degranulation is also inhibited by NO. [287]In contrast, murine bone marrow derived mast cell cytokine production is enhanced by incubation with MDSCs and MDSCs increase the severity of mouse airway hypersensitivity.[218] Therefore, the effects of human MDSCs on human mast cell function was assessed.

MC_{TC} cells were incubated overnight with sort purified MDSCs or non-MDSC myeloid cells, washed, and stimulated with 22E7, an IgE receptor crosslinking antibody. Degranulation was determined using the total tryptase assay as tryptase is a mast cell specific protease. Total

tryptase concentrations were determined by the G4 ELISA assay for both supernatant and lysed pellet. Net percent degranulation was determined as follows:

$$net\ \%\ degranulation =$$

$$\frac{([tryptase]_{degran\ releasate}) - [tryptase]_{quiescent\ releasate}}{([tryptase]_{degran\ retentate} + tryptase_{degran\ releasate}) - [tryptase]_{quiescent\ releasate}}$$

MC_{TC} incubation with MDSCs, but not myeloid cells, reduced FcεRIα mediated degranulation response compared to MC_{TC} alone at all 22E7 concentrations tested. (Figure 25) Although this experiment needs to be repeated to be validated, it is consistent with findings in human CD34+ derived mast cells. [287] Though significant alterations in surface FcεRI expression following overnight incubation are unlikely, many of the downstream signal mediators of the T cell receptor are structurally similar to those of FcεRI (e.g. Zap-70 and Syk) and alterations in CD3ζ chain function following exposure to L-arginine and ROS may be paralleled by alterations in FcεRI function due to such exposure.

n = 1

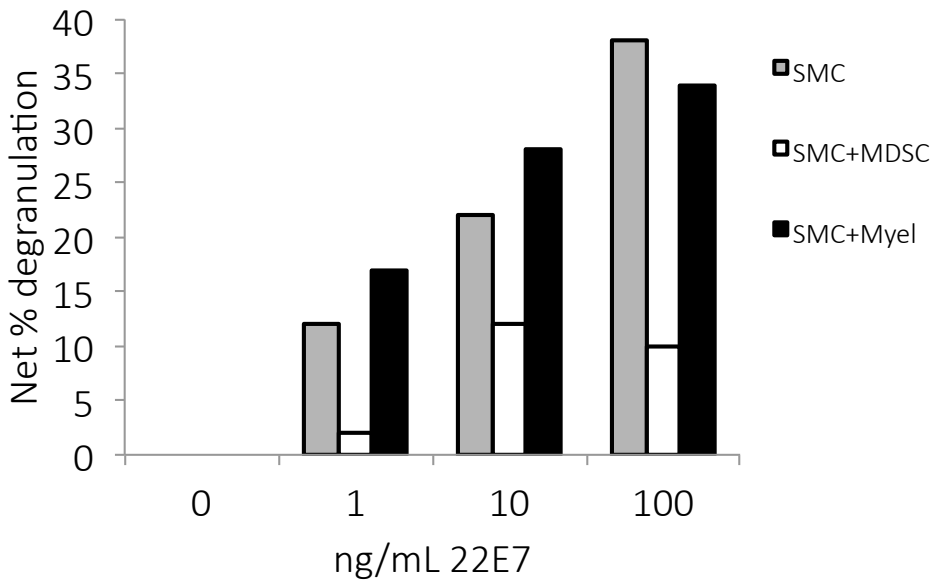

Figure 25. Mastocytosis MDSCs inhibit MC_{TC} IgE mediated degranulation following overnight culture.

Sort purified MDSCs and non-MDSC myeloid cells were incubated overnight at a 2:1, MC_{TC}:MDSC or non-MDSC myeloid cell ratio, then washed, resuspended in Tyrode's buffer, and stimulated for 20 min with 22E7. Releasate and retentates were separated and the retentate pellet was resuspended in equal volume as that of releasate. Triton-X 100 was added to both releasate and retentate at a final concentration of 1% to lyse pellet and solubilize all tryptase. Tryptase concentrations were evaluated by the G4 total tryptase ELISA. *n=1.*

