SOLUBLE T CELL MEDIATORS AND ALLERGIC DISEASES

SOLUBLE MEDIATORS RELEASED BY CD4+ T CELLS ACTIVATE BASOPHILS AND NEUTROPHILS IN THE CONTEXT OF IGE-MEDIATED ALLERGIC DISEASES

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LAY ABSTRACT

Allergies occur when the immune system overreacts to otherwise harmless substances in our environment. When this happens, innate immune cells, such as basophils, release histamine, causing allergic symptoms. Some people develop symptoms that can last several days, where other immune cells, such as T cells, get involved and contribute to inflammation. We looked at how basophils and neutrophils interacted with T cells, and whether T cells could directly activate basophils and neutrophils. We found that T cells produced substances that activated basophils and neutrophils, but this activation did not result in an increase in histamine. However, we saw that T cells produced substances that interacted with another component involved in allergies (a protein called IgE), which resulted in a supra-additive histamine response.

ABSTRACT

The current paradigm in allergic diseases is that it is driven by Th2 responses, causing the influx of cells including eosinophils, basophils, neutrophils, and Th2 cells, giving rise to the late-phase response. Our lab previously showed that peptide immunotherapy can reduce allergic symptoms – this relief of symptoms coincides with a reduction of IL-8 in allergen-specific T cells. We hypothesized that T cell-derived soluble mediators such as IL-8 contributes to allergic inflammation by stimulating basophils and neutrophils to release histamine. In this study, we determined that IL-8 alone was unable to stimulate basophils to release histamine; however, in the presence of IL-3, IL-3 and IL-8 acted synergistically with subsequent anti-IgE stimulation to release histamine (observed fractional response: 0.807, compared to predicted fractional response if the effects were additive: 0.453). Supernatants derived from CD4+ T cells stimulated with anti-CD3/28 and Immunocult acted synergistically with subsequent anti-IgE stimulation to release histamine (observed fractional response: 0.636 [anti-CD3/28 at $1.0x10^6$ cells/ml] and 0.754 [Immunocult at $2.5x10^6$ cells/ml], compared to predicted fractional response if the effects were additive: 0.364 and 0.434, respectively). Basophils primed with anti-CD3/28 and Immunocult-stimulated T cell supernatants plus anti-IgE did not statistically increase the amount of histamine released, compared to media-stimulated T cell supernatant plus anti-IgE. Basophils were activated by T cell supernatants alone and when used for priming with anti-IgE, by upregulating CD63/CD203c markers (anti-CD3/28 alone, anti-CD3/28+anti-IgE, Immunocult alone, and Immunocult+anti-IgE: 60.7%, 85.3%, 87.7%, and 93.7% CD63/CD203c+, respectively, compared to media+anti-IgE (46.36% CD63/CD203c+). Neutrophils contained histamine (63.71nM per 10^6 neutrophils) and released significant amounts in response to stimulation by fMLP. Neutrophils did not release histamine in response to stimulation by

supernatants derived from T cells. Neutrophils significantly downregulated CD62L when stimulated with supernatants derived from anti-CD3/28 and Immunocult-stimulated CD4+ T cells, and significantly up-regulated CD11b, compared to media-stimulated CD4+ T cells.

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In eternal love and memory.

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ABBREVIATIONS

AF	Alexa Fluor
AGEP	Acute generalized exanthematous pustulosis
AHR	Acute hyperresponsiveness
AIT	Allergen immunotherapy
ANOVA	Analysis of variance
AP-1	Activating protein 1
APC	Antigen presenting cell
AR	Allergic rhinitis
AUC	Area under curve
BAT	Basophil activation test
BMCP	Basophil-mast cell progenitors
BV	Brilliant Violet
C/EBP	CCAAT/enhancer binding protein
CAI	Chronic allergic inflammation
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
Con-A	Concanavalin A
CRTh2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
CX_3C	Fracktaline
CXCL	C-X-C motif ligand
CXCR	C-X-C motif receptor
CysLT	Cysteinyl leukotriene
DC	Dendritic cell
DHR123	Dihidrorhodamine 123
EDTA	Ethylenediaminetetraacetic
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay
ELR	Glu-Leu-Arg tripeptide motif
EPR	Early phase response
ERK	Extracellular signal-related kinase

FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FEV_1	Forced expiratory volume in the first second
FGF-2	Fibroblast growth factor 2
FITC	Fluorescein isothiocyante
FLT-3L	Fms-related tyrosine kinase 3 ligand
FMO	Fluorescence minus one
FSC	Forward scatter
GATA-3	GATA-binding protein 3
GFP	Green fluorescent protein
GM-CSF	Granulocyte-monocyte colony stimulating factor
GROα	Growth-regulated protein alpha
HDAC	Histone deacetylase
HDC	Histidine decarboxylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
IC	Intracellular
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon gamma
IgE-FAP	IgE-facilitated allergen presentation
IL	Interleukin
ILC	Innate lymphoid cell
ITAM	Immunoreceptor tyrosine-based activation motif
LAMP	lysosome-associated membrane proteins family
LAR	Late asthmatic response
LFA	Leukocyte function-associated antigen
LPR	Late phase response
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MCP-3	Monocyte chemotactic protein 3
MFI	Median fluorescence intensity
MHC	Major Histocompatibility Complex

MIP	Macrophage inflammatory protein
MLA	Mixed lymphocyte antigen
MMP9	Matrix metalloproteinase 9
MPO	Myeloperoxidase
NAb	Neutralizing antibody
NAP-1	Neutrophil attractant/activation protein-1
NIR	Near infrared
NK	Natural killer
OCT-1	Octamer 1
PAF	Platelet activating factor
PBCM	Peripheral blood mononuclear cell
PBS	Phosphate buffered solution
PE	Phycoerythrin
PGD_2	Prostaglandin D_2
PI3K	phosphatidylinositol 3 kinase
PIT	Peptide immunotherapy
PKC	Protein kinase C
PMA/I	Phorbol 12-myristate 13-acetate + Ionomycin
PMD	Piecemeal degranulation
PMN	Polymorphonuclear
pre-BMP	Bone marrow pre-basophil and mast cell progenitors
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SCIT	Subcutaneous immunotherapy
SD	Standard deviation
SEM	Standard error of mean
SLIT	Sublingual immunotherapy
SN	Supernatant
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TCR	T cell receptor

TET	Tetramer
TGFα	Transforming growth factor alpha
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
VEGF-A	Vascular endothelial growth factor A

DECLARATION OF ACADMIC ACHIEVEMENT

I, Mark Lychacz, performed all experiments described in this document with assistance from Dr. Tom Mu, Barbara Baker, Braeden Cowbrough, and Ana Chouvalova, in collecting blood samples from study participants. All sections in this document were written by me and reviewed by Dr. Mark Larché.

INTRODUCTION

Allergies, or allergic disorders, are a set of disorders caused by immune-hyperresponsiveness to non-infectious substances in the environment.[1] Allergic disorders include anaphylaxis, allergic rhinitis (hay fever), allergic conjunctivitis, atopic dermatitis (eczema), urticaria, and asthma.[2] Allergic diseases present as a global public health concern – it is estimated that 30-40% of the world's population is affected by one or more allergic disease, and the prevalence of allergic diseases is increasing worldwide in both developed and developing countries.[3] Furthermore, allergies are becoming more complex and severe, with polysensitization and the involvement in multiple organ systems.[3] Allergic rhinitis (AR) affects 10-30% of the population, and is a risk factor for developing asthma – it is one of the most common chronic conditions observed in children and adults.[3,4] AR is characterized by inflammation of the nasal mucosa, and is mediated by IgE.[3] The burden of AR goes beyond the psychological and social impact to the individual affected, and creates an economic burden for the family and society at large.[3] In the United States, costs in medical spending to treat AR almost doubled from 2000 ($6.1 billion) to 2005 ($11.2 billion), which include expenses such as over the counter medications and hospital inpatient care.[4] Indirect costs associated with AR include missed work, and reduced productivity at work, making it the fifth most costly disease.[4] In addition to being a risk factor for developing asthma, co-morbidities of AR include the development of other upper respiratory infections, sleep disorders, nasal polyposis, and conjunctivitis.[3] AR can be seasonal (occurring during a specific season) or perennial (occurring throughout the year).[5]

Allergies most often occur on the skin and in the airways and mucosal membranes.[3] Depending on which tissues are affected, allergic reactions manifest in different ways.[6] For example, allergic rhinitis involves inflammation of the nasal mucosa, whereas allergic asthma

involves inflammation and narrowing of the airways.[7,8] On the other hand, anaphylaxis is a systemic allergic reaction and affects multiple organs. It is particularly severe form of allergy, and can be fatal if not properly treated.[2,8]

1.1 Pathophysiology of Allergic Diseases

Often incorrectly referred to as "atopy" (which is actually just the genetic propensity to make IgE responses, but not necessarily associated with disease), IgE-mediated allergic diseases are characterized by the production of allergen-specific IgE upon exposing a susceptible person to an allergen, and the expression of clinical signs and symptoms upon subsequent exposure.[9] Individuals may be allergically "sensitized" (i.e. they have demonstrable allergen-specific IgE) without necessarily expressing symptoms. Such individuals are not referred to as "allergic," although they carry a high risk of progressing to allergic disease. In nonatopic individuals, exposure to aeroallergens derived from pollen, cat dander, and house-dust mites triggers an immunological response by producing allergen-specific IgG_1 and IgG_4 antibodies.[9] However, in atopic individuals, the predominant antibody isotype produced is IgE in response to exposure to allergens. IgE antibody is crucial in the process of sensitizing mast cells and basophils to respond to a particular allergen because 1) the IgE produced is specific to the allergen, and 2) the IgE epsilson (ε) heavy chain binds to the Fcε receptors on mast cells and basophils and primes them to respond upon subsequent exposure to the allergen.[2]

The production of IgE requires an orchestrated response from several different immune cells. Allergens that are inhaled or ingested must first be processed by professional antigen-presenting cells (APCs), most importantly dendritic cells (DCs), which drain into secondary lymphoid organs.[10] DCs are the sentinels of the immune system, responsible for initiating an immune response to potentially harmful substances by constantly sampling and recognizing

potential foreign antigens/allergens at mucosal sites. Following exposure of the skin, nose, or airway to an allergen allows epithelial DCs uptake the allergen, degrade the protein, and present processed peptide fragments on major histocompatibility complex (MHC) class II proteins which binds these peptide fragments on the cell surface. Allergen uptake by DCs kickstart the Th2 response, though it is still largely unknown how atopic and nonatopic individuals differ regarding how DCs respond to allergens. Nonetheless, DCs respond to major allergens by upregulating surface markers such as CD80, CD83, CD86, and human leukocyte antigen DR (HLA-DR) which are expressed by activated DCs and have been shown to enhance DC's antigen uptake capabilities.[11,12] The migration of DCs to the lymph nodes is mediated by lipid mediators and chemokines such as CCL3, CCL19, CXCL14, and CXCL12.[11] Furthermore, within the secondary lymph nodes, stromal cells within the T cell zone express ligands to CCR7, CCL19, and CCL21 that direct DC migration.[13]

T cells recognize peptides complexed with MHC class II molecules (TCRs) on naïve CD4+ T cells, thus priming naïve T cells (signal 1).[14] DCs also express the co-stimulatory molecules CD80 and CD86, which interact with CD28 on CD4+ T cells, providing additional activation signals (signal 2). Depending on the cytokine milieu, naïve CD4+ T cells can differentiate into several different T helper subsets (signal 3): Th1, Th2, Th9, Th17, and Tregs.[14] CD4+ Th2 cells are key in orchestrating the allergic response, due to their ability to release large amount of IL-4, IL-5, and IL-13 – cytokines which mediate isotype switching in B cells towards IgE- and IgG_1-producing B cells and the recruitment of eosinophils[15]. A group of endothelial-derived Th2-inducing cytokines, referred to as alarmins, include IL-25, IL-33 and TSLP (thymic stromal lymphopoietin) provide additional early signals to group 2 innate lymphoid cells

(ILC2s).[16] ILC2s are activated in response to these alarmins, and produce Th2 cytokines IL-4, IL-5, IL-9, and IL-13, which propagate the allergic response.

Following the production of type 2-dominated cytokines, a series of intracellular signalling events occur through the T cells receptor (TCR), which initiates the adaptive arm of the Th2 immune response towards extracellular parasites and is responsible for some asthmatic/allergic inflammatory diseases. Early sources of IL-4 derived from ILC2s[16] and basophils[17,18] binding to its receptor IL-4R on CD4+ T cells, coupled with TCR ligation with peptide:MHC class II molecules on DCs, activate the signal transducer and activator of transcription 6 (STAT6), which promotes the activation of the master Th2 regulator GATA-binding protein 3 (GATA-3).[19] GATA-3 binds to the gene loci for IL-4, IL-5, and IL-13, promoting the Th2 cytokine response.[19] STAT6 and GATA-3 additionally suppresses Th1 development by binding to and thus sterically hindering Th1-associated genes, and downregulating STAT4, respectively.[20] IL-2 plays a similarly critical role in driving T cell responses by stimulating the proliferation of CD4+ T cells, and inducing the expression of IL-4, which serves as a way to prime Th2 responses.[19] IL-2 binds to its receptor IL-2R and activates STAT5A, inducing IL-4 transcription in a GATA-3-independent manner.[19] Therefore, IL-4 is not strictly required for CD4+ Th2 cell differentiation, as IL-2 can up-regulate IL-4 expression, activating GATA-3 to induce Th2 differentiation.[19] IL-4 and IL-2 are essential in Th2 cell differentiation in-vitro, there are additional signalling pathways that induce Th2 gene expression in-vivo, such as the Notch-signalling pathway following binding of ligands Notch 1 and Notch 2 to *IL-4* and *GATA-3*, as well as IL-7 and the alarmin TSLP, which induce expression of STAT5.[15,19] IL-4 or STAT6-deficient mice showed significant impairment of Th2 responses to

parasitic infections.[19] GATA-3-deficient mice failed to develop Th2 cells and type 2 immune responses.[21]

The two main signals CD4+ T cells receive that drive differentiation into Th2 helper cells are: 1) the presence of IL-4, and 2) TCR-mediated ligation. IL-4 induces STAT6 and GATA-3 which stimulates IL-4 and IL-13 production. IL-4, though required in-vitro for Th2 differentiation, is not required in-vivo as there are redundant mechanisms that induce STAT6 and GATA-3 transcription factors to promote Th2 responses.[22] The strength of TCR ligation is an important factor in determining T cell fates. Low-dose peptide stimulation drives Th2 cell responses drive GATA-3 expression, while high-dose peptide stimulation inhibits GATA-3 expression.[15]

The key cytokines released by Th2-polarized CD4+ cells are IL-4, IL-5, IL-9, and IL-13. IL-4 promotes naïve CD4+ T cell differentiation into Th2 helper cells and promotes B cell isotype class switching and production of IgE (in addition to IgG_1). IL-4 also up-regulates the expression of low and high affinity receptors (FcεRII and FcεRI, respectively) present on mast cells and basophils.[22] IL-5 (along with GM-CSF and IL-3) mobilizes eosinophils and their progenitors in the bone marrow, enhances their maturation and survival, and primes them for migration to the site of inflammation via chemokines such as eotaxin-1 (which binds CCR3).[1] IL-9 stimulates epithelial cell mucus production, promotes proliferation and differentiation of mast cells, and promotes airway hyperresponsiveness.[22–24] IL-13 promotes mucus hypersecretion, sub-epithelial fibrosis, and airway hyperresponsiveness.[25]

The immune response to an allergen typically occurs in two phases. Firstly, there is a sensitization phase, where the first encounter of an allergen in a susceptible individual breaches the primary host defense systems, such as the epithelial barrier. DC migration, activation, and

polarization of naïve CD4+ T cells to Th2 helper cells, and the production of allergen-specific IgE are the body's immunological response during this phase of an allergic reaction. The allergen-specific IgE produced binds to high affinity FcεRI receptors on mast cells and basophils. The sensitization phase is unique in that not every individual who is sensitized to an allergen develops allergic symptoms when re-exposed to the sensitizing allergen. Secondly, there is a re-exposure phase, where re-exposure to the allergen results in binding of the allergen to the allergen-specific IgE which are bound to FcεRI receptors on the surface of mast cells and basophils. The binding of the allergen to IgE causes the FcεRI receptors on mast cells and basophils to crosslink. Crosslinking of the IgE bound to FcεRI by allergen causes the release of preformed and newly synthesized mediators, including vasoactive amines (histamine), lipid mediators, pro-inflammatory chemokines, and cytokines.[2,26,27] This characterizes the early phase reaction, which occurs within minutes of allergen exposure.[2,26] The inflammatory mediators released during the early phase response (EPR) result in changes to the host's physiology including promoting vasodilation, increased vascular permeability, and the local recruitment and activation of leukocytes.[2] These rapid changes present as clinical signs and may include rhinorrhea, conjunctivitis, and redness of the skin.

The re-exposure phase is further split into two phases: 1) the early phase response (EPR), and 2) the late-phase response (LPR). The EPR occurs within seconds or minutes of allergen exposure, the mechanisms and signs observed are the direct result of preformed and rapidly synthesized mediators being released from mast cells and basophils due to crosslinking IgE. The EPR resolves within 2 to 3 hours.[2,28] LPRs begin 3 to 8 hours following allergen exposure, and can take several days to fully resolve.[2] Unlike the EPR, the LPR involves the action of multiple, newly synthesized cytokines and chemokines, and lipid mediators (leukotriene C4, platelet

activating factor that are not immediately released by mast cells and basophils.[2] These mediators recruit other immune cells such as Th2 cells, eosinophils, neutrophils, and basophils, which propagates allergic inflammation. The pathophysiology of the LPR is heterogenous in nature and generally less well understood compared to the EPR – for example, it is not understood why only about 50% of individuals who develop an EPR will go on to develop an LPR. Additionally, LPRs can occur in the absence of EPRs (isolated LPRs).[28] The symptoms associated with the LPR depend on what organ is affected. In the skin, LPRs are characterized by pain, warmth, redness, and oedema, while in the lungs, LPRs are characterized by mucus hypersecretion and airway narrowing.[2] The EPR and the LPR will be discussed in more detail in sections **1.3** and **1.4**.

1.2 Characteristics of Allergens

For an antigen to be qualified as an allergen, it must not only induce IgE-production, but also elicit allergic symptoms via an IgE-mediated mechanism.[29] Introducing foods that are normally innocuous to mice in the presence of an adjuvant such as cholera toxin induces an immune response which can extend to other proteins (known as the bystander effect) and initiate the sensitization phase of the allergic response.[30] Although it has been argued that any antigen under the right conditions can become an allergen, most known allergens are limited to a small number of protein families (less than 2%).[30,31] Many allergens are soluble proteins that possess intrinsic adjuvant activity, such as inducing proteolysis, or containing enzymatic behaviour that may increase mucosal permeability.[9,32] Certain allergies initiate innate-immune pathways by activating pattern- or damage- associated molecular patterns such as TLR4 (in the case of the major house dust mite allergen Der p 2).[33]

The ability of an allergen to be airborne depends on the size of the molecule – smaller molecules (<5 kDa) tend to be airborne, while molecules greater than 5 kDa lose their capacity to become airborne.[34] The epithelium plays a primary role as one of the first barriers to the external environment – impairment of this barrier has been cited as a primary defect in allergic diseases.[35] Loss-of-function mutations of the filaggrin gene are a strong risk factor for the development of atopic dermatitis, asthma and other allergies – filaggrin is a protein that plays a key role in epithelial barrier function.[35,36] Furthermore, epithelial barrier function can be directly impaired as a consequence of proteolytic activity of allergens.

1.3 IgE and the Early Phase Response in Allergy

One of the primary drivers of the immediate hypersensitivity reaction underlying allergic conditions is IgE. IgE was the 5th and final antibody isotype discovered by Kimishige Ishikaka and his wife Teruko Ishizaka in 1967.[37] Serum IgE is typically present in small quantities due to the relatively short half life of free IgE (2-3 days), whereas cell-bound IgE is stable for several weeks.[38] Currently, total and specific IgE blood tests are employed as tools for diagnosing allergic disorders, though interpretation of results is not straightforward – high levels of IgE may not correlate with the extent of symptoms, and conversely, a normal IgE may not exclude allergic disorders.[39] Specific IgE above 0.35kU/L is used as a general guideline to suggest that an individual may be sensitized to an allergen, and some studies have showed specific IgE levels are correlated with the severity of allergic disease in patients.[39,40] An interesting approach was taken by Qiu et al (2020), published in *Scientific Reports*, demonstrating they were able to correctly diagnose 90% of allergic patients by taking into account total peripheral IgE (serum + cell-bound IgE).[38]

The binding of allergen-specific IgE following sensitization in allergic individuals triggers a cascade of events which may result in early and late phase responses. During the EPR, allergen-specific IgE cross links FcεRI receptors present on mast cells and basophils, resulting in their activation, causing them to degranulate and release preformed mediators. Early-phase IgE-mediated events result in the release of preformed and newly synthesized mediators (briefly discussed in section **1.1**). A wheal-and-flare allergic response is characteristic of an EPR in immediate hypersensitivity reactions following injection of small amounts of allergen into the dermis, nose, or airway. Wheal-and-flare reactions develop within minutes of exposure to the allergen – sneezing, wheezing, and a runny nose are common symptoms following exposure to an allergen and occur within minutes.[41] A wheal-and-flare reaction is a direct response the presence of histamine, causing swelling (wheal) and vasodilation (flare) at the injection site.[42] The skin-prick test is used clinically to diagnose IgE-mediated sensitization in asthma, AR, food allergies, and urticaria.[43] The crosslinking of FcεRI receptors on mast cells and basophils release a number of key mediators (Image 1A and Image 1B). Histamine increases vascular permeability, acts as a bronchial constrictor, and is even capable of chemoattracting eosinophils to the site of inflammation. Histamine is discussed more in section **1.4**.

In addition to binding mast cells and basophil high-affinity FcεRI, IgE binds a second receptor, FcεRII (CD23) with low affinity. CD23 is expressed on a variety of cells including B cells, T cells, follicular DCs, eosinophils, monocytes, platelets, and some thymic epithelial cells.[44] Although CD23 is the low affinity receptor for IgE, it plays a significant role in amplifying IgE-mediated allergic responses. Expression of CD23 has been shown to be expressed higher in atopic individuals compared to non atopic individuals, and its expression can be up-regulated in atopic subjects by IL-4.[45] CD23 binds IgE to initiate a process known as IgE-

facilitated allergen presentation (IgE-FAP). Typically, APCs in the local mucosa sample antigens through a process involving phagocytosis or pinocytosis – this is an passive and inefficient process that requires a large amount of antigen to trigger T cell activation.[46] However, B cells can amplify the T cell response through specific surface immunoglobulins binding the antigen, and internalizing the antibody-antigen complex for endosomal degradation – this is referred to as receptor-mediated "antigen focusing".[46] B cells can capture specific antigens this way, internalize them, and potentiate antigen-specific CD4+ T cell responses through peptide:MHC II-TCR interactions. B cell antigen focusing requires significantly less antigen to elicit T cell responses in comparison to passive antigen uptake via phagocytosis or pinocytosis (10^3 to 10^4-fold lower antigen concentrations required).[46] In allergic individuals, B cell antigen focusing is present, in addition to IgE-FAP. The presence of local specific-IgE causes APCs to up-regulate expression of a modified (trimeric) Fc epsilon receptor lacking the β subunit (FcεRIαγ_2).[47] This modified receptor is present on APCs in allergic individuals but nearly absent in healthy individuals.[46] Similar to the phenomenon of antigen focusing by B cells, antigen or allergen-antibody complexes form and bind to IgE receptors: the high affinity FcεRIαβγ_2 expressed on mast cells and basophils, the low affinity CD23 (FcεRII) expressed on a variety of cells, and a structurally related high affinity FcεRI-like receptor lacking the β-subunit (FcεRIαγ_2). This initiates IgE-FAP, where the allergen-IgE complexes get internalized by these receptors, degraded by endosomal compartments, and loaded onto MHC class II receptors to activate allergen-specific T cells.[46] IgE-FAP has been shown to activate specific T-cell clones via proliferation assays at 10^2-10^3-fold lower concentrations of Der p 2 allergen when the allergen was complexed to IgE-containing serum, compared to uncomplexed serum (where IgE-FAP cannot occur).[46]

The high affinity FcεRI receptor is constitutively expressed on mast cells and basophils and are critical for the immediate hypersensitivity reaction that is characteristic of allergic reactions. FcεRI exists as a tetramer ($\alpha\beta\gamma_2$) on mast cells and basophils and has a high affinity for IgE. Binding of allergen to IgE crosslinks FcεRI receptors, causing degranulation and the release of inflammatory mediators. FcεRI is also expressed as a trimer ($\alpha\gamma_2$) on various cells including DCs, monocytes, neutrophils, eosinophils, epithelial cells, among others.[48] As mentioned in the previous paragraph, the FcεRI$\alpha\gamma_2$ trimer variant of this receptor is involved in IgE-FAP to potentiate T cell responses to allergen. FcεRI expression on the surface of mast cells and basophils is modulated by serum IgE concentrations – higher serum IgE leads to higher receptor occupancy, which increases the receptor levels on the surface of these cells, amplifying effector responses.[49] IgE binding to FcεRI receptors 1) stabilizes receptor complexes, preventing them from being degraded, and 2) following maximal expression of intracellular FcεRI on the surface of cells, it is synthesized *de novo*.[49]

Mast cells and basophils are the primary effector cells in the EPR during an allergic reaction. These are granulated metachromatic cells – meaning they take on a different colour from that of certain basic dyes used to stain the cells. Toluidine blue is commonly used to identify mast cells and basophils via binding to acidic tissue components such as granules, which contain sulfates, carboxylates, and phosphate radicals, causing a colour change from blue-violet to yellow or red (metachromatic shift).[50] Although mast cells and basophils share some phenotypic features and functions, they represent distinct lineages. Both cell types are generated in the bone marrow and develop from common myeloid progenitors and granulocyte-monocyte progenitors.[51] Mast cells or basophils can differentiate from either spleen basophils-mast cell common progenitors (BMCPs), or bone marrow pre-basophil and mast cell progenitors (pre-

BMPs).[51] From BMCPs or pre-BMPs, basophil progenitors and mast cell progenitors gives rise to basophils and mast cells, respectively, thereby representing distinct lineages.[51] Both cells are associated with type 2 immunity and inflammatory responses to allergens, and constitutively express the high affinity FcεRI receptor on their cell surface, in addition to the low affinity CD23 (FcεRII) receptor. Upon cross-linking of these receptors by allergen-specific IgE, they release mediators which come in two flavours: preformed mediators, and newly synthesized lipid mediators/cytokines/chemokines.[44] These cells participate in the early phase response in immediate hypersensitivity reactions by releasing key mediators such as biogenic amines (histamine, serotonin), serine proteases (cathepsin G, elastase), metalloproteases (matrix metalloproteinase 9, or MMP9), lipid mediators such as prostaglandin D2 and cysteinyl leukotrienes (PGD_2, and cysLTs).[52] Mast cells, unlike basophils, secrete PGD_2 and several additional inflammatory cytokines (such as TNF-α).[52]

Due to their rarity (comprising less than 1% of circulating leukocytes) and their overlapping functionality with mast cells, basophils were largely thought to play a redundant role in allergic inflammation.[53,54] However, in a mouse model of IgE-mediated chronic allergic inflammation (IgE-CAI), depletion of basophils, but not mast cells or T lymphocytes, abrogated the development of IgE-mediated inflammation, demonstrating that basophils were nonredundant and essential for the development of allergic inflammation.[53] Adoptively transferring basophils reconstituted IgE-CAI. Furthermore, depleting basophils through anti-CD200R3 antibody abrogated the inflammatory response – as measured by decreased ear swelling and a drastic reduction in eosinophil, neutrophil and macrophage cellular infiltrate.[53]

Basophils are thought to orchestrate type 2 inflammatory responses by being early sources of IL-4 and IL-13, which promote IgE class switching in B cells and differentiation of

CD4+ T cells to a Th2 helper cell phenotype.[55] In addition to IgE-mediated activation, basophils can be activated through IgE-independent mechanisms – namely by TSLP, IL-3, IL-18 and IL33.[53] Generally, cytokine activated basophils (referred to as TSLP-elicited basophils) are known to release IL-4,[53] however, there is evidence that suggests IL-3 stimulation of basophils is capable of inducing direct histamine release in atopic individuals.[56] Pre-treatment of basophils with IL-3 potentiates histamine release by anti-IgE, fMLP, phorbol esters, and calcium ionophore A23187 stimulation in nearly all individuals (atopic or otherwise).[56]

1.4 Late Phase Response in Allergy

The LPR in allergic reactions occur in 66-85% of people who experience an early phase response.[57] The LPR develops anywhere between 2-6h and peaks 6-9h after allergen exposure, which may last up to several days.[2] It is not known why the LPR develops in some patients, but not others, although patients who experienced an isolated EPR or LPR (single-responders) showed a dual response (EPR and LPR) when the dose of allergen was increased.[58] The LPR represents a resurgence of signs that are similar to those observed during the EPR such as rhinitis and conjunctivitis (in the case of AR) and airway hyperresponsiveness (in the case of asthma).[59] In the skin, LPRs are characterized by edema (swelling) and redness.[9]

Seminal work by Bienenstock and co. in the 80s demonstrated that EPRs and LPRs can be elicited by intracutaneous injection of purified anti-IgE alone.[60] The EPR is a viewed as a result of an IgE-dependent release of preformed mediators from mast cells and basophils, whereas the LPR is accompanied by an influx of inflammatory cells to the site of inflammation (monocytes, neutrophils, eosinophils, basophils, and CD4+ Th2 cells) and a release of their mediators, causing a protracted type 2 inflammatory response.[2,54,61] The importance of IgE in

mediating the LPR is further evidenced by opiate-induced mast cell activation, which, unlike IgE-mediated degranulation, is infrequently followed by an LPR.[62]

The mechanism driving the symptoms observed during LPRs is complex and multifaceted. In subjects with type-2 asthma, eosinophils accumulate in the bronchoalveolar lavage fluid, and release toxic proteins such as major basic protein and eosinophil cationic protein.[63] However, the timing of recruitment appears to play an important role, because accumulation of eosinophils at 4 hours, but not 24 hours, resulted in the LPRs.[63] However, blocking eosinophil accumulation in the blood and sputum using an anti-IL-5 monoclonal antibody does not inhibit late asthmatic responses (LARs), suggesting eosinophils may not play a dominant role in mediating the LAR.[64] LPRs in the context of asthmatic reactions are referred to as late asthmatic reactions, or LARs.

We have discussed mast cells and eosinophils as they contribute to the LPR. However, basophils have been implicated in contributing to the inflammation seen during the LPR. Naclerio et al. in 1985 demonstrated that following allergen challenge in subjects, there was a significant increase in histamine during the LPR, without a concomitant increase of PGD_2, signifying that basophils, rather than mast cells, may be driving this phase of the disease pathogenesis.[65] This conclusion was drawn because while mast cells and basophils release histamine, only mast cells produce and release PGD_2 – the relative absence of PGD_2 suggests that mast cells may not be sustaining the symptoms that are observed in the LPR.

While antihistamines have been effective in treating allergic rhinitis and conjunctivitis, there is evidence that they do not consistently provide relief of clinical symptoms in allergic asthma, despite these being highly comorbid diseases and sharing common pathological mechanisms.[66–69] However, combination therapies utilizing antihistamines in conjunction with

leukotriene receptor antagonists were shown to provide a significant increase in forced expiratory volume (FEV_1) during the late asthmatic response.[67]

1.5 Late Phase Response: IgE-dependent or CD4+ T cell dependent?

There is conflicting evidence regarding which cells are the primary drivers of the LPR. On one hand, Watanabe et al. (1995) showed that adoptive transfer of allergen-challenged CD4+ T cells elicited LPRs in naïve recipient rats.[70] A study conducted by my supervisor, Dr. Mark Larché, demonstrated this phenomenon in humans, where a subset of allergic subjects experienced isolated LARs following intradermal injections of peptides derived from the major cat allergen Fel d 1.[28] It is important to note that the late asthmatic responses elicited were independent of IgE-mediated events such as the cross-linking of mast cells and basophils, because the peptides injected were too short to crosslink FcεRI receptors. However, these peptides directly bound to CD4+ T cell MHC class II complexes, causing CD4+ T cell activation and subsequently, an LAR measured by a decline in forced expiratory volume.[28]

On the opposite side of the debate, evidence has mounted which suggests the LPR is solely an IgE-dependent phenomenon. Intradermal injection of anti-IgE antibody induces both an immediate wheal and flare response, as well as a LPR in a variety of tissues, including the skin, lung, and nose.[60,62] Although approximately 6-14% of subjects experience isolated LPRs, the intensity of the LPR correlates with the intensity of the EPR in some studies.[57] The IgE-dependent nature of the LPR is likely mediated through mast cells and basophils, as skin testing with mast cell degranulator compound 48/80 produced similar biphasic reactions to anti-IgE administration.[57] Depleting basophils in a mouse model of AR abrogated the EPR and LPR in response to allergen-sensitized and challenged mice.[71]

Further evidence for the IgE-dependence of the LPR comes from studies using the humanized monoclonal anti-IgE antibody (generic name: Omalizumab). Omalizumab binds to the heavy chain (CHε3) region of IgE, which interacts with FcεRI and CD23 (FcεRII) on mast cells and basophils, thus preventing their interaction.[72] Omalizumab reduced the LPR in allergic subjects in response to allergen challenge.[73,74] This gives credence the LPR being a largely IgE-dependent phenomenon. However, in the context of facilitated antigen presentation (FAP), the relief of symptoms by blocking IgE can in fact be explained by an IgE-independent, CD4+ T cell-dependent manner. Circulating IgE up-regulates the trimer version of the high affinity IgE receptor (FcεRIαγ_2) expressed primarily on DCs, but also other APCs. Higher levels of FcεRIαγ_2, can capture and internalize allergen-antibody complexes as a mechanism (in addition to pinocytosis) to process and present peptides derived from allergen to CD4+ T cells, resulting in enhanced activation and proliferation of CD4+ T cells – it has been shown that IgE-FAP can amplify T cell responses 10-fold.[47] Therefore, anti-IgE can act via this mechanism by decreasing free IgE levels, reducing FcεRIαγ_2 expression on APCs, therefore inhibiting CD4+ T cell responses.

1.7 Histamine, Histamine Receptors, and their Role in Allergic Disease

Histamine is a biogenic amine released by mast cells and basophils upon cross-linking FcεRI receptors on their cell surface. Lesser known sources of histamine include gastric enterochromaffin cells, histaminergic neurons, neutrophils, macrophages, dendritic cells, and T cells.[75] Histamine is catalyzed by the enzyme histidine decarboxylase and immediately released – this type of histamine production is referred to as neosynthesized. Basophils and mast cells store histamine in granules and release it via degranulation.[75] In addition to contributing the symptoms in allergic diseases, histamine plays critical regulatory functions by acting on

histamine receptors H_1R-H_4R. The H_1 and H_2 receptors are expressed on many cell types, including mast cells, basophils, neutrophils, PBMCs, airway smooth muscle cells, T cells, and endothelial cells, to name several.[75,76] Comparative analyses of T cell subsets show that H_1R is mainly expressed on Th1 subsets, whereas H_2R is mainly expressed on Th2 subsets.[75] H_2R is also expressed on parietal cells of the stomach and activation through this receptor mediates hydrochloric acid secretion.[77] H_3R is restricted to histaminergic neurons (central nervous system) and H_4R, the most recently discovered histamine receptor, is expressed on immune-related tissues and a variety of leukocytes.[75] H_4R is preferentially expressed on Th2 cells, compared to naïve T cells and Th1 cell, but are also present on Th17 cells.[76] In mice, CD4+ T cells have been shown to express the enzyme histamine decarboxylase (HDC) and produced histamine in response to Con-A, IL-3, GM-CSF, and anti-CD3 antibody stimulation.[78,79] Jurkat T cells (a human leukemic T cell line) have also been reported to express HDC and release histamine upon treatment with phorbol 12-myristate 13-acetate (PMA).[80]

1.8 Current Treatments for Allergic Diseases

Management of allergies involves minimizing exposure of a sensitized person to an allergen, and treating sensitized individuals with therapeutics agents in order to ameliorate the disorder.[2] While it is easy enough to avoid contact with allergens such as certain foods or chemicals, it is much more difficult to avoid aeroallergens derived from sources such as pollen and house-dust mite.[81] Therapeutic agents include pharmacotherapies and immunotherapies. Antihistamines are a mainstay for treating AR, which work by binding to H1-receptors and "turn off" downstream processes (antihistamines are inverse agonists).[82] Intranasal corticosteroids are also a first line treatment option for people with moderate/severe symptoms, and can be used alone or in conjunction with antihistamines.[7] Leukotriene receptor agonists can also be effective

in treating AR, and should be considered as a treatment option when histamines and corticosteroids are ineffective or not well tolerated.[7] Pharmacological treatments are primarily aimed at relieving the symptoms associated with the disease, rather than providing curative measures.[7] Another drawback of pharmacological treatments for allergy is that they are not specific in targeting the pathology of the disease.

Immunotherapies try to address the underlying pathology in allergic disease. Unlike pharmacotherapies, allergen-specific immunotherapy (AIT) is a potentially disease-modifying therapy that is effective in a number of allergic diseases including AR, conjunctivitis and allergic asthma.[83,84] AIT involves administration of the patient's relevant allergen(s) until immunological tolerance to the allergen is achieved.[7] It is typically administered in weekly incremental doses over the course of 6-8 months, followed by monthly maintenance injections for 3-5 years.[7] AIT can be administered through subcutaneous injections of the allergen (subcutaneous immunotherapy; SCIT), or with tablets for the allergen that are placed under the tongue (sublingual immunotherapy; SLIT).[85]

The mechanisms of AIT are not completely understood, although it has been shown that there is a down-regulation of Th2 responses and/or an increased Th1 responses.[84] Enhanced regulatory T cell responses have also been described.[84] AIT is also associated with an increase in IgG_4 production against the allergen, which may compete with IgE for allergen binding.[86] Furthermore, it has been shown that protection is also associated with an allergen-specific T cell IL-10 response.[84] Despite the efficacy of AIT, this form of treatment carries the risk of causing adverse systemic reactions due to crosslinking allergen-specific IgE on mast cells and basophils, leading to their activation.[84] In order to circumvent the problem of cross-linking IgE receptors on these effector cells, vaccines have been developed that use short linear peptide sequences

containing the immunodominant T-cell epitopes of the major allergen protein – this form of immunotherapy is called peptide immunotherapy (PIT).[87] By using short linear peptide sequences, the likelihood of cross-linking IgE on mast cells and basophils, and thus activating them, is drastically reduced in comparison with using whole allergen.

1.9 IL-8 Characteristics

Interleukin 8 (IL-8) was among the first and best characterized chemotactic cytokines (chemokines) and was discovered in 1987.[88] IL-8 was originally named monocyte-derived neutrophil chemotactic factor because it was discovered to be released by monocytes in response to lipopolysaccharide (LPS) stimulation and its ability to chemoattract neutrophils, it was later renamed to IL-8 due to its ability to chemoattract a subset of T lymphocytes.[89]

There are four subfamilies of chemokines, they are named based on the location of the first two cysteine residues: CXC, CC, CX_3C, and C, where X denotes a non-cysteine amino acid residue.[90] CXC chemokines are further subdivided based on the presence or absence of the tripeptide motif Glu-Leu-Arg (ELR) at the N-terminus.[90] IL-8, in addition to CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL7 belong to the ELR+ chemokine subfamily.[90] ELR+ chemokines are distinguished from ELR- chemokines by their ability to specifically recruit polymorphonuclear (PMN) leukocytes into inflamed tissues[90] CXCR1 and CXCR2 are two receptors that are specific for ELR+ chemokines.[90]

1.10 IL-8 Transcription

The core IL-8 promoter contains a nuclear factor (NF)-κB binding element that is required for production, and activating protein (AP)-1, and a CAAT/enhancer-binding protein (C/EBP) binding sites that are not essential but contribute to maximal activation in some cell types.[91] In the absence of a stimulus, IL-8 is repressed via three mechanisms: 1) a negative

regulatory element that overlaps the NF-κB binding site, 2) the binding of octamer 1 (OCT-1) to a binding site on the complementary strand in the opposite direction of the C/EBP binding site, and 3) Deacetylation of histone proteins by histone deacetylase-1.[91]

1.11 IL-8 Expression and Function

IL-8 is generated as a 99 amino acid precursor containing a 20 amino acid signal sequence at the N-terminus.[92] Cleavage at the N-terminus yields a variety of IL-8 isoforms, including 79-, 77-, 72-, 71-, 70-, and 69-amino acid variants,[88,92] although the 77- and 72-amino acid variants represent the predominant forms.[92] Chemically synthesized analogues that are shorter in length exhibited greater potency *in-vitro*, although the relevance of these shorter isoforms *in-vivo* are unknown.[93] The 77-amino acid variant is the predominant isoform secreted by nonimmune cells, whereas the 72-amino acid variant is the predominant isoform secreted by macrophages, monocytes, and T cells.[94]

IL-8 is produced from virtually all nucleated human cells types given the appropriate stimulus, although it is produced primarily from nonepithelial cell types.[95] IL-8 is undetectable in healthy tissues and barely secreted from noninduced cells.[91,95] However, IL-8 is rapidly induced by 10-100-fold in response to proinflammatory cytokines or stimuli such as TNF-α, IL-1α, IL-1β, bacterial or viral products, and stress factors such as hypoxia, acidosis, nitric oxide and cell density.[91,96]

IL-8 mediates its effects by binding to CXCR1 and CXCR2. CXCR1/2 are G-protein coupled receptors containing 7 transmembrane domains.[97] CXCR1/2 are expressed on a variety of leukocytes and non-leukocytes. CXCR1 exhibits high affinity and specificity for IL-8 and with a lower affinity, binds CXCL5, CXCL6 (and does not bind other ELR+ chemokines).[98]

CXCR2 promiscuously binds all other ELR+ chemokines (CXCL1-3, 5-7) in addition to IL-8.[90,99]

1.12 IL-8 and the Existence of 'Th8' cells

There is mounting evidence that IL-8-producing CD4+ T cells may represent a novel subset of T helper cells: "Th8". There are a subset of CD4+ T cells that have been characterized as "naïve" (CD45RA+, CD45RO-, CD62L+, CCR7+) and express IL-8 in the absence of other effector Th cell cytokines such as IFN-γ, IL-4, IL-17, and IL-22.[100] These naïve T cells secreted biologically active IL-8 that 1) promoted tumorigenesis, and 2) promoted neutrophil migration. On the other hand, it has been shown that a distinct population of CD4+ T cells express IL-8 under Th17 polarizing conditions.[101] These CD4+ T cells co-express prototypical Th17 markers only to a very small extent, and primarily secrete IL-8.[101]

Up to 50% of CD4+ T cells from pre-term and term infants produced IL-8, without concomitant production of IFN-γ and IL-17A , demonstrating that IL-8-producing 'Th8' cells may play a role in early immune system responses against infections by recruiting and activating neutrophils.[102] In contrast, CD4+ IL-8+ T cells were rare in adults.

