ABSTRACT

Immune Profiles of Allergic Asthma Patients Treated with Anti-IgE

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Asthma is a chronic inflammatory disease of the airways characterized by bronchial hyper-reactivity, mucus overproduction and airway remodeling and narrowing. Of the various phenotypes of asthma, by far the most common is allergic asthma, in which the airway inflammation is triggered by allergen exposure in sensitized individuals. The diagnosis of allergic asthma is usually done through an allergen prick test; high allergen reactivity is correlated with allergy and these patients should also contain allergen-specific IgE antibodies (abs). One of the more effective medications for moderate-to-severe, uncontrollable allergic asthma is omalizumab, an anti-IgE ab that targets free IgE, thus preventing the continuation of IgE-dependent allergic responses. However, the mechanisms behind how anti-IgE treatment influences the pathophysiologic responses remain to be fully revealed. Additionally, only a 21-64% response rate is seen after 16 weeks, despite the underlying allergy pathogenesis. Thus, in order to better understand the role of IgE in the pathogenesis of human allergic asthma and to identify potential biomarkers for response to anti-IgE therapy, we studied the

mechanisms of action of anti-IgE abs in allergic asthma patients through the comparison of immune cell composition and activation status and whole blood transcriptional profiling. By acquiring patient samples before the start of anti-IgE treatment, we were also able to compare healthy donors with allergic asthma patients to glean additional insights into allergic asthma. This study offers a unique global examination on the impacts of anti-IgE on the immune response, as observed in peripheral blood and also gains a further step towards the development of a biomarker for response to anti-IgE treatment.

Immune Profiles of Allergic Asthma Patients Treated with Anti-IgE

by

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αGalCer	alpha-galactosylceramide
μL	microliter
Ab	antibody
ACT	asthma control test
ADCC	antibody-dependent cell-mediated cytotoxicity
Ag	antigen
AHR	airway hyperresponsiveness
AMϕ	aveolar macrophage
APC	antigen presenting cell
AS	asthma
BCA	B cell attracting
BHR	bronchial hyper-reactivity
Breg	regulatory B cell
BV	brilliant violet
CCR	C-C motif chemokine receptor
CD	cluster of differentiation
CM	central memory
COPD	chronic obstructive pulmonary disorder
cRPMI	complete RPMI
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CRTH2	chemoattractant receptor homologous molecule expressed on Th2 cells

CXCR	C-X-C motif chemokine receptor
DC	dendritic cell
DLDA	diagonal linear discriminant analysis
dLN	draining lymph node
EC	epithelial cell
EGF	epidermal growth factor
EM	effector memory
ENA-78	epithelial cell-derived neutrophil activating peptide-78
FcεR1	Fc epsilon receptor 1; high affinity IgE receptor 1
FcεR1α	Fc epsilon receptor 1 alpha; high affinity IgE receptor 1 alpha chain
FDA	Food and Drug Administration
FDR	false discovery rate
FE_{NO}	fractional exhaled nitric oxide
FEV_1	forced expiratory volume in 1 sec
FGF	fibroblast growth factor
Foxp3	forkhead box P3
FVC	forced vital capacity
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRO	growth regulated onocogene
HC	healthy control
HLA-DR	human leukocyte antigen – antigen D-related
ICS	inhaled corticosteroid
IDO	indoleamine 2,3-dioxygenase

IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
ILC-2	type-2 ILC
IPA	ingenuity pathway analysis
iNKT	invariant NKT
IP-10	interferon gamma-induced protein-10
LPS	lipopolysaccharide
MC	mast cell
MCP	monocyte chemotactic protein
mDC	myeloid dendritic cell
MDC	macrophage-derived cytokine
mIgE	membrane-bound IgE
MIP	macrophage inflammatory protein
MMP9	matrix metallopeptidase 9
MO	monocyte
MoDC	monocyte-derived DC
Mϕ	macrophage
NA	not assessable
NETs	neutrophil extracellular traps
NK	natural killer
NKT	natural killer T

NR	non-responder
n.s.	not significant
OX40L	OX40 ligand
PAM	partitioning around medoids
PB	plasmablasts
PBMC	peripheral blood mononuclear cell
PRR	pattern recognition receptor
RANTES	regulated-on-activation normal T-cell expressed and secreted
RNA	ribonucleic acid
ROS	reactive oxygen species
pDC	plasmacytoid dendritic cell
PGD_2	prostaglandin D_2
QuSage	quantitative set analysis of gene expression
R	responder
sCD40L	soluble CD40 ligand
SD	standard deviation
SVM	support vector machine
TARC	thymus and activation-regulated chemokine
TCR	T cell receptor
Tfh	T follicular helper
TGF	tumor growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor

Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin

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CHAPTER ONE

Introduction

Overview of Allergic Asthma

Allergic asthma is a highly heterogeneous disease. Since the various asthma endotypes will respond differently to treatment, it is important to try and define a patient's phenotype in order to aid in successful treatment. Generally, the asthma endotypes fall into two main groups: $Th2^{hi}$ and $Th2^{lo}$. This distinction is based on Th2-type cytokines and eosinophil presence (1, 2). The $Th2^{hi}$ group manifests as the typical representation of allergic asthma, with high levels of serum IgE, IL-4 and IL-5, and airway eosinophilia (3). This group is also more responsive to corticosteroid treatment and thus is generally less severe (4, 5). On the other hand, the $Th2^{lo}$ group is much more severe, largely owing to the fact that it is corticosteroid-resistant, driven by neutrophilia and IL-17 (6, 7). There is also an endotype that consists of a mixture of these two groups, with an overlap between eosinophilia and neutrophilia, and this group is also associated with poor asthma control (8). In order to understand the importance of each endotype, it is necessary to first identify the events involved in the allergic response and the cell types involved in each step.

Allergen Sensitization

Before allergic asthma can be induced, there must first be a sensitization to exogenous antigens in the airways. Sensitization is first initiated by the innate immune

system(9, 10). Signals from the airway epithelial cells, either through epithelial damage, such as by microbes or pollutants, or through pattern recognition receptor (PRR) activation. PRR activation may be caused by pollutants, bacteria, viruses or allergens. These resulting signals go on to induce the maturation of antigen-presenting cells (APCs), particularly dendritic cells (DCs) in the underlying mucosa. Once matured, these APCs have a greater capacity to take up, process and present antigens. Matured DCs in the airways have the capacity to sample the airway lumen (11, 12), thus allowing for the uptake, processing and presentation of these allergens. From here, these cells will migrate to the lymph node, where they can in turn start the recognition of the allergens by the adaptive immune system, namely T cells. Why this process is initiated in the first place is still unknown, however, it may be a result of lung injury coupled with a propensity for atopy. It has also been shown that some of the more allergenic allergens have an increased capacity to penetrate the lung epithelial barrier, and oftentimes exhibit protease activity (9).

Allergens by definition are mostly harmless antigens that manage to induce an immune response (13), and some of the most common are pollen, pet dander and dust. It may be that the combination of allergen presence along with epithelial damage caused by an infection is enough to start the process. However, there is most likely a large genetic component involved at multiple points throughout the process (14). It is also important