Conclusions

- MDSCs, particularly granulocytic MDSCs, are elevated in patients with mastocytosis compared to chronic urticaria and healthy controls.
- MDSCs, but not non-MDSC myeloid cells, from individuals with mastocytosis are able to suppress both T cell proliferation and mast cell degranulation.
- Although we hypothesize that MDSCs are recruited by mast cell mediators, analogous to tumors, there are additional explanations that must be evaluated
    1. Our primary hypothesis: Clonal mast cells in peripheral tissues produce mediators in excess, which are able to recruit MDSCs from the bone marrow
    2. A local hypothesis: clonal mast cells in the bone marrow produce mediators which act locally to induce MDSC production and exit from the bone marrow. Indeed, it is possible that antihistamines administered systemically are not efficient at inhibiting histamine receptors in the bone marrow. Thus, MDSCs may be a specific marker of bone marrow mast cell infiltration/expansion.
    3. MDSCs are progenitors of mast cells that harbor the D816V mutation and their increased numbers are due to clonal expansion and accumulation. This is a technically difficult but readily testable hypothesis that we are currently attempting to address.

Future/Ongoing studies

- Ongoing recruitment of healthy, chronic urticaria, and mastocytosis patients will enhance all the data presented thus far.
- In addition, all mastocytosis patients will be followed up for 10 years to determine the prognostic significance of blood MDSC concentration. In particular, we hope to determine if elevated MDSCs are predictive of adult forms of systemic mastocytosis for children currently diagnosed with CM and/or potential SM. If this is the case, MDSCs may represent both a diagnostic tool and a treatable target.
- The MDSC mechanism of T cell and mast cell suppression, assumed to be mediated by ROS and NO production, can be evaluated in vitro.
- The ability of histamine, mast cell mediators, and mast cells to prevent the rapid apoptosis and necrosis of MDSCs and/or to induce proliferation of MDSCs can be evaluated.

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Appendix.

Appendix I. Human Histamine receptor specificities

PDSP database ID	Receptor/ Aliases	Test Ligand Name/ Aliases	NCI ID	Ki (nM)	Hot Ligand/ Aliases	Source/ cell model	Reference
Amitryptiline/H1							
47185	HISTAMINE H1	AMITRIPTYLINE	N/A	0.5	3H-MEPYRAMINE	CLONED	Ghoneim OM, et al., 2006
				0.91	3H-MEPYRAMINE	Sf9	Appl et al 2012
Amitryptiline/H2							
				66.1	3H-tiotidine	Sf9	Appl et al 2012
Amitryptiline/H3							
55082	HISTAMINE H3	AMITRIPTYLINE	N/A	1,000	UNDEFINED	UNDEFINED	von Coburg Y et al., 2009
				75,858	3H-Nalphamethylhistamine	Sf9	Appl et al 2012
Amitryptiline/H4							
14421	HISTAMINE H4	AMITRIPTYLINE	N/A	34	3H-PYRILAMINE	CLONED	Nguyen T. et al., 2001
				26,303	3H-histamine	Sf9	Appl et al 2012
Cimetidine/H1							
12017	HISTAMINE H1	CIMETIDINE	335308	> 10,000	3H-doxepin	CORTICAL MEMBRANES	Kanba S, et al., 1984
				6,190			Bielory L, et al., 2005
Cimetidine/H2							
				2,377			Bielory L, et al., 2005
				630			Thurmond 2008 ****
Cimetidine/H3							
14405	HISTAMINE H3	CIMETIDINE	335308	> 10,000	3H-RAMHA	CLONED	Liu C., et al., 2000
				> 10,000			Bielory L, et al., 2005
Cimetidine/H4							
14461	HISTAMINE H4	CIMETIDINE	335308	>10,000	3H-Histamine	CLONED	Liu C., et al., 2000