A phenotype of T cells consistent with the idea of a 'Th8' subset of CD4+ T cells has been reported during disease states. In a drug hypersensitivity reaction (referred to as acute generalized exanthematous pustulosis, or AGEP), 80% of drug-specific CD4+ T cells from patients were found to secrete high levels of IL-8.[103] These drug-specific IL-8+ T cells also produced GM-CSF, IFN-γ, and TNF-α. Neutrophils were shown to have a 40% reduction in apoptosis when treated with supernatant from drug-specific IL-8+ T cells.[103] This data suggests that not only are drug-specific IL-8+ T cells strongly chemotactic for neutrophils, but that they

are able to promote neutrophil survival, providing evidence that these T cells are involved in neutrophil infiltration and disease progression.

The current literature suggests there may be a novel 'Th8' CD4+ T cell that predominantly secretes IL-8, however, more work needs to be done to characterize them, especially regarding the differences and similarities between 'Th8' cells present during infancy and 'Th8' cells that may be involved in developing tumors and AGEP.

1.13 Interaction between CD4+ T cells and Basophils/Neutrophils and their role in allergic diseases

Basophil-CD4+ T cell interaction

There is a considerable amount of literature demonstrating that basophils interact with CD4+ T cells in the pathogenesis of allergic diseases. Some of this work has identified basophils as contributing to allergic diseases through their early expression of *IL-4*, and expression of MHC class II, showing that basophils contribute to allergies by activating CD4+ T cells and promoting their proliferation.[104–106] There have been limited studies which evaluated how CD4+ T cells activate basophils in the context of allergies. Some studies have looked at how CD4+ T cells or CD4+ T cell-derived cytokines stimulate basophils to express IL-4.[107,108] However, these studies did not evaluate histamine release in basophils, or CD63/CD203c upregulation (a surrogate for basophil activation and a correlate for histamine release). As previously discussed, basophils can be stimulated through IgE-dependent or IgE-independent mechanisms – the latter mechanism of activation is primarily responsible for inducing IL-4 expression in basophils, but not histamine release. Therefore, our aim was to evaluate the role CD4+ T cells had in stimulating basophils to release histamine, in addition to measuring activation markers CD63/CD203c.

Neutrophil-CD4+ T cell interaction

As with basophils, investigators have looked at neutrophils-CD4+ T cells interactions. However, similarly to basophils, much of this work has evaluated neutrophils as acting on CD4+ T cells, causing them to become activated.[109–112] Relative to basophils, more work has been done on neutrophils, where neutrophil activation was assessed following interactions with CD4+ T cells – these studies examined MHC class II expression,[113,114] and expression of surface markers such as CD11b, CD62L and CD64.[115] However, neutrophils have recently been shown to contain and release histamine in response to contain and release histamine in allergic subjects using antigens the subjects were sensitized to, and LPS.[116] Since this paper was released in 2013, investigators have not looked at what other stimuli is capable of stimulating neutrophil histamine release. Our aim was to evaluate the role CD4+ T cells had in stimulating neutrophils to release histamine, as well measuring expression of neutrophil activation markers CD11b and CD62L.

Basophil Activation by IL-8

CD4+ T cells express a vast array of cytokines and other proteins, which may or may not be contributing to or synergizing basophils and neutrophil activation. Previous work done by our lab guided our search for possible CD4+ T cell-derived cytokines to investigate (elaborated in **PROJECT RATIONALE & HYPOTHESIS)**, in addition to the publicly available literature.

Two papers which set the groundwork for the rationale for this project were produced in 1989 by 2 groups – Dahinden et al. and White et al.[117,118] White et al. demonstrated that IL-8 alone could stimulate basophils to release histamine in a dose-dependent manner (range tested: $3x10^{-7}$ M to $4x10^{-6}$ M).[118] Notably, histamine releasability of the basophils was independent of their allergic status.[118] Dahinden et al., however, demonstrated that IL-8 required the presence of IL-3 to stimulate basophils to release histamine.[117] IL-8 alone (concentration ranges: 1nM to

1000nM, or $1x10^{-9}$ M to $1x10^{-6}$ M) was ineffective in inducing histamine release from basophils.[117] By briefly (~5 minutes) priming basophils with IL-3 (20ng/ml), they were able to induce basophils to release histamine – notably, IL-3 alone was not sufficient in stimulating basophils to release histamine.[117] Despite the differences in the findings between these two papers, they both demonstrate that IL-8 was capable of stimulating basophils to release histamine. As previously mentioned, IL-3 is secreted by basophils and mast cells in response to IgE receptor crosslinking, suggesting a role for IL-3 in IgE-dependent allergic inflammation. IL-8 is not a typical Th2 cytokine, however, it has been shown to be produced during both the EPR and LPR in allergic rhinitis following allergen challenge.[119] In addition to activating basophils, IL-8 is a potent chemoattractant for granulocytes such as neutrophils, eosinophils, and basophils via interacting with receptors CXCR1 and CXCR2 expressed on these cells.[119] Therefore, IL-8 may play a more significant role in contributing to the allergic response than previously appreciated.

Neutrophil Activation by IL-8

IL-8 was originally referred to as neutrophil attractant/activation protein 1 (NAP-1) because it was one of the first chemokines that activated neutrophils.[120] Neutrophils express the high affinity IL-8 receptors CXCR1 and CXCR2.[120] Following ligation of IL-8 to its receptor, neutrophil activation occurs in the following manner: 1) rapid and transient rise in cytosolic calcium, which is necessary for many of the subsequent activation steps[120,121] 2) shape change and activation of the motile apparatus[122] 3) enhanced expression of adhesion molecules CD11b/CD18[123] 4) The respiratory burst (production of H_2O_2 and O^-_2)[124] and exocytosis of granules (secretory, specific, and azurophilic granules).[122,124]

PROJECT RATIONALE & HYPOTHESIS

Our lab has developed a safe and clinically efficacious PIT for cat allergy tested in phase IIb clinical trials. During a clinical study aimed at understanding the mechanism of action of PIT, allergen-specific T cells derived from patients before and after receiving PIT were sequenced at the mRNA level (RNA-seq).[125] It was found that IL-8 mRNA transcripts were reduced 98.5% post-treatment – this was mirrored by a similar decrease in secreted IL-8 protein.[125] These data suggests that IL-8 may play a central role in the pathogenesis of allergic diseases. Specifically, these allergen-specific T cells may produce IL-8 in response to allergens, which may contribute to the late-phase response in an allergic reaction. IL-8 can recruit and activate basophils and neutrophils to the site of inflammation, further contributing to the inflammatory response. This is supported by complete blood counts taken from these patients after treatment which show a decrease in allergy-induced neutrophilia.

Based on these observations, we hypothesized that (a) allergen-specific T cells drive the pathogenesis of allergic disease through the release of IL-8, and (b) IL-8 produced by allergen-specific T cells can recruit and activate basophils and neutrophils and that these two populations of cells are able to contribute to the pathophysiology of allergic disease in a T cell-dependent manner. More broadly, however, we hypothesized that T cells interact directly with basophils and neutrophils in the LPR to stimulate basophils and neutrophils to release histamine. Based on previous observation, we specifically hypothesized that this T-cell mediated activation of basophils and neutrophils was due to T-cell derived IL-8.

OBJECTIVES OF THE PROJECT

Aim 1: Identifying IL-8-producing (IL-8+) CD4+ T cells and factors which stimulate T cells to produce IL-8

1.1 Identifying the frequency of allergen-specific T cells in whole blood using MHC class II tetramers without magnetic enrichment and no additional tetramers

Work done by a previous master's student in our lab attempted to investigate the frequency of IL-8+ allergen-specific CD4+ T cells (IL-8+ TET+) present in cat allergic and non allergic subjects. The technique that was used to identify IL-8+ allergen-specific T cells involved the use of magnetic enrichment to isolate cells that are bound to a peptide-MHC tetramer. This technique was useful to visualize, by flow cytometry, low-frequency populations such as allergen-specific CD4+ T cells, but it introduced biases in the calculation of the absolute number of these cells in the sample.

TCR downregulation is the phenomenon by which engagement of the TCR complex by specific ligands (such as through recognition of a peptide:MHC complex), resulting in the internalization of the TCR.[126] It has been shown that adding a protein kinase inhibitor (Dasatinib) may enhance staining by inhibiting TCR internalization.[127] In the previous study, in order to compensate for TCR downregulation, the additional tetramers were added during the 24 hour stimulation period to the magnetically enriched TET+ cell fraction collected after initial tetramer staining. Based on the abnormally high frequency of tetramers in the both the cat allergic and non-allergic subjects (Figure 1) it was suspected that the addition of excess tetramers during incubation resulted in nonspecific binding of tetramers. The high percentage of tetramer positive cells found was inconsistent with the literature that suggests the frequency of allergen-specific CD4+ T cells in tissue and blood was typically very low, ranging from 10^{-5} to 10^{-3} percent.[128]

This is especially true for non-allergic controls, which have been demonstrated to have a lower frequency of TET+ CD4+ T cells.

Therefore, in this first aim, we performed tetramer staining, as what had been previously done, with the exception that we did not further enrich this population. Most importantly, additional tetramers were not added in subsequent steps. The primary objective was to capture the number of allergen-specific CD4+ T cells in allergic subjects and detect these using flow cytometry. CD4+ T cells were isolated and stained immediately with peptide-MHC class II tetramers, conjugated to phycoerythrin (PE), and T cells that were specific for this peptide were analyzed using flow cytometry.

1.2 Identifying the frequency of IL-8+ allergen-specific T cells in cat allergic subjects

A similar approach was taken with this approach as with ***aim 1.1***, where our objective was to determine the frequency of allergen-specific T cells that secrete IL-8 without the use of magnetic enrichment and adding additional tetramers. In preliminary experiments, we stimulated CD4+ T cells with PMA and ionomycin (PMA/I), anti-CD3/28 beads (Dynabeads, Thermofisher Scientific), or media to induce IL-8 production by CD4+ T cells and captured as many events as possible using flow cytometry. Our objective was to capture tetramer positive cells that co-expressed IL-8.

1.3 Identifying factors which stimulate T cells to produce IL-8

To better characterize IL-8-producing T cells, we investigated cytokines and other factors which stimulated CD4+ T cells to produce IL-8. First, we established that CD4+ T cells produce IL-8 in response to antibody stimulation to CD3 and CD28 to model T cell activation without the need for APCs. To do this, we used an immortalized T cell line, Jurkat (clone E6.1) and freshly isolated CD4+ T cells cultured in cRPMI. CD2 stimulation was used in conjunction to CD3 and

CD28 stimulation to investigate if the CD2 activation pathway can elicit stronger IL-8 responses from CD4+ T cells.

Prostaglandin D_2 (PGD_2) and leukotriene E4 (LTE_4) have been shown to up-regulate IL-8 production in Th2 cells by interacting with chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2) and cysteinyl leukotriene receptors 1 and 2 ($CysLT_1$ and $CysLT_2$), respectively.[129] It was shown that PGD_2 and LTE_4, in addition to promoting Th2 cytokine production, stimulated Th2 cells to produce IL-8.[129] This finding suggests that IL-8 could play a role in allergic inflammation, given that PGD_2 and LTE_4 are lipid mediators released by mast cells. Our objective was to compare naïve and Th2-differentiated CD4+ T cells in their ability to respond to PGD_2 and LTE_4 by producing IL-8.

Aim 2: Demonstrate IL-3+/-IL-8 and supernatants derived from stimulated Jurkat and CD4+ T cells can activate basophils

2.1 Basophil activation test

The basophil activation test (BAT) is a flow cytometric technique to measure expression of activation markers on the surface of basophils. The BAT is used clinically, though not routinely, to assess patients with suspected IgE-mediated allergic diseases. CD63 is a 4-transmembrane protein that is associated with vesicle fusion events.[130,131] CD63 is expressed on the surface of basophils and is strongly correlated with histamine release.[131] CD203c is an ecto-nucleotide pyrophosphatase/phosphodiesterase that is expressed on resting cells and is rapidly up-regulated upon activation.[130]

IL-8 activated basophils, through a receptor mediated mechanism similar to that in neutrophils, leads to a transient rise in cytosolic calcium.[132] IL-3 pre-treatment led to a significant increase in histamine release from basophils when subsequently stimulated with IL-

8.[132] Central to our research question was to ask and answer the question whether CD4+ T cell-derived IL-8 was capable of stimulating basophils to release histamine.

Therefore, our objective was to utilize flow cytometry to measure basophil activation. Activated CD4+ T cells, which have been shown to produce IL-8, were incubated with basophils and assessed for expression of activation markers CD63 and CD203c. Neutralizing IL-8 antibody (nAbIL-8 obtained from Sigma-Aldrich) was introduced into the cultures to block the effect of IL-8, to determine the relative contribution IL-8 had in activating these cells.

2.2 Basophil histamine release

Basophils express the high affinity receptor for IgE (FcεRI) that, upon cross-linking by IgE by allergen or anti-IgE antibody, release histamine (among other mediators).[77] Basophils can also be stimulated through IgE-independent means, i.e. by cytokines, TLRs, and proteases.[77] IL-3 has been described as the most potent activator of basophils, capable of inducing histamine release from basophils from select atopic individuals.[56] However, pre-treatment of basophils with IL-3 can prime basophils for enhanced responsiveness to anti-IgE, among other stimuli.[56] Notably, IL-3 is capable of priming basophils for histamine release when subsequently stimulated with IL-8.[117] CD4+ T cell-derived IL-8 was the focus of our investigation, as the means by which basophils are activated by CD4+ T-cell dependent mechanisms.

Therefore, our objective was to measure histamine release from basophils following incubation with supernatants derived from stimulated CD4+ T cell supernatants, containing IL-8. NAbIL-8 was introduced into the cultures to block the effect of IL-8, which determined the relative contribution IL-8 had in activating these cells and causing them to release histamine. In addition to using CD4+ T cell supernatants, recombinant human IL-8 was used to replicate

White et al. and Dahinden et al.'s finding that IL-8 (+/-IL-3) stimulated basophils to release histamine.

Aim 3: Demonstrate that supernatants from stimulated CD4+ T cells can activate neutrophils

3.1 Neutrophil activation test

Neutrophils express CXCR1 and CXCR2, the receptors for IL-8. IL-8 has been shown to activate neutrophils and is a potent neutrophil chemoattractant. Neutrophils express a variety of receptors involved in cell adhesion (CD11b, CD62L, CD49d, CD54) that are altered in expression in disease states, or when neutrophils become activated.[133] Dihydrorhodamine 123 (DHR 123) is a fluorogenic dye that is oxidized to rhodamine 123 in the presence of reactive oxidative species (ROS) produced by neutrophils.[134] Myeloperoxidase (MPO) is a peroxidase expressed predominantly in neutrophils, which catalyzes the formation ROS intermediates such as hypochlorous acid (HClO).[135] There is controversy in the literature whether IL-8 alone can initiate the respiratory burst, although IL-8 has been shown to prime neutrophils for enhanced oxidative burst in response to fMLP.[92,136]

Our objective was to build a suitable panel for detecting neutrophil activation using flow cytometry. To reduce the number of antibodies used, we selected antibodies to CD11b and CD62L to measure altered of cell adhesion during activation. CD11b expression is up-regulated upon neutrophil activation,[137] whereas CD62L is shed upon activation.[112] Because DHR 123 and intracellular MPO are markers for ROS production, they were used to assess if neutrophils activated in our system were producing ROS, which would suggest additional functional roles activated neutrophils had that contribute to inflammation.

3.2 Neutrophil histamine release

Human neutrophils have recently (2013) been shown to express the enzyme histidine decarboxylase (HDC) and store and release histamine in response to antigen and other neutrophil agonists.[116] Neutrophils stored approximately 0.29pg/cell and release ~50% of histamine content upon stimulation with agonists.[116] Given that IL-8 had been demonstrated to activate neutrophils to some capacity, our objective was to determine if 1) neutrophils released histamine in response to IL-8 stimulation, and/or 2) neutrophils released histamine in response to stimulation with activated T cell supernatants.

METHODS

Aim 1: Identifying IL-8-producing (IL-8+) T cells and factors which stimulate T cells to produce IL-8

1.1 Identifying the frequency of allergen-specific T cells in cat allergic subjects

PBMCs were isolated from 40ml of whole blood using Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation and suspended in RPMI + 5% autologous plasma. Autologous plasma was collected, and heat inactivated at 56℃ for 30-minutes. PBMCs were counted and resuspended to $50x10^6$ cells/ml for CD4+ T cell isolation using EasySep Human CD4+ T cell Enrichment Kit (StemCell Technologies) according to manufacturers instructions. Isolated CD4+ T cells were resuspended to a concentration of $150x10^6$ cells/ml and incubated with PE-conjugated tetramers (Benaroya) of the appropriate MHC class II haplotype for 100 minutes at 37℃ and 5% CO_2 at a final concentration of 10ug/ml. Dasatinib was added to the media to a final concentration of 50nM.

Immediately after incubation, cells were washed with PBS for flow cytometry staining. Cells were stained with the dead cell marker APC-Cy7, for 15 minutes at room temperature in

the dark. Next, cells were washed with FACS buffer (PBS containing 1% FBS and 0.1% sodium azide) and stained with a combination of AF488-CD3, AF700-CD4, BV510-CD14, BV510-CD19 antibodies for 30 minutes at 4℃. Antibodies were purchased from Biolegend unless otherwise stated. Cells were fixed with IC Fixation Buffer (Invitrogen). Cells were washed and resuspended in FACS buffer and filtered into 5ml polystyrene tubes. Samples were acquired on the flow cytometer (BD LSRII), and data was analyzed with FlowJo V10.7.2.

1.2 Identifying the frequency of IL-8+ allergen-specific T cells in cat allergic subjects

One hundred twenty millilitres (mls) of blood were collected and processed identically to ***methods 1.1***. CD4+ T cells were isolated and stained with tetramers. One third of the cells obtained were stained for flow cytometry immediately after tetramer staining to determine the frequency of allergen-specific CD4+ T cells. Another third fraction of cells obtained were stimulated for 24 hours at 37℃ with Dynabeads Human T-Activator CD3/28 beads (Thermofisher). The final third fraction of cells obtained were stimulated with media alone (RPMI + 5% autologous plasma). An additional $1x10^6$ cells were stimulated with PMA/I (50ng/ml PMA; 1ug/ml ionomycin) for 24 hours at 37℃. In all stimulation conditions, cells were resuspended to a final concentration of $10x10^6$ cells/ml. After 24 hours, cells were stained for flow cytometry. After washing in at least 1ml of PBS, cells were stained with the dead cell marker Zombie NIR Fixabile Viability dye (Biolegend), for 15 minutes at room temperature in the dark. Next, cells were washed with FACS buffer. Before surface staining, cells were incubated with Fc block (BD Biosciences) for 5 minutes, in the dark. All conditions were stained using a combination of AF488-CD3 (1.25ul), AF700-CD4 (2.5ul), BV510-CD14 (1.25ul), BV510-CD19 (1.25ul) antibodies for 30 minutes at 4℃ (all antibodies purchased from Biolegend). Stimulated conditions were further permeabilized with Permeabilization Buffer

(eBioscience) for intracellular staining and stained with APC-IL-8 (5ul) (clone E8N1) for 40 minutes at 4°C. Samples were washed, resuspended in FACS buffer, and filtered into 5ml round bottom polystyrene tubes with cell strainer cap (Corning Falcon). Samples were acquired on the flow cytometer (BD LSRII), and data was analyzed with FlowJo V10.7.2.

1.3 Identifying factors which stimulate T cells to produce IL-8

1.3.1 Jurkat T cell Preparation

Jurkat T cells were purchased from ATCC (clone E6.1) and cultured in RPMI containing 10% heat inactivated fetal bovine serum, 2mM L-glutamine, 1500mg/L sodium bicarbonate, 10mM HEPES, and 1mM sodium pyruvate (hereafter referred to as cRPMI).

1.3.2 PBMC and Isolated CD4+ T cell Preparation

Peripheral blood was obtained by a neighboring lab at McMaster University. Thirty mls of peripheral blood were collected in BD Vacutainer tubes containing 5mls acid citrate dextrose (used as an anticoagulant). The blood was centrifuged for 20 minutes at 800 rpm. The platelet rich plasma was then removed, and the remaining blood was layered over Ficoll-Paque PLUS (GE Healthcare) and centrifuged for 30 minutes at 400xg, at room temperature.

The PBMC fraction was collected and washed in RPMI containing 1 unit/ml heparin. Following an additional wash, PBMCs were resuspended in EasySep Buffer (StemCell Technologies) to a final concentration of $50x10^6$/ml for CD4+ T cell isolation following the manufacturer's instructions (StemCell Technologies). In several experiments, PBCMs were not further processed, and cells were resuspended in cRPMI. Following CD4+ T cell isolation, cells were washed, counted, and resuspended in cRPMI for downstream experiments. All experiments using PBMCs and isolated CD4+ T cells were stained with a combination of APC-Cy7-Live/dead (1:500 diluted in PBS), AF488-CD3 or PerCP-CD3 (1.25ul), AF700-CD4 or PE/Dazzle594-CD4

(2.5ul), BV510-CD14 (1.25ul), BV510-CD19 (1.25ul) to assess the purity of cells following CD4+ T cell isolation.

In several experiments, previously frozen PBMCs were used as the starting material. To prepare previously frozen PBMCs for CD4+ T cell isolation and stimulation, the vial containing frozen cells was submerged in a 37°C-water bath for 2 minutes to thaw. Next, cells were incubated with DNase I solution (100ug/ml) for 15 minutes, washed once in cRPMI, and resuspended in cRPMI to a concentration of 2-5x10^6 cells/ml. Cells were left to sit overnight in the 37°C incubator, 5% CO_2. The next day, CD4+ T cell isolation was performed (protocol identical to above)

1.3.3 Flow cytometry staining protocol

Following isolation, stimulation or other experiments, flow cytometry was performed to assess the purity and/or activation state of the cells. This involved washing the cells in at least 1ml of sterile PBS (centrifuge at 400xg for 5-minutes, at room temperature). If applicable, cells were stained with Zombie NIR Fixabile Viability dye (Biolegend), which has a similar emission to APC-Cy7 (here on out referred to as APC-Cy7-Live/dead) by diluting the dye 1:500 in PBS and resuspending cells in 100ul of diluted Zombie NIR, for 15-minutes at room temperature, protected from light. Next, cells were washed with FACS buffer (400xg, 4°C for 5-minutes). Next, 2ul of Fc block (BD Biosciences) was added to cells for 5-minutes, protected from light. Fc block was used to block nonspecific binding of fluorescently labeled antibodies to Fc receptors on B cells, NK cells, etc. Next, 50ul of a previously titrated antibody mixture were added stain cells for expression of surface markers. These would vary depending on the purpose of the experiment and which cells were being analyzed. For example, PBMC and CD4+ T cell surface included the following fluorophore-antibody mixture: AF488-CD3 or PerCP-CD3

(1.25ul), AF700-CD4 or PE/Dazzle594-CD4 (2.5ul), BV510-CD14 (1.25ul), and BV510-CD19 (1.25ul). All antibodies were purchased from Biolegend. The cells would be incubated with the antibody master mix for 30-minutes at 4°C, protected from light. Following surface staining, cells were washed with FACS buffer (1ml was added to tubes, and centrifuged at 400xg for 5-minutes, 4°C). Next, cells were fixed with 200ul IC Fixation Buffer (eBioscience) for 20-minutes at room temperature, protected from the light. If no intracellular staining was performed, cells were washed using 1ml FACS buffer, resuspended in 200ul FACS buffer and filtered into 5ml round bottom polystyrene tubes with cell strainer cap (Corning Falcon). If intracellular staining was needed, cells were permeabilized using Permeabilization Buffer (eBioscience) by centrifuging cells with at least 1ml of Permeabilization Buffer (400xg, 5-minutes at 4°C). Next, 50ul of diluted fluorophore-antibody mixture (in Permeabilization Buffer) was added to cells and incubated 40-minutes at 4°C, protected from light. Samples were washed, resuspended in FACS buffer, and filtered into 5ml round bottom polystyrene tubes with cell strainer cap (Corning Falcon). Samples were acquired on the flow cytometer (BD LSRII), and data was analyzed using FlowJo V10.7.2.

1.3.4 CD3/28/2 stimulation of T cells and IL-8 ELISA

For CD3/28 stimulation, 96-, 12- and 6-well flat-bottomed plates (Corning Falcon) were coated in 70ul (in 96-well plates) or 1ml (in 12- and 6-well plates) of 10ug/ml anti-CD3 antibody (clone OTK3) in PBS overnight at 4°C (eBioscience). The remaining wells were coated in sterile PBS. Only anti-CD3/28-stimulated conditions were coated with anti-CD3 (10ug/ml).

Jurkat or isolated CD4+ T cells were plated at varying concentrations (typically $1x10^6$ and $2.5x10^6$ cells/ml, respectively, though the densities used in experiments ranged from 0.5 to $5x10^6$ cells/ml) into 96-, 12-, and 6-well flat-bottom plates containing immobilized anti-CD3

(10ug/ml) or PBS which was left in the 4℃ fridge, overnight. The wells were washed at least twice prior to the addition of cells. Media-only conditions contained cRPMI only, anti-CD3/28-stimulated conditions contained the immobilized anti-CD3 (10ug/ml) in addition to soluble anti-CD28 (2ug/ml) (clone CD28.2, eBioscience), and Immunocult-stimulated conditions contained 25ul/ml Immunocult Human CD3/28/2 T cell activator (StemCell Technologies).

Following the addition of cRPMI, anti-CD28 or Immunocult, cells were mixed and incubated at 37℃, 5% CO_2 for 24 hours. After 24 hours, the supernatants were collected and stored at -20℃.

Jurkat T cells were plated in a 6-well or 96-well flat-bottomed plate and stimulated with 70ul of immobilized anti-CD3 (10ug/ml) and soluble anti-CD28 (2ug/ml). The optimal concentration of Jurkat T cells plated was $1x10^6$ cells/ml as determined by IL-8 production (Figure 4B). Time-course and dose-response curves were established to determine the optimal concentration of antibodies, and the optimal stimulation time for IL-8 production. However, in some experiments, the concentration of Jurkat T cells stimulated were changed to evaluate the effect that T cell densities had in generating supernatants that stimulated basophils and/or neutrophils – concentrations ranged from 0.5 to $5x10^6$ cells/ml. Next, an anti-CD2 monoclonal antibody (Invitrogen, clone RPA-2.10) was added to anti-CD3 and anti-CD28 antibodies to determine if CD2 stimulation, in conjunction with CD3 and CD28 stimulation, enhanced IL-8 production. The IL-8 ELISA kit was purchased from Thermofisher Scientific, and samples were read on the iMark Microplate Absorbance Reader (Bio-Rad) with the absorbance set to 450nm.

1.3.5 IL-12 and IL-18 stimulation

Jurkat T cells and freshly isolated PBMCs were stimulated with IL-12 (Peprotech, 10ng/ml) and IL-18 (MBL International, 100ng/ml) for 6h and 24h to see if T cells produced IL-

8 in response to stimulation with IL-12 +/- IL-18. Four hours before samples were collected, Brefeldin A (Invitrogen) was added to the samples so that cells could be intracellularly stained for IL-8. After 6h or 24h, supernatants were collected for quantification of IL-8 by ELISA, and cells were stained for flow cytometry. The protocol for flow cytometry staining was identical to section ***methods 1.3.3*** with several modifications. Cells were stained using the fluorophore-antibody mixture: APC-Cy7-Live/dead, AF488-CD3, AF700-CD4, BV510-CD14, BV510-CD19 (all antibodies purchased from Biolegend). Cells were subsequently intracellularly stained for IL-8 (APC-IL-8, clone E8N1 purchased from Biolegend).

1.3.6 PGD_2 and LTE_4 stimulation

Jurkat T cells and isolated CD4+ T cells were incubated with PGD_2 (100nM) and LTE_4 (50nM) with or without the addition of IL-4 (100ng/ml) for up to 24h. Following 24h incubation with IL-4, CD4+ T cells were isolated from frozen PBMCs using EasySep Human CD4+ T cell Enrichment Kit (StemCell Technologies) according to manufacturer's instructions. Isolated CD4+ T cells were stimulated for 24h with PGD_2, LTE_4 or the appropriate controls. Following stimulation, cells were stained for flow cytometry. The protocol for flow cytometry staining was identical to ***methods 1.3.3*** with the following modifications: the fluorophore-antibody mixture included APC-Cy7-Live/dead, PerCP-CD3, AF700-CD4, BV510-CD14, BV510-CD19, FITC-CRTh2 (5ul) and BV605-CRTh2 (5ul) antibodies (all antibodies were purchased from Biolegend).

The protocol in ***methods 1.3.6*** was repeated with a few modifications: CD4+ T cells were isolated from PBMCs prior to incubation with IL-4 (100ng/ml) for 24h or 48h. Cells were collected and incubated with PGD_2/LTE_4 for an additional 24h. Supernatants were collected for quantification of IL-8 and cells were stained for flow cytometry.

Aim 2: Demonstrate that supernatants from stimulated CD4+ T cells can activate basophils

2.1 Jurkat T cell Preparation

Jurkat T cells were prepared as per ***1.3.1***

2.2 Isolated CD4+ T cell Preparation

Isolated CD4+ T cells were prepared as per ***1.3.2***

2.3 T cell Stimulation

The protocol for T cell stimulation was described in ***1.3.4***

2.4 Whole blood basophil activation test

Unlike CD4+ T cell preparation, blood used for basophil activation tests and histamine release assays was not pre-processed by a neighboring lab, as we found the preprocessing procedure affected basophil viability.

The procedure for the basophil activation test (BAT) in whole blood was done according to the following protocol: whole blood was obtained from healthy volunteers at McMaster University and collected in BD Vacutainer tubes containing EDTA anti-coagulant. Fifty microlitres of stimulant(s) and 15ul of fluorophore-antibody mixture (CCR3-AF647, CD63-FITC, and CD203-PE (all antibodies were purchased from Biolegend) were directly added to 5ml polystyrene tubes containing 100ul of whole blood and incubated for 30 minutes in a 37℃-water bath, protected from light. Following incubation, 2ml of 1X red blood cell (RBC) lysis buffer (Biolegend) was added to each tube and incubated for 15-minutes at room temperature, protected from the light. Cells were washed in at least 1ml of FACS buffer and fixed (IC Fixation Buffer, Invitrogen). Following a final wash, cells were resuspended in FACS buffer and acquired on the flow cytometer (BD LSRII). Flow cytometry data was analyzed using FlowJo V10.7.2.

2.5 Isolated basophil activation test

The protocol for BATs using isolated basophils was identical to the whole blood BAT protocol (see ***methods 2.4***) with the following modifications: following the collection of blood, basophils were isolated from blood using immunomagnetic negative selection according to instructions (EasySep Direct Human Basophil Isolation Kit (StemCell Technologies). Next, basophils were resuspended to $4x10^5$/ml in cRPMI and 85ul was aliquoted into 5ml polystyrene tubes containing 50ul of stimulant(s) and 15ul of antibody mixture AF647-CCR3 (5ul), FITC-CD63 (5ul), and PE-CD203c (5ul) (Biolegend). Isolated basophils were incubated for 30 minutes in a 37℃-water bath, protected from light. Isolated basophils were prepared for flow cytometric analysis following the method used in ***methods 1.3.3***. For basophil priming experiments, basophils were incubated for 15-minutes with supernatants derived from T cells, followed by the addition of anti-IgE for 15-minutes, for a total of 30-minute incubation period (in a 37℃-water bath, protected from light).

2.6 Basophil histamine release

Basophils were collected and prepared identically to ***methods 2.4*** or ***methods 2.5*** depending on if whole blood or isolated basophils were used for the experiment. Following incubation, cells were diluted 5.25x with histamine release buffer (provided in histamine ELISA kit, Beckman Coulter). In parallel, a total histamine release sample was prepared. For this, an equal number of cells and concentration (relative to the number and concentration used in the experimental conditions) was aliquoted into a 1.5ml Eppendorf tube and frozen/thawed twice to lyse the cells. Cells were centrifuged at 400xg, for 5-minutes at 4℃ and 100ul of supernatant was collected from tubes and plated in 96-well flat-bottomed plate containing 25ul acylation buffer (provided in histamine ELISA kit, Beckman Coulter). Next, 25ul of acylation reagent

(provided in ELISA kit, Beckman Coulter) was added to each well, and the 96-well plate was stored in -20℃ until ready for histamine measurement. Histamine in the samples were quantified via a competitive ELISA (Beckman Coulter). Absorbance was measured using the iMark Microplate Absorbance Reader (Bio-Rad) with the absorbance set at 405nm.

2.7 Basophil-T cell contact-dependent activation (basophil activation test and histamine release in bulk PBMCs)

PBMCs were prepared from whole blood identically to ***methods 1.3.2*** with the following modifications: Approximately 50mls of whole blood was collected from healthy volunteers at McMaster University in BD Vacutainer tubes using EDTA as an anticoagulant. Twenty-five mls of blood was layered over 15mls of Ficoll-Paque PLUS (GE Healthcare) and centrifuged at for 30-minutes at 400xg, room temperature (acceleration – low, break – low). The buffy coat containing PBMCs were collected and washed in approx. 40ml of wash media (RPMI 1640, 1unit/ml heparin) for 10-minutes at 400xg, at room temperature. PBMCs were resuspended in culture media (cRPMI) and counted using the Countess Automated Cell Counter (Thermofisher Scientific).

PBMCs were resuspended to a concentration of $5x10^6$ cells/ml and plated in 96-well flat-bottom plates pre-coated with 70ul of anti-CD3 (10ug/ml, eBioscience) or PBS. Prior to plating, the wells were washed at least 2x with sterile PBS. Two hundred microlitres of PBMCs were plated in each well of 96-well flat-bottomed plate, representing 1 condition ($1x10^6$ PBMCs at a concentration of $5x10^6$ cells/ml). Five microlitres of stimulant(s) were added to the wells containing PBMCs. Conditions included: cRPMI, immobilized anti-CD3 (10ug/ml) + soluble anti-CD28 (2ug/ml, both antibodies from eBioscience), Immunocult (25ul/ml, StemCell Technologies), anti-human IgE (1ug/ml, Sigma-Aldrich). In several experiments, cat-allergic

subjects were recruited and bulk PBMCs were prepared as previous described. In addition to cRPMI, anti-CD3/28, Immunocult and anti-IgE stimulation, PBMCs were stimulated with the major cat allergen Fel d 1 (25ug/ml), and a mixture of 7 short peptides derived from Fel d 1, referred to as Cat-PAD (for composition of Cat-PAD, see Appendix S1). The plate containing PBMCs and stimulant(s) was placed in 37℃, 5% CO_2 incubator for various timepoints. At each timepoint, 100ul of supernatant was collect from each condition and transferred to another 96-well flat-bottomed plate and acylated (protocol for acylation and histamine measurement identical to ***methods 2.6*** with the following modification: supernatants collected from PBMCs were not diluted in histamine release buffer.

The cell fraction from each condition at each timepoint was collected and stained according to the protocol outlined in ***methods 1.3.3*** with several modifications. The surface staining panel included the following fluorophore-antibody mixture: APC-Cy7-Live/dead (1:500), PerCP-CD3 (1.25ul), AF700-CD4 (2.5ul), BV605-CD8 (2.5ul), BV510-CD14, BV510-CD19 (1.25ul), AF647-CCR3 (5ul), FITC-CD63 (5ul), and PE-CD203c (5ul). This protocol did not require intracellular staining.

Aim 3: Demonstrate that supernatants from stimulated CD4+ T cells can activate neutrophils

3.1 Isolation and staining of neutrophils

Similarly to basophil functional tests, it was found that whole blood preprocessed by removing platelets affected neutrophil viability, therefore only freshly isolated blood was used for neutrophil functional assays. Healthy volunteers at McMaster University were recruited to donate blood for assays. Neutrophils were isolated from whole blood using the MACSxpress Human Whole Blood Neutrophil Isolation Kit (Miltenyi Biotec), according to manufacturer's

instructions. Cells were washed in at least 1ml PBS (centrifuged at 400xg, 4℃, for 5-minutes) and stained according to the protocol outlined in ***methods 1.3.3*** with several modifications. The surface staining panel included the following fluorophore-antibody mixture: APC-Cy7-Live/dead (1:500), PerCP-CD15 (2.5ul), PE/Dazzle594-CD16 (2.5ul), AF647-CD62L (2.5ul), PerCP/Cy5.5-CD66b (2.5ul), BV421-CD11b (2.5ul), (all antibodies were purchased from Biolegend). Isolated neutrophils were not intracellularly stained.

3.2 Whole blood staining of neutrophils

Whole blood was obtained from healthy volunteers around McMaster University. One hundred microlitres of whole blood was incubated with 10ul of 1:1000 diluted dihydrorhodamine 123 (DHR123, Cayman Chemical) in PBS for 15-minutes in a 37℃-water bath, protected from the light. Following DHR123 staining, samples were then incubated for 45-minutes with 50ul of stimulant or appropriate control. Following stimulation, 2ml of 1X RBC lysis buffer (Biolegend) were added and samples were incubated for 15-minutes at room temperature, protected from light. Cells were washed in at least 1ml PBS (centrifuged at 400xg, 4℃, for 5-minutes) and stained according to the protocol outlined in ***methods 1.3.3*** with several modifications. The surface staining panel included the following fluorophore-antibody mixture: APC-Cy7-Live/dead, PerCP-CD15, PE/Dazzle594-CD16, AF647-CD62L, PerCP/Cy5.5-CD66b, BV421-CD11b, (all antibodies were purchased from Biolegend). Volumes of antibodies used were identical to ***methods 3.1***. Next, cells were intracellularly stained according to the protocol outlined in ***methods 1.3.3*** using a fluorophore-antibody specific to myeloperoxidase (20ul of PE-MPO, BD Biosciences).

3.3 Neutrophil histamine release

Neutrophil histamine release assays were performed identically to ***methods 2.6***, albeit the cells used were isolated neutrophils rather than whole blood or isolated basophils.

RESULTS

Aim 1: Identifying IL-8-producing (IL-8+) CD4+ T cells and factors which stimulate T cells to produce IL-8

1.1 Identifying the frequency of allergen-specific CD4+ T cells in cat allergic subjects

In our preliminary study, we observed the frequency of allergen-specific T cells in a cat-allergic subjects and established a gating strategy (Figure 2). Isolated CD4+ T cells were gated based on forward scatter (FSC) and side scatter (SSC) to eliminate debris and eliminate non-lymphocytes (Figure 2A). Lymphocytes were further gated to eliminate doublets and dead cells (Figure 2B, C). Next, B cells (expressing CD19) and monocytes (expressing CD14) were gated out and cells expressing CD3 were included (Q3) (Figure 2D). CD4+ TET+ cells (Figure 2E) were gated with the help of a PE fluorescence minus one (FMO) control (Figure 2F). Out of approximately 850,000 events in total, 5 tetramer positive cells were detected. The frequency of TET+ cells in the first cat-allergic subject was 0.000662% (6.62×10^{-4}%).

The protocol was repeated with the same cat-allergic subject. Gating strategy was identical as just described; however, more cells were recovered. Only the final gates were shown (Figure 2G-I). Figure 2G, H were identical but acquired separately. Figure 2I showed the FMO control. Approximately 3.5×10^{6} events were acquired, with a total of 37 tetramer positive cells detected. The frequency of tetramer positive cells in this experiment is 0.00114% (1.14×10^{-3}%).