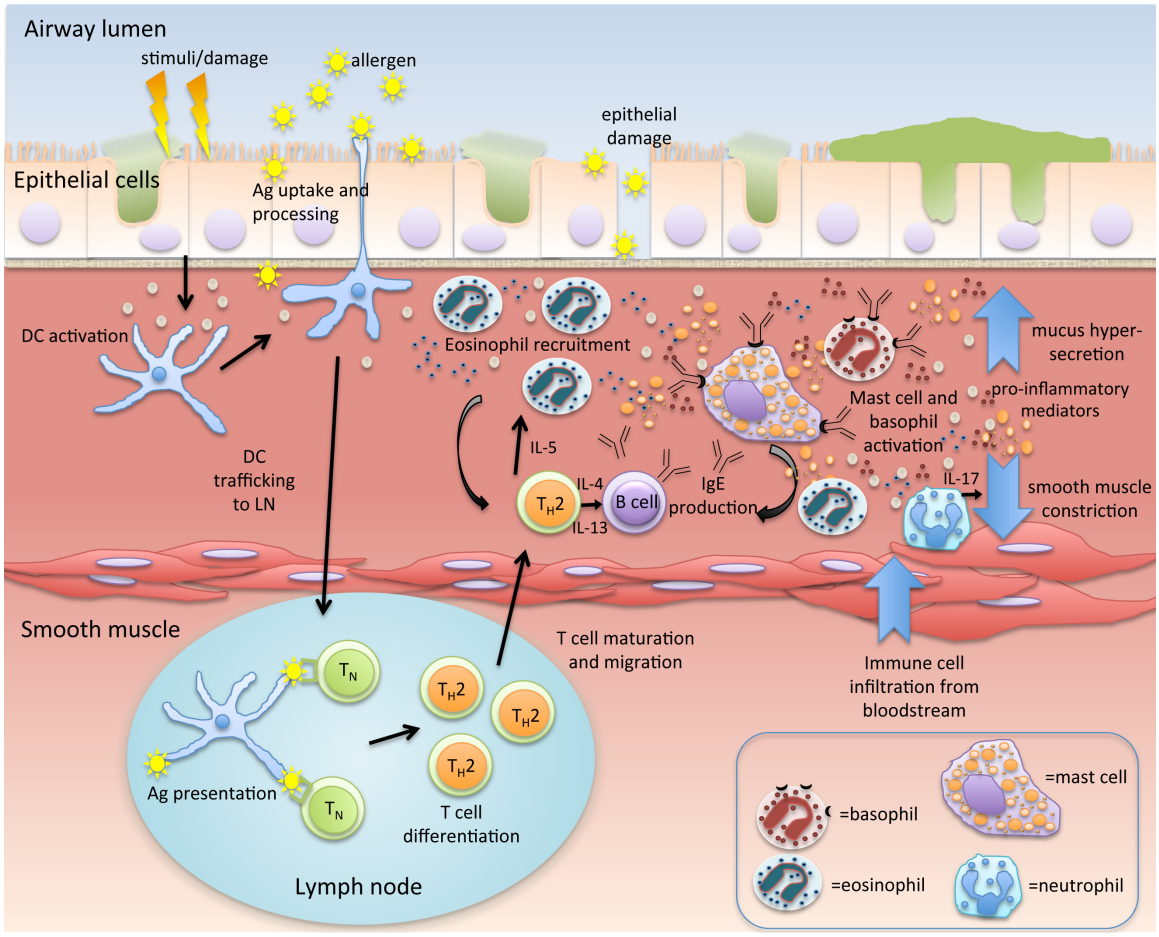


Figure 1.1. Sensitization of the airways and the induction of allergic asthma**.**
Damage or stimulation of the airway epithelial cells leads to the activation and maturation of DCs, in turn leading to their increased capacity for antigen uptake and processing. Once these DCs process antigens, such as allergens, they can than migrate to the local lymph node where they induce T cell differentiation, generally towards a Th2 phenotype. These Th2 cells than migrate to the lung, where they can induce B cell IgE class-switching through the production of IL-4 and IL-13 and attract eosinophils through the production of IL-5. IgE can than activate basophils and mast cells, causing the release of pro-inflammatory mediators. Cytokines and chemokines produced by basophils, eosinophils and mast cells further enhance Th2 and IgE production and the infiltration of inflammatory cells. This leads to changes in the airway environment, including airway hyperreactivity, mucus hyper-secretion, smooth muscle constriction and airway remodeling. Neutrophils present in the lung epithelium can also contribute to smooth muscle constriction and airway remodeling.

to note that, while up to half the population worldwide is sensitized to at least one allergen, only a small percentage go on to develop asthma (15), further complicating the understanding of the initiation process of allergic asthma. Of the cell types involved,

dendritic cells (DCs) are of critical importance. An overview of this process is portrayed in Figure 1.1.

Cellular Players in Allergic Asthma

Airway DCs

Residing within the lungs are BDCA-1^+ myeloid DCs (mDCs), BDCA-2^+ plasmacytoid DCs (pDCs), monocytes and, in the alveoli, alveolar macrophages (AMØs)(16). DCs are able to send dendrites into the airway lumen where they can sample the local environment (17). These dendrites express PRRs and will endocytose bound allergens (18). Most lung DCs are immature, meaning that they can efficiently recognize and uptake antigens. Upon antigen/allergen recognition, the DCs become activated and leave the lung to migrate to the regional lymph nodes, where they become mature DCs (19). These mature DCs acquire the ability to polarize the immune response, which in the case of allergic asthma, is generally toward Th2-type responses. DCs have been shown to be critical for the allergic response. A study by van Rijt et al (20) showed that when mouse lung $CD11c^+$ DCs are depleted during allergen challenge, no asthma characteristics are seen. This includes eosinophilic inflammation, goblet cell hyperplasia and bronchial hyperactivity. They also observed that $CD4^+$ T cells stopped producing IL-4, IL-5 and IL-13. All the characteristics of allergic asthma were restored when $CD11c^+$ DCs were adoptively transferred. Thymic stromal lymphopoietin (TSLP), which is expressed by bronchial epithelial cells (21), can stimulate DCs to express OX40 ligand (OX40L), which is a Th2 cell-polarizing signal (22). DCs activated by TLSP (TSLP-DC)

can initiate the production of inflammatory Th2 cells (23). They can also upregulate Th2 expression of proallergic genes, which further increases the asthmtic environment (24).

Monocytes and macrophages (Mϕ)

One of the main cellular types residing in the lumen of the lung alveoli is the resident alveolar macrophages (AMϕs). Similar to the DCs, they can endocytose antigens/allergens. There have been mixed results on their capacity to leave the lumen (25). Their potential ability to migrate to the lymph nodes would implicate them in the activation of allergen-specific T cells; however, if they are unable to do so, they would have a limited impact on allergic progression. Little is known about the roles of these cells in allergic asthma; nonetheless, it has been shown that asthmatic lungs have an increased amount of AMϕ (26). It is known that Mϕ in the airways can produce pro-inflammatory mediators, such as IL-17, IL-8 and TNF, and further exacerbate the allergic response, especially by aiding in the recruitment of neutrophils to the lung (27). Monocytes circulating in the blood are capable of migrating to the lung and differentiating into AMϕ or monocyte-derived DCs (moDCs)(28). Monocytes can be divided into three main types, based on their expressions of CD14 and CD16: classical ($CD14^{+}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{dim}CD16^{+}$). As monocytes mature, they express higher levels of CD16, and as may be expected, the level of $CD16^{+}$ monocytes increases in inflammatory conditions (29). It has been shown that severe asthmatic patients have enhanced levels of peripheral intermediate monocytes (30). These intermediate monocytes seem to have a greater capacity to promote a pro-inflammatory environment (31) than the other subtypes, which may be why there are increased in the setting of allergic asthma.

Basophils

DCs are not the only cells in the lung that can present antigen. Basophils can express HLA-DR and act as an APC. Basophils are a type of granulocyte terminally differentiated in the bone marrow. They express the high affinity IgE receptor, FcεR1, and contain granules in the cytoplasm that are released upon FcεR1 cross-linking. They can also release Th2-type cytokines, such as IL-4, IL-5 and IL-13, and proinflammatory molecules such as histamine and lipid mediators (32). The major growth factor for basophils is IL-3, and this is required for optimal IL-4 and IL-13 production. In addition the release of cytokines through FcεR1 cross-linking, they can also produce cytokines in response to Toll-like receptor (TLR) stimulation (33). They express TLR1, TLR2, TLR4 and TLR6. TLR4 stimulation by LPS, as well as by inhaled proteins, has been shown to be required for inducing Th2 responses(34, 35).

Eosinophils

Similar to basophils, eosinophils have the capacity to express HLA-DR and act as an APC. Eosinophil infiltration is one of the defining features of allergic asthma, with patients showing eosinophilia in the lung tissue. The Th2-type cytokines, IL-4, IL-5 and IL-13, play a role in regulating the migration of eosinophils to the inflamed lung (36). Eosinophils can release granule proteins and inflammatory mediators, which add to the symptoms of allergic asthma. Jacobsen, EA et al (37) have shown that eosinophils can induce the accumulation of mature mDCs to the lung draining lymph nodes (dLN) during allergen challenge. The accumulation of mDCs than leads to the activation of T cells, suggesting that the eosinophils are not being used as APCs in this scenario; however,

they seem to be critical for the induction of Th2-type responses. IL-5 is the main growth factor for eosinophils, and is involved in regulating their development, survival and activation. IL-5 is so critical to eosinophils that a recently FDA-approved IL-5 blocking antibody, Mepolizumab, has been used to successfully treat patients with eosinophilic asthma (38). Eosinophils have also been shown to produce IL-4 and store it in their cytoplasm for rapid release upon activation (39). They also secrete Idoleamine 2,3-dioxygenase (IDO), which is an enzyme that catalyzes the oxidative catabolism of tryptophan to kynurenines, and is able to inhibit proliferation and promote preferential apoptosis of Th1 cells (40). It has been shown that allergic donor eosinophils constitutively express IDO, which works to promote the Th2 environment (41). Eosinophils have been shown to be critical in allergic asthma, as mice without eosinophils have greatly reduced T cell migration and cytokine expression in the lung (42).

Mast Cells

In addition to basophils and eosinophils, mast cells (MCs) also play a large role in allergic inflammation. They have been difficult to study, as they circulate in the blood in a progenitor state, and do not mature until they reach the tissue (43). MCs are found in all mucosal tissues in healthy subjects, but have been shown to be present at elevated levels in asthmatic lungs (44). Along with basophils, MCs are the main expresser of FcεR1, and cross-linking of these receptors can lead to mast cell degranulation. MCs release inflammatory mediators such as histamine, tryptase, granulocyte macrophage colony-stimulating factor (GM-CSF) and various proteases (45). They have also been shown to localize within the bronchial smooth muscle bundles in asthmatic patients, but

not in those with eosinophilic bronchitis or in normal subjects, and are able to play a role in the remodeling of the airways after injury (46).