14436	HISTAMINE H4	CIMETIDINE	335308	>10,000	3H-PYRILAMINE	CLONED	Nguyen T. et al., 2001
Cetirizine/H1							
11036	HISTAMINE H1	Cetirizine	N/A	6	3H-MEPYRAMINE	CLONED	Moguilevsky N et al, 1994
11909	HISTAMINE H1	Cetirizine	N/A	6	3H-MEPYRAMINE	CLONED	Moguilevsky N, et al., 1995
17393	HISTAMINE H1	Cetirizine	N/A	6	3H-MEPYRAMINE	CLONED	Gillard M, et al., 2002
Cetirizine/H4							
	HISTAMINE H4			>10,000	3H-MEPYRAMINE	SK-N-MC/hH4 cells.	Lim et al 2005
Diphenhydramine/H1							
18968	HISTAMINE H1	Diphenhydramine	33299	14	3H-MEPYRAMINE	CLONED	Booth RG et al., 2002
47171	HISTAMINE H1	Diphenhydramine	33299	10	3H-MEPYRAMINE	CLONED	Ghoneim OM, et al., 2006
				13			Bielory L, et al., 2006
Diphenhydramine/H3							
14408	HISTAMINE H3	Diphenhydramine	33299	> 10,000	3H-RAMHA	CLONED	Liu C., et al., 2000
14416	HISTAMINE H3	Diphenhydramine	33299	> 10,000	3H-RAMHA	CLONED	Lovenberg, TW et al., 1999
Diphenhydramine/H4							
14464	HISTAMINE H4	Diphenhydramine	33299	> 10,000	3H-Histamine	CLONED	Liu C., et al., 2000
Doxepin/H1							
18971	HISTAMINE H1	DOXEPIN	N/A	0.3	3H-MEPYRAMINE	CLONED	Booth RG et al., 2002
47178	HISTAMINE H1	DOXEPIN	N/A	0.2	3H-MEPYRAMINE	CLONED	Ghoneim OM, et al., 2006
	HISTAMINE H1	DOXEPIN		1.2	3H-MEPYRAMINE	Sf9	Appl et al 2012
	HISTAMINE H1	DOXEPIN		0.1	3H-MEPYRAMINE		Bakker et al2007
Doxepin/H2							
	HISTAMINE H2	DOXEPIN		162	3H-tiotidine	Sf9	Appl et al 2012
Doxepin/H3							
	HISTAMINE	DOXEPIN		39811	3H-	Sf9	Appl et al 2012

	H3				Nalphamethylhistamine		
Doxepin/H4							
14423	HISTAMINE H4	DOXEPIN	N/A	106	3H-PYRILAMINE	CLONED	Nguyen T. et al., 2001
	HISTAMINE H4	DOXEPIN		15135	3H-histamine	Sf9	Appl et al 2012
Fexofenadine/H1							
17389	HISTAMINE H1	Fexofenadine	N/A	10	3H-MEPYRAMINE	CLONED	Gillard M, et al., 2002
				83			Bielory L., et al., 2005
Fexofenadine/H4							
	HISTAMINE H4	Fexofenadine		>10,000	3H-Histamine	SK-N-MC/hH4 cells.	Lim et al 2005
Histamine/H1							
	HISTAMINE H1	Histamine		2,060	3H-MEPYRAMINE	Sf9	Seifert et al 2003
	HISTAMINE H1	Histamine		1,260	3H-MEPYRAMINE		Moguilevsky et al 1994
	HISTAMINE H1	Histamine		2,399	3H-MEPYRAMINE		Strasser et al 2008
	HISTAMINE H1	Histamine		63,095	3H-MEPYRAMINE		Lim et al 2005
	HISTAMINE H1	Histamine		2,398	3H-MEPYRAMINE	Sf9	Appl 2011
Histamine/H2							
	HISTAMINE H2	Histamine		1,100	125I-AMINOPOTENTIDINE	Sf9	Kuhn et al 1996
	HISTAMINE H2	Histamine		50,118	3H-Iodoaminopotentidine		Lim et al 2005
Histamine/H3							
	HISTAMINE H3	Histamine		6.31			Schnell et al 2010
	HISTAMINE H3	Histamine		13	125I-IODOPROXYFAN		Ligneau et al 2000
	HISTAMINE H3	Histamine		10		SK-N-MC cells	Lim et al 2005
Histamine/H4							

