Histidine Decarboxylase Expression in Human CD4+ T Lymphocytes

Histidine Decarboxylase Expression in Human CD4+ T Lymphocytes

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LAY ABSTRACT

Histamine is a compound primarily released from mast cells and basophils during the early phase reaction of allergic manifestations. Prior studies showed that mouse and human immortalized T lymphocytes can release histamine following stimulation. This project set out to investigate whether primary human T lymphocytes can express HDC. The expression of this gene likely indicates that primary human T lymphocytes can synthesize histamine. The results of this project found that rare human T cells can express HDC, and this expression was not upregulated following activation.

Abstract

The early phase of allergic reactions is largely dominated by IgE-mediated degranulation of mast cells and basophils. Mast cells and basophils release mediators which include histamine, prostaglandins, leukotrienes, cytokines, etc. Histamine is a biogenic compound that can directly cause physiological changes that ultimately contribute to allergy and asthma symptoms. The current literature focused on the study of histamine production from "professional histamine producers", such as mast cells and basophils in allergic diseases. Previous research had shown that activation of allergic specific T cells led to airway narrowing independent of basophil activation 6-hour post exposure. It was therefore possible that the activation of T cells may release histamine which contributed to the airway narrowing observed. This project aimed to determine expression of histidine decarboxylase (HDC) in T lymphocytes, which is the gene encoding the enzyme solely responsible for the production of histamine. We found that HDC is expressed in rare T cell populations by the bioinformatic analysis of publicly available datasets, and we found that the activation of human primary CD4+ T cells by anti-CD3/CD28 did not lead to the upregulation of HDC by qPCR. The activation of CD4+ T cells in non-allergic donors led to a LOG_2FC of HDC to B2M housekeeping gene of: -1.0 +/- 0.48 at 6-hour, -1.3 +/- 0.23 at 24-hour, -2.2 +/- 0.32 at 72-hour. The activation of CD4+ T cells in allergic donors led to a LOG_2FC of HDC of: -0.48 +/- 0.13 at 6-hour, -2.1 +/- 0.35 at 24-hour, -4.1 +/- 1.1 at 72-hour. In conclusion, HDC expressing T cells were rare and of low expression level. The activation of CD4+ T cells did not upregulate HDC and therefore it was unlikely that T cell derived histamine contribute to allergic manifestations.

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ABBREVIATIONS

APC	Antigen Presenting Cell
AR	Allergic Rhinitis
BALF	Bronchoalveolar Lavage Fluid
CCL	C-C Motif Chemokine Ligand
CCR	C-C Motif Chemokine Receptor
CD	Cluster of Differentiation
CGRP	Calcitonin Gene Released Peptide
Con A	Concanavalin A
CRTH2	Chemoattractant Receptor Homologous Molecule expressed on Th2 cells
CXCL	C-X-C Motif Ligand
CXCR	CXC Chemokine Receptor
DCs	Dendritic Cells
ELISA	Enzyme-linked Immunosorbant Assay
EPR	Early phase reaction
FACS	Fluorescence-activated Cell Sorting
FBS	Fetal Bovine Serum
FcεR1	High Affinity IgE Receptor
FEV1	Forced Expiratory Volume in The First Second
FMO	Fluorescence Minus One
FSC	Forward Scatter
GATA	GATA-binding Protein
GM-CSF	Granulocyte-monocyte Colony Stimulating Factor
HDC	Histidine Decarboxylase
I	Ionomycin
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cells
LPS	Lipopolysaccharide
M - CSF	Monocyte Colony Stimulating Factor
MHC	Major Histocompatibility Complex
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PMA	Phorbol 12-myristate 13-acetate
PKC	Protein Kinase C
qPCR	Quantitative Polymerase Chain Reaction
scRNA	Single cell RNA
TCR	T cell Receptor
TGFb	Tumor Growth Factor Beta
Th cells	T-helper cells

TSLP	Thymic Stromal Lymphopoietin
UCP	Uncoupling protein

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Binh Nguyen, performed all experiments described in this thesis. Dr. Anna Dvorkin performed bioinformatics analysis of publicly available datasets and contributed to the results and conclusion of this research. Dr. Tom Mu collected blood samples from study participants. All sections in this document were written by me and reviewed by Dr. Mark Larché.

Introduction

1.0 Allergic Disorders.

Allergies are disorders characterized by immune hyperresponsiveness to non-infectious exogenous antigens (allergens) that are usually harmless in the majority of the human population[1]. The sources of these allergens can include but are not limited to: foods, pollen, house dust mites, medication, etc[2]. Contacts with allergens typically occurs through skin contact, the digestive system, or the respiratory system. Dependent on the source of contact, allergic disorders can manifest as allergic rhinitis, allergic asthma, atopic dermatitis, hives, and anaphylaxis[3]. Allergic rhinitis is the most common disorder, with common symptoms of rhinorrhea, sneezing, itchy nose and nasal congestion[4]. The prevalence of allergic rhinitis has increased rapidly in the previous 3 decades. It is estimated to affect 25% of children and 40% of adults globally as of 2022[5]. Allergic rhinitis by itself already presents a substantial public health concern. Allergic rhinitis causes an economic burden to society, as well as detracting from psychological and social well being of individuals[6]. Allergic asthma is another increasing prevalent disorder, affecting roughly 10% of the population in developed countries[7]. Symptom manifestations of allergic asthma include cough, shortness of breath, and chest tightness. These symptoms result from bronchoconstriction of the airway, leading to reduced gas exchange[8]. Severe bronchoconstriction is one of the allergic reaction manifestations that may contribute to the severity of anaphylaxis, with life threatening consequences if proper medical assistance cannot be provided. Anaphylaxis is characterized as a systemic reaction presenting symptoms such as: angioedema of the skin, nasal congestion, bronchoconstriction leading to chest tightness and wheezing, dizziness, vomiting, and light-headedness[9]. Anaphylaxis may also

include the cardiovascular collapse due to a fall in blood caused by systemic vasodilation and as a result of an overreacting immune system. The lifetime prevalence of anaphylaxis is estimated to be between 0.3% to 5.1% of the population, varying across geographical area[10]. All of these allergic disorders are examples of the Type 1 hypersensitivity reactions, which are rooted in immunoglobulin E (IgE) antibody mediated reactions to allergens.

1.1 Pathophysiology of Allergic Diseases.

Allergic reactions are primarily Type 1 hypersensitivity reactions and are initiated by the action of allergen specific IgE antibody in triggering an immune cascade to the allergen. Sensitization to an allergen is the first step of developing an allergy against a non-infectious substance[11]. Sensitization can occur at any point in human and animal life. In healthy individuals, initial exposure to environmental allergens through the respiratory tract lead to a protective immune response as orchestrated by T regulatory cells (Treg)[10]. This protective immune response does not manifest symptoms. In contrast, other individuals may develop a population of allergen-specific T helper type 2 (Th2) cells, which lead to allergic sensitization and manifestation of symptoms[12]. This interplay between Treg and Th2 cells can be best seen as a population ratio between allergen-specific Treg cells and Th2 cells. Healthy individuals would have a bigger pool of Treg cells as compared to Th2 cells for the same allergen[12]. The development of allergen specific Th2 cells during the sensitization phase is necessary for the production allergen specific IgE for type 1 hypersensitivity reactions following subsequent allergen exposure.

The immune cascade leading to the production of IgE begins at the extrinsic barriers relevant to the route of allergen exposure. Respiratory allergens enter through the airway, and

are picked up by dendritic cells (DCs) extending their dendrites between the epithelial cells[13]. Professional antigen presenting cells reside in this layer and can take up and process the allergens and present them to T cell receptors on their Major Histocompatiblity Complex 1/2 (MHC 1/2) surface molecules for antigen presentation. Dendritic cells are crucial APC for the initiation of the adaptive immune response. Immature DCs in the subepithelial layer internalize and process the allergen to present as peptide fragments on their MHC 2 surface molecule[14]. Allergen processing and loading onto MHC 2 is coupled with DCs migration to the lymph nodes by entering the draining lymphatic vessel[15]. This migration to the lymph node is dependent on expression of the CCR7 surface receptor, which follows the chemotactic gradient of CCL19 and CCL21 produced by lymphatic endothelial cells[15]. Migration is coupled with the increase expression of CD80 and CD86 surface receptors. These receptors function as co-stimulatory receptors for subsequent CD4+ T cell activation once the DCs have entered the lymph nodes[15].

Naïve CD4+ T cells residing within the lymph node use their T cell receptors to recognize processed allergen loaded on MHC2 of DCs. If the naïve CD4+ T cells recognize the cognate antigen on the DC, this TCR-MHC interaction is the main interaction that is required for T cell activation[16]. A secondary interaction between co-stimulatory receptors CD80 or CD86 on DCs and the CD28 receptor on the naïve CD4+ T cell is also required for a productive activation of the CD4+ T cells[16]. The combination of both these interactions provides the necessary signals for the T cell to become activated, leading to division of this monoclonal allergen-specific T cell population and preparation for differentiation. Activation of T cells lead to the production of IL-2 in an autocrine fashsion[17]. IL-2 is not only crucial for the proliferation of T cells, but also for the expression of IL-4 receptors on the T cells. The increased responsiveness to IL-4 promotes the

Th2 differentiation in T cells[17]. Activated CD4+ T cells can differentiate into different phenotypes such as Th1, Th2, Th9, Th17, and iTreg depending on the cytokines available, the co-stimulatory molecules interaction on DCs, and the amount of antigen encountered in their microenvironment[18]. The Th2 differentiated T cells is the major phenotype of interest in allergic disorders due to its contribution to the production of allergen specific IgE antibody[19].

Th2 cell differentiation is heavily dependent on the presence of IL-4 cytokine. In the initial sensitization event, sources of IL-4 are reported to be from lymph node trafficked basophils, innate lymphoid cells type 2 (ILC2), and of activated CD4+ cells themselves[19–21]. Epithelial cells at the site of allergen exposure release thymic stromal lymphopoietin (TSLP). TSLP can bind to the heterodimeric receptor composed of a TSLP receptor and an IL-7 receptor alpha chain on basophils[22]. In human, the binding of TSLP and IL-3 to basophils leads to upregulation of cytokine and chemokine receptors such as CRTH2, IL-33R, CCR2, CCR3 or CXCR4[23]. Basophils have been reported to travel to the lymph node through their expression of CCR7, but it is currently unknown if TSLP directly act on the basophil for lymph node chemotaxis, or through TSLP activation of DCs or T cells[23]. TSLP activated basophils are involved in an interplay with DCs to release IL-4, leading to Th2 differentiation[20]. ILC2 are also activated by epithelial derived cytokines in response to allergen exposure, namely IL-33, IL-25 and TSLP[24]. Activated ILC2s produce IL-4 which leads to Th2 differentiation[24]. The binding of IL-4 to CD4+ T cells in conjunction with TCR-MHC interaction and co-stimulatory interactions leads to the expression of the Th2 gene master regulator GATA3[25]. GATA3 is a transcription factor that binds to the promoter of IL-4, IL-5 and IL-13 genes among others, allowing the characteristic cytokines of Th2 cells to be expressed. GATA3 also inhibits the process of Th1 differentiation, helping the T

cell to commit to its lineage of Th2 differentiation[25]. From here on, allergen specific Th2 cells continue to proliferate.

The function of Th2 in the sensitization event of allergy is through assisting B cells differentiation into allergen specific IgE antibody producing plasma cells[20]. B cells are also APCs and can process and present allergens to T cells[21]. In allergies, allergen specific B cells present their captured allergens to Th2 cells, forming a cell-to-cell interaction and leading to the activation of the Th2 cells resulting in the release of IL-4, IL-5 and IL-13[20]. This interaction also includes co-stimulatory receptor binding such as CD40L on Th2 cells to CD40 on the B cells. The combination of IL-4 exposure and CD40-CD40L binding leads to immunoglobulin class switching in the B cells, becoming short-lived IgE secreting plasma cells or enter the germinal centre to become memory B cells[22]. Upon re-exposure, these memory B cells can class switch into IgE secreting plasma cells[23,24]. These differentiated plasma cells produce allergen specific IgE for circulation, allowing for the manifestation of allergic pathophysiology on subsequent exposure.

1.2 The Early and Late Phase Reaction

Re-exposure to an allergen in a sensitized host leads to a range of signs and symptoms. In a re-exposure event, there can exist 2 temporal phases of allergic inflammatory responses and symptoms: the early phase reaction and the late phase reaction[3]. The early phase reaction has a rapid onset and is the classical type 1 hypersensitivity reaction. This early phase reaction is characterized by the involvement of innate immune cells, mainly mast cells and basophils. Allergen specific IgE are bound to the high affinity FceRI receptors on mast cells and basophils[25]. Once allergen penetrates the extrinsic barrier, the allergen can encounter the allergen specific IgE on the high affinity FceRI receptors, leading to IgE crosslinking of the FceRI receptors on

mast cells or basophils[26,27]. This receptor activation leads to the mast cell/basophil degranulation of preformed mediators or de novo generation of lipid mediators[28]. Some of the released mediators include histamine, proteases, leukotrienes, prostaglandins, IL-4, IL-5, IL-9 and IL-13[29,30]. These mediators are responsible for the immediate allergic symptom manifestations. Of note, histamine and leukotrienes release leads to bronchoconstriction, vasodilation, mucosal edema, pruritus, rhinorrhea and other immediate hypersensitivity manifestations[31].

The early phase reaction represents a well-studied phenomenon in the literature. The second temporal phase of an allergic reaction is the late phase reaction and is much less well understood. The late phase reaction does not manifest in every allergic individual, nor does it manifest following after an early phase reaction, although this lack of manifestation can be explained by an insufficient load of antigen exposure[32]. It can manifest without the hypersensitivity manifestations from the early phase reaction as well[33]. The late phase reaction typically occurs between 2 to 6 hours post allergen exposure with physical manifestations similar to the early phase reaction[3]. The late phase reaction is characterized by immune cell activation and recruitment to the area of allergen exposure between 2 to 6 hours post exposure. These cells include Th2 cells, ILC2s, eosinophils, basophils, and other immune cells[3]. The activation of these cells leads to the release of inflammatory cytokines and mediators, creating a relapse in allergic symptoms[34]. The activation of ILC2 by mast cell derived prostaglandin D2, cysteinyl leukotrienes, IL-25 or by epithelial cells' TSLP, IL-24, and IL-33 lead to the release of ILC2 derived Th2 cytokines (IL-4, IL-5, IL-13)[35–37]. IL-4 contributes to further the Th2 differentiation process, while IL-5 and IL-13 contributes to allergic manifestations such as

eosinophilia, mucus overproduction, and airway hypersensitivity[38,39]. IL-13 in particular was reported to increase the potency of histamine and leukotrienes D4 as contractile agonists[40].

The mechanism leading to the development of late phase reaction after the initial exposure remains elusive. The recruitment, maturation and activation of eosinophils by IL-4, IL-5, IL-13, and IL-33 leads to the damaging of the nasal mucosa through the degranulation of granules containing major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil derived neurotoxin[41,42]. Activated eosinophils also releases leukotrienes C4 and D4, which are potent bronchoconstrictors[43]. Released of CCL2 (epithelial cells, endothelial cells, smooth muscle cells) and eotaxins (epithelial cells) from the early phase reaction can recruit basophils through the CCR2 and CCR3 receptor[44,45]. Basophils can be activated by various cytokines (IL-3, IL-18, IL-33 and TSLP), in addition to the IgE crosslinking of the high affinity FcεR1 receptors[46]. IL-3, IL-18, IL-33 activated basophil led to the release of IL-4, and IL-3 was capable of inducing histamine release from basophil in some atopic individuals[47]. IL-3 in particular primes basophil for degranulation, which lead to an increase in histamine release following non-cytokine induced activation[47]. Sources of IL-3 in the late phase reaction is likely from activated T cells or from mast cells secretion from the early phase reaction[48]. The full activation basophils and release of all basophil products in the late phase reaction remains elusive.

The involvement of allergen specific IgE is debated in the context of the late phase reaction. There is evidence that the late phase reaction is IgE-dependent. Intradermal injection of anti-IgE antibody led to a late phase reaction in the skin of most atopic human subjects[49]. The transfer of serum from atopic subjects to non-atopic subjects led to manifestation of late phase

reactions in non-atopic subjects[49,50]. The competitive inhibition of IgE binding and direct inactivation of IgE by heating atopic serum led to a lack of symptom development in both the early phase reaction and late phase reaction[49]. In another study, the administration of a drug (Omalizumab) that prevents IgE from binding to the high affinity FceRI receptors on mast cells reduced the severity of the late phase reaction in response to an allergen challenge[51]. While a correlation between allergen-specific IgE and late phase reactions exists, it is also entirely possible that the mediators released in the early-phase reaction are sufficient to activate cells in the late phase reaction. Other triggers of mast cell degranulation in allergies can include IgG, as well as complement C3a and C5b[52,53].

On the other hand, evidence also exists that late phase reactions can occur completely independent of the early phase reaction, and of IgE antibody involvement. Haselden et al, demonstrated that specific activation of Fel d1 - specific T cells using peptide epitopes led to the manifestation of a late phase reaction in some allergic asthmatic subjects[33]. This late phase reaction was defined as a forced expiratory volume (FEV) reduction that began at 2-3 hours post peptide challenge. The peptides were shown to not induce IgE crosslinking of basophils as they were designed to be shorter than the required length for FcεR1 crosslinking. From this study, it is likely that T cell activation is involved in inducing a late phase reaction. The exact mechanism of how T cells are contributing to this reduction in FEV1 is currently unknown. One explanation is through the release of calcitonin gene-released peptide (CGRP) from activated T cells. Injection of allergen derived T-cell peptides into human subjects led to a concomitant increased in CGRP+ T cells and the magnitude of the allergic reaction, peaking at 6-hour post injection[54]. Following the gradual resolution of late phase reaction, the number of CGRP+ cells also

decreased. CGRP is a potent vasodilator, akin to histamine. As such, it was theorized that CGRP can contribute to the airway wall edema in the late phase reaction[55]. CGRP deficient mice also showed attenuation of airway hyperresponsiveness[56].

Differentiating and differentiated T cells release specific cytokines based on their current phenotype when activated[57–59]. These cytokines may exert their effects directly, for example on airway smooth muscle cells to cause the reduction in FEV1, or indirectly by activating other immune cells to exert the drop in FEV1. A previous study by Hakonarson and Grunstein (1999) showed that incubation of rabbit airway smooth muscle cells with the addition of exogenous IL-5 led to an enhanced maximal isometric contractility in response to an acetylcholine challenge[60]. The same study also showed that atopic asthmatic human airway smooth muscle cell upregulates and release IL-1b in response with stimulation with atopic sera, and with exogenous IL-5. The same authors then co-cultured activated T cells with human airway smooth muscle cells, which lead to an increase in expression of IL-5 and IL-1b mRNA in the airway smooth muscle cells[61]. These results indicated that the action of IL-5, an activated Th2 cell product have a direct effect on airway smooth muscle cells. On the other hand, it is also likely that activated T cell products may indirectly lead to a drop in FEV1 through the activation of other immune cells. As mentioned above, IL-3 has been reported to induce basophil release of histamine in certain atopic individuals and IL-3 can prime basophil to release more histamine. IL-3 is a product of activated T cells and as such it is possible that IL-3 releasing T cells can contribute to the late phase reaction through IL-3 to basophil signaling[62]. Another example is of activated Th2 cell release of IL-5 which contribute to the maturation and activation of eosinophils. Eosinophils activation releases major basic protein, which is an antagonist of

parasympathetic nerve M2 receptors. The presence of eosinophils' major basic protein lead to M2 receptor being unable to regulate acetylcholine release from parasympathetic nerves, leading to parasympathetic nerve-mediated bronchoconstriction[63].

Direct consequences of T cell activation on bronchoconstriction and FEV1 reduction led to the formulation of this thesis. Current immunological understanding highlights leukotrienes, histamine, and prostaglandins, produced by cells such as mast cells and basophils as mediators that contribute to bronchoconstriction[64]. Previous literature also indicates that histamine can be produced by murine T cells. The purpose of the experimental and analytical worked described in this thesis was to determine whether human T lymphocytes are capable of synthesizing histamine and thereby contributing to the late phase reaction.

1.3 Histamine

Histamine is an immunomodulatory amine known for its involvement in the allergic cascade. Most of the histamine release comes from mast cells and basophils[65]. Other cell types have been observed to produce histamine including dendritic cells, monocytes, neutrophils, and epithelial cells[66].

The synthesis of histamine requires the decarboxylation of the amino acid histidine in the Golgi apparatus[67]. This decarboxylation is catalyzed by histidine decarboxylase (HDC). As HDC expression and activity are necessary for the synthesis of histamine, detecting HDC expression is crucial for the determination of histamine production. A widely utilized approach is to deduce histamine production in different cells through transcriptional analysis for HDC expression[68].

Histamine exerts its effects by binding specific histamine receptors found on a variety of cells. Notable effects include the modulation of vascular and smooth muscle activity, along with immunoregulation of immune cells[69,70]. Elevated histamine levels in plasma or urine is associated with the diagnosis of anaphylaxis[71]. Bronchoalveolar lavage fluid (BALF) of asthmatic individuals has a higher histamine concentration as compared to individuals with allergic rhinitis [72]. Similarly, BALF of asthmatic individuals had a significantly higher histamine level compared to non asthmatic individuals[73]. Administration of histamine at low dose induced bronchoconstriction in asthmatic individuals when the same dose did not have an effect the healthy control group[73].

As an early mediator in the allergic cascade, histamine is an important molecule in the pathogenesis of allergies and allergic asthma. The primary trigger of histamine release is through antigen binding with antigen-specific IgE, followed by crosslinking of IgE receptors found on mast cell and basophil surfaces[28]. Histamine released from mast cell and basophils degranulation accounts for the immediate rise in histamine concentration in nasal secretion or in blood plasma[74,75]. Histamine has a paracrine effect on nearby cells such as airway smooth muscle cell and vascular smooth muscle cells, and endocrine effect following distribution through the bloodstream[70]. Endocrine effects include the function as a chemotactic agent to recruit other immune cells using the histamine H4 receptors[70].

Histamine effects are pleiotropic as they are dependent on the type of histamine receptors. There are 4 histamine receptors: H1, H2, H3 and H4[31]. The diversity of histamine receptors allows histamine to act as an immunoregulatory molecule[31]. Selective receptor activation can lead to proinflammatory or anti-inflammatory responses. Histamine binding to

H1 receptors is responsible for bronchoconstriction and vasoconstriction, mucosal edema, and other immediate hypersensitivity manifestations such pruritus and rhinorrhea[31]. H1 activation has also been observed to polarize the immune system towards a Th1/M1 profile through the effect of histamine on dendritic cells[76]. Histamine binding to H2 receptors antagonizes the function of the H1 receptor. H2 activation leads to relaxation of smooth muscle cells, leading to bronchodilation and vasodilation[77]. H2 stimulation of dendritic cells skews the T lymphocyte profile towards a Th2 profile[78]. The H3 receptor is primarily found in the nervous system and its function has not been clearly explored[79]. The H4 receptor is the latest addition to the repertoire of histamine receptors and its function has mainly been described for the chemotaxis of immune cells, including basophils[80,81]. Overall, histamine exhibits complex biology. Its function is largely regulated by receptor specificity and abundance.

1.4 Histidine Decarboxylase

HDC is broadly expressed in many cell and tissue types and therefore it is proposed that these different cell types can produce histamine. HDC is expressed constitutively in mast cells, basophils and histaminergic neurons[82]. Expression of HDC can be positively or negatively regulated. IgE crosslinking on mast cells has been shown to increase HDC expression and histamine synthesis. Phorbol 12-myristate 13-acetate (PMA) is another stimulating agent known to induce HDC expression in Jurkat T lymphocytes. Several cytokines have been implicated in the induction of HDC and histamine, including IL-3, IL-18, IL-33, GM-CSF and SCF[28]. These cytokines share a common functional role in mast cell and basophil growth and differentiation and further consolidate the role of mast cells and basophils in histamine release. It is important to note that it is unknown whether these cytokines are directly involved in promoting HDC

activity, or whether they upregulate other genes that lead to HDC expression[28]. Negative regulation of HDC also exists; Mitochondrial uncoupling protein 2 (UCP2) expression has been shown to inhibit HDC expression in human mast cells as observed in a UCP2 knock out study[83].

Based on sequencing analysis, HDC sequence has 8 predicted isoforms through alternative splicing. The main isoform of HDC is a protein of 74-kDa[66]. This isoform is active and functional in the decarboxylation of histidine, but it is unstable. Post translational carboxyl terminal truncation modification, mediated by caspase 9, of this 74 kDa isoform led to a 53-55 kDa isoform[82]. This 53-55 kDa isoform shared similar enzymatic activity to its 74 kDa isoform in human mast cells, but studies using mouse cells demonstrated the 53-55 kDa isoform was more enzymatically active[82]. The literature divides histamine producing cells into 2 classes: professional and non-professional producers. Konttinen et al defined professional histamine producers as cells that produce “copious” amount of histamine, and they store histamine in granules until release[66]. Examples of professional histamine producers are mast cells and basophils. Non-professional histamine producers are producing histamine at a 100 to 1000-fold lower “pace”, they also constantly release histamine into the extracellular space as opposed to storing histamine in granules like professional histamine producers[66]. Examples of non-professional histamine producers are monocytes and neutrophils. It has been proposed that professional histamine producers in the mouse are capable of this HDC post translational modification while nonprofessional histamine cannot. This was demonstrated through an HDC expression study using RAW264 macrophage cells stimulated with LPS or PMA[62]. By definition, RAW264 macrophage cells are non-professional histamine producers. Researchers found maximal HDC expression was reached after 4 hours post stimulation and western blot analysis

showed only the 74 kDa isoform of HDC. The 53-55 kDa isoform was found localized in granules of mouse mast cells and basophils[84].

Transcriptional regulation of HDC remains poorly understood. Prior studies in mouse RAW 264 cell line and gastric cancer cell line demonstrated the binding of SP1 transcription factor to the promoter region of the HDC gene locus[28,85]. SP1 binding to the promoter region led to an upregulation in HDC expression following LPS stimulation[85]. The transcription factors YY1 and KLF4 in turn negatively control HDC through the suppression of SP1 as seen in a gastric cancer cell line[28]. The expression of transcription factor GATA2 has also been seen to upregulate HDC expression. GATA2 is crucial in the development and differentiation of mast cells and basophils[86,87]. This further reinforces the link between mast cell and basophil maturation and their capacity to produce histamine.

1.5 Histamine sources and production

An assortment of cell types can produce and release histamine. In the scope of allergies and asthma, mast cells and basophils are primary histamine producers while dendritic cells, neutrophils, monocytes, epithelial cells, and T lymphocytes (mouse) are non-professional histamine producers.

Mast cell degranulation is the major source of histamine in the early phase reaction as part of the immediate hypersensitivity response. Mast cells continuously produce and store histamine within intracellular granules until degranulation. When allergens are introduced to the target organs, allergen can bind to its antigen-specific IgE antibody bound to high affinity IgE receptors on mast cells and basophils. This antigen IgE crosslinking is the predominant trigger of

histamine release[28]. Complement components C3a and C5a activation can also bind to G protein coupled receptors on mast cells to induce degranulation of mast cells, although this degranulation cascade is not as potent as the IgE mediated degranulation[88].

Similar to mast cells, basophil belong to the class of professional histamine producers. As with mast cells, complement C3a and C5a have been known trigger degranulation in basophils[89]. Unlike mast cells, basophils are not tissue resident cells but are circulating immune cells within the blood and accumulate in tissues at site of inflammation. The recruitment and chemotaxis of basophils to inflammatory sites is often required to exert their functions. Interestingly, histamine can function as a chemokine for basophil recruitment and this function can be seen as a positive feedback loop in increasing local histamine concentration[81].

In this project, we address whether human T lymphocytes, like their murine counterparts, can produce histamine. Previous studies concluded that CD4+ and CD8+ T lymphocytes in mice can produce histamine following stimulation with the mitogen Concanavalin A (Con A)[90,91]. Both studies were conducted using isolated spleen cells. The first study used mast cell deficient mice and measured HDC enzymatic activity and histamine concentration from the medium of the spleen cells cultures. Researchers depleted the spleen cells of non-T lymphocytes and stimulated T lymphocytes with Con A. They determined that Con A induced HDC activity and histamine release[90]. The second study was conducted by the same investigators and focused on isolating CD4+ and CD8+ cells from mouse. The authors concluded that both CD4+ and CD8+ cells released histamine in response to Con A stimulation[91]. CD8+ cells released more histamine as compared to CD4+ cells. CD4+ appeared to release small amount of histamine in the absence of Con A stimulation. Interestingly, the authors noted that

recombinant mouse IL-3 markedly stimulated histamine release even without presence of Con A. There are two interpretations of this result. The first interpretation is that IL-3 induced proliferation and degranulation of contaminating basophils leading to the production histamine[92]. The second interpretation is that IL-3 is a stimulus that can influence lymphocytes to release histamine. Given the involvement of IL-3 in regulating Th2 cytokines, it is possible that histamine production is a function of Th2 cells[93]. In human cells, IL-3 has been shown to induce basophils but not mast cells to release histamine[92].

One study has been conducted using the human acute leukemia lymphocyte cell line Jurkat to study histamine production in human T cells[94]. Nagashima et al noted previous research on T cell release of histamine and investigated the potential of Jurkat T cells to express HDC and produce histamine. Histamine production was determined by mass spectroscopy from the Jurkat T cells supernatant, following stimulation with PMA/Ionomycin. Through PCR analysis, stimulation with PMA was found to stimulate HDC expression in Jurkat T cells. This study created a precedent for HDC expression by human T cell linage cells, suggesting that non-transformed human T cells may also express HDC and this have the potential to contribute to allergic reactions via histamine production.

The determination of histamine production is reliant on HDC expression and the detection of the released histamine. HDC expression is necessary for the decarboxylation of histidine into histamine, and as such is the prerequisite for histamine production. Evaluating HDC expression hinges on qPCR, bulk RNA sequencing and single cell sequencing analysis. All these methods need stringent requirements to validate the results. Given the variety of histamine sources from a human blood sample, it is imperative to ensure other HDC positive

cells are not skewing the results. Theoretically, a single HDC mRNA transcript from a contaminating cell could give a positive qPCR signal. Single cell sequencing data can partially circumvent the contamination issue if the entire cell population can be correctly assigned to their specific cell types and phenotypes. Analysis using cell specific markers can identify the cell types and phenotypes. The difficulty with relying on single cell datasets is that of data selection. A suitable sample size of cell along with the methodology to stimulate/harvest the cell must be evaluated in the context akin to that of allergies and asthma. In addition, 100% purification of human CD4+/CD8+ is currently not possible, both for the purpose of qPCR and single cell sequencing.

As a result, this project aims to determine whether histamine production from non-transformed human T lymphocytes can occur. A combination of methods has been used to evaluate HDC expression. The first is through qPCR analysis to establish HDC expression. The second method is through the bioinformatic analysis of bulk RNA sequencing and single cell sequencing datasets of T lymphocytes for HDC expression from publicly available datasets.

Research Question

Do primary human T lymphocytes transcribe HDC?

Project Rationale

In previous research conducted by Dr. Larché, direct activation of allergen specific T lymphocytes resulted in bronchoconstriction without IgE crosslinking on mast cells or basophils. As histamine is known to contribute to the process of bronchoconstriction, it is possible that the

release of histamine from T lymphocytes could contribute to the IgE independent bronchoconstriction following activation of allergic-specific T cells. Furthermore, a previous study demonstrated an elevated histamine concentration in the late phase reaction that was greater as compared to the early phase reaction in BALF of individuals post-allergen challenge[33]. This spike in histamine concentration between 6-9 hours post challenge coincided with the increase in T lymphocyte infiltration to the lung during the late phase reaction[74,95]. More recently, work done by Mark Lychacz, a previous graduate under Dr. Mark Larché again showed histamine production by Jurkat T lymphocytes when stimulated with soluble anti-CD3/anti-CD28 and soluble anti-CD3/anti-CD28/anti-CD2 Immunocult[96].

With the focus on histamine release, it is necessary to find prior observations of T lymphocytes producing histamine. Histamine production from mouse T lymphocyte have been studied previously using concanavalin A as stimulant. The authors determined that CD4+ and CD8+ T lymphocyte from mouse expressed HDC along with releasing histamine into the medium. Concanavalin A is a mitogen that functions as a T cell activator through the activation of T lymphocytes surface receptors. This receptor binding activity allow Concanavalin A to act as an antigen independent TCR activator. The downstream consequence is similar to that of antigen activation with cell proliferation and cytokine secretion. The results of these studies using this stimulant could possibly be transferable to human T lymphocytes.

As mentioned above, a past study showed the induction of HDC expression in human leukemic cell line Jurkat T lymphocyte using PMA and ionomycin. PMA stimulation is associated with the activation of the Protein Kinase C (PKC) pathways found in many cell types, including T lymphocytes. The PKC pathway has also been linked to the activation of HDC expression in

gastric cancer cells in both rats and humans, along with transformed macrophage line RAW264[97–99]. CD3 stimulation of T cell receptor also activates the PKC pathway.

Combining these prior observations from mouse and human cell line studies, it is possible that activation of human T lymphocytes can result in the expression of HDC and histamine production.

Hypothesis

Stimulation of human CD4+ T lymphocytes can result in the transcription of histidine decarboxylase (HDC).

Objectives/Aims

There are 2 major aims of this study. Aim 1 is the determination of whether human T cells expresses HDC. This aim can be divided into the evaluation of HDC expression from human Jurkat T lymphocyte cell following stimulation, and the evaluation of primary human CD4+ lymphocytes as isolated from peripheral mononuclear blood cells. Aim 2 is to determine HDC expression in T lymphocytes through the usage of bioinformatics analysis.

Methods

Aim 1: The evaluation of T lymphocyte expression of HDC

1.0 Jurkat T lymphocytes Stimulation and Collection

Jurkat T lymphocytes clone E6.1 (ATCC) were cultured in flasks to appropriate cell number (>12 millions) before being stimulated. Jurkat T cells were grown in Gibco™ RPMI 1640 Medium supplemented with Gibco™ 10% FBS, 2mM L-glutamine, 2mM Pen/Strep, 25 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose and 1500 mg/L sodium bicarbonate. This media will be referred to as cRPMI from now on.

Cells were seeded at the final concentration of 1.0e6/ml to a final volume of one ml per well in 12-well flat-bottomed culture plate (Corning Falcon, USA). The volume added per well was 500ul of cells in cRPMI, as such, cells were resuspended to 2.0e6/ml (2x).

There were 3 stimulation conditions across 3 timepoints. The experimental condition was that of stimulation with anti-CD3/anti-CD28 antibodies. Dynabeads™ Human T-Activator CD3/CD28 beads (Gibco™, USA) was used as the reagent of choice. The preparation of stimulation beads was as followed. Anti-CD3/anti-CD28 beads were briefly vortexed and 80 ul (~3 million beads) of beads were pipetted into a 5ml polystyrene round bottom tube. 1ml of sterile PBS (Gibco) was added onto the bead solution and vortexed to wash the beads. The polystyrene tube was inserted into a EasySep™ magnet (Stemcell, USA) and incubated for one min, the PBS solution was then decanted by picking up the magnet and inverting the polystyrene tube. The tube was then removed from the magnet and 1.5ml of cRPMI was added to resuspend the beads.

Stimulation beads were now ready for the addition into culture. 500ul of bead in cRPMI solution was added per well for the anti-CD3/anti-CD28 stimulation condition.

The positive control was stimulated with Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldritch, USA) and Ionomycin (I) (Sigma-Aldritch, USA) at PMA (10 ng/ml) and Ionomycin (1 ug/ml). PMA and Ionomycin were prepared separately at 4x concentration in cRPMI (PMA: 40 ng/ml, Ionomycin: 4 ug/ml). 250ul of each PMA or Ionomycin were added per positive control well, to the final desired concentration. PMA and Ionomycin will now be referred to as PMA/I.

The negative control only contained Jurkat T cells in cRPMI. 500ul of cRPMI were added on top of the 500ul of cells in cRPMI to the final concentration of 1e6/ml, or 1 million cells in each well.

Stimulations were taken place in 37-degree Celsius incubators at 5% CO_2 in air. Cells and supernatant were collected after 6-hour, 24-hour and 72-hour stimulation timepoints. Cells and supernatants were collected by mixing each wells using a pipette, followed by centrifugation at 400xg for 5 mins. Supernatant were carefully removed using a pipette.