Neutrophils

The role of neutrophils in allergic asthma is still poorly understood. It has been shown that patients with severe asthma tend to have more neutrophils in their sputum, and that the levels of eosinophils do not always correlate with the amount of neutrophils (6). Since neutrophils have been shown to be steroid insensitive (47, 48), this means that inhaled corticosteroids will not be very useful as the main line of defense against asthmatic inflammation for those with primary neutrophilic inflammation. As their role in the first line of defense against infection, neutrophils express a wide range of toll-like receptors (TLRs) and as such can respond to a multitude of various pathogens and stimuli. They contain within their cytoplasm granules filled with pro-inflammatory proteins, such as lactoferrin and matrix metalloproteinase 9 (MMP9) (49). They also produce reactive oxygen species (ROS) and extracellular traps (NETs), which serve to damage invading pathogens, but which can also harm host cells, as they act indiscriminately (50). They can also modify the environment of the lungs through various secreted proteins, such as elastase, which can induce mucus secretion and induce proliferation of smooth muscle cells (51). Recruitment of neutrophils to the lung can be enhanced by the production of IL-17, secreted by T cells and natural killer cells (NK)(25, 52). They can also be stimulated by Th2 cells in the airways to express high levels of FcεR1 (53, 54). The activation of FcεR1 on their surface leads to the release of IL-8, which can attract additional neutrophils and induce the degranulation of other granulocytes (51). The complex involvement of neutrophils in allergic asthma points to

the high degree of interplay between the various inflammatory types, and indicates that it is more than a Th2-type disease.

T Cells in Allergic Asthma

Of the immune cells known to be involved in the progression and maintenance of allergic asthma, T cells are critical. Naïve T cells that become activated by allergen-presenting APCs will grow into allergen-memory cells. Based on the APC-T interaction and cytokine milieu, these T cells will polarize towards a specific type; in the context of allergy this is generally Th2-like. These T cells can than migrate to the sites of inflammation in the airways and aid in the recruitment of additional proinflammatory cells. For example, IL-5 production by T cells leads to the migration of eosinophils (55), IL-13 production leads to bronchial hyperactivity (56, 57), and IL-4 or IL-13 production leads to the generation of IgE-producing B cells (1, 58), all of which escalate the allergic response in the lung. In addition to Th2 cells, a few other Th subtypes are known to be involved in the pathogenesis of allergic asthma. Th9 cells, in some ways considered a subtype of Th2, are known to be increased in allergic asthma (59). While little is known about this cell type, as it has only recently been differentiated from Th2 cells, it has been shown that IL-9 produced by these cells can stimulate increased mucus production by bronchial epithelial cells (60). Th9 cells have also been shown to be required for mast cell accumulation in the lung through the production of IL-9 (61, 62). In addition to Th9 cells, Th17 cells have shown an interesting involvement in the pathogenesis of allergic asthma. As noted above, IL-17 production by T cells can lead to the recruitment of neutrophils to the airways. Additionally, IL-17 can act on various airway tissue cells to induce increase mucus production and airway remodeling (63, 64). Th17 cells, like

neutrophils, have also been shown to be corticosteroid-resistant (65). This again implicates them in the pathogenesis of steroid-refractory allergic asthma. In addition to the alterations in Th2, Th9 and Th17 cells in allergic asthma, there also seems to be a dysfunction in regulatory T cells (Treg). Tregs are defined by the expression of the transcription factor Foxp3, production of the suppressive cytokines IL-10 and TGF-β, and expression of the surface marker CD25 (66, 67). It is unclear how they are subverted in the context of allergic asthma, some studies have shown that allergic patients have a decreased amount of Tregs, while others have shown an increase, however, it is clear that these Tregs are functionally impaired to suppress inflammation (1).

IgE-Producing B Cells

While B cells on their own do not appear to play much of a role in allergic asthma, the IgE that they produce is critical. IgE-producing B cells are exceedingly rare and IgE antibodies are found at ng/mL levels in the serum, with a half-life of only a couple days (68). This is opposed to IgG, which can be found at mg/mL levels in the serum and with a half-life of almost a month (69). B cells develop in the bone marrow and leave as immature B cells expressing IgM (70). By circulating through the peripheral lymphoid organs, B cells can encounter antigens. When these antigen-presenting B cells encounter a T cell specific for that same allergen, it can undergo maturation and differentiation. In order for B cells to switch from IgM to IgE synthesis, they must interact with their corresponding T cells in the presence of additional helper signals from the ligation of CD40 (on the surface of the B cell) with CD40 ligand (CD40L, on the T cell), in addition to the presence of IL-4 and IL-13 (9). IgE produced by these B cells is either high or low affinity. Generally, it is considered that high affinity IgE is produced

by B cells that had an intermediary IgG1 phase before switching to IgE (71). By having an indirect class switching, the resulting antibody has additional somatic hypermutation and as a result has increased affinity. IgE produced by these B cells is able to exert its activity through binding to its high and low affinity IgE receptors (FcεR1: high affinity, CD23: low affinity) (54, 72-79).

NK and NKT Cells

Natural killer (NK) and natural killer T (NKT) cells are innate immune cells and have a complicate involvement in allergic asthma. NK and NKT cells both express the surface marker CD56, however, they differ in their expression of CD3; NK cells are $CD3^-$, while NKT cells are $CD3^+$. NKT cells can be further divided into type I classical invariant NKT (iNKT) and type II non-classical NKT. iNKT cells express invariant TCR α-chains, while NKT express various TCR α-chains, but both iNKT and NKT can recognize lipids presented by CD1d (9, 25). Since iNKT cells have a highly characterized ligand (αGalCer) that can be utilized for study, they are better studied in the context of allergic asthma than variant NKT. Activation of iNKT cells leads to the generation of both Th1- and Th2-type cytokines. Their role in human allergic asthma is highly contentious; multiple studies have found either an increase or decrease in iNKT in BAL, lungs or sputum of patients (80). Similar to iNKT cells, NK cells are capable of producing both Th1- and Th2-type cytokines. Some studies have indicated an increase in NK cells in the blood of allergic asthma patients, and they are capable of responding to IgE through CD16 (81). Since both these cell types are critical in viral immunity, their

role may be in aggravating asthma during patient viral infection. They are also cytotoxic and may contribute to tissue damage in the lung.

Innate Lymphoid Cells (ILCs)

The presence of ILCs in allergic asthma was first discovered when it was shown that mice deficient in B and T cells could still generate lung eosinophilia in response to allergen administration (1). While ILCs express similar cytokines to T helper cells, they do not express antigen-specific receptors. Based on their shared similarities with T cells, they have thus far been divided into three subtypes: ILC-1, which includes NK cells and produce Th1-type cytokines, ILC-2, which produce Th2-type cytokines, and ILC-3, which produce Th17-type cytokines. In allergic asthma, ILC-2 is most involved; they can be activated by cytokines produced by the lung epithelial cells, and produce large amounts of Th2-type cytokines. Since ILC-2s seem to be a highly heterogeneous population, there are a wide range of surface markers that can be used to identify them. Similar to Th2 cells, ILC-2s can express CRTH2 (82), indicating their capacity to migrate to the lung. They are found mainly in tissues, with only a very small presence in the blood (<0.03%)(83). As they are found in such small percentages, little is known about their role in human allergic asthma.

Airway Epithelium

In order to fully understand the process of allergen sensitization and the asthmatic response, one must also know the role of the airway epithelium. Indeed, one integral component that is often over-looked in the study of allergic asthma is the role of the airway epithelium. The lung tissue is made up of the airway epithelial cells (ECs),

underlined by the basement membrane. Within the epithelium, there are the classic ciliated cells, along with Clara cells and the mucus-producing goblet cells. The space between the epithelial basement membrane and the smooth muscle cells is the lamina propria. This is where the majority of the infiltrating immune cells will be found. In an asthmatic lung, there will be structural changes to the lung tissue, also known as airway remodeling. The smooth muscles underlying the epithelium will proliferate, the epithelial basement membrane will become thickened and goblet cells in the epithelium will produce increased amounts of mucus. These all work to decrease the amount of free space that can be occupied by air. Since the ECs are the first to encounter the environment, they express a large variety of pattern recognition receptors (PRRs) that allow them to sense and respond to their environment. Activation of the epithelial PRRs will lead to the release of numerous cytokines, chemokines and antimicrobial peptides that can attract the notice of immune cells. This epithelial activation is considered to be the key event in the initiation of allergen sensitization. Chemokines released by the ECs can recruit immature DCs into the lung and prime them to generate Th2 responses by the release of additional cytokines such as TSLP, GM-CSF, IL-25 and IL-33 (84). Pro-inflammatory mediators released by both the ECs and other immune cells will drive the differentiation of ciliated and Clara cells to goblet cells, induce proliferation in the smooth muscle cells, and lead to thickening of the basement membrane (85-87). Some of these pro-inflammatory mediators, in addition to the proteolytic activity of certain allergens and infections, will lead to the disruption of the epithelial membrane integrity (84, 88-91). Once the epithelial membrane becomes compromised, it becomes

increasingly easy for invading inhalants to cause further damage to the lung tissue and to directly activate immune cells residing in the lamina propria.