1.2 Identifying the frequency of IL-8+ allergen-specific CD4+ T cells in cat allergic subjects

To determine the frequency of allergen-specific IL-8+ T cells, we recruited another cat allergic subject to measure the frequency of these cells in their blood under stimulatory and non-stimulatory conditions. One third of the CD4+ T cells collected were used to determine the number of tetramer positive cells using an identical gating strategy to ***results 1.1***. In the immediate staining condition, nearly $2.5x10^6$ events were collected, of which there were 29 tetramer positive events collected (CD4+ TET+, Figure 3B). An FMO control was included in this experiment as well (Figure 3A). The frequency of tetramer positive cells was $1.46x10^{-3}$%. This fraction was unstimulated and not intracellularly stained for IL-8.

The remaining CD4+ T cells were stimulated with RPMI + 5% autologous plasma (media), Dynabeads Human T-cell Activator CD3/28 (αCD3/28 Dynabeads, Thermofisher Scientific), or PMA (50ng/ml) + Ionomycin (1ug/ml) for 24 hours (Figure 3C-E), and intracellularly stained for IL-8 (with the exception of Figure 3E). The frequency of tetramer positive events in the media- and the αCD3/28 Dynabead-stimulated CD4+ T cells were 0.00108% and 0.000921%, respectively (Figure 3C, D). The frequency of CD4+ T cells expressing IL-8 in the media-, the αCD3/28 Dynabead-, and the PMA/I-stimulated CD4+ T cells were 0.00837%, 0.0892%, and 0.22%, respectively (Figure 3C-E). These preliminary results show that there was no allergen-specific IL-8+ CD4+ T cells (Figure 3C-E).

1.3 Jurkat T cells and isolated CD4+ T cells produce IL-8 in response to anti-CD3/CD28 and anti-CD3/CD28/CD2 (Immunocult) stimulation

Time course and dose-response curves were performed with Jurkat T cells to determine the optimal stimulation conditions (Figure 4). Jurkat T cells were resuspended in log 10-fold dilutions ($1x10^{5-7}$/ml) and the amount of IL-8 produced followed bell-shaped dose-response

curve (Figure 4A). Jurkat T cells were also incubated with a variety of stimuli for up to 120hrs (Figure 4B). Stimulation beyond 24 hours did not result in an appreciable increase in IL-8 (Figure 4B). Next, immobilized anti-CD3 and soluble anti-CD28 were titrated – the optimal dose of anti-CD3 was 10ug/ml, while the optimal dose of anti-CD28 did not make a noticeable difference in IL-8 produced (Figure 4C) and was kept at a concentration of 2ug/ml for downstream experiments (previously established concentration).

To further enhance IL-8 production by Jurkat T cells, we examined whether stimulating CD2, in conjunction with CD3/28, influenced IL-8 production (Figure 5). Soluble anti-CD2 stimulation provided better stimulation than immobilized anti-CD2 (Figure 5A). Titrating anti-CD2 resulted in a bell-shaped dose-response curve, with the optimal stimulation at 1ug/ml of anti-CD2 (Figure 5A). However, Immunocult anti-CD3/28/2 T cell Activator (StemCell Technologies) proved to be a more potent stimulator of Jurkat T cells compared to making up our own anti-CD3/28/2 antibody mixture, and we opted to use this product for future T cell stimulation experiments (Figure 5B). A summary of Jurkat T cells stimulated in 96-well flat-bottomed and 6-well flat-bottomed plates (Figure 6A, B).

Media-stimulated Jurkat T cells in 96-well or 6-well plates across all Jurkat T cell densities ($0.5\text{-}5x10^6$ cells/ml) did not produce any IL-8. Jurkat T cells stimulated with anti-CD3/28 in 96-well plates produced 23.19±20.24pg/ml IL-8, while Jurkat T cells stimulated with Immunocult in 96-well plates produced 124.61±40.60pg/ml IL-8. Jurkat T cell experiments in 96-well plates were stimulated and incubated at a density of $1x10^6$ cells/ml.

Anti-CD3/28-stimulated Jurkat T cells in 6-well plates produced approximately 10-times the IL-8 compared to Jurkat T cells stimulated with anti-CD3/28 in 96-well plates (238.51±104.16 pg/ml, compared to 23.19±20.24 pg/ml) at the same density ($1x10^6$ cells/ml).

Possible explanations for the enhanced IL-8-production in 6-well plates compared to 96-well plates were discussed later. Anti-CD3/28-stimulated Jurkat T cells in 6-well plates at densities of $0.5x10^6$, $1x10^6$, $2.5x10^6$, and $5x10^6$ cells/ml produced 130.27±7.85, 238.51±104.16, 464.32±100.55, and 313.20 pg/ml IL-8, respectively. Interestingly, Jurkat T cells ($1x10^6$ cells/ml stimulated with anti-CD3 alone produced 68.62pg/ml IL-8, without the need for co-stimulation.

Immunocult-stimulated Jurkat T cells in 6-well plates produced approximately twice the IL-8 compared to Jurkat T cells stimulated with Immunocult in 96-well plates (266.76±151.43pg/ml, compared to 124.61±40.60pg/ml) at the same density of Jurkat T cells ($1x10^6$ cells/ml). Immunocult-stimulated Jurkat T cells in 6-well plates at densities of $0.5x10^6$, $1x10^6$, $2.5x10^6$, and $5x10^6$ cells/ml produced 175.28±19.32, 266.76±151.43, 756.65±136.03, and 673.45pg/ml IL-8, respectively.

Supernatants from activated CD4+ T cells isolated from PBMCs were also generated by stimulating CD4+ T cells with media, anti-CD3/28, and Immunocult (Figure 6C). CD4+ T cells were stimulated using 96-, 12-, and 6-well plates, depending on the number (and therefore volume) of CD4+ T cells acquired after separation.

Media-stimulated CD4+ T cells at densities of $0.5x10^6$, $1x10^6$, $2.5x10^6$, and $5x10^6$ cells/ml produced 0, 0.368±0.74, 6.53±6.65, and 0pg/ml IL-8, respectively.

Anti-CD3/28-stimulated CD4+ T cells at densities of $0.5x10^6$, $1x10^6$, $2.5x10^6$, and $5x10^6$ cells/ml produced 27.11±26.2, 100.17±68.84, 146.30±130.84, and 328.53 pg/ml IL-8, respectively.

Immunocult-stimulated CD4+ T cells at densities of $0.5x10^6$, $1x10^6$, $2.5x10^6$, and $5x10^6$ cells/ml produced 85.50±61.60, 170.85±54.77, 212.86±187.97, and 520.39 pg/ml IL-8, respectively. The decision to use $2.5x10^6$ cells/ml CD4+ T cells (in most of our experiments to

generate supernatants) was due to 1) previous master's student choice to stimulate CD4+ T cells at this density and 2) relatively large variability in the amount of IL-8 produced from stimulated CD4+ T cells.

Aim 2 (Part 1: Histamine Release). Demonstrate that supernatants from stimulated T cells can induce histamine release from basophils, and determine if this effect was mediated through IL-3 and/or IL-8

2.1 IL-3 and/or IL-8 did not stimulate basophils to release histamine

Whole blood was stimulated with IL-3 and IL-8 to determine if basophils were stimulated to release histamine. Established doses of IL-3 and IL-8 that induced histamine release from basophils in the literature, did not induce histamine release from basophils in our hands (see Appendix S2).[117,118]

2.2 T cell supernatants did not stimulate basophils to release histamine

Next, we utilized T cell supernatants derived from Jurkat T cells and isolated CD4+ T cells to see if they contained mediators that could induce basophils to release histamine (Figure 7). Isolated basophils incubated with supernatants derived from Jurkat T cells stimulated with anti-CD3/28 and Immunocult released 22.65±9.36% and 25.26±5.10% of the total histamine in the samples, respectively, following 30-minutes of incubation (Figure 7A). This is comparable with the positive control, anti-IgE-stimulated basophils, which released 28.46±19.43% of the total histamine content from basophils, following a 30-minute incubation period (Figure 7A). However, the source of histamine in these samples was not basophils (discussed below). Basophils incubated with Jurkat T cells stimulated with media released 1.28±0.97% of total histamine in the basophils. Isolated basophils incubated with supernatants derived from isolated CD4+ T cells did not release any noticeable amount of histamine above baseline (Figure 7B).

Supernatants derived from media-, anti-CD3/28-, and Immunocult-stimulated CD4+ T cells incubated with basophils released 2.660±1.94%, 2.50±1.35%, 2.75±9.88% of the total histamine released (Figure 7B). These values did not significantly vary from the baseline (media only) stimulation condition (2.30±1.23% total histamine release) (Figure 7B). A two-way mixed ANOVA with Dunn's multiple comparisons was performed ($p>0.05$ across all conditions).

2.3 Jurkat T cells, but not CD4+ T cells, produced histamine when activated by anti-CD3/28 and Immunocult

While investigating if PLB-985 (Leibniz Institute DMSZ), a neutrophil-like cell line, contained histamine, it was discovered that Jurkat T cell supernatants themselves synthesized and produced histamine (Appendix S3). There was no detectable histamine release in the total histamine release condition of PLB-985 (0nM), indicating that all the histamine present in the samples was derived from the Jurkat T cell supernatants (Appendix S3). Because the supernatant-only conditions (100ul) were undiluted, while the PLB-985 (100ul) plus supernatants (50ul) were diluted, if the supernatants contained histamine, the dilution factor would be 3. Anti-CD3/28 and Immunocult-stimulated Jurkat T cell supernatant-only conditions released 47.40±8.06nM and 49.06±1.91nM histamine, respectively, whereas PLB-985 incubated with anti-CD3/28 and Immunocult stimulated Jurkat T cell supernatants released 14.00±3.36nM and 16.43±9.99nM histamine respectively. Dividing the supernatant-only conditions by the PLB-985 plus supernatant conditions yield: 3.39 and 2.99 for anti-CD3/28 and Immunocult conditions, respectively. The variability between the expected value (3) and the actual values (3.39, 2.99) may be due to random error. The percent deviation from expected values are 12.85% and 0.0033%, respectively. The experiment was repeated with several additional batches of

Jurkat T cell supernatants (batches #5-8) to confirm that histamine was present in the Jurkat T cell supernatants (Appendix S3 B).

To tease out the source of histamine in our samples with respect to basophils, T cell supernatants (Jurkat T cells and isolated CD4+ T cells) were generated at varying concentrations from 0.5-5x10^6 cells/ml and incubated with, and without isolated basophils (supernatants alone) (Appendix S4). Isolated basophils stimulated with CD4+ T cell supernatants (from 2 different subjects) did not release significant amounts of histamine compared to baseline (cRPMI) (Appendix S4A, B). There was no effect observed between the amount of histamine released from CD4+ T cell supernatants alone, or CD4+ T cell supernatants incubated with basophils. Jurkat T cell supernatants alone contained more histamine than Jurkat T cell supernatants incubated with basophils (due to diluting the culture with basophils which did not release histamine), suggesting that the predominant source of histamine in the samples was from the Jurkat supernatants, rather than basophils releasing histamine (Appendix S4).

A concern with extended time-course experiments is that histamine degrades overtime. To determine if histamine degradation could impact our results, several different total histamine samples were prepared and acylated at different timepoints (1h, 2h, 4h, and 8h). The histamine content in the prepared samples did not significantly degrade over the span of 7 hours (p=0.74), (Appendix S5). Kruskal-Wallis test with Dunn's multiple comparisons test was performed (n=3 at timepoints 1h, 2h, 4h, and n=2 at timepoint 8h).

2.4 Basophils primed with IL-3 and/or IL-8 did not significantly enhance basophil responsiveness to anti-IgE stimulation by stimulating histamine release.

Our findings in ***results 2.1*** and ***2.2*** demonstrated that directly activating basophils with IL-3, IL-8, and bulk (activated) CD4+ T cell supernatants did not stimulate basophils to release

significant amounts of histamine. However, there are several cytokines and other soluble mediators that, while unable to directly induce histamine release from basophils, have been shown in the literature to potentiate (or markedly enhance histamine release from) basophils and enhance responsiveness to anti-IgE.[56] IL-3 was the most potent cytokine, which has even been shown to stimulate basophils to release histamine in a subset of atopic patients.[56] However, IL-3, among other cytokines that CD4+ T cells release, including GM-CSF and IL-5, also enhance histamine release from basophils when stimulated with anti-IgE, fMLP, calcium ionophore A23187, and phorbol esters.[56] Therefore, there are soluble factors within CD4+ T cell supernatants that can sensitize basophils for enhanced histamine release (we will use the word 'priming' to describe the process sensitizing basophils to a positive control, such as anti-IgE or fMLP, in the context of these next experiments.

Basophils were primed for 15-minutes with IL-3 (20ng/ml) and/or IL-8 (100nM) and subsequently stimulated with anti-IgE for an additional 15-minutes (Figure 8). In our hands, priming basophils with IL-3 and/or IL-8 did not significantly up-regulate histamine. Basophils stimulated with IL-3 alone released 3.56±1.87nM histamine, whereas basophils primed with IL-3 and stimulated with anti-IgE released 8.50±6.03nM histamine ($p=0.404$). There was a larger anti-IgE response when basophils were primed with IL-3 and IL-8 together, however, this was still not significant. Basophils stimulated with IL-3 and IL-8 alone released 4.73±3.80nM histamine, whereas basophils primed with IL-3 and IL-8 and stimulated with anti-IgE released 14.05±4.75nM histamine ($p=0.236$). IL-3+/-IL-8-primed anti-IgE-stimulated basophil histamine release experiments were summarized in Figure 8.

2.5 Basophils primed with supernatants derived from bulk activated CD4+ T cells did not significantly enhanced basophil responsiveness to anti-IgE stimulation by increasing histamine release.

Supernatants derived from bulk CD4+ T cells contain IL-3, IL-8, and many other cytokines that may contribute to priming basophils for enhanced responsiveness to anti-IgE stimulation. We hypothesized that supernatants derived from CD4+ T cells may contain other factors that are capable of priming basophils for enhanced responsiveness to anti-IgE (such as GM-CSF and IL-5 mentioned earlier, or perhaps other factors that have not been identified).

Similar to ***methods 2.4***, basophils were primed for 15-minutes and subsequently stimulated with anti-IgE for 15-minutes, and histamine release was measured (Figure 9). However, rather than using IL-3 and IL-8 to prime basophils, supernatants derived from bulk (activated) CD4+ T cells were used to prime basophils. CD4+ T cells were stimulated with media (cRPMI), anti-CD3/28, or Immunocult, and supernatants were collected (see ***methods 1.3.4*** for stimulation protocol).

Priming basophils with supernatants (SNs) derived from anti-CD3/28 and Immunocult-stimulated CD4+ T cells did not significantly increase their responsiveness to anti-IgE stimulation (compared to media SN+anti-IgE, p=0.134 and p=0.0712, respectively) (Figure 9). Compared to basophils stimulated with media SN+anti-IgE alone (positive control plus background from media), priming basophils with SNs derived from activated CD4+ T cells did not result in significantly higher histamine release. Anti-CD3/28 SN-primed and Immunocult SN-primed basophils stimulated with anti-IgE did not significantly increase histamine release compared to basophils stimulated with media SN+anti-IgE alone (8.65±7.67% and 9.46±6.55 vs. 4.51±2.81nM, respectively) (p=0.134 and p=0.0712, respectively) (Figure 9A). However, with a

p-value less than 0.1, Immunocult SN-primed basophils stimulated with anti-IgE trended towards significance. The results from basophils primed with SNs derived from media-, anti-CD3/28-, and Immunocult-stimulated CD4+ T cells are show in Figure 9A.

To further elaborate on basophil priming, the percent change in basophil histamine release was compared between the anti-IgE stimulated basophils and the priming alone/priming plus anti-IgE stimulated basophils (Figure 9B). The histamine release values in all conditions were divided by the histamine release in the anti-IgE stimulated basophils to get a percent change value compared to the positive control (similar to fold-change). While anti-CD3/28 and Immunocult SN priming alone did not markedly increase the percent histamine release from basophils compared to anti-IgE (129.9% and 139.6%, respectively), basophils primed with SN derived from anti-CD3/28 and Immunocult-stimulated T cells, when subsequently stimulated with anti-IgE, resulted in a sharp increase in percent histamine release (421.5% and 631.7%, respectively) compared to anti-IgE alone (Figure 9B).

Finally, experiments were performed using a neutralizing antibody to IL-8 (Sigma-Aldrich, clone 6217, here on out referred to as nAbIL-8). The purpose of using a nAbIL-8 antibody in T cell supernatants was to evaluate the relative contribute IL-8 had in stimulating basophils to release histamine, by blocking the action of IL-8 and seeing if there are similar levels of histamine release. In our hands, we did not see any significant effect that neutralizing IL-8 had in attenuating or augmenting basophil activation (results summarized in Appendix S6).

Aim 2 (Part 2: CD63 and CD203c). Demonstrate that supernatants from stimulated T cells can activate basophils by upregulating surface markers CD63 and CD203c, and determine if this effect is mediated through IL-3 and/or IL-8

The function of CD63 and CD203c basophil surface markers were briefly introduced in **OBJECTIVES OF THE PROJECT**. Upregulation of CD63 and CD203c following stimulation were used for this project as additional dependent variables, as these markers potentially reflect different mechanisms for activation (see ***discussion 2.6***). CD63 is an activation marker on basophils that has been used as a reporter molecule for many allergens, however, studies using CD63 upregulation to identify IgE-mediated allergies have showed it lacks sensitivity (63% and 54% in two studies).[138,139] For this reason, CD203c has been included more recently because of enhanced sensitivity in diagnosing IgE-mediated allergies.[140,141]

2.6 IL-3-primed basophils stimulated with IL-8 significantly up-regulated activation markers CD63/CD203c

Basophil gating strategy was shown in Figure 10. The strategy for whole blood (Figure 10A) and isolated basophils (Figure 10B) gating was identical. After gating out debris and doublets (top left and right plots, respectively), basophils were gated on CCR3+ and SSClow (bottom left). Activated basophils up-regulated CD63 and CD203c cell surface markers. The threshold for positivity for basophils was 5% CD63+ and 5% CD203c+ in the unstimulated (or baseline) condition. Five percent CD63+ in the unstimulated cells is commonly used in the literature,[130] however, CD203c typically uses median fluorescence intensity (MFI) to measure activation. Standardizing MFI across experiments is difficult without an internal control and repeatability is an issue.[142] Therefore, a 5% CD203c+ in the unstimulated cells was used as a threshold for positivity as well.

Basophil activation tests were performed for basophils incubated with increasing concentrations of IL-8 (with and without IL-3-priming) to see if these cytokines up-regulated basophil activation markers (Figure 11). IL-3-was used to briefly prime the basophils (5-minutes) prior to IL-8-stimulation for an additional 15-minutes (Figure 11A) or 25-minutes (Figure 11B). Basophils primed with IL-3 and subsequently stimulated with the two highest doses of IL-8 (200nM and 2000nM) significantly up-regulated activation markers CD63 and CD203c compared unstimulated basophils (49.00±24.79% vs. 4.08±1.54% and 58.08±19.31% vs. 4.08±1.54, respectively) ($p=0.0138$ and $p=0.0121$, respectively). (Figure 11A). Basophils incubated with IL-8 alone at any concentration did not significantly up-regulate activation markers CD63/CD203c ($p>0.999$).

2.7 T cell supernatants stimulated basophils to up-regulate activation markers CD63/CD203c

The procedure for stimulating basophils with T cell supernatants was performed identically to ***methods 2.5***: basophils were stimulated with T cell supernatants for 30-minutes, and expression of CD63 and CD203c was measured using flow cytometry (Figure 12Figure 9). Jurkat and CD4+ T cells were stimulated with media (cRPMI), anti-CD3/28, or Immunocult, and supernatants were collected (see ***methods 1.3.4*** for stimulation protocol).

Basophils were incubated with Jurkat T cell SNs (Figure 12A) and CD4+ T cell SNs (Figure 12B) for 30-minutes and subsequently stained for CD63/CD203c activation markers. Compared to media-only stimulated T cells (media stim.), basophils stimulated with SN derived from anti-CD3/28 and Immunocult-activated Jurkat T cells significantly up-regulated activation markers CD63/CD203c (5.06±2.52% media-stim. vs. 27.74±18.87% anti-CD3/28 stim. and 43.52±28.90% Immunocult stim.) ($p=0.0334$ and $p=0.0020$, respectively) (Figure 12A). Basophils stimulated with SN derived from CD4+ T cells activated with anti-CD3/28 and

Immunocult significantly up-regulated basophil activation markers CD63/CD203c (38.98±28.57% and 42.28±28.03%, respectively) compared to media stim. (5.06±2.52%) (p=0.0082 and p=0.0053, respectively) (Figure 12B).

2.8 Basophils primed with IL-3 or IL-3+IL-8 significantly enhanced basophil responsiveness to anti-IgE stimulation by upregulating CD63/CD203c activation markers. IL-3 alone, but not IL-8 alone, significantly up-regulated CD63/CD203c activation markers compared to baseline

We previously evaluated the effect that priming basophils with IL-3 and/or IL-8, with and without subsequent stimulation with anti-IgE, had on histamine release (see ***results 2.4***). Here, we applied the same protocol but looked at basophil upregulation of CD63 and CD203c following priming with IL-3+/-IL-8 (Figure 13). Note: the Boolean operator 'OR' refers to basophils which were either CD63 or CD203c positive, or both (double positive) determined by our flow cytometry gating strategy.

IL-3 alone and IL-3+IL-8 alone, but not anti-IgE alone, significant up-regulated CD63 and CD203c expression on basophils compared to baseline (84.45±10.00%, 92.95±4.82% and 35.57±35.06% compared to 8.42±0.73% CD63 OR CD203c+) (p=0.0003, p=0.006, and p=0.0516, respectively). IL-8 alone did not significantly up-regulate basophils compared to baseline (11.39±3.85 vs. 8.42±0.729, p>0.999) (Figure 13).

Compared to basophils stimulated with anti-IgE alone, basophils primed with IL-3 or IL-3+IL-8 first, and subsequently stimulated with anti-IgE, significantly up-regulated CD63/CD203c (35.57±35.06% CD63/CD203c+ vs. 93.30±4.20% and 92.95±4.82% CD63/CD203c+ (p=0.0139 and p=0.0147, respectively). IL-3-alone-stimulated basophils nearly significantly up-regulated CD63/CD203c markers (trended towards significance, p<0.1) compared to anti-IgE-alone-stimulated basophils (84.45±10.00% vs. 35.57±35.06%, p=0.0557).

IL-8 did not significantly up-regulate surface activation markers compared to baseline ($p>0.999$) (Figure 13).

2.9 Basophils primed with anti-CD3/28-stimulated T cell-derived supernatants significantly enhance basophil responsiveness to anti-IgE stimulation as measured by the upregulation of activation markers CD63/CD203c.

The procedure in ***results 2.5*** was repeated with a change in dependent variable: basophil surface activation markers CD63/CD203c were measured, rather than basophil histamine release. The upregulation of CD63/CD203c from SN-primed basophils subsequently stimulated with anti-IgE are summarized in Figure 14. Data from Jurkat T cell SN-priming and CD4+ T cell SN-priming were combined.

Compared to media SN-primed basophils stimulated with anti-IgE, basophils primed with SN from anti-CD3/28 and Immunocult-stimulated T cells stimulated with anti-IgE significantly up-regulated CD63/CD203c (46.36±20.75% CD63/CD203c+ compared to 85.31±12.02% and 93.70±4.55% CD63/CD203c) ($p<0.0001$ and $p<0.0001$, respectively). In fact, SN from anti-CD3/28 and Immunocult-stimulated T cells alone was sufficient to significantly up-regulate CD63/CD203c on basophils compared to media SN+anti-IgE-stimulated basophils (60.70±15.74% and 87.69±5.59% CD63/CD203c+ compared to 46.36±20.75% CD63/CD203c+, $p=0.0098$ and $p<0.0001$, respectively) (Figure 14).

Aim 2 (Part 3). Demonstrate basophils, directly in contact with activated CD4+ T cells, are stimulated to produce histamine and up-regulate CD63/CD203c activation markers

2.10 Basophils in direct contact with activated CD4+ T cells are not stimulated in response to anti-CD3/28, but are stimulated in response to Immunocult (anti-CD3/28/2) antibodies

Having determined that that soluble T cell mediators were not able to induce basophil histamine release, we assessed whether activated CD4+ T cells, in direct contact with basophils, could stimulate basophils to release histamine. PBMCs containing CD4+ T cells and basophils were isolated and T cell specific antibodies (anti-CD3/28) and Immunocult (anti-CD3/28/2) added to the cultures to stimulate CD4+ T cells. Aggregate results are shown in Figure 15 (n=7). PBMCs were stimulated up to 10 hours with antibodies to T cell-specific surface molecules, to see if activated T cells can stimulate basophils to up-regulate CD63/CD203c (Figure 15A, B). Compared to baseline, Immunocult and anti-IgE-stimulated PBMCs caused a significant upregulation of CD63/CD203c on basophils (9.31% CD63/CD203c+ baseline compared to 22.36% and 37.15% CD63/CD203c+ Immunocult and anti-IgE stimulated, respectively) ($p=0.0069$ and $p<0.0001$, respectively). Area under curve (AUC) was plotted for the 4 conditions: baseline (cRPMI), anti-CD3/28, Immunocult, and anti-IgE-stimulation (Figure 15C). However, the AUC analysis showed that compared to baseline, only basophils stimulated with anti-IgE significantly up-regulated expression of CD63/CD203c (AUC 100.6 vs. 328, respectively) ($p=0.0023$) (Figure 15C). Supernatants were also collected and analyzed for histamine release (Figure 15D). Statistics were not performed because it was clear from the graph that basophils in the presence of anti-CD3/28 and Immunocult-stimulated T cells did not release histamine (Figure 15D, green and red bars). AUC analysis showed there was no

significant increase in histamine release in any conditions, including anti-IgE-stimulated conditions (Figure 15E).

Because CD63 and CD203c were distinct molecules expressed on basophils, and they ultimately reflect different underlying processes, we repeated the analyses, but looked at these molecules individually using the AUC approach (Figure 16A, C) as well by comparing the means between the conditions (via one-way ANOVA) (Figure 16E, F). AUC analysis showed that only anti-IgE-stimulated basophils resulted in a significant upregulation of CD203c – this was not the case for CD63 expression ($p=0.0003$ and $p=0.9993$, respectively) (Figure 16B, D). On the other hand, we did see significant differences between conditions when taking the means between groups (Figure 16E, F). Basophils significantly up-regulated CD63 when PBMCs were stimulated with Immunocult (6.74±2.25%, compared to 14.69±5.47% CD63+. Basophils stimulated with anti-IgE did not up-regulate CD63 on basophils ($p=0.9961$) (Figure 16E). Similar to our AUC analysis, anti-IgE-stimulated basophils significantly up-regulated CD203c, compared to baseline (3.55±0.72% CD203c+ at baseline vs. 30.12±9.47% CD203c+ in the anti-IgE-stimulated condition ($p<0.0001$) (Figure 16F).

2.11 Basophils in direct contact with activated T cells were stimulated in response to a mixture of cat peptides

Next, cat-allergic subjects were specifically recruited to assess if synthetic peptides derived from the major cat allergen Fel d 1 (referred to as Cat-PAD, see Appendix S1 for composition) could be presented to CD4+ T cells in an MHC-restricted manner, causing them to become activated and in turn activate basophils. Importantly, the Cat-PAD peptides were previously shown to be too short to cross-link IgE (bound to FcεRI receptors) on the surface of basophils, ensuring that basophils would only be activated in an IgE-independent manner.[87]

Additionally, basophils are known to express MHC class II molecules capable of binding these peptides.[87] The procedure for these experiments were identical to ***results 2.10***, however, Cat-PAD peptides were included as additional conditions and added at various concentrations (0.1-25ug/ml). Fel d 1 (25ug/ml) was included as a positive control. A total of 4 cat-allergic subjects were recruited. However, data from one subject is excluded due to compromised basophil viability.

Histamine release data from the 3 cat-allergic subjects were shown in Figure 17A while the CD63/CD203c flow cytometric data were shown in Figure 17B. We observed a great deal of variability in terms of basophil histamine release, even at baseline (cRPMI only conditions). Therefore, the data were normalized by dividing histamine release values by the values obtained at baseline, at each timepoint, and obtaining fold-change values (Figure 17C). Flow cytometry data was simply analyzed by comparing the mean values for each condition across all timepoints to the baseline (Figure 17D).

Compared to baseline, anti-IgE stimulation resulted in a significantly higher fold-increase in histamine release from PBMCs (2.67-fold increase, $p<0.0001$). Cat-PAD-treated PBMCs at 0.1ug/ml, 1ug/ml, 10ug/ml, and 25ug/ml also caused a significant increase in histamine release from PBMCs compared to baseline (1.81-fold, 1.65-fold, 1.97-fold, and 1.84-fold increase) ($p=0.0087$, $p=0.0471$, $p=0.0051$, and $p=0.0471$, respectively) (Figure 17C). Basophils also significantly up-regulated CD63/CD203c in response to Cat-PAD peptides compared to baseline, although CD63/CD203c were only significantly up-regulated at 0.1ug/ml and 10ug/ml Cat-PAD concentrations (9.72% CD63/CD203c+ at baseline, compared to 17.86% and 17.78% CD63/CD203c+ Cat-PAD at 0.1ug/ml and 10ug/ml, respectively) ($p=0.0354$ and $p=0.0306$, respectively).

Aim 3: Demonstrate that supernatants from stimulated CD4+ T cells could activate neutrophils

3.1 Neutrophils contained and released histamine in response to fMLP. Histamine was not induced from neutrophils in response to IL-8 stimulation

Neutrophils were found to contain histamine (Figure 18A). Across 7 neutrophil isolations, it was found that the average total histamine content per million neutrophils was 63.7nM (Figure 18A). A preliminary experiment was done to see if IL-8, LPS, and fMLP could act as positive controls in stimulating neutrophils to release histamine. LPS was not used further as a positive control for neutrophil histamine release due to minimal histamine release in neutrophils stimulated with LPS (percent of total histamine release=1.83) (data not shown). Compared to the spontaneous release condition, fMLP-stimulated neutrophils caused a significant increase in histamine release from neutrophils (4.436% vs. 27.976% histamine release, p=0.0478) (Figure 18B). IL-8-stimulated neutrophils did not significantly increase the amount of histamine released compared to spontaneous release (9.63% IL-8-stimulated vs. 3.22% baseline histamine release, p=0.152) (Appendix S10 B).

3.2 Neutrophils did not release histamine in response to stimulation by supernatants derived from activated Jurkat and CD4+ T cells

Neutrophils were incubated with stimulated Jurkat T cell supernatants and the appropriate controls. However, similar to our analysis looking at basophil histamine release (see ***results 2.3***), it was discovered that Jurkat T cells, rather than neutrophils, were the predominant source of histamine in the cell culture supernatants (Appendix S3). When neutrophils were incubated with supernatants derived from activated CD4+ T cells, there was no histamine release observed (Appendix S7 B).

3.3 Neutrophil up-regulated CD11b and downregulated CD62L in response to Jurkat and CD4+ T cell supernatant stimulation

Downregulation of CD62L (L-selectin) and upregulation of CD11b are often used as surrogate markers for neutrophil activation. Neutrophil gating strategy was shown in Figure 19 and Figure 20. The strategy for isolated neutrophil (Figure 19) and whole blood neutrophil (Figure 20) gating was identical. The debris and non-granulocytes were gated out (Figure 19A, Figure 20A). Next, doublets were excluded (Figure 19B, Figure 20B). Dead cells expressing the dead cell marker (APC-Cy7+) were excluded/gated out (Figure 19C, Figure 20C). Neutrophils were identified as expressing both CD15 and CD16 (Figure 19D, Figure 20D). Neutrophil activation was depicted in Figure 20E-H. Figure 20E and Figure 20G show unstimulated whole blood neutrophils (media-stimulated only), whereas Figure 20F and Figure 20H show activated neutrophils (fMLP-stimulated).

Neutrophils were stained for activation markers following stimulation with activated CD4+ T cell supernatants (Figure 21). Summary of experiments showed neutrophils incubated with isolated CD4+ T cell supernatants significantly decreased expression of CD62L (Figure 21A) and increased expression of CD11b (Figure 21B) compared to neutrophils incubated with supernatants derived from CD4+ T cells stimulated with media only (media-only stim.). Neutrophils incubated with supernatants derived from anti-CD3/28 and Immunocult-stimulated CD4+ T cells significantly downregulated expression of CD62L, compared to media stim. (78.77% and 78.18% CD62L-, compared to 29.48% CD62L- ($p=0.0035$ and $p=0.0039$, respectively) (Figure 21A). Similarly, neutrophils incubated with supernatants derived from anti-CD3/28 and Immunocult-stimulated CD4+ T cells significantly up-regulated expression of

CD11b, compared to baseline (77.28% and 83.57%, compared to 10.77%) ($p<0.0001$ and $p<0.0001$, respectively) (Figure 21B).

Neutrophils were also stained for activation markers following incubation with supernatants derived from activated Jurkat T cells (Figure 22). There was no statistically significant decrease in CD62L expression on neutrophils incubated with supernatants derived from anti-CD3/28 and Immunocult-stimulated Jurkat T cells, compared to media stim. (39.36% and 52.07%, compared to 23.31%) ($p=0.249$ and $p=0.0845$, respectively) (Figure 22A). However, neutrophils incubated with supernatants derived from anti-CD3/28 and Immunocult-stimulated Jurkat T cells did significantly up-regulate expression of CD11b, compared to baseline (45.23% and 51.99%, compared to 11.43%) ($p=0.0140$ and $p=0.0032$, respectively) (Figure 22B).

DISCUSSION

Aim 1: Identifying IL-8-producing (IL-8+) CD4+ T cells and factors which stimulate T cells to produce IL-8

1.1 Identifying the frequency of allergen-specific CD4+ T cells in cat allergic subjects

The downregulation of IL-8 mRNA transcripts and reduction in IL-8 protein in allergen-specific T cells from allergic subjects following treatment with PIT suggested that IL-8 may play an important role in the pathogenesis of allergic disease – it was a good starting point to further investigate the role IL-8 had in IgE-mediated allergic diseases. Based on these results and the literature available, we sought to ask whether T cell-derived IL-8 can contribute to the pathogenesis of allergies by activating basophils and neutrophils to release histamine.

We previously reviewed the role and capacity IL-8 had in activating neutrophils and basophils, however, IL-8 has also been shown to play a role in IgE-mediated diseases. Th2-

helper allergen-specific T cells, one of the main cell types orchestrating allergic inflammation, were shown to produce appreciable amounts of IL-8 following stimulation with monoclonal antibodies to CD3, CD28 and CD2.[143] More direct evidence for the role of IL-8 in IgE-mediated allergic inflammation comes from a study demonstrating that following anti-IgE stimulation of human lung samples, IL-8 was produced in sufficient quantities and induced chemotaxis of neutrophils and eosinophils.[144]

A previous master's student had done a lot of work characterizing IL-8-producing CD4+ T cells, which may represent a distinct lineage of T cells that mediated allergic reactions (referred to as 'Th8' cells). They had performed an intermediary magnetic enrichment step and staining with additional tetramers, which had increased the frequency of allergen-specific CD4+ T cells (TET+) (Figure 1C, D) compared to non-enriched cells (Figure 1A, B). Our aim was to repeat the tetramer staining protocol with cat-allergic subjects to compare the frequency of allergen-specific CD4+ T cells present in peripheral blood, without magnetic enrichment and without the subsequent addition of tetramers. Two volunteers were recruited for this purpose (Figure 2G, H and Figure 3B show the frequency of allergen-specific CD4+ T cells, or TET+ CD4+ T cells). The percent allergen-specific CD4+ T cells acquired from cat-allergic subjects in our preliminary findings were 0.1094% and 0.146%. Fel d 1-specific CD4+ T cells have been previously identified and quantified in PBMCs in cat-allergic and non-cat allergic subjects. The frequency of Fel d 1-specific CD4+ T cells in cat allergic subjects has been reported in the literature and is in the range of 1:7000 to 1:300000 (0.014% to 0.00033%) while the frequency of Fel d 1-specific CD4+ T cells in healthy controls was barely detectable.[145]

1.2 Identifying the frequency of IL-8+ allergen-specific CD4+ T cells in cat allergic subjects

Our intention of using IL-8-producing allergen-specific CD4+ T cells was further complicated by the fact that we did not observe any detectable IL-8+ TET+ cells (Figure 3C, D). IL-8 alone was not strongly expressed in CD4+ T cells following stimulation with anti-CD3/28 (Figure 3D, and Figure 5C, D). Considering these preliminary findings, i.e., low frequency of Fel d 1-specific CD4+ T cells and low frequency of IL-8-expressing CD4+ T cells, we opted to modify our original research question. Rather than looking specifically at IL-8-derived allergen-specific CD4+ T cells, we decided to utilize polyclonal CD4+ T cells. In addition to using peripheral blood CD4+ T cells, we used an immortalized line of human T lymphocyte cells (clone E6.1). Jurkat T cells are widely used as a model to study T cell signalling in CD4+ T cells – they highly express the TCR, the CD3 co-receptor, low levels of CD4, but no CD8 or MHC class II molecules.[146] Therefore, our amended research question was: can IL-8 derived from polyclonal CD4+ T cells activate basophils and neutrophils in an IgE-independent manner. Later, we can establish the relative role IL-8 had in activating neutrophils and basophils by using nAbIL-8 in the supernatant cultures and see if there was a change in activation. Polyclonal CD4+ subsets were present in peripheral blood leukocytes at the following frequencies: Th1 (7.3±2.9%), Th2 (5.4±2.5%), Th17 (10.5±4.4%), Treg (0.7±0.4%). It has been shown that adult blood contains few naïve IL-8+ CD4+ T cells (5%, although this was only after stimulation with PMA/I).[102]

1.3 Jurkat T cells and isolated CD4+ T cells produce IL-8 in response to anti-CD3/CD28 and anti-CD3/CD28/CD2 (Immunocult) stimulation

Jurkat T cell stimulation was optimized, and IL-8 was quantified using an ELISA (Figure 4). Log10-fold concentrations of Jurkat T cells were stimulated for 24-hours with T cell specific

markers (anti-CD3/28) at cell densities ranging from 10^5 to 10^7 cells/ml (Figure 4A). It was determined that the optimal density to stimulate Jurkat T cells was $1x10^6$ cells/ml. A limitation in this experimental design was that using log-10 for increasing doses resulted in densities in-between being omitted. Later experiments demonstrated that stimulating Jurkat T cells at a density of $2.5x10^6$ cells/ml was more optimal for IL-8 production from Jurkat T cells, compared to $1x10^6$ cells/ml (for anti-CD3/28-stimulated Jurkat T cells: 464.32pg/ml vs. 238.51pg/ml, respectively) (Figure 6B).

A time course experiment was performed to determine the kinetics of IL-8-production from Jurkat T cells (Figure 4B). Beyond 24-hours of stimulation, there was no appreciable increase in IL-8-for either PMA/I or anti-CD3/28-stimulated Jurkat T cells. Anti-CD3 and anti-CD28 doses were titrated (Figure 4C). Anti-CD3 doses had a larger difference than anti-CD28 on IL-8 production of Jurkat T cells, where Jurkat T cells responded best (produced the most IL-8) at the highest anti-CD3 dose used (10ug/ml). Anti-CD28 doses ranging from 0-5ug/ml had a similar effect on Jurkat T cell stimulation, while the highest dose (10ug/ml) was sub-optimal. Therefore, a dose of 2ug/ml was chosen, consistent with the dose determined previously by a student in our lab.

We discussed the addition of a monoclonal antibody to the membrane receptor CD2 which has been shown to increase IL-8 production in T cells.[143] CD2 is a co-stimulatory molecule expressed on T cells, NK cells, DCs, and thymocytes.[147] CD2 ligation to its cognate receptor leukocyte function-associated antigen-3 (LFA-3) expressed on APCs augments T cell-APC interaction by facilitating the formation of the immune synapse and stabilizing cell-cell contact between T cells and APCs.[147] Injection of monoclonal antibodies to CD2 (anti-CD2) resulted in an in-vivo inhibition of T cell antigen-specific responses.[148]

Anti-CD2, in addition to its role in mediating cell-adhesion between T cells and APCs, acts as a co-stimulatory signal for T cells.[149] CD2, in conjunction with CD3 stimulation, enhanced CD3-mediated tyrosine phosphorylation of Syk. Furthermore, CD2 co-stimulation enhanced phosphorylation of adaptor proteins Shc and Cbl.[149] CD3 signalling requires phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by Lyn and Syk, therefore stimulation of CD2 augments CD3 stimulation by helping recruit these kinases and aiding their interaction with TCR-CD3ζ (zeta) complex.[149] This in turn recruits ZAP-70 and other protein kinases which recruit adaptor proteins (such as Shc and Cbl). Co-stimulation of T cells by anti-CD2 (with and without anti-CD3/28) demonstrated that anti-CD2 alone was more effective at inducing IL-8 production in both bulk CD4+ T cells and allergen-specific T cell clones.[143] When anti-CD2 was used in conjunction with anti-CD28, there was even more IL-8 produced, which, again, was significantly higher than that achieved by anti-CD3+anti-CD28-stimulation.[143]

Similar to the anti-CD3 and anti-CD28 titrations, we titrated anti-CD2 (0.1-10ug/ml) with and without anti-CD3 and anti-CD28. Anti-CD2 was more effective in stimulating Jurkat IL-8 production as a soluble antibody, compared to when it was immobilized to the surface of the wells (Figure 5A). It was interesting to find that, unlike in the Spinozzi et al. (1996) paper, anti-CD3 was necessary for T cell production of IL-8 (Figure 5A, absence of black bars). We repeated the titration with doses of anti-CD2 between 1 and 10ug/ml (Figure 5B). We found that varying the doses of anti-CD2 between 1 and 10ug/ml did not significantly alter Jurkat IL-8 production (Figure 5B).