Cells were lysed in 350ul of buffer RLT solution (RNAeasy Mini Extraction Kit, Qiagen, Germany). Lysed cells were stored in a -80C freezer until RNA extraction. Supernatant were frozen in a -80C freezer.

1.1 Human CD4+ T lymphocyte Stimulation and Collection

Blood from self reported allergic and non allergic donors were collected and used to explore the factor of allergic status on T lymphocyte HDC expression and histamine

production. 60ml of blood were collected from individuals in BD Vacutainer™ 10mL Plastic Blood Collection Tubes with Sodium Heparin (Becton Dickinson, USA). PBMCs were isolated from human peripheral blood using Ficoll-Paque (GE Healthcare, USA) density gradient centrifugation. 20ml of peripheral blood were lightly layered over 15ml of Ficoll-Paque in 50ml Falcon tubes. The tubes were then centrifuged at 400xg for 30 mins with low acceleration and no brake. PBMCs were collected from the middle buffy layers using a transfer pipette and move into a new 50ml Falcon tube containing 30ml of wash media (RPMI1640, 2.5% FBS, 1% Pen/Strep and 1 IU/ml Heparin). The tubes were then centrifuged at 250xg for 10 mins and supernatant decanted. CD4+ T cells were isolated from human PBMCs by negative isolation using StemCell EasySep™ Human CD4+ Enrichment Kit (StemCell, Canada). PBMCs were counted, washed, and resuspended in EasySep™ Buffer at concentration close to but less then 50e6 cells/ml in 5ml polystyrene round-bottom tube. If the volume of PBMCs resuspension was greater then 1ml, but less then 2ml, the sample was filled up to 2ml using EasySep™ Buffer and divided into two 5ml polystyrene round-bottom tube. 50ul of EasySep™ enrichment cocktail was added to the samples and incubated at room temperature for 10 mins. Next, 100ul of EasySep™ magnetic particles were added, mixed by pipetting, and incubated at room temperature for five minutes. The samples were filled up to 2.5 ml of total volume using additional EasySep™ Buffer and placed into the EasySep™ magnet for five minutes for incubation at room temperature. After the final incubation, the magnet was picked up and inverted to pour the tube containing the isolated human CD4+ T cells into a 15 ml Falcon tube. Contaminating cells were stuck to the magnetic beads and therefore trapped to the side

of polystyrene tubes by the magnet. Collected CD4+ T cells were then centrifuged at 400xg for five mins and decanted to remove EasySep™ buffer.

Cells were then resuspended in cRPMI and plated onto plastic petri dishes. Petri dishes were incubated for 2 hours in 37C incubator to remove monocyte contamination. Monocytes are semi-adherence cells and therefore should adhere to the plastic bottom of the petri dishes[100]. Petri dishes were lightly washed using cRPMI to collect the CD4+ T cells. Cells were resuspended to a final concentration of 1.0e6/ml in each well and stimulated. The stimulation protocol was identical to the Jurkat stimulation protocol as per section **1.0** of methods. A sample of cells were set aside and collected for flow cytometry analysis of CD3+/CD4+ cell purity. In samples with IL-33 stimulation, a final concentration of 20ng/ul of IL-33 (PeproTech, USA) were added. IL-33 stimulation samples required adjusting the initial CD4+ T cell concentration to 4.0e6/ml (4x) and adding 250ul of cell in cRPMI in each wells instead. IL-33 were resuspended to 80ng/ul (4x) and 250ul were added per stimulation wells.

Cells and supernatant were collected after the stimulation period. Three stimulation times of interests were 6-hour, 24-hours, and 72-hours stimulation. Stimulations were taken place in 37-degree Celsius incubators at 5% CO2. Cells were lysed in 350ul of buffer RLT solution (RNAeasy Mini Extraction Kit, Qiagen, Germany). Lysed cells were stored in a -80C freezer until RNA extraction. Supernatant were frozen in a -80C freezer.

1.2 Human Th2 T cells Differentiation

Th2 lymphocytes differentiation were performed using the CellXVivo™ Th2 differentiation kit (Bio-techne, USA). Briefly, 10 wells of 24-well flat-bottomed culture plates (Corning Falcon, USA) were coated with soluble mouse anti-human CD3 antibody overnight as prepared per kit's instructions. The final concentration anti-CD3 coating was unknown to the user. 3 vials of blood were collected in BD Vacutainer™ 10mL Plastic Blood Collection Tubes with Sodium Heparin (Becton Dickinson) per self-reported allergic or non-allergic donors. CD4+ T cells were negatively isolated as per section **1.1** of methods and resuspended in Lonza™ X-VIVO 15 growth media supplemented with IL-2, IL-4, IL-6 and anti-IFNγ antibody as per kit's instructions. The resuspension concentration was 0.25e5/ml CD4+ T cells. The final concentration of IL-2, IL-4, IL-6 and anti-IFNγ was unknown to the user. T cells were seeded at 0.25e6/ml in 1ml of total volume per well in the mouse anti-human CD3 antibody coated wells. 10 wells were used for each donor. IL-2, IL-4, IL-6, and anti-IFNγ supplemented Lonza™ X-VIVO 15 media were changed every 3-4 days, or when the cells reached a confluency of 1.5e6/ml. This first media change did not remove any cells by carefully removing media from the top layer and slowly working downwards with the pipet tip pulling away from the centre of the well (cells should be settled at the bottom). Subsequent media change included the splitting of the cells. The first split was of 1:10, and subsequent split was of 1:2. Cells were resuspended by pipetting. Cells were then counted, and appropriate volume of media was removed (900ul for 1:10 split, 500ul for 1:2 split) and replaced with the appropriate volume of new media. In the final 1:2 split, the number of cells and wells were adjusted to anticipate a minimum

of 13 million Th2 cells in total. The culture concluded on day 13 and cells were harvested for stimulation and flow cytometry.

In the case of T cells were dividing slowly, additional cells at any split were kept, their media were changed, and the cells were reseeded onto newly coated-anti CD3 wells if necessary.

In the event of Th2 differentiation culture were started using frozen PBMC, additional steps were performed. Frozen PBMCs vials were thawed by submerging in a 37C water bath for 2 mins. Thawed cells in freezing media were transferred (RPMI 1640, 20% FBS, 10% DMSO) into 10ml of pre-warmed cRPMI. PBMCs were then centrifuged at 130xg for 8 mins and supernatant were removed by decanting. PBMCs were resuspended in cRPMI at a 5.0e6/ml concentration and plated 1ml of volume into wells of a 12-well culture plate. PBMCs were incubated in 37C incubator overnight. PBMCs were then ready to proceed with human CD4+ negative isolation as per section **1.1** of methods and followed by Th2 differentiation culture protocol. The cultures were extended to 14 days.

1.3 Human Th2 T Cells Stimulation and Collection

Th2 cells were resuspended to a final concentration of 1.0e6/ml and stimulated. The stimulation protocol was identical to the Jurkat T lymphocytes stimulation protocol as per section **1.0** of methods.

Cells and supernatant were collected after the stimulation period. Three stimulation times of interests were 6-hour, 24-hours, and 72-hours stimulation. Stimulations were taken place in 37-degree Celsius incubators at 5% CO2. Cells were lysed in 350ul of RLT

solution (Qiagen). Lysed cells were stored in a -80C freezer until RNA extraction. Supernatant were frozen in a -80C freezer.

1.4 Flow cytometry to identify CD3+/CD4+ purity and identifying Th2 phenotype.

A population of CD4+ T lymphocytes collected after the monocyte adherence step were stained for flow cytometry. Cells were first washed in 1ml of PBS, centrifuged at 400xg for 5 mins at 4C, and then resuspended in 200ul of FACS buffer (Biolegend, USA) for an additional wash using the same centrifuge settings. FACS buffer was then decanted, and cells were then stained with antibodies for extracellular stain. CD3-BV421 (Biolegend, USA) and CD4-BV605 (Biolegend, USA) staining antibodies were used to determine CD4+ T lymphocyte purity level. 1:50 dilution of both antibodies was used to stain for 30 minutes on ice and in the dark. Cells were then washed twice using 300ul of FACS buffer to remove non-binding and excess antibodies using flow cytometry centrifugation settings outlined above. Cells were then resuspended in 200ul of FACS buffer. 2ul of eBioscience™ 7-AAD viability staining solution (eBioscience™, USA) were then added and incubated for 10 mins to determine cell viability. Samples were filtered into 5ml round bottom polystyrene tubes with cell strainer cap (Corning Falcon, USA) and were now ready for flow cytometry. Compensation beads (Invitrogen™, USA) for CD3 and CD4 antibodies were used for compensation, PBMCs were used for 7-AAD viability stain compensation.

Samples were run on the BD LSRFortessa flow cytometer. Data was analyzed using the FlowJo software version 10.8 with appropriate gatings (live cells → lymphocytes population → single cells → CD3+/CD4+).

Th2 cells pre and post differentiation were collected and stained for flow cytometry to identify Th2 population. Before staining, ~4 million cells were collected and set aside for intracellular staining stimulation. Cells were washed and resuspended in cRPMI at 1ml of total volume, 500ul of cells were added per wells in a 12-well flat bottom culture plate for stimulation. PMA and Ionomycin were added to a final concentration of 10 ng/ul PMA and 1 ug/ml Ionomycin in 1ml (method section **1.0**). Brefeldin A (Biolegend, USA) was added at a 1:1000 dilution (1ul) to inhibit intracellular protein transport. Incubation took place in 37C incubator for 6 hours.

Cells were then washed using 1ml of PBS, followed by resuspension in FACS buffer in 5ml round polystyrene bottom tubes. Cells were then centrifuged at 400xg for 5 mins at 4C and FACS buffer was decanted. Cells were first stained with Fixable Viablity Dye eFluor 780 viability dye (eBioscience, USA), CD3-PerCP (Biolegend, USA), and CD4-BV605 (Biolegend, USA) at the same time for 30 mins on ice and in the dark for viability and extracellular stains. 1:1000 dilution of eFluor 780 viability dye was used to stain for viability, 1:50 dilution for both CD3-PerCp and CD4-BV605 was used to extracellular stain in 50ul total of FACS buffer per tube. Cells were then washed twice with 500ul of FACS buffer to remove any non-binding antibodies and excess viability dye using the same centrifugation setting outlined above. FACS buffer was decanted post centrifuge.

Cells were then fixed using 200ul of eBioscience™ IC Fixation solution for 20 mins on ice and in the dark. Cells were then washed twice with 500ul of eBioscience™ Permeabilization Buffer to remove excess fixation buffer. Permeabilization buffer was decanted post centrifuge.

Cells were then stained with IL-4-BV421 (Biolegend, USA), IL-5-PE (Biolegend, USA) and anti-IFNγ-BV650 (Biolegend, USA) in permeabilization buffer. 1:25 dilution for IL-4-BV421, IL-5-PE, and 1:50 dilution for anti-IFNγ-BV650 was used to stain in 50ul total of permeabilization buffer. Cells were then washed twice using 300ul of FACS buffer and resuspended in 200ul of FACS buffer. Samples were filtered into 5ml round bottom polystyrene tubes with cell strainer cap (Corning Falcon, USA) and were ready for flow cytometry. Fluorescent minus one (FMO) samples were prepared with the same procedure. FMO samples were IL-4 FMO, IL-5 FMO, and IFNγ FMO. Compensation beads for CD3, CD4, IL-4, IL-5, IFNγ antibodies were used for compensation, Th2 cells were used for eFluor 780 viability dye compensation.

Samples were run on the BD LSRFortessa flow cytometer. Data was analyzed using the FlowJo software version 10.8 using these gatings (live cells → lymphocyte population → single cells → CD3+/CD4+ → IFNγ- population → IL4+/IL-5+) to identify IL4+/IL-5+ and IFNγ- cells.

1.5 RNA extraction

RNA extractions were performed using the RNeasy Mini Kit (Qiagen, Germany). Frozen lysed cells were thawed at room temperature on ice and RNA extraction proceeded per the manufactures' instructions, followed by an RNA clean up step. Samples were processed in racks placed on ice to reduce RNA degradation over the procedure. Centrifuge were set at 4C. RNA extractions were performed as followed. A 1 to 1 ratio of 70% ethanol was added to RNA suspended in buffer RLT (350ul 70% ethanol to 350ul buffer RLT). Samples were mixed using by pipetting and 700ul of the sample was

transferred onto RNAeasy Mini spin column placed in 2 ml collection tube. The lid on the spin column was closed, and the spin column was centrifuged at 11000xg for 15 seconds. The flow through of the spin column was in the 2ml collecting tube below the spin column was discarded. Next, 700ul of buffer RW1 was added (RNeasy Mini Kit, Qiagen) into the spin column, the lid of the column was closed, followed by centrifugation at 11000xg for 15 seconds and flow through discarded. 500ul buffer RPE was added (RNeasy Mini Kit, Qiagen) to the RNAeasy spin column, the lid was closed, and the column was centrifuged at 11000xg for 15 seconds and flow through discarded. 500ul buffer RPE was added to the RNAeasy spin column, lid closed and spin column centrifuged at 11000xg for 2 minutes, and the flow through discarded. The empty spin column was centrifuged again at 11000xg for 1 min to remove any reagents residue. Spin column was then transferred into 1.5ml Eppendorf tube. 50ul of DNA/RNA free water was added into spin column by pipette directly onto the membrane. Pipetting directly onto the membrane was an important step as this step increased the RNA purity of the sample. The spin column was centrifuged at 11000xg for 1 min to elute the RNA. Repeat with another 50ul of DNA/RNA free water into column for a total of 100ul of eluted volume. RNA clean up was the next step. 100ul of eluted RNA in 1.5 ml Eppendorf tube was mixed by pipetting with 350ul of buffer RLT and 250ul of 100% ethanol. 700ul of the sample was transferred onto a new RNAeasy Mini spin column placed on top of a new 2 ml collection tube. The lid of the spin column was closed, and column centrifuged at 11000xg for 15 seconds. The flow through was then discarded. Next, 500ul of buffer RPE was added into the column, spin column lid was closed, and the column centrifuged at 11000xg for 15 seconds and the flow through

discarded. This step was repeated with another 500ul of buffer RPE and centrifuge dat 11000xg for 2 mins followed by flow through removal. The spin column with no reagent inside was centrifuged at 11000xg for 2 mins to eliminate any reagents residue. The spin column was placed into a new 1.5ml Eppendorf tube. 50ul of DNA/RNA free water was added into the spin column, pipetted directly onto the membrane. The spin column was centrifuged at 11000xg for 1 min to elute the RNA. Pipette the eluted 50ul from the Eppendorf tube and back into spin column at the membrane layer. The spin column was centrifuged again at 11000xg to for 1 min to elute the RNA.

RNA quality and concentration were measured using a Nanovue spectrophotometer (GE HealthCare, USA). 2ul of each RNA samples were pipetted into the spectrophotometer for measurement. RNA concentrations were adjusted to desired concentration based on the concentration measured using the Nanovue. Concentrations were adjusted to avoid adding <1ul of RNA volume for cDNA synthesis. Samples were then stored in -80C freezer or immediately used for cDNA synthesis.

1.6 Primer Design

HDC primers were designed using the NCBI Primer-Blast program. HDC DNA sequence data was used as template for the program (Gene ID: 3067). Primers were designed to span exon-exon boundaries to prevent amplification of genomic DNA. The designed primers were checked through the NCBI Blast program and exhibited specificity to only the HDC gene. The primer specificity to RNA was validated by running a RT-PCR using RNA extracted from samples along side genomic DNA collected from other Jurkat T

lymphocytes. Gel electrophoresis was used to see if results aligned with the expected sequence length (151 bp) of the HDC gene amplified.

Forward Primers: 5’ GGA CAG CAT CTT TGG GGA CAT 3’

Reverse primers: 5’ GAA TCC CAA GCA GTT GAT GGC 3’

Endogenous housekeeping control chosen was B2M. This gene was chosen as an endogenous control because T cell stimulation was reported to have no effect on the regulation of B2M[101]. B2M primers were designed using the NCBI Primer-Blast program. B2M DNA sequence data was used as template for the program (Gene ID: 567). Primers were designed to span exon-exon boundaries to prevent genomic DNA amplification. Endogenous control B2M gel electrophoresis was conducted prior to qPCR and confirmed amplified B2M sequence were of the expected length (192 bp).

Forward Primers: 5’ GCT CGC GCT ACT CTC TCT TT3’

Reverse primers: 5’ TCT GAA TGC TCC ACT TTT TCA A3’

Two positive control genes were employed: IL-2 and IL-8. These genes were chosen as positive controls because T cell stimulation was known to induce IL-2 and IL-8[102,103]. IL-2 is a classical T cell activation product with function to induce T cell proliferation in an autocrine fashion[104]. IL-8 has been reported to be released from activated T cells to create a chemotactic gradient, leading to chemotaxis in other T cells and granulocytes[105,106]. IL-

2/IL-8 primers were designed using the NCBI Primer-Blast program (Gene ID: 3558 and 3576). Primers were designed to span exon-exon boundaries to prevent genomic DNA amplification. Gel electrophoresis of IL-2 (202 bp) and IL-8 (118 bp) products were conducted prior to qPCR and confirmed amplified sequences are of the expected length.

L-2 Forward Primers: 5’ TTA CAT GCC CAA GAA GGC CA 3’

IL-2 Reverse primers: 5’ TGG TTG CTG TCT CAT CAG CAT 3’

IL-8 Forward Primers: 5’ CTG CGC CAA CAC AGA AAT TA 3’

IL-8 Reverse primers: 5’ TGA ATT CTC AGC CCT CTT CA 3’

Primers were synthesized by Mobix lab at McMaster University and delivered as freeze-dried oligonucleotides. Appropriate volume of DNA/RNA free water was added to adjust primers concentration to 100uM. An aliquot of primers was further diluted to 10uM. Primers diluted in 100uM were stored in -20C freezer, primers diluted to 10uM were stored in 4C fridge for use. 10uM primer should be replaced every 3 months.

1.7 Reverse Transcription

Reverse transcription of RNA samples was conducted using High-Capacity cDNA Reverse Transcription (Applied Biosystem, USA) kit according to the manufactures’ instructions. To allow for consistent qPCR results, only ~150ng of RNA was used in each reverse transcription process. The total volume of PCR tubes for reverse transcription were 30ul (150ng of cDNA total in 30ul,

5ng cDNA per ul). The total cDNA was inferred from a 1:1 ratio of RNA to cDNA conversion in the cDNA synthesis step.

The procedures were as follows. Each reagents were briefly vortexed and centrifuged before proceeding. A master mix of reagents was made with the composition of: 2.0ul of Reverse Transcription (RT) buffer (Applied Biosystem), 0.8ul of dNTP (100 mM) mix (Applied Biosystem), Reverse Transcription Random Primers (Applied Biosystem), and 4.2ul of DNA/RNA free water. The total volume of this master mix was 10ul per sample. The final 20ul of volume was that of the addition of appropriate RNA volume to make up 150 ng of RNA. DNA/RNA free water were added to fill the reaction to 30ul. For example: if the RNA sample had a concentration of 75ng/ul, 2ul of the RNA sample were added, along with 18ul of DNA/RNA free water to the final volume of 30ul total with the master mix. The samples were briefly centrifuged to ensure the volume was settled at the bottom. Reverse transcription were carried out using Applied Biosystem™ Veriti model thermal cycler (Applied Biosystem, USA). The thermal cycler was programed as followed: 25C for 10 minutes, 37C for 120 minutes, and 85C for 5 mins per manufacturer instructions. cDNA samples were then stored in -80C freezer.

1.8 Quantitative Polymerase Chain Reaction

cDNA samples were used with qPCR using Promega GoTaq™ qPCR Master Mix kit to perform qPCR as per manufacture's instructions. 2ul of cDNA samples from the reverse transcription steps was used per well (10ng of cDNA per qPCR well). Master mixes were made for each of the genes of interest. All reagents were briefly vortexed and centrifuged: the primers of the genes of interest, the GoTaq™ qPCR Master Mix (Promega, USA) and CxR reference dye (Promega, USA) before proceeding. The

composition of the master mix was as followed: 10ul of GoTaq qPCR Master Mix, 1ul of forward primer (10uM), 1ul of reverse primer (10uM), 0.2ul of CxR reference dye, and 5.8ul of DNA/RNA free water. 2ul of cDNA samples were plated, followed by 18ul of Master Mix based on the gene of interest onto Applied Biosystems™ MicroAmp Fast Optical 96-Well Reaction Plate.

Each sample was evaluated for 4 genes of interest. HDC as main result output, B2M as housekeeping endogenous control, and IL-2/IL-8 as positive stimulation control. Comparative deltadelta CT analysis was used as the main method of analysis[107]. The qPCR instrument used was the Applied Biosystems™ StepOnePlus Real Time PCR (Applied Biosystems, USA) and was programed as follows: Denature (95C for 45 secs), Annealing (57C for 30 secs) and Extension (72C for 1 min). Optimal annealing temperature were determined through prior temperature gradient PCR for all primers. Extension time were kept short to further prevent gDNA contamination from being amplified.

Gel electrophoresis was conducted using samples after qPCR. The gel was cut, and the DNA bands was sent for sequence analysis at the Mobix lab at McMaster University to confirm the amplified sequence to be that of HDC.

1.9 DeltaDelta Ct Analysis

qPCR data were outputted as Cycle Threshold (CT) values per well. CT values were normalized to housekeeping control (B2M) using the deltadelta CT analysis as developed by Livak and Schmittgen (2001)[107]. The overall formula for this method is:

$$= 2^{-\Delta\Delta Ct}$$

$$= 2^{(CT\ \textbf{Stimulated (HDC)} - CT\ \textbf{Stimulated (B2M)})_{TimeX} - (CT\ \textbf{Control (HDC)} - CT\ \textbf{Control (B2M)})_{TimeX}}$$

The Livak method was chosen for this project's analysis due to the experiment design of multiple timepoints and stimulation conditions. The Livak method calculated the difference between a target gene (HDC) relative to a stable housekeeping gene (B2M) in stimulated samples in each stimulation timepoint. The value of 2 to the power of deltadeltaCT indicates doubling of cDNA transcript each cycle of qPCR. First, the technical replicates of each sample for each gene of interest were averaged (n=3). The first deltaCT is the difference between HDC/IL-2/IL-8 and B2M cycle threshold in a stimulated sample. The CT values of HDC/IL-2/IL-8 in stimulated samples (anti-CD3/CD28, PMA/I) subtracts the CT values of B2M in stimulated samples of a given stimulation time (6-hour, 24-hour, 72-hour). The second deltaCT is the difference between HDC and B2M cycle threshold in unstimulated samples. The CT values of HDC/IL-2/IL-8 in unstimulated samples (media control) subtracts the CT values of B2M in unstimulated samples of a given stimulation time (6h, 24h, 72h). The deltadeltaCT of that stimulation time was obtained by taking the stimulated deltaCT and subtract the unstimulated deltaCT. The entire deltadeltaCT was treated as a negative for to allow the relative fold change to be positive. The fold change is then Log_2 transformed to showcase the upregulation or downregulation of the target genes (HDC/IL-2/IL-8) as compared to B2M in fold changes (LOG_2FC). A positive LOG_2FC indicates upregulation, a negative LOG_2FC indicates downregulation relative to the stable housekeeping gene. The Livak method was used to determine whether or

not the relative expression of a gene is induced or reduced, it was not used to determine whether a gene is expressed or not.

Aim 2: The evaluation of T lymphocyte HDC expression from publicly available single cell and bulk RNA sequencing datasets.

2.0 RNA Sequencing Analysis

Publicly available single cell and bulk RNA sequencing datasets of human T lymphocytes were evaluated to determine if the expression of HDC had been previously reported. As no previous studies have focused on the expression of HDC in human T lymphocytes, bioinformatics analysis was used to support the findings of this project. Bioinformatic databases were curated based on criteria mimicking the wet lab procedure in this project, as well as procedure criteria appropriating allergic diseases. These criteria were studies using asthmatic subjects, allergic subjects, followed by studies on T lymphocytes in a range of non-allergic diseases. Studies with large cell samples analysed were prioritized to evaluate the overall prevalence of HDC expression.

Dr. Anna Dvorkin worked on the bioinformatic analysis for this project and was responsible for every analysis done and as well as the construction of any figures related to bioinformatic analysis. Single cell dataset with mixed population of T lymphocytes and other cell types were evaluated by determining the expression of unique lineage markers to that cell linage.

Results

Aim 2: The evaluation of T lymphocyte HDC expression from publicly available single cell sequencing dataset

2.0 Bulk RNA sequencing of human T lymphocytes showed HDC expression.

Dr. Dvorkin analyzed a bulk RNA sequencing by (Single-cell transcriptomics identifies an effectorness gradient shaping the response of $CD4^+$ T cells to cytokines, https://doi.org/10.1038/s41467-020-15543-y) and found the expression of HDC in different CD4+ T cell phenotypes (**Figure 1A**). The authors of the dataset isolated human CD4+ T cells through the usage of Ficoll-Paque density gradient, as well as StemCell EasySep™ negative selection kit for memory CD4+ T cells and naïve CD4+ T cells. These lymphocytes were then activated using anti-CD3/CD28 beads, with the addition of appropriate cocktails to induce CD4+ T cells differentiation. The phenotypes and their respective cytokine cocktails utilized were as follow: Th0 (no additional cytokines), Th1 (IL-12, anti-IL-4), Th2 (IL-4, anti-IFNγ), Th17 (TGFb, IL-6, IL-23, IL-1b, anti-IFNγ, anti-IL-4), iTreg (TGFb, IL-2), IFNβ (IFNβ). The stimulation timepoints were either 16-hour, or 5-days, at which the sample were collected for bulk RNA sequencing analysis. Dr. Dvorkin found that CD4+ T cells belonging to almost every differentiated phenotype had varied level of HDC expression, with exception of resting naive CD4+ T cells at 5-day stimulation (**Figure 1A**). Memory CD4+ T cells showed a higher level of HDC expression as compared to any naïve CD4+ T cells phenotype. Activation of T cells using anti-CD3/CD28 beads did not lead to an upregulation in HDC expression in any T cell phenotype (**Figure 1A**). Isolated T cells activated for 16-hour showed a higher normalized expression of HDC as compared to the 5-day stimulation group (**Figure 1A**). This analysis was applied to measure the expression of IL-8

and IL-2 to mimic the experimental procedure of our experiments. Resting CD4+ T cells of either memory or naïve phenotype did not appear to express IL-2 (**Figure 1B**). All activated phenotype of CD4+ T cells upregulated IL-2 when activated. This upregulation was strongest at 16-hour and was lowered at 5-day of stimulation (**Figure 1B**). Analysis of IL-8 expression showed the expression of IL-8 in every phenotype, as well as any stimulation time, and including resting CD4+ T cells (**Figure 1C**). The strongest expression of IL-8 was from memory CD4+ T cells of all phenotypes. Interestingly, resting 16-hour and 5-day CD4+ T cells express this similar strength of IL-8 expression, with an overall higher expression for the 16-hour timepoints accounting for standard errors (**Figure 1C)**.

2.1 Single cell RNA sequencing of human T lymphocytes showed HDC expression in rare and heterogenous population of T lymphocytes.

Dr. Dvorkin analyzed 3 different scRNA datasets from T cell single cell sequencing analysis studies for the identification of HDC+ T cells. It was found that rare T cells of different phenotypes were detected to express HDC at a low level.

The first dataset (Single-cell transcriptomics identifies an effectorness gradient shaping the response of CD4+ T cells to cytokines, https://doi.org/10.1038/s41467-020-15543-y) had samples of memory and naïve CD4+ T cells at the 16-hour stimulation and at the 5-day stimulations. The pre-annotated T cell phenotypes were determined by the authors using lineage markers and as well as the corresponding stimulation conditions as outlined in **2.0 Bulk RNA sequencing of human T lymphocytes showed HDC expression.** Analysis of this dataset showed different T cell phenotype to lowly express HDC. The frequency of HDC+ T cell per annotated phenotype were as follows: Natural T regulatory cell - nTreg (Th0): 0.56%, T central

memory cell of cluster 1 - TCM1 (Th0): 0.625%, T central memory cell of cluster 1 - TCM1 (Th17/iTreg): 0.69%, T central memory cell of cluster 2 - TCM2 (Th0): 0.17%, T central memory cell of cluster 2 - TCM2 (Th17/iTreg): 0.56%, T effector memory - TEM (Th0): 0.17%, T effector memory - TEM (Th17/iTreg): 0.88%, T effector memory re-expressing CD45RA - TEMRA (Th0): 0.29%, T effector memory re-expressing CD45RA - TEMRA (Th17/iTreg): 0.77%, Naïve T cells - TN (iTreg): 0.71%, Naïve T cells - TN (Th0): 0.81%, Naïve T cells - TN (Th17): 0.79%, Naïve T cells - TN (Th17/iTreg): 1.06%, Naïve T cells - TN (Th2): 0.264%. Resting T cells phenotypes were not detected to express HDC (**Figure 2)**.

The second dataset (Single-cell transcriptomics of human T cells reveals tissue and activation signatures in health and disease, https://doi.org/10.1038/s41467-019-12464-3) utilized mechanical tissue disruption, Ficoll-Paque density gradient, followed by CD3+ negative selection (Human CD3+ T cell Isolation Kit, BioLegend) to isolate human T cells. The isolated T cells were then activated using soluble anti-CD3/CD28 for 16 hours and mRNA was collected for single cell sequencing by 10x Chromium. Analysis of this dataset showed HDC+ cells from resting or activated CD4 and CD8 T cells as classified by the author (**Figure 3A**). Breaking down the annotated cell populations showed the percentage of HDC+ from each population: activated CD4+ T cells: 0.12% (23/18979 cells), activated CD8+ T cell: 0.21% (14/6515 cells), unclassified cells “none”: 3.09% (907/29394 cells), resting CD4+ T cells: 0.15% (11/7102 cells), resting CD8+ T cells: 0.039% (7/17706 cells), unassigned resting and activated T cells were not positive for HDC expression. 55 out of 51130 T cells were HDC+, a total of 962 out of 80524 cells were HDC+. 29394 cells (36.5%) were not classified by the authors as T cells and this population was

likely composed of contaminating cells (**Figure 3A**). The expression level of activated T cells and resting T cells varied but appeared to be of similar level.

Using different markers to identify differentiated T cells allowed Dr. Dvorkin to identify a population of HDC+ cells to also be IFNγ+, implying that HDC expression was found in the Th1 phenotype (**Figure 4**). Some HDC+ cells were also co-expressing GATA3 (master transcription regulator of Th2 differentiation), IL-4 (Th2 cytokine), IL-5 (Th2 cytokine), and IL-2 (activated T cells cytokine). HDC+ expression in these co-expressing cells represent a small population of overall "T cells", with HDC+/GATA3+ cells representing 0.07% of the total population, HDC+/IL-4+ cells representing 0.03% of the total population, HDC+/IL-5+ cells representing 0.062% of the total population, HDC+/IFNγ+ cells representing 0.163% of the total population, and HDC+/IL-2+ cells representing 0.022% of the total population (**Figure 4**).

Interestingly, the majority of HDC+ cells were not classified as T cells by the authors. 18.08% of this "none" population was found to co-express HDC and IL-8. Attempts to classify these mystery HDC+ cells found most of these mystery cells to resemble ILC2, NK cells and basophils. The marker utilizes were as follows: CCR3, FcεR1 for basophils, IL-7R, KLRG1 for ILC2 cells and NCR1, KLRB1, NKG7, CD3e negative for NK cells. Basophil was the predominant HDC+ contaminating cells (**Figure 3B)**. Additional analysis to identify the specific phenotype of the mystery "NK cell" found that it was unlikely that these were true NK cells (**Supplementary Figure 8**). Analysis also found possible contaminating cells with some markers of plasmacytoid DCs and myeloid DCs. The existence of contaminating basophil when utilizing negative selection as a mean to isolate T cells has implication for the rest of the result of this project (**Figure 3B)**. Regardless, rare but classifiable T cells were found to be positive for HDC expression.

The third study (Single-cell transcriptomic analysis of allergen-specific T cells in allergy and asthma, https://doi.org/10.1126/sciimmunol.aba6087) utilized fluorescent activated cell sorting to isolated house dust-mite allergen specific T lymphocytes from human PBMC samples. Analysis done by Dr. Dvorkin once again found rare T cells of the T cell effector phenotype to express HDC (**Figure 5**). 6 out of 28181 T effector cells were positive for HDC expression. The clustering of some HDC+ T effector cells suggested that the expression landscape resembles that of Th2 cells. Low-level of activated T cells (CD154-) were excluded from the analysis by the authors.

Further correlation analysis of these HDC+ cells found these cells were likely to co-expressed the IL1RL1 gene (IL-33 receptor gene) (**Supplementary Table 6**). Other genes that co-expressed with significant correlation in HDC+ cells include GZMB (granzyme B – expressed in CD8+ T cells, Treg cells, Th1 cells, Th2 cells, Th17 cells[108]), C10orf54 (VISTA – V-Domain Ig Suppressor of T cell activation – expressed in CD4+ T cells, regulatory T cells[109]), GNLY (granulysis - expressed in CD8+ T cells, NK cells[110]), OPA1 (maintenance of mitochondrial DNA, ubiquitously expressed[111]), HDAC7 (histone deacetylase 7 – highly expressed in CD4+ and CD8+ T cells[112]), NQO2 (enzyme that catalyze the reduction of quinone substrate – highly expressed in liver and kidney[113]), VPS25 (Vacuolar protein sorting associated protein – ubiquitously expressed[114]), GADD45G (growth arrest and DNA damage inducible gene – expressed in T cells, preferentially in Th1 and CD8+ cells[115]), SLC39A6 (zinc transporter - ubiquitously expressed[116]).

Aim 1: The evaluation of T lymphocyte expression of HDC

1.0 Jurkat T lymphocytes stimulated with anti-CD3/CD28 beads demonstrated an upregulation of HDC expression.

The activation of Jurkat T lymphocytes using anti-CD3/CD28 beads or PMA/Ionomycin led to induction of HDC at 6-hour, 24-hour, and 72-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 30.04 +/- 1.48 CT at 6-hour, 25.57 +/- 2.57 CT at 24-hour, and 27.33 +/- 3.65 CT at 72-hour of stimulation (n=7, **Supplementary Table 1A**). The cycle threshold (CT) values for PMA/I stimulation were averaged at 29.13 +/- 1.13 CT at 6-hour, 25.14 +/- 2.73 CT at 24-hour, and 23.67 +/- 2.29 CT at 72-hour of stimulation (n=7, **Supplementary Table 1A**). Unstimulated media control samples averaged 33.13 +/- 1.10 CT at 6-hour, 33.81 +/- 1.78 CT at 24-hour, and 34.19 +/- 1.96 CT at 72-hour. CT values represent the number of qPCR cycle when the amplified product fluoresces at a threshold of detection. Therefore, lower cycle threshold corresponds to a higher abundance of the transcript of interest.

Following normalization of HDC's CT values to the B2M housekeeping gene, the degree of HDC induction was calculated as a $\log_2$ fold change (LOG_2FC) as compared to B2M expression. The averaged LOG_2FC for the expression of HDC when Jurkat T lymphocytes were stimulated with anti-CD3/CD28 was 3.2 +/- 0.79 at 6-hour, 7.6 +/- 0.67 at 24-hour, and 6.5 +/- 1.5 at 72-hour (**Figure 6, Table 1**). The averaged LOG_2FC for the expression of HDC when Jurkat T lymphocytes were stimulated with PMA/I was 3.6 +/- 0.38 at 6-hour, 7.7 +/- 1.0 at 24-hour, and 8.3 +/- 1.1 at 72-hour (**Figure 6, Table 1**).