	HISTAMINE H4	Histamine		9.8			Schneider et al 2009
	HISTAMINE H4	Histamine		4.7	3H-HISTAMINE		Gbahou et al 2006
	HISTAMINE H4	Histamine		12.9			Brunskole et al 2011
	HISTAMINE H4	Histamine		8.5			deml et al 2009
	HISTAMINE H4	Histamine		15.8			Lim et al 2005
Hydroxyzine/H1							
17384	HISTAMINE H1	Hydroxyzine	N/A	2	3H-MEPYRAMINE	CLONED	Gillard M, et al., 2002
Loratidine/H1							
	HISTAMINE H1	Loratidine		35			Bielory, et al., 2005
Loratidine/H4							
	HISTAMINE H4	Loratidine		>10,000	3H-Histamine	SK-N-MC/hH4 cells.	Lim et al 2005
Ranitidine/H1							
11039	HISTAMINE H1	RANITIDINE	N/A	> 10,000	3H-MEPYRAMINE	CLONED	Moguilevsky N et al, 1994
				> 10,000			Bielory L, et al., 2005
Ranitidine/H2							
	HISTAMINE H2	RANITIDINE		187			Bielory L, et al., 2005
	HISTAMINE H2	RANITIDINE		79			Thurmond 2008
Ranitidine/H3							
14404	HISTAMINE H3	RANITIDINE	N/A	> 10,000	3H-RAMHA	CLONED	Liu C., et al., 2000
14415	HISTAMINE H3	RANITIDINE	N/A	> 10,000	3H-RAMHA	CLONED	Lovenberg, TW et al., 1999
	HISTAMINE H3	RANITIDINE		> 10,000			Bielory L, et al., 2005
Ranitidine/H4							
14460	HISTAMINE H4	RANITIDINE	N/A	> 10,000	3H-Histamine	CLONED	Liu C., et al., 2000

Vita

Sahar Lotfi-Emran was born on April 12, 1983, in Ghahemshahr, Mazandaran, Iran. She and her family immigrated to the United States in 1989 and she is currently a naturalized citizen of the United States of America. Sahar attended The Park School of Baltimore in Brooklandville, MD, and graduated with her high school diploma in 2001. She received her Bachelor of Arts in Sociology with a minor in Cellular Biology in 2005 from Vassar College, Poughkeepsie, NY. She then entered and completed one year of the Drexel University Interdisciplinary Medical Sciences (IMS) Program, Philadelphia, PA, finishing in 2006. Sahar entered Virginia Commonwealth University School of Medicine as a MD candidate in 2006 and was accepted to the MD/PhD program in 2007.

Sahar has had diverse research experiences. In high school, Sahar was a lab technician in the laboratory of Dr. Daniel Drachman at Johns Hopkins Hospital in the department of Neurology where she assisted in conducting research on Myasthenia Gravis. Later, while still in high school, she worked in the office of Dr. Mark Noar and assisted in accumulation of clinical data on the Stretta procedure. She continued this research into college and her work was published in 2007, "Sustained improvement in symptoms of GERD and antisecretory drug use: 4-year follow-up of the Stretta procedure."[288] During college, she also had the opportunity to work as a research technician to develop a student protocol for use of MALDI-TOF in the classroom with Dr. Eric Eberhardt. Following her year in the Drexel IMS program, Sahar worked as a technician in the laboratory of Dr. William Baldwin, III where she assisted with research pertaining to

chronic heart rejection post-transplant and the role of complement in chronic heart rejection. Her work resulted in second authorship in 2007, “The involvement of FcR mechanisms in antibody-mediated rejection.”[289]

Since joining the laboratory of Dr. Lawrence Schwartz, Sahar has had the opportunity to assist with several ongoing projects which have resulted in co-authorship of two papers: “Total tryptase levels indicate risk for systemic reactions to rush immunotherapy and mast cell activation”[290] and “Intracellular adenosine inhibits IgE-dependent degranulation of human skin mast cells.”[127] Articles derived from the work presented in this dissertation are forthcoming. Sahar has received several honors during her time in the laboratory including selection for Phi Kappa Phi Honor Society award in 2011, Phi Kappa Phi membership from 2011 to the present, selection for the Chrysalis Program of the American Academy of Allergy, Asthma, and Immunology in 2011, selection for the Virginia Commonwealth University Thesis/Dissertation Award in 2014, and selection for funded participation at the Mucosal Immunity Modeling School taking place at Virginia Technical Institute in 2014.