Therefore, anti-CD3 (10ug/ml), anti-CD28 (2ug/ml), and anti-CD2 (1ug/ml) were deemed the optimal concentrations of antibodies for T cell stimulation. We included Immunocult

Human CD3/28/2 T cell activator produced from StemCell Technologies, to compare it to our titrated anti-CD3/28/2 doses. At the recommended dose by StemCell, Immunocult-stimulated Jurkat T cells produced more than double the amount of IL-8 compared to our reagents and titrated anti-CD3/28/2 (Figure 5B). Therefore, we replaced our in-house titrated anti-CD3/28/2 antibody mixture with the Immunocult product for downstream Jurkat and CD4+ T cell stimulation experiments. This product contained soluble antibody complexes that are specific to CD3, CD28, and CD2, and result in cross-linking of these cell-surface molecules – however it was not known at what concentrations these antibodies were present in the product.

In addition to measuring IL-8 with ELISAs, we utilized flow cytometry for titrating anti-CD3/28/2 doses and measuring the percent of CD4+ T cells that expressed IL-8. Previously, anti-CD3/28/2 were titrated and used at 5ug/ml each for flow cytometry applications (Figure 5C). We wanted to compare this dose to our IL-8 ELISA-optimized dose (anti-CD3/28/2 = 10ug/ml, 2ug/ml, 1ug/ml, respectively) (Figure 5C, D). There was a higher percentage of IL-8+ CD4+ T cells in the previously titrated doses compared to the newly titrated doses (2.05% vs. 0.46%) in the anti-CD3/28/2 conditions. However, in the anti-CD28/2-only conditions, there was a higher percentage of IL-8+ CD4+ T cells (1.61%), compared to 0.62% IL-8+ CD4+ T cells in the previously titrated doses. 5.56% of CD4+ T cells expressed IL-8 in the Immunocult-stimulated condition, corroborating our ELISA findings that the Immunocult reagent was more effective in stimulating T cells to produce IL-8, compared to our in-house titrated anti-CD3/28/2 mixture.

The entire repertoire of Jurkat T cell stimulation experiments and IL-8 ELISAs were summarized in Figure 6. The first 5 batches of Jurkat T cells prepared were stimulated in 96-well flat-bottomed plates, immobilized with 70ul of 10ug/ml anti-CD3 antibody (Figure 6A). However, larger batches were prepared for use in later experiments to generate more

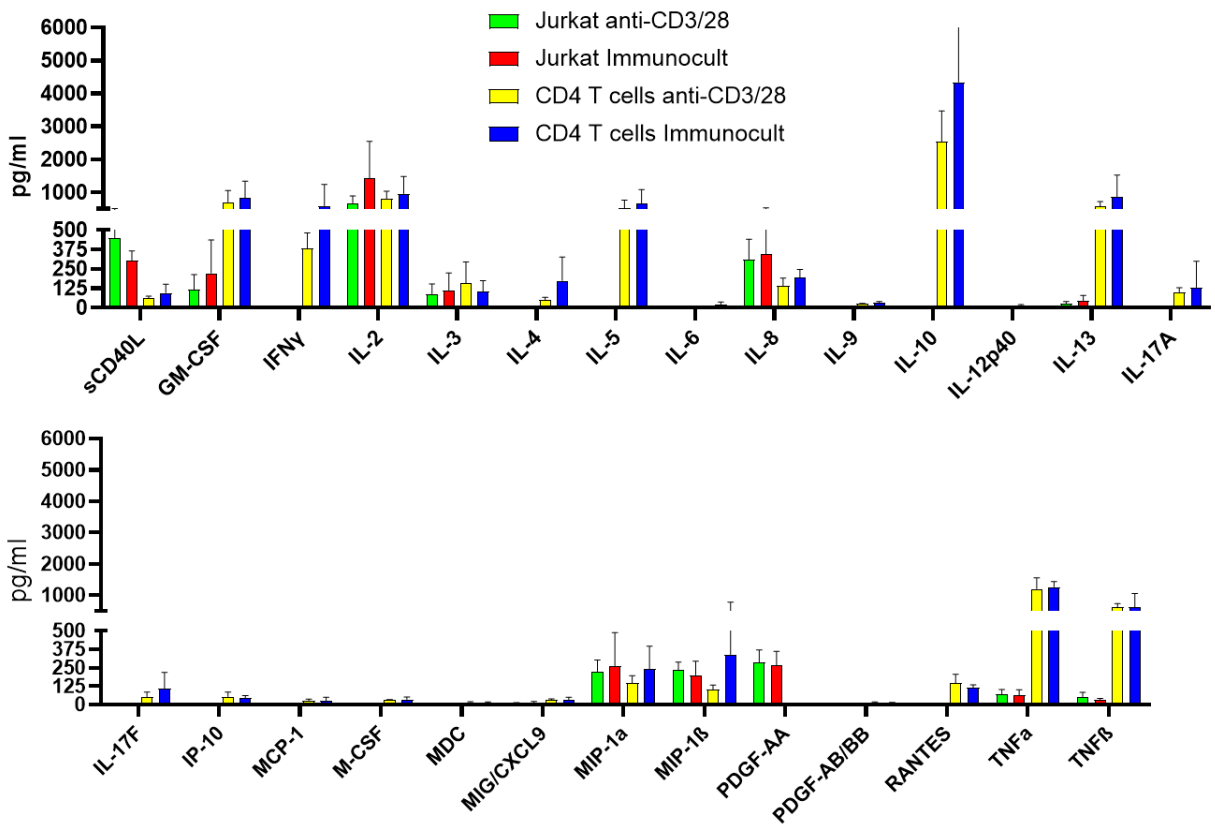
supernatants (batches 6-10). These were prepared in 6-well flat-bottomed plates, precoated with 1ml of 10ug/ml anti-CD3 antibody (Figure 6B). Jurkat T cell stimulation was more effective in 6-well plates, compared to 96-well plates. There were two possible explanations to this: 1) 96-well plates have a growth area of $0.32cm^2$ while 6-well plates have a growth area of $9.5cm^2$. Because anti-CD3 is immobilized to the plate, there is substantially more surface area in 6-well plates for the T cells to encounter the immobilized anti-CD3 and receive signal 1 required for activation (potentially circumventing anergy). The increased area can accommodate Jurkat T cell expansion in the culture as well – an important consideration because stimulation times were 24-hours (Jurkat T cells double once every 24-hours). Option 2) Although the concentration of anti-CD3 was identical between both stimulation conditions, the absolute amount of anti-CD3 used was different: 10ug of anti-CD3 was added to the 6-well plates, whereas 0.7ug of anti-CD3 was added to the 96-well plates. Therefore, there was a lot more anti-CD3 immobilized to the bottom of the wells in the 6-well plates compared to the 96-well plates. However, the microgram amount of anti-CD3 antibody per unit of surface area (ug/cm^2) was less in the 6-well plate than the 96-well plate ($1.05ug/cm^2$ vs. $2.19ug/cm^2$, respectively). A limitation in how set-up the stimulation conditions was that we did not account for and normalize the amount of immobilized anti-CD3 between different sized wells (using ug/cm^2). Based on the available literature and our results, we are unable to definitively explain why the Jurkat T cells stimulated in larger, 6-well plates, produced more IL-8 compared to Jurkat T cells stimulated in 96-well plates.

CD4+ T cells were not optimized in the same way as Jurkat T cells, with varying doses of anti-CD3/28/2 and stimulation conditions. We used the Jurkat T cell-optimized conditions and antibody concentrations and applied them to CD4+ T cell stimulation conditions. Our lab had previously isolated CD4+ T cells and stimulated them at a concentration of $2.5x10^6$/ml, and so

we opted to use this concentration and measure the concentration of IL-8 in the supernatants. The entire repertoire of CD4+ T cell stimulation experiments were shown in Figure 6C (n=10). It was apparent that, like in stimulated Jurkat T cells, isolated CD4+ T cells released more IL-8 when stimulated in larger wells (6- or 12-well flat-bottomed plates), even at the same concentration of cells. There were two reasons that a higher concentration of CD4+ T cells were used for stimulation experiments, relative to Jurkat T cells: 1) unlike Jurkat T cells, isolated CD4+ T cells do not rapidly divide in culture in the absence of growth factors (IL-2 being the most important[150]), and 2) there was significantly more variability in the amount of IL-8 produced following CD4+ T cell isolation and stimulation with anti-CD3/28 and/or anti-CD2. This was due several factors, including but not limited to the proportion of T helper subsets that make-up the polyclonal CD4+ T cells from the donor (i.e. the relative proportion of T helper subsets Th1, Th2, and Th17, which may be related to their health status[151]), and the purity of the CD4+ T cells isolated from PBMCs. To mitigate the influence that isolation purity may have on the generation T cell-derived IL-8, each sample was stained for common cell markers (CD14, CD19, CD3, CD4, and viability dye) and analyzed on the flow cytometer to assess purity of the isolated fraction. A representative plot was shown in Appendix S8**.** We consistently achieved >95% purity of CD4+ T cells, which we used as an unofficial cut-off. Isolations which did not yield >95% purity of CD4+ T cells were discarded. Nonetheless, it was not possible to achieve 100% purity, and the possibility of contaminating monocytes, NK cells, and other cells that contribute to background IL-8 would persist. For example, Figure 6C shows one experiment where anti-CD3/28-stimulated CD4+ T cells produced 394.3pg/ml IL-8, and another experiment where 20.06pg/ml IL-8 was produced, despite both samples being >95% CD4+ T cells.

1.4 Human cytokine 48-plex assay was performed to measure mediators in Jurkat and isolated CD4+ T cell supernatants

A human cytokine 48-plex assay was performed to determine possible soluble mediators contained in T cell supernatants which contributed to basophil and neutrophil activation (see



Appendix S9). The soluble mediators up-regulated in both Jurkat and isolated CD4+ T cells included: sCD40L, GM-CSF, IL-2, IL-3, IL-8, IL-13, MIP-1α, MIP-1β, TNF-α, and TNF-β. Cytokines which were specifically up-regulated in isolated CD4+ T cells included: IFN-γ, IL-4, IL-5, IL-10, IL-17A, IL-17F, IP-10 (CXCL10), MCP-1, M-CSF, and RANTES (CCL5). There were no cytokines released that were specific to Jurkat T cells.

We have previously discussed IL-3 as being the most potent activator of basophils, in addition to IL-5 and GM-CSF contributing to basophil priming and enhancing histamine release when subsequently stimulated with anti-IgE. Earlier studies demonstrated preincubation of

basophils with IL-2 did not enhance histamine release,[152] however, a study done more recently (2020) demonstrated that IL-2-stimulated basophils up-regulated numerous inflammatory cytokines such as IL-5, IL-13, GM-CSF, and CCL17.[153] IL-2 was one of several cytokines that was up-regulated in both Jurkat and CD4+ T cells following stimulation with anti-CD3/28 and Immunocult. Macrophage inflammatory protein-1 alpha (MIP-1α) and MIP-1β were also secreted by T cells (typically secreted by macrophages as well) and have been shown to activate both basophils and neutrophils. MIP-1α caused a dose-dependent release of histamine from basophils in 14 out of 20 allergic subjects (mean 13.5±2.9% of total release)[154]. Both MIP-1α and MIP-1β were shown to induce neutrophilic infiltration and contribute to overall inflammation in human diseases by stimulating the release of pro-inflammatory cytokines such as IL-1α and IL-6 from macrophages.[155] In the presence of TNF-α, the integrin CD11b (expressed on neutrophils) was up-regulated and consequently, neutrophils were chemoattracted towards MIP-1α.[156] TNF-α and TNF-β have also been shown to stimulate basophils, although by themselves, these cytokines only induced basophils to release approximately 10% of their total histamine content.[157] Even in combination with other cytokines (IL-1, IL-3, IL-8, GM-CSF) – basophils only released approximately 20% of their total histamine. However, pre-treatment of basophils with a combination of cytokines (IL-3, IL-6, IL-7, IL-8, TNF-α, and GM-CSF) with subsequent anti-IgE stimulation resulted in a marked increase in histamine release.[157] Based on our multiplex analysis, anti-CD3/28 and Immunocult-stimulated Jurkat and CD4+ T cells released IL-3, IL-8, TNF-α, and GM-CSF (which were present in both our supernatants and the cytokines shown in the literature to stimulate basophils). This finding was corroborated by our finding that soluble mediators in T cells alone do not strongly induce basophils to release

histamine, but rather interact together to prime basophils to enhance histamine release in response to anti-IgE stimulation.

Aim 2: Demonstrate that supernatants from stimulated T cells can induce histamine release from basophils, and determine if this effect is mediated through IL-3 and/or IL-8

2.1-2.2 IL-3 and/or IL-8 did not stimulate basophils to release histamine. T cell supernatants did not stimulate basophils to release histamine

Our results from ***results 2.1*** demonstrated that basophils did not significantly increase histamine release when incubated with IL-3 and/or IL-8 (Figure 8).

Our results from ***results 2.2*** demonstrated that when basophils were incubated with supernatants derived from activated T cells, they did not release histamine (Figure 9).

2.3 Jurkat T cells, but not CD4+ T cells, produced histamine when activated by anti-CD3/28 and Immunocult

Our finding that Jurkat T cells produced histamine in response to anti-CD3/28 and Immunocult (anti-CD3/28/2) stimulation was unexpected (see Appendix S3). While it had been previously reported that Jurkat T cells produced histamine in response to PMA stimulation,[80] PMA bypasses the T cell membrane receptor complex to directly activate protein kinase C (PKC), whereas anti-CD3/28 stimulation interacts with cell surface proteins on T cells to initiate intracellular signalling cascades.[158] Interestingly, we failed to see histamine release in our isolated CD4+ T cells stimulated with anti-CD3/28 or Immunocult (Appendix S7 B) despite previous literature demonstrating mouse CD4+ T cells produced histamine in response to ConA, LPS, Compound 48/80, A23187, C5a, Substance P, IL-1α, IL-3, and GM-CSF.[79,159,160] However, two out of three of the studies which looked at this (Kubo & Nakano, and Nakashima et al.) cultured CD4+ T cells at $\underline{6x10^6\ cells/ml}$ and $\underline{2x10^7\ cells/ml}$ (respectively) – high cell

concentrations relative to the concentrations used in our experiments. Kubo & Nakano, who stimulated CD4+ T cells with IL-1α, IL-3, and GM-CSF, observed less than 0.5nM per 10^7 cells. Nakashima et al. similarly observed relatively low amounts of histamine – ConA stimulated T lymphocytes released approximately 2nM. At the highest concentration of human CD4+ T cells used in our stimulation experiments ($5x10^6$ cells/ml), even after subtracting relatively high baseline histamine release (3.21nM), there was 1.68nM of histamine present in the supernatant following anti-CD3/28 stimulation (data not displayed, however, the data is derived from the experiment summarized in Appendix S4). Stimulated CD4+ T cells obtained from another subject (density $2.5x10^6$ cells/ml) did not release any histamine (<0.2nM, even without subtracting spontaneous release) (Appendix S4), demonstrating that histamine release may be donor dependent. Therefore, although we have concluded that CD4+ T cells did not release histamine based on statistical analysis of aggregate data (Figure 7B), we observed some histamine present in isolated CD4+ T cell supernatants following activation of cell surface CD3, CD28, and CD2 molecules. This was consistent with the previous literature showing CD4+ T cells are capable of producing histamine de novo, possibly through activating the enzyme HDC (this was previously established, but not demonstrated in our experiments).[79,159] It remains to be determined why Jurkat T cells synthesized and released histamine to a much greater extent than freshly isolated CD4+ T cells.

A possible explanation is that synthesizing histamine is a by-product of tumor development and progression, rather than a consequence of CD4+ T cell phenotype. Most malignant cell lines have been shown to express HDC and contain high concentrations of histamine.[161] Jurkat T cells are an immortalized T cell leukemic cell line, and therefore its

expression of HDC and release of histamine following stimulation may reflect the fact that HDC is commonly expressed in cancer cell lines as well as melanoma, colon, and breast cancers.[161]

2.4 Basophils primed with IL-3 and/or IL-8 did not significantly enhance basophil responsiveness to anti-IgE stimulation by stimulating histamine release. However, IL-3-primed basophils interacted in a synergistic manner with anti-IgE to enhance basophil histamine release. IL-8 priming alone did not interact synergistically with anti-IgE, although in the presence of IL-3, produced a stronger synergistic response with anti-IgE compared to IL-3 on its own

We previously discussed the phenomena whereby basophils can be primed by certain cytokines and stimuli for enhanced responsiveness to various stimuli – we limited the stimuli to anti-IgE because of its relevance to IgE-mediated allergies. IL-3 is by far the most potent cytokine in its ability to activate mature basophils, as well as potentiate basophil degranulation (histamine release) by anti-IgE.[56] In our experiments, we did not see a significant increase in basophil histamine release following stimulation with IL-3 or IL-8 (Figure 8).

Although the histamine release did not reach statistical significance, there did appear to be a noticeable increase in histamine released from basophils stimulated with anti-IgE following priming with IL-3, and IL-3+IL-8. Anti-IgE was used as a positive control, and stimulated basophils to release 4.33±3.99nM histamine, although IL-3+IL-8-alone stimulated basophils to release 4.73±3.80nM histamine (similar histamine release as with the positive control). We looked at whether there was an additive and/or synergistic effect between the priming stimuli and the subsequent anti-IgE stimulation. For example, if the effect was additive, basophils primed with IL-3+IL-8 and subsequently stimulated with anti-IgE would be expected to release 9.06nM histamine. However, basophils released 14.05nM±4.75nM histamine – more than three times the

positive control alone, and a 55.1% increase in histamine released compared to the expected released if the individual histamine release values (IL-3+IL-8-priming) and (anti-IgE stimulation) were additive.

The Bliss Independence Model was used to mathematically evaluate synergy between anti-IgE and priming with IL-3, IL-8, or IL-3+IL-8. The Bliss Independence Model is expressed as:

$$Y_{ab,P} = Y_a + Y_b - Y_a Y_b \text{ (162)}$$

Where $Y_{ab,P}$ was the predicted total response if our two agents interacted independently

a was the concentration of anti-IgE alone required to produce response Y_a

b was the presence of priming agent (IL-3, IL-8, or IL-3+IL-8) required to produce response Y_b

We define $Y_{ab,O}$ as the observed combined effect of anti-IgE and priming agent

$$Y_{ab,O} \begin{cases} > Y_{ab,P} & synergistic \\ = Y_{ab,P} & additive \\ < Y_{ab,P} & antagonistic \end{cases} \text{ (162)}$$

Anti-IgE (alone)-stimulated basophils produced 0.249 of the total histamine response (responses were graded between 0 and 1, with 0 representing the minimal response and 1 representing the maximal response). The maximal response was referenced to the conditions which produced the most histamine (in this series of experiments, IL-3+IL-8-primed basophils stimulated with anti-IgE produced the most histamine, releasing 17.41nM). All values were divided by the maximal response value to attain fractional response values. Replicates were averaged for each condition.

IL-3 (+/-) IL-8-primed basophils stimulated with or without anti-IgE

	Anti-IgE (1ug/ml) alone	IL-3 (20ng/ml)	IL-3+anti-IgE	IL-8 (100nM)	IL-8+anti-IgE	IL-3+IL-8	IL-3+IL-8+anti-IgE
Fractional Response (Observed) $Y_{ab,O}$	0.249	0.205	0.488	0.109	0.257	0.272	0.807
Fractional Response (Predicted) $Y_{ab,P}$			0.403		0.331		0.453
Relationship			$Y_{ab,O} > Y_{ab,P}$ Synergistic		$Y_{ab,O} < Y_{ab,P}$ Antagonistic		$Y_{ab,O} > Y_{ab,P}$ Synergistic

Our results showed that IL-3 acted synergistically with anti-IgE, resulting in supra-additive basophil histamine release. Synergy is the interaction between two agents (IL-3, or IL-3+IL-8 and anti-IgE) that work in combination to produce an effect that is greater than what would be expected if the effect of said agents were additive.[163] IL-8 alone did not act synergistically with anti-IgE to enhance basophil histamine release (it was labeled as antagonistic in the table because of convention by the model). Interestingly, there was a stronger synergy present when IL-8 was included as a priming agent, in conjunction with IL-3 (IL-3+IL-8). The observed response between IL-3+IL-8 and anti-IgE was nearly double the observed response between IL-3 and anti-IgE, despite a similar expected response.

2.5 Basophils primed with supernatants derived from bulk activated CD4+ T cells did not statistically significantly increase basophil histamine release to subsequent anti-IgE stimulation, compared to non-primed basophils (anti-IgE only). However, activated CD4+ T

cell supernatants interacted in a synergistic manner with anti-IgE, resulting in supra-additive histamine release compared to what would be expected if the effects were additive

Looking at our data from the human cytokine 48-plex assay ***(***see ***discussion 1.4)***, T cells produce many more soluble mediators besides IL-3 and IL-8, some of which may contribute to activating and/or priming basophils for enhanced histamine release by anti-IgE. IL-3, despite being a strong activator of basophils (as purported in the literature), did not statistically increase basophil responsiveness to anti-IgE (measured via histamine release) in our hands. Therefore, we explored whether basophils required additional cytokines and mediators present in the supernatants in activated CD4+ T cells to significantly prime basophils for enhanced histamine release. As reported in ***results 2.5***, we found that basophils primed with supernatants derived from activated T cells and subsequently stimulated with anti-IgE did not significantly increase the amount of histamine released from basophils compared to media SN+anti-IgE (Figure 9).

However, there seemed to be a synergistic effect anti-CD3/28 and Immunocult-stimulated CD4+ T cell supernatants had in potentiating basophil response to anti-IgE. Anti-IgE (positive control) stimulation alone caused basophils to release 3.29±4.17nM histamine, however, anti-CD3/28 and Immunocult-stimulated CD4+ T cell supernatants alone caused basophils to release 2.18±1.54nM and 2.13±1.98nM histamine, respectively. If priming and stimulation of basophils resulted in an additive effect in activation, basophils would be expected to release 5.45nM histamine (the average was taken between anti-CD3/28 and Immunocult SN stimulated basophil histamine release because values were very similar, 2.155nM histamine). However, anti-CD3/28 and Immunocult SN-primed basophils stimulated with anti-IgE released 8.65±7.67 and 9.46±6.55nM histamine, respectively. These values represent a 58.7% and 73.6% increase from the expected histamine release values if the priming effect were additive.

As with ***discussion 2.4***, we utilized the Bliss Independent Model to mathematically evaluate synergy between anti-IgE and CD4+ T cell supernatant priming. The Bliss Independent Model is expressed as:

$$Y_{ab,P} = Y_a + Y_b - Y_a Y_b \ ^{(162)}$$

Where $Y_{ab,P}$ was the predicted total response if our two agents interacted independently

a was the concentration of anti-IgE plus background (media SN+anti-IgE condition) required to produce response Y_a

b was the concentration (or density) of anti-CD3/28 or Immuncoult-stimulated CD4+ T cell supernatants alone (SN) required to produce response Y_b

We defined $Y_{ab,O}$ as the observed combined effect of anti-IgE and CD4+ T cell SN

$$Y_{ab,O} \begin{cases} > Y_{ab,P} & synergistic \\ = Y_{ab,P} & additive \\ < Y_{ab,P} & antagonistic \end{cases} \ ^{(162)}$$

Anti-IgE-stimulated basophils produced 0.117, 0.269, and 0.288 of the total histamine response (responses were graded between 0 and 1, with 0 representing the minimal response and 1 representing the maximal response) when primed with media-only (0.5, 1.0 and 2.5x10^6 cells/ml, respectively). The maximal response is referenced to the conditions which produced the most histamine (in this series of experiments, Immunocult SN-primed basophils stimulated with anti-IgE at 2.5x10^6 cells/ml produced the most histamine, releasing 20.27nM histamine. All values were divided by the maximal response value to attain fractional response values. Replicates were averaged for each concentration of SN (0.5 to 2.5x10^6 cells/ml). Anti-IgE was used at a constant concentration of 1ug/ml.

Basophils primed with supernatants derived from anti-CD3/28-stimulated T cells with and without subsequent anti-IgE stimulation

	Media SN ($0.5x10^6$ cells/ml + Anti-IgE (1ug/ml) alone	Anti-CD3/28 SN ($0.5x10^6$ cells/ml)	Anti-CD3/28 SN ($0.5x10^6$ cells/ml) + anti-IgE	Media SN ($1x10^6$ cells/ml + Anti-IgE (1ug/ml) alone	Anti-CD3/28 SN ($1x10^6$ cells/ml)	Anti-CD3/28 SN ($1x10^6$ cells/ml) + anti-IgE	Media SN ($2.5x10^6$ cells/ml + Anti-IgE (1ug/ml) alone	Anti-CD3/28 SN ($2.5x10^6$ cells/ml)	Anti-CD3/28 SN ($2.5x10^6$ cells/ml) + anti-IgE
Fractional Response (Observed) $Y_{ab,O}$	0.117	0.0463	0.0835	0.269	0.130	0.636	0.288	0.156	0.523
Fractional Response (Predicted) $Y_{ab,P}$			0.158			0.364			0.399
Relationship			$Y_{ab,O} < Y_{ab,P}$ Additive or Antagonistic			$Y_{ab,O} > Y_{ab,P}$ Synergistic			$Y_{ab,O} > Y_{ab,P}$ Synergistic

Basophils primed with supernatants derived from Immunocult-stimulated T cells with and without subsequent anti-IgE stimulation

	Media SN ($0.5x10^6$ cells/ml + Anti-IgE (1ug/ml) alone	Immunocult SN ($0.5x10^6$ cells/ml)	Immunocult SN ($0.5x10^6$ cells/ml) + anti-IgE	Media SN ($1x10^6$ cells/ml + Anti-IgE (1ug/ml) alone	Immunocult SN ($1x10^6$ cells/ml)	Immunocult SN ($1x10^6$ cells/ml) + anti-IgE	Media SN ($2.5x10^6$ cells/ml + Anti-IgE (1ug/ml) alone	Immunocult SN ($2.5x10^6$ cells/ml)	Immunocult SN ($2.5x10^6$ cells/ml) + anti-IgE
Fractional Response (Observed) $Y_{ab,O}$	0.117	0.0242	0.190	0.269	0.116	0.307	0.288	0.205	0.754
Fractional Response (Predicted) $Y_{ab,P}$			0.138			0.354			0.434

Relationship		$Y_{ab,O} \approx Y_{ab,P}$ Additive or Synergistic		$Y_{ab,O} \approx Y_{ab,P}$ Additive or Antagonistic		$Y_{ab,O} > Y_{ab,P}$ Synergistic

Supernatants derived from CD4+ T cells stimulated at the highest concentrations (1.0 and 2.5x10^6 cells/ml for anti-CD3/28-stimulated T cells and 2.5x10^6 cells/ml for Immunocult-stimulated T cells) acted synergistically with anti-IgE to enhance basophil histamine release (light yellow shaded regions). Synergy is the interaction between two agents (T cell SN and anti-IgE) that work in combination to produce an effect that is greater than the expected additive effect of said agents.[163] At lower concentrations of SN, the effect between anti-IgE and SN was not clearly synergistic (nor do we suspect it was antagonistic, even though $Y_{ab,O} < Y_{ab,P}$). Where the difference in fractional response between $Y_{ab,O}$ and $Y_{ab,P}$ was less than 0.1, we did not conclude either a synergistic or antagonistic relationship. At higher concentrations of SN, the synergistic effect between anti-IgE and SN was more clear (for example, at 1x10^6 cells/ml anti-CD3/28-primed SN plus anti-IgE, the observed combined effect $Y_{ab,O}$ was nearly twice (1.75x) that which would be expected if the SN and anti-IgE acted in an additive manner ($Y_{ab,P}$) ($Y_{ab,O}$ was 1.75x that of $Y_{ab,P}$).

Fractional responses by supernatants derived from both anti-CD3/28 and Immunocult-stimulated CD4+ T cells increased as concentration increased 0.0463, 0.130, and 0.156 for anti-CD3/28 SN at 0.5, 1.0, and 2.5x10^6 cells/ml (respectively) and 0.0242, 0.116, and 0.205 for Immunocult SN at 0.5, 1.0, and 2.5x10^6 cells/ml (respectively), indicating the concentration of the soluble mediators in the supernatants had a dose-dependent effect on basophil histamine release (albeit this relationship was modest and was not statistically significant compared to baseline, see Figure 9). However, in the presence SN derived from activated CD4+ T cells, basophils demonstrated an enhanced responsiveness to anti-IgE (via histamine release),

compared to anti-IgE+media SN alone. We described the interaction between the priming stimuli (the SN) and anti-IgE as acting synergistically, where the combined effects of the SN and anti-IgE were greater than what would be expected if the two stimuli interacted independently (or additively). Our findings demonstrated that CD4+ T cells produced soluble mediators that could prime basophils for enhanced responsiveness to anti-IgE. We demonstrated that CD4+ T cell supernatants acted synergistically with anti-IgE to induce basophils to release supra-additive levels of histamine.

2.6 IL-3, but not IL-8, stimulated basophils to up-regulate activation markers CD63/CD203c

Our previous results demonstrated that IL-3 and/or IL-8 failed to significantly stimulate basophils to release histamine or significantly prime basophils for enhanced responsiveness to histamine release by anti-IgE stimulation (although IL-3 and IL-3+IL-8 acted synergistically with anti-IgE to induce basophils to release supra-additive levels of histamine). In this section, we have demonstrated that IL-3, but not IL-8, was capable of significantly upregulating basophil activation markers CD63/CD203c (Figure 13).

In addition to basophil histamine release, upregulation of CD63/CD203c surface markers was used as a surrogate for basophil activation. CD63 is a membrane protein belonging to the lysosome-associated membrane proteins family (LAMP) and was found to be involved in vesicle fusion events.[130] CD63 was used as an indirect measure of histamine granule fusion events and histamine release. Under resting conditions, CD63 is bound to intracellular granule membranes and plasma membrane.[164] Following stimulation of basophils by antigen, these granules rapidly (within minutes) move towards the cell surface and fuse with the plasma membrane.[164] Therefore, measuring cell-surface expression of CD63 is an indirect measure of basophil vesicle fusion events and histamine release. Activation of CD63 requires phosphorylation of p38

mitogen-activation protein kinases (MAPK). However, several studies have shown that surface expression of CD63 does not strictly reflect histamine release – a study evaluated the correlation between histamine release and CD63, and found the correlation between histamine release and percent CD63 expression in nonatopic subjects was 0.70, while the correlation in atopic subjects was 0.91 (Spearman correlation coefficients).[165] Therefore, we utilized an additional surface activation marker, CD203c. CD203c is an ecto-nucleotide pyrophosphatase/phosphodiesterase with an unknown function – however it is expressed to some degree on resting basophils (constitutively expressed) and rapidly up-regulated following activation by allergen-specific and anti-IgE-induced cross-linking.[130,166] More recent studies have shown that CD203c is also up-regulated in response to IL-3.[167]

Upon activation, basophils degranulate via two different processes: anaphylactic degranulation (AND), and piecemeal degranulation (PMD). AND involves the release of histamine, IL-4, and LTC_4, and is associated with CD63 expression on the basophil surface.[168] This is because AND involves granule fusion with each other and the plasma membrane, where histamine and other granule contents are expelled. Piecemeal degranulation, on the other hand, is associated with neither (or low levels of) histamine release nor CD63 expression on the surface – this is because PMD involves small vesicles forming from granules, which shuttle granule contents to the plasma membrane (recall CD63 is expressed on granules, which are not themselves trafficked to the plasma membrane).[168] However, both anaphylactic degranulation and piecemeal degranulation are reflected by upregulation of CD203c on the surface of basophils.[168] For this reason we utilized both CD63 and CD203c in our basophil activation tests.

In our experiments, IL-3, but not IL-8, significantly up-regulated CD63 and CD203c on basophils (Figure 13). Although IL-3 was not sufficiently potent to induce basophil histamine

release, IL-3 was able to significantly up-regulate basophil activation markers CD63/CD203c, relative to the positive control anti-IgE (84.4±10.00% CD63/CD203c+ in IL-3-stimulated conditions compared to 35.57±35.06% CD63/CD203c+ in anti-IgE-sitmulated conditions, respectfully) (p=0.0393). One striking difference between anti-IgE-stimulated basophils and IL-3-stimulated basophils was the inter-subject variability in activation levels to anti-IgE stimulation. It has been established in the literature that approximately 20% of subjects are designated as anti-IgE 'non-responders' and do not respond to anti-IgE stimulation with an influx of intracellular calcium and activation of PKC – this was reflected by a lack of histamine release following anti-IgE stimulation.[169] However, priming basophils from non-responders with IL-3 (prior to stimulating with anti-IgE) resulted in a drastic increase in histamine release (one study showed an increase in mean levels from 37% to 92%).[169] Although basophil expression of CD63/CD203c has not been characterized as 'responder' vs. 'non-responder' in the literature (like histamine-release has been), our data suggested that CD63/CD203c similarly showed a responder vs. non-responder outcome to anti-IgE cross-linking. There was a great deal of variability in CD63/CD203c responses to anti-IgE cross-linking (35.57±35.06%). One out of 4 subjects' basophils did not increase expression of CD63/CD203c following stimulation with anti-IgE (6.29% double positive) whereas another subject's basophils were 86.40% double positive. On the other hand, basophils from all the subjects responded strongly and consistently to IL-3 stimulation alone, as indicated by a high percent double positive and low standard deviation (84.45±10.00%+ CD63/CD203c).

IL-8, on the other hand, failed to induce histamine release or up-regulate CD63/CD203c in basophils, suggesting that IL-8 alone does not activate basophils. However, basophils primed with IL-8 showed an enhanced responsiveness to subsequent anti-IgE stimulation, compared to

anti-IgE alone (63.84±26.00% vs. 35.57±35.06% CD63/CD203c+), although this difference was not significant (p=0.461). Furthermore, while anti-IgE alone did not significantly up-regulate CD63/CD203c on basophils compared to baseline, priming basophils with IL-8 first (15-minutes) and subsequently stimulating with anti-IgE resulted in basophils significantly upregulating CD63/CD203c compared to baseline (63.84±26.00% vs. 8.42±0.73, p=0.0030) (Figure 13).

In another set of (preliminary) experiments, basophils were primed with IL-3 (5-minutes) and subsequently stimulated with increasing doses of IL-8 ($2x10^{-8}$ to $2x10^{-6}$ nM, or $5x10^{-8}$ to $5x10^{-6}$ ng/ml) for 15-minutes (Figure 11A) or 30-minutes (Figure 11B). IL-3-stimulated basophils did not significantly up-regulate CD63/CD203c compared to baseline, however, IL-8-stimulated basophils pre-treated with IL-3 significantly up-regulated CD63/CD203c compared to baseline at the two highest doses ($2x10^{-7}$ and $2x10^{-6}$nM) (p=0.0138 and p=0.0121, respectively) (Figure 11A). It's worth noting that the series of experiments shown in Figure 11A were preliminary and not powered to the same degree as subsequent IL-3+/-IL-8 experiments (n=3). IL-8 alone, at any concentration, did not significantly up-regulate CD63/CD203c on basophils.

Basophils express the receptors for IL-8 (CXCR1 and CXCR2) and, although only demonstrated in neutrophils[170] and endothelial cells,[94] binding of IL-8 to these receptors triggers phosphorylation of phosphatidylinositol 3 kinases (PI3Ks) and extracellular signal-regulated protein kinases (ERK1/2), and subsequent activation of NF-κB. This is similar to the mechanism of action of how IL-3 activates basophils, and therefore it is unclear why IL-3 alone, but not IL-8 alone, significantly up-regulated basophil activation markers CD63/CD203c. Still, it was clear IL-8 had some capacity to prime basophils for enhanced responsiveness to anti-IgE, as indicated by the significant upregulation of CD63/CD203c following stimulation with IL-8 in combination

with anti-IgE (compared to baseline). More work needs to be done to explore the downstream signalling pathway of IL-8 in basophils specifically.

2.7 T cell supernatants stimulated basophils to up-regulate activation markers CD63/CD203c

Our results demonstrated that basophils significantly up-regulated activation markers CD63/CD203c when stimulated with supernatants derived from activated T cells (Figure 12 and Figure 14). Two sets of basophil activation test data were collected to generate these two figures. Figure 12 represents an earlier set of experiments performed with (predominantly) isolated basophils stimulated with supernatants derived from activated Jurkat (Figure 12A) and isolated CD4+ (Figure 12B) T cells. Figure 14 represents a set of experiments performed later, where supernatants were used as a priming stimulus for basophils for subsequent stimulation with anti-IgE (see ***discussion 2.9***).

We demonstrated basophils strongly up-regulated CD63/CD203c in response to stimulation with activated T cell supernatants ($p<0.05$ for all comparisons, see ***results 2.7***). Therefore, we see that CD63 and CD203c expression on basophils is not concordant with histamine release in basophils stimulated with T cell supernatants. Basophils significantly up-regulated CD63/CD203c on their surface without a concomitant increase in histamine release (see ***results 2.2***). Previous literature reported that CD63 and CD203c may not strictly reflect histamine release,[130,141] which may depend on factors such as allergic status and the stimuli used. In a study where basophils from venom-allergic subjects were stimulated with venom in the presence of IL-3, the correlation between histamine release and CD63 expression was very significant.

According to the PMD and AND model of basophil histamine release, histamine release could occur in the absence of increased CD63 expression (via piecemeal degranulation), but

increased CD63 expression without histamine release could not occur, since CD63 is associated with histamine-containing granules.[171] Although this model had been previously challenged, it was shown that inhibition of histamine and CD63 by SB203580 (a p38 MAPK inhibitor) was concordant.[171] Interestingly, our results showed that SN derived from T cells significantly up-regulated CD63/CD203c on basophils, but did not significantly increase histamine release (Figure 14 vs. Figure 9A). To better understand the relationship between CD63/CD203c and histamine release, we looked at the correlation between fractional responses for CD63/CD203c and fractional responses for histamine release (Appendix S11). Interestingly, in basophils primed with supernatants and subsequently stimulated with anti-IgE, CD63/CD203c expression and histamine release were moderately and positively correlated (Spearman's r=0.5746, p=0.0017) (Appendix S11 A). However, in basophils stimulated with supernatants alone, there was no correlation between CD63/CD203c and histamine responses (Spearman's r=0.1249, p=0.561) Appendix S11 B). When we looked at CD63 and CD203c markers individually, and how they correlated to histamine release, we saw that there was a stronger correlation between CD203c and histamine release in basophils primed with SNs and stimulated with anti-IgE (r=0.614, p=0.0007) (Appendix S12 A), but not in basophils stimulated with SNs alone (r= -0.00490, p=0.981) (Appendix S12 B). In basophils primed with SNs and stimulated with anti-IgE, CD63 was only weakly (but positively) correlation with histamine release (r=0.386, p=0.0468) (Appendix S13 A), but in the absence of anti-IgE, was weakly but negatively correlated with histamine release, although this was not statistically significant (r=-0.336, p=0.0867) (Appendix S13 B), suggesting that the negative correlation had not been established.

Taken together, our results suggested stimulation of basophils by T-cell soluble mediators, and cross-linking of receptor-bound IgE by anti-IgE on basophils, represented distinct

mechanisms of basophil activation.[172] T-cell mediators significantly up-regulated CD63/CD203c on basophils (Figure 12), but on their own, failed to stimulate basophils to release histamine (Figure 14). For this reason, we saw a discordance between expression of CD63/CD203c and histamine release. This was significant because although CD63/CD203c expression is known to reflect histamine release, we saw that these markers can be useful in quantifying basophil activation which does not strictly result in histamine release. The methods we utilized did not investigate alternative variables in basophil activation – non-IgE-mediated basophil activation typically results in cytokine production from basophils (notably IL-4, IL-13, but also IL-6, TNF-α, CCL3, and CCL4) without concomitant degranulation and histamine release.[53] We were therefore unable to determine the consequence of CD63/CD203c upregulation in basophils following activation by T-cell-derived soluble mediators.

Consistent with the literature, we also observed that FcεRI receptor crosslinking by anti-IgE on basophils resulted in both an increase in histamine release and upregulation in CD63/CD203c, given that these two processes were shown to be moderately and positively correlated (even though the histamine increase was not statistically significant, Figure 9A). It has been found that CD203c upregulation is induced in response to more diverse set of stimuli – IL-3 and PGD_2, for example, induced CD203c but not CD63 expression on basophils.[173] This broader responsiveness to stimuli may be what occurs in basophils stimulated with T cell supernatants, where there was a significant increase in activation markers CD63/CD203c but not increase in histamine release. Recall that AND, but not PMD, results in the release of histamine and upregulation of CD63 on the surface of basophils. It was possible, therefore, that T cell-derived supernatants activated basophils specifically via the PMD mechanism, which was reflected by an increase in CD203c (but not necessarily detectable by measuring histamine release or CD63

expression).[130,168] However, when we looked at CD63 and CD203c separately (Appendix S14), we saw that, except for the basophils stimulated with supernatants derived from Immunocult stimulated T cell condition, there were no significant differences between CD63 and CD203c expression following supernatant stimulation of basophils (Appendix S14). Therefore, both CD63 and CD203c were significantly up-regulated on basophils following T cell supernatant stimulation, meaning that basophils were activated via PMD and AND mechanisms of degranulation.