IL-8 and IL-2 expression were measured as positive controls for Jurkat T cell stimulation. Activation of Jurkat T lymphocytes using anti-CD3/CD28 beads or PMA/Ionomycin led to induction of IL-8 and IL-2 at 6-hour, 24-hour, and 72-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 27 +/- 1.3 CT for IL-8, 26.81 +/- 2.67 CT for IL-2 at 6-hour, 27.98 +/- 0.76 CT for IL-8 and 27.24 +/- 4.11 CT for IL-2 at 24-hour, 33.57 +/- 1.62 CT for IL-8 and 30.79 +/- 1.53 CT for IL-2 at 72-hour of stimulation (n=7, **Supplementary Table 1A**). The cycle threshold (CT) values for PMA/I stimulation were averaged at 22.47 +/- 1.65 CT for IL-8 and 23.06 +/- 2.3 CT for IL-2 at 6-hour, 25.67 +/- 2.4 CT for IL-8 and 21.52 +/- 3.76 CT for IL-2 at 24-hour, 27.86 +/- 2.12 CT for IL-8 and 25.81 CT for IL-2 at 72-hour of stimulation (n=7, **Supplementary Table 1A**). Unstimulated media control samples averaged 33.19 +/- 1.75 CT for IL-8 and 33.22 +/- 1.32 CT for IL-2 at 6-hour, 34.77 +/- 1.62 CT for IL-8 and 32.93 +/- 0.56 CT for IL-2 at 24-hour, 33.29 +/- 2.8 CT for IL-8 and 32.66 +/- 1.21 CT for IL-2 at 72-hour (n=7, **Supplementary Table 1A**).

Normalization to B2M housekeeping gene showed the degree of IL-8 and IL-2 upregulation. The averaged LOG_2FC for the expression of IL-8 when Jurkat T lymphocytes were stimulated with anti-CD3/CD28 was 6.3 +/- 1.8 at 6-hour, 6.1 +/- 1.1 at 24-hour, and -0.69 +/- 1.5 at 72-hour (**Figure 6, Table 1**). The averaged LOG_2FC for the expression of IL-8 when Jurkat T lymphocytes were stimulated with PMA/I was 10 +/- 0.7 at 6-hour, 8.2 +/- 0.72 at 24-hour, and 3.2 +/- 0.8 at 72-hour (**Figure 6, Table 1**). The averaged LOG_2FC for the expression of IL-2 when Jurkat T lymphocytes were stimulated with anti-CD3/CD28 was 6.6 +/- 1.7 at 6-hour, 5.0 +/- 1.7 at 24-hour, and 1.5 +/- 0.8 at 72-hour (**Figure 6, Table 1**). The averaged LOG_2FC for the

expression of IL-2 when Jurkat T lymphocytes were stimulated with PMA/I was 9.8 +/- 1.2 at 6-hour, 10 +/- 3.7 at 24-hour, and 4.6 +/- 1.2 at 72-hour (**Figure 6, Table 1**).

Melt curve analysis of Jurkat T lymphocyte post qPCR for HDC showed a distinct peak at 85.9C (**Figure 12**). It was impossible to confirm the product identity of this peak but given the upregulation of HDC when compared to media control, it is most likely that this peak represented the amplified HDC product. This peak was present in both unstimulated and stimulated samples, but unstimulated samples also frequently possess multiple peaks of less than 85C, which is indicative of non-specific binding and primer dimer formation (**Figure 12**).

LinRegPCR is a mathematical program designed by Ruijter et al to evaluate qPCR data based on the mathematical modeling of the amplification curve in qPCR cycling. A function of this program is to use the fluorescent value at the qPCR detection threshold and work backwards to determine an arbitrary starting fluorescence value (N0 value) of a sample. Thus, the program can interpolate the absolute abundance of the starting material of a gene of interest (cDNA in this project) using this arbitrary starting fluorescent value. The N0 in AFU (arbitrary fluorescence unit) for Jurkat T lymphocytes were averaged out across every biological replicate (n=7).

The average N0 for HDC expression in Jurkat T lymphocytes stimulated with anti-CD3/CD28 were as follows: 1.539e-008 +/- 4.028e-009 at 6-hour, 2.362e-007 +/- 7.192e-008 at 24-hour, and 3.105e-007 +/- 1.251e-007 at 72-hour (**Supplementary Figure 1, Table 2**). The average NO for HDC expression in Jurkat T lymphocytes stimulated with PMA/I were as follows: 2.568e-008 +/- 6.474e-009at 6-hour, 5.037e-007 +/- 1.286e-007 at 24-hour, and 1.330e-006 +/-

4.054e-007 at 72-hour (**Supplementary Figure 1, Table 2**). The average N0 for HDC expression in unstimulated Jurkat T lymphocytes were as follows: 2.259e-009 +/- 5.438e-010 at 6-hour, 9.061e-010 +/- 2.076e-010 at 24-hour, and 1.037e-009 +/- 2.487e-010 at 72-hour (**Supplementary Figure 1, Table 2**). The average N0 for IL-8 expression in Jurkat T lymphocytes stimulated with anti-CD3/CD28 were as follows: 7.572e-008 +/- 1.899e-008 at 6-hour, 1.899e-008 +/- 5.776e-009 at 24-hour, and 2.063e-009 +/- 8.782e-010 at 72-hour (**Supplementary Figure 1, Table 2**). The average N0 for HDC expression in Jurkat T lymphocytes stimulated with PMA/I were as follow: 3.879e-007 +/- 8.678e-008 at 6-hour, 6.959e-008 +/- 1.684e-008 at 24-hour, and 1.227e-008 +/- 5.575e-009 at 72-hour (**Supplementary Figure 1, Table 2**). The average N0 for IL-8 expression in unstimulated Jurkat T lymphocytes were as follows: 3.144e-010 +/- 1.070e-010 at 6-hour, 6.984e-010 +/- 2.327e-010 at 24-hour, and 4.280e-010 +/- 1.391e-010 at 72-hour (**Supplementary Figure 1, Table 2**).

Based on the data shown, Jurkat T lymphocytes express HDC and this expression can be upregulated when stimulated using anti-CD3/CD28 or PMA/I at 6-hour, 24-hour, and 72-hour.

1.1 Stimulation of human CD4+ T cells did not induce HDC expression, regardless of allergic status.

The activation of human CD4+ T lymphocytes using from non-allergic donors anti-CD3/CD28 beads or PMA/Ionomycin did not lead to induction of HDC at 6-hour, 24-hour, and 72-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 32.38 +/- 0.53 at 6-hour, 32 +/- 0.89 CT at 24-hour, and 33 +/- 0 CT at 72-hour of stimulation (n=6, **Supplementary Table 1B**). The cycle threshold (CT) values for PMA/I stimulation were averaged at 32.50 +/- 0.5 CT at 6-hour, 31.65

+/- 0.53 CT at 24-hour, and 32.01 +/- 0.66 CT at 72-hour of stimulation (n=6, **Supplementary Table 1B**). Unstimulated media control samples averaged 32.48 +/- 0.4 CT at 6-hour, 32.81 +/- 0.78 CT at 24-hour, and 32.94 +/- 0.91 CT at 72-hour.

The activation of human CD4+ T lymphocytes from allergic donors using anti-CD3/CD28 beads or PMA/Ionomycin did not lead to induction of HDC at 6-hour, 24-hour, and 72-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 31.02 +/- 2.27 CT at 6-hour, 31.76 +/- 2.38 CT at 24-hour, and 33.11 +/- 2.01 CT at 72-hour of stimulation (n=6, **Supplementary Table 1C**). The cycle threshold (CT) values for PMA/I stimulation were averaged at 31.96 +/- 2.09 CT at 6-hour, 31.15 +/- 1.9 CT at 24-hour, and 31.65 +/- 1.49 CT at 72-hour of stimulation (n=6, **Supplementary Table 1C**). Unstimulated media control samples averaged 30.76 +/- 2.65 CT at 6-hour, 31.54 +/- 1.9 CT at 24-hour, and 31.45 +/- 2.36 CT at 72-hour.

Following normalization of HDC's CT values to the B2M housekeeping gene, the averaged LOG_2FC for the expression of HDC when human CD4+ T lymphocytes from non-allergic donors were stimulated with anti-CD3/CD28 was -1.0 +/- 0.48 at 6-hour, -1.3 +/- 0.23 at 24-hour, and -2.2 +/- 0.32 at 72-hour (**Figure 7, Table 2).** The averaged LOG_2FC for the expression of HDC when human CD4+ T lymphocytes from non-allergic donors were stimulated with PMA/I was -0.42 +/- 0.76 at 6-hour, -1.2 +/- 0.63 at 24-hour, and -1.4 +/- 0.54 at 72-hour (**Figure 7, Table 2**).

The averaged LOG_2FC for the expression of HDC when human CD4+ T lymphocytes from allergic donors were stimulated with anti-CD3/CD28 was -0.48 +/- 0.13 at 6-hour, -2.1 +/- 0.35 at 24-hour, and -4.1 +/- 1.1 at 72-hour (**Figure 8, Table 3**). The averaged LOG_2FC for the expression

of HDC when allergic human CD4+ T lymphocytes were stimulated with PMA/I was -1.3 +/- 0.58 at 6-hour, -1.5 +/- 0.42 at 24-hour, and -2.9 +/- 1.0 at 72-hour (**Figure 8, Table 3**).

IL-8 and IL-2 expression were measured as positive controls for human CD4+ T cells stimulation. Activation of human CD4+ T lymphocytes from non-allergic donors using anti-CD3/CD28 beads or PMA/Ionomycin led to induction of IL-8 and IL-2 at 6-hour, 24-hour, and 72-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 24.48 +/- 1.4 CT for IL-8, 16.76 +/- 1.02 CT for IL-2 at 6-hour, 25.77 +/- 2.27 CT for IL-8 and 16.19 +/- 1.11 CT for IL-2 at 24-hour, 29.43 +/- 1.98 CT for IL-8 and 21.72 +/- 1.91 CT for IL-2 at 72-hour of stimulation (n=6, **Supplementary Table 1B**). The cycle threshold (CT) values for PMA/I stimulation were averaged at 22.08 +/- 2.03 CT for IL-8 and 16.44 +/- 2.17 CT for IL-2 at 6-hour, 23.48 +/- 1.33 CT for IL-8 and 15.73 +/- 0.49 CT for IL-2 at 24-hour, 27.6 +/- 1.67 CT for IL-8 and 19.97 +/- 3.67 CT for IL-2 at 72-hour of stimulation (n=6, **Supplementary Table 1B**). Unstimulated media control samples averaged 28.44 +/- 2.99 CT for IL-8 and 29.08 +/- 3.52 CT for IL-2 at 6-hour, 31.29 +/- 2.76 CT for IL-8 and 30.28 +/- 0.31 CT for IL-2 at 24-hour, 30.45 +/- 2.94 CT for IL-8 and 29.91 +/- 1.04 CT for IL-2 at 72-hour (n=6, **Supplementary Table 1B**).

Activation of human CD4+ T lymphocytes from allergic donors using anti-CD3/CD28 beads or PMA/Ionomycin led to induction of IL-8 and IL-2 at 6-hour, 24-hour, and 72-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 23.94 +/- 2.23 CT for IL-8, 17.56 +/- 1.68 CT for IL-2 at 6-hour, 24.84 +/- 3.05 CT for IL-8 and 15.94 +/- 1.51 CT for IL-2 at 24-hour, 28.44 +/- 1.46 CT for IL-8 and 21.17 +/- 1.82 CT for IL-2 at 72-hour of stimulation (n=6, **Supplementary Table 1C**). The cycle

threshold (CT) values for PMA/I stimulation were averaged at 20.19 +/- 1.75 CT for IL-8 and 15.58 +/- 1.69 CT for IL-2 at 6-hour, 23.00 +/- 1.73 CT for IL-8 and 15.83 +/- 1.36 CT for IL-2 at 24-hour, 27.06 +/- 3.33 CT for IL-8 and 21.17 +/- 5.29 for IL-2 at 72-hour of stimulation (n=6, **Supplementary Table 1C**). Unstimulated media control samples averaged 28 +/- 1.9 CT for IL-8 and 29.51 +/- 0.58 CT for IL-2 at 6-hour, 31 +/- 2.92 CT for IL-8 and 29.34 +/- 0.79 CT for IL-2 at 24-hour, 29.99 +/- 2.96 CT for IL-8 and 28.97 +/- 0.95 CT for IL-2 at 72-hour (n=6, **Supplementary Table 1C**).

The averaged LOG_2FC for the expression of IL-8 when human CD4+ T lymphocytes from non-allergic donors were stimulated with anti-CD3/CD28 when normalized to B2M was 2.8 +/- 0.82 at 6-hour, 3.5 +/- 0.47 at 24-hour, and –1.2 +/- 1.3 at 72-hour (**Figure 7, Table 2**). The averaged LOG_2FC for the expression of IL-8 when human CD4+ T lymphocytes from non-allergic donors were stimulated with PMA/I was 6 +/- 0.77 at 6-hour, 5.4 +/- 1.6 at 24-hour, and 0.48 +/- 0.76 at 72-hour (**Figure 7, Table 2**). The averaged LOG_2FC for the expression of IL-2 when human CD4+ T lymphocytes from non-allergic donors were stimulated with anti-CD3/CD28 was 11 +/- 1.3 at 6-hour, 12 +/- 0.49 at 24-hour, and 6 +/- 0.52 at 72-hour (**Figure 7, Table 2**). The averaged LOG_2FC for the expression of IL-2 when human CD4+ T lymphocytes from non-allergic donors were stimulated with PMA/I was 12 +/- 1.2 at 6-hour, 12 +/- 0.49 at 24-hour, and 7.6 +/- 1.4 at 72-hour (**Figure 7, Table 2**).

The averaged LOG_2FC for the expression of IL-8 when human CD4+ T lymphocytes from allergic donors were stimulated with anti-CD3/CD28 and normalized to B2M was 3.8 +/- 0.89 at 6-hour, 4.3 +/- 0.95 at 24-hour, and -0.92 +/- 0.76 at 72-hour (**Figure 8, Table 3**). The averaged LOG_2FC for the expression of IL-8 when human CD4+ T lymphocytes from allergic donors were

stimulated with PMA/I was 7.7+/- 0.7 at 6-hour, 6.1 +/- 0.76 at 24-hour, and 0.21 +/- 1.2 at 72-hour (**Figure 8, Table 3**). The averaged LOG_2FC for the expression of IL-2 when human CD4+ T lymphocytes from allergic donors were stimulated with anti-CD3/CD28 was 12 +/- 0.71 at 6-hour, 11 +/- 0.61 at 24-hour, and 5.3 +/- 1.3 at 72-hour (**Figure 8, Table 3**). The averaged LOG_2FC for the expression of IL-2 when human CD4+ T lymphocytes from allergic donors were stimulated with PMA/I was 14 +/- 0.65 at 6-hour, 12 +/- 0.76 at 24-hour, and 5.1 +/- 2.5 at 72-hour (**Figure 8, Table 3**).

Melt curve analysis of human CD4+ T lymphocyte from non-allergic and allergic donors post qPCR for HDC showed a distinct peak at 85.9C akin to that of Jurkat T lymphocytes (**Figure 13**). This peak was present in both unstimulated and stimulated samples, but unstimulated and stimulated samples also frequently possess multiple peaks of less than 85C, which is indicative of non-specific binding and primer dimer formation (**Figure 13**).

The average N0 for HDC expression in combined human CD4+ T lymphocytes from non-allergic and allergic donors stimulated with anti-CD3/CD28 were as follows: 3.073e-009 +/- 4.680e-010 at 6-hour, 3.490e-009 +/- 4.159e-010 at 24-hour, and 1.393e-009 +/- 1.538e-010 at 72-hour (**Supplementary Figure 2, Table 3**). The average N0 for HDC expression in human CD4+ T lymphocytes stimulated with PMA/I were as follow: 1.931e-009 +/- 2.308e-010 at 6-hour, 3.100e-009 +/- 3.100e-009 at 24-hour, and 3.412e-009 +/- 4.746e-010 at 72-hour (**Supplementary Figure 2, Table 3**). The average N0 for HDC expression in unstimulated human CD4+ T lymphocytes were as follows: 4.401e-009 +/- 9.732e-010 at 6-hour, 2.374e-009 +/- 3.576e-010 at 24-hour, and 2.924e-009 +/- 7.455e-010 at 72-hour (**Supplementary Figure 2, Table 3**).

The average N0 for IL-8 expression in combined human CD4+ T lymphocytes from non-allergic and allergic donors stimulated with anti-CD3/CD28 were as follows: 2.838e-007 +/- 6.582e-008 at 6-hour, 2.278e-007 +/- 4.847e-008 at 24-hour, and 8.951e-009 +/- 1.640e-009 at 72-hour (**Supplementary Figure 2, Table 3**). The average N0 for IL-8 expression in human CD4+ T lymphocytes stimulated with PMA/I were as follow: 1.571e-006 +/- 3.741e-007 at 6-hour, 4.298e-007 +/- 8.721e-008 at 24-hour, and 3.106e-008 +/- 4.724e-009 at 72-hour (**Supplementary Figure 2, Table 3**). The average N0 for IL-8 expression in unstimulated human CD4+ T lymphocytes were as follows: 1.205e-007 +/- 5.031e-008 at 6-hour, 9.666e-008 +/- 4.947e-008 at 24-hour, and 1.048e-008 +/- 2.995e-009 at 72-hour (**Supplementary Figure 2, Table 3**).

Comparing the CT values, LOG_2FC as normalized to B2M, and LinRegPCR analysis N0 values of HDC expression in non-allergic and allergic individuals showed a consistent flatline between HDC expression of stimulated and unstimulated samples. This is indicative of a lack of HDC upregulation when stimulated with anti-CD3/CD28 or PMA/I. Self-reported allergic status did not influence the upregulation of HDC.

1.2 Stimulation of human CD4+, Th2 cells did not induce HDC expression, regardless of allergic status.

The activation of differentiated human Th2 lymphocytes from non-allergic donors using anti-CD3/CD28 beads or PMA/Ionomycin did not lead to induction of HDC at 6-hour, 24-hour, and 72-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 34.4 +/- 0.89 at 6-hour, 34.4 +/- 0.55 CT at 24-hour, and 34.19 +/- 0.76 CT at 72-hour of stimulation (n=5, **Supplementary Table 1D**). The

cycle threshold (CT) values for PMA/I stimulation were averaged at 34.4 +/- 0.86 CT at 6-hour, 33.87 +/- 0.9 CT at 24-hour, and 35.13 +/- 0.66 CT at 72-hour of stimulation (n=5, **Supplementary Table 1D**). Unstimulated media control samples averaged 34.83 +/- 0.45 CT at 6-hour, 34.73 +/- 0.43 CT at 24-hour, and 34.27 +/- 0.72 CT at 72-hour.

The activation of allergic human differentiated human Th2 lymphocytes using anti-CD3/CD28 beads or PMA/Ionomycin did not lead to induction of HDC at 6-hour, 24-hour, and 72-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 33.63 +/- 1.58 CT at 6-hour, 34.03 +/- 1.48 CT at 24-hour, and 34.07 +/- 1.61 CT at 72-hour of stimulation (n=6, **Supplementary Table 1E**). The cycle threshold (CT) values for PMA/I stimulation were averaged at 33.56 +/- 1.38 CT at 6-hour, 33.81 +/- 1.1 CT at 24-hour, and 34.14 +/- 1.02 CT at 72-hour of stimulation (n=6, **Supplementary Table 1E**). Unstimulated media control samples averaged 34.25 +/- 1.19 CT at 6-hour, 34.07 +/- 1.61 CT at 24-hour, and 33.78 +/- 1.44 CT at 72-hour.

Following normalization of HDC's CT values to the B2M housekeeping gene, the averaged LOG_2FC for the expression of HDC when human differentiated human Th2 lymphocytes from non-allergic were stimulated with anti-CD3/CD28 was -0.17 +/- 0.46 at 6-hour, 0.51 +/- 0.88 at 24-hour, and -1.1 +/- 0.7 at 72-hour (**Figure 9, Table 4**). The averaged LOG_2FC for the expression of HDC when human differentiated human Th2 lymphocytes from non-allergic donors were stimulated with PMA/I was 0.45 +/- 1 at 6-hour, 0.5 +/- 0.82 at 24-hour, and -2.2 +/- 0.66 at 72-hour (**Figure 9, Table 4**).

The averaged LOG_2FC for the expression of HDC when human differentiated human Th2 lymphocytes from allergic donors were stimulated with anti-CD3/CD28 was 0.8 +/- 0.94 at 6-hour, 1.2 +/- 0.45 at 24-hour, and -1.6 +/- 0.64 at 72-hour (**Figure 10, Table 5**). The averaged LOG_2FC for the expression of HDC when human differentiated human Th2 T lymphocytes from allergic donors were stimulated with PMA/I was 1.7 +/- 0.69 at 6-hour, 1.1 +/- 0.41 at 24-hour, and -2.1 +/- 0.73 at 72-hour (**Figure 10, Table 5**).

IL-8 and IL-2 expression were measured as positive controls for human differentiated human Th2 T cells stimulation. The activation of human differentiated human Th2 lymphocytes from non-allergic donors using anti-CD3/CD28 beads or PMA/Ionomycin led to induction of IL-8 and IL-2 at 6-hour, 24-hour, and 72-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 24.99 +/- 1.3 CT for IL-8, 26.07 +/- 2.2 CT for IL-2 at 6-hour, 26.13 +/- 1.04 CT for IL-8 and 25.56 +/- 2.62 CT for IL-2 at 24-hour, 24.83 +/- 3.25 CT for IL-8 and 29.37 +/- 2.51 CT for IL-2 at 72-hour of stimulation (n=5, **Supplementary Table 1D**). The cycle threshold (CT) values for PMA/I stimulation were averaged at 18.53 +/- 2.17 CT for IL-8 and 20.11 +/- 3.26 CT for IL-2 at 6-hour, 22.67 +/- 1.02 CT for IL-8 and 22.4 +/- 2.51 CT for IL-2 at 24-hour, 26.15 +/- 0.74 CT for IL-8 and 27.16 +/- 4.3 CT for IL-2 at 72-hour of stimulation (n=5, **Supplementary Table 1D**). Unstimulated media control samples averaged 31.53 +/- 2.77 CT for IL-8 and 32.16 +/- 0.59 CT for IL-2 at 6-hour, 35.93 +/- 2.13 CT for IL-8 and 31.67 +/- 0.41 CT for IL-2 at 24-hour, 36.41 +/- 2.94 CT for IL-8 and 30.66 +/- 1.71 CT for IL-2 at 72-hour (n=5, **Supplementary Table 1D**).

Activation of human differentiated human Th2 lymphocytes from allergic donors using anti-CD3/CD28 beads or PMA/Ionomycin led to induction of IL-8 and IL-2 at 6-hour, 24-hour,

and 72-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 22.89 +/- 2.13 CT for IL-8, 24.1 +/- 1.78 CT for IL-2 at 6-hour, 27.24 +/- 2.99 CT for IL-8 and 25.92 +/- 3.84 CT for IL-2 at 24-hour, 27.93 +/- 3.39 CT for IL-8 and 28.17 +/- 1.82 CT for IL-2 at 72-hour of stimulation (n=6, **Supplementary Table 1E**). The cycle threshold (CT) values for PMA/I stimulation were averaged at 18.39 +/- 1.29 CT for IL-8 and 21.29 +/- 3.28 CT for IL-2 at 6-hour, 23.45 +/- 0.92 CT for IL-8 and 23.86 +/- 3.6 CT for IL-2 at 24-hour, 24.41 +/- 2.96 CT for IL-8 and 25.62 +/- 4.05 for IL-2 at 72-hour of stimulation (n=6, **Supplementary Table 1E**). Unstimulated media control samples averaged 29.76 +/- 2.29 CT for IL-8 and 32.61 +/- 0.94 CT for IL-2 at 6-hour, 34.18 +/- 1.07 CT for IL-8 and 32.27 +/- 0.71 CT for IL-2 at 24-hour, 35.17 +/- 1.31 CT for IL-8 and 31.62 +/- 0.47 CT for IL-2 at 72-hour (n=6, **Supplementary Table 1E**).

Normalization to B2M housekeeping gene showed upregulation of IL-8 and IL-2 in human differentiated Th2 lymphocytes from non-allergic donors. The averaged LOG_2FC for the expression of IL-8 when human differentiated human Th2 lymphocytes from non-allergic donors were stimulated with anti-CD3/CD28 was 5.8 +/- 0.79 at 6-hour, 10 +/- 0.7 at 24-hour, and 10 +/- 2.5 at 72-hour (**Figure 9, Table 4**). The averaged LOG_2FC for the expression of IL-8 when human differentiated human Th2 lymphocytes from non-allergic donors were stimulated with PMA/I was 13+/- 0.38 at 6-hour, 13 +/- 0.5 at 24-hour, and 8.9+/- 1.4 at 72-hour (**Figure 9, Table 4**). The averaged LOG_2FC for the expression of IL-2 when human differentiated Th2 lymphocytes from non-allergic donors were stimulated with anti-CD3/CD28 was 5.5 +/- 0.92 at 6-hour, 6.3 +/- 1.3 at 24-hour, and 0.093 +/- 1.3 at 72-hour (Figure, Table). The averaged LOG_2FC for the expression of IL-2 when differentiated human Th2 T lymphocytes from non-allergic donors were stimulated

with PMA/I was 12 +/- 1.5 at 6-hour, 8.9 +/- 1.4 at 24-hour, and 2.2 +/- 1.9 at 72-hour (**Figure 9, Table 4**).

Normalization to B2M housekeeping gene also showed IL-8 and IL-2 induction in differentiated human Th2 lymphocytes from allergic donors. The averaged LOG_2FC for the expression of IL-8 when differentiated human Th2 lymphocytes from allergic donors were stimulated with anti-CD3/CD28 was 7.1 +/- 0.96 at 6-hour, 8.1 +/- 1 at 24-hour, and 6 +/- 1.5 at 72-hour (**Figure 10, Table 5**). The averaged LOG_2FC for the expression of IL-8 when human CD4+ T lymphocytes from allergic donors were stimulated with PMA/I was 12 +/- 0.97 at 6-hour, 12 +/- 0.71 at 24-hour, and 9 +/- 1.5 at 72-hour (**Figure 10, Table 5**). The averaged LOG_2FC for the expression of IL-2 when differentiated human Th2 lymphocytes from allergic donors were stimulated with anti-CD3/CD28 was 8.7 +/- 0.83 at 6-hour, 7.6 +/- 1at 24-hour, and 2.2 +/- 0.71 at 72-hour (**Figure 10, Table 5**). The averaged LOG_2FC for the expression of IL-2 when human differentiated Th2 lymphocytes from allergic donors were stimulated with PMA/I was 12 +/- 1.5 at 6-hour, 9.2 +/- 1.4 at 24-hour, and 4.3 +/- 2 at 72-hour (**Figure 10, Table 5**).

Melt curve analysis of human differentiated human Th2 lymphocyte from non-allergic and allergic donors post qPCR showed a distinct peak at 85.9C similar to that of Jurkat T lymphocytes (**Figure 14**). This peak was present in both unstimulated and stimulated samples, but unstimulated samples also frequently possess multiple peaks of less than 85C, which is indicative of non-specific binding and primer dimer formation (**Figure 14**).

The average N0 for HDC expression in combined differentiated human Th2 lymphocytes from non-allergic and allergic donors stimulated with anti-CD3/CD28 were as follows: 7.099e-

010 +/- 1.463e-010 at 6-hour, 4.572e-010 +/- 7.254e-011 at 24-hour, and 4.322e-010 +/- 8.486e-011 at 72-hour (**Supplementary Figure 3, Table 4**). The average N0 for HDC expression in differentiated human Th2 lymphocytes stimulated with PMA/I were as follow: 3.793e-010 +/- 4.427e-011 at 6-hour, 4.220e-010 +/- 5.415e-011 at 24-hour, and 3.259e-010 +/- 3.464e-011 at 72-hour (**Supplementary Figure 3, Table 4**). The average N0 for HDC expression in unstimulated differentiated human Th2 lymphocytes were as follows: 3.237e-010 +/- 4.068e-011 at 6-hour, 3.147e-010 +/- 2.630e-011 at 24-hour, and 4.089e-010 +/- 4.237e-011 at 72-hour (**Supplementary Figure 3, Table 4**).

The average N0 for IL-8 expression in combined differentiated human Th2 lymphocytes from non-allergic and allergic donors stimulated with anti-CD3/CD28 were as follows: 6.599e-008 +/- 2.039e-008 at 6-hour, 1.524e-008 +/- 4.651e-009 at 24-hour, and 2.745e-008 +/- 7.212e-009 at 72-hour (**Supplementary Figure 3, Table 4**). The average N0 for IL-8 expression in differentiated human Th2 lymphocytes stimulated with PMA/I were as follow: 9.770e-007 +/- 1.758e-007 at 6-hour, 5.987e-008 +/- 1.221e-008 at 24-hour, and 6.436e-008 +/- 2.924e-008 at 72-hour (**Supplementary Figure 3, Table 4**). The average N0 for IL-8 expression in unstimulated differentiated human Th2 lymphocytes were as follows: 5.930e-010 +/- 9.941e-011 at 6-hour, 2.597e-010 +/- 8.913e-011 at 24-hour, and 1.067e-010 +/- 2.253e-011 at 72-hour (**Supplementary Figure 3, Table 4**).

Comparing the CT values, LOG_2FC as normalized to B2M, and LinRegPCR analysis NO values of HDC expression in non-allergic and allergic individuals showed a consistent flatline between HDC expression of stimulated and unstimulated samples. This is indicative of a lack of HDC upregulation when stimulated with anti-CD3/CD28 or PMA/I.

1.3 Stimulation of human CD4+ T cells with IL-33 and anti-CD3/28 did not induce HDC expression.

The activation of human CD4+ T lymphocytes with IL-33 using anti-CD3/CD28 beads or PMA/Ionomycin did not lead to induction of HDC at 6-hour or 24-hour-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 31.84 +/- 0.73 at 6-hour and 31.33 +/- 0.88 CT at 24-hour of stimulation (n=3, **Supplementary Table 1F**). The cycle threshold (CT) values for PMA/I stimulation were averaged at 33.9 +/- 0.61 CT at 6-hour and 33.42 +/- 1.37 CT at 24-hour of stimulation (n=3, **Supplementary Table 1F**). Unstimulated media control samples averaged 31.97 +/- 2.48CT at 6-hour and 32.11 +/- 2.5CT at 24-hour.

Following normalization of HDC's CT values to the B2M housekeeping gene, the averaged LOG_2FC for the expression of HDC when human CD4+ T lymphocytes with IL-33 were stimulated with anti-CD3/CD28 was -0.32+/- 2.1 at 6-hour and 0.99 +/- 1.8 at 24-hour (**Figure 11, Table 6**). The averaged LOG_2FC for the expression of HDC when human CD4+ T lymphocytes with IL-33 were stimulated with PMA/I was -3.1 +/- 0.7 at 6-hour and -1.7 +/- 1.7 at 24-hour (**Figure 11, Table 6**).

IL-8 and IL-2 expression were measured as positive controls for human CD4+ T lymphocyte stimulation with IL-33. The activation of human CD4+ T lymphocytes with IL-33 using anti-CD3/CD28 beads or PMA/Ionomycin led to induction of IL-8 and IL-2 at 6-hour and 24-hour timepoints as compared to negative control samples. The cycle threshold (CT) values for anti-CD3/CD28 stimulation were averaged at 21.67+/- 1.53 CT for IL-8, 20.42 +/- 0.7 CT for IL-2 at 6-hour, 18.31 +/- 4.12 CT for IL-8 and 20 +/- 1.6 CT for IL-2 at 24-hour (n=3, **Supplementary Table**

1F). The cycle threshold (CT) values for PMA/I stimulation were averaged at 16.78+/- 2.41 CT for IL-8 and 19.22 +/- 1.13 CT for IL-2 at 6-hour, 18.33 +/- 5.86 CT for IL-8 and 19.67 +/- 1.77 CT for IL-2 at 24-hour (n=3, **Supplementary Table 1F**). Unstimulated media control samples averaged 27.56 +/- 2.22 CT for IL-8 and 31.44 +/- 1.02 CT for IL-2 at 6-hour, 28.21 +/- 5.17 CT for IL-8 and 31.62 +/- 0.62 CT for IL-2 at 24-hour. (n=3, **Supplementary Table 1F**).

DeltadeltaCT normalization to B2M housekeeping gene showed IL-8 and IL-2 degree of upregulation. The averaged LOG_2FC for the expression of IL-8 when human CD4+ T lymphocytes with IL-33 were stimulated with anti-CD3/CD28 was 5.84+/- 2.4 at 6-hour and 10 +/- 3.8 at 24-hour (**Figure 11, Table 6**). The averaged LOG_2FC for the expression of IL-8 when human CD4+ T lymphocytes with IL-33 were stimulated with PMA/I was 9.6 +/- 3.2 at 6-hour and 9.5 +/- 3.5 at 24-hour (**Figure 11, Table 6**). The averaged LOG_2FC for the expression of IL-2 when human CD4+ T lymphocytes with IL-33 were stimulated with anti-CD3/CD28 was 11 +/- 1.7 at 6-hour and 12 +/- 0.37 at 24-hour (**Figure 11, Table 6**). The averaged LOG_2FC for the expression of IL-2 when human CD4+ T lymphocytes with IL-33 were stimulated with PMA/I was 11 +/- 0.66 at 6-hour and 12 +/- 1.6 at 24-hour (**Figure 11, Table 6**).

Melt curve analysis of human CD4+ T lymphocytes with IL-33 post qPCR showed a distinct peak at 85.9C akin to that of Jurkat T lymphocytes (**Figure 15**). This peak was present in both unstimulated and stimulated samples, but unstimulated samples also frequently possess multiple peaks of less than 85C, which is indicative of non-specific binding and primer dimer formation (**Figure 15**).

The average N0 for HDC expression in human CD4+ T lymphocytes with IL-33 stimulated with anti-CD3/CD28 were as follows: 2.794e-008 +/- 5.463e-009 at 6-hour and 1.264e-008 +/- 1.702e-009 at 24-hour (**Supplementary Figure 4, Table 5**). The average N0 for HDC expression in human CD4+ T lymphocytes with IL-33 stimulated with PMA/I were as follow: 2.514e-009 +/- 4.756e-010 at 6-hour and 2.896e-009 +/- 5.087e-010 at 24-hour (**Supplementary Figure 4, Table 5**). The average N0 for HDC expression in human CD4+ T lymphocytes with IL-33 were as follows: 2.410e-008 +/- 9.153e-009 at 6-hour and 7.898e-009 +/- 1.741e-009 at 24-hour (**Supplementary Figure 4, Table 5**).

The average N0 for IL-8 expression in human CD4+ T lymphocytes with IL-33 stimulated with anti-CD3/CD28 were as follows: 6.599e-008 +/- 2.039e-008 at 6-hour and 1.524e-008 +/- 4.651e-009 at 24-hour (**Supplementary Figure 4, Table 5**). The average N0 for IL-8 expression in human CD4+ T lymphocytes with IL-33 stimulated with PMA/I were as follow: 9.770e-007 +/- 1.758e-007 at 6-hour and 5.987e-008 +/- 1.221e-008 at 24-hour (**Supplementary Figure 4, Table 5**). The average N0 for IL-8 expression in unstimulated human CD4+ T lymphocytes were as follows: 5.930e-010 +/- 9.941e-011 at 6-hour and 2.597e-010 +/- 8.913e-011 at 24-hour (**Supplementary Figure 4, Table 5**).

Comparing the CT values, LOG_2FC as normalized to B2M, and LinRegPCR analysis NO values of HDC expression in human CD4+ T lymphocyte samples stimulated with the addition of IL-33 showed a consistent flatline between HDC expression of stimulated and unstimulated samples. This is indicative of a lack of HDC upregulation when stimulated with anti-CD3/CD28 or PMA/I.

Discussion

0.0 Rationale for the methodology of the project

The objective of this project was to explore if human T lymphocytes can transcribe the HDC gene, and if it was possible that stimulation of the CD4+ subset of T lymphocytes can upregulate the expression of HDC. The data presented in this thesis suggested that human T lymphocytes can transcribe HDC in rare cells, but bulk stimulation of human T lymphocytes by anti-CD3/CD28 or PMA/I did not lead to an upregulation of HDC. Previous research has shown the capacity of mouse and human transformed T lymphocytes to upregulate HDC when stimulated[90,91,94]. As such, it was likely that human T lymphocytes can also upregulate as part of an activation response.

It was important to investigate the potential of this phenomenon as it contributes to knowledge of how histamine may play a role in T cell biology. The function of histamine is pleiotropic, and histamine can influence many different cellular functions[31]. Immunological understanding of histamine focused on the effects of histamine as an inflammatory inducer in an allergic responses[65]. The release of histamine leads to changes in the bronchial and vascular smooth muscle, which directly contribute to different allergic manifestations such as sneezing, pruritus, rhinorrhea, and congestion[117]. The infiltration and accumulation of activated T cells is a hallmark of the allergic late phase reaction[3]. It was then possible that the activation of T cells can lead to the release of histamine, and thus T cell derived histamine may contribute to the late phase reaction. Outside of the scope of allergic diseases, T cell derived histamine may also contribute to the immune response of different diseases, such as infections and cancer[118].