The Development of an Anti-IgE Antibody for the Treatment of Allergic Asthma

There are multiple different classes of drugs that are used for the treatment of asthma, all with varying levels of efficacy. There are both quick-relief medications, as well as long-term control prescriptions. What all these drugs have in common is their targeting of a specific inflammatory compound. For example, one of the most well known is antihistamine. They target histamine released by cells such as mast cells and basophils that can cause sensorial neural stimulation and DC activation through the ligation with its histamine receptors (92). Many asthma patients also use β_2-agonists and inhaled corticosteroids (ICS). Long- and short-acting β_2-agonists can reverse bronchoconstriction by binding to the β_2-adrenoceptor causing the relaxation of the bronchial smooth muscle cells, and this is often combined with ICS which work to inhibit multiple inflammatory cell types present in the airway (93). While the combination of ICS and long-acting β_2-agonists is sufficient medication to control most asthma, some patients still exhibit uncontrollable asthma. Oral steroids can be given to further dampen the inflammatory response occurring during allergic asthma, but the toxicity of this treatment means that it can only be used for a defined duration. This unmet need for patients with corticosteroid-resistant allergic asthma led to the generation of an IgE blocking antibody. First reported in 1993, a group from Genentech developed a humanized IgE-blocking monoclonal antibody (anti-IgE) (94). It was critically important

that the anti-IgE antibody not crosslink to IgE already bound by the IgE receptor on the surface of mast cells and basophils, thus the antibody they developed bound to the same

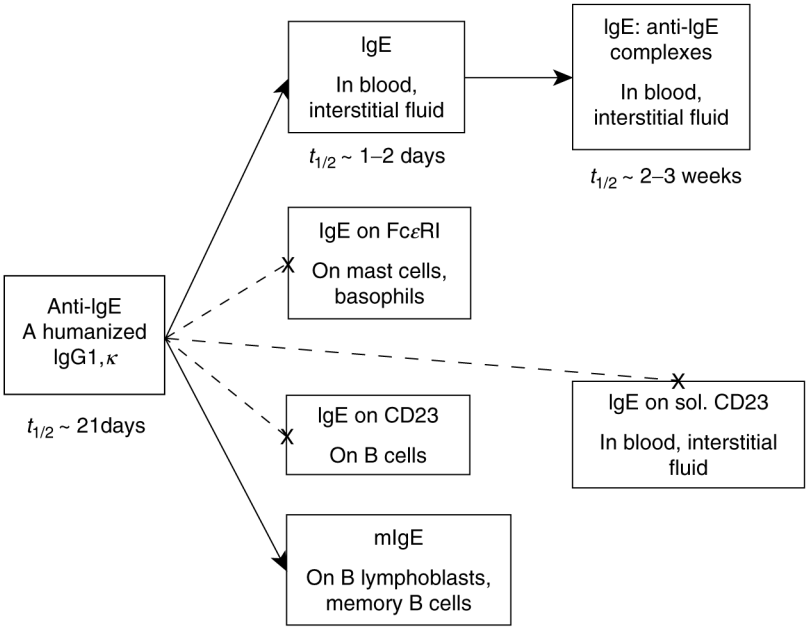


Figure 1.2. Binding specificities of anti-IgE antibody (omalizumab).
The half-lives of IgE, anti-IgE, and the immune-complexes of IgE and anti-IgE are also indicated. Reprint by permission form Elsevier: Advances in Immunology, copyright 2007(95). mIgE: membrane-bound IgE.

region of IgE as the IgE receptor, causing competition between αIgE and FcεR1 for binding free IgE. The resulting αIgE antibody (omalizumab, trade name Xolair) was approved in the United States in 2003 for the treatment of moderate-to-severe allergic asthma. A schematic of the action of anti-IgE is shown in Figure 1.2.

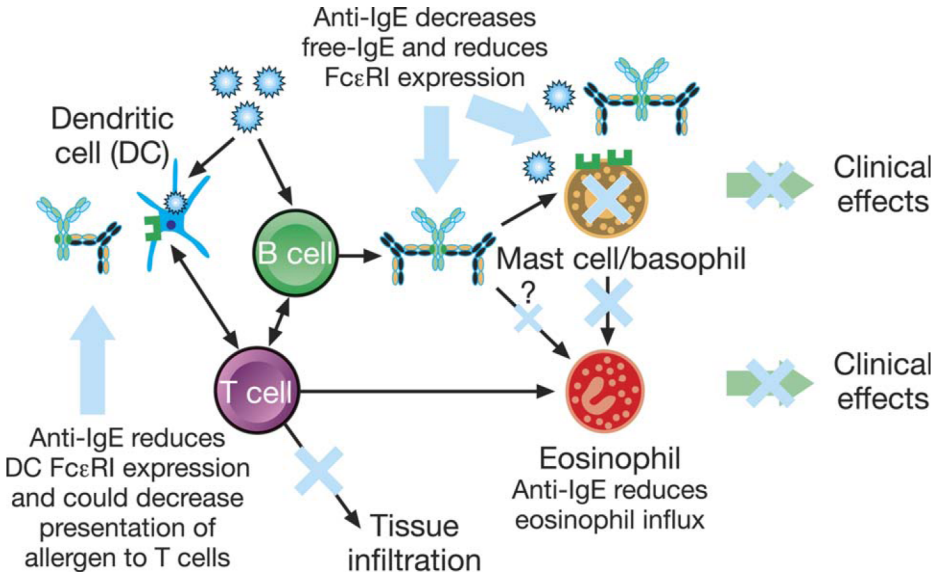


Figure 1.3. Proposed mechanism of action of anti-IgE antibody (omalizumab).
Anti-IgE decreases free IgE levels and reduces FcεR1 expression on mast cells and basophils. This results in decreased mast cell activation and sensitivity, leading to a reduction in eosinophil influx and activation. Anti-IgE treatment may also result in decreased mast cell survival. Anti-IgE also reduces DC FcεR1 expression. Reprint by permission form Elsevier: Journal of Allergy and Immunology, copyright 2005(96).

By targeting IgE, anti-IgE is able to prevent IgE cross-linking on mast cells and basophils, thus preventing their degranulation and release of inflammatory mediators. This bypasses the need for therapeutics that targets these inflammatory mediators, such as antihistamines and leukotriene modifiers. The proposed action of anti-IgE on immune cells is shown in Figure 1.3. That anti-IgE is highly effective indicates the fundamental involvement of IgE in the pathogenesis of allergic asthma. Interestingly, however, not all patients with allergic asthma will respond to anti-IgE treatment. There appears to be a response rate around 20-90%, with patients who exhibit more severe asthma and have a serum IgE range from 30-700 IU/mL having the greatest chance of response (95). It is curious that not all patients with allergic asthma would respond to the blocking of IgE, and this may represent a unique IgE-independent mechanism.

Author Contributions

Chapter Three

K.C.U. analyzed the data, and wrote the manuscript. S.B. helped acquire the data. S.O. supervised the project, analyzed the data, and wrote the manuscript.

Chapter Four

K.C.U. performed the experiments, analyzed the data, and wrote the manuscript. J.E., J.C. and Y.X. helped in experiments. M.M. and B.L provided clinical samples. J.T. and H.J. aided in data analysis. S.O. supervised the project, analyzed the data, and wrote the manuscript.

Chapter Five

K.C.U. performed the experiments, analyzed the data, and wrote the manuscript. J.E., J.C. and Y.X. helped in experiments. M.M. and B.L provided clinical samples. J.T. and H.J. aided in data analysis. S.O. supervised the project, analyzed the data, and wrote the manuscript.

Chapter Six

K.C.U. performed the experiments, analyzed the data, and wrote the manuscript. J.E., J.C. and Y.X. helped in experiments. M.M. and B.L provided clinical samples. J.T. and H.J. aided in data analysis. S.O. supervised the project, analyzed the data, and wrote the manuscript.

Chapter Seven

K.C.U. performed the experiments, analyzed the data, and wrote the manuscript. J.E., J.C. and Y.X. helped in experiments. M.M. and B.L provided clinical samples. J.T. and H.J. aided in data analysis. S.O. supervised the project, analyzed the data, and wrote the manuscript.

Chapter Eight

K.C.U. performed the experiments, analyzed the data, and wrote the manuscript. J.E., J.C. and Y.X. helped in experiments. M.M. and B.L provided clinical samples. J.T. and H.J. aided in data analysis. S.O. supervised the project, analyzed the data, and wrote the manuscript.

Chapter Nine

K.C.U. performed the experiments, analyzed the data, and wrote the manuscript. J.E., J.C. and Y.X. helped in experiments. M.M. and B.L provided clinical samples. J.T. and H.J. aided in data analysis. S.O. supervised the project, analyzed the data, and wrote the manuscript.

Chapter Ten

K.C.U. performed the experiments, analyzed the data, and wrote the manuscript. J.C., J.S and D.N. helped in experiments. M.M. and B.L provided clinical samples. J.T.

and H.J. aided in data analysis. S.O. supervised the project, analyzed the data, and wrote the manuscript.

CHAPTER TWO

Objectives

Much is still unknown about the underlying mechanisms behind the pathogenesis of allergic asthma, especially why some people will become sensitized to a harmless allergen and mount a highly pathogenic response against it. By studying allergic asthma patient response to anti-IgE treatment, we may be able to glean more information about the process of IgE neutralization on various inflammatory cell types. Even more interesting is the differences that may be seen between those that respond and do not respond to anti-IgE. Herein, we try to understand these differences by fully characterizing and analyzing peripheral blood immune cells through multicolor flow cytometry, gene expression microarray, and serum cytokine and chemokine expression in allergic asthma patients being treated with Xolair.