2.8 Basophils primed with IL-3 or IL-3+IL-8 significantly enhanced basophil responsiveness to anti-IgE stimulation by upregulating CD63/CD203c activation markers. IL-3 alone, but not IL-8 alone significantly up-regulated CD63/CD203c activation markers compared to baseline

Our results corroborated with the established literature that IL-3 remained a potent stimulator of basophils. Following priming of basophils with IL-3 alone, basophils were strongly stimulated – the percent CD63/CD203c positive IL-3-primed+anti-IgE-stimulated basophils more than doubled compared to anti-IgE (alone) stimulated basophils (93.3% vs. 35.57% CD63/CD203c+, p=0.0139) (Figure 13).

In fact, IL-3-alone stimulated basophils were strongly activated – the percent CD63/CD203c positive cells more than doubled compared to anti-IgE alone-stimulated basophils (84.45% vs. 35.57% CD63/CD203c+) although this was not statistically significant (p=0.0557).

We had determined that IL-3-stimulated basophils did not significantly up-regulate histamine (Figure 8) but had a synergistic effect on basophil histamine release when IL-3 was used to prime basophils when they were subsequently stimulated with anti-IgE (see ***discussion 2.4***). There was a discordance once again between histamine release and the expression of CD63/CD203c – IL-3 did not cause a significant increase in histamine from basophils yet

strongly up-regulated both CD63 and CD203c markers (Figure 13). The effect that stimulating basophils with IL-3 has in the literature is debated, some studies demonstrated that CD203c, but not CD63, were up-regulated in response to IL-3[167] while other studies have showed both CD63 and CD203c were up-regulated following incubation with IL-3[174] – our findings have supported the latter, that IL-3 alone can stimulate basophils to express both CD63 and CD203c.

2.9 Basophils primed with anti-CD3/28-stimulated T cell-derived supernatants significantly enhance basophil responsiveness to anti-IgE stimulation as measured by the upregulation of activation markers CD63/CD203c.

This series of experiments were performed were similar to the set of experiments discussed in ***discussion 2.6***. However, rather than evaluating if T cell supernatants stimulated basophils on their own (compared to baseline), we looked at whether basophils could be primed to produce an enhanced response to IgE-mediated cross-linking of receptors by pre-incubating basophils with supernatants derived from activated T cells, before subsequent anti-IgE stimulation (Figure 14). The literature suggested that incubating basophils from nonresponders with IL-3 allows these basophils to overcome nonresponsiveness to anti-IgE (see ***discussion 2.8***). In this set of experiments, we used the supernatants from bulk activated T cells, as they may contain mediators in addition to IL-3 that prime or activate basophils. The experiments are summarized in Figure 14.

We reported that, compared to anti-IgE-stimulated basophils, basophils stimulated with supernatants derived from anti-CD3/28 or Immunocult-activated T cells (Figure 14A, green and red bars) significantly up-regulated CD63/CD203c, compared to anti-IgE stimulated basophils. Basophils also significantly up-regulated CD63/CD203c when they were primed with

supernatants, and subsequently stimulated with anti-IgE (Figure 14A, dark green and dark red bars).

2.10 Basophils in direct contact with activated CD4+ T cells are stimulated in response to anti-CD3/28 and anti-CD3/28/2 (Immunocult) T cell-specific antibodies

Next, we wanted to capture an additional dimension of CD4+ T cell-basophil interaction that had not been previously addressed: physical contact. Until this point, we had utilized soluble factors that activated CD4+ T cells produced which may activate or prime basophils, however, in an in-vivo situation, both basophils and CD4+ T cells are recruited to the site of allergic inflammation, making contact between these two cells a likely event. Direct cell-cell contact between CD4+ T cells and basophils was shown to be required for optimal IL-4 production by basophils.[175] Furthermore, circulating basophils were reported to increase MHC class II expression in Th2-associated diseases, highlighting basophil interaction with CD4+ T cells through antigen presentation.[176] While basophils are known to enhance T cell responses through IL-4 and IL-6,[177] a less explored subject area is how CD4+ T cell-mediated effects on basophil activation. We explored this topic by isolating PBMCs containing CD4+ T cells and basophils, and selectively stimulating CD4+ T cells via T cell specific antibodies anti-CD3/28 and/or anti-CD2 (Immunocult) and measuring basophil histamine release and basophil expression of surface activation markers CD63/CD203c.

We found that basophils significantly up-regulated CD63/CD203c in response to anti-IgE (positive control) and Immunocult-stimulated PBMCs, relative to baseline (Figure 15B). Area under curve analysis was used in this series of experiments because CD4+ T cells take approximately 6 hours to become activated in response to TCR stimulation with CD28 co-stimulation, so the increase in CD63/CD203c would not be immediately evident.[178] Anti-IgE-

stimulated PBMCs, but not Immunocult-stimulated PBMCs significantly up-regulated CD63/CD203c in the AUC analysis (Figure 15C). Histamine release was not significantly enhanced across any conditions, compared to baseline (Figure 15D, E). It was interesting to note that CD63 and CD203c responded differently to different stimuli. For example, CD63 was not significantly up-regulated in response to anti-IgE-stimulation, while CD203c was significantly up-regulated (Figure 16E, F). On the other hand, Immunocult-stimulated PBMCs significantly up-regulated CD63, but not CD203c (Figure 16E, F). We have discussed in some length the differences between CD63 and CD203c, and how they relate to the two types of degranulation basophils undergo (see ***discussion 2.7***). We see here that CD63 was not up-regulated in response to anti-IgE stimulation, while histamine to some extent was (though this was not statistically significant). However, when we looked at allergic subjects (n=3) (who were more likely to have 'responder' basophils, which respond to anti-IgE) (Appendix S15), we saw a discordance between CD63 expression (Appendix S15 A) and histamine release (Appendix S15 B). In these subjects, basophils stimulated by anti-IgE released significantly more histamine compared to anti-CD3/28 and Immunocult-stimulated PBMCs ($p=0.0127$ and $p=0.0022$, respectively). Histamine that was released in the absence of CD63 upregulation meant that histamine was released via piecemeal degranulation, rather than anaphylactic degranulation (according to the PMD/AND model). CD203c was significantly up-regulated in anti-IgE-stimulated basophils from these subjects, compared to baseline (Figure 16F).

It was interesting to find that in Immunocult-stimulated PBMCs, basophils significantly up-regulated CD63, but not CD203c or histamine. This finding contradicts the proposed PMD/AND model of basophil degranulation because although histamine may be released from basophils via PMD or AND, detecting CD63 on the surface of basophils necessitates the

trafficking and fusing of intracellular histamine-containing granules to the plasma membrane (so-called AND).[171] In this case, we saw a significant increase in CD63 expression on the surface of basophils, but no concordant histamine release.

2.11 Basophils in direct contact with activated T cells are stimulated in response to a mixture of cat peptides

To close out our investigation into contact-dependent activation of basophils by activated CD4+ T cells and to tie it back to IgE-mediated allergies, we incubated PBMCs with Cat-PAD peptides – a mixture of 7 synthetic peptides derived from the major cat allergen Fel d 1 (to see composition of peptides, see Appendix S1). Bulk PBMCs containing APCs could uptake these peptides on MHC class II molecules and present them to CD4+ T cell-receptors, resulting in CD4+ T cell activation. This assay is one step closer in simulating what happens in an in-vivo scenario during allergic inflammation, where the presence of Fel d 1-derived peptides are taken up by APCs and present allergen-derived peptides to CD4+ T cells, in the presence of basophils. Subjects recruited for these series of experiments were self-reported cat-allergic subjects. The summary of experiments were shown in Figure 17. Histamine release in all conditions were normalized to baseline (cRPMI only) release values shown in Figure 17C due to high baseline levels in some subjects recruited.

We found that basophils significantly increased histamine release compared to baseline when PBMCs were incubated with Cat-PAD at 0.1, 10, and 25ug/ml, as well as anti-IgE ($p=0.0237$, $p=0.007$, $p=0.0211$, and $p<0.0001$, respectively). Histamine release was normalized to baseline because between different subjects there was a lot of variability in histamine release (evident in Figure 17A). Therefore, all values were divided by the histamine in the baseline condition, at each timepoint.

We also saw a significant increase in basophil expression of CD63/CD203c in two of the Cat-PAD conditions (10ug/ml and 0.1ug/ml), compared to baseline (Figure 17D). However, when CD63 and CD203c were analyzed individually, we saw that (consistent to what we saw previously in ***discussion 2.10***) CD203c, but not CD63, was up-regulated in response to anti-IgE stimulation (Figure 16D). When analyzed individually, neither CD63 nor CD203c were significantly up-regulated in any Cat-PAD conditions, compared to baseline (data not shown). There also seemed to be a trend in increased CD63 expression in all conditions over the course of 10 hours, including the baseline condition. Due to a low number of subjects recruited (n=3), as well as inter-subject variability in histamine release, additional cat-allergic subjects would need to be recruited to substantiate the claim that Cat-PAD peptides are capable of stimulating CD4+ T cells, which in turn stimulate basophils to release histamine and up-regulate CD63/CD203c markers. Another limitation in this set of experiments (which also applies for the set of experiments in ***discussion 2.10***) that may account for the differences in histamine release was that the number of basophils was not standardized between subjects. Some subjects may have more basophils than other subjects, and this was not taken into account – as well as different frequencies of CD4+ T cells and dendritic cells. It would be possible to separately isolate basophils, CD4+ T cells, and dendritic cells from PBMCs and culture these cells together in a fixed ratio (but equivalent numbers across subjects), thereby removing a great deal of variability between subjects.

Aim 3: Demonstrate that supernatants from activated T cells can stimulate neutrophils

Neutrophils are one of the first innate immune cells that migrate to the site of inflammation following allergen exposure, and there is evidence that local cytokines involved in the allergic reaction, such as IL-8, can increase their lifespan well beyond their expected lifespan

of a few hours.[103,179] Similarly, basophils are recruited by IL-8 (and with the help of IL-3, activated) and have been shown to play an important role in both the early and late phase response.[132,180]

3.1-3.2 Neutrophils contained and released histamine in response to fMLP. Histamine was not induced from neutrophils in response to IL-8 stimulation or stimulation by supernatants derived from activated Jurkat and CD4+ T cells

Our first objective was establishing that neutrophils contained and released histamine in response to stimulation by a positive control (Figure 18). Unlike the original research paper by Alcañiz et al. in 2013, LPS in our hands did not induce histamine release in neutrophils (data not shown). However, neutrophils stimulated with the bacterial-derived peptide fMLP released significant amounts of histamine (Figure 18B).

Although IL-8 has been shown to stimulate neutrophils to some extent in the literature, we did not see a significant increase in histamine release in neutrophils following stimulation with IL-8 (Appendix S10 A). Similarly, we did not see any appreciable amount of histamine release following incubation of neutrophils with supernatants derived from CD4+ T cells Appendix S7 B). Our findings that Jurkat T cells produced histamine in response to stimulation with anti-CD3/28 and Immunocult (see ***discussion 2.3***) meant we could not interpret our results from neutrophils stimulated with supernatants derived from activated Jurkat T cells (summarized in Appendix S7 A).

3.3 Neutrophil up-regulated CD11b and downregulated CD62L in response to stimulation with Jurkat and CD4+ T cell supernatants

Although activated T cell supernatants did not induce histamine release from neutrophils, we found that there was a significant change in expression of activation markers CD11b and

CD62L on neutrophils. Neutrophils display this altered phenotype (↓CD62L and ↑CD11b) in systemic inflammatory diseases such as sepsis,[181] and integrins such as CD11b play an important role in neutrophil migration by binding to ICAM-1 and ICAM-2 on endothelium.[182] Downregulation of CD62L in neutrophils indicates they are in an activated state, and $CD62L^{low}$ neutrophils have been shown to enhance T cell priming and eosinophil migration in allergic rhinitis.[112]

We were not able to detect expression of markers that indicated neutrophils increased ROS production in response to supernatants from T cells – these markers included surface expression of DHR123 and intracellular expression of MPO (data not shown). The literature has suggested that pro-inflammatory cytokines such as IL-1β, GM-CSF, and TNF-α activate neutrophil NADPH oxidase via phosphorylation of Ser345 on p47-phox, leading to the activation of MAPK/extracellular signal-regulated kinases (MEK)-ERK and the MAPK kinase 3/6 (MKK3/6)-p38 MAPK pathway.[183] Anti-CD3/28 and/or anti-CD2-stimulated Jurkat and CD4+ T cells produced both TNF-α and GM-CSF, but not IL-1β, according to the data obtained from our multiplex assay. All 3 cytokines (on their own) were shown to induce superoxide (O_2^-) release from neutrophils; therefore, it remains to be determined if the concentration of these cytokines in our anti-CD3/28 and Immunocult-stimulated CD4+ T cell supernatants was too low (TNF-α: 1.205ng/ml and 1.257ng/ml for anti-CD3/28- and Immunocult-stimulated CD4+ T cells; GM-CSF: 0.711ng/ml and 0.828ng/ml for anti-CD3/28- and Immunocult-stimulated CD4+ T cells.

Another consideration in our ROS measurements was that while DHR123 responded to both PMA and fMLP positive controls, MPO did not (data not shown). Therefore, in the future an alternative assay to measure ROS activity in neutrophils stimulated with T cell supernatants would be advised, such as the dichlorodihydrofluorescein diacetate staining assay.[184]

Alternatively, measuring phosphorylation of proteins upstream of NADPH, such as ERK1/2 and p38 MAPK[185] in neutrophils following stimulation with T cell supernatants could be considered.

CONCLUSION AND FUTURE DIRECTIONS

The main finding from this work was that isolated polyclonal CD4+ T cells and Jurkat T cells release soluble mediators, following ligation of cell-surface molecules CD3/CD28 and/or CD2, that activate basophils and neutrophils. Unexpectedly, we observed that Jurkat T cells produced and released histamine in response to anti-CD3/28 and/or anti-CD2 stimulation. It was not known whether the histamine produced by Jurkat T cells reflects its phenotype as a T cell, given that CD4+ T cells have been demonstrated to express HDC and release histamine, or its phenotype as a leukemic cell line, given that most malignant cell lines highly express HDC.

We observed that T cell-derived soluble mediators significantly up-regulated basophil activation markers CD63/CD203c, and significantly altered neutrophil activation markers CD62L (↓) and CD11b (↑). However, the soluble mediators were 1) not sufficiently potent, or 2) did not activate the appropriate signalling cascades, to induce basophil and neutrophil histamine release. We demonstrated that T cell-derived mediators acted synergistically with anti-IgE to induce supra-additive levels of histamine release from basophils.

We found that basophils stimulated with supernatants derived from activated T cells up-regulated both CD63 and CD203c but did not significantly up-regulate histamine. According to the current model of anaphylactic and piecemeal degranulation, histamine may be released without a concomitant increase in CD63 (AND), but the reverse (CD63 upregulation without a concomitant increase in histamine), is not possible. In our stimulation experiments, we saw that CD63 was strongly up-regulated in response to T cell supernatant stimulation, but histamine release was not significantly increased. This example of discordance between CD63 expression

and histamine release is at odds with the PMD/AND model of basophil degranulation. Another example of discordance between surface markers and histamine release was that basophils within PBMCs stimulated with Immunocult T cell stimulator significantly up-regulated CD63, but CD63 was not significantly up-regulated following stimulation with anti-IgE – although this observation was consistent with the PMD/AND model because histamine was also not significantly increased (in any conditions). It remains to be determined why basophils stimulated with T cell supernatants significantly altered CD63 and CD203c without a significant change in histamine release – additional mediators will need to be assessed in follow-up experiments.

IL-8, on its own, did not contribute to histamine release or upregulation of activation markers CD63/CD203c on basophils. IL-3 was corroborated with previous findings that alone it was a potent stimulator of basophils (CD63/CD203), however, future insight into the relative contribution that IL-3 had in the presence of other T cell-derived cytokines in stimulating basophil activation markers is needed. This could be done by introducing a neutralizing IL-3 antibody into the supernatants prior to stimulating basophils with the supernatants.

Neutrophil activation by T cell-derived supernatants should also be further characterized. We began evaluating cell surface markers beyond activation markers CD62L and CD11b, such as MPO and DHR123 which measure the presence of reactive oxygen species intermediates in neutrophils. However, we did not see any difference in MPO and DHR123 expression following neutrophil activation by T cell supernatants. The oxidative burst, and more broadly neutrophil phenotypic changes, following incubation by T cell-derived supernatants should be explored. Similar to how basophils can be primed for enhanced histamine release, neutrophils can be primed for enhanced respiratory burst by a variety of stimuli (prior to the stimulation by fMLP), including LPS,[186] IL-8,[187] TNF-α,[188] as well as other pro-inflammatory cytokines.[183]

IMAGES

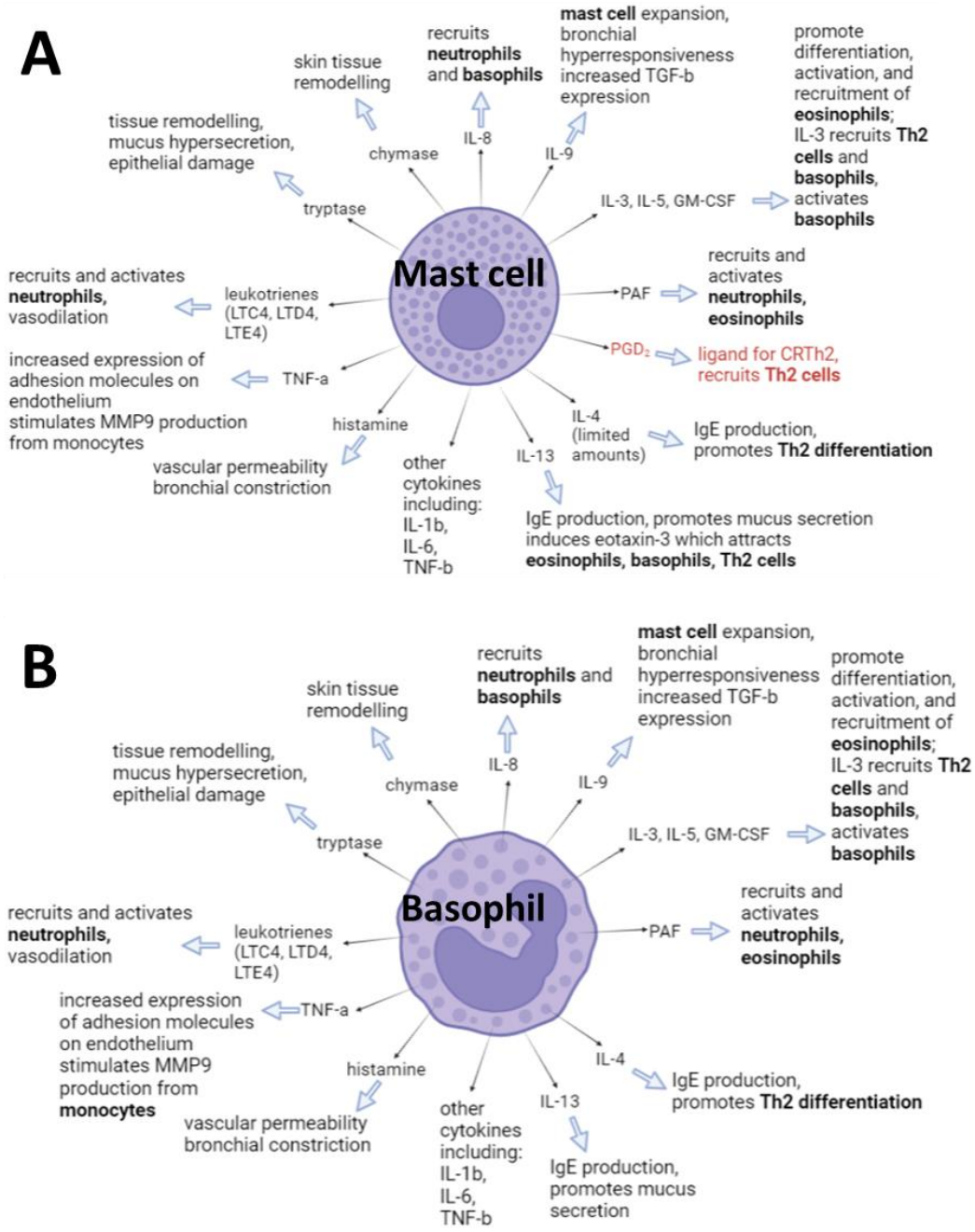


Image 1. Mediators released by (A) mast cells and (B) basophils during the early phase response of an allergic reaction (not a comprehensive list). Both cell types release many mediators that have numerous effector functions, however, there are two important distinctions: 1) mast cells, but not basophils, release PGD_2, and 2) mast cells produce relatively little IL-4 compared to basophils. PGD_2, prostaglandin D2; PAF, platelet activating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MMP9, matrix metalloproteinase 9; TNF, tumor necrosis factor; TGF, transforming growth factor; CRTh2, chemoattractant receptor-homologous molecule expressed on Th2 cells.

FIGURES

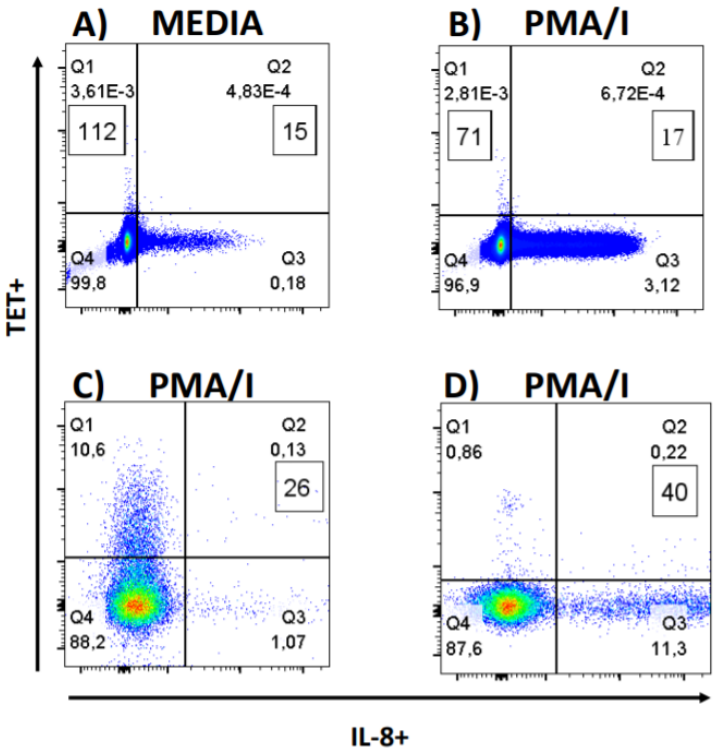


Figure 1. Detection of CD4+ T cells in allergic subjects, 6-hour incubation. A, B) CD4+ T cells stained with tetramers without subsequent enrichment in one cat-allergic subject. C, D) CD4+ T cells stained with tetramers and enriched for tetramers in two cat-allergic subjects. Total n=3. PMA/I, phorbol 12-myristate 13-acetate + ionomycin; TET+, tetramer positive.

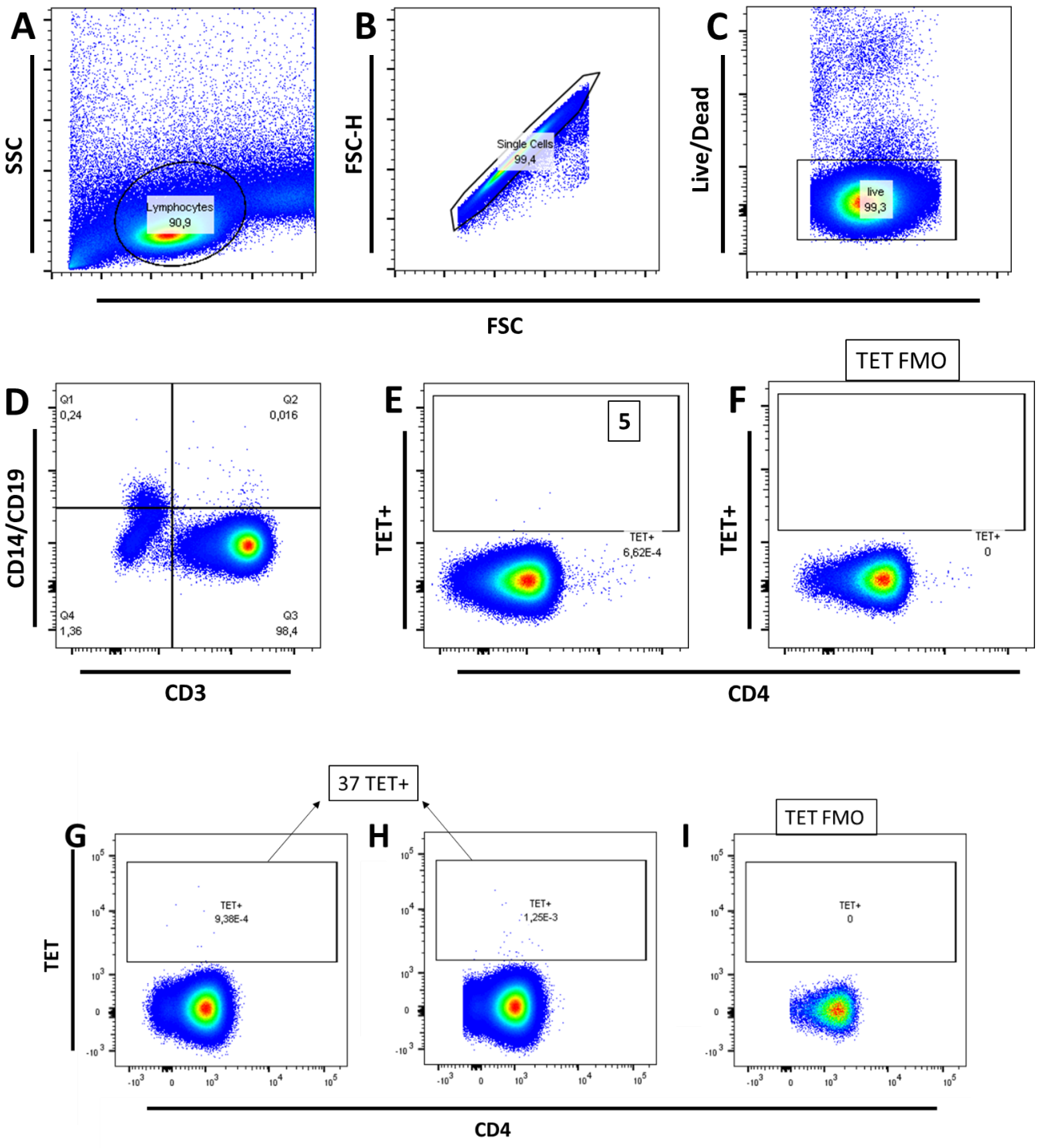


Figure 2. Gating strategy for tetramer staining protocol (Aim 1) using a cat-allergic subject. A) Isolated CD4+ T cells were gated based on FSC and SSC to exclude debris and non-lymphocytes. B) Doublets were excluded, and C) live cells were gated. D) CD14-CD19- cells were gated out and CD3-expressing cells gated in. E) CD4 and PE to gate for CD4+ TET+ cells. F) PE FMO control was used to select a positive gate for TET+ events. G-I) The protocol was repeated using the same gating strategy; the final gates are displayed. Samples were unstimulated and not intracellularly stained. G, H) CD4+ tetramer+ cells were gated, samples were identical but acquired separately. I) PE FMO control was to set the positive gate for TET+ events. PE, phycoerythrin; FMO, fluorescence minus one

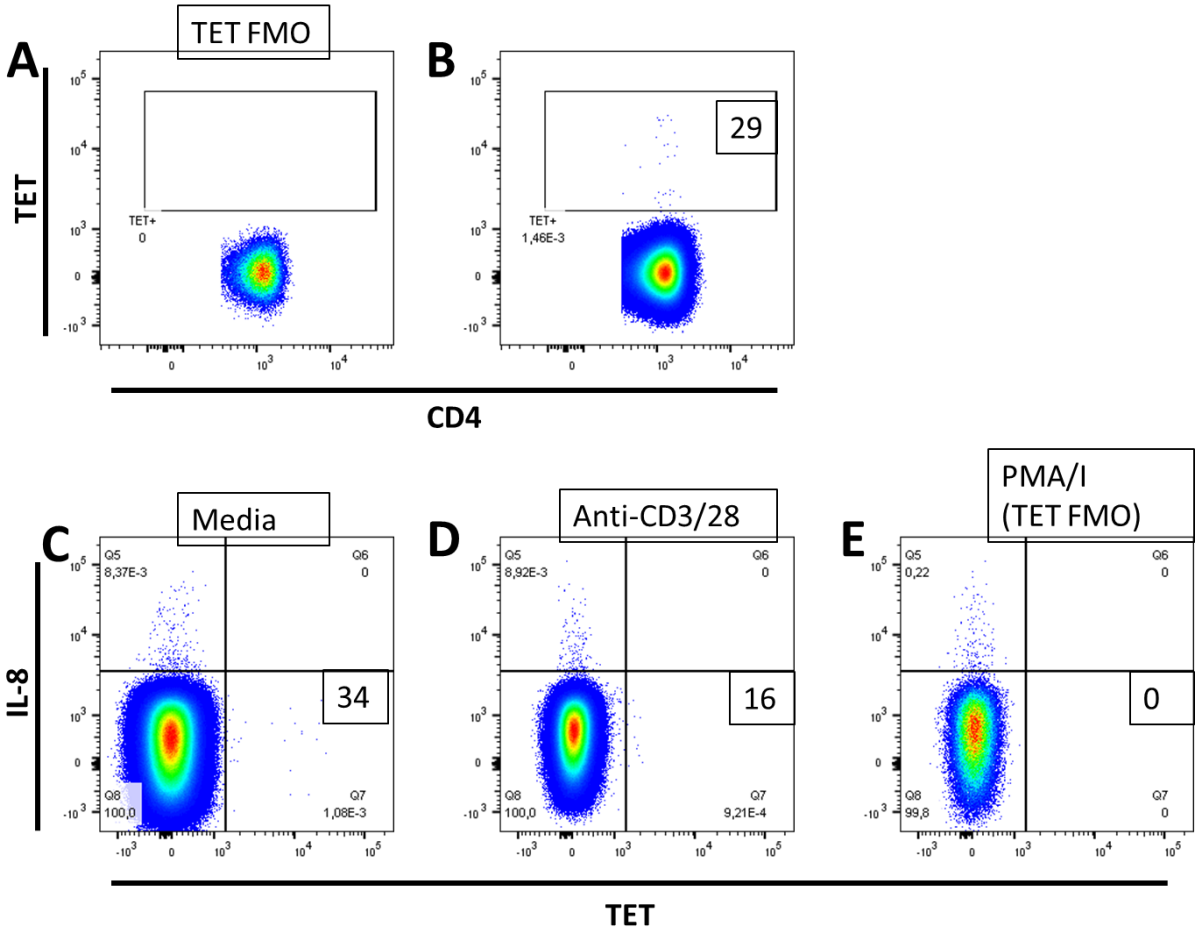


Figure 3. Tetramer staining protocol with stimulated conditions and IL-8 IC staining with a cat-allergic subject. Gating strategy identical to **Figure 2.** A) PE FMO control was used to set the positive gate for tetramer+ CD4+ T cells. B) TET+ cells were captured. C-D) CD4+ T cells were stained with tetramers and stimulated for 24 hours with media (C), anti-CD3/28 beads (D). E). PMA/I-stimulated condition was not stained for tetramers. IC, intracellular; PE, phycoerythrin; FMO, fluorescence minus one; PMA/I, phorbol 12-myristate 13-acetate + ionomycin

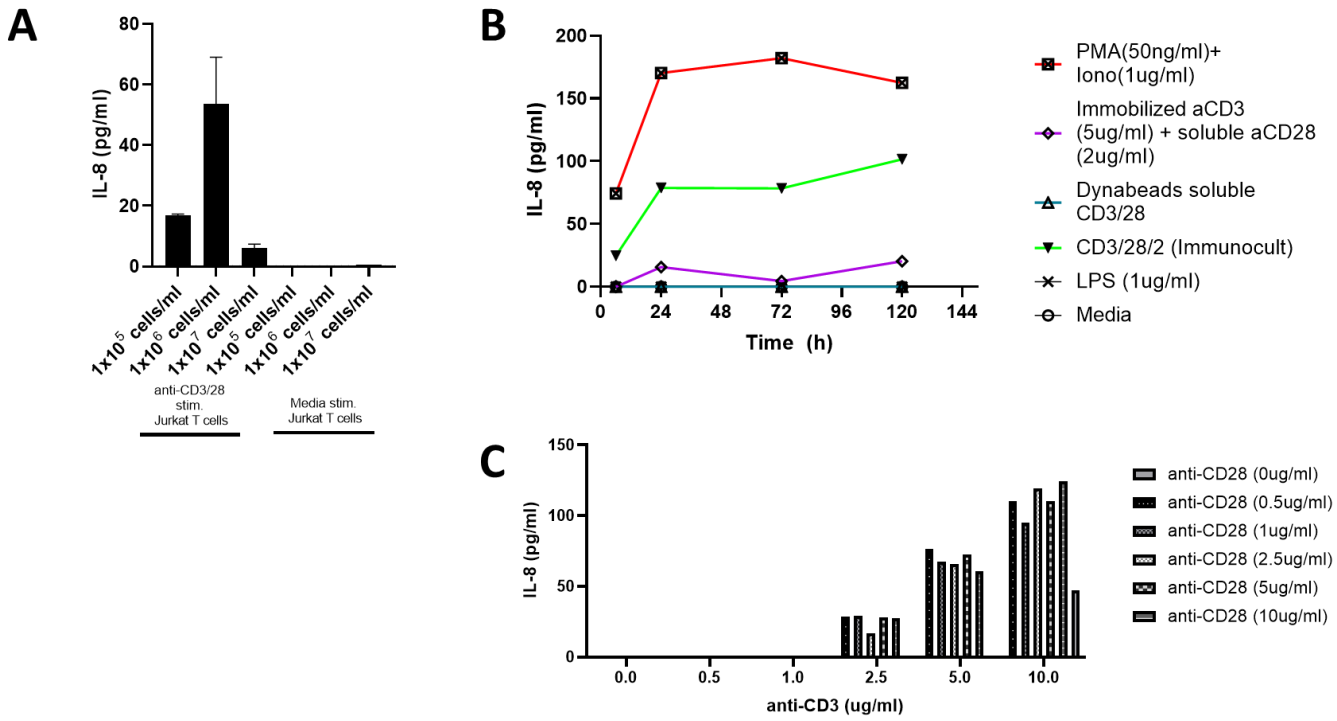


Figure 4. Jurkat T cells were stimulated at different densities and with various stimuli to determine optimal conditions for IL-8 production. 70ul anti-CD3 diluted in PBS was immobilized to a flat-bottomed 96-well plate and let sit in 4°C overnight. Wells were washed with sterile PBS at least twice. A) and B) Jurkat T cells were stimulated for 24hrs at $1x10^6$ cells/ml. A) Jurkat T cells were plated in log 10-fold serial dilution of densities (10^5-10^7cells/ml and stimulated with anti- CD3/28 for 24 hours to determine the optimal concentration of Jurkat T cells for IL-8 production (y-axis). B) Time course experiment was performed to determine the kinetics of IL-8 production (y-axis) from Jurkat T cells and choose the optimal stimulation time. C) Jurkat T cells were stimulated with varying concentrations of anti-CD3 (x-axis) and anti-CD28 (bars) to determine the optimal dose of anti-CD3 and anti-CD28. Data bars are shown as mean±SD. LPS, lipopolysaccharide; PMA/Iono, phorbol 12-myristate 13-acetate + ionomycin; PBS, phosphate buffered saline.

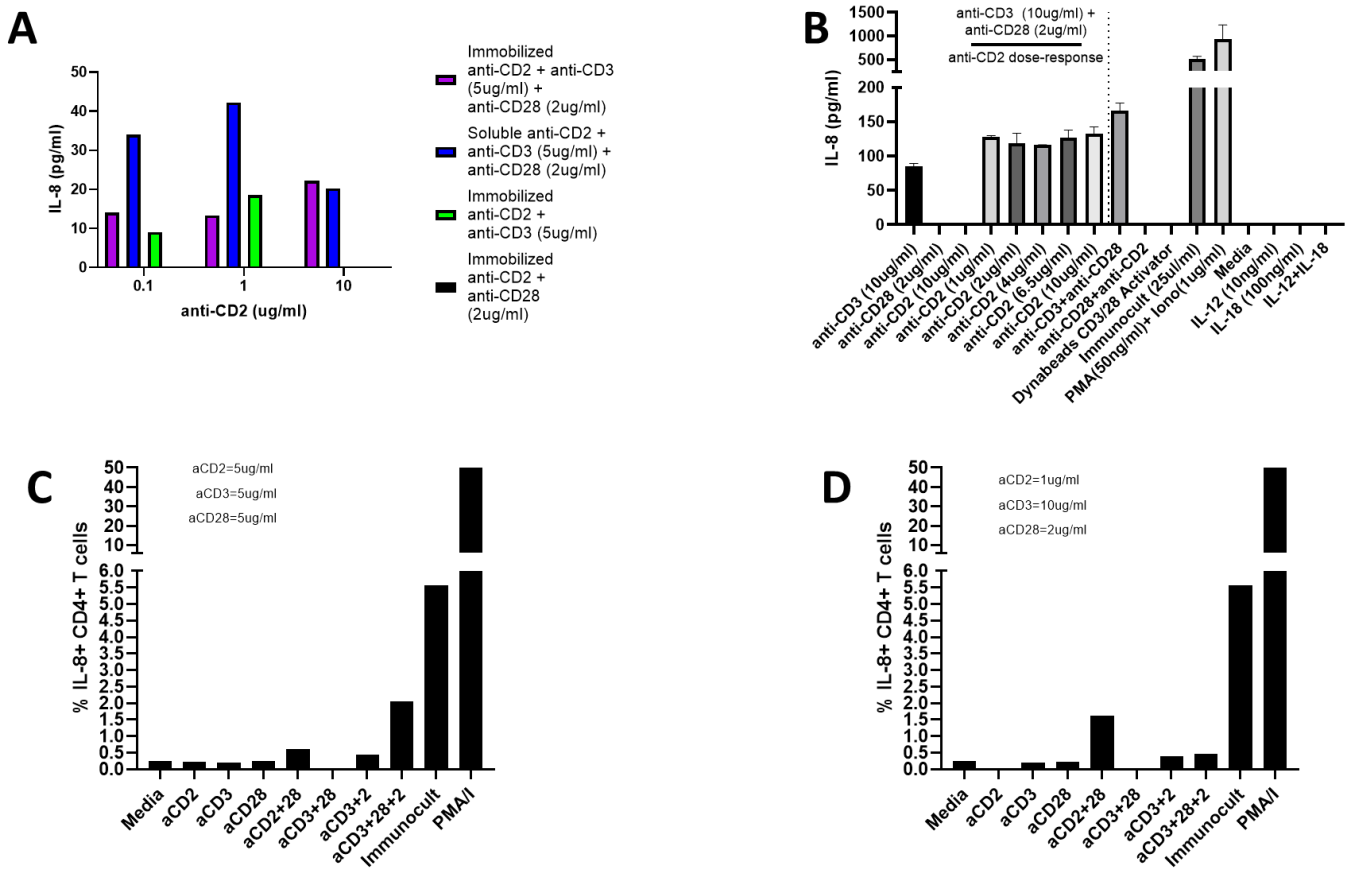


Figure 5. Jurkat T cells and CD4+ T cells were stimulated with anti-CD2 to determine optimal stimulation conditions for IL-8 production and expression. Anti-CD3 was immobilized to a flat-bottomed 96-well plate, as well as anti-CD2 (where indicated) by adding 70ul to well and letting the plate sit in 4°C overnight. Wells were washed with sterile PBS at least twice. A) and B) Jurkat T cells were stimulated for 24hrs at $1x10^6$ cells/ml. A) Jurkat T cells were stimulated with anti-CD2 at 0.1, 1, and 10ug/ml to determine the optimal concentration of anti-CD2 antibody to use. Soluble (blue bars) vs. immobilized (purple, green, and black bars) anti-CD2 formulations were also compared to see if there was a difference in IL-8 produced (y-axis). B) Soluble anti-CD2 was titrated in combination with the optimal concentrations of anti-CD3 and anti-CD28 antibodies (10ug/ml and 2u/ml, respectively) to determine the optimal dose. Furthermore, we compared the use of these antibodies to Immunocult Human CD3/28/2 T cell Activator. IL-12 (10ng/ml) and IL-18 (100ng/ml)-stimulated Jurkat T cells were included to assess IL-8 production (y-axis). Data bars are shown as mean±SD. C) and D) Protocol from above repeated, except Brefeldin A was added 4 hours before stimulation was finished and anti-CD2 was not immobilized. Cells were stained for surface markers and intracellularly for IL-8. Cells were CD4+ T cells were isolated from PBMCs and stimulated combinations of anti-CD3/28/2 for 24hrs, then subsequently stained intracellularly for IL-8. C) Repeat of doses previously titrated. D) Recently titrated doses (based on IL-8 ELISA data). PMA/I, phorbol 12-myristate 13-acetate + ionomycin.