We set out to answer the research question and hypothesis through 2 main methods: bioinformatics and quantitative PCR analysis. Bioinformatic analysis of bulk RNA sequencing and single cell RNA sequencing represent the most specific and state of the art technology approach for the analysis of gene expression[119]. qPCR analysis was then used to quantify the relative abundance of the HDC transcript and to determine whether stimulation of human T lymphocytes can upregulate HDC expression. Bioinformatic analysis of single cell datasets had the advantage of directly answering our research question as it provided HDC expression data at a single cell level. Bulk RNA sequencing and qPCR were then used to address the proposed hypothesis of whether stimulation of human T lymphocytes can lead to the expression of HDC.

Preliminary work for this project showed that the HDC transcript was of extremely low relative abundance as analyzed by qPCR. This result led to the reliance on bioinformatics as a means to definitively answer our research question. Analysis by Dr. Dvorkin showed the existence of HDC+ cells in different T cell populations (populations as defined by the authors of the bioinformatics datasets). Dr. Dvorkin analyzed the pre-annotated T cell populations for the co-expression of HDC and found rare T cells with the expression of HDC. This data was the confirmation for the expression of HDC at a single cell level, and that the preliminary qPCR results were not of background noise or nonspecific binding.

Since the analysis for HDC by qPCR for T lymphocytes had never been done, we utilized bulk RNA sequencing analysis as a companion to the qPCR data obtained. The chosen dataset of bulk RNA sequencing for T cells utilized similar stimulation methods as the qPCR experiments. The data from the bulk RNA sequencing for HDC expression showed an identical trend to that of the qPCR and that the activation and stimulation of human T lymphocytes did not lead to any

upregulation of HDC. Another function of analyzing bulk RNA sequencing data was to determine the overall strength of expression of HDC as compared to the IL-2 and IL-8 control genes.

The original objective of the project was to determine whether human T lymphocytes can produce and release histamine when stimulated. Histamine ELISA was an additional procedure we thought would contribute to this project. Bioinformatics results showed the rarity of HDC expressing cells and as such it was possible that the concentration of T cell derived histamine would be too low to detect on ELISA. qPCR and bioinformatics were then chosen as the focus of the project. The detection of HDC from either method was then deemed to be the substitute for verifying the production of histamine.

The main stimulation method chosen was of anti-CD3/CD28 as this method was sufficient to provide signal 1 (TCR) and signal 2 (co-stimulation, CD28) for T cell activation[120]. Traditional T cell activation and intracellular signaling requires TCR-MHC complex ligation, which is immediately followed by the signal transduction through different immunoreceptor tyrosine-based activation motifs (ITAMs) in the CD3 receptor complexes to downstream targets[121–123]. Therefore, the artificial stimulation by anti-CD3/CD28 antibody in this project allowed for the activation of polyclonal T cell populations, while maintaining the full length of any intracellular signaling pathway downstream of the TCR. PMA/I stimulation of T cells was used a pseudo positive control as this stimulation method bypasses the TCR and led to the activation of several intracellular signaling pathways[124]. Both anti-CD3/CD28 and PMA/I stimulation led to the activation of the protein kinase C pathway (PKC) which has been implicated in the upregulation of HDC in human and murine gastric cancer cell, as well as murine bone marrow derived mast cells and transformed RAW264 cells[84,99,125,126]. Therefore, both these stimulation methods

should be suitable for inducing HDC expression if T lymphocytes rely on the PKC pathways for HDC upregulation.

It was also considered that stimulation of T cells with antigen peptides or by APCs may contribute to the expression of HDC. The method could be redesigned to stimulate the T cell with random pool of antigen peptides along with soluble anti-CD28 to mimic a more accurate activation in vivo. This change in stimulation variable may not lead to a more definitive answer as the frequency of antigen-specific T cells are low and if a HDC signal were to be significantly upregulated, it is possible that qPCR signal will be indistinguishable from the control at the current sample size. An enrichment for these antigen specific T cells by increasing the initial lymphocytes collection can solve this problem, but the volume of blood required from each donor is unfeasible. Introducing APCs as a co-culture to model T cell activation in vivo also runs into the problem of directly introducing more contaminating cells that may express HDC, even if these APCs will be removed using negative selection.

1.0 Jurkat T lymphocytes stimulated with anti-CD3/CD28 beads led to an upregulation of HDC expression.

Previous research showed that Jurkat T lymphocytes express HDC and produce histamine when stimulated with PMA. As such, it was necessary to confirm this HDC expression in our experimental design to serve as a positive control. qPCR data obtained suggested that Jurkat E6.1 T lymphocytes likely express HDC at a low level basally or have rare cells that express HDC. This was observed from samples from Jurkat T lymphocyte experiments where gel electrophoresis of amplified HDC products displayed expected bands for the HDC product in stimulated and unstimulated conditions (**Supplementary Figure 5**). This HDC expression was

upregulated when Jurkat T lymphocytes were stimulated with anti-CD3/CD28 beads, or with PMA/Ionomycin. This increased in HDC expression in Jurkat T lymphocytes likely led to an increase histamine release as demonstrated by Mark Lychacz, a previous M.Sc student from our lab. Mark Lychacz stimulated Jurkat T lymphocytes with anti-CD3/CD28 or anti-CD3/CD28/CD2 and found a significantly higher histamine released as compared to the unstimulated Jurkat T lymphocytes[96]. In our experiment, anti-CD3/CD28 and PMA/I stimulation led to a similar upregulation of HDC. This upregulation was detected at every stimulation timepoints, starting at 6-hour and persisting at 72-hour. The averaged CT values for unstimulated Jurkat T lymphocytes were 33.13 at 6-hour, 33.81 at 24-hour, and 34.19 at 72-hour. These CT values served as the baseline to determine whether stimulation influenced HDC upregulation. Stimulated Jurkat T lymphocytes resulted in lower CT values on average as compared to unstimulated Jurkat T lymphocytes. As such, it was likely that stimulation had an effect on the upregulation of HDC.

Comparing the data from this project to the original Jurkat T lymphocytes HDC expression paper by Nagashima et al (2012) showed very similar results. Nagashima et al did not utilize qPCR as a mean of quantitation, but used the fluorescence intensity of their HDC bands post-PCR to show an increase in HDC signal as PCR cycle went on. Their result suggested that the minimum PCR cycle required for a distinct band of HDC product was 24 cycles for a 16-hour stimulation with PMA. Without knowing the concentration of their starting PCR product, their result at the 16-hour timepoint was very similar to that of this project at 24-hour timepoint stimulation by PMA/I[94]. The average CT for detection by qPCR at 24-hour for PMA/I stimulation was 25.14.

It was important to be able to quantify the relative upregulation of HDC. The deltadeltaCT method was chosen as the method of analysis to calculate the fold changes of HDC as compared to the housekeeping gene B2M. This normalization process allowed for a consistent calculation of the fold changes between an upregulated gene (HDC) compared to stable gene (B2M). The fold changes were then Log_2 transformed. The LOG_2FC of this upregulation was calculated at every timepoint for both anti-CD3/CD28 and PMA/I condition. The LOG_2FC was similar at the 24-hour stimulation, and the 72-hour stimulation, suggesting that Jurkat T lymphocytes can continually upregulate HDC without exhaustion. A potentially important difference between Jurkat T cells and primary T cells is that Jurkat T cells are transformed/malignant cells. Jurkat T cell HDC mRNA expression was compared to that of mouse T cell derived histamine from Kubo and Nakano (1998). Kubo and Nakano performed histamine ELISAs on isolated mouse CD4+ T cells and found histamine release at ~12-hour, ~22-hour, and 46-hour of stimulation with Con A[91]. The trend for the CD4+ T cells showed the initial rise in histamine concentration at ~12-hour, followed by a slightly larger rise at ~22-hour, followed by the largest rise at 46-hour. The detected concentration of histamine was negligible, ranging from ~0.1 to 0.5 nmol/10e7 cells[91]. The positive trend of histamine release over time was similar to the data from this project, with Jurkat T cells having an initial HDC expression at 6-hour but increase to the maximum upregulation at 24-hour, and a slight decrease at 72-hour.

qPCR amplification measurement follows logarithmic growth, which includes a plateau phase[127]. This PCR logarithmic growth is called the “amplification curve”, in which the fluorescence value is correlated with the abundance of amplified product. LinRegPCR was a program devised by Ruijter et al (2009) to calculate the amplification efficiency of a given qPCR

run[128]. This program utilizes the mathematical modeling of the qPCR amplification curve to determine the starting abundance of a gene of interest (cDNA in our experimental design). This so called the N0 value in arbitrary fluorescent units represents the lowest fluorescent value of a sample, and as such is a representation of the starting cDNA material. N0 was calculated as N0 = Nq / (Emean^CT), where Nq was the fluorescent threshold for detection, Emean was the PCR efficiency as determined by the program, and CT being cycle threshold number to reach fluorescent threshold. Raw qPCR data for each of the Jurkat T lymphocytes biological replicates were computed using the program to obtain the N0 for each of the samples run. In a sense, this N0 can be seen as a way to measure the bulkRNA of a sample, although the methodology is different. Other studies have utilized this method in place of the deltadeltaCT method to quantify the relative abundances of their target genes[129–131].

The results of this method of analysis in Jurkat T lymphocytes align with what was expected based on the deltadeltaCT analysis for HDC expression. The starting abundance of HDC in a stimulated sample was vastly higher than that of an unstimulated sample. PMA/I stimulation consistently led to higher N0 value, and PMA/I was a stronger stimulus for the upregulation of HDC.

The upregulation of HDC was showcased based on these analyses, but it was important to put this upregulation of HDC in context of other T cell genes. The premise of this project was that upregulated HDC and the subsequent release of histamine from human T lymphocytes can lead to bronchoconstriction. Therefore, the upregulation of HDC must be compared to that of other activated T cell genes. IL-2 and IL-8 were the positive gene controls for the qPCR assay as both Jurkat T cells and human primary T cells are known to express/produce IL-2 and IL-8 when

stimulated[102,105,132,133]. The average CT values for both IL-8 and IL-2 showed a similar trend to that of HDC. Stimulated Jurkat T lymphocytes led to lower CT values for both IL-8 and IL-2, indicative of a higher abundance of these genes as compared to media control. PMA/I stimulation consistently led to a stronger upregulation of both IL-8 and IL-2, as observed using the LOG_2FC (**Figure 6**). PMA/I stimulation also led to a longer persistence of IL-8 and IL-2 upregulation as compared to anti-CD3/CD28 stimulation (**Figure 6**). The upregulation of IL-8 and IL-2 as calculated by LOG_2FC were higher than that HDC at 6-hour, but lower than that of HDC at 72-hour. Upregulation of IL-8 and IL-2 versus HDC was comparable at 24-hour, depending on the method of stimulation. There is currently no literature focused on the qPCR analysis of IL-2 or IL-8 mRNA expression in Jurkat T cells but there was evidence that anti-CD3/CD28 and PMA/I stimulation led to the release of IL-2 and IL-8[134]. The authors of this paper stimulated Jurkat T lymphocytes "overnight" with anti-CD3/CD28 and showed the release of IL-2 and IL-8 post stimulation. IL-2 stimulated by anti-CD3/CD28 led to upregulation of 8 LOG_2FC, while PMA/I led to an upregulation of ~128 LOG_2FC. The anti-CD3/CD28 stimulation for IL-2 was alike to the data from this project at the 24-hour timepoint (LOG_2FC: 7.6). The authors did not show data for IL-8 release. We only found one study in the literature that measured the expression kinetic of IL-2 in stimulated Jurkat T cells. The study compared the expression of IL-2 at 1-hour and 8-hour of stimulation and showed a large upregulation at 8-hour as compared to 1-hour of stimulation[135]. This spike and persistence of IL-2 expression at 8-hour from the study supported the results obtained in this project. The lack of literature took away from fully interpreting the kinetics of IL-2 and IL-8 expressions in Jurkat T lymphocyte from this project, but the data presented in the literature validated our approach in using IL-2 and IL-8 as the positive control for Jurkat cells.

The LinRegPCR program was unable to determine the N0 values for IL-2 for unknown reasons. It was suspected this error had to do with the amplification curve for IL-2 to not have a baseline value (as determined by the program), this occurs when the log-linear phase was too short or an excessive measurement noise was present. Regardless, the program was able to calculate the N0 for IL-8, and as such IL-8 was the positive control for this method of analysis. Comparing the N0 value for HDC at 6-hour showed a lower HDC abundance at 6-hour compared to IL-8, but a higher abundance at both 24-hour and 72-hour. This aligned with the results of the deltadeltaCT analysis as HDC were continually upregulated, while IL-8 upregulation tapered off over time. The deltadeltaCT analysis at the 24-hour stimulation showed that HDC and IL-8 have similar upregulation, but this was not reflected in the N0 value as the N0 value for IL-8 was less then that of HDC. This highlighted a noticeable flaw in using this particular N0 approach as these values did not undergo any process of normalization, unlike the deltadeltaCT method. The N0 values were essentially transformed CT values and reflected the trend of the raw CT analysis.

Melt curve analysis was also performed for every qPCR run. Melt curve analysis measured the temperature at which the fluorescence level decreases, represented as a peak and is indicative of amplified products being broken down. A longer gene sequence would require a higher temperature to breakdown, and shorter gene sequence would require a lower temperature to break down[136]. Both stimulated and unstimulated Jurkat T lymphocyte melt curves resulted in a distinct peak at 85.9C. This peak likely represents the amplified HDC product being broken down. There were also other melt curve peaks at <80C, which is indicative of non-specific binding and primer dimer formation (Life Technology – Real-Time PCR handbook). This data was supported by the gel electrophoresis of amplified HDC product.

Through the analysis of this data, Jurkat T lymphocytes were capable of upregulating HDC when stimulated. Therefore, we can use Jurkat T lymphocytes as a pseudo-positive control for the investigation of human primary T cells.

1.1 Stimulation of human CD4+ T cells did not induce HDC expression, regardless of allergic status.

To investigate whether human CD4+ T cells can express or upregulate HDC, we collected peripheral blood from self-reported non-allergic and allergic individuals' for PBMC and CD4+ T cell isolation.

Allergic individuals may have a different immune cell phenotype landscape and expression of cytokines as compared to non-allergic individuals[137,138]. The referenced study by Haselden et al (1999) showed that bronchoconstriction resulting from peptide-specific activation of human T cells in the late phase reaction only occurred in some subjects with allergic asthma[33]. This conclusion led this project to investigate whether the expression or upregulation of HDC may be dependent on allergic status.

PBMCs were isolated using Ficoll-Paque centrifuge gradient. The gradient contains 4 distinct layers: the bottom layers composed of heavy cells (red blood cells, granulocytes), the Ficoll layer, the buffy layer containing PBMCs and the plasma layer. This buffy layer is removed to isolate PBMCs which includes CD4+ T cells. However, basophils are also found within the buffy layer (1-3% of cells are basophils), and thus can contaminate the purity of lymphocytes recovered[139]. Basophils are professional histamine producers, and express high level of HDC[62]. Furthermore, monocytes have also been reported to express HDC[140,141], and monocytes are also

found within the buffy layer for PBMCs isolation[142]. Both cell types can skew the results of this project and therefore the purity of T lymphocytes must be as high as possible.

It was imperative to design our experimental methodology to avoid T cell activation during isolation and to minimize contaminating cells. Ficoll-Paque density gradient for the isolation of PBMCs is the standard method for lymphocyte isolation and was chosen for this project. This method left room for the contamination of non-lymphocytes (monocytes, dendritic cells, NK cells, basophils), and this method has been reported once in the literature to lead to lymphocyte proliferation[143,144]. Given the widespread use of Ficoll-Paque isolation in the literature studying T cell activation, it was unlikely that any non-specific activation as caused by the Ficoll-Paque method would contribute to the expression of HDC. CD4+ T cells were isolated using a negative selection method instead of a positive isolation method to prevent any T cell activation prior to stimulation[145]. The kit functioned to remove all non CD4+ cells using magnetic selection beads. The choice of negative selection left an inherent flaw as the procedure required the removal of the untouched CD4+ cells by pouring off liquid that may contain contaminating cells that escaped magnetic beads binding. The addition of a plastic monocyte adherence step likely removed some contaminating monocytes, but the effect would be lesser to that of utilizing a CD14 positive selection kit to remove monocytes[100].

The usage of CD4+ positive selection may have reduced the contamination issue that plagued this project. A redesign of the project methods to include a CD4+ positive selection step after the initial negative selection allows for a theoretical increase in CD4+ cell purity. The positive selection procedure uses the magnetic beads to trap the CD4+ cells and therefore it is possible to keep washing the tube repeatedly to remove any non-CD4+ cells. However, the

positive isolation of CD4+ T cells uses antibody specific for the CD4 receptor, which may lead to an increase in intracellular calcium level and T cell activation[146]. Since the nature of CD4 is of a co-receptor to the TCR, it was best to minimize any external disturbance to the cell to maintain T cell quiescence. Ultimately, it was still possible to have contaminating cells and this method was not necessary. FACS sorting was another method that was considered for this project, but it may lead to an upregulation of different gene expressions as stress response. Furthermore, antibody binding FACS sorting of the cells may also inadvertently activate the cells[145,147].

The activation of non-allergic and allergic individuals isolated CD4+ T lymphocytes did not lead to an upregulation of HDC at any timepoint. The CT values for the stimulated conditions compared to the unstimulated condition were similar and displayed a flatline across all timepoints. Gel electrophoresis of amplified HDC product from an example CD4+ T lymphocyte qPCR showed bands of varying intensity across anti-CD3/CD28 stimulation and unstimulated cells. This data supports the idea that there are rare CD4+ T lymphocytes expressing HDC as discovered in bioinformatics analysis of Aim 2. The other explanation is that T lymphocytes may express HDC at an extremely low level basally. Given the design of the HDC primers to span exon-exon boundary and the short extension time (1 min) of qPCR run (full amplified gDNA length: 4868bp), it was highly unlikely that CT values of the samples were a result of gDNA amplification. The DNA polymerase used for qPCR in our methodology was the Taq DNA polymerase (Promega, USA), which has a reported extension time of 30 seconds to 2 minutes per 1000 bp[148]. More so, gDNA amplification would lead to a significantly lowered CT values as gDNA are abundant. Lastly, the primers were also tested in negative reverse transcriptase samples prior to qPCR assay to confirm the lack of gDNA amplification.

Analysis using the deltadeltaCT method for non-allergic and allergic individuals showed a lack of upregulation. The data for HDC suggested that HDC expression is downregulated following stimulation. This was unlikely as the raw averaged CT values were of similar values to that of unstimulated CD4+ T cells. The stability of the B2M housekeeping gene was the reason in creating the negative values in data. The deltadeltaCT method normalized a gene to B2M using: deltadeltaCT= $2^{(\text{CT } \textbf{Stimulated (HDC)} - \text{CT } \textbf{Stimulated (B2M)})_{\text{Time(X)}} - (\text{CT } \textbf{Control (HDC)} - \text{CT } \textbf{Control (B2M)})_{\text{Time(X)}}}$. This equation is dependent on B2M expression being stable across stimulation conditions. We found that B2M was inconsistently unstable and was upregulated in T cells stimulated using anti-CD3/CD28 or PMA/I (**Supplementary Table 1**). The result was that the CT value for the left bracket had a bigger gap (difference in CT between HDC and B2M of stimulated condition), while the right bracket remained stable (difference in CT between HDC and B2M of unstimulated). This gives an effect of HDC downregulation, which was false as observed by the raw averaged CT of HDC across stimulation conditions, timepoints and allergic status. The instability of B2M was unexpected as the literature frequently showed B2M as a stable reference gene for T lymphocyte activation[149–151]. No current study exists investigating the effect of anti-CD3/CD28 or PMA/I on the upregulation of B2M. Since B2M is a protein subunit of the MHC1 receptor presented on all cells, this instability can be due to the nature of T cell preparing for cell division following activation and therefore was increasing the expression of B2M[152]. Anti-CD3/CD28 and PMA/I are stimulators that are sufficient to induce T cell proliferation[153]. T cells may require at least 20 hour of consistent activation signal to commit to proliferation[154,155]. It could be assumed that B2M upregulation immediately follows the commitment of the T cells to proliferation. If this hypothesis was correct, then the unstable raw CT values for B2M data of all

the CD4+ experiments can be explained by this commitment to proliferation. The value for the 6-hour timepoint of B2M were slightly unstable across stimulated vs unstimulated samples. But this instability was much more pronounced at 24-hour and 72-hour timepoints, which was after the minimum threshold for the T cells commitment to proliferation. It was then likely that the upregulation of B2M was the product of cell division. Curiously, this instability was less pronounced in differentiated Th2 cells, but was mostly present at the 72-hour timepoint. Since T cells require activation (anti-CD3 in the differentiation procedure) to initiate differentiation, it was possible that these recently activated T cells required a longer restimulation time to commit to proliferation[156,157]. Lastly, the B2M instability was only observed in PMA/I stimulated Jurkat T cells, which suggest that PMA/I stimulation may directly activate pathways leading to B2M expression, or by pushing Jurkat T cells to divide more frequently.

Combining the non-allergic and allergic individuals for LinRegPCR analysis once again showed a complete flatline for HDC upregulation. N0 values for HDC expression in unstimulated T cells was like that of the stimulated T cells. The N0 values for HDC was substantially lower compared to that of IL-8, including to that of unstimulated CD4+ T cells for IL-8. This data suggested that human CD4+ T cells expressed IL-8 basally at a higher level than HDC, or that there were more IL-8 expressing CD4+ T cells at rest, but more likely that contaminating cells were also a source of IL-8 expression.

Similar to the Jurkat T lymphocytes, IL-2 and IL-8 were chosen as the gene positive controls. Primary human CD4+ T cells have been shown to express IL-2 with the highest peak around 7–8 hour post-stimulation by PMA/I, followed by a slow tapering off in further stimulation timepoints[158,159]. This observation did not align with the data obtained in this

project for the 6-hour and 24-hour timepoints as the detected abundance of IL-2 mRNA was similar between 6-hour and 24-hour. The expression of IL-2 at 72-hour timepoint was largely reduced and was expected as previously reported[160]. Regardless, the use of IL-2 as a positive control for T cell activation has precedence in the literature and was an appropriate control for the qPCR analysis. The inclusion of IL-8 as a positive control served as a redundancy for the qPCR system. IL-8 has been reported to be strongly expressed in recent thymic emigrant T cells and naïve CD4+ T cells from adults and neonates[161,162]. IL-8 has also been known to be produced from bulk T cells, and allergen specific T cells (including Th2 cells) using a combination of anti-CD3/CD28/CD2 stimulation[163,164]. There is currently no literature that studied the kinetics of IL-8 mRNA expression, but these previous studies showed IL-8 release as measured by ELISAs at 8h, and up until 5 days post stimulation[164]. The kinetic of IL-8 release was relative linear across the first 4 days (96-hour stimulation), with the highest spike in IL-8 release between day 4 and day 5. Combining the qPCR data from this project and the IL-8 ELISA from the paper suggested that the secretion of IL-8 from CD4+ T cells was steady from to 0 to 96-hour, even though the IL-8 mRNA was largely upregulated by the 24-hour timepoint. Allergic status did not influence the upregulation of IL-2 or IL-8. The frequency of IL-2+ and IL-8+ T cell following stimulation has been reported to be similar between allergic and non-allergic individuals and thus agreed with our findings[165,166].

It was interesting to note how high the resting IL-8 expression was in isolated CD4+ T cells using the LinRegPCR program. This was different to the differentiated human Th2 samples where resting Th2 cells did not seem to express IL-8. It was possible that IL-8 expressing cells like monocytes, dendritic cells, basophils and NK cells were contaminating the population of

CD4+ T cells post isolation[167–170]. This data was supported by the bulk RNA sequencing analysis (**Figure 1C)** which showed that IL-8 was expressed in every T cell phenotype, including T cells at rest. Contaminating monocytes, basophils and dendritic cells have been known to express HDC, which leads to our theory that this abundance of IL-8 in resting T cell samples may be from contaminating cells, which is then contributing to the false HDC signal as well[141,171–175]. Basophils were the major cells of interest as basophils continuously produce histamine[28,176]. This IL-8 signal from contaminating cells could be the result of a basal transcription level as these cells were not activated, nor were the T cell activated in unstimulated samples.

To note, Dr. Dvorkin's analysis of the co-expression of NK cells markers and HDC also seems to indicate that NK cells may also express HDC. A pilot experiment for this project was performed aimed at evaluating the expression of HDC in cultured NK cells and found that PMA/I stimulation did not induce HDC expression at 6-hour or 24-hour timepoints. Thus, we can attribute some of the IL-8 signal to that of contaminating cells, as well as HDC.

The other explanation for the IL-8 expression in unstimulated sample was that freshly isolated T cells from PBMCs were basally expressing IL-8 or the T cells were randomly activated by being in cRPMI. This basal expression diminished overtime following the removal of T cells from physiological conditions and into culture conditions.

The 13-14 days culture of differentiated CD4+ T cells into Th2 cells would likely drastically reduce number of contaminating cells, and thus IL-8 expression at rest was not detected. This difference in IL-8 expression between CD4+ T cell samples and differentiated Th2 samples highlighted the important problem of maintaining CD4+ T cell purity that plagued this project.

Melt curve analysis of CD4+ T cells qPCR showed a similar trend to that of Jurkat T lymphocytes. The 85.9C peak that is representative of HDC products still existed in both unstimulated and stimulated samples. But the stimulated samples in CD4+ T cells had more frequent peaks corresponding to non-specific binding and primer-dimer formation. This data supports the gel electrophoresis of amplified HDC products in human CD4+ T cell samples, where the HDC bands usually existed across all experimental conditions to varying intensity.

Overall, applying the same analyses to isolated human CD4+ T cells showed that HDC expression was not upregulated following stimulation using anti-CD3/CD28 or PMA/I. While primary human cells are not a one to one comparable to Jurkat T lymphocytes, it was clear that Jurkat T lymphocyte data showed a consistent trend of HDC upregulation while human CD4+ T cells did not. It is therefore unlikely that primary human CD4+ T lymphocytes are capable of upregulating HDC expression when stimulated using anti-CD3/CD28 or PMA/I regardless of allergic status. The expression of rare transcripts of HDC in rare cells or basally was still a possibility.

1.2 Stimulation of human CD4+, Th2 cells did not induce HDC expression, regardless of allergic status.

To further push our experimental design in a direction supporting allergic hypersensitivity, human CD4+ T lymphocytes were differentiated to Th2 cells in vitro using a commercial kit (Human Th2 Cell Differentiation Kit – Bio-techne). Self-reported allergic and non-allergic individuals' T cells were isolated using a CD4+ negative isolation kit, and the isolated CD4+ T cells were supplemented with IL-2, IL-4, IL-6 and anti-IFNγ for 13-14 days. IL-6 is not a cytokine associated with Th2 differentiation unless combined with exogenous IL-4. IL-6 then functions to

inhibit the Th1 differentiation pathway in concert with the anti-IFNγ blocking antibody[177]. A long culture time of 14 days should theoretically reduce the survival of contaminating cells. Basophils were reported to have a lifespan of 1-2 days, and some reported that basophil survival with neglect in vitro has been reported to drastically reduce after 6 days (~10% of total population)[178,179]. Monocyte survival is for far longer according to the literature. Total neglect of isolated monocytes (without M – CSF, GM – CSF) was reported to be minimum (4% of total population remained) at 7 days (earlier time course data was not provided)[180]. However, because the negative selection kit isolated all CD4+ T cell subsets, memory T cells were also selected for and activated within the culture. Their release of cytokines may have prolonged the survival of contaminating cells. The addition of GM-CSF allowed for prolonged monocyte survival (20% of the total population remained at 7 days)[180]. Since M – CSF and GM – CSF are both activated T cell products, it was possible that some of IL-8 signal from activated memory T cells samples could be from contaminating monocytes (**Figure 1A,B,C**)[181]. This explanation does not entirely align with our data for HDC expression from the publicly available bulk RNA sequencing dataset if we were to use resting T cells samples for IL-8 expression to correlate with HDC signal from contaminating cells. **Figure 1A** showed an elevated in HDC expression in resting CD4+ T cell samples for both 16-hour and 5-days stimulation samples, with the 5 days HDC expression to be slightly lowered. Resting CD4+ T cells should not release GM-CSF, and the survival of monocytes contributing to HDC signal should be ablated after 5 days of neglect. More so, GM – CSF also enhances basophil survival, which may prolong the HDC signal[181]. The survival of both monocytes and basophils after 5 days of culture should be minimal in resting T cell samples. The main concern of the data was that HDC signals from activated CD4+ T cell

phenotypes were lower than that of resting CD4+ T cells at 5-days stimulation (similar trend for IL-8). This brought in the question of whether the proposed theory of using IL-8 in resting T cell samples as an indicator of contaminating cells to be logical, as the IL-8 signal should be higher in activated T cell samples (from the survival of contaminating cells) (**Figure 1C)**. Looking at the LinRegPCR obtained from this project (**Supplementary Figure 2**) showed that this theory was also maybe not applicable to our results. Raw HDC N0 values for anti-CD3/CD28 stimulated CD4+ T cells decreased from 6-hour to 72-hour, while PMA/I stimulation led to N0 increase from 6-hour to 72-hour, and unstimulated decreased from 6-hour to 72-hour. The activation of T cells by either anti-CD3/CD28 or PMA/I should lead to the release of GM-CSF[182]. And yet HDC expression for anti-CD3/CD28 decreased overtime while PMA/I increased overtime. It was important to keep in mind that values of these data are at an extremely miniscule amount (**Supplementary Table 3**) and perhaps no trend should be drawn from these data.

Regardless, the idea that activated T cell products leading to HDC expression was not without a direct precedent from the rationale for this project. Kubo and Nakano (1999) of the referenced 2nd mouse T lymphocytes study showed that the addition of IL-3 in both unstimulated and Con A stimulated mouse CD4+ T cells led to an upregulation in histamine release to a similar degree at 48-hour of stimulation[91]. IL-3 is a cytokine that contributes to the survival of basophils and happens to prime basophil for histamine release (or reportedly to induce histamine release from basophils)[183]. The elevated histamine release because of IL-3 addition from both unstimulated and stimulated samples strongly implies that some histamine release may have been a result of contaminating basophils. In this same experiment, the addition of GM-CSF also led to an increase in histamine release in Con A stimulated samples[91].

Overall, human differentiated Th2 cells from allergic and non-allergic individuals did not upregulate HDC when stimulated using anti-CD3/CD28. The averaged CT values for individual conditions and samples were higher than that of Jurkat T lymphocytes and human CD4+ T cells. If the assumption of the Th2 culture being purely T cells, then some extremely rare T cells do express HDC or that T cells expresses HDC at a low level basally. Flow cytometry was performed to determine the percentage of Th2 cells by gating on IL-4+ or IL-4+/IL-5+ cells. The results of the differentiation procedure led to an increased in the percentage of Th2 cells, but we were unsuccessful at obtaining >90% Th2 population. Because the purity of Th2 cells was not 100%, but the purity of CD4+ T cells (which includes Th2 cells) was assumed to be 100% (if all contaminating cells did not survive), it was unclear if the amplified HDC signal came from a CD4+ T cell population, or a Th2 population. Regardless, it was unlikely that amplified HDC signal came from contaminating cells.

DeltadeltaCT analysis once again highlighted the lack of upregulation of HDC in activated allergic or non-allergic differentiated Th2 cells. This was supported by LinRegPCR analysis, where the N0 values for HDC abundance were less than that of Jurkat T lymphocytes and human CD4+ T cells.

It was interesting to note that gel electrophoresis of an example Th2 qPCR showed bright HDC bands for the anti-CD3/CD28 stimulation condition, while faint bands were observed for the unstimulated condition. This singular result implied that activation of Th2/CD4+ cells led to an upregulation of HDC. This was further supported by another example melt curve analysis of a different sample where stimulation using anti-CD3/CD28 and PMA/I led to a more frequent singular melt peak corresponding to the HDC product. The CT values, LOG_2FC and LinRegPCR

results for this sample showed similar results to that of media control. It was unlikely that stimulation influenced the upregulation of HDC.

If HDC expression was a characteristic of certain T cell phenotypes, it was impossible to know whether the differentiation process, activation and proliferation process can alter this phenotype. Exposure to IL-2, IL-4 or IL-6 may have altered CD4+ T cells capacity to express HDC. Division may lead to a loss of the HDC phenotype in CD4+ T cell progeny as the activated T cell product changes the microenvironment. Lastly, differentiation may also remove the expression of HDC. All these explanations can contribute to the reduction in amplified HDC signal from Th2 qPCR analyses. But it was more likely that the lowered HDC signal was a result of a reduction in contaminating cells.

IL-2 and IL-8 positive controls once again showed trends as seen from the human CD4+ T cell data. Outside the scope of this project, IL-8 expression in Th2 differentiated cells were stronger and persisted for longer compared human CD4+ T cells samples. This upregulation and persistence were independent of allergic status.

1.3 Stimulation of human CD4+ T cells with IL-33 and anti-CD3/28 did not induce HDC expression.

Single cell sequencing analysis of (Single-cell transcriptomic analysis of allergen-specific T cells in allergy and asthma, Seumois et al, 2020) led to the detection of HDC+ T cells. Correlation analysis of these HDC+ T cells showed that HDC+ cells were likely to co-express the IL-33 receptor gene. We decided to add soluble IL-33 into our stimulation conditions to determine whether IL-33 co-stimulation is crucial to upregulating HDC expression in human

CD4+ T lymphocytes. The addition of IL-33 into either stimulation of anti-CD3/CD28 or PMA/I did not lead to an upregulation of HDC. The averaged CT values for anti-CD3/CD28 and PMA/I stimulation with IL-33 were similar to those of stimulation conditions without additional IL-33. LOG_2FC values for these experiments once again showed a lack of upregulation in HDC expression as compared to B2M. LinRegPCR analysis suggested that there was a higher starting abundance of HDC expression as compared to human CD4+ T cell stimulated without IL-33, but there was no upregulation of HDC. The lower sample size of these experiments (n=3) was associated with data variability. This was most evident by examining the averaged CT values outputted with the addition of the standards error of the means.

In this experimental design, we did not check for the frequency of IL-33R expression in human CD4+ T cell samples. IL-33R expression in CD4+ T cell has been reported to be found on Th2 cells, activated Treg cells, and activated Th1 cells[184,185]. These T cell differentiation phenotypes are rare within PBMCs (Th1: 5.51%, Th2: 4.72%, Treg: 1.77%), as such it was entirely possible that these rare IL-33R expressing T cells are HDC+ T cells[186]. However, if IL-33 had any effect on the upregulation of HDC, it was likely that qPCR analysis would have been able to detect a difference in HDC expression compared to the unstimulated media control without IL-33.

As the co-expression data was analyzed from scRNA samples isolated by FACS sorting, it was unlikely but not impossible that the IL-33R expression came from contaminating cells. FACS sorting has been reported to yield 95-100% cell purity of the desired population[187], while the averaged CD4+ cell purity of the negative selection performed in this project was 94.02%. Considering that FACS sorting can detect individual cell markers and sort them accordingly, the

chance for the introduction of contaminating cells should be lower as compared to negative selection by magnetic beads by methodology alone. If contaminating cells were wrongly sorted into the T cell population, they would have presented as a false positive for the co-expression of HDC and IL-33R. Monocytes, basophils, and dendritic cells express the IL-33 receptor along with expressing HDC[185,188,189]. It was then not impossible that the analysis was misled by contaminating cell expression of IL-33R and HDC.