Aim 1: To Identify Anti-IgE Treatment Responders and Non-Responders in Asthma Patients

a) *To identify anti-IgE responders from non-responders using clinical information*
b) *To identify variations in clinical and demographic information between responders and non-responders at baseline and at week 26 of anti-IgE treatment*

In Chapter Three, we identified anti-IgE non-responders using methods similar to other studies that analyzed response to anti-IgE. Once the criteria for response and non-response was determined, patients were split into their respective groups and their clinical

and demographic information was analyzed, both at baseline (before treatment) and at week 26 of treatment.

Aim 2: To Assess the Effects of Anti-IgE Treatment on Dendritic Cells and Monocytes

a) *To investigate variations between healthy controls and allergic asthma DCs and monocytes*
b) *To investigate the longitudinal effects of anti-IgE treatment on DCs and monocytes, including variations in changes between anti-IgE responders and non-responders*

In Chapter Four, we analyzed the variations between asthma patient and healthy control APCs, including DCs and monocytes. We than followed the asthma patients over the course of treatment with anti-IgE to observe the effects of free IgE neutralization on their population frequency and marker expression. Lastly, variations in APC profiles were compared between anti-IgE responders and non-responders.

Aim 3: To Assess the Effects of Anti-IgE Treatment on B Cells

a) *To study the variations between healthy control and allergic asthma B cells*
b) *To investigate the effects of anti-IgE treatment on B cell profiles*
c) *To determine differences in B cells between anti-IgE responders and non-responders*

In Chapter Five, we developed several comprehensive flow cytometry panels for the analysis of peripheral blood B cells. We analyzed and compiled cellular frequencies, numbers and activation marker expression and compared asthmatic patients with healthy

controls. In addition, we followed the asthma patients over the course of anti-IgE treatment to observe the effects of IgE neutralization on B cell profiles and to determine differences between anti-IgE responder and non-responder B cells,

Aim 4: To Assess the Effects of Anti-IgE Treatment on T Cells

a) *To investigate the variations between allergic asthma patient and healthy control T cells*
b) *To investigate the effects of anti-IgE treatment on T cell profiles*
c) *To determine differences in T cells between anti-IgE responders and non-responders*

In Chapter Six, we developed several comprehensive flow cytometry T cell panels to assess the variations between asthmatic patient and healthy control T cells. We also analyzed the effects of IgE neutralization on the T cell profiles of these asthmatic patients. In addition, we were able to compare T cell populations between anti-IgE responders and non-responders, both before the start of treatment (baseline) and at week 26 (W26) of treatment.

Aim 5: To Assess the Effects of Anti-IgE Treatment on Cytokine Expression from PBMCs and in Sera

a) *To assess cytokine concentration from both serum and stimulated PBMC supernatants*
b) *To compare cytokine expression between asthma patients and healthy controls*
c) *To compare cytokine expression in asthmatic patients over time with anti-IgE treatment*

d) *To investigate variations in cytokine secretion between anti-IgE responders and non-responders*

In Chapter Seven, we calculated cytokine expression using Luminex multiplex analysis. We analyzed cytokines from sera and from anti-CD3/anti-CD28-stimulated PBMC supernatants. We compared asthmatic patients to healthy controls, the effects of anti-IgE treatment on the asthmatic patients cytokine expression, and the variations in cytokine expression between anti-IgE responders and non-responders, both at baseline and at week 26 of treatment.

Aim 6: To Assess the Effects of Anti-IgE Treatment on Innate Lymphoid Cells

a) *To investigate variations between healthy controls and allergic asthma innate lymphoid cells, including NK, NKT and ILC-2*
b) *To investigate the effects of anti-IgE on ILC populations and determine variations between ILCs in anti-IgE responders and non-responders*

In Chapter Eight, we analyzed the variations between asthma patient and healthy control ILC: NK, NKT and ILC-2. We than followed the asthma patients over the course of treatment with anti-IgE to observe the effects of free IgE neutralization on their population frequency and marker expression. Lastly, variations in ILC profiles were compared between anti-IgE responders and non-responders.

Aim 7: To Assess the Effects of Anti-IgE Treatment on Granulocytes

a) *To investigate variations between healthy controls and allergic asthma granulocytes, including basophils, mast cell precursors, eosinophils and neutrophils*
b) *To investigate the longitudinal effects of anti-IgE treatment on granulocyte populations, including the variations between anti-IgE responders and non-responders*

In Chapter Nine, we analyzed the variations between asthma patient and healthy control granulocytes, including basophils, mast cell precursors, eosinophils and neutrophils. We than followed the asthma patients over the course of treatment with anti-IgE to observe the effects of free IgE neutralization on their population frequency and marker expression. Lastly, variations in granulocyte profiles were compared between anti-IgE responders and non-responders.

Aim 8: To Assess Blood Transcription Profiles of Asthma Patients Treated with Anti-IgE

a) *To study the transcriptional fingerprint of anti-IgE treatment*
b) *To compare differences in transcriptional profiles between anti-IgE responders and non-responders*
c) *To discover biomarkers for response using gene expression and canonical pathway analysis*

In Chapter Ten, we analyzed gene expression using microarray. We generated and annotated a set of gene clusters using transcripts statistically significant in at least one comparison between responders or non-responders at any time point versus healthy

controls. We used cross-validation analysis to produce potential biomarkers of response using probe expression at baseline.

CHAPTER THREE

Evaluating Response to Anti-IgE

Abstract

Anti-IgE antibodies (omalizumab, trade name Xolair®) are used for the treatment of moderate-to-severe allergic asthma that is not controlled despite optimal controller therapy. In order to better target patients for optimal response, we investigated and compared patient clinical characteristics both before treatment and at week 26 of anti-IgE therapy. We distinguished anti-IgE responders (N=34) from non-responders (N=11) using criteria similar to other studies that have analyzed response to anti-IgE, including changes in asthma control and the number of exacerbations. We observed that anti-IgE non-responders exhibited a higher comorbidity of obesity than responders. This was reflected in both the average BMI value and in the percent of patients that identified as obese before treatment. This study may aid in patient selection for anti-IgE treatment.

Introduction

IgE is critical to the pathogenesis of allergic asthma, and this has been reinforced by the success of IgE neutralization for the treatment of allergic asthma (96). Anti-IgE works by reducing the availability of free IgE, which in turn reduces IgE cross-linking on basophils and mast cells and prevents their activation and degranulation, attenuates their survival and decreases their sensitivity to IgE stimulation (97-100). There are also indications that anti-IgE therapy can decrease IgE production (101) and induce B cell

anergy in cells bearing surface IgE (102). However, despite its many successes (103-105), there are still patients that do not respond to treatment (106, 107). There have been many studies done to determine biomarkers for response, but thus far nothing substantial has been found (106-111).

In this study, we recruited adult patients (N=45) with moderate-to-severe allergic asthma that qualified for anti-IgE (omalizumab) treatment. After 26 weeks of treatment, we evaluated changes in patient clinical information and divided patients into anti-IgE responders (N=34) and non-responders (N=11). We than compared the clinical and demographic features of responders and non-responders, both before treatment (baseline) and at week 26 of treatment to determine if any patient variables could be used as a marker for success.

Methods

Study Subjects

Moderate-to-severe asthma patients (N=45) were recruited under protocols approved by the Institutional Review Board of Baylor Research Institute. Disease severity was defined based on a combination of low asthma control test (ACT) score (below a score of 19), low lung function (as defined by the forced expiratory volume in 1 sec, FEV_1, score of less than 80% of predicted), and by their frequency of symptoms, including total numbers days with symptoms per week and of nighttime sleep disruption (more than once per week). Patients were excluded if they were pregnant, under the age of 18 or recently on omalizumab (Xolair®, Genentech). Healthy subjects (N=21) were enrolled to match age, ethnicity and sex of the allergic asthma patients and were also

recruited under protocols approved by the Institutional Review Board of Baylor Research Institute. Subject characteristics are summarized in Table 3.1.

Study Design

Patients were prescribed omalizumab (provided by Genentech) based on their physician's recommendation, and were dosed as per the manufacturer's dosing table

Table 3.1. Characteristics of asthma patients and healthy controls subjects recruited in this study

Characteristics	Asthma Patients	Healthy Subjects	*p*-value
Demographics			
Total Population, n	45	21	
Age (years)	56.7 (±11.72)	43.59 (±12.89)	***0.0001
Sex (M/F) (%M)	15/30 (33)	8/13 (38)	0.7039
Total IgE (IU/mL)	323.6 (±456.6)	N/A	
Past Smoker, n (%)	14 (40)	N/A	
Race			
Caucasian, n (%)	41 (91)	16 (76)	0.0989
Black, n (%)	3 (7)	4 (19)	0.1285
Asian, n (%)	1 (2)	1 (5)	0.5755
Clinical			
ACT Score	12.24 (±4.53)	N/A	
Asthma-related symptoms - no. of days in previous wk			
Total symptoms	5.39 (±2.24)	N/A	
β-agonist usage	7.41 (±5.51)		
Nighttime sleep disruption	3.06 (±2.26)	N/A	
Lung Function			
FEV_1 (% of predicted)	72.65 (±16)	N/A	
FVC (% of predicted)	77.36 (±15)	N/A	

Data represent means with standard deviations, where applicable

ACT: asthma control test, F: female, FEV_1: forced expiratory volume in 1 sec, FVC: forced vital capacity

(based on serum IgE and subject body weight). Blood was collected one week before the initiation of treatment and again on the day of initial treatment. Blood was than collected on week 6, week 14 and week 26 after the initiation of treatment. A schematic of anti-IgE treatment schedule and blood draws is shown in Figure 3.1. At each blood draw,

patients filled out the ACT questionnaire and an evaluation form for total numbers of symptoms per week, inhaled steroid/β-agonist usage per week, and nighttime awakenings per week. Patients were also asked to perform the lung spirometer test to evaluate lung function.