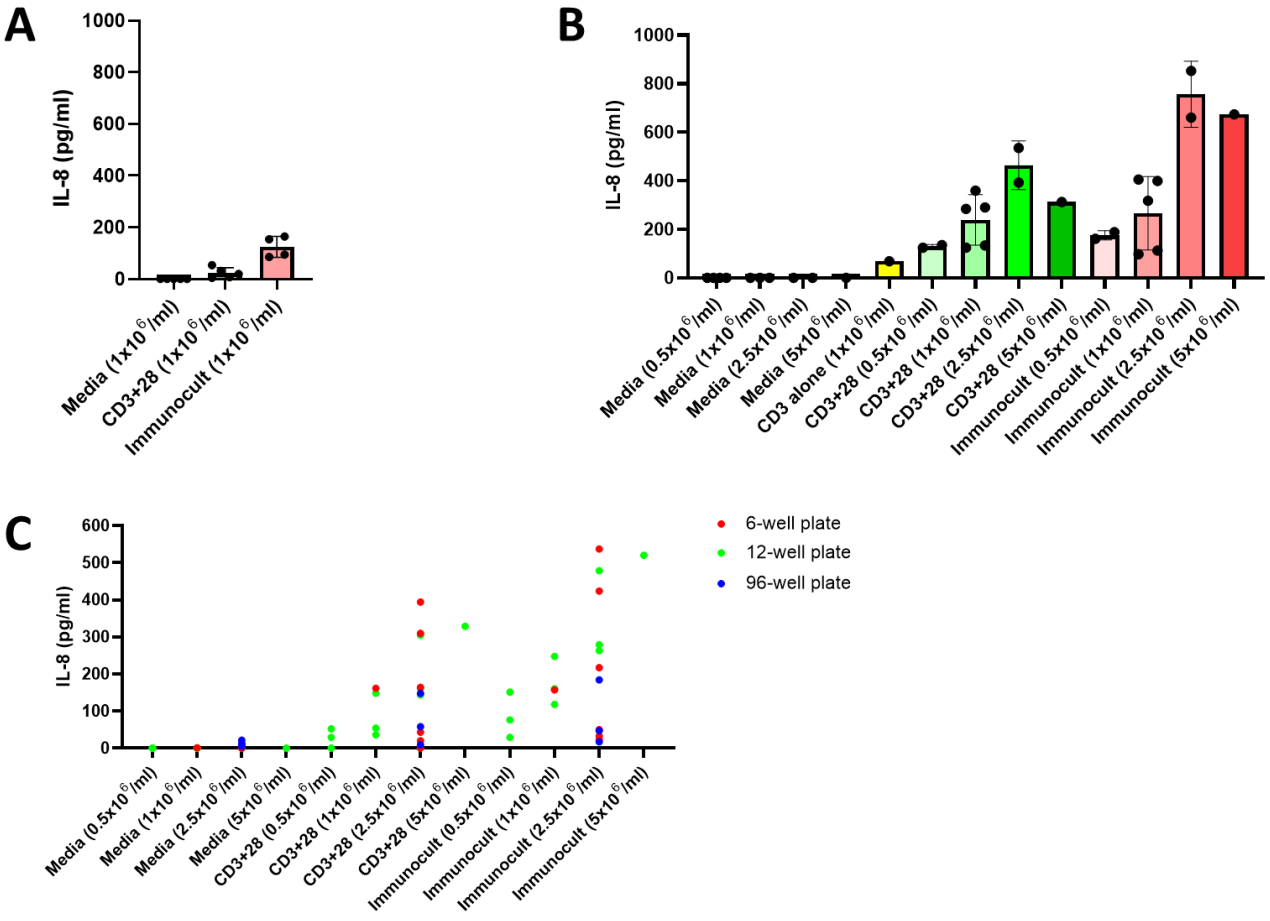


Figure 6. Jurkat and CD4+ T cells produced IL-8 in response to anti-CD3/28 and Immunocult stimulation. A) Jurkat T cells were stimulated at $1x10^6$/ml in 96-well flat-bottomed plates or (B) at concentrations ranging from $0.5\text{-}5x10^6$/ml in 6-well flat-bottomed plates for 24hrs. Supernatants were collected after 24hrs and stored in -20C until used for stimulation experiments. In total, 10 different Jurkat T cell supernatant batches were prepared (5 batches in 96-well plates, 5 batches in 6-well plates). C) Isolated CD4+ T cells were stimulated at concentrations ranging from $0.5\text{-}5x10^6$/ml in 6-well, 12-well, or 96-well flat-bottomed plates for 24hrs. Prior to stimulation, plates were coated in 10ug/ml of anti-CD3 antibody in PBS for immobilization. 6-well and 12-well plates were coated in 1ml of 10ug/ml anti-CD3, and 96-well plates were coated in 70ul of 10ug/ml anti-CD3 and left overnight at 4°C. Wells were washed at least twice in PBS before plating. CD4+ T cells were isolated from the peripheral blood of 12 donors (3 in 96-well plates, 3 in 12-well plates, and 6 in 6-well plates). Data bars are shown as mean±SD.

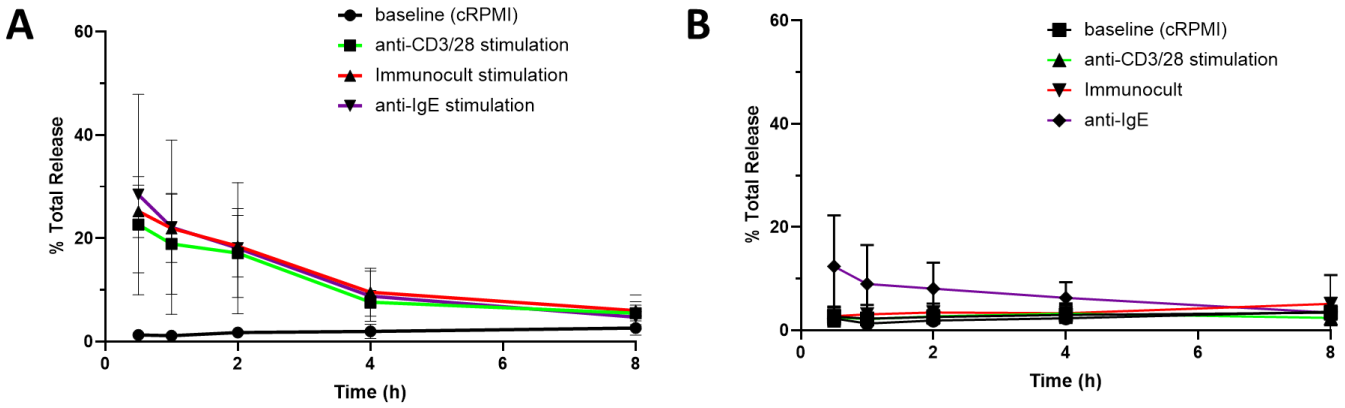


Figure 7. Isolated basophils were incubated with supernatants derived from Jurkat T cells or isolated CD4+ T cells over the course of 8 hours. A) Basophils were isolated from whole blood using immunomagnetic negative selection. Supernatants were prepared by stimulating Jurkat T cells at $1x10^6$/ml for 24hrs in media (cRPMI), immbolized anti-CD3 (10ug/ml) and soluble anti-CD28 (2ug/ml), or Immunocult (25ul/1ml) using 96-well flat-bottomed plates. Supernatants were collected at stored at -20℃. 100ul of isolated basophils ($4x10^4$/ml) were resuspended in 100ul media and 50ul of media, anti-CD3/28, or Immunocult-stimulated Jurkat T cell media for 0.5h, 2h, 4h, and 8h (total vol=150ul) in 37℃ incubator, 5% CO_2. Following stimulation, samples were centrifuged, and supernatants were collected, acylated, and stored at -20℃. The cells were stained for flow cytometry using antibodies to CCR3, CD63, and CD203c. The concentration of histamine in the sample was quantified by ELISA. Total histamine release was determined by freezing and thawing an equivalent number of cells to determine the total amount of histamine in the samples. Percent total histamine was determined by dividing the histamine released in each condition by the total histamine. Data points are shown as mean±SD (n=4). B) Basophils were isolated from whole blood using immunomagnetic negative selection. CD4+ T cell supernatants were prepared by isolating CD4+ T cells from PBMCs. Supernatants were prepared by stimulating CD4+ T cells at $2.5x10^6$/ml for 24hrs in media (cRPMI), immobilized anti-CD3 (10ug/ml) and soluble anti-CD28 (2ug/ml), or Immunocult (25ul/ml) in 96-well flat-bottomed plates. The remainder of the experiment is identical to part A. Data points are shown as mean±SD (n=3). PBMC, peripheral blood mononuclear cells; cRPMI, complete Roswell Park Memorial Institute.

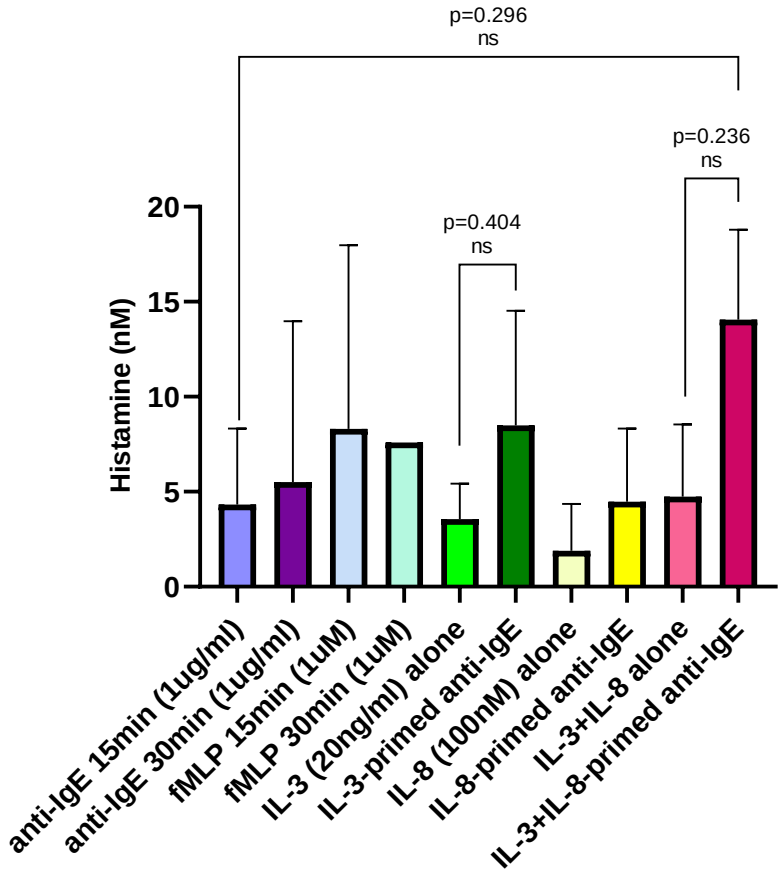


Figure 8. Whole blood basophils did not significantly increase histamine release compared to controls when primed with IL-3 and/or IL-8 (15-min) and subsequently stimulated with positive control (anti-IgE, 15-min). Whole blood basophils did not significantly increase histamine release compared to controls when primed with IL-3 and/or IL-8 (15-min) and subsequently stimulated with positive control (anti-IgE or fMLP, 15-min). 100ul whole blood basophils were incubated with 20ng/ml IL-3, 100nM IL-8, or both (vol=50ul) for 15-minutes at 37℃ in a water-bath. 15ul antibodies to CCR3, CD63, and CD203c were added for flow cytometric analysis. After 15-minutes, 1ug/ml anti-IgE (10ul) was added to the appropriate conditions, and basophils were incubated for an additional 15-minutes at 37℃ in a water-bath. Total incubation time was 30-minutes for most conditions. Following incubation, RBCs were lysed with 2ml RBC lysis buffer. Cells were washed and fixed for flow cytometric analysis. (minimum n=2). Supernatants were collected, acylated, and stored at -20℃ until ready for histamine quantification by ELISA (y-axis). Spontaneous release values were subtracted from all conditions. Data bars are shown as mean ± SD. Statistics: one-way ANOVA with Holm-Sidak's multiple comparisons test. fMLP, N-formyl-methionyl-leucyl-phenylalanine; RBC, red blood cell.

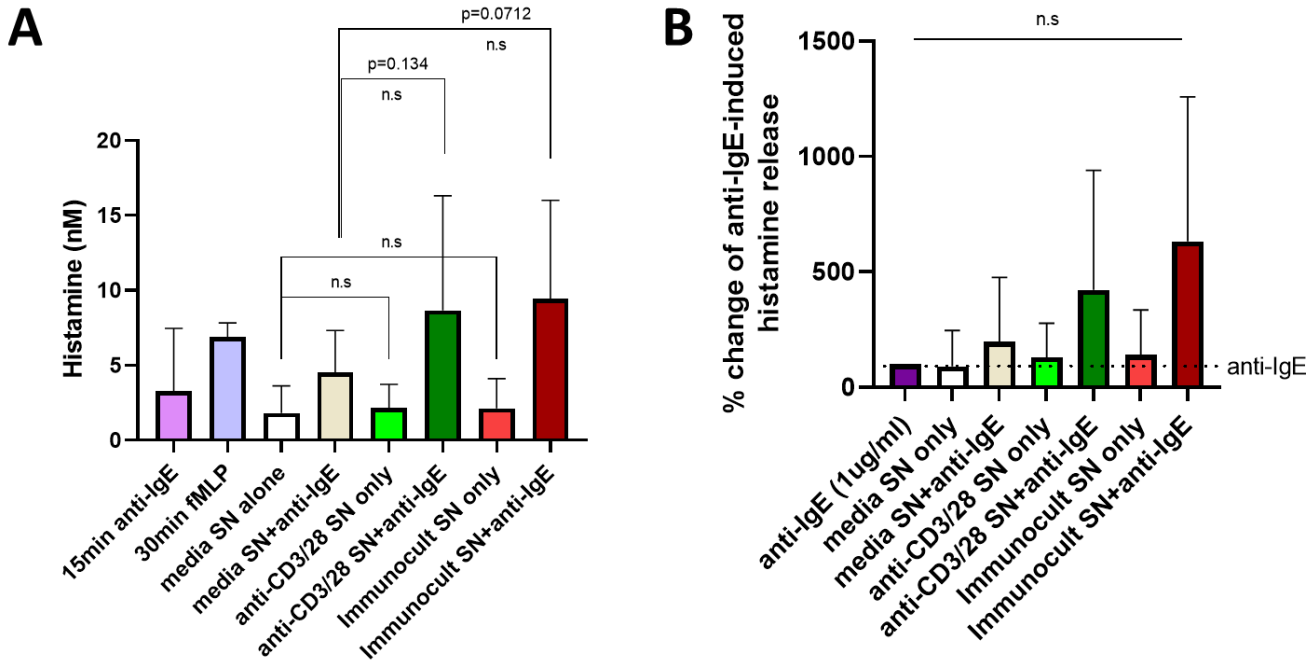


Figure 9. Whole blood basophils primed with supernatants derived from activated CD4+ T cells did not significantly increase histamine release compared to media-stimulated basophils, whether or not basophils were subsequently stimulated with anti-IgE. A) Isolated CD4+ T cells or Jurkat T cells were stimulated at concentrations ranging from 0.5-2.5x10^6/ml in 6-well, 12-well, or 96-well flat-bottomed plates for 24hrs. Prior to stimulation, plates were coated in 10ug/ml anti-CD3 antibody in PBS – 6-well and 12-well plates were coated in 1ml of 10ug/ml anti-CD3, and 96-well plates were coated in 70ul of 10ug/ml anti-CD3. Supernatants were collected and stored at -20°C. For stimulation: 100ul of whole blood was incubated with 50ul of supernatant for 15-minutes in a 37°C-water bath (priming). Next, anti-IgE was added to the conditions as a positive control for an additional 15-minutes (total incubation time = 30-minutes). Histamine concentration was reported in nanomolar (measured by ELISA, y-axis). Histamine release in all conditions was subtracted from histamine in the spontaneous release condition (media only condition). Data shows a summary of supernatant priming experiments. n=5. B) Replicates from each condition were averaged and divided by the average values from anti-IgE-stimulated basophils (histamine release) and multiplied by 100 to express them as a percent. n=6. Statistics: one way ANOVA with Holm Sidak's multiple comparison. Data bars are shown as mean±SD. SN, supernatant; fMLP, N-formyl-methionyl-leucyl-phenylalanine; RBC, red blood cell.

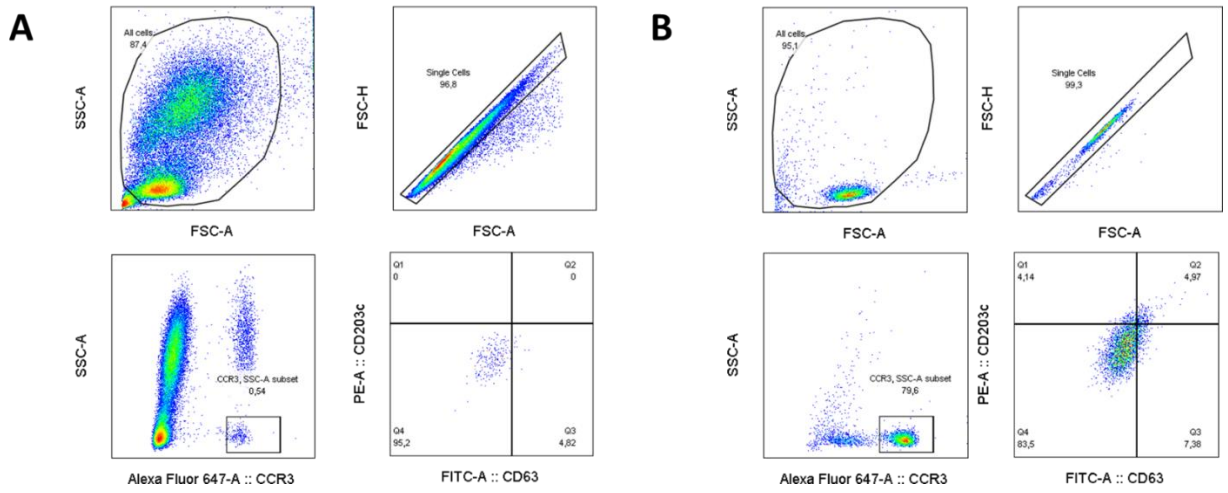


Figure 10. Representative basophil gating strategy (unstimulated cells). After gating out doublets (top right plots) basophils are defined as CCR3+ and side scatter low (bottom left plot). When basophils are activated, they up-regulate CD63 and CD203c surface markers (bottom right plot). A) Whole blood. B) Isolated basophils.

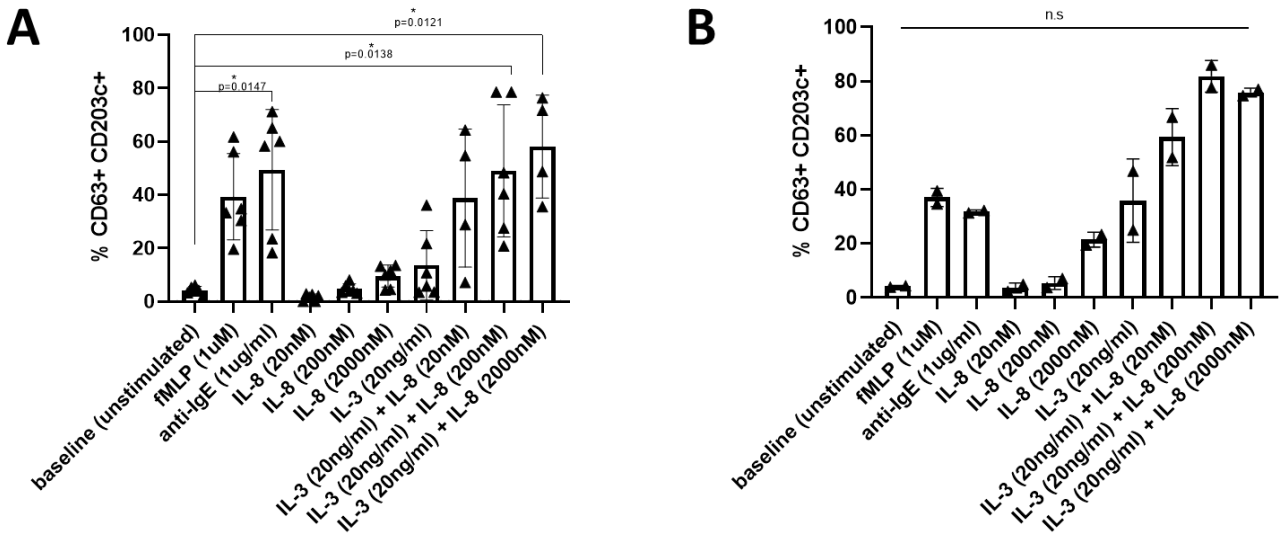


Figure 11. Whole blood basophils significantly up-regulated activation markers CD63/CD203c at the two highest doses of IL-8 when they were pre-incubated with IL-3. A) 100ul of whole blood basophils were primed with 25ul IL-3 (20ng/ml) for 5-minutes in a 37°C water-bath, then subsequently with 25ul IL-8 (20-2000nM) for an additional 15-minutes (20-minute incubation time). Antibodies to CCR3, CD63, and CD203c were added for flow cytometric analysis (total vol=175). Following stimulation, 2ml RBC lysis buffer was added. Cells were washed and fixed for flow cytometric analysis. Percent positivity in the baseline conditions for CD63 and CD203c was set at 5%. (n=3) B) Protocol identical to part A, except that whole blood was incubated for 25-minutes following IL-3-priming (30-minute total incubation time). (n=1) with two replicates. Statistics used: Kruskal-Wallis test with Dunn's multiple comparisons. Experiments were performed in replicates. Data bars are shown as mean±SD. fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; RBC, red blood cell. 20, 200, 2000nM IL-8 is approximately equivalent to 50, 500, 5000ng/ml IL-8 (respectively).

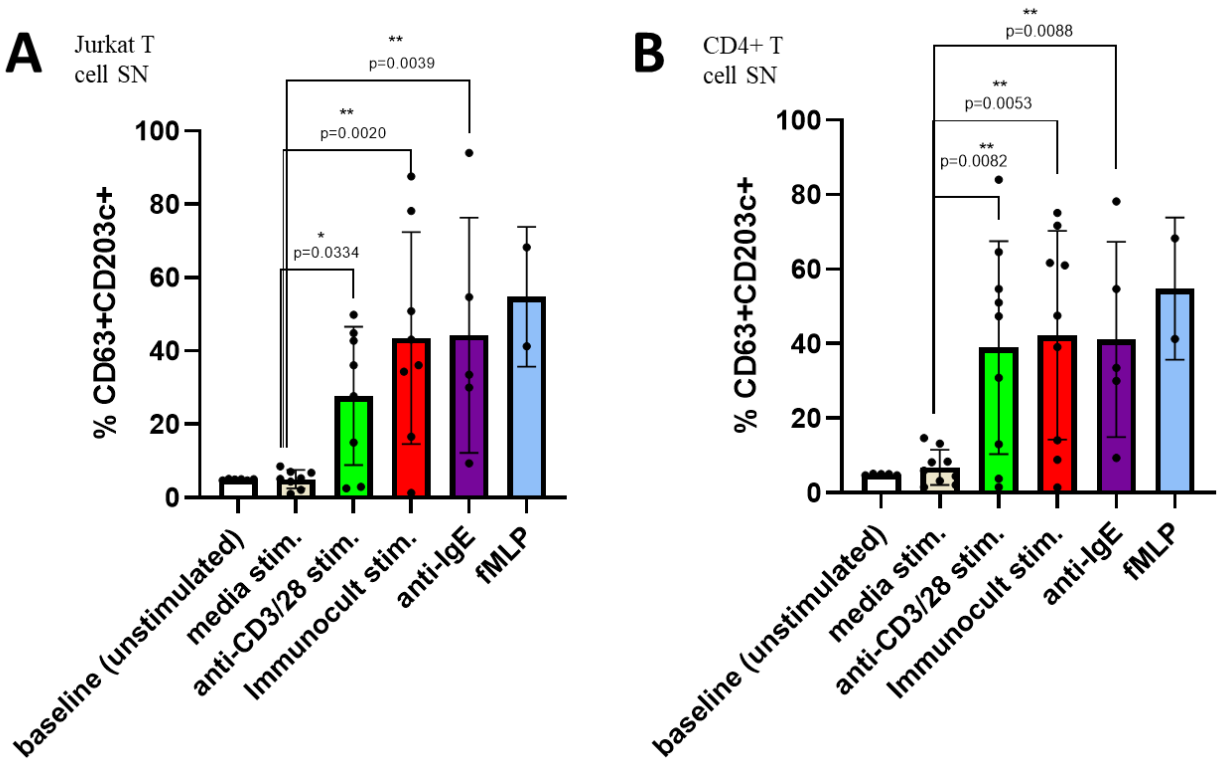


Figure 12. Basophils significantly up-regulated CD63 and CD203c surface markers following incubation with stimulated T cell supernatants. A) 100ul isolated basophils ($4x10^3$ cells/ml) or whole blood basophils were incubated with 50ul Jurkat T cell supernatants (A) or 50ul isolated CD4+ T cell supernatants (B). Supernatants were prepared by stimulating Jurkat T cells at various concentrations ($0.5\text{-}5x10^6$/ml) for 24hrs in media (cRPMI), anti-CD3 (10ug/ml) and anti-CD28 (2ug/ml), or Immunocult (5ul reagent per 1ml) using 96-well or 6-well flat-bottomed plates. Supernatants were collected at stored at -20°C. Next, 100ul of isolated basophils ($4x10^4$/ml) or whole blood were resuspended in 100ul media and 50ul media, anti-CD3 (10ug/ml) and anti-CD28 (2ug/ml), or Immunocult (5ul reagent per 1ml)-stimulated for 30-minutes at 37°C in a water-bath. 15ul antibody master mix were added (CCR3, CD63, and CD203c) for flow cytometric analysis (total vol=165ul). Following stimulation, samples were centrifuged, and RBC lysis was performed where applicable. Cells were washed and prepared for flow cytometric analysis. Data points are shown as mean±SD (n=4). B) Protocol identical to part A, except isolated CD4+ T cells were used to generate the supernatants, at various concentrations ($0.5\text{-}5x10^6$ cells/ml) to stimulate 100ul isolated basophils ($4x10^3$ cells/ml) or whole blood basophils. Data points are shown as mean±SD (n=4). Threshold for positivity for CD63 and CD203c were set at 5% in the unstimulated conditions (y-axis). Statistics: one-way ANOVA with Holm-Sidak's multiple comparisons test. fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; RBC, red blood cell; SN, supernatant

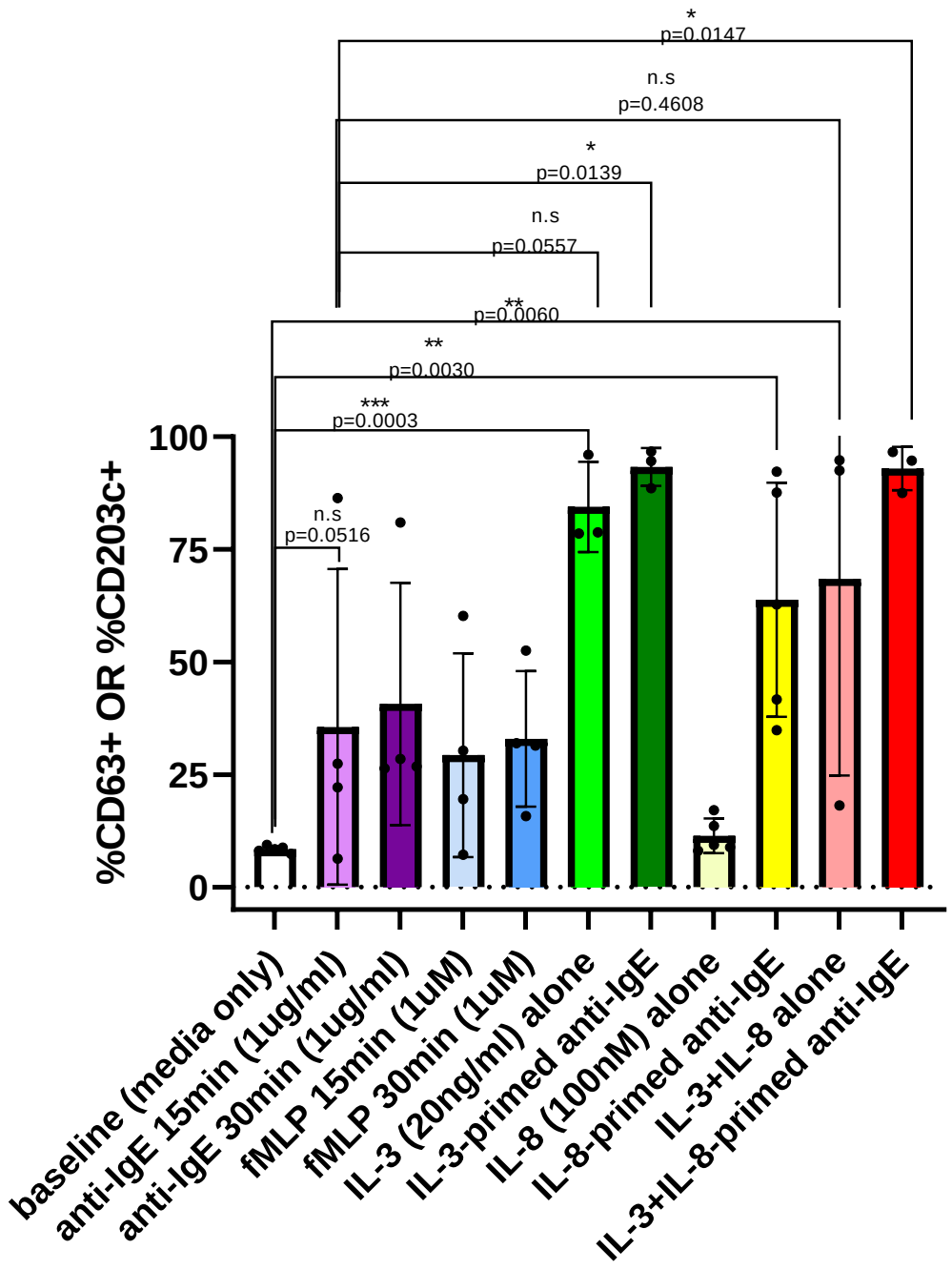


Figure 13. Whole blood basophils were primed with IL-3 and/or IL-8 (15-min) and subsequently stimulated with positive control (anti-IgE or fMLP, 15-min). 100ul whole blood basophils were incubated with 20ng/ml IL-3, 100nM IL-8, or both (50ul) for 15-minutes at 37°C in a water-bath. 15ul antibodies to CCR3, CD63, and CD203c were added for flow cytometric analysis. After 15-minutes, 1ug/ml anti-IgE (10ul) was added was added to the appropriate conditions, and basophils were incubated for an additional 15-minutes at 37°C in a water-bath. Total incubation time was 30-minutes for most conditions. Following incubation, RBCs were lysed with 2ml RBC lysis buffer. Cells were washed and fixed for flow cytometric analysis. (minimum n=3). Data bars are shown as mean±SD. Each data point represents 1 experiment. Threshold for positivity for CD63 and CD203c were set at 5% in the unstimulated conditions (y-axis). Statistics: one-way ANOVA with Holm-Sidak's multiple comparisons test. fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; RBC, red blood cell.

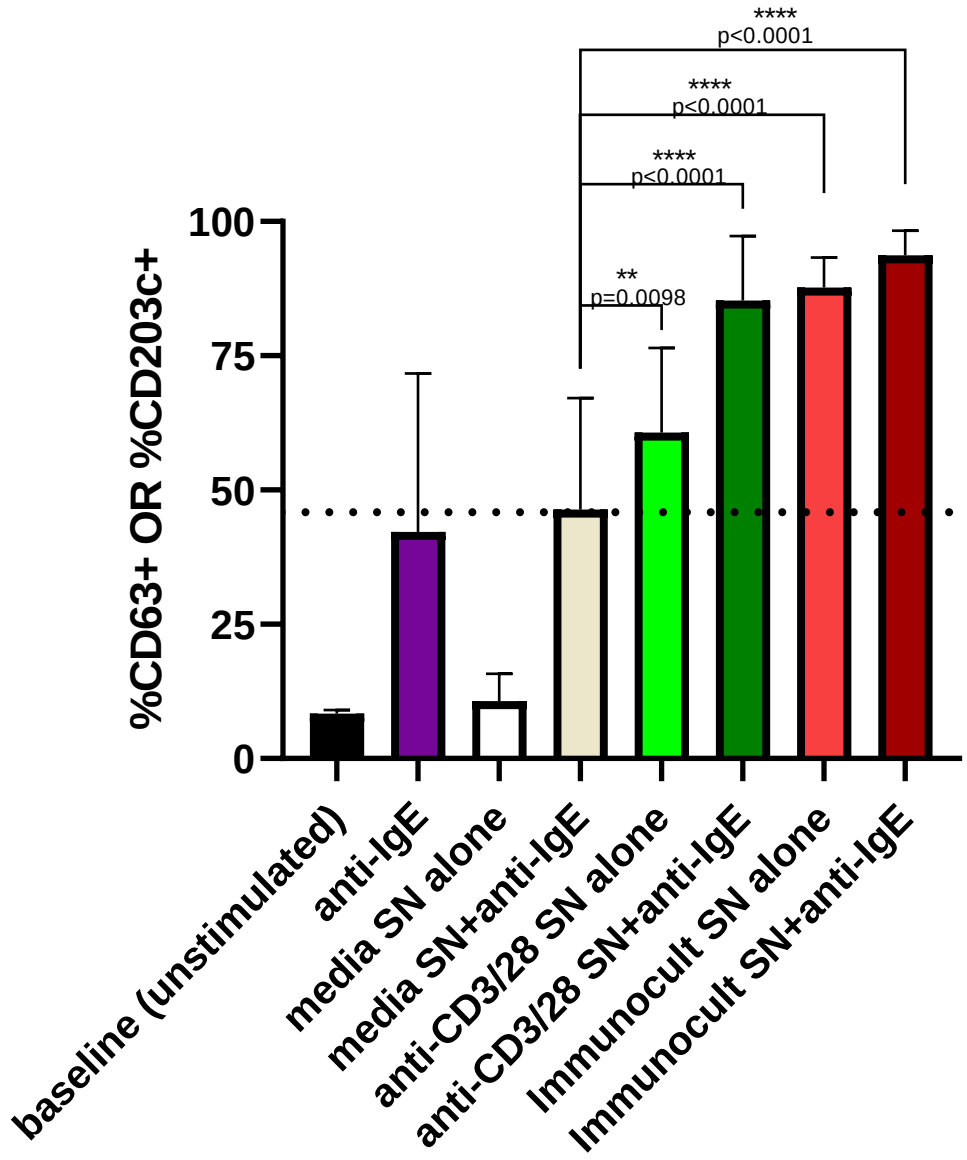


Figure 14. Whole blood basophils primed with SNs derived from activated Jurkat and CD4+ T cells showed an increased response to anti-IgE. T cells were stimulated at concentrations ranging from 0.5-2.5x10^6/ml in 6-well, 12-well, or 96-well flat-bottomed plates for 24hrs. Prior to stimulation, plates were coated in 10ug/ml of anti-CD3 antibody in PBS for immobilization. 6-well or 12-well plates were coated in 1ml of 10ug/ml anti-CD3. T cells were plated and stimulated with cRPMI, anti-CD3 (10ug/ml) and anti-CD28 (2ug/ml) or Immunocult (5ul reagent per 1ml) for 24h at 37°C, 5% CO_2. Supernatants were collected and stored at -20°C. Stimulation protocol: 100ul of whole blood was incubated with 50ul of supernatant or PBS for control, for 15-minutes in a 37°C-water bath (priming). 15ul antibodies to CCR3, CD63, and CD203c were added for flow cytometric analysis. Next, anti-IgE was added to the conditions as a positive control for an additional 15-minutes. Following incubation, RBCs were lysed with 2ml RBC lysis buffer. Cells were washed and fixed for flow cytometric analysis. A) Threshold for CD63 and CD203c positivity was set at 5% in unstimulated cells (y-axis). n=7. Statistics: one way ANOVA with Holm-Sidak's multiple comparisons. SN, supernatant; RBC, red blood cell; SN, supernatant

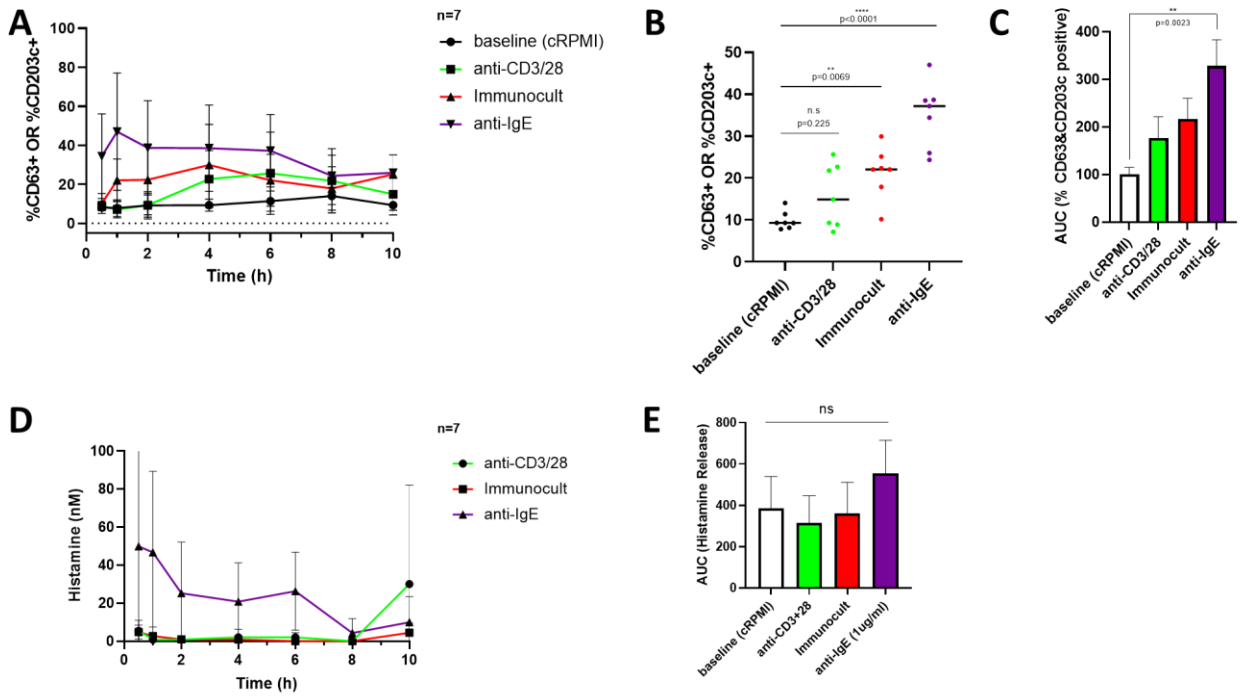


Figure 15. Contact-dependent activation of basophils in the presence of activated-CD4+ T cells did not significantly up-regulate CD63/CD203c markers on basophils. Prior to stimulation, 96-well flat-bottomed plates were coated in 10ug/ml of anti-CD3 antibody in PBS (vol=70ul) and left overnight at 4℃. Stimulation: PBMCs were isolated from whole blood and plated in 96-well flat-bottomed plates at a concentration of $5x10^6$/ml (vol=200ul). Anti-CD28 (2ug/ml, added to the anti-CD3-coated wells), Immunocult (25ul per 1ml culture), and anti-IgE (1ug/ml) were added (vol=5ul) to PBMCs and they were stimulated for up to 10hrs in a 37℃ incubator, 5% CO_2. Following stimulation, supernatants were collected, acylated, and stored at -20℃ for histamine quantification. Cells were stained with CCR3, CD63, and CD203c for flow cytometric analysis. n=7. A) Activated CD4+ T cells in the presence of basophils over the course of 10hrs were measured for expression of CD63/CD203c. Threshold for CD63 and CD203c positivity was set at 5% in the unstimulated condition (y-axis). B) Data identical to (A) but the average value at each timepoint was taken. Each point represents a single timepoint (0.5h, 2h, etc.). Statistics: One-way ANOVA with Dunnett's multiple comparisons test was performed. C) AUC was performed for data shown in (A) to determine the area under each condition. One-way ANOVA with Dunnett's multiple comparisons test was performed. Data bars are shown as mean±SEM. D) Activated CD4+ T cells in the presence of basophils over the course of 10hrs were measured for histamine release. Histamine release values were subtracted from baseline (cRPMI) values. E) AUC was performed for data shown in (D) to determine the area under each condition. One-way ANOVA with Dunnett's multiple comparisons test was performed. Data bars are shown as mean±SEM. AUC, area under curve; PBMC, peripheral blood mononuclear cells; cRPMI, complete Roswell Park Memorial Institute