2.0 Bulk RNA sequencing of human T lymphocytes showed HDC expression.

The analysis done by Dr. Anna Dvorkin on bulk RNA sequencing showed HDC expression in different differentiation of CD4+ T cell phenotypes. From the analysis, Dr. Dvorkin found HDC expression in memory CD4+ T cells and naïve CD4+ T cells populations of different phenotypes in both 16-hour and 5 days culture of CD4+ T cells. The memory CD4+ T cells population had a higher expression of HDC as compared to naïve. The activation and differentiation of these CD4+ T cells did not lead to an upregulation of HDC in any phenotype. HDC expression in the cells found in the bulk RNA sequencing was higher at 16-hour of stimulation compared to 5-day stimulation. This higher HDC expression can be attributed to contaminating cells (monocytes, basophils, dendritic cells). As mentioned above, basophils and monocyte can survive in these cultures for an extended period, which contributes to the higher expression of HDC. This theory was supported by the elevated expression of IL-8 within the resting memory and naive CD4+ T lymphocytes. Activation of memory CD4+ T cells could also be releasing cytokines to sustain the survival of contaminating cells, primarily basophils. If this was the case, it would be expected that the HDC expression in activated memory CD4+ T cell cultures to be higher at 5-days as compared to resting T cells, but the results were shown otherwise. The most interesting data

from this analysis was the lack of HDC expression in the unstimulated naive CD4+ T cells in the 5-day culture while the rest of the naïve CD4+ T cell phenotype expresses HDC at a low level. This result implied that activation and differentiation of naive T cells led to either upregulation or maintenance of HDC expression. Alternatively, this data also somewhat supported the theory of how activated memory CD4+ T cells are sustaining HDC expression in contaminated cells as activated naive CD4+ T cells can release cytokines, including GM - CSF[190,191]. This led to the maintenance of HDC expression in contaminating signal in activated naïve T cell phenotypes at the 5-days timepoint. This same logic can explain why the sample of naïve T cells at 5-day resting did not express HDC. Resting naïve CD4 at 5-day stimulation should not release any cytokine that may prolong contaminating cells survival, and the lack of HDC was a result of contaminating cells dying off.

It was curious as to why the expression of IL-8 from this data was very different from the qPCR data obtained from our project, but IL-2 expression to follow the expected trend. Resting T cells expressed very similar level of IL-8 as the activated T cells, including for the Th0 phenotype where no other cytokines could have activated any contaminating cells. We hypothesized above that the IL-8 expression level in resting T cell likely involved IL-8 expressing contaminant cells. This theory likely holds true as the IL-8 expression level did reduce at the 5-day timepoint. However, the majority of cells should still be T lymphocytes, and as such should upregulate IL-8 following activation. This irregular trend can only be explained by that the sample of T cells collected did not contain any T cells capable of producing IL-8, although this was extremely improbable. Furthermore, the expression strength for IL-8 was similar to that of HDC, which was highly unlikely as evident by the qPCR results from this project.

Bulk RNA sequencing measures the total mRNA of particular gene in a pooled sample by sequencing every mRNA transcript and quantifying the number of transcript repeats. LinRegPCR analysis accomplishes a similar goal but through the usage of mathematical modeling to determine the starting abundance of a gene. These analyses however cannot be compared in this example as the methodology is too different. The study used for bulk RNA sequencing analysis also included different cytokine cocktail in the stimulation of CD4+ T cells, as well as a division between memory and naïve CD4+ T cells, while our experimental procedure did not.

Taken together, bulk RNA sequencing analysis detected HDC expression in both the memory and naïve CD4+ phenotype, as well as different differentiated CD4+ phenotypes.

2.1 Single cell RNA sequencing of human T lymphocytes showed HDC expression in rare and heterogenous population of T lymphocytes.

Dr. Dvorkin also analyzed 3 scRNA sequencing datasets. The first dataset analyzed a variety of differentiated T cell phenotypes, corresponding to the additions of cytokines cocktails to induce differentiation. The second dataset analyzed a general selection of CD4+ T cells isolated from human peripheral blood, lungs, and bone marrow. The third dataset analyzed FACS sorted house dust-mite specific T cells of different phenotype. Dr. Dvorkin found rare HDC+ T cells in all these datasets.

Dataset 1 used Ficoll-Paque gradient followed by memory and naïve CD4+ negative selection to isolate CD4+ T cells. As discussed, magnetic selection cannot guarantee a 100% purity of the isolated cell type. The addition of different cytokines cocktails allowed for the determination of whether several additional stimuli may induce HDC+ expression. While these

cytokines may not encompass all possible cytokines or combinations of cytokines, the data seems to suggest that HDC can be expressed by numerous different T cell phenotypes (including different differentiations) (**Figure 2**). Cell classification was annotated by the authors of the paper and many different T cell phenotypes were identified. The data showed that resting T cells of different subsets were not detected to express HDC and activated T cells of different subsets did express HDC in rare cells. Resting T cells that did not express HDC does not align with the rest of the data analyzed in this thesis and of the bulk RNA sequencing data from the exact same study (**Results and Discussion 2.0 Bulk RNA sequencing of human T lymphocytes showed HDC expression**). The same cell cultures were split between scRNA analysis, and bulk RNA sequencing analysis. As such, the lack of HDC expression in resting T cells samples of the scRNA analysis points towards the potential prevalence of contaminating cells that were contributing to the HDC signal found in the resting sample of the bulk RNA sequencing analysis. This has implication on the rest of the bulk RNA sequencing data as the level of resting HDC+ expression was very similar to that of other activated T cells (and higher in the 5-days stimulation). This suggested that the majority of HDC expression signal were coming from contaminating cells, and the HDC+ T cells contributed very little to the overall HDC mRNA landscape, as well as the overall strength of expression. Other scRNA sequencing data, gel electrophoresis and melt curves data also suggested that resting T cells did express HDC. Any explanation using low sequencing depth to explain the lack of expression was not possible as bulk RNA sequencing and qPCR data suggested that stimulation does not upregulate HDC.

Naïve T cells of the Th2 subset were detected to be HDC+ at a low frequency. This data aligned with the qPCR data collected as it appeared that differentiated human Th2 T cells were

expressing HDC at an extremely low level, as based on the gel electrophoresis and melt curve analysis. The Th17, iTreg and Th17/iTreg T cell phenotype repeatedly showed up as the phenotype with HDC expression. Th17 cells participate in the defense against extracellular pathogens, so it was possible that the function of Th17-derived histamine was to exert its effect through the H1 or H4 receptor, contributing to inflammation and chemotaxis[31]. Th17 cells also possess the H4 receptor, and activation of this receptor led to an increase in IL-17 release by Th17. HDC expression and histamine released by Th17 may then participate to increase IL-17 release and the mobilizing and activation of neutrophils. Given the role of histamine in immune regulation through the H2 receptor, it was possible that the Treg cells may express HDC as part of their function as immune regulatory cells[31]. Combining both these phenotypes, the double positive Th17/iTreg (IL-10+/IL-17+) cells were theorized to play a protective role and pathogenic role in different diseases. The potential function of Th17/iTreg derived histamine was then likely also immunomodulatory.

Dataset 2 utilized mechanical dissociation to isolate T cells from lung tissues and bone marrow, and utilized Ficoll-Paque gradient to isolate PBMCs, both followed by a CD3+ negative selection kit. This left room for potential contamination of non T cells. The data from this dataset showed a surprising 36.5% of all cells to not be classified as T cells by the authors. The authors noted to have used CD3D (CD3 delta) and TRAC (TCR alpha subunit) expression to identify T cells, which should include all T cell variants (and NK T cells)[192]. Their method for T cell isolation seemingly led to 36.5% of the total cells to be contaminating cells. Since the kit was designed to remove non-CD3 cells from PBMC, it was likely that the majority of contaminating cells to be stem cells originated from the bone marrow samples.

The HDC expression level and frequency of HDC+ cells for the authors annotated activated CD4/CD8 cells and resting CD4/CD8 cells varied. It was clear that these HDC+ cells were rare (0.107% of all T cells), and stimulation once again did not seem to upregulate the HDC expression level. The sample size of these HDC+ cells was likely too low to give a concluding statement, but this trend followed all the qPCR results obtained from this project, and as well as the bulk RNA sequencing dataset.

The authors did not specify the criteria for the "unassigned T cell" population. But since the authors divided the rest of the population into CD4+ or CD8+, it was likely that the unassigned T cells to be CD3+/CD4-/CD8- T cells. These double negative T cells are present within the peripheral blood and the lungs and this phenotype appeared to lack HDC expression according to our analysis[193].

Dr. Dvorkin found HDC+ cells in this dataset that were not classified as any cell type by the authors of the paper (cells were classified as "none"). Further analysis identified these mystery HDC+ cells as contaminating ILC2, NK cells and basophils. Out of these 3 cell types, NK cells and ILC2 have not been reported to express HDC. This was a lead we briefly pursued as mentioned above. We found that NK cells also did not upregulate when stimulated with PMA/I at 6-hour and 24-hour of stimulation. A majority of these mystery HDC+ cells were identified as basophil contamination. This supported our theory of contaminating cells leading to the detection of HDC transcript within bulk RNA sequencing, and of the human CD4+ T cell experiments. More interestingly, after classification of these mystery cells into ILC2, NK cells, and basophils, there were still HDC+ cells that Dr. Dvorkin was unable to identify. These unclassifiable cells expressed FcεR1, but not the other basophil markers used for identification

(CCR3). These cells were likely to be basophil precursors. HDC expression is tied to the differentiation process of basophils and mast cell, as such it is likely that HDC expression is a step in which pre-basophil must possess to mature into functional basophils[194].

In an early attempt to investigate whether HDC+ cells may belong to the Th1 or Th2 phenotype, we utilized GATA-3 (Th2 master regulator), IL-4 (Th2 cytokines), IL-5 (Th2 cytokines), IFNγ (Th1), and IL-2 (activated T cell cytokine) as co-expression marker for HDC (**Figure 4**). The data obtained once again highlighted the problem of false HDC+ signal from contaminating cells. GATA3, IL-4, IL-5 are also expressed in ILC2, and IFNγ is also expressed in NK cells[19,195].

HDC expression in identified ILC2 cells could be another novel path for research. No current study exists that examined the capacity for ILC2 to produce histamine. Although it was possible that these identified ILC2 may not be ILC2 at all, but NK cells. Dr. Dvorkin utilized identification markers IL-7R and KLRG1 to find ILC2 cells in non-T cell populations. Assuming lymphocytes were fully excluded, the expression of IL-7R and KLRG1 was not sufficient to identify ILC2 cells as these markers are may also co-express in NK cells[196,197].

As mentioned in **Discussion 1.1: Stimulation of human CD4+ T cells** and supported by **Discussion 1.2: Stimulation of human Th2 T cells**, it was interesting that the expression of IL-8 in unstimulated T cells was higher in the CD4+ T cells samples compared to the Th2 cells samples as analyzed by the LinRegPCR program. We proposed the explanation that this difference in IL-8 expression was due to the presence of IL-8 expressing contaminating cells. Further analysis by Dr. Dvorkin showed that the contaminating cells (the “none” population of this dataset) had a population of IL-8+ cells. As mentioned, monocytes, dendritic cells, basophils

and NK cells can express IL-8. Further co-expression analysis for HDC and IL-8 showed that 18.08% of the HDC+ cells were co-expressing IL-8 (**Supplementary Figure 9B**), which represented a considerable population of the HDC+ cells from the "none" population. This left 743 HDC+ cells (81.92%) to not co-express IL-8 but are still likely to be composed of contaminating cells known to express HDC (monocyte, dendritic cells, basophils). Since the stimulation method for this study should not activate any of these cells, perhaps these HDC+/IL-8- cells lacked the basal transcription for IL-8. More likely, the remaining 81.92% of HDC+ contaminating cells is largely composed of basophils. This theory is supported by the previous co-expression analysis where we found that the majority of HDC+ cells of the "none" population expressed markers akin to basophil markers. (**Figure 3B**). This single cell analysis further supported our hypothesis that contaminating cells could be responsible for the basal expression of IL-8 in unstimulated CD4+ T cell qPCR samples, as well being a minor contribution to the false HDC+ signal.

Dataset 3 used FACS sorting to isolate house dust-mite allergen specific human T lymphocytes after PBMCs isolation by Ficoll-Paque gradient centrifugation. The samples were pooled from multiple individuals of either allergic rhinitis, mild asthmatic, or healthy controls. FACS isolation represented the highest theoretical purity of samples that was analyzed for this project. Analysis once again found very rare pre-annotated T cells for HDC expression. Approximately 0.02% (6/28181 cells) of the T effector phenotypes were positive for HDC expression, and no other phenotypes were expressing HDC (T negative/unactivated, T regulatory, Th2). This rarity of HDC expression in allergen specific T cells, in the theoretically highest purity sample highlighted the unlikely potential of T cell derived histamine to contribute

to any allergic manifestation. Allergen-specific T cells are already rare within the human body, with the estimated house-dust mite specific T cells to be 1-6 cells per 10000 CD4+ T cells in PBMC (pending epitopes)[198]. HDC+ cells then represented a fraction of an already rare antigen specific T cells in the body, and as such they represented a miniscule fraction of the total pool of T cells available to react to a given antigen.

However, it was possible that the method used to enrich house dust-mite specific T cells may have led to induction of HDC in rare cells. To sort for house dust-mite specific T cells, the authors stimulated the PBMC pools with house dust-mite peptides for 6-hour before harvest. But the cDNA amplification step post sorting happened on day 3, approximately 48 hours after the end of stimulation. This timepoint represented the state of the cells for the mRNA expression landscape. In this case, it is entirely possible that the initial peptide stimulation of these T cell was required for the upregulation of HDC, and when some cells returned to their resting state, the HDC expression disappeared.

Co-expression analysis found HDC+ cells to express other genes with high confidence. The co-expression of granzyme B with HDC was expected as some T cells phenotypes have been known to express granzyme B[108,199]. Unfortunately, contaminating monocytes, basophils, and dendritic cells also happen to express granzyme B[200–202]. Yet so, the strength of granzyme B co-expression with HDC was at a 4.35-fold change as compared to control (HDC fold change was 2). T cells are known to upregulate granzyme B following TCR activation[108,203]. Because the method of T cell activation was by the addition of HDM peptides to the culture, it was unknown whether these peptides could activate the contaminating monocytes, basophils, or dendritic cells. If we operate under the assumption that the stimulation was specific to T cells, then the granzyme B

co-expression was likely from true T cells, since granzyme B should not be upregulated by contaminating cells. Interestingly, very recent research discovered that granzyme B expression and release was upregulated by exposure to histamine through the H2 receptor in human Th2 polarized CD4+ T cells in atopic dermatitis and control donors[204]. It was then possible that either that granzyme B from our analyzed dataset could have been partially upregulated by histamine derived from contaminating cells, or by histamine derived from T cells.

The other two co-expressing genes that been known to express in T cells were HDAC7 and GADD45G. Similarly, HDAC7 and GADD45G expression are not unique to T cells, but also in monocytes according to the Human Protein Atlas database. However, GADD45G has been studied to be upregulated following TCR activation in Th1 cells and Th2 cells[205]. This means that the co-expression of GADD45G with HDC could be from a true T cell population.

Conclusion and Future Directions

The combination of bioinformatics and qPCR data showed that human T lymphocytes express HDC in rare cells of different phenotypes. T cells classified by single cell RNA sequencing dataset showed rare HDC expression in both resting and activated T cells and of different differentiated CD4+ T cells phenotypes. Bulk RNA sequencing dataset analysis supported the findings with the detection of HDC expression in both activated T cells and resting T cells of induced differentiated T cell phenotypes. This dataset also showed that memory T cells expressed a higher level of HDC expression and showed activation by anti-CD3/CD28 did not lead an upregulation of HDC in any T cell phenotypes.

Working simultaneously with bioinformatics, we found that stimulation using anti-CD3/CD28 or PMA/I of human CD4+ T cells and differentiated Th2 cells did not upregulate HDC. The allergic status of the donors did not influence HDC upregulation. The expression level of HDC was consistent across any stimulation conditions using different methods of analyzing qPCR data. Directly comparing the CT values for stimulated T cells (CD4+, Th2) to unstimulated cells showed a flatline of expression across any conditions. Analysis using deltadeltaCT also showed a lack of upregulation in HDC. Lastly, analysis using the LinRegPCR program to determine the starting abundance of HDC also showed the lack of HDC upregulation. The addition of gel electrophoresis and melt curve analysis confirmed HDC expression within the samples. Sequencing analysis of cut out HDC bands confirmed the identity to be of the intended product. From qPCR data alone, it was impossible to discern whether the amplified HDC product was from rare HDC+ T cells, or from T cells expressing low transcript of HDC basally, or from contaminating cells such as basophils. Direct comparison between data collected from Jurkat T

lymphocytes to primary human T cells was not a “fair” comparison. Jurkat T lymphocyte cancerous nature was a result of irregular gene expression, and thus should not be compared to healthy human T cells. But the data collected from Jurkat T lymphocytes allowed for a pseudo-positive control in what HDC upregulation might look like when compared to data obtained from primary cells. It was Jurkat T lymphocyte characterization as a leukemic immortal cell line that likely allowed the cells to express HDC. Thus, it was possible that the immortalization of cells may lead to the expression of HDC. From earlier references, transformed monocyte cell line (RAW 264), human gastric cancer cells and murine colon cancer cells were utilized to study the transcription regulation of HDC. Other examples of HDC expression in immortal cells include the HT168 melanoma cell line, human small cell lung carcinoma, human pancreatic cancer cells, and K562 (another leukemic T cell line)[206–209]. Histamine was demonstrated to be an autocrine/paracrine growth factors for cancer cells, and the abolishment of HDC expression led to a suppression of cancer cell proliferation[208]. The expression of histamine is likely a method that speed up proliferation in cancerous cells, and histamine has been used as a biomarker for some cancer types[210].

Combining bioinformatics and qPCR analysis allowed each methodology to cover each other's pitfalls. scRNA pitfalls included varying sequencing depth, small cell number, and short number of mRNA amplification cycle to generate cDNA library[211]. Sequencing depth refers to the number of times a single nucleotide was read in sequencing. A low depth (read number) would therefore provide a worse resolution of the genetic landscape as rare transcripts might not be detected, or detected in insufficient quantity for a strong correlation[211]. qPCR does not have this flaw as qPCR has very specific targets as guided by primers design. Primer

concentration often eclipsed the abundance of their target, and as such primers likely saturate any possible target for amplification. scRNA can also only utilize a small number of cells per sample to maintain resolution, and not many cells might be in a state that allow for specific phenotype (like HDC+) to express, or very small number of cells will be of the rare phenotype. qPCR was not limited by cell numbers as the starting product is the isolated RNA/DNA from any number of cells. The number of rare cells within a sample can be scaled up to where qPCR can reliably detect the transcript. Lastly, some methods of scRNA use a short amplification cycle (~13-16 cycles) to amplify the sample RNA into cDNA (10x Chromium – Chromium NeXt GEM Automated Single Reagent Kits v2). qPCR in theory can have as many amplifications cycle as necessary, with the limiting factors being reagents availability and nonspecific amplification. The detection range of HDC expression also required mostly 31+ cycle of amplification, implying the amplification of a rare transcript. A practical example that reflects the rarity of 30+ CT for HDC detection was the use of qPCR as a diagnosis for COVID-19. A CT values of >33 was used to characterize non-infectious cases, and CT of ~30 was used to characterize low viral RNA[212,213]. This may lead to very rare transcripts not being abundant enough after a short amplification cycle for scRNA preparation to be picked up by sequencing and analysis. Pitfall of qPCR was the inability for the full characterization of a cell's mRNA landscape at an individual cell resolution, which was then covered by the usage of single cell sequencing. scRNA sequencing can also analyze the co-expression of the gene of interest with multiple lineage markers to characterize the identity of the cells.

Even with the combination of qPCR and bioinformatics, the biggest challenge of this project was to obtain a pure population of T cells for analysis. T cells isolation by Ficoll-Paque

gradient centrifugation followed by magnetic bead negative isolation was the preferred method of all the bioinformatic datasets analyzed, and for this project's protocol. These methods do not guarantee the exclusion of non-T cells purely due to the nature of the procedure. It was not guaranteed that every contaminating cell would bind to their magnetic bead for exclusion, or the contaminating cells would not be transferred with the T cells when the solutions were moved. If resources allowed, it is theoretically possible to obtain a higher purity of T cells by subjecting the sample through multiple rounds of negative selection or by using positive selection for potential contaminating cells. However, this would still not guarantee a perfectly pure population of T cells. The addition of FACS sorting was employed in one of the datasets analyzed and represented the highest purity of sample obtained. Although the process of staining and FACS sorting may have altered the transcription landscape of the cell, there was HDC+ T cells detected from the sample. It was possible that the sorting process led to HDC expression, but this would still contribute to the project's hypothesis as HDC was expressing in human T cells.

Attempts to exclude contaminating cells by scRNA sequencing proved to be less straightforward than applying lineage markers to the analysis, and represented an analysis pitfall to the method. The transcriptional profile varies between cell to cell, even if they are of the same lineage. A cell is defined by its lineage markers, but the expression of these lineage markers is dependent on which state of differentiation the cell may be in. An apt example is that of myeloid progenitor cells which lack the FcεR1 receptor but can be further differentiated or is differentiating into mast cells or basophils[214]. Myeloid progenitors also happen to express HDC and can be found rarely in peripheral blood[215,216]. In this project, the expression of HDC may

precede the temporal regulation of the lineage marker expression, which leads to identification of HDC+ cells without being able to accurately identify the identity of this HDC+ cell.

These bioinformatic pitfalls should be considered when interpreting the results of any bioinformatic analyses. The physiological importance of rare transcripts and rare cells must be tested in assays that evaluate their relevance in the overall pathogenesis of a disease. The results of this project showed a lack of upregulation of HDC in human T cells. It was therefore unlikely that histamine derived from T cells contributes to the bronchoconstriction in the late phase reaction of allergies.

There are several future directions for the extension of this project. The expression of HDC does not guarantee that functional histamine is being released from the T cell and it is necessary to determine whether HDC expression is leading to histamine release. Performing histamine ELISAs will allow for the quantification of the released histamine. Previous work by Mark Lychacz showed that unstimulated Jurkat T lymphocyte did not lead a detectable level of histamine using ELISAs (Histamine ELISA – Beckman Coulter), but stimulated Jurkat T lymphocyte did release a detectable level of histamine. Comparing the averaged CT values between unstimulated Jurkat T lymphocytes and human T lymphocytes showed similar values. It was therefore possible that the released histamine from human T lymphocytes to also be below the detection range for ELISA.

A flow cytometry-based procedure to detect histamine was developed in the past to observe histamine released from basophil. Ebo et al, 2012 developed a technique coined “HistaFlow” where diamine oxidase is coupled with a fluorescence antibody and is used to

detect histamine[217]. Diamine oxidase functions as the opposite of HDC where diamine oxidase breaks down histamine in the environment. The method relies on the binding of diamine oxidase to histamine to detect histamine within basophil granules. To measure histamine inside the basophil, permeabilization buffer can be used to allow the antibody conjugated diamine oxidase to enter the cell. This method can be used with any of the T cell experiments of this project. We can stain the culture with basophil markers (ex: CD11b), or other contaminating cell markers, any T cell markers of interests, and the antibody coupled diamine oxidase. Flow analysis would exclude CD11b+ cells to exclude basophils, exclude other potential contaminants, include cells with T cell markers and plot for diamine oxidase signal. Since basophils store histamine in granules, but only some T cells have granules (cytotoxic T cells, Treg), it was theorized that T cells would release their histamine into the extracellular constantly. Monensin A or another protein transport blockers can be used to retain the histamine within the T cells allowing this method to work. Monensin A would be the ideal candidate as the drug does not block intracellular protein synthesis, and only blocks transfer of products formed within the Golgi Apparatus. Since histamine is synthesized within the Golgi, the use of Monensin A may localize histamine to the Golgi, allowing the diamine oxidase to concentrate and provide a stronger signal by MFI measurement for flow cytometry measurement[67]. Utilization of this method allows for the simultaneous detection of T cell derived histamine, as well as the pinpointing histamine producing cells. The specificity of diamine oxidase for histamine within the cell is a hurdle of this technique for T cells. Diamine oxidase expression in basophils is likely to be minimal as basophils continuously produce and store histamine, but the expression level of diamine oxidase is not known in T cells. If T cells

naturally express diamine oxidase, then the addition of diamine oxidase may counteract any detection of histamine. This will then lead to false positive data where the fluorescent signal from T cells results from free diamine oxidase labeled with antibody binding non-specifically. And if T cells naturally express diamine oxidase, it may effectively block any histamine from being produced/release from HDC+ T cells.

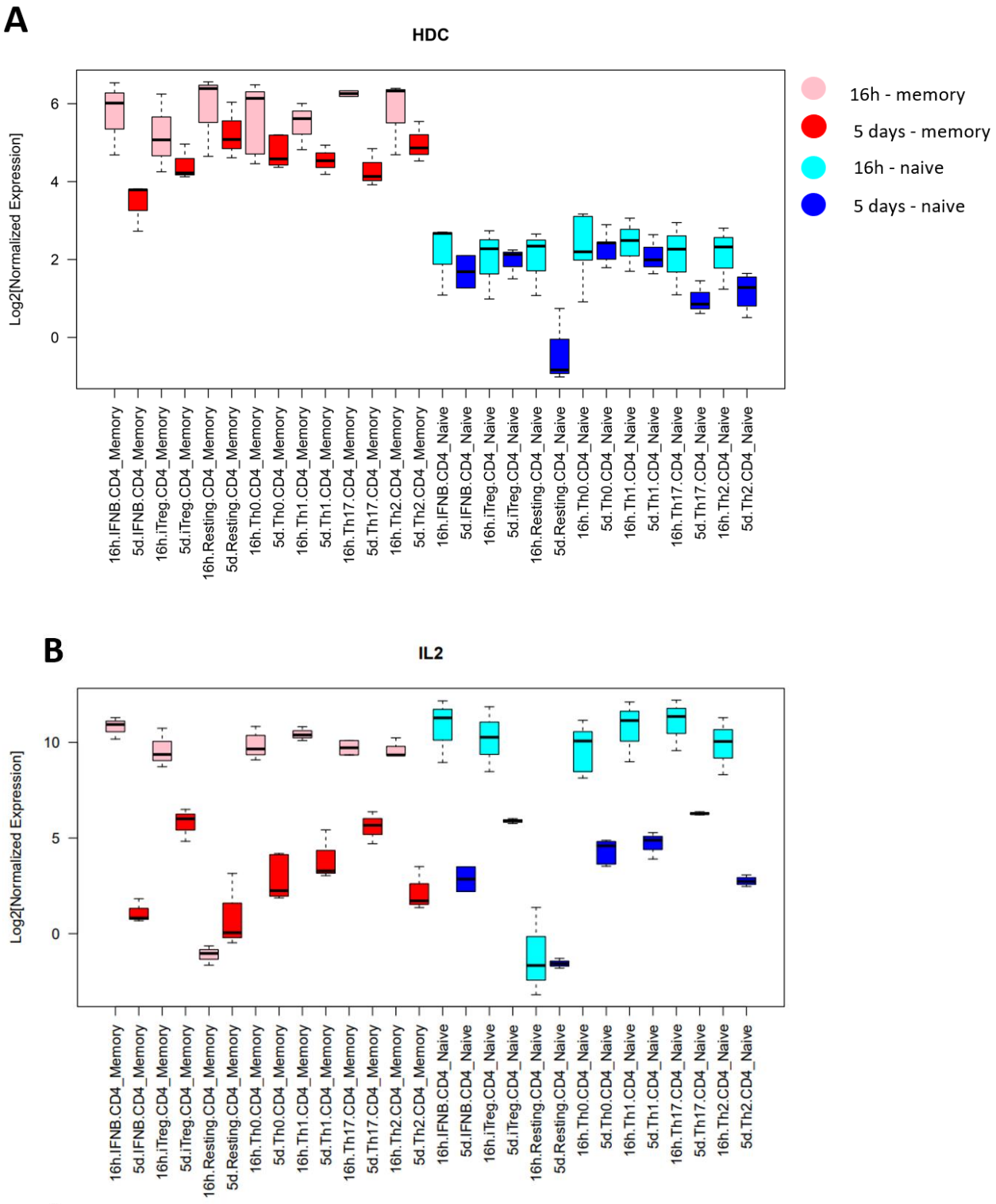
Beyond the scope of this project, it is important to isolate these HDC+ T cells and determine what allowed these T cells to be HDC+, as well as determining if this phenotype is kept after proliferation. The data from this project showed that it was unlikely the HDC+ phenotype is kept after proliferation. Stimulation at the 72-hour timepoint did not lead to any upregulation of HDC, nor did the 13 days culture for Th2 differentiation. Both cultures' procedure was not reflective of the physiological conditions that may induce HDC expression. To concretely determine if the HDC expression is maintained after proliferation, HDC+ T cells must be isolated by FACS sorting, cultured, and analyzed for HDC expression. Stimulation should cover a variety of methods, such as anti-CD3/CD28 beads, PMA/I, mitogens, or co-culture with DCs loaded with allergen peptides. If the HDC+ phenotype is not maintained, then it is more likely that the expression of HDC is a genetic regulation artefact, or HDC expression requires very specific signaling with multiple intracellular signaling pathway. In this sense, the expression of HDC may be a product of some type of differentiation, inducible in a very specific microenvironment. Determining which signaling pathways allow for HDC expression requires a more in-depth look at the genetic regulation of HDC. Current literature on this topic was sparse and our understanding is very limited. Research done in different transformed cell lines elucidated the PKC pathway activation in upregulating HDC. The method of stimulation

deployed in this project should be sufficient to activate the PKC pathway in T cells, but no upregulation was observed. It was then possible that multiple signaling pathways, perhaps independent of the traditional TCR signaling pathway are required for HDC expression. The choice of PMA/I stimulation in this project was supposed to act as a positive control given PMA/I activation of multiple T cells pathways[124]. These PMA/I activated pathways may still be insufficient to induce HDC. To nail down the signals required for HDC expression, it is best to determine the transcription factor governing the promoter sequence for HDC, and thus determining which intracellular signal is responsible for the import of the specific transcription factor into the nucleus. Prior literature showed the involvement of the SP1 transcription factor in the promoter binding of HDC, and SP1 can be phosphorylated by the PKC pathway downstream of TCR signaling[85,218]. SP1 transcriptional activity is also upregulated in dividing T cells and in IL-21R expressing activated T cells [219,220]. Taken together, it was unlikely that SP1 alone governed the transcription of HDC as the project methodology should have been sufficient to phosphorylate and upregulate SP1. The transcription factors YY1 and KLF4 were also implicated in the negative regulation of SP1, which lead to the repression of HDC[28]. YY1 transcription factor is upregulated following TCR stimulation and is heavily associated with the Th2 phenotye[221]. Overexpression of YY1 led to the production of Th2 cytokines, and YY1 directly regulated GATA3 expression (Th2 master regulator)[222]. This precedent in the literature can also explain the lack of HDC expression from Th2 cells (qPCR and single cell data) being due to the expression of YY1 potentially blocking SP1 promoter binding of HDC. KLF4 expression is another transcriptional repressor for HDC and is involved in the Th17 differentiation pathways in mice through its positive regulation of IL-17[223]. If KLF4 functions to repress HDC, then this result does

not align with the data obtained from single cell sequencing as the Th17 phenotype was the highest population with HDC+ cells. Ultimately, the interplay between transcription factors for the regulation of HDC in T cells requires additional investigation to obtain any meaningful understanding. If all methods of T cell stimulation and co-stimulation were exhausted without inducing HDC, it is then possible that genetic/epigenetic regulation/dysregulation through histone acetylation maybe required for the expression of HDC. Histone dysregulation is linked to cancer development, and HDC expression was linked to the immortalization of cells, then it is possible that histone dysregulation is the potential cause for HDC expression[224].

In conclusion, this project aimed to determine the expression of HDC in human T lymphocytes and the results showed that rare T cells of various phenotypes were detected to express HDC. This expression of HDC was not upregulated using TCR signaling through anti-CD3/CD28 beads stimulation, or multiple intracellular pathways signaling through PMA/I stimulation at the 6-hour, 24-hour, and 72-hour stimulation timepoints. The current requirement for HDC expression in human T lymphocytes is unknown and the function of these HDC+ T cell phenotypes is unknown. The lack of upregulation of HDC through the TCR signaling pathways as well as the rarity of HDC T cells are contributing evidence that T cell derived histamine is likely not a contributor in late phase reaction bronchoconstriction.

Figures and Tables



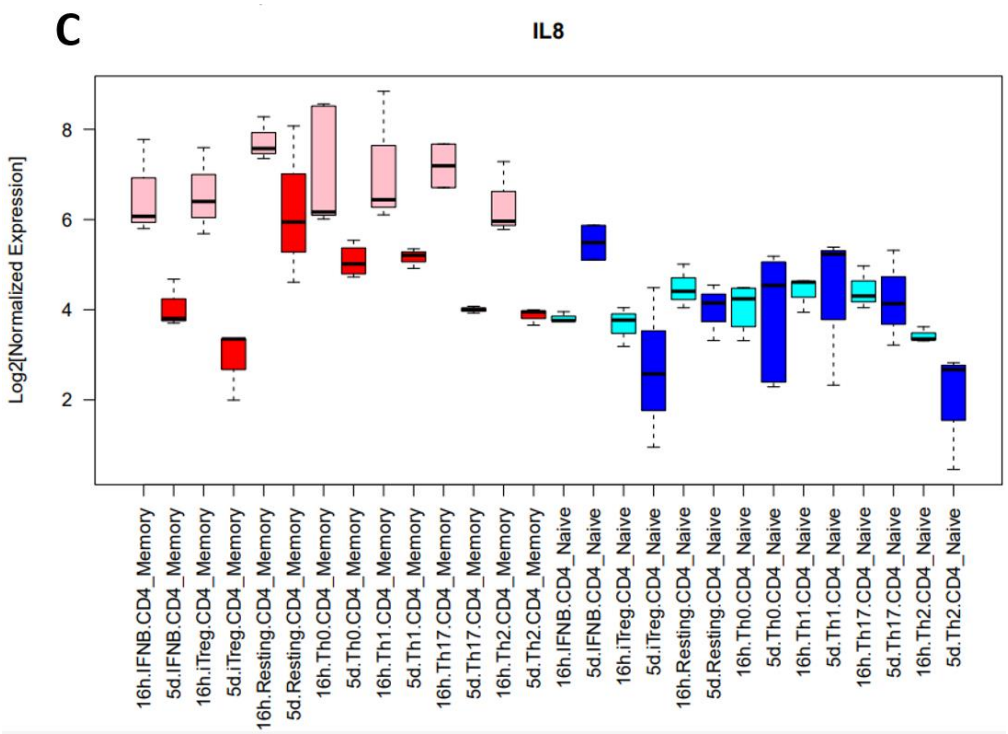
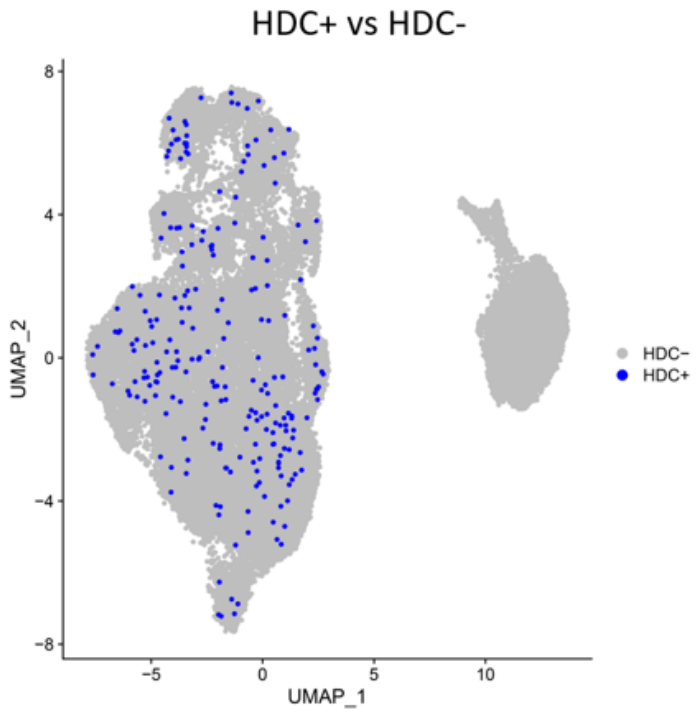


Figure 1. HDC gene expression in human T lymphocytes: Dataset 1 (Single-cell transcriptomics identifies an effectorness gradient shaping the response of CD4⁺ T cells to cytokines, https://doi.org/10.1038/s41467-020-15543-y): Bulk RNA sequencing analysis of isolated human CD4+ T cells activated with anti-CD3/CD28 beads with differentiation cocktails for 16 hours or 5 days. Human PBMCs were isolated using Ficoll-Paque (GE Healthcare) gradient. Memory CD4+ T cells were isolated using EasySep™ memory T cell enrichment kit (StemCell). Naïve CD4+ T cells were isolated using EasySep™ naïve T cell isolation kit (StemCell). Purity of CD4+ T lymphocytes were not directly reported, an example flow cytometry gating for CD4+ cells showed a 96% purity. T cells differentiation were as followed: Th0 (no additional cytokines), Th1 (IL-12, anti-IL-4), Th2 (IL-4, anti-IFNγ), Th17 (TGFb, IL-6, IL-23, IL-1b, anti-IFNγ, anti-IL-4), iTreg (TGFb, IL-2), IFNβ (IFNβ). T cells phenotypes were determined by RNA sequencing for 5 days culture samples. HDC, IL-8 and IL-2 normalized expression were measured. **A.** HDC expression level in different activation state, differentiation environment and phenotypes. **B.** IL-8 expression level in different activation state, differentiation environment and phenotypes. **C.** IL-2 expression level in different activation state, differentiation environment and phenotypes.