Statistical Analysis

Statistical significance between patient groups continuous data was determined using the student's two-tailed unpaired *t*-test with Prism 5 (GraphPad Software, California). Comparisons between population proportions were calculated using a *z* score test. Significance was set at $P<0.05$.

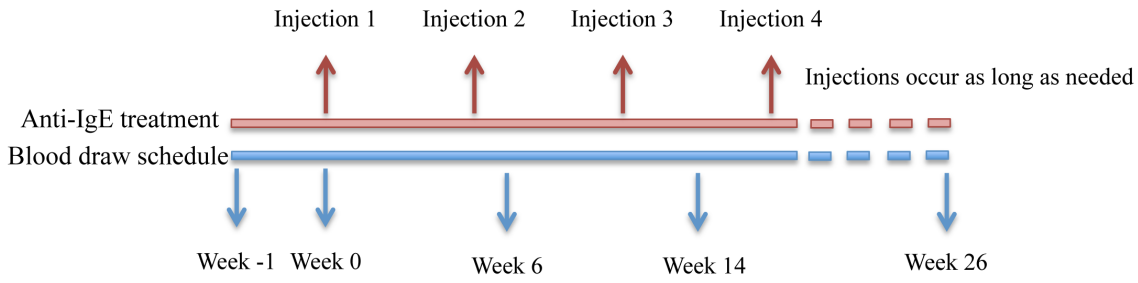


Figure 3.1. Timeline of patient blood draw.

Results

Determination of Responders versus Non-Responders

Response to omalizumab was defined based on improvements in asthma control, with non-responders still exhibiting uncontrolled asthma. Asthma was considered not well controlled if patients had a combination of an ACT score less than 19, with asthma symptoms and inhaled steroid/β-agonist usage at least twice per week and nighttime

awakenings at least once per week, lack of change in asthma control medication, and indications of lack of asthma improvement by physicians, similar to other studies conducted analyzing omalizumab response (105, 107, 108, 111, 112). Since lung function has not been shown to be an indicator of response to omalizumab (103), it was not included in our responder classification. Table 3.2 shows summaries of responders and non-responders both before and at week 26 of treatment. Based on our criteria for response to anti-IgE, we observed a 76% response rate. Compared with responders, non-responders exhibited significantly greater BMI and a larger percentage with BMI indicating obesity. We observed no other variances between patients before treatment, including age, sex, serum IgE, past smoking status, asthma severity or lung function. Our criteria for response included asthma control indications, which is evident in the significant differences between responders and non-responders at week 26 of treatment. We observed that non-responders had significantly lower ACT scores and significantly greater numbers per week of asthma symptoms, β-agonist/rescue inhaler usage and nights disrupted by symptoms than responders at week 26. There was no difference in lung function between responders and non-responders. This indicates that obesity may be a limiting factor for response to anti-IgE treatment.

Discussion

In this study, we identified obesity as a potential determinant to response to anti-IgE, since patients that do not respond to anti-IgE were significantly more likely to be obese. While we observed that our average patient BMI was quite high and that non-responders were more likely to be obese than responders, all body weights were within

Table 3.2. Characteristics of anti-IgE responders and non-responders

	Baseline			Week 26 of Treatment		
	Responder (N=34)	Non-Responder (N=11)	*p*-value	Responder	Non-Responder	*p*-value
Demographic						
Age (years)	56.79 (±12.02)	56.09 (±11.19)	0.8648			
Sex (M/F) (%M)	11/23 (32)	4/7 (36)	0.8026			
BMI	29.07 (±6.21)	33.77 (±7.29)	*0.0426			
Obese, n (%)	10 (29)	8 (73)	*0.0108			
Total IgE (IU/mL)	356.1 (±501.6)	244.7 (±272.9)	0.4886			
Past Smoker, n (%)^	9 (35)	4 (44)	0.5961			
Race						
Caucasian, n (%)	32 (94)	9 (82)	0.2113			
Black, n (%)	1 (3)	2 (18)	0.0784			
Asian, n (%)	1 (3)	0 (0)	0.5619			
Clinical						
ACT Score	12.41 (±4.61)	11.73 (±4.45)	0.6681	20.88 (±3.02)	15.82 (±5.33)	***0.003
Asthma-related symptoms - no. of days in previous wk						
Total symptoms	5.35 (±2.33)	5.09 (±2.35)	0.7482	1.70 (±1.85)	3.96 (±3.00)	**0.0048
β-agonist usage	7.71 (±5.64)	6.41 (±5.38)	0.5059	2.08 (±2.86)	4.46 (±3.86)	*0.0299
Nighttime sleep disruption	2.96 (±2.36)	3.36 (±2.01)	0.6089	0.15 (±0.49)	2.00 (±2.37)	***<0.0001
Lung Function						
FEV_1 (% of predicted)	71.65 (±16.01)	76.73 (±18.39)	0.3823	76.74 (±13.46)	78.09 (±17.28)	0.7924
FVC (% of predicted)	77.53 (±14.62)	76.82 (±14.99)	0.8898	83.03 (±12.97)	80.45 (±14.23)	0.5838
FEF_{25-75} (% of predicted)	62.76 (±31.63)	76.55 (±36.37)	0.2344	65.23 (±27.94)	73.73 (±32.51)	0.4109

Values represent mean with standard deviation, where applicable. Obesity is defined by a BMI >30
^Incomplete data
ACT: asthma control test, F: female, FEF_{25-75}: forced expiratory flow during the mid (25-75%) of the FVC, FEV_1: forced expiratory volume, FVC: forced vital capacity, M: male, N/A: not applicable, % of pred: percent of predicted for age, sex and height
*P<0.05, **P<0.01, ***P<0.001

the dosing guidelines, which are determined based on weight and serum IgE. Obseity is a relatively common comorbidity with severe asthma (113, 114) and it has been shown that

patients with IgE and weight above the recommended range can still benefit from anti IgE treatment (115, 116). However, in a study by Novelli et al (106), they observed that patients who experienced asthma exacerbations even while on anti-IgE treatment exhibited a significantly higher BMI and comorbidity of obesity. This study also observed that patients that did not respond well to anti-IgE treatment also had lower lung function capacity. While our study did not observe such variation, it may be because our study was smaller, but we also had a tighter restriction on anti-IgE response, as indicated by the differences in our ACT score between responders and non-responders. Thus it is likely that obesity induces a chronic inflammatory state that is not receptive to IgE neutralization. This may also act as a biomarker that could eventually allow us to predict responders and non-responders before the initiation of anti-IgE.

CHAPTER FOUR

Longitudinal Analysis of Blood Dendritic Cells and Monocytes of Asthma Patients Treated with Anti-IgE Antibody (Omalizumab)

Abstract

Allergen-specific IgE plays a central role in the pathogenesis of asthma. Neutralization of free IgE with anti-IgE antibody (omalizumab, trade name Xolair) can thus clinically benefit patients. However, the mechanisms of action of anti-IgE treatment are not fully understood. In this study, we investigated changes occurring in blood dendritic cells (DCs) and monocytes during anti-IgE treatment of moderate-to-severe adult asthma patients (N=45). We found that the frequency of myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) was similar in patients and healthy controls (N=21) at baseline. Moreover, anti-IgE treatment did not alter the frequency of DCs in patients. However, anti-IgE treatment significantly reduced CCR7 and HLA-DR expression on DCs, which are critical for DC migration into lymph nodes and elicitation of allergen-specific $CD4^+$ T cell responses. Anti-IgE also decreased the expression levels of costimulatory molecules (CD86, CD83, and CD80). Such decreases were more significant in patients who responded to anti-IgE therapy (N=34) than non-responders (N=11). It is also of note that responders have greater numbers of mDCs and higher mDC/pDC ratios (but not higher pDCs) than non-responders at baseline. We also found that patients have a greater frequency of $CD14^+$ monocytes than healthy controls, although anti-IgE treatment did not alter their frequency. However, anti-IgE treatment significantly reduced monocyte activation, along with decreased CD88 expression on

monocytes, with more significant effects on responders than non-responders. Data from this study support new pathways for the mechanisms of action of anti-IgE treatment in asthma patients.