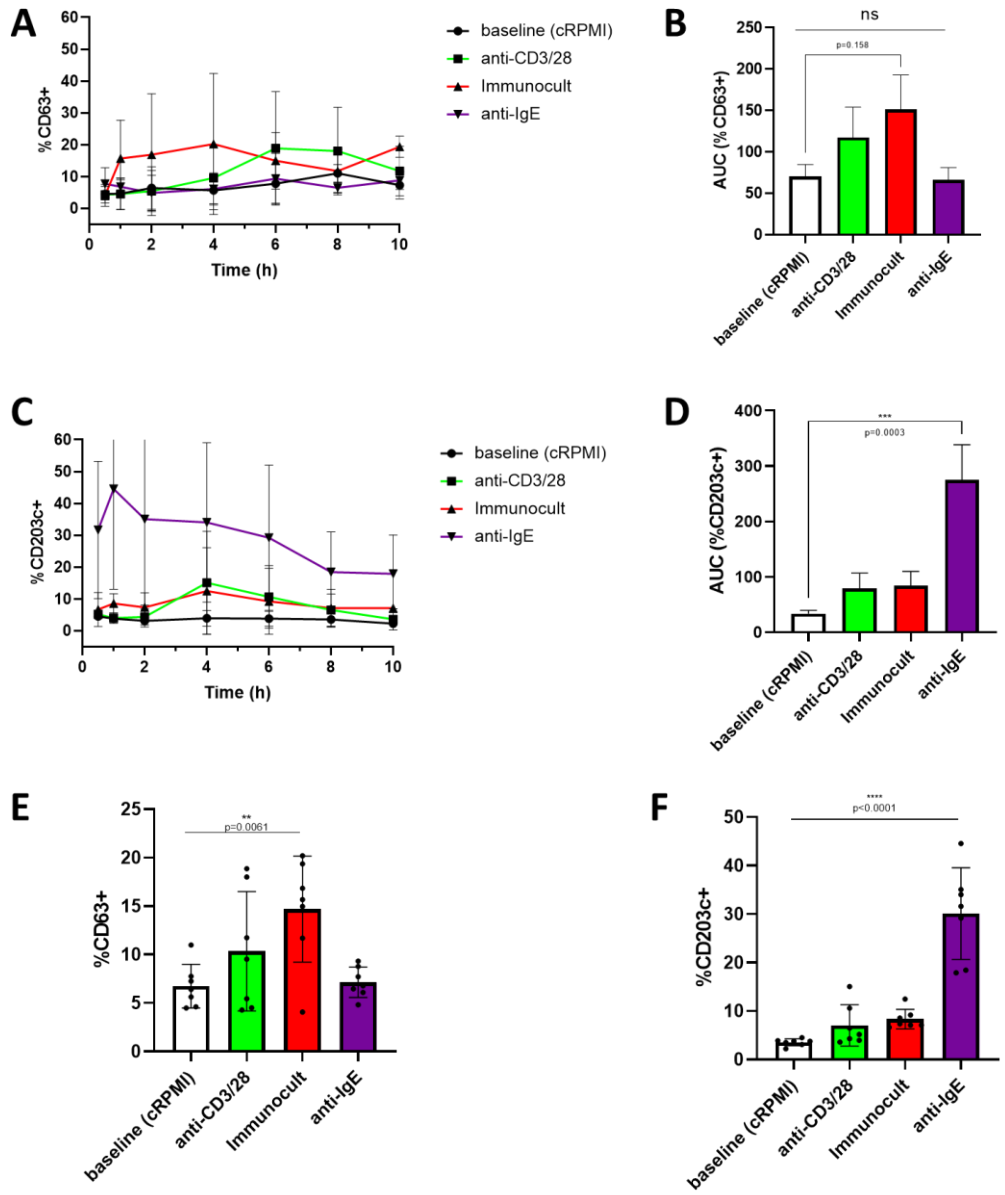


Figure 16. Contact-dependent activation of basophils in the presence of activated-CD4+ T cells significantly up-regulated CD63 on basophils, but not CD203c. Prior to stimulation, 96-well flat-bottomed plates were coated in 10ug/ml of anti-CD3 antibody in PBS (vol=70ul) and left overnight at 4°C. Stimulation: PBMCs were isolated from whole blood and plated in 96-well flat-bottomed plates at a concentration of $5x10^6$/ml (vol=200ul). Anti-CD28 (2ug/ml, added to the anti-CD3-coated wells), Immunocult (25ul per 1ml culture), and anti-IgE (1ug/ml) were added (vol=5ul) to PBMCs and they were stimulated for up to 10hrs in a 37°C incubator, 5% CO_2. Following stimulation, supernatants were collected, acylated, and stored at -20°C for histamine quantification. Cells were stained with CCR3, CD63, and CD203c for flow cytometric analysis. n=7. A) Activated T cells in the presence of basophils over the course of 10hrs were measured for expression of CD63. Threshold for CD63 positivity was set at 5% in the unstimulated condition (y-axis). B) AUC was performed for data shown in (A) to determine the area under each condition. One-way ANOVA with Dunnett's multiple comparisons test was performed. Data bars are shown as mean±SEM. C) Activated T cells in the presence of basophils over the course of 10hrs were measured for expression of CD203c. Threshold for CD203c positivity was set at 5% in the unstimulated condition (y-axis). D) AUC was performed for data shown in (C) to determine the area under each condition. One-way ANOVA with Dunnett's multiple comparisons test was performed. Data bars are shown as mean±SEM. E) and F) Data from (A) and (C) were organized to show average value at each timepoint (0.5h, 2h, etc.). One-way ANOVA with Dunnett's multiple comparisons. Data bars are shown as mean±SD. AUC, area under curve; PBS, phosphate buffered solution; PBMC, peripheral blood mononuclear cells; cRPMI, complete Roswell Park Memorial Institute

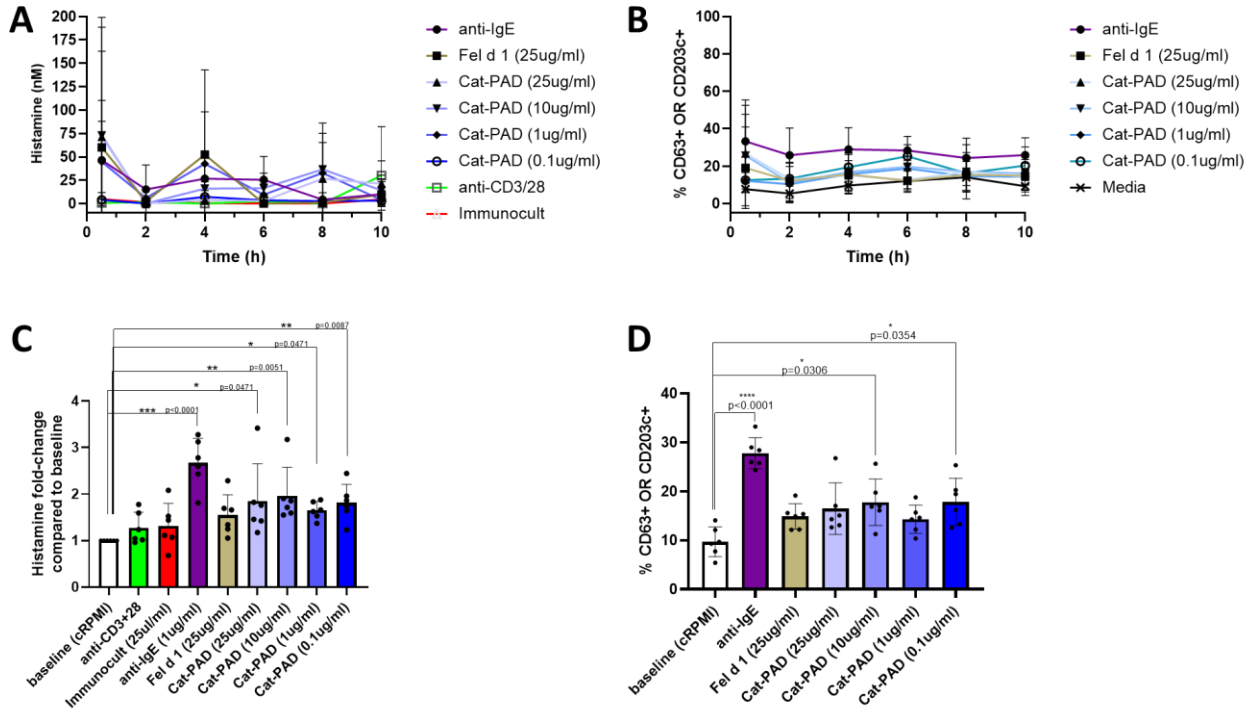


Figure 17. Basophils significantly increased histamine release following stimulation with Cat-PAD peptides but did not significantly up-regulate activation markers CD63/CD203c. Prior to stimulation, 96-well flat-bottomed plates were coated in 10ug/ml of anti-CD3 antibody in PBS (vol=70ul) and left overnight at 4°C. Stimulation: PBMCs were isolated from whole blood and plated in 96-well flat-bottomed plates at a concentration of $5x10^6$/ml (vol=200ul). Anti-CD28 (2ug/ml, added to the anti-CD3-coated wells), Immunocult (25ul per 1ml culture), anti-IgE (1ug/ml), Fel d 1 (25ug/ml), Cat-PAD (0.1, 1, 10, and 25ug/ml) were added (vol=5ul) to PBMCs and they were stimulated for up to 10hrs in a 37°C incubator, 5% CO_2. Following stimulation, supernatants were collected, acylated, and stored at -20°C for histamine quantification. Cells were stained with CCR3, CD63, and CD203c for flow cytometric analysis (n=3). A) histamine release was measured in nanomolar at each timepoint, n=3 (y-axis). Histamine release was subtracted by spontaneous histamine release condition (baseline condition). B) Expression of CD63/CD203c was measured using flow cytometry (n=3) Threshold for CD63 and CD203c positivity is set at 5% in the unstimulated condition (y-axis). A) and B) Data points are shown as mean±SD. C) Fold-change compared to baseline (cRPMI) for histamine release was calculated for each condition (y-axis). Data bars are shown as mean±SD. Each dot represents a timepoint. n=3. D) Percent CD63/CD203c (y-axis) was averaged between each condition across all timepoints. Statistics: Kruskal-Wallis test with Dunn's multiple comparisons test was performed. Data bars are shown as mean±SD. Each dot represents one timepoint. Concentration of anti-IgE used in all experiments was 1ug/ml. Fel d 1, Felis domesticus polypeptide chain 1; AUC, area under curve; cRPMI, complete Roswell Park Memorial Institute

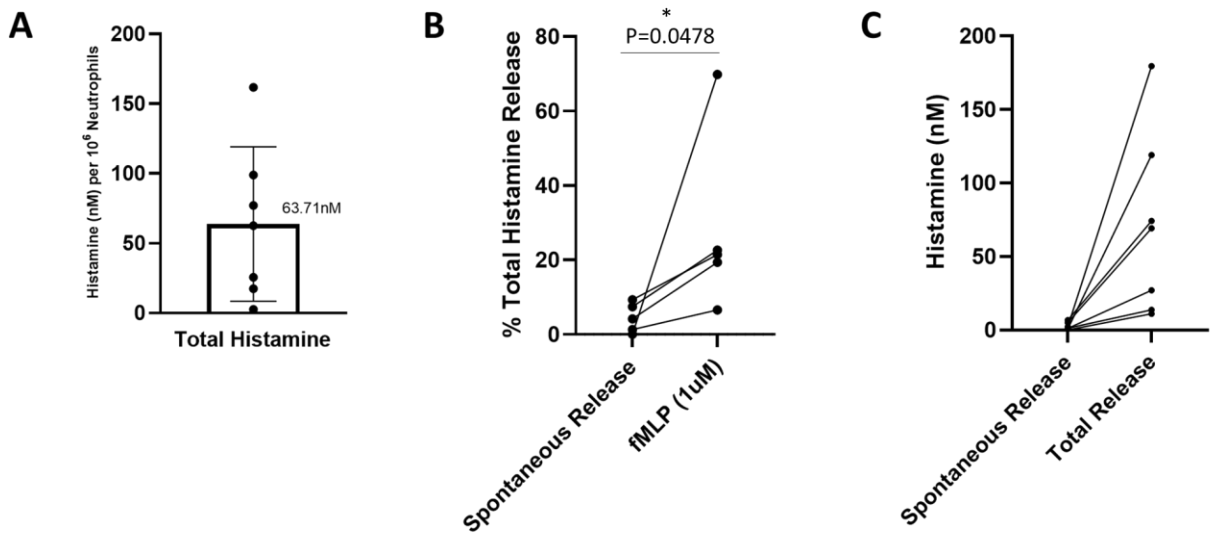


Figure 18. Neutrophils contain histamine and release histamine in response to stimulation with fMLP. Neutrophils were isolated from human peripheral blood via MACS and plated in 96-well flat-bottomed plates for stimulation with fMLP (30-minutes, in 37°C incubator, 5% CO_2). Following stimulation, cells were stained with antibodies to CD15, CD16, CD62L, CD66b, CD11b, and a live/dead marker. Supernatants were collected, acylated, and stored in -20°C for histamine quantification. A) Total histamine release was determined by freeze/thawing an equivalent number of isolated neutrophils to determine the total amount of histamine the samples contained. Due to varying purities obtained from isolation, histamine release was normalized to determine the quantity of histamine per 10^6 cells (y-axis). Data bars are shown as mean±SD. n=7, each point represents one experiment. B) Following isolation (identical to part A), neutrophils were incubated with fMLP as a positive control for 30-minutes at 37°C, 5% CO_2. Percent total histamine release was determined by dividing the histamine released in the fMLP condition by the total histamine release and multiplying by 100% (y-axis). Data points shown individual experiments. n=5. Statistics: One-tailed unpaired t test with Welch's correction was performed. C) Histamine release is shown compared to spontaneous histamine release across 7 experiments (each point represents one experiment). MACS, magnetic-activated cell sorting; fMLP, N-formyl-methionyl-leucyl-phenylalanine.

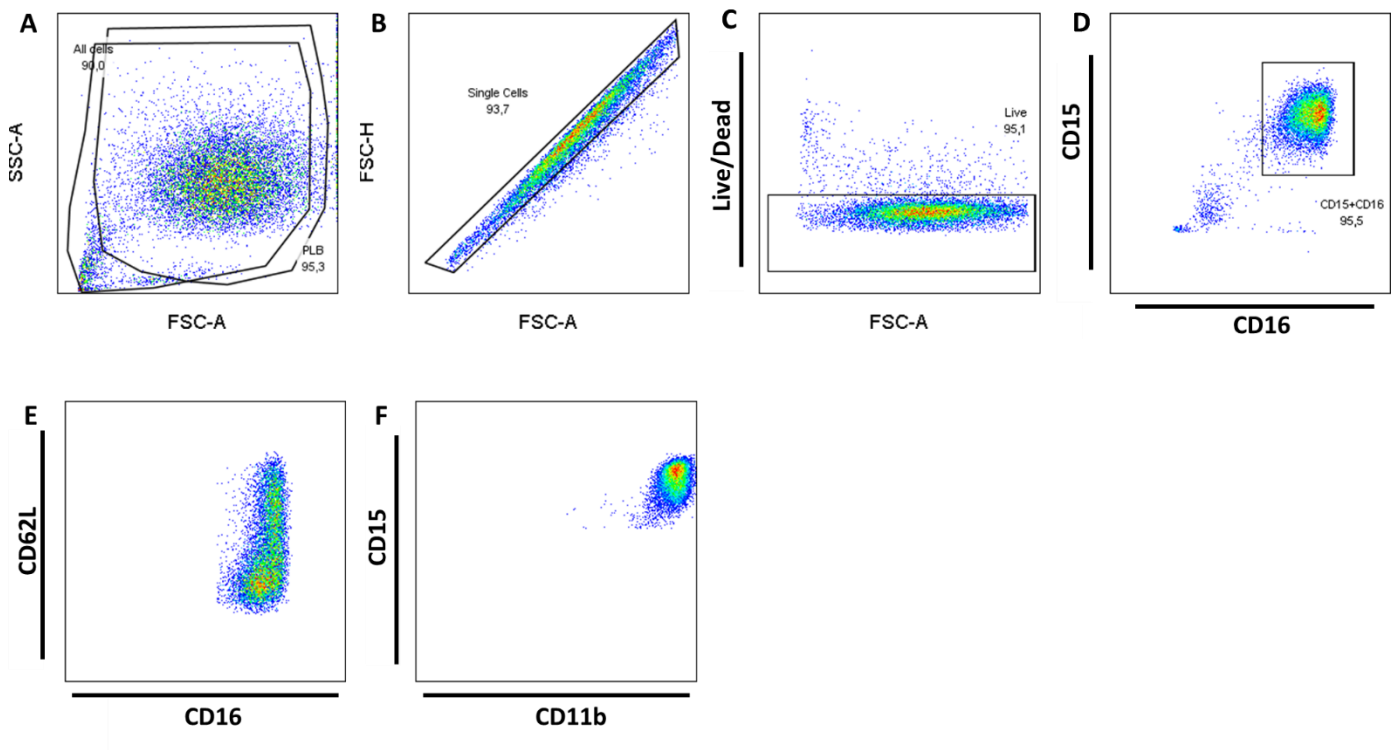


Figure 19. Representative gating strategy for isolated neutrophils. A) Debris and other contaminating cells are gated out. B) Doublets are gated out. C) Live cells are gated. D) Neutrophils express CD15 and CD16. E, F) unstimulated neutrophils.

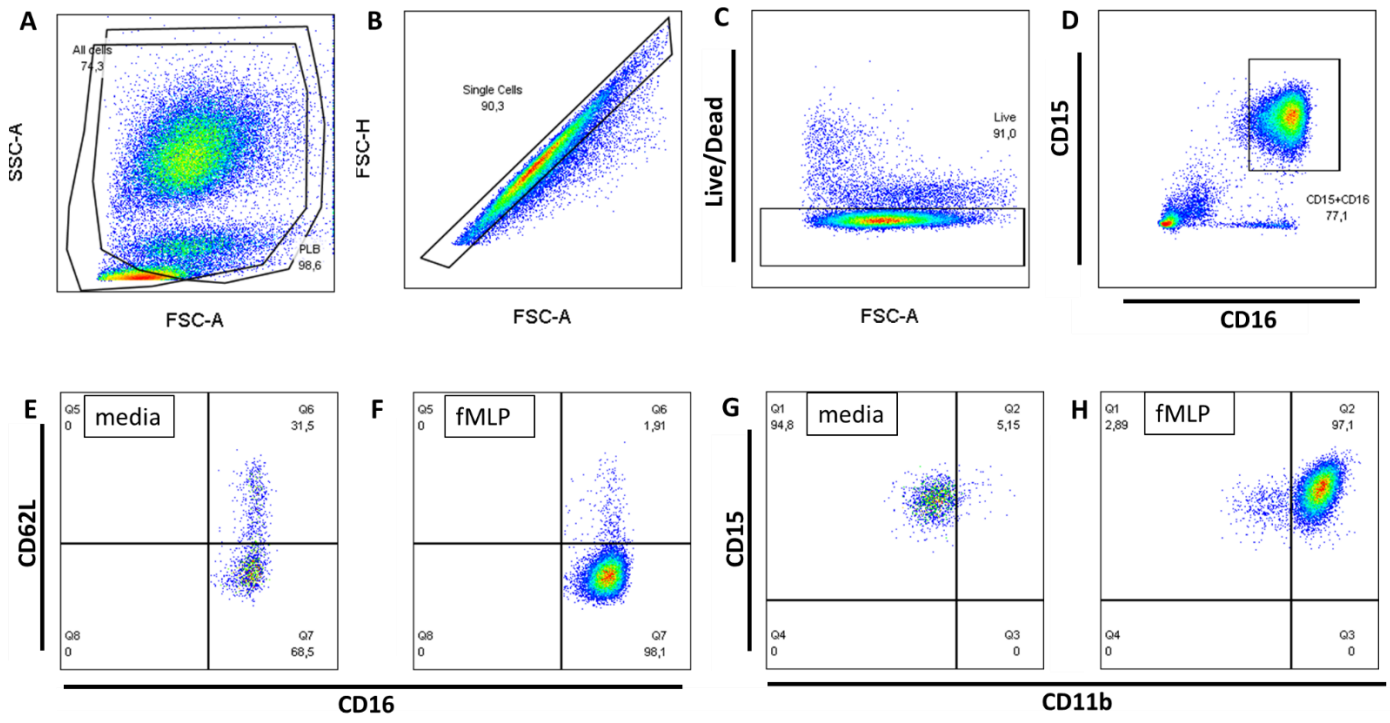


Figure 20. Representative gating strategy for whole blood. A) Debris and other contaminating cells are gated out. B) Doublets are gated out. C) Live cells are gated. D) Neutrophils express CD15 and CD16. E, G) unstimulated neutrophils (media). F, H) activated neutrophils (fMLP). fMLP, N-formyl-methionyl-leucyl-phenylalanine

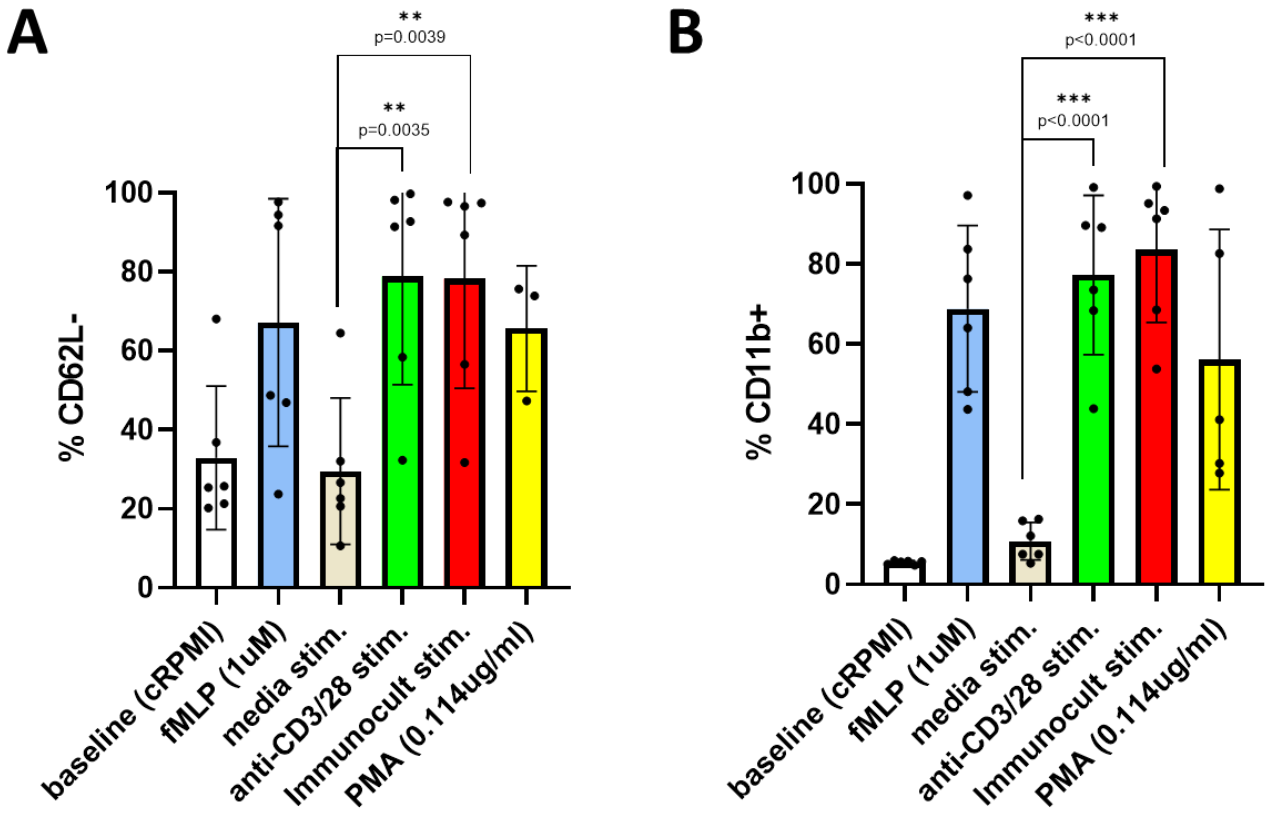


Figure 21. Whole blood neutrophil stimulation experiments with isolated CD4+ T cell supernatants – neutrophils significantly altered expression of CD62L and CD11b following incubation with CD4+ T cell supernatants. Supernatants were prepared by stimulating isolated CD4+ T cells at various concentrations (0.5-2.5x10^6 cells/ml) for 24hrs in media (cRPMI), immobilized anti-CD3 (10ug/ml) and soluble anti-CD28 (2ug/ml), or Immunocult (5ul/ml) using 6-well and 12-well flat-bottomed plates. Supernatants were collected at stored at -20℃. For stimulation: 100ul whole blood was incubated with 50ul activated T cell supernatants (media-, anti-CD3/28- or Immunocult-stimulated T cells) or control conditions and incubated for 40-minutes in a water-bath set to 37℃. Following incubation, red blood cells were lysed with RBC lysis buffer, and cells were stained for flow cytometric analysis using antibodies to CD15, CD16, CD62L, CD66b, CD11b, and a viability dye. A) Percent neutrophils (gated on CD15+ CD16+) that lost or did not express CD62L following incubation with T cell supernatants and controls. Threshold for the CD62L negative population was ~25% in the unstimulated conditions (outlier present). n=6. Statistics: one way ANOVA with Dunnett's multiple comparisons. Data bars are shown as mean±SD. B) Percent neutrophils (gated on CD15+ CD16+) that expressed CD11b following incubation with CD4+ T cell supernatants and controls. Threshold for positivity was set a 5% in the unstimulated condition. n=6. Statistics: one way ANOVA with Dunnett's multiple comparisons. Data bars are shown as mean±SD. PMA, phorbol 12-myristate 13-acetate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; RBC, red blood cell.

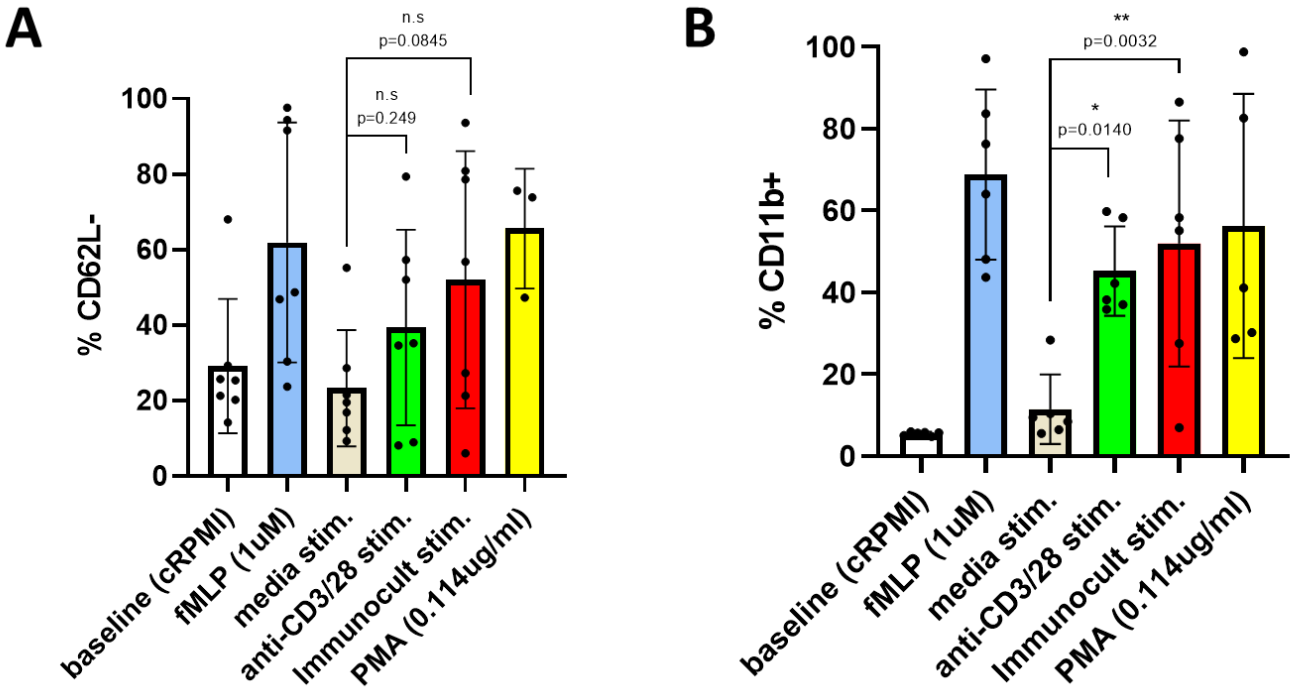
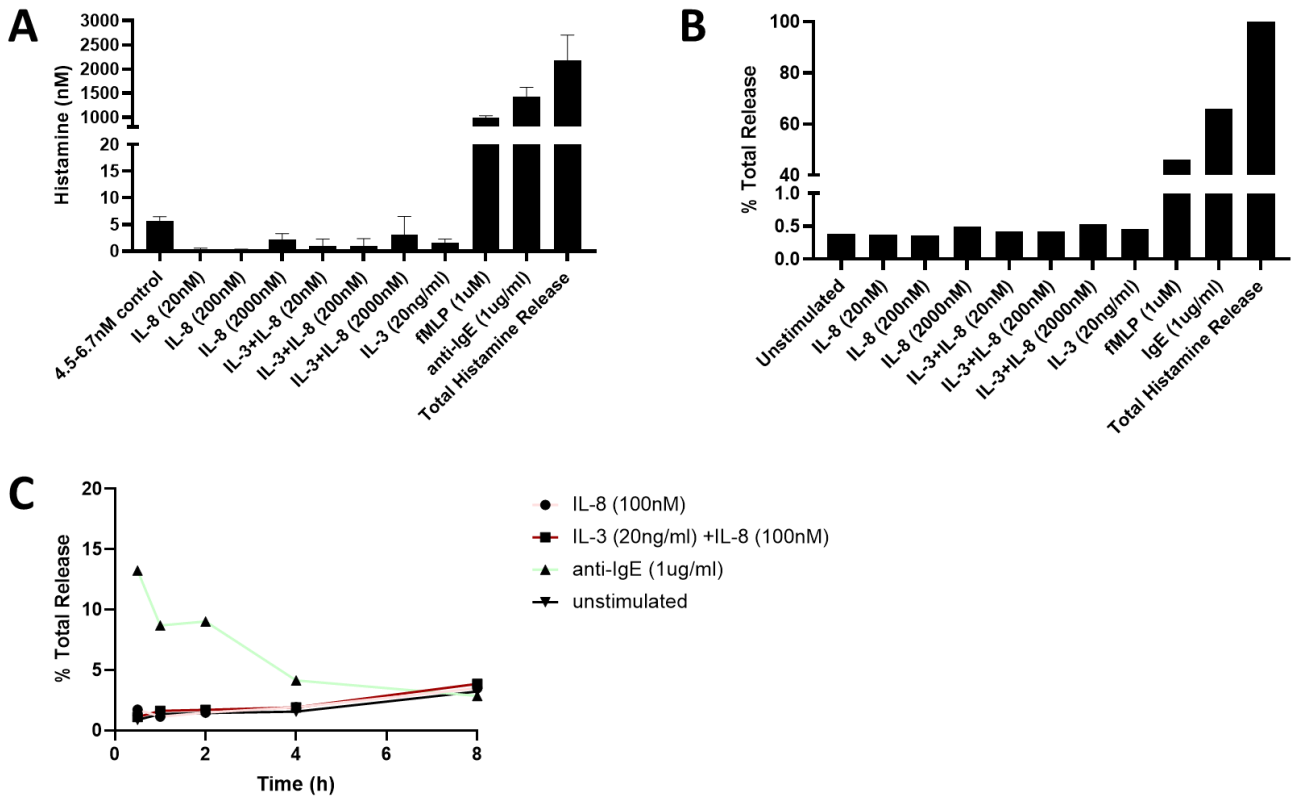


Figure 22. Whole blood neutrophil stimulation experiments with Jurkat T cell supernatants – neutrophils significantly up-regulated expression of CD11b following incubation with Jurkat T cell supernatants. Supernatants were prepared by stimulating Jurkat T cells at various concentrations (0.5-2.5x10^6 cells/ml) for 24hrs in media (cRPMI), immobilized anti-CD3 (10ug/ml) and soluble anti-CD28 (2ug/ml), or Immunocult (5ul reagent per 1ml) using 6-well, 12-well flat-bottomed plates. Supernatants were collected at stored at -20°C. For stimulation: 100ul whole blood was incubated with 50ul activated Jurkat T cell supernatants (media-, anti-CD3/28- or Immunocult-stimulated Jurkat T cells) or control conditions and incubated for 40-minutes in a water-bath set to 37°C. Following incubation, red blood cells were lysed with RBC lysis buffer, and cells were stained for flow cytometric analysis using antibodies to CD15, CD16, CD62L, CD66b, CD11b, and a viability dye. A) Percent neutrophils (gated on CD15+ CD16+) that downregulated CD62L following incubation with Jurkat T cell supernatants or control conditions. Threshold for the CD62L negative population was ~25% in the unstimulated conditions (outlier present). n=6. Statistics: one way ANOVA with Dunnett's multiple comparisons. Data bars are shown as mean±SD. B) Percent neutrophils (gated on CD15+ CD16+) that up-regulated CD11b following incubation with T cell supernatants and controls. Threshold for CD11b positivity was set a 5% in the unstimulated condition. n=6. Statistics: one way ANOVA with Dunnett's multiple comparisons. Data bars are shown as mean±SD. PMA, phorbol 12-myristate 13-acetate; fMLP, N-formyl-methionyl-leucyl-phenylalanine; RBC, red blood cell.

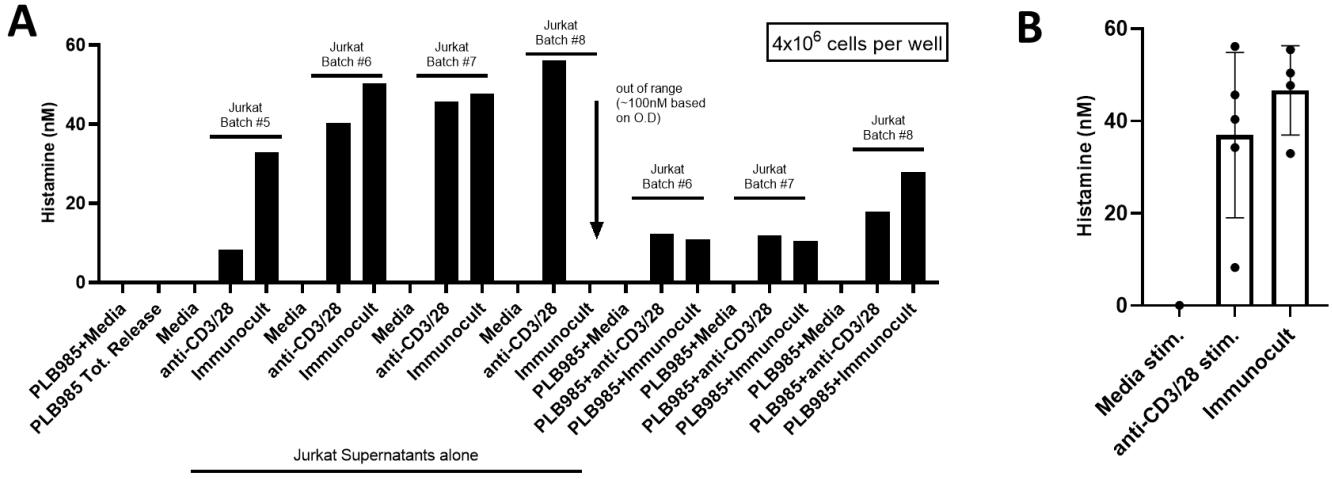
APPENDIX

Product	Batch/Lot#	Manufactured By	Sequence
MLA01	CIRy026	Bachem AG	CPAVKRDVDLFLT
MLA03	1030164	Bachem AG	EQVAQYKALPVVLENA
MLA04	1030157	Bachem AG	KALPVVLENARILNCV
MLA05	1036151	Bachem AG	RILKNCVDAKMTEEDKE
MLA07	1030162	Bachem AG	KENALSLLDKIYTSPL
MLA12	1030165	Bachem AG	TAMKKIQDCYVENGLI
MLA14	1030163	Bachem AG	SRVLDGLVMTTISSSK

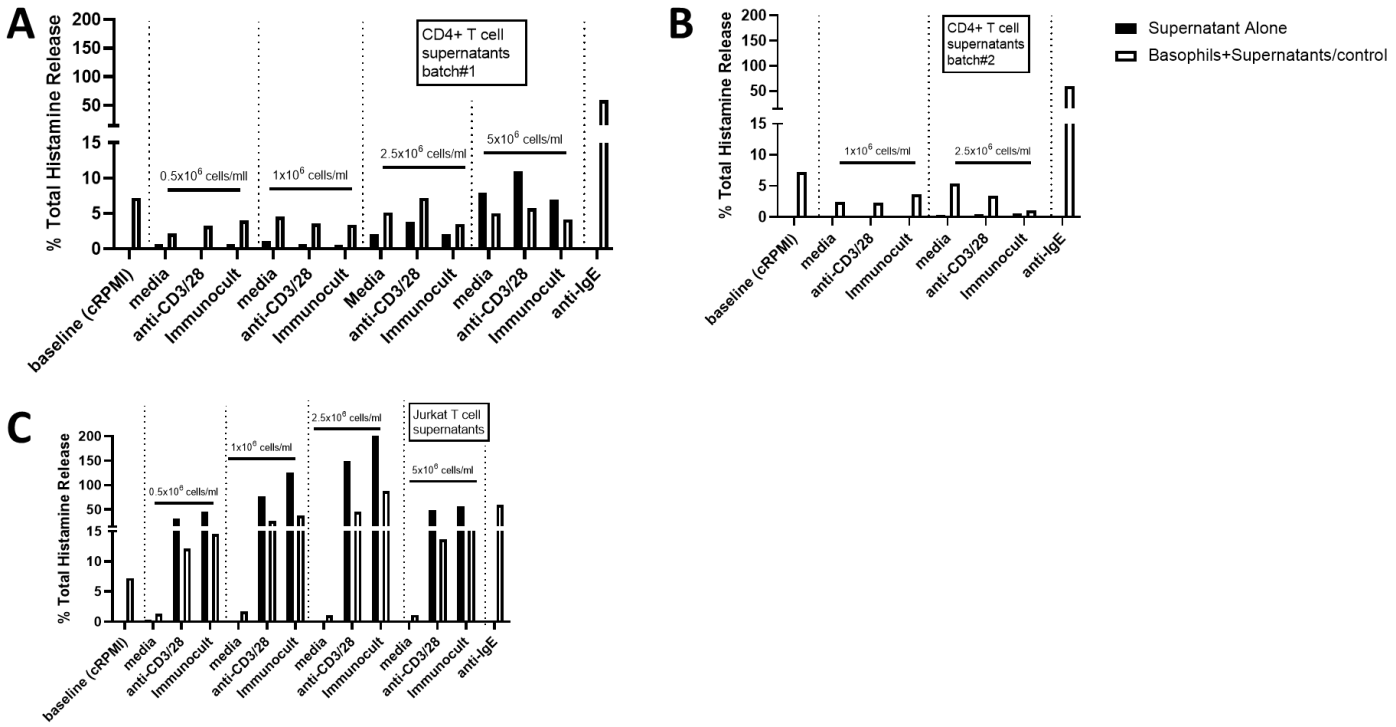
Appendix S1. Cat-PAD peptide composition



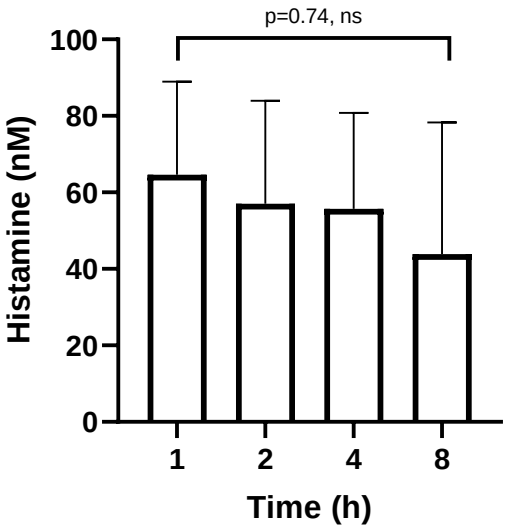
Appendix S2. Basophils stimulated with IL-3 and/or IL-8 did not induce basophils to release histamine. Whole blood and isolated basophils were incubated with IL-3 (20ng/ml) and IL-8 (100nM) for histamine release assay. A) and B) Representative figures from one experiment. 100ul of whole blood basophils were primed with 25ul of IL-3 for 5 minutes in 37°C water bath followed by 15-minute incubation with 25ul IL-8, plus 15ul of antibodies for flow cytometry analysis (total volume=175ul). Following stimulation, samples were centrifuged, and supernatants were collected, acylated, and stored at -20°C. The concentration of histamine in the sample was quantified by ELISA. A) Spontaneous release values were subtracted from all conditions (y-axis). Experiment was performed in duplicates. Data points are shown as mean±SD. B) Total histamine release (y-axis) was calculated by dividing all conditions by the total histamine release (2174.99nM) and multiplied by 100. Experiment was performed as a singlet. C) Before stimulation, basophils were isolated from whole blood using immunomagnetic negative selection and resuspended in media at $4x10^4$/ml (volumes are identical to protocol used in part A, B). Basophils were stimulated for 0.5h, 2h, 4h, 8h. Samples were processed and collected for histamine release identical to part A, B. Total histamine release was averaged between all timepoints (47.24nM).