Numbers of HDC+ and HDC- cells

Cell cluster	HDC+	HDC-
HSP.high	9	1088
IFN.high	9	1196
Mitotic	18	1480
nTreg (resting)		248
nTreg (Th0)	5	887
TCM (resting)		1855
TCM1 (Th0)	10	1599
TCM1 (Th17/iTreg)	7	1001
TCM2 (Th0)	9	2248
TCM2 (Th17/iTreg)	19	3365
TEM (resting)		419
TEM (Th0)	2	1157
TEM (Th17/iTreg)	23	2622
TEMRA (resting)		188
TEMRA (Th0)	1	345
TEMRA (Th17/iTreg)	7	907
TN (iTreg)	29	4061
TN (resting)		2336
TN (Th0)	17	2086
TN (Th17)	31	3920
TN (Th17/iTreg)	24	2253
TN (Th2)	8	3019

Figure 2. HDC gene expression in human T lymphocytes phenotypes: Dataset 1 (Single-cell transcriptomics identifies an effectorness gradient shaping the response of CD4$^+$ T cells to cytokines, https://doi.org/10.1038/s41467-020-15543-y). Single cell RNA sequencing analysis of human CD4+ T cells differentiated in vitro. Analysis characterized T cells into different T cell subsets based on lineage markers as annotated by authors. HDC+ cells were highlighted and classified into T cell subsets. The frequency of HDC+ T cell per annotated phenotype were as follows: Natural T regulatory cell - nTreg (Th0): 0.56% (5/892 cells), T central memory cell of cluster 1 - TCM1 (Th0): 0.625% (10/1609 cells), T central memory cell of cluster 1 - TCM1 (Th17/iTreg): 0.69% (7/1008 cells), T central memory cell of cluster 2 - TCM2 (Th0): 0.17% (9/2267 cells), T central memory cell of cluster 2 - TCM2 (Th17/iTreg): 0.56% (19/3384 cells), T effector memory - TEM (Th0): 0.17% (2/1159 cells), T effector memory - TEM (Th17/iTreg): 0.88% (23/2345 cells), T effector memory re-expressing CD45RA - TEMRA (Th0): 0.29% (1/346 cells), T effector memory re-expressing CD45RA - TEMRA (Th17/iTreg): 0.77% (7/914 cells), Naïve T cells - TN (iTreg): 0.71% (29/4090 cells), Naïve T cells - TN (Th0): 0.81% (17/2103 cells), Naïve T cells - TN (Th17): 0.79% (31/3951 cells), Naïve T cells - TN (Th17/iTreg): 1.06% (24/2277 cells), Naïve T cells - TN (Th2): 0.264% (8/3027 cells).

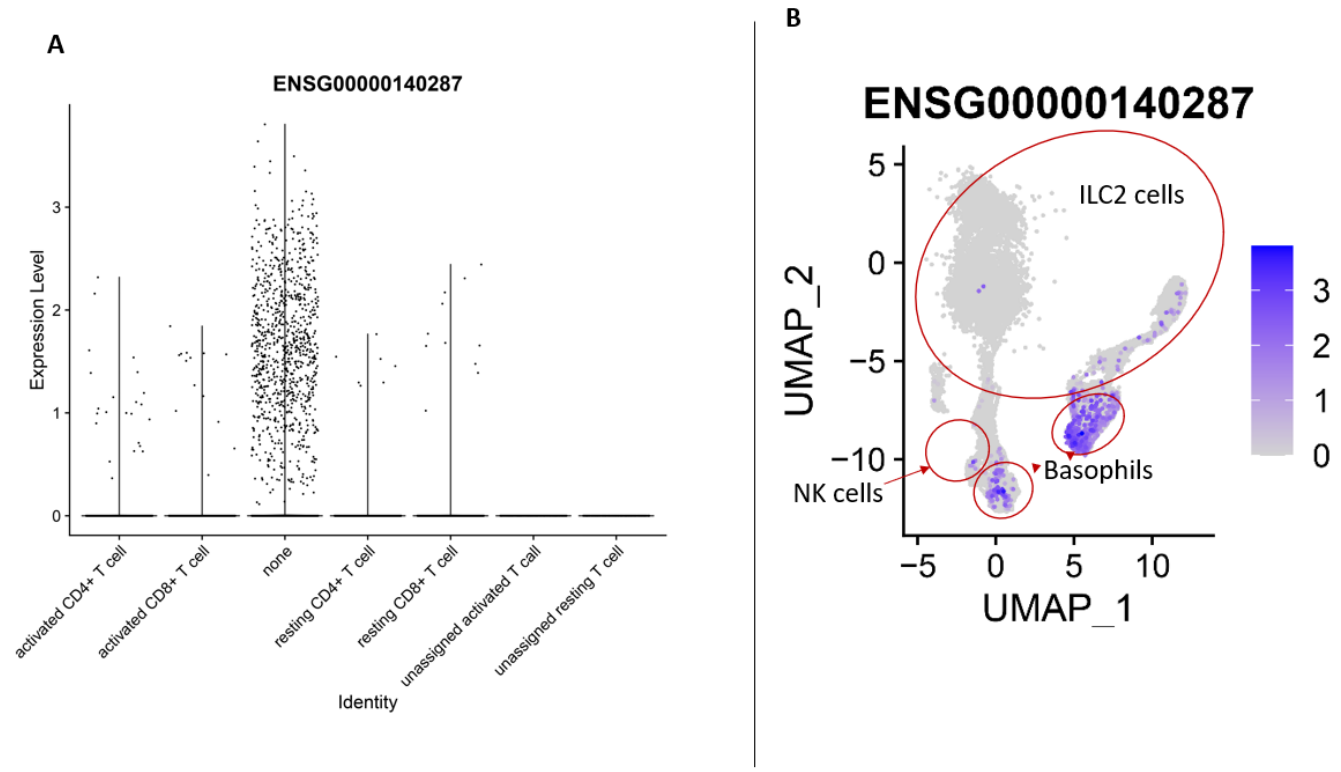


Figure 3A. Single cell analysis of HDC (ENSG00000140287) expression in human T lymphocytes: Dataset 2: (Single-cell transcriptomics of human T cells reveals tissue and activation signatures in health and disease, https://doi.org/10.1038/s41467-019-12464-3). Single-cell RNA sequencing analysis of isolated human CD4+ T cells from human lungs, lymph nodes, peripheral blood, and bone marrow. Cells were isolated through mechanical tissue dissociation (lung tissues, lymph nodes, bone marrow) or Ficoll gradient (PBMCs), followed by CD3+ negative isolation using MojoSort Human CD3+ T cell Isolation Kit (BioLegend). Cells were activated for 16 hours using soluble anti-CD3/CD28 antibodies. Cells positive for HDC expression were grouped into different T cell subsets as annotated by the authors. These subsets and their corresponding percentage of HDC+ cells were: activated CD4+ T cells: 0.12% (23/18979 cells), activated CD8+ T cell: 0.21% (14/6515 cells), unclassified cells "none": 3.09% (907/29394 cells), resting CD4+ T cells: 0.15% (11/7102 cells), resting CD8+ T cells: 0.039% (7/17706 cells), unassigned resting and activated T cells were not positive for HDC expression. 55 out of 51130 T cells were HDC+, a total of 962 out of 80524 cells were HDC+.

Figure 3B. Identification of HDC positive cells of unknown cell type as assigned as "none" by the authors: Dataset 2: (Single-cell transcriptomics of human T cells reveals tissue and activation signatures in health and disease, https://doi.org/10.1038/s41467-019-12464-3). Attempts to classify HDC+ cells belong to the "none" category using lineage markers identified different cells akin to NK cells, basophils, ILC2. Markers used were: CCR3, FCER1 for basophils, IL-7R, KLRG1 for ILC2 cells, NCR1, KLRB1, NKG7, CD3e for NK cells.

Cell counts

	+/+	-/+	+/-	-/-
HDC/GATA3	56	9,084	906	70,478
HDC/IL4	24	665	938	78,897
HDC/IL5	50	306	912	79,256
HDC/INFG	131	10,405	831	69,157
HDC/IL2	18	2,072	944	77,490

% out of all cells

	+/+	-/+	+/-	-/-
HDC/GATA3	0.070	11.281	1.125	87.524
HDC/IL4	0.030	0.826	1.165	97.979
HDC/IL5	0.062	0.380	1.133	98.425
HDC/INFG	0.163	12.922	1.032	85.884
HDC/IL2	0.022	2.573	1.172	96.232

Figure 4. Co-expression of typical T helper markers in HDC+ cells. Dataset 2: (Single-cell transcriptomics of human T cells reveals tissue and activation signatures in health and disease, https://doi.org/10.1038/s41467-019-12464-3). Analysis for the co-expression of GATA3 (Th2), IL-4 (Th2), IL-5 (Th2), IFNγ (Th1), and IL-2 (activated T cells) in HDC+ cells. Yellow highlighted columns highlight double positive cells.

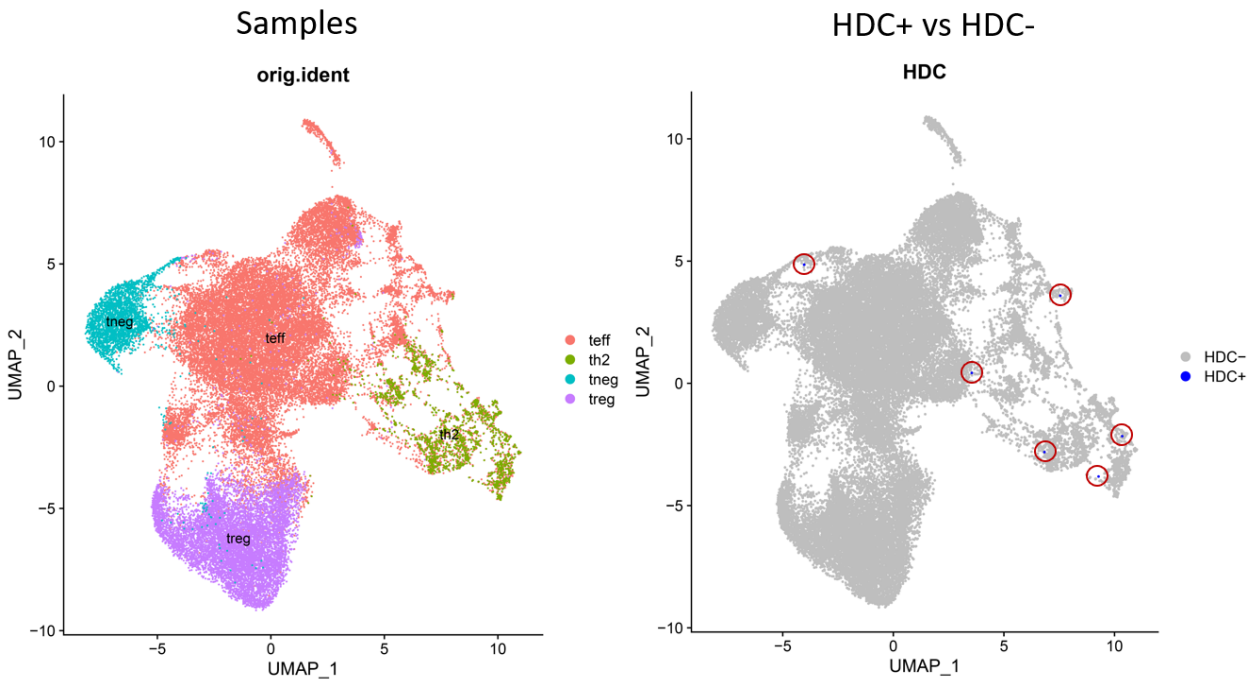


Figure 5. HDC positive cells in human house dust-mite allergen specific T lymphocytes: Dataset 3: (Single-cell transcriptomic analysis of allergen-specific T cells in allergy and asthma, https://doi.org/10.1126/sciimmunol.aba6087). Single cell sequencing analysis of FACS isolated HDC specific CD4+ T cells from human allergic rhinitis, mild asthmatic, and healthy individuals. T cells were stimulated with HDM peptides for 6h, followed by CD154+ enrichment and single cell analysis. Analysis characterized T cells into different T cell subsets based on markers as annotated by authors. HDC+ cells were found in the T effector phenotypes (6/28181 cells were HDC+, 0.02%).

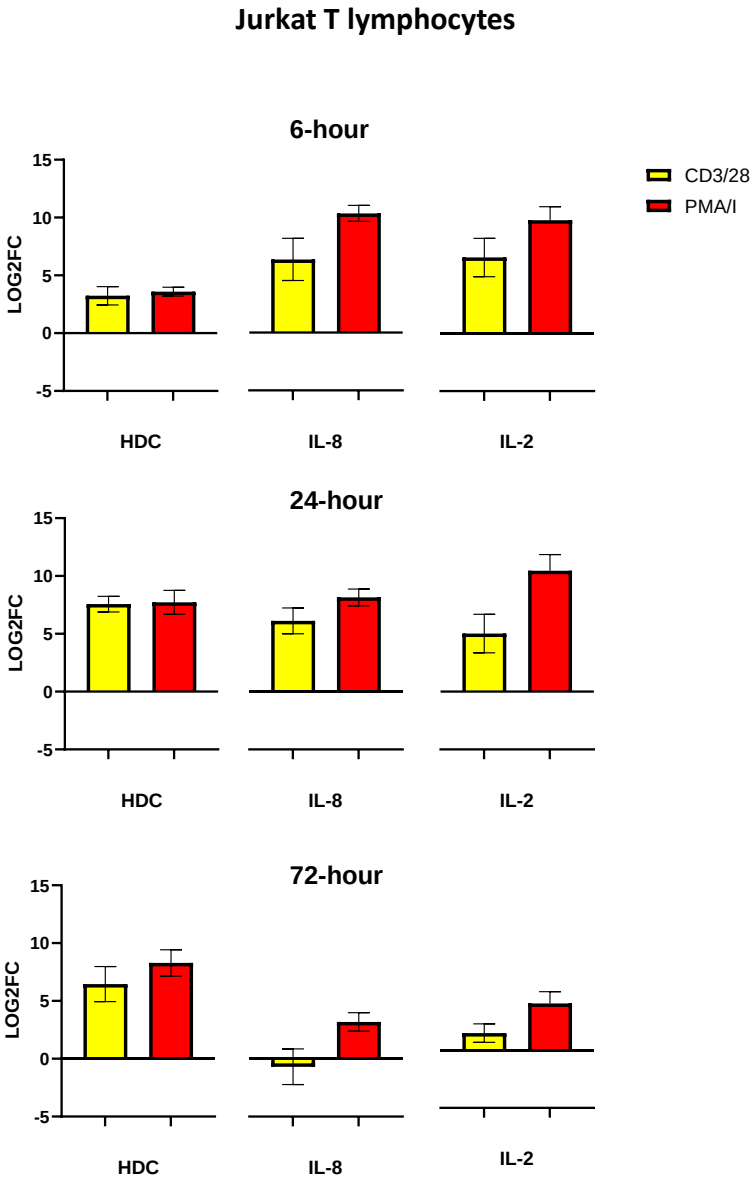


Figure 6. HDC, IL-8 and IL-2 gene upregulation in activated Jurkat T lymphocytes: Jurkat T lymphocytes stimulated with anti-CD3/CD28 beads (yellow) or PMA/Ionomycin (red) for 6 hours, 24 hours, and 72 hours. RNA was extracted and qPCR performed with primers targeting HDC, IL-8, IL-2, and B2M. Data were normalized to B2M using the deltadeltaCt method. Data reported were Log2 transformed and represent the Log2 Fold Changes as compared to B2M and HDC expression in media control (n=7). Error bars represent SEM.

Table 1: Log2 fold changes of HDC as normalized to housekeeping gene B2M in cultured Jurkat T lymphocytes. Data accompanies **Figure 6.**

	HDC LOG_2FC	
	Jurkat T lymphocytes	
	aCD3/28	PMA/I
6-hour stimulation	3.2 +/- 0.79	3.6 +/- 0.38
24-hour stimulation	7.6 +/- 0.67	7.7 +/- 1.0
72-hour stimulation	6.5 +/- 1.5	8.3 +/- 1.1

	IL-8 LOG_2FC	
	Jurkat T lymphocytes	
	aCD3/28	PMA/I
6-hour stimulation	6.3 +/- 1.8	10 +/- 0.7
24-hour stimulation	6.1 +/- 1.1	8.2 +/- 0.72
72-hour stimulation	-0.69 +/- 1.5	3.2 +/- 0.8

	IL-2 LOG_2FC	
	Jurkat T lymphocytes	
	aCD3/28	PMA/I
6-hour stimulation	6.6 +/- 1.7	9.8 +/- 1.2
24-hour stimulation	5.0 +/- 1.7	10 +/- 3.7
72-hour stimulation	1.5 +/- 0.8	4.6 +/- 1.2

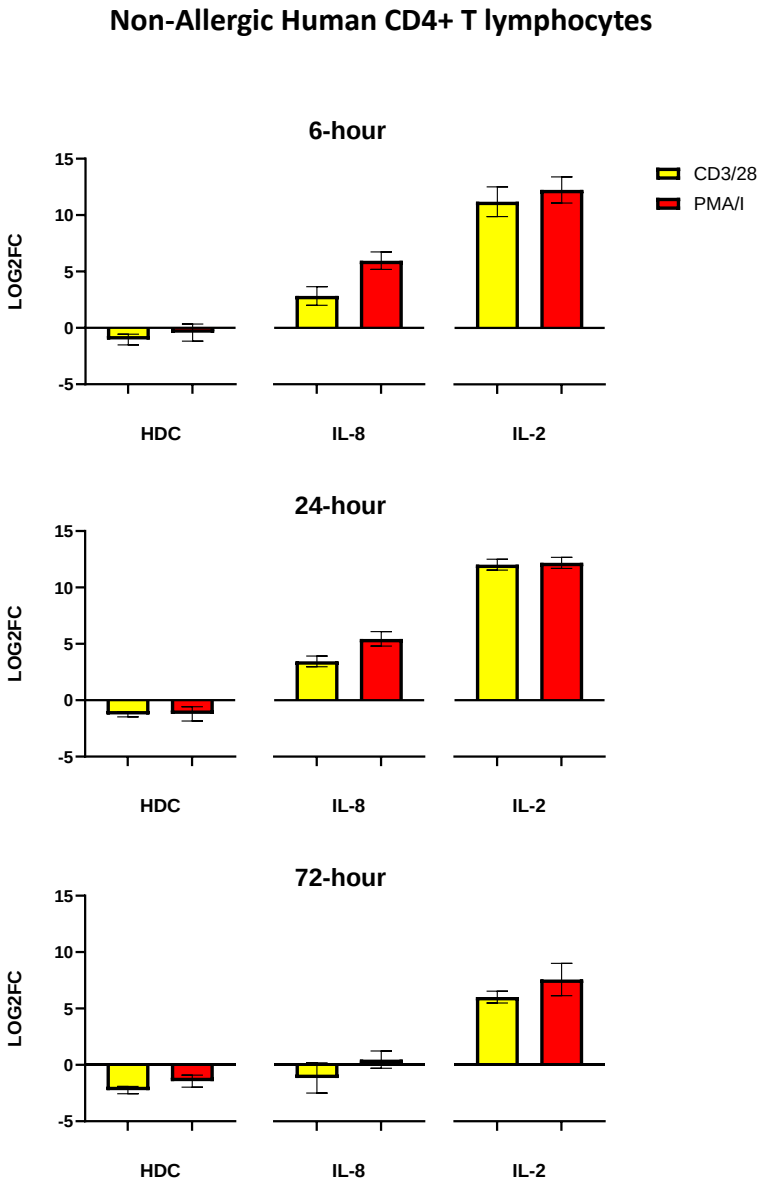


Figure 7. HDC expression was not upregulated in activated non-allergic human CD4+ T lymphocytes: Human CD4+ T cells from non-allergic individuals (n=6) were negatively isolated and stimulated with anti-CD3/CD28 beads (yellow) or PMA/Ionomycin (red) for 6 hour, 24 hours, and 72 hours. RNA was extracted and qPCR performed with primers targeting HDC, B2M, IL-8, and IL-2. Data were normalized to B2M using the deltadeltaCt method. Data reported were Log2 transformed and represent the Log2 Fold Changes as compared to B2M expression. Error bars represent SEM.

Table 2: Log2 fold changes of HDC as normalized to housekeeping gene B2M in isolated non- allergic human CD4+ T lymphocytes stimulated with anti-CD3/CD28 beads or PMA/Ionomycin. Data accompanies **Figure 7.**

	HDC LOG_2FC	
	Human CD4+ T lymphocytes (Non-allergic)	
	aCD3/28	PMA/I
6-hour stimulation	-1.0 +/- 0.48	-0.42 +/- 0.76
24-hour stimulation	-1.3 +/- 0.23	-1.2 +/- 0.63
72-hour stimulation	-2.2 +/- 0.32	-1.4 +/- 0.54

	IL-8 LOG_2FC	
	Human CD4+ T lymphocytes (Non-allergic)	
	aCD3/28	PMA/I
6-hour stimulation	2.8 +/- 0.82	6.0 +/- 0.77
24-hour stimulation	3.5 +/- 0.47	5.4 +/- 1.6
72-hour stimulation	-1.2 +/- 1.3	0.48 +/- 0.76

	IL-2 LOG_2FC	
	Human CD4+ T lymphocytes (Non-allergic)	
	aCD3/28	PMA/I
6-hour stimulation	11 +/- 1.3	12 +/- 1.2
24-hour stimulation	12 +/- 0.49	12 +/- 0.49
72-hour stimulation	6 +/- 0.52	7.6 +/- 1.4

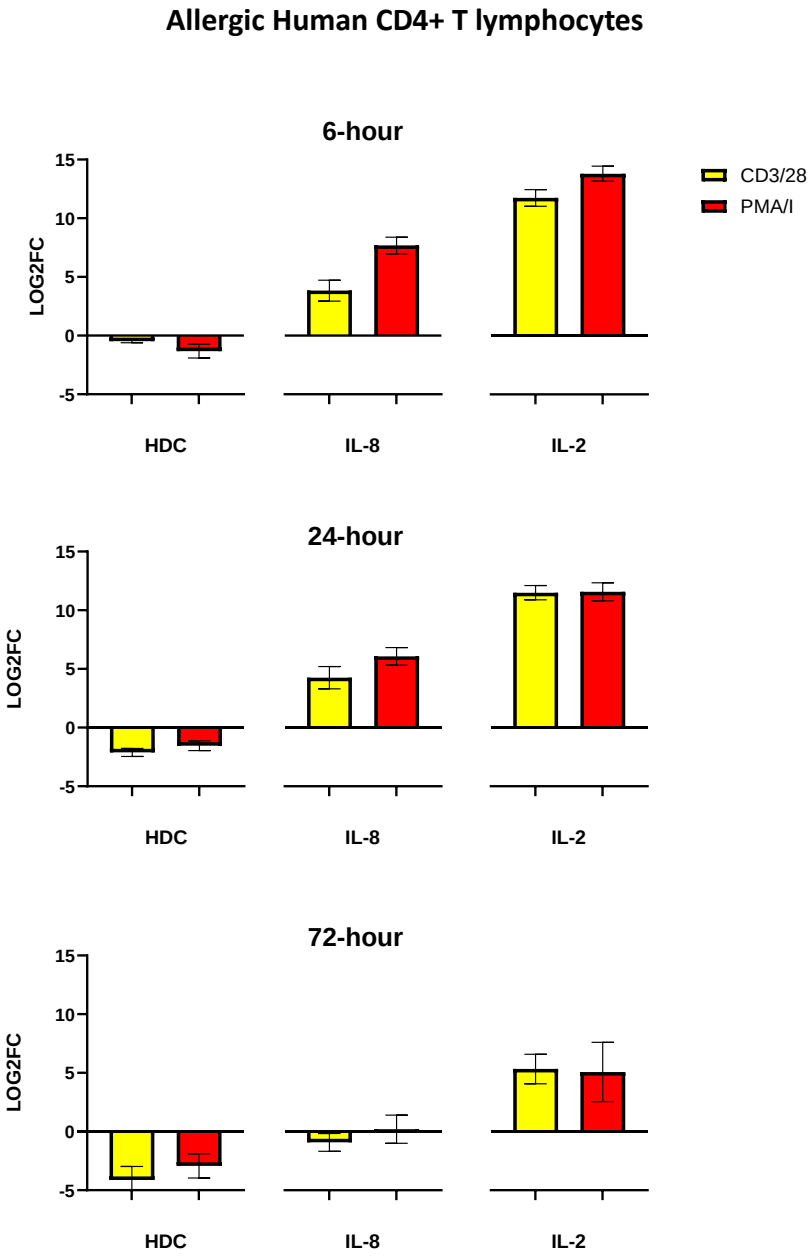


Figure 8. HDC expression was not upregulated in activated allergic human CD4+ T lymphocytes: Human CD4+ T cells from allergic individuals (n=6) were negatively isolated and stimulated with anti-CD3/CD28 beads (yellow) or PMA/Ionomycin (red) for 6 hour, 24 hours, and 72 hours. RNA was extracted and qPCR performed with primers targeting HDC, B2M, IL-8, and IL-2. Data were normalized to B2M using the deltadeltaCt method. IL-2 and IL-8 expressions were used as positive control. Data reported were Log2 transformed and represent the Log2 Fold Changes as compared to B2M expression. Error bars represent SEM.

Table 3: Log2 fold changes of HDC as normalized to housekeeping gene B2M in isolated allergic human CD4+ T lymphocytes stimulated with anti-CD3/CD28 beads or PMA/Ionomycin. Data accompanies **Figure 8.**

	HDC LOG_2FC	
	Human CD4+ T lymphocytes (Allergic)	
	aCD3/28	PMA/I
6-hour stimulation	-0.48 +/- 0.13	-1.3 +/- 0.58
24-hour stimulation	-2.1 +/- 0.35	-1.5 +/- 0.42
72-hour stimulation	-4.1 +/- 1.1	-2.9 +/- 1.0

	IL-8 LOG_2FC	
	Human CD4+ T lymphocytes (Allergic)	
	aCD3/28	PMA/I
6-hour stimulation	3.8 +/- 0.89	7.7 +/- 0.7
24-hour stimulation	4.3 +/- 0.95	6.1 +/- 0.76
72-hour stimulation	-0.92 +/- 0.76	0.21 +/- 1.2

	IL-2 LOG_2FC	
	Human CD4+ T lymphocytes (Allergic)	
	aCD3/28	PMA/I
6-hour stimulation	12 +/- 0.71	14 +/- 0.65
24-hour stimulation	11 +/- 0.61	12 +/- 0.76
72-hour stimulation	5.3 +/- 1.3	5.1 +/- 2.5

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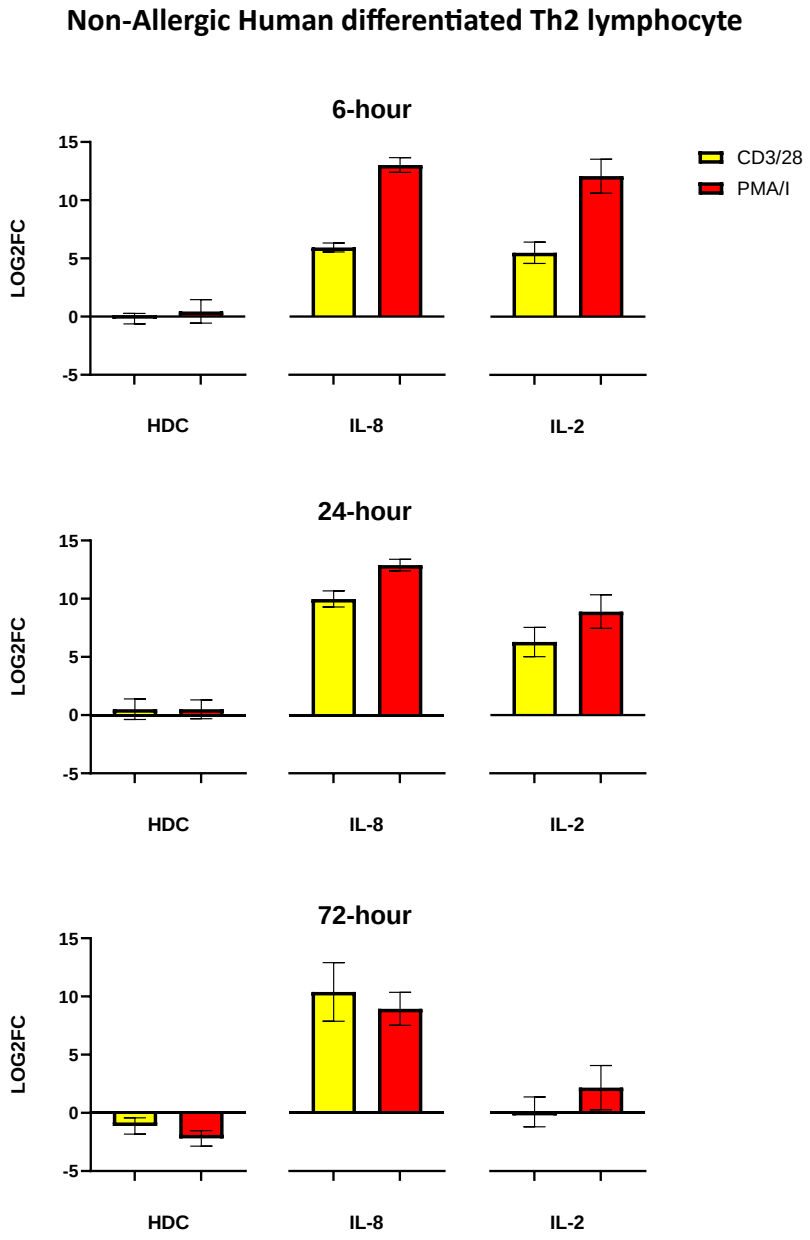


Figure 9. HDC expression was not upregulated in activated non-allergic human differentiated Th2 lymphocytes: Human CD4+ T cells from non-allergic individuals (n=5) were negatively isolated and differentiated into Th2 cells using IL-2. IL-4, IL-6, and anti-IFNγ in culture for 13 days. Th2 cells were stimulated with anti-CD3/CD28 beads (yellow) or PMA/Ionomycin (red) for 6 hours, 24 hours, and 72 hours. RNA was extracted and qPCR performed with primers targeting HDC, B2M, IL-8, and IL-2. Data were normalized to B2M using the deltadeltaCt method. IL-2 and IL-8 expressions were used as positive control. Data reported were Log2 transformed and represent the Log2 Fold Changes as compared to B2M expression. Error bars represent SEM.

Table 4: Log2 fold changes of HDC as normalized to housekeeping gene B2M in differentiated non-allergic human Th2 lymphocytes stimulated with anti-CD3/CD28 beads or PMA/Ionomycin. Data accompanies **Figure 9.**

	HDC LOG_2FC	
	Human Th2 lymphocytes (Non-allergic)	
	aCD3/28	PMA/I
6-hour stimulation	-0.17 +/- 0.46	0.45 +/- 1
24-hour stimulation	0.51 +/- 0.88	0.50 +/- 0.82
72-hour stimulation	-1.1 +/- 0.7	-2.2 +/- 0.66

	IL-8 LOG_2FC	
	Human Th2 lymphocytes (Non-allergic)	
	aCD3/28	PMA/I
6-hour stimulation	5.9 +/- 0.79	13 +/- 0.38
24-hour stimulation	10 +/- 0.7	13 +/- 0.5
72-hour stimulation	10 +/- 2.5	8.9 +/- 1.4

	IL-2 LOG_2FC	
	Human Th2 lymphocytes (Non-allergic)	
	aCD3/28	PMA/I
6-hour stimulation	5.5 +/- 0.92	12 +/- 1.5
24-hour stimulation	6.3 +/- 1.3	8.9 +/- 1.4
72-hour stimulation	0.093 +/- 1.3	2.2 +/- 1.9

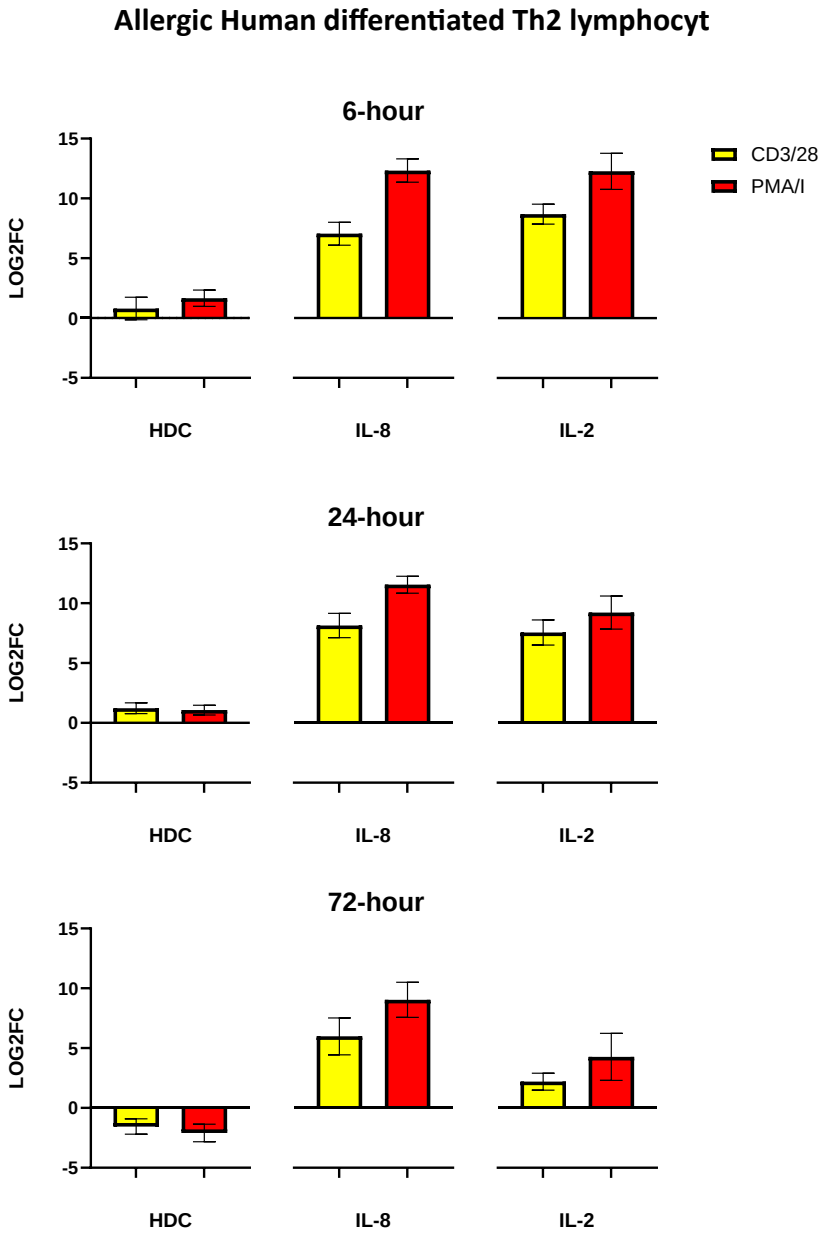


Figure 10. HDC expression was not upregulated in activated allergic human differentiated Th2 lymphocytes: Human CD4+ T cells from allergic individuals (n=6) were negatively isolated and differentiated into Th2 cells using IL-2. IL-4, IL-6, and anti-IFNγ in culture for 13 days. Th2 cells were stimulated with anti-CD3/CD28 beads (yellow) or PMA/Ionomycin (red) for 6 hours, 24 hours, and 72 hours. RNA was extracted and qPCR performed with primers targeting HDC, B2M, IL-8, and IL-2. Data were normalized to B2M using the deltadeltaCt method. IL-2 and IL-8 expressions were used as positive control. Data reported were Log2 transformed and represent the Log2 Fold Changes as compared to B2M expression. Error bars represent SEM.