Introduction

Critical to the pathogenesis of allergen-induced asthma is allergen-specific IgE. IgE interacts with its receptors, including FcεR1 and the low affinity IgE receptor (CD23). FcεR1, as a tetramer ($\alpha\beta\gamma_2$) (72), has the greatest presence on basophils and mast cells (117). Stimulation by IgE cross-linking on these cells leads to degranulation, release of pro-inflammatory mediators and enhanced survival (118, 119). The activation of mast cells and basophils by IgE can further perpetuate the activation and accumulation of additional immune cells at the site of inflammation, including eosinophils, DCs and T cells (9, 118). This can promote mucus hypersecretion, airway inflammation and hyperresponsiveness (120), which is known as the late allergic/asthmatic response. Therefore the use of an anti-IgE antibody (omalizumab) that can prevent the binding and cross-linking of IgE to FcεR1 (78, 97) for the treatment of asthma is reasonable. Studies have also shown that anti-IgE can gradually downregulate FcεRI expression on basophils and mast cells (98, 121, 122). This is of great importance, as it renders these cells less sensitive to allergen stimulation. This negative effect on FcεRI expression was also demonstrated on DCs (123), affecting their ability to promote Th2-type T cell responses and subsequent inflammation (124). Anti-IgE can also target surface IgE-bearing B cells, resulting in B cell anergy (102).

DCs are major APCs that play an important role in both induction and progression of allergic immune responses. Upon allergen exposure, DCs sensitize naive T cells and also direct them towards Th2-type immune responses, which can eventually lead to the generation of IgE-producing B cells (9, 120, 125). In the late phase when allergen-specific IgE is available, DCs can further facilitate allergic immune responses with increased uptake of allergen-IgE complexes via FcεR1 (126, 127). However, there is evidence that DCs in asthma patients can have distinct functions in the context of ongoing allergic inflammatory responses. DCs express an $\alpha\gamma_2$ trimer form of FcεR1, lacking the signal-enhancing β-chain found in the tetrameric FcεR1 on basophils and mast cells (72, 128). However, IgE binding to the FcεR1 on DCs can still induce intracellular signals. As a result, reduced signaling via FcεR1 leads to a decrease in DC activation and allergen-specific immune responses (124). Furthermore, the expression level of FcεR1 on the surface of DCs is highly sensitive to the concentration of serum IgE (129), and anti-IgE treatment can thus decrease FcεR1 expression on DCs (123), as is also observed for mast cells and basophils (98, 121, 122). Therefore, it is plausible to postulate that clinical outcomes of anti-IgE treatment could also be associated with the biological functions of DCs in patients who received anti-IgE treatment. Modification of DC functions by anti-IgE could be one of the major mechanisms of action of anti-IgE for controlling allergic immune responses in asthma patients. Since monocytes are precursors of both DCs and macrophages, their expression of FcεR1 may also have significant potential to influence allergic inflammatory responses in asthma patients (130). However, the correlation between FcεR1 expression on DCs and serum IgE level is still debatable (131, 132).

In this study, we recruited adult patients (N=45) with moderate-to-severe asthma and treated them with anti-IgE (omalizumab). In 26 weeks of the treatment, we investigated the characteristics and changes of different subsets of blood DCs and monocytes. We than compared the characteristics of such APC subsets in patients who showed clinical improvement with those in patients who did not respond to the treatment. Additionally, we also compared characteristics of APC subsets from asthma patients with those from healthy controls (N=24).

Methods

Study Subjects

Described in methods section of Chapter Three

Study Design

Described in methods section of Chapter Three.

Determination of Responders versus Non-Responders

Described in methods section of Chapter Three.

Whole Blood Staining

Whole blood (200 μL) was stained with the indicated antibodies, and 50 μL/well of brilliant stain buffer (BD, California) for brilliant violet (BV) fluorochrome stability was added. Blood was lysed and cells were fixed with BD lysing solution (BD). Stained cells were analyzed on an LSR Fortessa flow cytometer (BD), and the results were analyzed with Flow Jo (TreeStar, Oregon). Detailed information for antibodies used in this study is summarized in Table 4.1. To count cell numbers, 20 μL of CountBright

absolute counting beads (Life Technologies, California) was added to each well. Cell counts (determined as number per μL) were calculated using the number of cell events (A) divided by the number of bead events (B) multiplied by the assigned number of counting beads added based on lot (C) divided by the volume of the sample (D):

$$\frac{A}{B} \times \frac{C}{D} = concentration\ of\ sample$$

Table 4.1. Antibodies used in DC/monocyte analysis

Marker	Clone	Company
Lin1		BD
HLA-DR	L243	BioLegend
CD11c	B-ly6	BD
CD123	9F5	BD
CD14	M5E2	BD
CD16	3G8	BD
CD2	RPA-2.10	BD
CD141 (BDCA-3)	AD5-14H12	Miltenyi Biotec
FcεR1α	CRA1	BioLegend
CCR7	G043H7	BioLegend
CD80	2D10	BioLegend
CD83	HB15e	BD
CD86	IT2.2	BioLegend
CD88	S5/1	BIoLegend
mIgG1	MOPC-21	BD/BioLegend
mIgG1	IS5-21F5	Miltenyi Biotec
mIgG2a	MOPC-173	BD/BioLegend
mIgG2b	MCP-11	BioLegend

Statistical Analysis

Two sample *t*-tests were used to assess the differences between responder status groups (Responder, Non-Responder, and Healthy Control) at each time point (weeks -1 and 0 for baseline and weeks 6, 14, and 26), as well as the difference between all baseline patients and HC. Paired *t*-tests were used to answer the question about differences from

baseline within each group as well as the differences between all baseline patients and all patients at each time point. All tests were conducted on $\log_2$ transformed data using R studio. Significance was set at $P<0.05$.

Results

Asthma Patients and Healthy Controls have Similar Frequencies of Blood mDCs

We measured the frequency of mDCs ($Lin^-HLA^-DR^+CD11c^+CD123^-$) (Fig. 4.1A) in the blood of healthy controls (N=21) and than compared them with those of asthma patients (N=45) at baseline as well as during treatment with anti-IgE (weeks 6, 14 and 26). As shown in Figure 4.1B and 4.1C left, there was no significant difference between the two groups of subjects in either percentage (Fig. 4.1B, left) or number (Fig. 4.1C, left) of blood mDCs. In addition, the frequency of blood mDCs in asthma patients was not significantly altered by treatment with anti-IgE.

Anti-IgE-Responders have a Higher Frequency of Blood mDCs Compared to Non-Responders at Baseline

We next analyzed the frequencies of blood mDCs in responders (N=34) and than compared them with those of non-responders (N=11) at baseline as well as during treatment. Interestingly, we found that responders have a greater percentage (Fig. 4.1B, right) and number (Fig. 4.1C, right) of blood mDCs than non-responders at baseline.