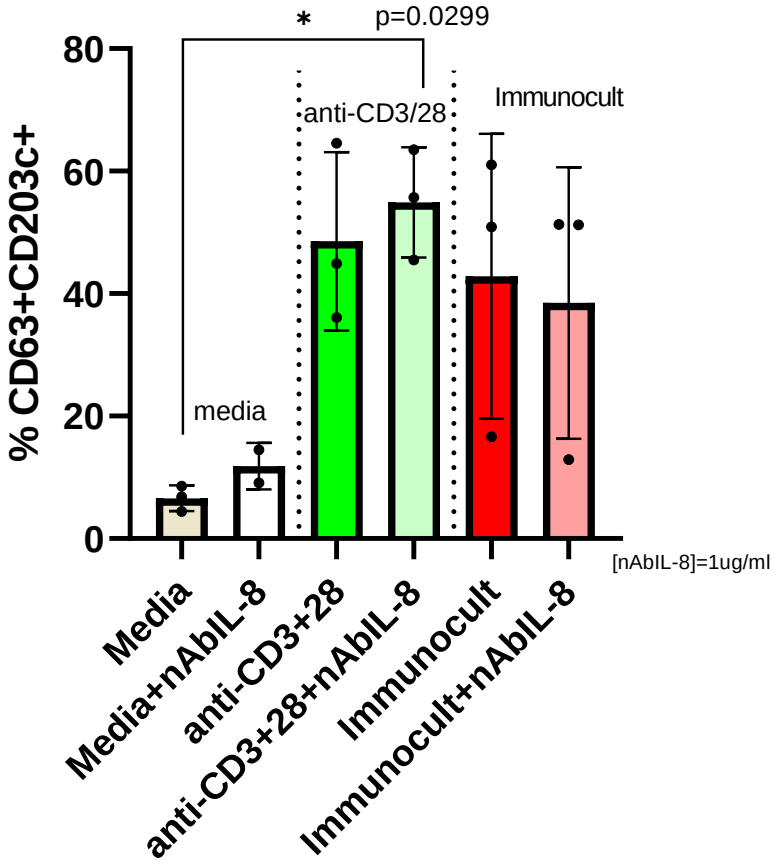
Appendix S3. Neutrophil-like cell line PLB985 histamine release assay demonstrated Jurkat T cells contain histamine. A) 100ul of $4x10^6$ cells/ml of a neutrophil-like cell line, PLB985, was plated in a 96-well plate and incubated with 50ul of Jurkat T cell supernatants previously prepared for 30-minutes in a 37°C-water bath (vol=50ul, PBS was used as control). Supernatants were prepared by stimulating Jurkat T cells at $1x10^6$/ml for 24hrs in media (cRPMI), immobilized anti-CD3 (10ug/ml) and anti-CD28 (2ug/ml), or Immunocult (5ul/ml) using 96-well flat-bottomed plates. Immobilized anti-CD3 was prepared by coating the wells in 70ul of 10ug/ml anti-CD3 and leaving in 4°C fridge overnight. Wells were washed with PBS at least twice. Following 24h stimulation, supernatants were collected at stored at -20°C. Samples were then centrifuged, and supernatants were collected, acylated, and stored at -20°C. The concentration of histamine in the sample was quantified by ELISA. Total histamine release was determined by freezing/thawing an equivalent number of PLB-965 cells ($4x10^6$) to determine the total amount of histamine the samples. One data bar was missing in Immunocult condition because it was above the detection range of the assay. B) In a subsequent experiment, protocol was repeated using supernatants from multiple batches of Jurkat T cells (#5-9). Data points are shown as mean±SD. Histamine was quantified by ELISA. Histamine was quantified in Jurkat supernatants alone (batches #5-8). Data bars are shown as mean±SD. 100ul of isolated basophils ($4x10^6$ cells/ml) were resuspended in 100ul media (cRPMI) and 50ul of cRPMI, anti-CD3/28, or Immunocult-stimulated Jurkat T cell media for 30-minutes (total vol=150ul).



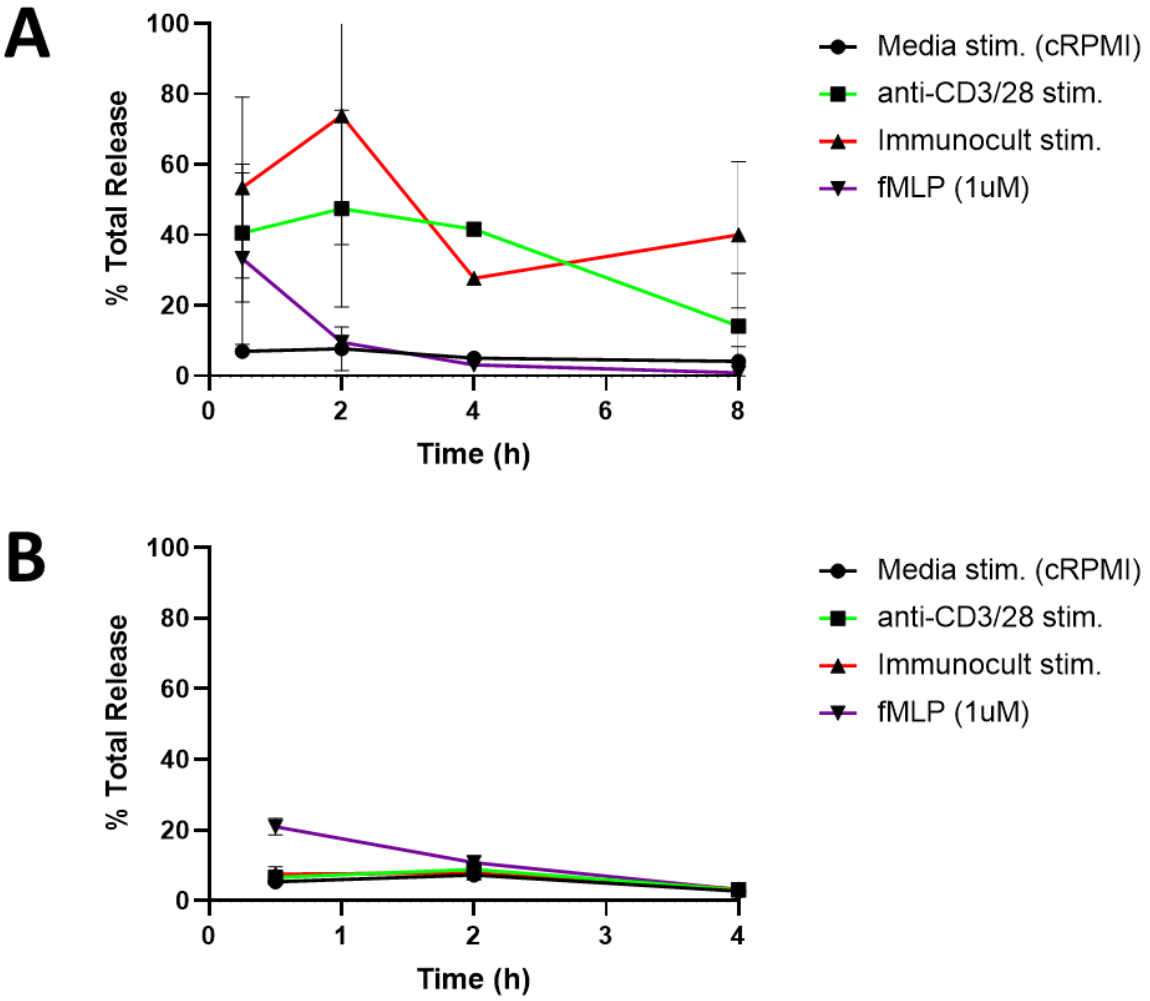
Appendix S4. Supernatants from T cells did not induce histamine release from basophils. Basophils were isolated from whole blood from subject with unknown allergy status using immunomagnetic negative selection. Supernatants were prepared by stimulating Jurkat or isolated CD4+ T cells at 0.5-5x10^6 cells/ml for 24hrs in media (cRPMI), anti-CD3 (10ug/ml) and anti-CD28 (2ug/ml), or Immunocult (25ul/ml) using 96-well flat-bottomed plates. Supernatants were collected at stored at -20°C. 100ul of isolated basophils (4x10^4/ml) were resuspended in cRPMI and an additional 50ul of cRPMI, anti-CD3/28, or Immunocult-stimulated Jurkat T cell media for 0.5h at 37°C. 15ul of antibody master mix to CCR3, CD63, and CD203c were added for flow cytometric analysis (total vol=165ul). Following stimulation, samples were centrifuged, and supernatants were collected, acylated, and stored at -20°C. The cells were stained for flow cytometry using antibodies to CCR3, CD63, and CD203c. The concentration of histamine in the sample was quantified by ELISA. Total histamine release was determined by freeze/thawing an equivalent number of basophils to determine the total amount of histamine the samples contained. Percent total histamine was determined by dividing the histamine released in each condition by the total histamine (y-axis). Basophils incubated with 1ug/ml of anti-IgE was used as a positive control. Basophils incubated with cRPMI alone are the baseline conditions. A) and B) Two different CD4+ T cell supernatants were used to generate results. A) Basophil histamine release graph showing Jurkat T cell supernatants at various concentrations incubated with basophils. B) Basophil histamine release graph showing isolated CD4+ T cell supernatants at various concentrations incubated with basophils. C) Basophil histamine release graph showing Jurkat T cell supernatants at various concentrations incubated with basophils. Experiment was performed in singlicate (n=1). Black bars: 100ul T cell supernatants alone. White bars: 100ul basophils, 50ul T cell supernatants. cRPMI, complete Rosewell Park Memorial Institute. Statistics: two-way mixed model ANOVA.



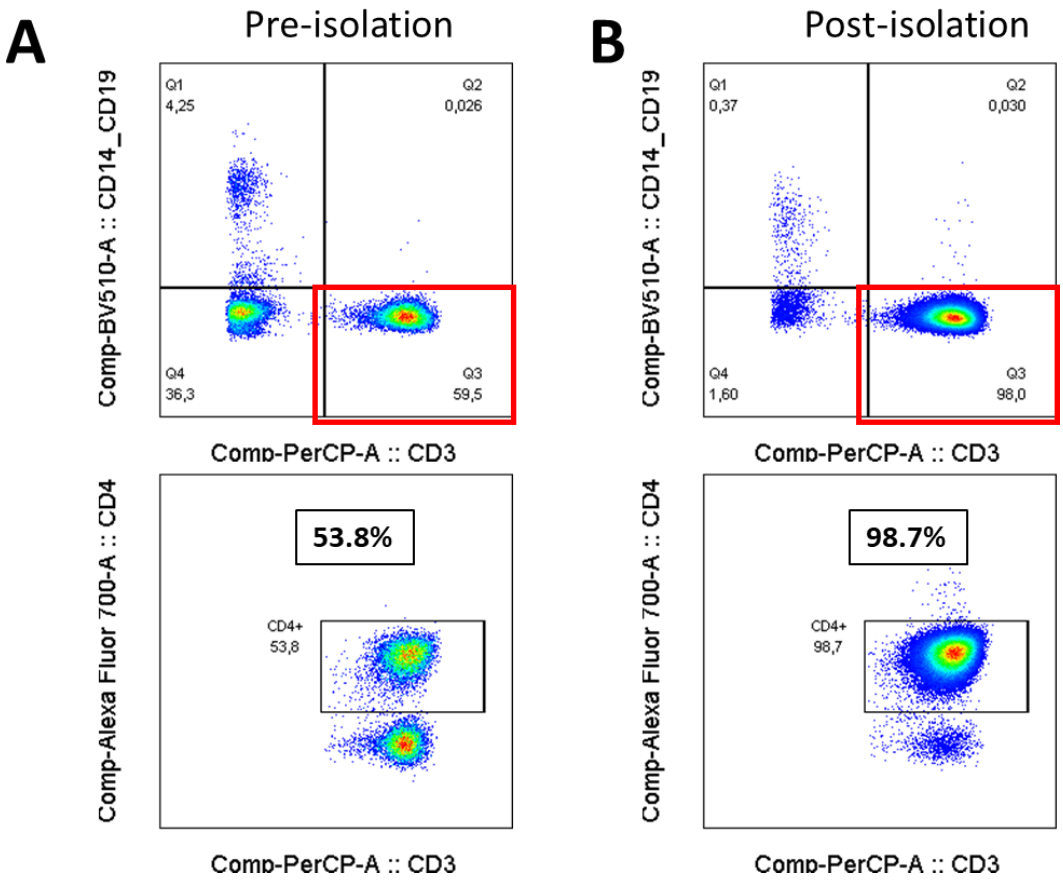
Appendix S5. Total histamine released from isolated basophils does not significantly degrade in samples over the course of 8h. 100ul of isolated basophils in cRPMI were freeze/thawed twice at time 0, and acylated at the following time points (1, 2, 4, 8h). Basophils were stored a room temperature until ready for acylation. Following acylation, samples were stored at -20°C. Data bars represent mean±SD. Statistics: Kruskal-Wallis test using Dunn's multiple comparisons. n=3 at timepoints 1h, 2h, 4h, and n=2 at timepoint 8h. cRPMI, complete Roswell Park Memorial Institute.



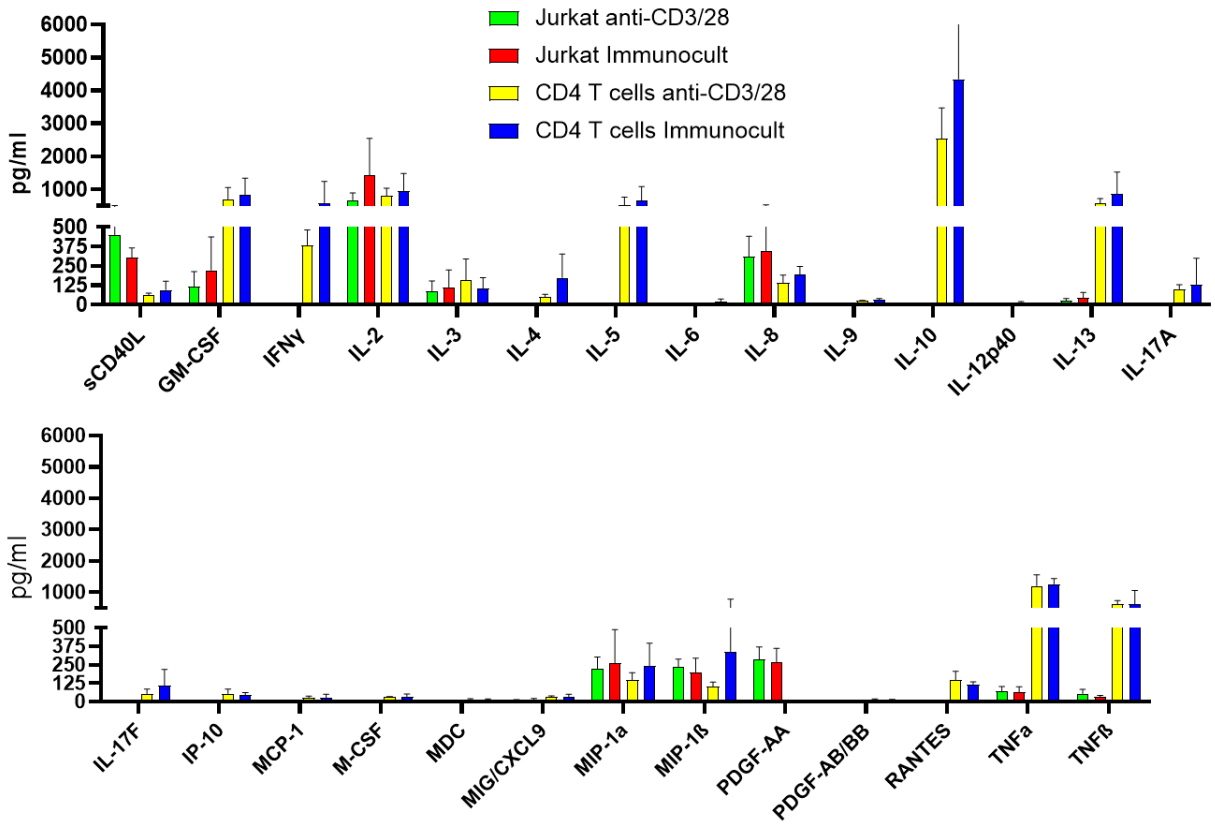
Appendix S6. Neutralizing IL-8 did not affect expression of basophil activation markers CD63/CD203c. Supernatants from stimulated T cells were prepared as previously described. Prior to stimulation experiment, supernatants were pre-incubated with or without 1ug/ml neutralizing anti-IL-8 antibody (nAbIL-8) for 30-minutes at room temperature, then incubated with isolated basophils at 37°C for 15-minutes. NAbIL-8 was previously titrated, and 1ug/ml was determined to be more than the minimum required concentration to completely neutralize IL-8 in supernatants. Data bars are shown as mean±SD. n=2, minimum. To determine the relative contribution IL-8 had in activating basophils, Jurkat and CD4+ T cell supernatants were pre-incubated with 1ug/ml nAbIL-8 to neutralize IL-8, then supernatants were incubated with isolated basophils (basophils were isolated from whole blood using immunomagnetic negative selection) for 30-minutes in a 37°C water-bath. Cells were stained following incubation as previously described. By comparing basophil activation to supernatants with and without the addition of nAbIL-8, it was possible to see if IL-8 had a role in activating basophils. Threshold for CD63 and CD203c positivity is set at 5% in unstimulated cells (y-axis). nAbIL-8, neutralizing anti-IL-8 antibody



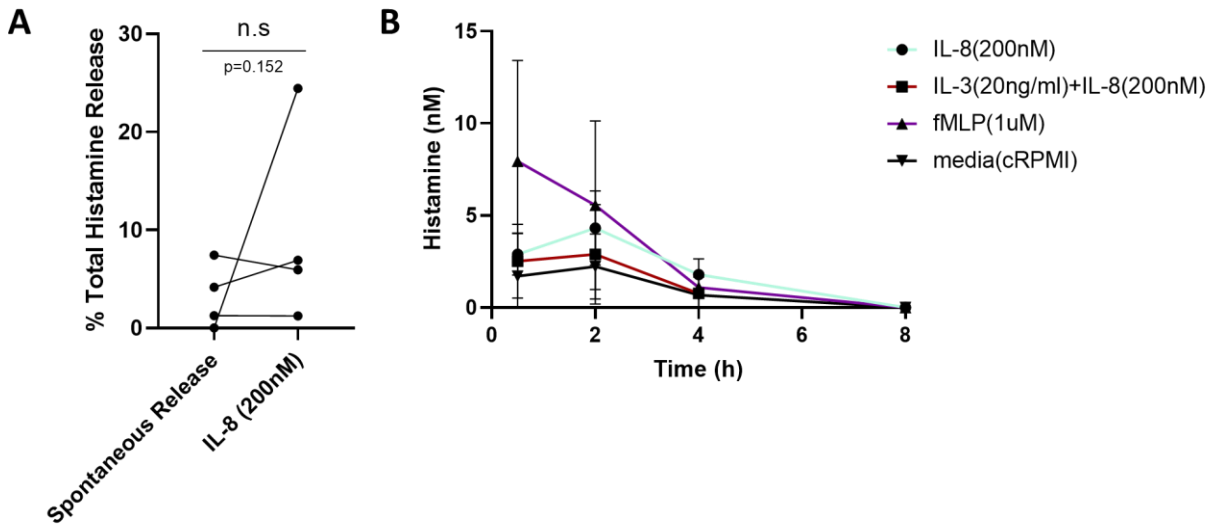
Appendix S7. Isolated neutrophils were stimulated with supernatants derived from activated Jurkat T cells (A) and CD4+ T cells (B). The concentration of histamine in the sample was quantified by ELISA. Total histamine release was determined by freezing and thawing an equivalent number of cells to determine the total amount of histamine in the samples. Percent total histamine release was determined by dividing the histamine released in each condition by the total histamine and multiplying by 100 (% total release, y-axis). 100ul of neutrophils were plated and stimulated with 50ul T cell supernatant. Samples were collected and acylated at 0.5h, 2h, 4h, 8h and/or 0.5h, 2h, 4h. A) Supernatant derived from media-, anti-CD3/28-, or Immunocult-stimulated Jurkat T cells. n=5 total, n=1 at 4h timepoint. B) Supernatant derived from media, anti-CD3/28, or Immunocult-stimulated isolated CD4+ T cells. n=3. Statistics: one-way ANOVA with Sidak's multiple comparisons test. Data points are shown as mean±SD.



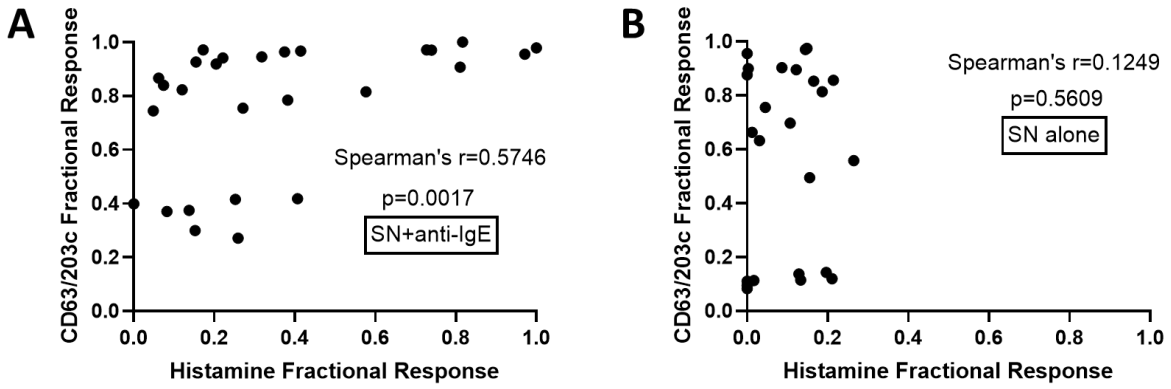
Appendix S8. Representative flow cytometric analysis of purity of isolated CD4+ T cells from human peripheral blood. Following isolation of PBMCs, CD4+ T cells were separated via immunomagnetic negative selection. Cells shown were gated on live cells. The two bottom plots were gated on CD3+ CD14- CD19- (red box). Cells were stained prior to isolation (A) and following isolation (B). Isolation purities which obtained less than 95% CD4+ T cells were excluded from downstream experiments. PBMCs, peripheral blood mononuclear cells.



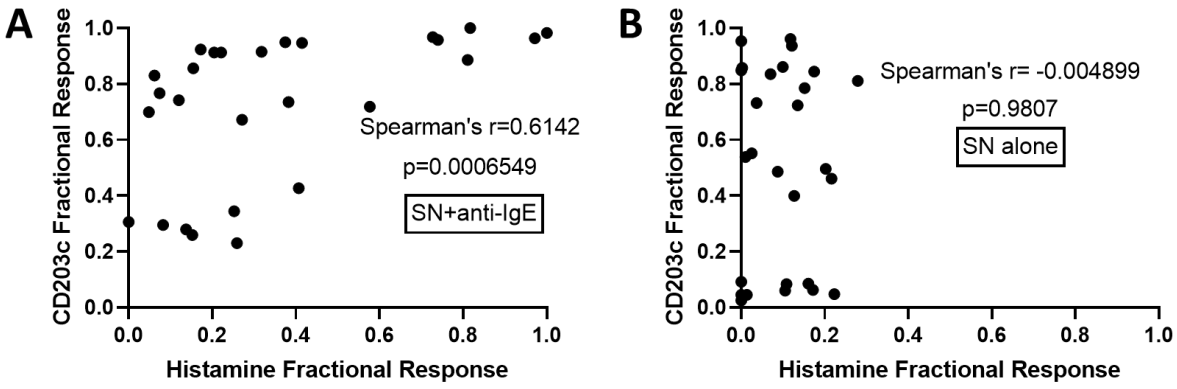
Appendix S9. Human Cytokine 48-plex assay was performed to determine which cytokines and chemokines are released from Jurkat and isolated CD4+ T cells. Isolated CD4+ T cells and Jurkat T cells were stimulated at $2.5x10^6$ cells/ml and $1x10^6$ cells/ml (respectively) 6-well or 12-well flat-bottomed plates for 24hrs. Prior to stimulation, plates were coated in 10ug/ml of anti-CD3 antibody in PBS for immobilization. 6-well or 12-well plates were coated in 1ml of 10ug/ml anti-CD3 overnight at 4°C. Plates were washed 3x with sterile PBS and Jurkat T cells ($1x10^6$ cells/ml) or isolated CD4+ T cells ($2.5x10^6$ cells/ml) were plated. Anti-CD28 was added to the anti-CD3-coated wells at a concentration of 2ug/ml, and Immunocult was added to non-anti-CD3 coated wells (5ul reagent per 1ml culture). Jurkat or isolated CD4+ T cells were incubated for 24hrs at 37°C, 5% CO2. Following incubation, supernatants were collected and stored in -20°C until ready to be sent for multiplex assay analysis by EveTechnologies. Forty-eight cytokines and chemokines were measured. Supernatants with a detectable signal were plotted in the bar graph. Because isolated CD4+ T cells were stimulated at 2.5 times the concentration of Jurkat T cells, cytokine concentrations were divided by 2.5. Spontaneous release values (cRPMI) were subtracted from all conditions. The following mediators were excluded from the graph due to undetectable signals: EGF, Eotaxin, FGF-2, FLT-3L, Fractalkine, G-CSF, GROα, IFN-a2, IL-1a, IL-1b, IL-1Ra, IL7, IL-12p70, IL-15, IL-17E/IL-25, IL-18, IL-22, IL-27, MCP-3, TGFα, VEGF-A. Data bars are shown as mean±SD. n=3. Three replicates per condition



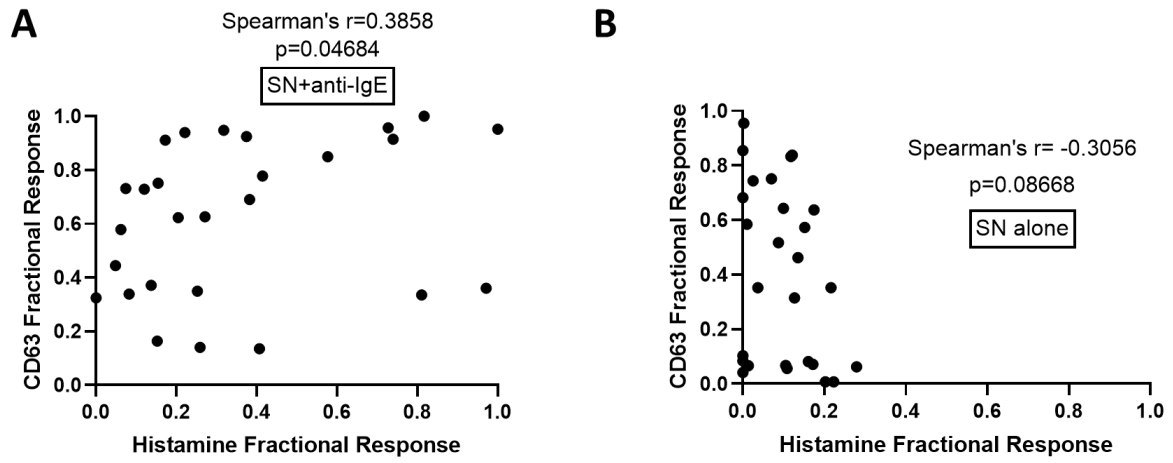
Appendix S10. IL-8-stimulated neutrophils did not significantly increase histamine release in isolated neutrophils, compared to spontaneous release. A) Neutrophils were isolated from human peripheral blood via MACS and plated in 96-well flat-bottomed plates for stimulation with IL-8 (30-minutes in 37°C incubator, 5% CO_2.) Following stimulation, cells were stained with antibodies to CD15, CD16, CD62L, CD66b, CD11b, and a live/dead marker. Supernatants were collected, acylated, and stored in -20°C for histamine quantification. A) Total histamine release was determined by freeze/thawing an equivalent number of isolated neutrophils to determine the total amount of histamine the samples contained. Percent total histamine release was determined by dividing the histamine released in the fMLP condition by the total histamine release and multiplying by 100% (y-axis). Data bars are shown as mean±SD. n=4, each point represents one experiment. Statistics: One-tailed Unpaired t test with Welch's correction. B) Procedure identical to (A) except neutrophils were incubated for up to 8 hours with fMLP, IL-8, or IL-3+IL-8. At 30-minutes, 2h, and 8h, supernatants were collected, acylated, and stored in -20°C for histamine quantification. Data points are shown as mean±SD, n=3 minimum.



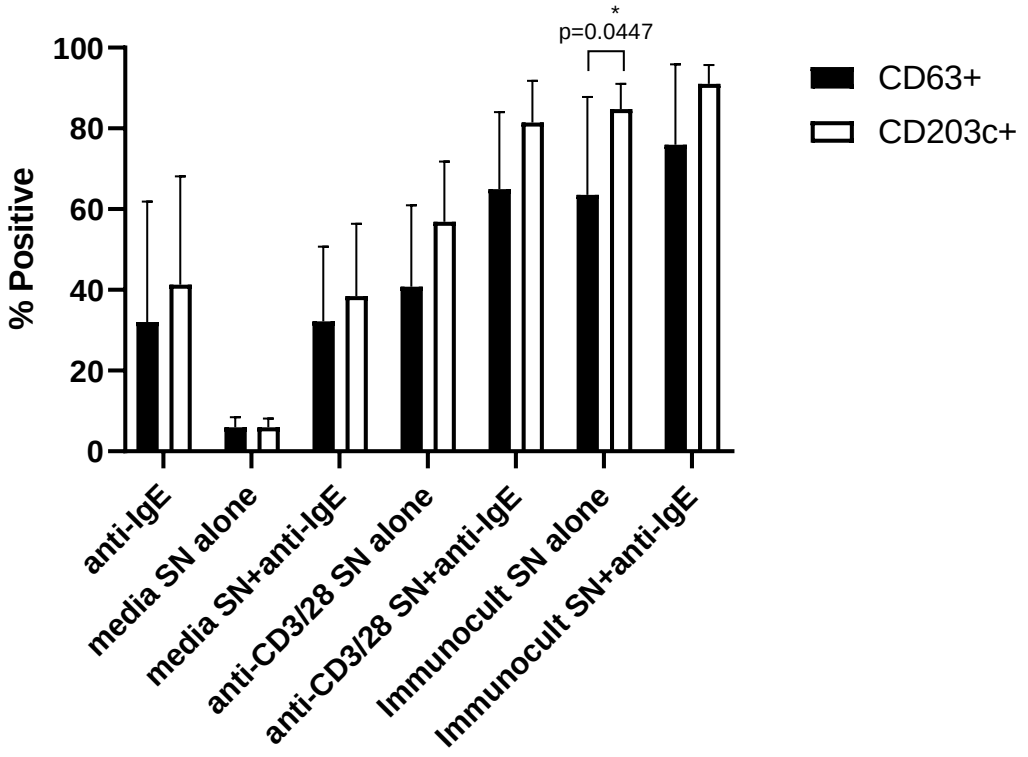
Appendix S11. Correlation between histamine release (x-axis) and expression of CD63/CD203c (y-axis). Supernatant generation: T cells were stimulated at concentrations ranging from 0.5-2.5x10^6/ml in 6-well, 12-well, or 96-well flat-bottomed plates for 24hrs. Prior to stimulation, plates were coated in 10ug/ml of anti-CD3 antibody in PBS for immobilization. 6-well or 12-well plates were coated in 1ml of 10ug/ml anti-CD3. T cells were plated and stimulated with cRPMI, anti-CD3 (10ug/ml) and anti-CD28 (2ug/ml) or Immunocult (5ul reagent per 1ml) for 24h at 37°C, 5% CO_2. Supernatants were collected and stored at -20°C. Stimulation protocol: 100ul of whole blood was incubated with 50ul of supernatant or PBS for control, for 15-minutes in a 37°C-water bath (priming). 15ul antibodies to CCR3, CD63, and CD203c were added for flow cytometric analysis. Next, anti-IgE was added to the conditions as a positive control for an additional 15-minutes. Following incubation, RBCs were lysed with 2ml RBC lysis buffer. Cells were washed and fixed for flow cytometric analysis. Threshold for CD63 and CD203c positivity was set at 5% in unstimulated cells (y-axis). n=4. Baseline (unstimulated) values excluded in correlation assessment. Analysis: Fractional response for histamine release (x-axis) and CD63/CD203c expression (y-axis) was determined by dividing all values by the highest response value. Conditions: media-stimulated, anti-CD3/28-stimulated, and Immunocult-stimulated T cells Spearman's r was used to determine the correlation between histamine response and CD63/CD203c response in basophils. A) Basophils were primed with T cell supernatants for 15-minutes, following by anti-IgE stimulation for 15-minutes. B) Basophils were stimulated with T cell supernatants alone. SN, supernatant; RBC, red blood cell; cRPMI, complete Rosewell Park Memorial Institute.



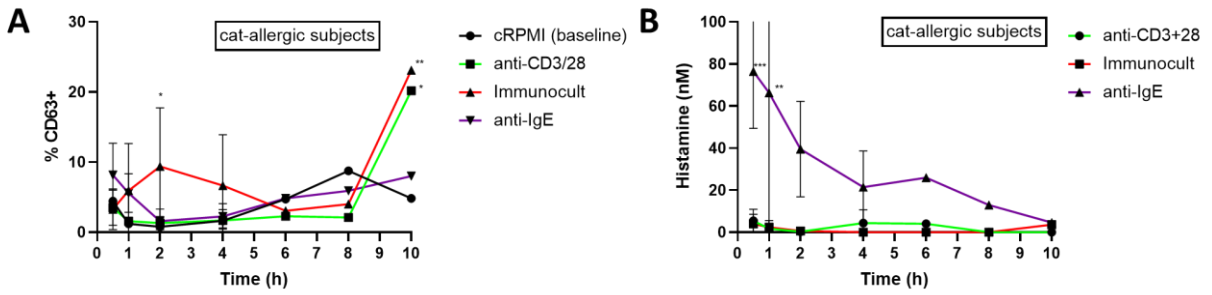
Appendix S12. Correlation between histamine release (x-axis) and expression of CD203c (y-axis). Supernatant generation: T cells were stimulated at concentrations ranging from 0.5-2.5x10^6/ml in 6-well, 12-well, or 96-well flat-bottomed plates for 24hrs. Prior to stimulation, plates were coated in 10ug/ml of anti-CD3 antibody in PBS for immobilization. 6-well or 12-well plates were coated in 1ml of 10ug/ml anti-CD3. T cells were plated and stimulated with cRPMI, anti-CD3 (10ug/ml) and anti-CD28 (2ug/ml) or Immunocult (5ul reagent per 1ml) for 24h at 37℃, 5% CO_2. Supernatants were collected and stored at -20℃. Stimulation protocol: 100ul of whole blood was incubated with 50ul of supernatant or PBS for control, for 15-minutes in a 37℃-water bath (priming). 15ul antibodies to CCR3, CD63, and CD203c were added for flow cytometric analysis. Next, anti-IgE was added to the conditions as a positive control for an additional 15-minutes. Following incubation, RBCs were lysed with 2ml RBC lysis buffer. Cells were washed and fixed for flow cytometric analysis. Threshold for CD203c positivity was set at 5% in unstimulated cells (y-axis). n=4. Baseline (unstimulated) values excluded in correlation assessment. Analysis: Fractional response for histamine release (x-axis) and CD203c expression (y-axis) was determined by dividing all values by the highest response value. Conditions: media-stimulated, anti-CD3/28-stimulated, and Immunocult-stimulated T cells Spearman's r was used to determine the correlation between histamine response and CD203c response in basophils. A) Basophils were primed with T cell supernatants for 15-minutes, following by anti-IgE stimulation for 15-minutes. B) Basophils were stimulated with T cell supernatants alone. SN, supernatant; RBC, red blood cell; cRPMI, complete Rosewell Park Memorial Institute.



Appendix S13. Correlation between histamine release (x-axis) and expression of CD63 (y-axis). Supernatant generation: T cells were stimulated at concentrations ranging from 0.5-2.5x10^6/ml in 6-well, 12-well, or 96-well flat-bottomed plates for 24hrs. Prior to stimulation, plates were coated in 10ug/ml of anti-CD3 antibody in PBS for immobilization. 6-well or 12-well plates were coated in 1ml of 10ug/ml anti-CD3. T cells were plated and stimulated with cRPMI, anti-CD3 (10ug/ml) and anti-CD28 (2ug/ml) or Immunocult (5ul reagent per 1ml) for 24h at 37℃, 5% CO_2. Supernatants were collected and stored at -20℃. Stimulation protocol: 100ul of whole blood was incubated with 50ul of supernatant or PBS for control, for 15-minutes in a 37℃-water bath (priming). 15ul antibodies to CCR3, CD63, and CD203c were added for flow cytometric analysis. Next, anti-IgE was added to the conditions as a positive control for an additional 15-minutes. Following incubation, RBCs were lysed with 2ml RBC lysis buffer. Cells were washed and fixed for flow cytometric analysis. Threshold for CD63 positivity was set at 5% in unstimulated cells (y-axis). n=4. Baseline (unstimulated) values excluded in correlation assessment. Analysis: Fractional response for histamine release (x-axis) and CD63 expression (y-axis) was determined by dividing all values by the highest response value. Conditions: media-stimulated, anti-CD3/28-stimulated, and Immunocult-stimulated T cells Spearman's r was used to determine the correlation between histamine response and CD63 response in basophils. A) Basophils were primed with T cell supernatants for 15-minutes, following by anti-IgE stimulation for 15-minutes. B) Basophils were stimulated with T cell supernatants alone. SN, supernatant; RBC, red blood cell; cRPMI, complete Rosewell Park Memorial Institute



Appendix S14. Differences in expression between CD63 and CD203c were compared for each stimulation condition. Supernatant generation: T cells were stimulated at concentrations ranging from 0.5-2.5x10^6/ml in 6-well, 12-well, or 96-well flat-bottomed plates for 24hrs. Prior to stimulation, plates were coated in 10ug/ml of anti-CD3 antibody in PBS for immobilization. 6-well or 12-well plates were coated in 1ml of 10ug/ml anti-CD3. T cells were plated and stimulated with cRPMI, anti-CD3 (10ug/ml) and anti-CD28 (2ug/ml) or Immunocult (5ul reagent per 1ml) for 24h at 37℃, 5% CO_2. Supernatants were collected and stored at -20℃. Stimulation protocol: 100ul of whole blood was incubated with 50ul of supernatant or PBS for control, for 15-minutes in a 37℃-water bath (priming). 15ul antibodies to CCR3, CD63, and CD203c were added for flow cytometric analysis. Next, anti-IgE was added to the conditions as a positive control for an additional 15-minutes. Following incubation, RBCs were lysed with 2ml RBC lysis buffer. Cells were washed and fixed for flow cytometric analysis. Threshold for CD63 and CD203c positivity was set at 5% in unstimulated cells (y-axis). n=4. Baseline (unstimulated) values excluded. Analysis: The percent positive CD63 and CD203c were separated and plotted on the y-axis to see if expression of CD63 and CD203c were significantly different from each other in any stimulation condition. Statistics: Two-way ANOVA mixed-effects analysis with Sidak's multiple comparisons test was performed. SN, supernatant; RBC, red blood cell; cRPMI, complete Rosewell Park Memorial Institute.



Appendix S15. Discordance between expression of CD63 and histamine release in allergic subjects. PBMCs were isolated from whole blood and plated in 96-well flat-bottomed plates at a concentration of $5x10^6$/ml (vol=200ul). Prior to stimulation, 96-well flat-bottomed plates were coated in 10ug/ml of anti-CD3 antibody in PBS (vol=70ul) and left in 4°C overnight. Wells were washed in PBS at least twice prior to stimulation. PBMCs were plated and 5ul of stimulants were added: to the immobilized anti-CD3 coated wells, soluble anti-CD28 (2ug/ml) was added; to separate wells: Immunocult (25ul/ml), anti-IgE (1ug/ml). PBMCs were stimulated for up to 10hrs in a 37°C incubator, 5% CO_2. Following stimulation, supernatants were collected, acylated, and stored at -20°C for histamine quantification. Cells were stained with CCR3, CD63, and CD203c for flow cytometric analysis (n=3). A) Expression of CD63 was measured using flow cytometry (n=3). Threshold for CD63 positivity was set at 5% in the unstimulated condition (y-axis). B) histamine release was measured at each timepoint, n=3 (y-axis). Histamine release was subtracted by spontaneous histamine release condition (baseline condition). A) and B) Data points are shown as mean±SD. Statistics: 2-way ANOVA with Tukey's multiple comparisons was performed. A) At 2hr timepoint: * denotes cRPMI vs. Immunocult. At 10hr timepoint: * and ** denote cRPMI vs. anti-CD3/28 and Immunocult, respectively. B) At 0.5hr timepoint: *** denotes anti-CD3/28 and Immunocult vs. anti-IgE. At 1hr timepoint: ** denotes anti-CD3/28 and Immunocult vs. anti-IgE. Concentration of anti-IgE used in all experiments was 1ug/ml. cRPMI, complete Roswell Park Memorial Institute

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