Table 5: Log2 fold changes of HDC as normalized to housekeeping gene B2M in differentiated allergic human Th2 lymphocytes stimulated with anti-CD3/CD28 beads or PMA/Ionomycin. Data accompanies **Figure 10.**

	HDC LOG_2FC	
	Human Th2 lymphocytes (Allergic)	
	aCD3/28	PMA/I
6-hour stimulation	0.80 +/- 0.94	1.7 +/- 0.69
24-hour stimulation	1.2 +/- 0.45	1.1 +/- 0.41
72-hour stimulation	-1.6 +/- 0.64	-2.1 +/- 0.73

	IL-8 LOG_2FC	
	Human Th2 lymphocytes (Allergic)	
	aCD3/28	PMA/I
6-hour stimulation	7.1 +/- 0.96	12 +/- 0.97
24-hour stimulation	8.1 +/- 1	12 +/- 0.71
72-hour stimulation	6 +/- 1.5	9 +/- 1.5

	IL-2 LOG_2FC	
	Human Th2 lymphocytes (Allergic)	
	aCD3/28	PMA/I
6-hour stimulation	8.7 +/- 0.83	12 +/- 1.5
24-hour stimulation	7.6 +/- 1	9.2 +/- 1.4
72-hour stimulation	2.2 +/- 0.71	4.3 +/- 2

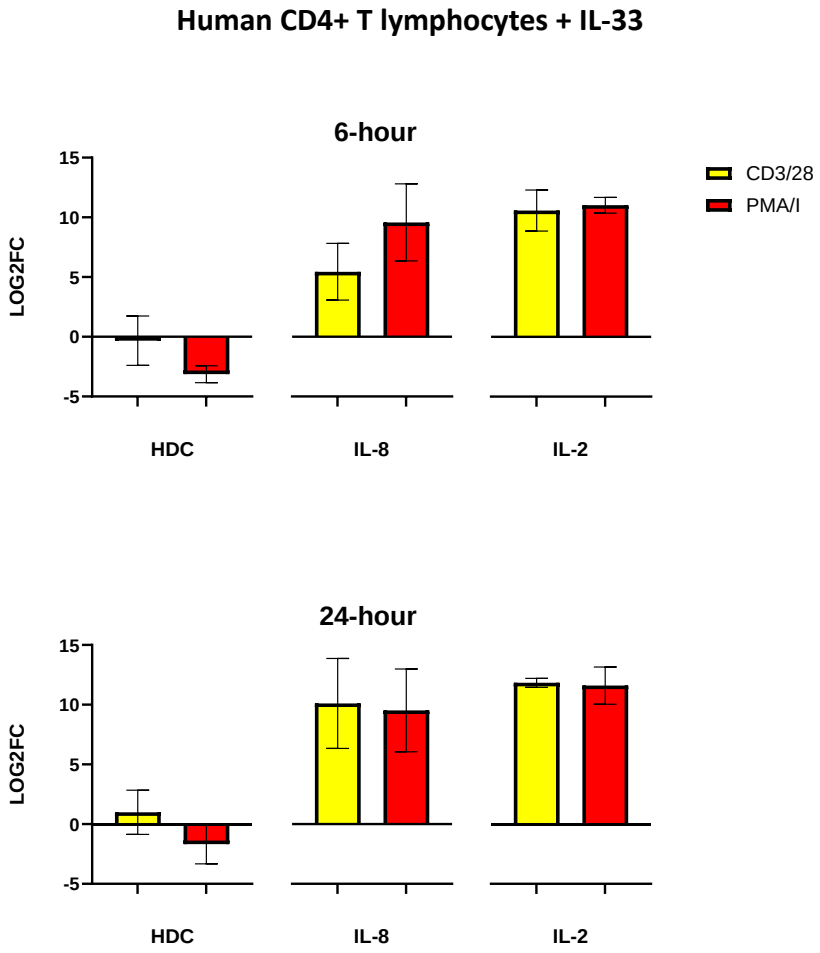


Figure 11. HDC expression was not upregulated in activated human CD4+ lymphocytes with the addition of IL-33: Human CD4+ T cells from donors (n=3) were negatively isolated and stimulated with anti-CD3/CD28 beads (yellow) or PMA/Ionomycin (red) with the addition of IL-33 for 6 hours and 24 hours. RNA was extracted and qPCR performed with primers targeting HDC, B2M, IL-8, and IL-2. Data were normalized to B2M using the deltadeltaCt method. IL-2 and IL-8 expressions were used as positive control. Data reported were Log2 transformed and represent the Log2 Fold Changes as compared to B2M expression. Error bars represent SEM.

Table 6: Log2 fold changes of HDC as normalized to housekeeping gene B2M in isolated human CD4+ T lymphocytes stimulated with IL-33 and anti-CD3/CD28 beads or PMA/Ionomycin. Data accompanies **Figure 11.**

	HDC LOG_2FC	
	Human CD4+ T lymphocytes (IL-33)	
	aCD3/28	PMA/I
6-hour stimulation	-0.32 +/- 2.1	-3.1 +/- 0.7
24-hour stimulation	0.99 +/- 1.8	-1.7 +/- 1.7

	IL-8 LOG_2FC	
	Human CD4+ T lymphocytes (IL-33)	
	aCD3/28	PMA/I
6-hour stimulation	5.4 +/- 2.4	9.6 +/- 3.2
24-hour stimulation	10 +/- 3.8	9.5 +/- 3.5

	IL-2 LOG_2FC	
	Human CD4+ T lymphocytes (IL-33)	
	aCD3/28	PMA/I
6-hour stimulation	11 +/- 1.7	11 +/- 0.66
24-hour stimulation	12 +/- 0.37	12 +/- 1.6

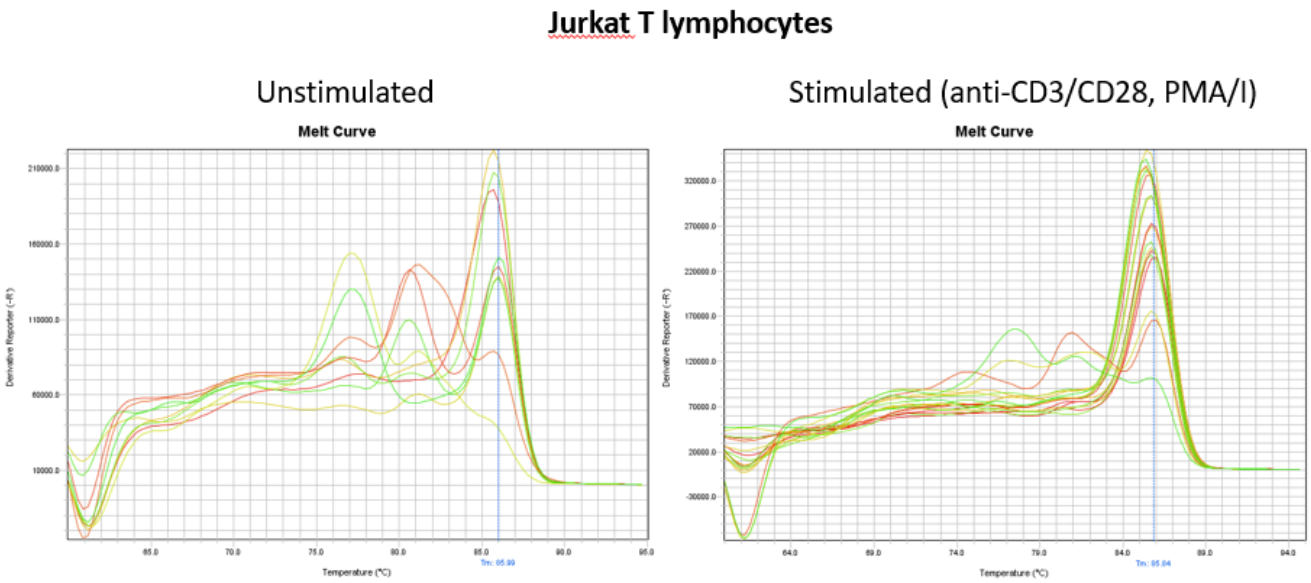


Figure 12. Melt Curve analysis for HDC amplification specificity in Jurkat T lymphocytes: Example **of** Jurkat T lymphocytes qPCR analysis for unstimulated (cRPMI control) and stimulated (anti-CD3/CD28, PMA/I) samples. Synthesized double stranded products were continuously heated up at an increment of 0.5C until 95C while the fluorescent level is measured. Peaks represent the melting temperature of a target; longer DNA products require a higher melting temperature.

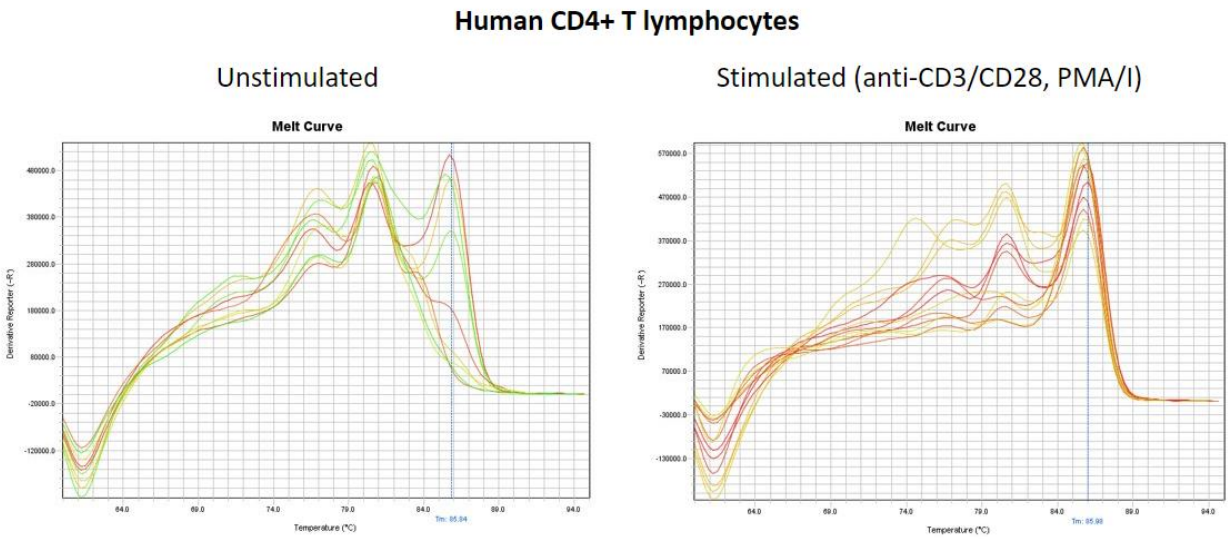


Figure 13. Melt Curve analysis for HDC amplification specificity in human CD4+ T lymphocytes: Example of human CD4+ T lymphocytes qPCR analysis for unstimulated (cRPMI control) and stimulated (anti-CD3/CD28, PMA/I) samples. Synthesized double stranded products were continuously heated up at an increment of 0.5C until 95C while the fluorescent level is measured. Peaks represent the melting temperature of a target; longer DNA products require a higher melting temperature.

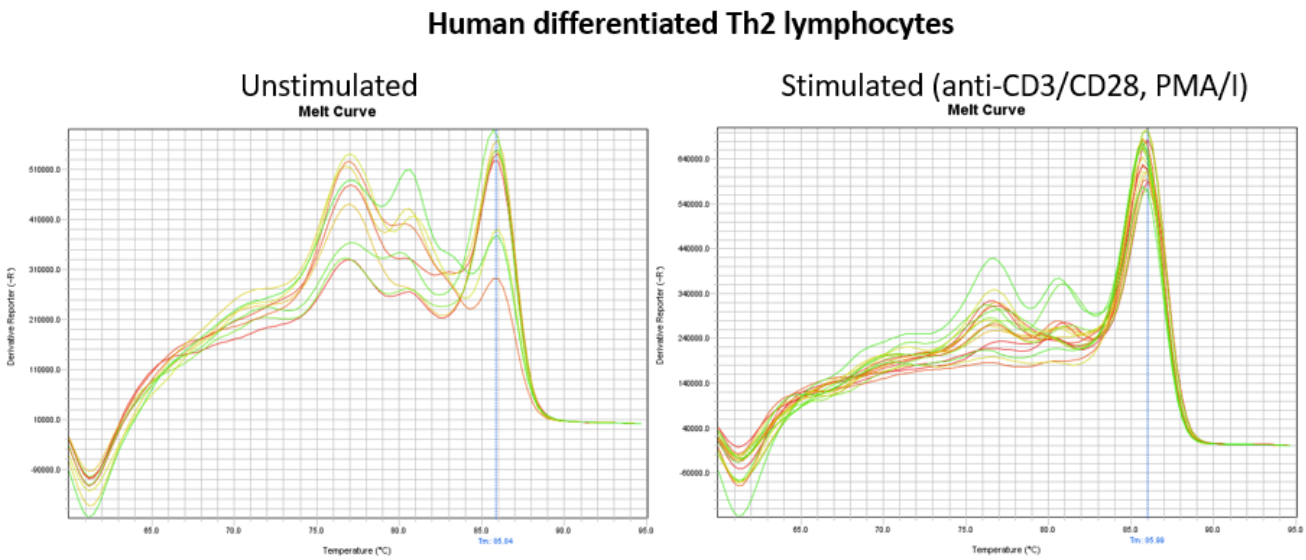


Figure 14. Melt Curve analysis for HDC amplification specificity in human differentiated Th2 lymphocytes: Example of human The lymphocytes qPCR analysis for unstimulated (cRPMI control) and stimulated (anti-CD3/CD28, PMA/I) samples. Synthesized double stranded products were continuously heated up at an increment of 0.5C until 95C while the fluorescent level is measured. Peaks represent the melting temperature of a target; longer DNA products require a higher melting temperature.

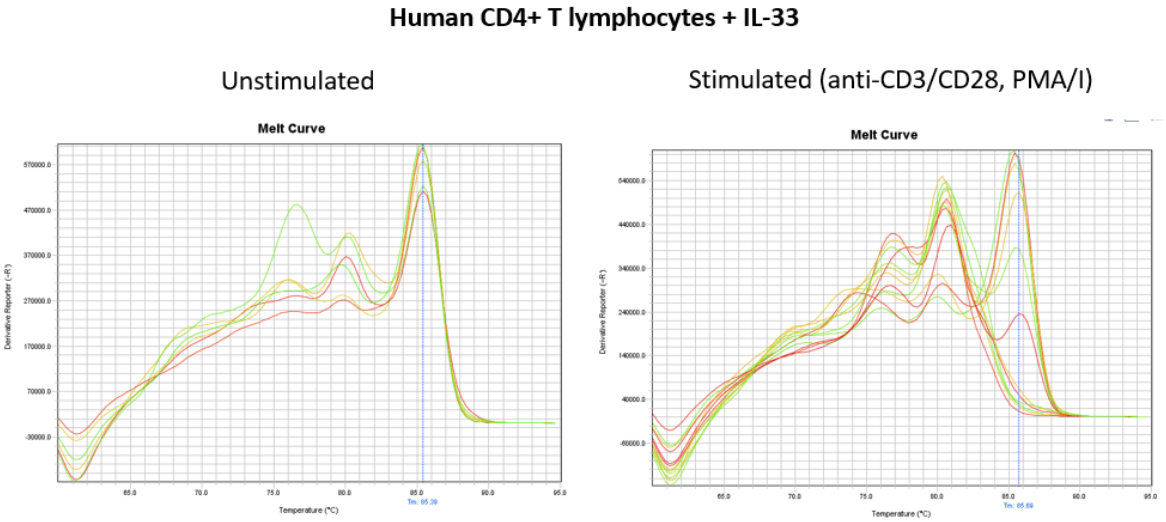


Figure 15. Melt Curve analysis for HDC amplification specificity in human CD4+ T lymphocytes stimulated with the addition of IL-33: Example of human The lymphocytes qPCR analysis for all stimulation conditions. Synthesized double stranded products were continuously heated up at an increment of 0.5C until 95C while the fluorescent level is measured. Peaks represent the melting temperature of a target; longer DNA products require a higher melting temperature.

Supplementary Data

Supplementary Table 1 Summary of the average Cycle Threshold (CT) values for different cell types and stimulation conditions: Table 1A: CT values for anti-CD3/CD28 (aCD3/CD28) or PMA/Ionomycin (PMA/I) stimulated Jurkat T lymphocytes or non-stimulated (media) Jurkat T lymphocytes. **Table 1B**: CT values for anti-CD3/CD28 (aCD3/CD28) or PMA/Ionomycin (PMA/I) stimulated non-allergic human CD4+ T lymphocytes or non-stimulated (media) non-allergic human CD4+ T lymphocytes. **Table 1C**: CT values for anti-CD3/CD28 (aCD3/CD28) or PMA/Ionomycin (PMA/I) stimulated allergic human CD4+ T lymphocytes or non-stimulated (media) allergic human CD4+ T lymphocytes. **Table 1D**: CT values for anti-CD3/CD28 (aCD3/CD28) or PMA/Ionomycin (PMA/I) stimulated non-allergic human differentiated Th2 lymphocytes or non-stimulated (media) non-allergic human differentiated Th2 lymphocytes. **Table 1E**: CT values for anti-CD3/CD28 (aCD3/CD28) or PMA/Ionomycin (PMA/I) stimulated allergic human differentiated Th2 lymphocytes or non-stimulated (media) allergic human differentiated Th2 lymphocytes. **Table 1F**: CT values for anti-CD3/CD28 (aCD3/CD28) with IL-33 or PMA/Ionomycin (PMA/I) with IL-33 stimulated human CD4+ T lymphocytes or non-stimulated (media) CD4+ T lymphocytes.

	Average Cycle Threshold (CT) values								
	Stimulation Conditions								
	aCD3 /CD28 6h	**PMA/I 6h**	**Media 6h**	**aCD3 /CD28 24h**	**PMA/I 24h**	**Media 24h**	**aCD3 /CD28 72h**	**PMA/I 72h**	**Media 72h**

A

Gene	**Jurkat T lymphocytes (n=7)**								
HDC	30.04 +/- 1.48	29.13 +/- 1.13	33.13 +/- 1.10	25.57 +/- 2.57	25.14 +/- 2.73	33.81 +/- 1.78	27.33 +/- 3.65	23.67 +/- 2.29	34.19 +/- 1.96
B2M	19.62 +/- 1.38	19.07 +/- 2.49	19.48 +/- 2.52	19.05 +/- 3.61	18.77 +/- 3.28	19.62 +/- 3.06	19.40 +/- 2.88	17.57 +/- 3.26	19.81 +/- 3.27
IL-8	27.00 +/- 1.3	22.47 +/- 1.65	33.19 +/- 2.77	27.98 +/- 0.76	25.67 +/- 2.4	34.77 +/- 2.27	33.57 +/- 1.62	27.86 +/- 2.12	33.29 +/- 2.8
IL-2	26.81 +/- 2.67	23.05 +/- 2.3	33.22 +/- 1.32	27.24 +/- 4.11	21.52 +/- 3.76	32.93 +/- 0.56	30.79 +/- 1.53	25.81 +/- 3.29	32.66 +/- 1.21

	Average Cycle Threshold (CT) values								
	Stimulation Conditions								
	aCD3 /CD28 6h	**PMA/I 6h**	**Media 6h**	**aCD3 /CD28 24h**	**PMA/I 24h**	**Media 24h**	**aCD3 /CD28 72h**	**PMA/I 72h**	**Media 72h**

B

Gene	**Non-Allergic Human CD4+ T lymphocytes (n=6)**								
HDC	32.38 +/- 0.53	32.50 +/- 0.5	32.48 +/- 0.4	32.00 +/- 0.89	31.65 +/- 0.53	32.81 +/- 0.78	33.00 +/- 0.00	32.01 +/- 0.66	32.94 +/- 0.91
B2M	16.05 +/- 2.08	16.78 +/- 3.02	17.18 +/- 2.73	15.37 +/- 2.25	15.07 +/- 1.58	17.43 +/- 2.43	15.20 +/- 2.22	15.00 +/- 1.67	17.37 +/- 1.77
IL-8	24.48 +/- 1.4	22.08 +/- 2.03	28.44 +/- 2.99	25.77 +/- 2.27	23.48 +/- 1.33	31.29 +/- 2.76	29.43 +/- 1.98	27.60 +/- 1.67	30.45 +/- 2.94
IL-2	16.76 +/- 1.02	16.44 +/- 2.17	29.08 +/- 3.51	16.19 +/- 1.11	15.73 +/- 0.49	30.28 +/- 0.31	21.72 +/- 1.91	19.97 +/- 3.67	29.91 +/- 1.04

	Average Cycle Threshold (CT) values								
	Stimulation Conditions								
	aCD3 /CD28 6h	**PMA/I 6h**	**Media 6h**	**aCD3 /CD28 24h**	**PMA/I 24h**	**Media 24h**	**aCD3 /CD28 72h**	**PMA/I 72h**	**Media 72h**

C

Gene	**Allergic Human CD4+ T lymphocytes (n=6)**								
HDC	31.02 +/- 2.27	31.96 +/- 2.09	30.76 +/- 2.65	31.76 +/- 2.38	31.15 +/- 1.9	31.54 +/- 2.54	33.11 +/- 2.01	31.65 +/- 1.49	31.45 +/- 2.36
B2M	17.12 +/- 0.69	17.21 +/- 1.12	17.33 +/- 0.78	15.78 +/- 0.75	15.75 +/- 0.46	17.68 +/- 0.56	16.49 +/- 0.52	16.23 +/- 0.96	18.96 +/- 2.13
IL-8	23.94 +/- 2.23	20.19 +/- 1.75	28.00 +/- 1.90	24.84 +/- 3.05	23.00 +/- 1.73	31.00 +/- 2.92	28.44 +/- 1.46	27.06 +/- 3.33	29.99 +/- 2.96
IL-2	17.56 +/- 1.68	15.58 +/ 1.69-	29.51 +/- 0.58	15.94 +/- 1.51	15.83 +/- 1.36	29.34 +/- 0.79	21.17 +/- 1.82	21.17 +/- 5.29	28.97 +/- 0.95

	Average Cycle Threshold (CT) values								
	Stimulation Conditions								
	aCD3 /CD28 6h	**PMA/I 6h**	**Media 6h**	**aCD3 /CD28 24h**	**PMA/I 24h**	**Media 24h**	**aCD3 /CD28 72h**	**PMA/I 72h**	**Media 72h**

D

Gene	**Non - Allergic Human Differentiated Th2 lymphocytes (n=5)**								
HDC	34.40 +/- 0.89	34.40 +/- 0.86	34.83 +/- 0.45	34.40 +/- 0.55	33.87 +/- 0.9	34.73 +/- 0.43	34.19 +/- 0.76	35.13 +/- 0.36	34.27 +/- 0.72
B2M	19.13 +/- 2.1	19.75 +/- 1.94	19.73 +/- 2.64	18.80 +/- 1.92	18.25 +/- 1.5	18.66 +/- 2.01	18.13 +/- 1.51	18 +/- 1.58	19.33 +/- 1.22
IL-8	24.99 +/- 1.3	18.53 +/- 2.17	31.53 +/- 0.77	26.13 +/- 1.04	22.67 +/- 1.02	35.93 +/- 2.13	24.83 +/- 3.25	26.15 +/- 0.74	36.41 +/- 2.94
IL-2	26.07 +/- 2.2	20.11 +/- 3.26	32.16 +/- 0.59	25.56 +/- 2.62	22.40 +/- 2.51	31.67 +/- 0.41	29.37 +/- 2.51	27.16 +/- 4.3	30.66 +/- 1.71

	Average Cycle Threshold (CT) values								
	Stimulation Conditions								
	aCD3 /CD28 6h	**PMA/I 6h**	**Media 6h**	**aCD3 /CD28 24h**	**PMA/I 24h**	**Media 24h**	**aCD3 /CD28 72h**	**PMA/I 72h**	**Media 72h**

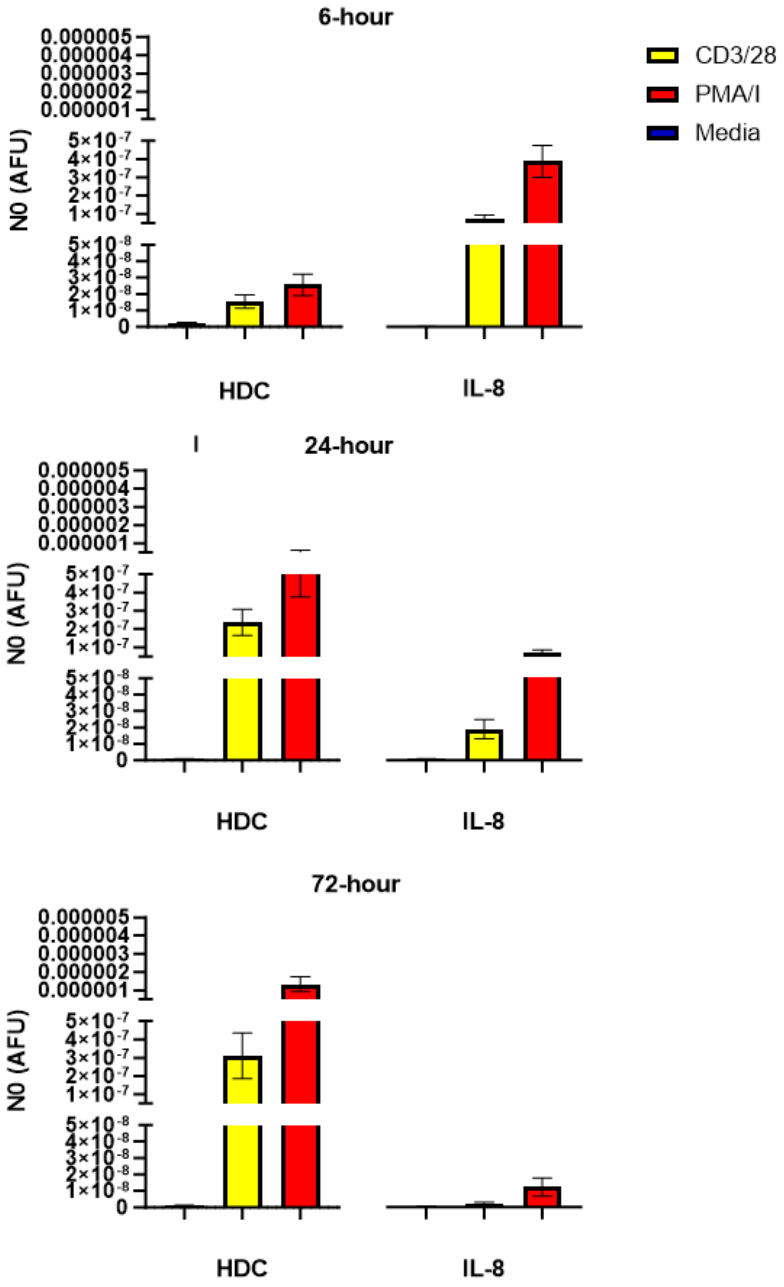
E

Gene	**Allergic Human Differentiated Th2 lymphocytes (n=6)**								
HDC	33.63 +/- 1.58	33.56 +/- 1.38	34.25 +/- 1.19	34.03 +/- 1.48	33.81 +/- 1.1	34.06 +/- 1.02	34.07 +/- 1.61	34.14 +/- 1.02	33.78 +/- 1.44
B2M	18.67 +/- 1.52	19.44 +/- 2.35	18.48 +/- 3.34	20.27 +/- 1.11	19.89 +/- 2.64	19.04 +/- 1.59	18.91 +/- 2.84	18.44 +/- 2.71	20.17 +/- 1.83
IL-8	22.89 +/- 2.13	18.39 +/- 1.29	29.76 +/- 2.29	27.24 +/- 2.99	23.45 +/- 0.92	34.18 +/- 1.07	27.93 +/- 3.39	24.41 +/- 2.96	35.17 +/- 1.31
IL-2	24.10 +/- 1.78	21.29 +/- 3.28	32.61 +/- 0.94	25.92 +/- 3.84	23.86 +/- 3.6	32.27 +/- 0.71	28.17 +/- 1.82	25.62 +/- 4.05	31.62 +/- 0.47

	Average Cycle Threshold (CT) values								
	Stimulation Conditions								
	aCD3 /CD28 6h	**PMA/I 6h**	**Media 6h**	**aCD3 /CD28 24h**	**PMA/I 24h**	**Media 24h**			

F

Gene	**Human CD4+ T lymphocytes with IL-33 (n=3)**								
HDC	31.84 +/- 0.73	33.90 +/- 0.61	31.97 +/- 2.48	31.33 +/- 0.88	33.42 +/- 1.37	32.11 +/- 2.5			
B2M	18.28 +/- 0.35	17.52 +/- 2.09	18.72 +/- 2.18	17.54 +/- 1.02	16.98 +/- 2.39	17.33 +/- 0.58			
IL-8	21.67 +/- 1.53	16.78 +/- 2.41	27.56 +/- 2.22	18.31 +/- 4.12	18.33 +/- 5.86	28.21 +/- 5.17			
IL-2	20.42 +/- 0.7	19.22 +/- 1.13	31.44 +/- 1.02	20.00 +/- 1.6	19.67 +/- 1.77	31.62 +/- 0.62			

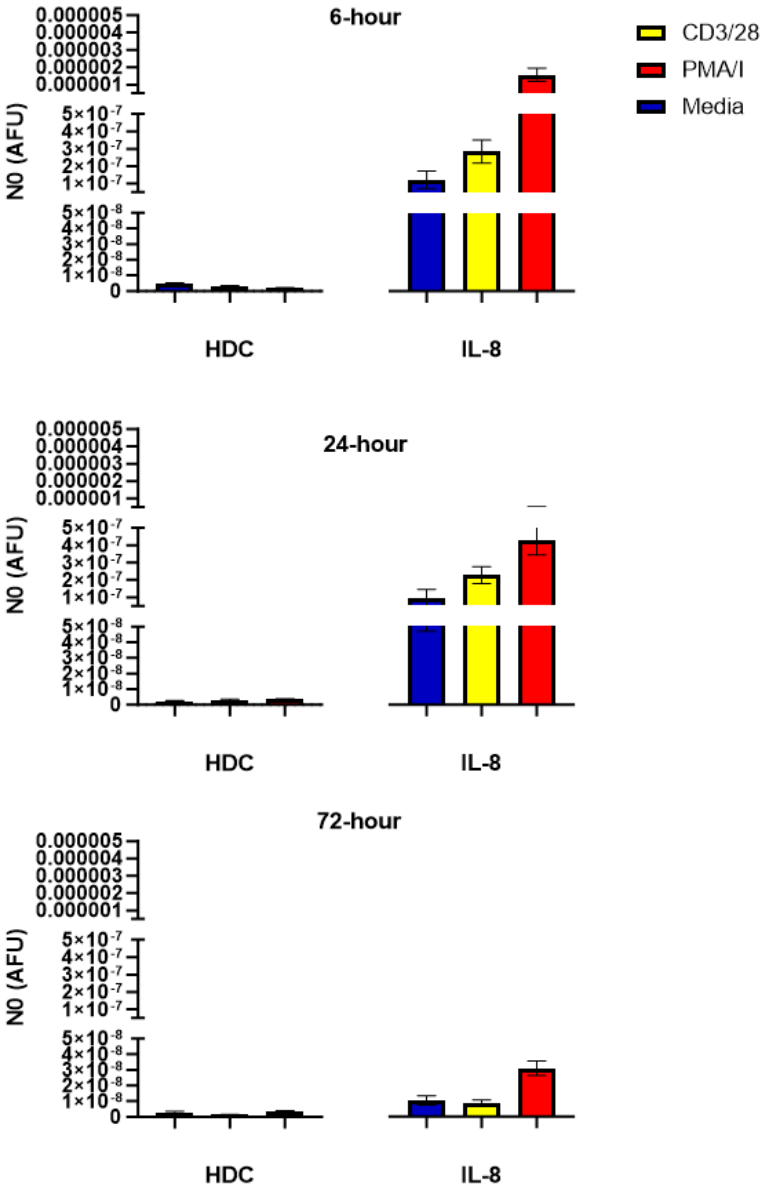


Supplementary Figure 1. Starting abundance (N0) of HDC and IL-8 cDNA transcript in Jurkat T lymphocytes as determined by LinRegPCR program: LinRegPCR program was used to analyze the starting fluorescent values (N0 in Arbitrary Fluorescence Unit) of HDC and IL-8 expression of Jurkat T lymphocytes activated with anti-CD3/anti-CD28 or PMA/I or media control. Error bars represent SEM. (n=7)

Supplementary Table 2: Starting abundance (N0) of HDC and IL-8 cDNA transcript in Jurkat T lymphocytes as determined by LinRegPCR program. LinRegPCR program was used to determine the starting abundance of gene of interests (HDC, IL-8) in qPCR samples. The starting abundance (N0) was measured in arbitrary fluorescent unit (AFU) and is representative of the theoretical minimum fluorescent value of a sample pre-amplification. Table accompanies **Supplementary Figure 1** (n=7)

	HDC N0		
	Jurkat T lymphocytes		
	aCD3/28	PMA/I	Media
6-hour stimulation	1.539e-008 +/- 4.028e-009	2.568e-008 +/- 6.474e-009	2.259e-009 +/- 5.438e-010
24-hour stimulation	2.362e-007 +/- 7.192e-008	5.037e-007 +/- 1.286e-007	9.061e-010 +/- 2.076e-010
72-hour stimulation	3.105e-007 +/- 1.251e-007	1.330e-006 +/- 4.054e-007	1.037e-009 +/- 2.487e-010

	IL-8 N0		
	Jurkat T lymphocytes		
	aCD3/28	PMA/I	Media
6-hour stimulation	7.572e-008 +/- 1.899e-008	3.879e-007 +/- 8.678e-008	3.144e-010 +/- 1.070e-010
24-hour stimulation	1.899e-008 +/- 5.776e-009	6.959e-008 +/- 1.684e-008	6.984e-010 +/- 2.327e-010
72-hour stimulation	2.063e-009 +/- 8.782e-010	1.227e-008 +/- 5.575e-009	4.280e-010 +/- 1.391e-010

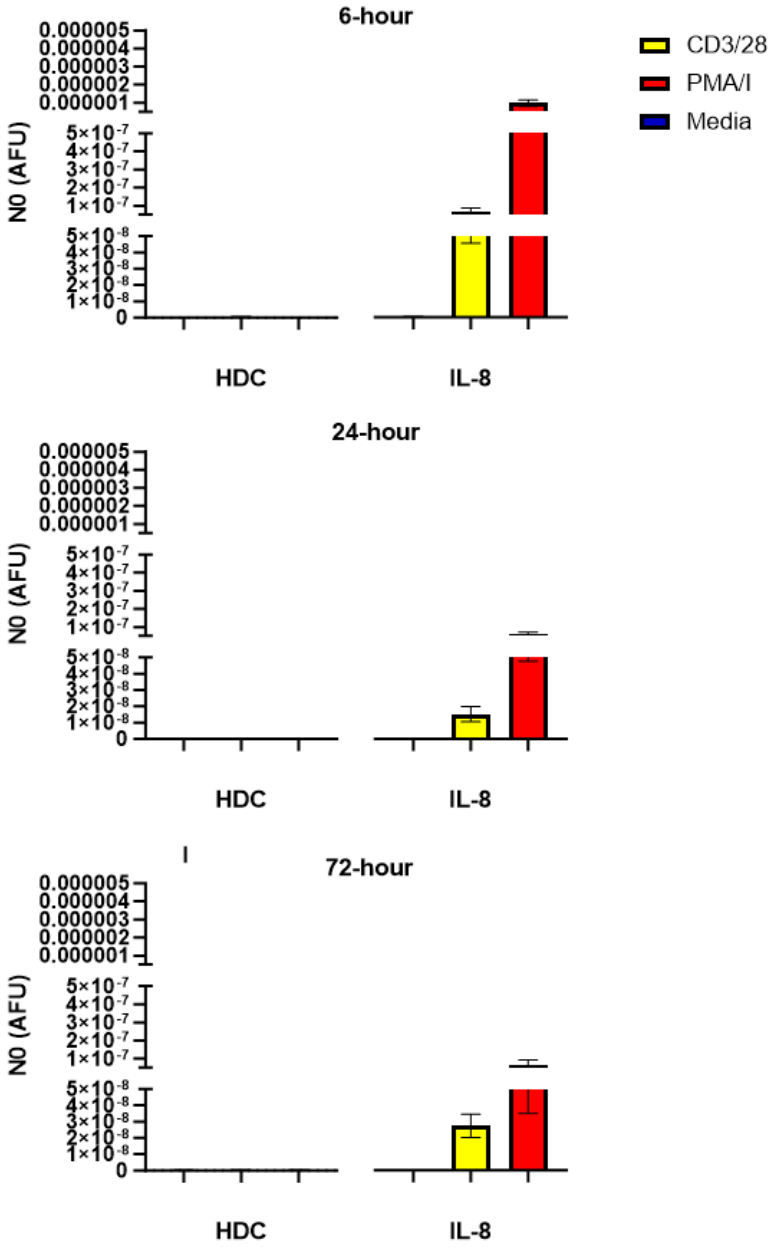


Supplementary Figure 2. Starting abundance (N0) of HDC and IL-8 cDNA transcript in Human CD4+ T lymphocytes as determined by LinRegPCR program: LinRegPCR program was used to analyze the starting fluorescent values (N0 in Arbitrary Fluorescence Unit) of HDC and IL-8 expression of isolated human CD4+ T lymphocytes activated with anti-CD3/anti-CD28 or PMA/I or media control. Error bars represent SEM. (n=12)

Supplementary Table 3. Starting abundance (N0) of HDC and IL-8 cDNA transcript in Human CD4+ T lymphocytes as determined by LinRegPCR program: LinRegPCR program was used to determine the starting abundance of gene of interests (HDC, IL-8) in qPCR samples. The starting abundance (N0) was measured in arbitrary fluorescent unit (AFU) and is representative of the theoretical minimum fluorescent value of a sample pre-amplification. Table accompanies **Supplementary Figure 2**. (n=12)

	HDC N0		
	Human CD4+ T Lymphocytes		
	aCD3/28	PMA/I	Media
6-hour stimulation	3.073e-009 +/- 4.680e-010	1.931e-009 +/- 2.308e-010	4.401e-009 +/- 9.732e-010
24-hour stimulation	3.490e-009 +/- 4.159e-010	3.100e-009 +/- 3.100e-009	2.374e-009 +/- 3.576e-010
72-hour stimulation	1.393e-009 +/- 1.538e-010	3.412e-009 +/- 4.746e-010	2.924e-009 +/- 7.455e-010

	IL-8 N0		
	Human CD4+ T Lymphocytes		
	aCD3/28	PMA/I	Media
6-hour stimulation	2.838e-007 +/- 6.582e-008	1.571e-006 +/- 3.741e-007	1.205e-007 +/- 5.031e-008
24-hour stimulation	2.278e-007 +/- 4.847e-008	4.298e-007 +/- 8.721e-008	9.666e-008 +/- 4.947e-008
72-hour stimulation	8.951e-009 +/- 1.640e-009	3.106e-008 +/- 4.724e-009	1.048e-008 +/- 2.995e-009

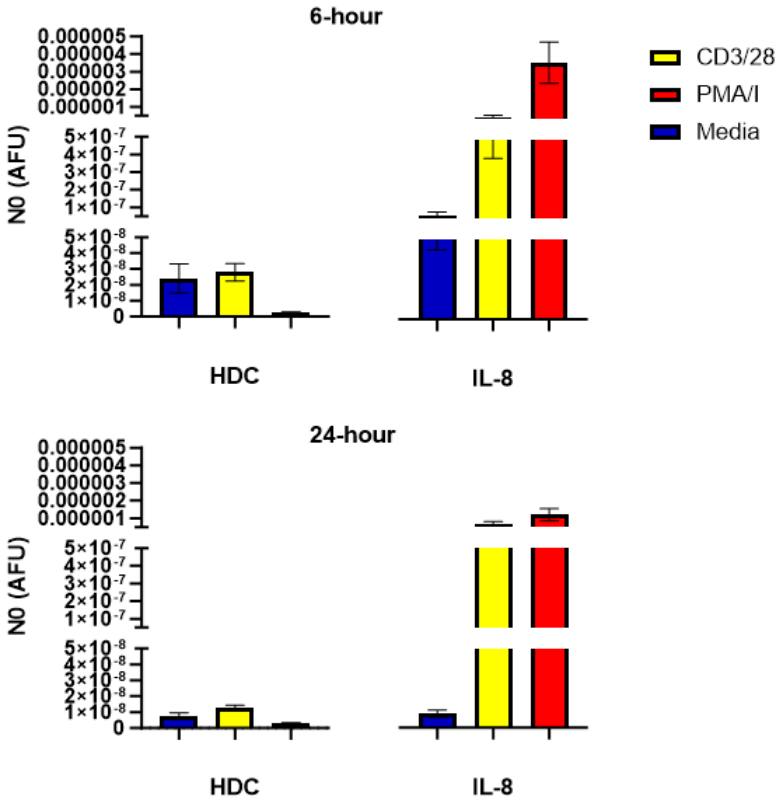


Supplementary Figure 3. Starting abundance (N0) of HDC and IL-8 cDNA transcript in differentiated Human Th2 lymphocytes as determined by LinRegPCR program: LinRegqPCR program was used to analyze the starting fluorescent values (N0 in Arbitrary Fluorescence Unit) of HDC and IL-8 expression of human differentiated Th2 lymphocytes activated with anti-CD3/anti-CD28 or PMA/I or media control. Error bars represent SEM. (n=11)

Supplementary Table 4. Starting abundance (N0) of HDC and IL-8 cDNA transcript in differentiated Human Th2 lymphocytes as determined by LinRegPCR program: LinRegPCR program was used to determine the starting abundance of gene of interests (HDC, IL-8) in qPCR samples. The starting abundance (N0) was measured in arbitrary fluorescent unit (AFU) and is representative of the theoretical minimum fluorescent value of a sample pre-amplification. Table accompanies **Supplementary Figure 3**. (n=11)

	HDC N0		
	Human differentiated Th2 Lymphocytes		
	aCD3/28	PMA/I	Media
6-hour stimulation	7.099e-010 +/- 1.463e-010	3.793e-010 +/- 4.427e-011	3.237e-010 +/-4.068e-011
24-hour stimulation	4.572e-010 +/- 7.254e-011	4.220e-010 +/- 5.415e-011	3.147e-010 +/- 2.630e-011
72-hour stimulation	4.322e-010 +/- 8.486e-011	3.259e-010 +/- 3.464e-011	4.089e-010 +/- 4.237e-011

	IL-8 N0		
	Human differentiated Th2 Lymphocytes		
	aCD3/28	PMA/I	Media
6-hour stimulation	6.599e-008 +/- 2.039e-008	9.770e-007 +/- 1.758e-007	5.930e-010 +/- 9.941e-011
24-hour stimulation	1.524e-008 +/- 4.651e-009	5.987e-008 +/- 1.221e-008	2.597e-010 +/- 8.913e-011
72-hour stimulation	2.745e-008 +/- 7.212e-009	6.436e-008 +/- 2.924e-008	1.067e-010 +/- 2.253e-011



Supplementary Figure 4. Starting abundance (N0) of HDC and IL-8 cDNA transcript in Human CD4+ T lymphocytes stimulated with the addition of IL-33 as determined by LinRegPCR program: LinRegqPCR program was used to analyze the starting fluorescent values (N0 in Arbitrary Fluorescence Unit) of HDC and IL-8 expression of human differentiated Th2 lymphocytes activated with anti-CD3/anti-CD28 with the addition of IL-33 or PMA/I with the addition of IL-33 or media control. Error bars represent SEM. (n=3)

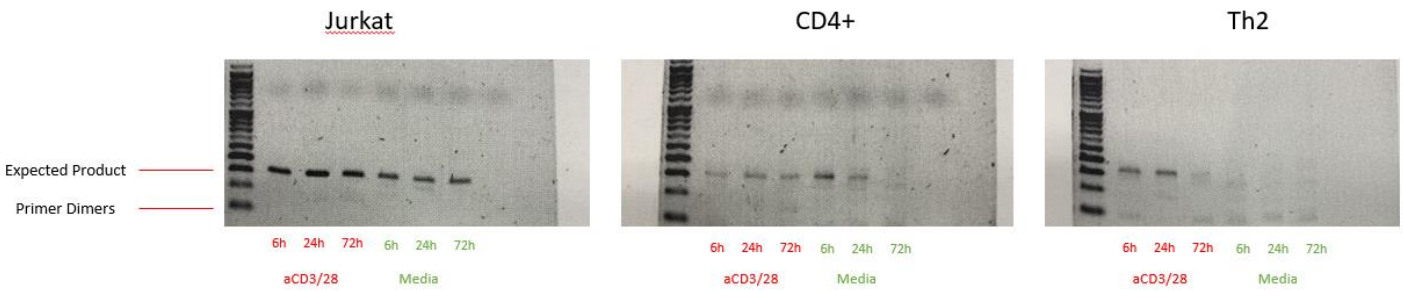
Supplementary Table 5 Starting abundance (N0) of HDC and IL-8 cDNA transcript in Human CD4+ T lymphocytes stimulated with the addition of IL-33 as determined by LinRegPCR program: LinRegPCR program was used to determine the starting abundance of gene of interests (HDC, IL-8) in qPCR samples. The starting abundance (N0) was measured in arbitrary fluorescent unit (AFU) and is representative of the theoretical minimum fluorescent value of a sample pre-amplification. Table accompanies **Supplementary Figure 4**. (n=3)

	HDC N0		
	Human CD4+ T lymphocytes with IL-33		
	aCD3/28	PMA/I	Media
6-hour stimulation	2.794e-008 +/- 5.463e-009	2.514e-009 +/- 4.756e-010	2.410e-008 +/- 9.153e-009
24-hour stimulation	1.264e-008 +/- 1.702e-009	2.896e-009 +/- 5.087e-010	7.898e-009 +/- 1.741e-009

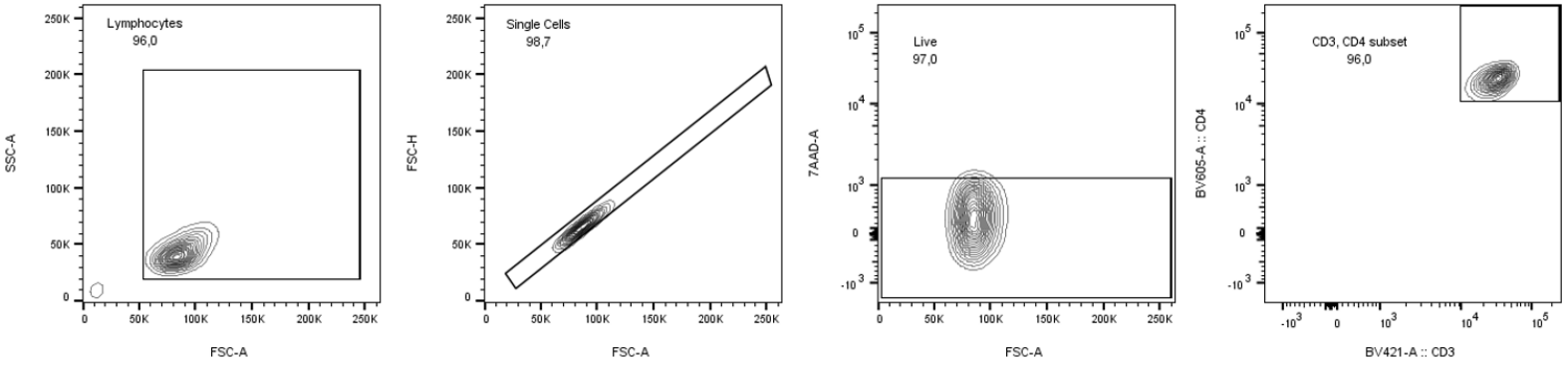
	IL-8 N0		
	Human CD4+ T lymphocytes with IL-33		
	aCD3/28	PMA/I	Media
6-hour stimulation	5.383e-007 +/- 1.468e-007	3.665e-006 +/- 1.168e-006	6.615e-008 +/- 2.253e-008
24-hour stimulation	6.918e-007 +/- 1.222e-007	1.196e-006 +/- 3.509e-007	8.865e-009 +/- 2.292e-009

Supplementary Table 6: Co-expression analysis of HDC+ cells: Dataset 3: (Single-cell transcriptomic analysis of allergen-specific T cells in allergy and asthma, https://doi.org/10.1126/sciimmunol.aba6087). Analysis of HDC+ cells found these cells to co-express different genes with high confidence.

Gene	Fold Change	P-Value
HDC	**2**	**0**
GZMB	4.35	9.34e-06
IL1RL1	2.67	0.000198
C10orf54	1.94	0.013617
GNLY	1.76	0.005184
OPA1	1.74	1.27e-07
HDAC7	1.64	2.88e-06
NQO2	1.6	1.41e-11
VPS25	1.59	6.63e-08
GADD45G	1.57	1.7e-07
SLC39A6	1.53	0.008066

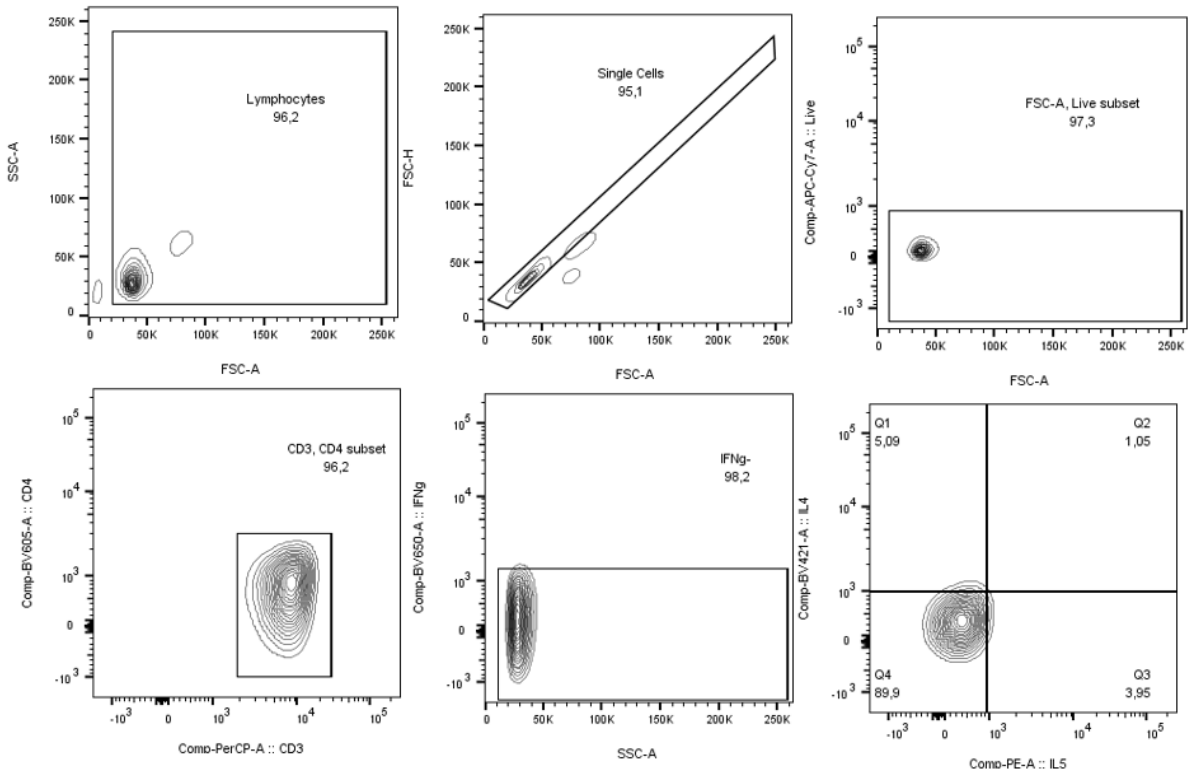


Supplementary Figure 5. Example gel electrophoresis of amplified HDC products from Jurkat T lymphocytes, human CD4+ T lymphocytes, and differentiated human Th2 lymphocytes: Verification of HDC transcript post qPCR. HDC amplicons were expected to be 157 bp. Nonspecific bands were likely due to primer dimers formation as the length were <50bp.



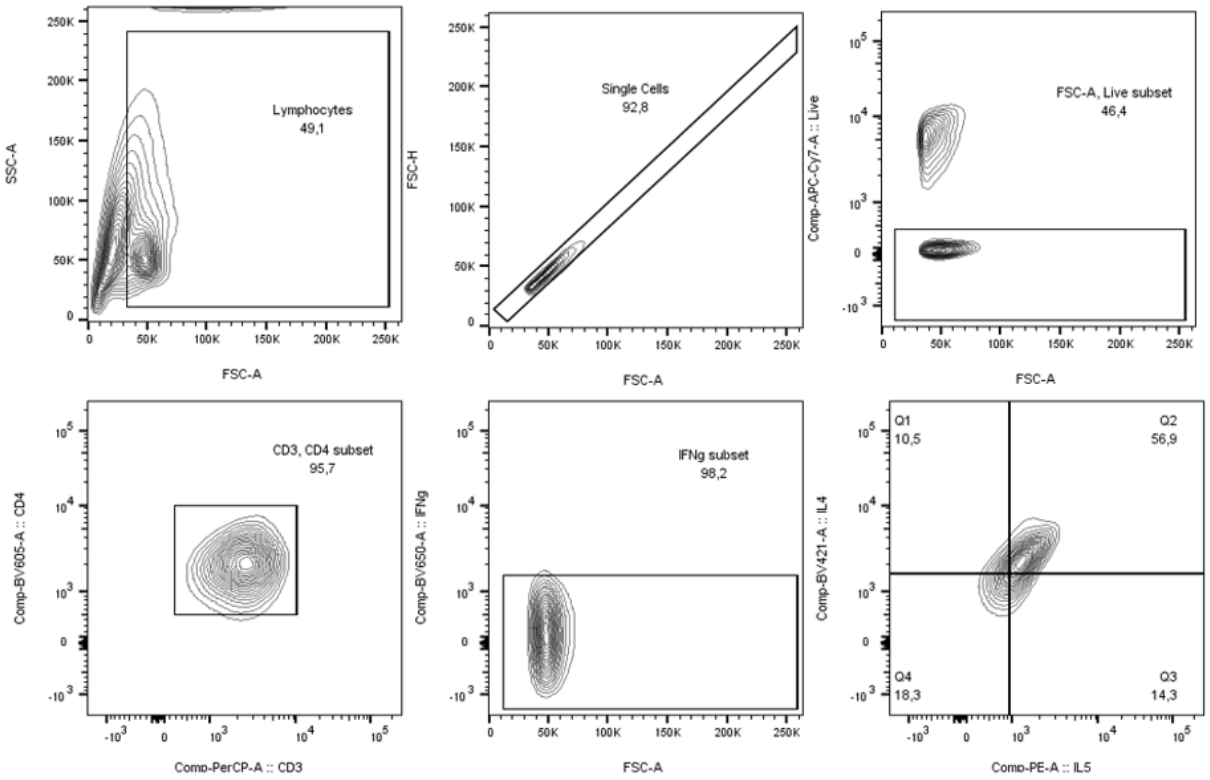
Supplementary Figure 6 Flow cytometry gating and analysis for CD3+/CD4+ T cell purity post isolation and monocyte adherence: Human CD4+ T lymphocytes were negatively isolated using StemCell EasySep™ Human CD4+ Enrichment Kit, followed by a plastic monocyte adherence step. Samples were run on the BD LSRFortessa flow cytometer. Data was analysed using FlowJo v10.8 software. Gating strategy were as follows: FSC-A vs SSC-A (lymphocytes gating) à FSC-A vs FSC-H (Single cell gating) à FSC-A vs 7-AAD (viability gating) à CD3 vs CD4 (CD3+/CD4+ purity gating). The average CD3+/CD4+ purity was 94.02%

A



Supplementary Figure 7A. Flow cytometry gating and analysis for IFNγ- , IL-4+/IL-5+ human differentiated Th2 cells pre Th2 differentiation culture for 13 or 14 days: Human Th2 lymphocytes were differentiated using the CellXVivo™ Th2 differentiation kit. Samples were run on the BD LSRFortessa flow cytometer. Data was analysed using FlowJo v10.8 software. Gating strategy were as follows: FSC-A vs SSC-A (lymphocytes gating) à FSC-A vs FSC-H (Single cell gating) à FSC-A vs APC-Cy7 (viability gating) à CD3 vs CD4 (CD3+/CD4+ purity gating) à FSC-A vs IFNγ (IFNγ- gating) à IL-5 vs IL-4 (IL-4+/IL-5+ gating). IL-4, IL-5 and IFNγ gating were determined by FMO samples. Averaged IFNγ-, IL4+/IL-5+ population was: 8.63%.

B



Supplementary Figure 7B. Flow cytometry gating and analysis for IFNγ- , IL-4+/IL-5+ human differentiated Th2 cells post Th2 differentiation culture for 13 or 14 days: Human Th2 lymphocytes were differentiated using the CellXVivo™ Th2 differentiation kit. Samples were run on the BD LSRFortessa flow cytometer. Data was analysed using FlowJo v10.8 software. Gating strategy were as follows: FSC-A vs SSC-A (lymphocytes gating) à FSC-A vs FSC-H (Single cell gating) à FSC-A vs APC-Cy7 (viability gating) à CD3 vs CD4 (CD3+/CD4+ purity gating) à FSC-A vs IFNγ (IFNγ- gating) à IL-5 vs IL-4 (IL-4+/IL-5+ gating). IL-4, IL-5 and IFNγ gating were determined by FMO samples. Averaged IFNγ-, IL4+/IL-5+ population was: 71.98%.

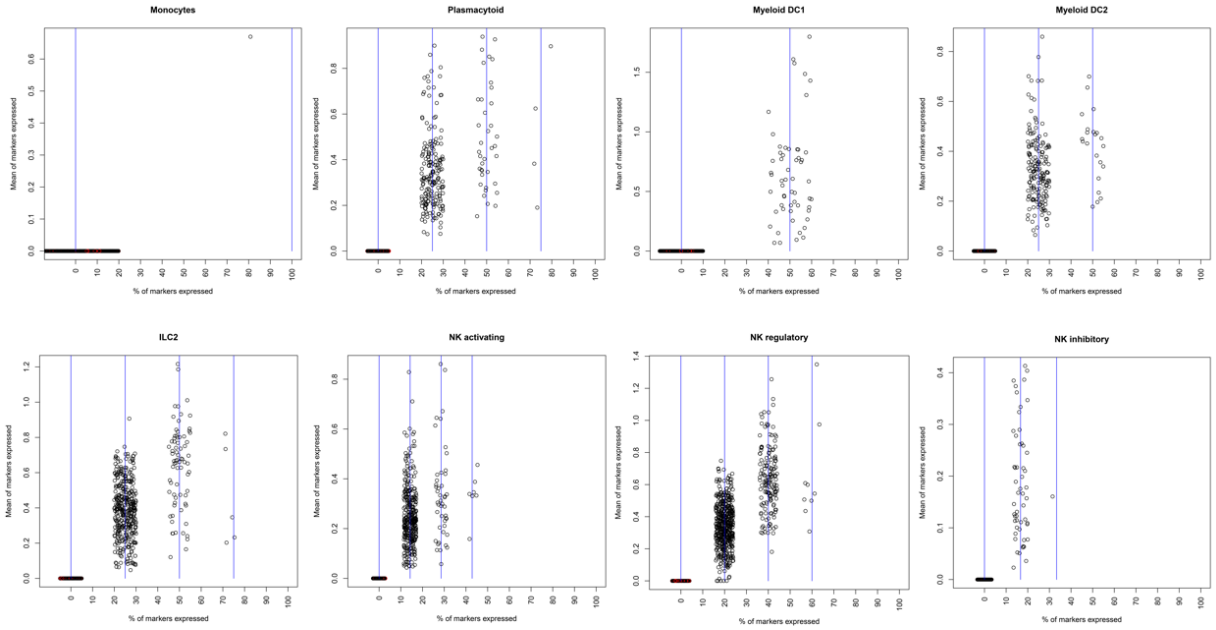
NK cells:
- Activating receptors:
  - CD56 (NCAM1)
  - CD16 (FCGR3A)
  - NKG2D (KLRK1)
  - NKp30 (NCR3)
  - NKp44 (NCR2)
  - NKp46 (NCR1)
  - CD69
- Inhibitory receptors:
  - CD56 (NCAM1)
  - CD16 (FCGR3A)
  - NKG2A (KLRC1)
  - CD158a (KIR2DL1)
  - CD158b – could find only CD158B2 (KIR2DL3)
  - CD158e1 (KIR3DL1)
- Regulatory NK cell markers:
  - CD56 (NCAM1)
  - CD16 (FCGR3A)
  - CD9
  - CD49a (ITGA1)
  - CD73 (NT5E)

Dendritic cells:
- Plasmacytoid DC:
  - CD123 (IL3RA)
  - CD303 (CLEC4C)
  - CD304 (NRP1)
  - IRF8
  - IRF4
- Myeloid DC1:
  - CD141 (THBD)
  - IRF8
- Myeloid DC2:
  - CD1C
  - CD11C (ITGAX)
  - CD11B (ITGAM)
  - IRF4

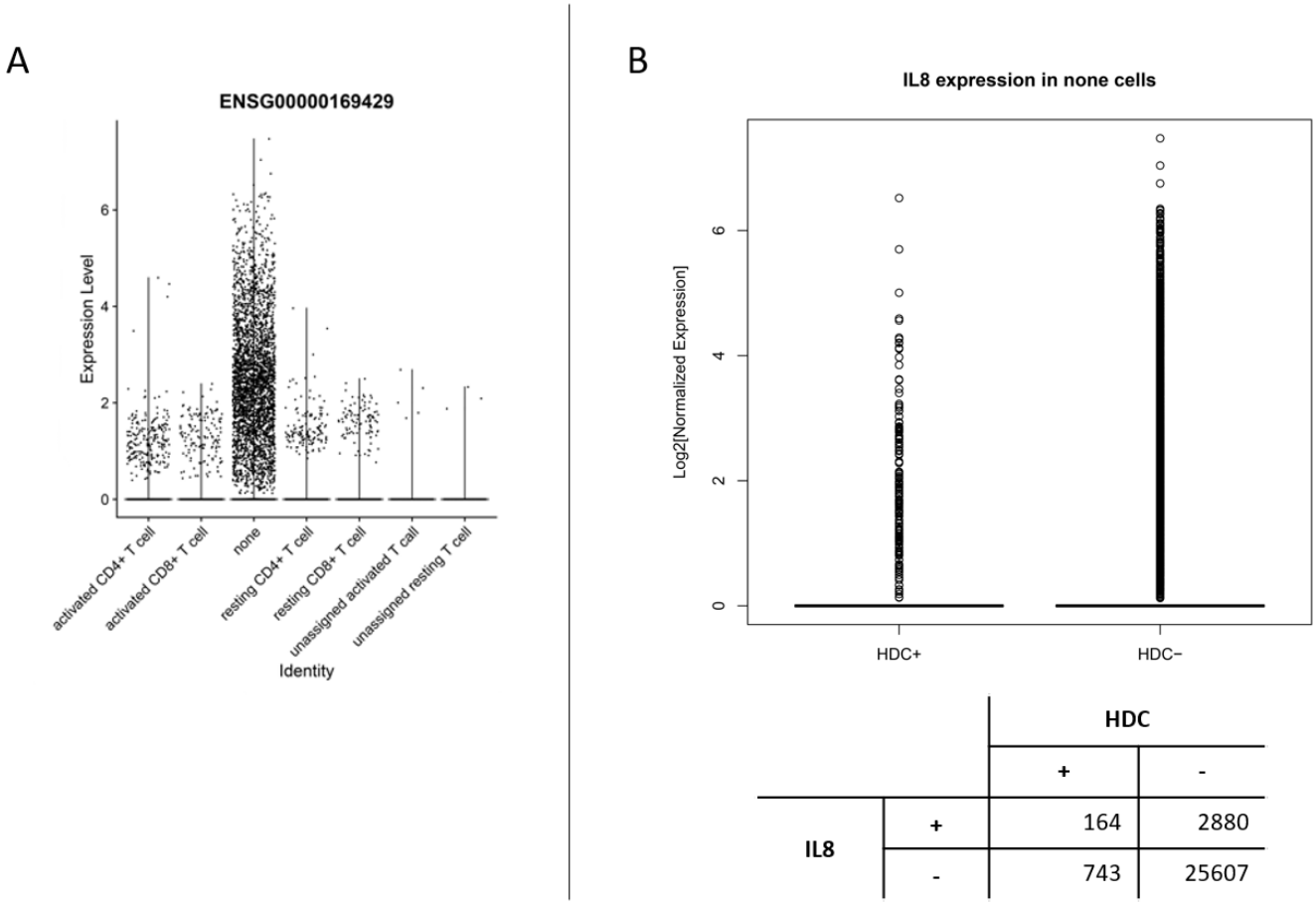
Basophils:
- CD193 (CCR3)
- FCER1A

Gamma/delta T cells:
- TRG - could not find
- TRD - could not find

ILC2 cells:
- CD127 (IL7R)
- KLRG1
- CRTh2 (PTGDR2) (not unique)
- CD117 (KIT) (on some)



Supplementary Figure 8. Analysis of annotated "none" cells with additional markers for contaminating cells. Dataset 2: (Single-cell transcriptomics of human T cells reveals tissue and activation signatures in health and disease, https://doi.org/10.1038/s41467-019-12464-3) Additional markers were used for analysis of "none" cells to determine the cell identity of possible contaminating cells. Violin plots representing classified cells based on markers used, and the percentage of these markers expressed within these cells.

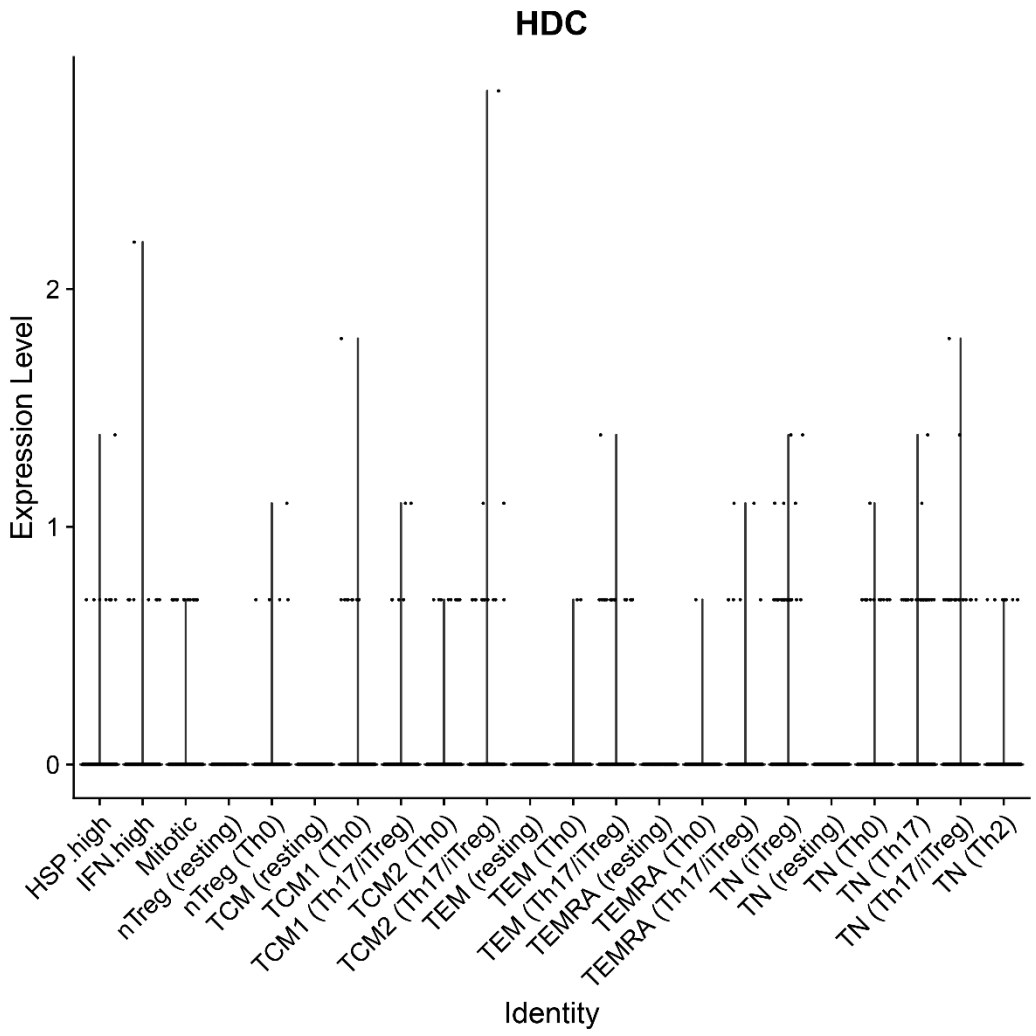


		HDC	
		+	-
IL8	+	164	2880
	-	743	25607

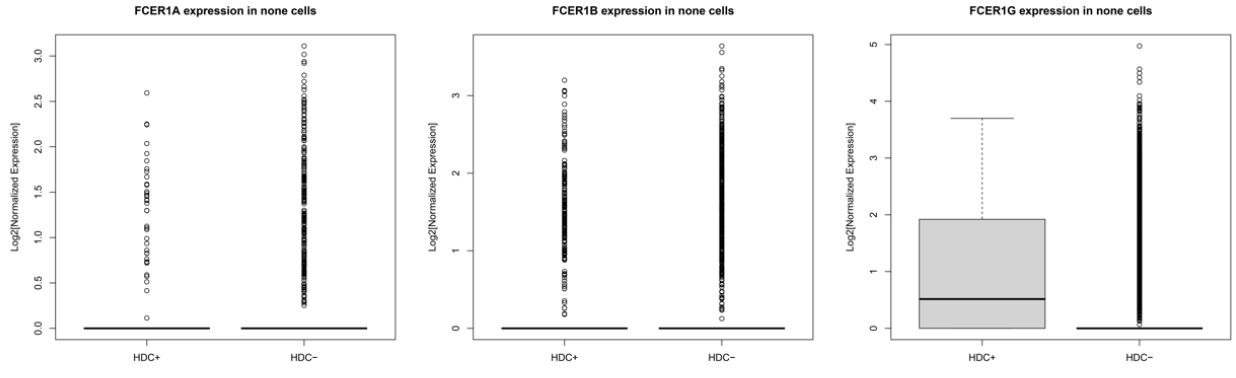
Supplementary Figure 9A. Analysis of the annotated "none" cells for expression the of IL-8 (ENGSG00000169429) as a strategy to determine contaminating monocytes, dendritic cells, and NK cells: Dataset 2: (Single-cell transcriptomics of human T cells reveals tissue and activation signatures in health and disease, https://doi.org/10.1038/s41467-019-12464-3). Analysis of the "none" population showed of IL-8.

Supplementary Figure 9B. Analysis of the annotated "none" cells for the co-expression of HDC and IL-8 as a strategy to determine contaminating monocytes, dendritic cells, and NK cells: Dataset 2: (Single-cell transcriptomics of human T cells reveals tissue and activation signatures in health and disease, https://doi.org/10.1038/s41467-019-12464-3). Analysis of the "none" population showed the co-expression of HDC and IL-8. HDC+/IL-8+ population represents 18.08% of the total HDC+ population from the annotated "none" population (P-value < 1.182e-12)

Appendix



Appendix Figure 1. HDC Expression level of house dust-mite human T cells. Dataset 3: (Single-cell transcriptomic analysis of allergen-specific T cells in allergy and asthma, https://doi.org/10.1126/sciimmunol.aba6087)



		HDC +	HDC -
FceR1A	+	37	234
	-	870	28253

		HDC +	HDC -
FceR1B	+	173	436
	-	734	28051

		HDC +	HDC -
FceR1G	+	468	2484
	-	439	26003

Appendix Figure 2. Co-expression of HDC with FceR1 alpha, beta, gamma in the "none" population. Dataset 2: (Single-cell transcriptomics of human T cells reveals tissue and activation signatures in health and disease, https://doi.org/10.1038/s41467-019-12464-3. (P-values: FceR1A: < 3.766e-14, FceR1B: < 2.2e6-16, FceR1G: < 2.2e-16)

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