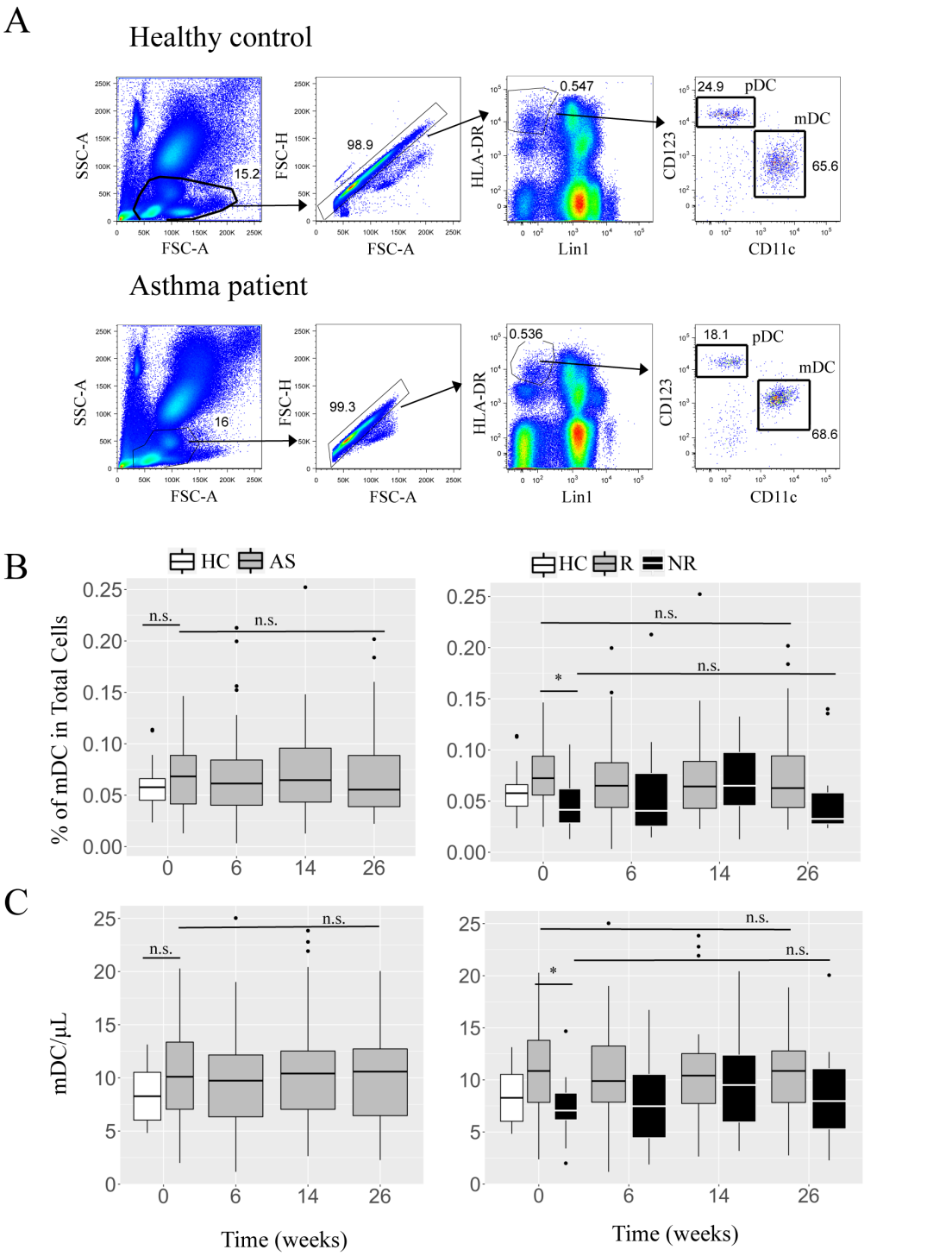


Figure 4.1. Anti-IgE responders have an increased frequency of blood mDCs at baseline that is not changed by treatment.
(A) Gating strategy for mDCs and pDCs after staining fresh whole blood. (B) Average percentage of mDCs in whole blood and (C) average number of mDCs per μL with standard deviation. The left panels of (B) and (C) indicate all patients over the time course of anti-IgE treatment, and the right panels indicate anti-IgE responders and non-responders over time. *P<0.05 and n.s.: not significant.

However, anti-IgE treatment did not significantly alter the frequencies of blood mDCs in either responders or non-responders over time (through week 26).

We thus concluded that the frequency of blood mDCs in moderate-to-severe adult asthmatic patients and healthy controls is similar, but patients who responded to anti-IgE treatment have increased an frequency of blood mDCs compared to non-responders at baseline, although anti-IgE treatment did not significantly alter the frequency of blood mDCs over time (through week 26).

Anti-IgE Treatment Decreases CCR7 and HLA-DR Expression on Blood mDCs, Particularly from Responders

Given our observations in Figure 4.1 that 1) patients and healthy controls have similar frequencies of blood mDCs and 2) anti-IgE treatment does not significantly alter the frequency of blood mDCs in patients, we further investigated surface expression levels of CCR7 and HLA-DR, which play key roles in DC migration into lymph nodes and in eliciting allergen-specific $CD4^+$ T cell responses, respectively.

As previously described (123), Figure 4.2A (left) shows that anti-IgE treatment resulted in a significant decrease of FcεR1α expression on blood mDCs. This decrease was observed from week 6 to week 26. The expression levels of FcεR1α on blood mDCs from asthma patients and healthy controls were similar at baseline. Figure 4.2A (right) further demonstrates that both responders and non-responders show decreases of FcεR1α expression over time during anti-IgE treatment. Interestingly, however, we found that anti-IgE treatment significantly decreased CCR7 expression on blood mDCs. This decrease was observed from week 6 to week 26 (Fig. 4.2B, left). More importantly, anti-IgE treatment significantly decreased CCR7 expression on blood mDCs from responders,

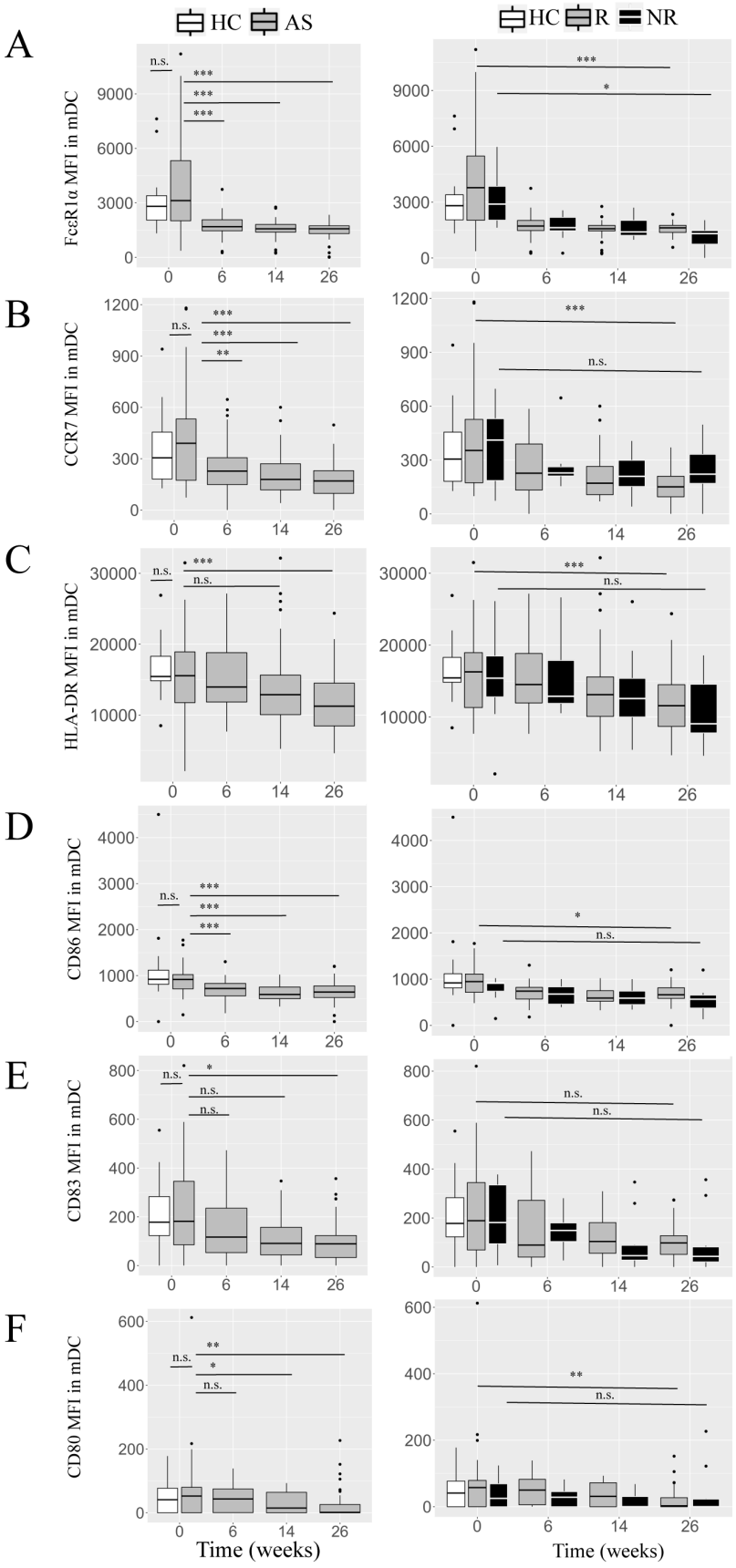


Figure 4.2. Anti-IgE treatment decreases CCR7, HLA-DR and co-stimulatory molecule expression in mDCs, particularly from anti-IgE responders.
(A-F) Average marker expression, determined as mean fluorescent intensity (MFI) in mDCs. The left panels indicate all patients over the time course of anti-IgE treatment, and the right panels indicate anti-IgE responders and non-responders over time. **P*<0.05, ***P*<0.01, ****P*<0.001, and n.s.: not significant. Error bars indicate SD.

while this was not significant in non-responders (Fig. 4.2B, right). In line with the changes in CCR7 expression, anti-IgE treatment also decreased HLA-DR expression on blood mDCs (Fig. 4.2C, left), and this decrease was also significant only in responders (Fig. 4.2C, right). We further found that anti-IgE treatment decreased the expression levels of costimulatory molecules CD86 (Fig. 4.2D), CD83 (Fig. 4.2E), and CD80 (Fig. 4.2F). Aside from CD83, these decreases were also more significant in responders than in non-responders (right panels in Fig. 4.2D and 4.2F).

Taken together, we concluded that anti-IgE treatment does not alter the frequency of blood mDCs (Fig. 4.1), but it can significantly decrease the activation of blood mDCs, particularly for CCR7 and HLA-DR expression in responders. Non-responders have decreased frequency of blood mDCs (Fig. 4.1), and these mDCs were more resistant to anti-IgE treatment for the downregulation of CCR7 and HLA-DR than mDCs from responders.

Asthma Patients have Decreased Numbers of Blood pDCs, which are not Altered by Anti-IgE Treatment

pDCs are one of the major subsets of DCs in the blood. They are the major type 1 IFN producers and are also known to promote regulatory T cell responses that can suppress inflammatory immune responses (133, 134). We thus assessed the frequency (percentage and numbers) of pDCs ($Lin^-HLA^-DR^+CD11c^-CD123^+$, Fig. 4.1) in the blood of healthy controls and asthma patients at baseline and during treatment with anti-IgE. As shown in Figure 4.3A (left) and 4.3B (left), asthma patients have a decreased number of blood pDCs at baseline when compared to healthy controls (Fig. 4.3B, left). We also observed that anti-IgE treatment resulted in a slightly increased frequency of pDCs in

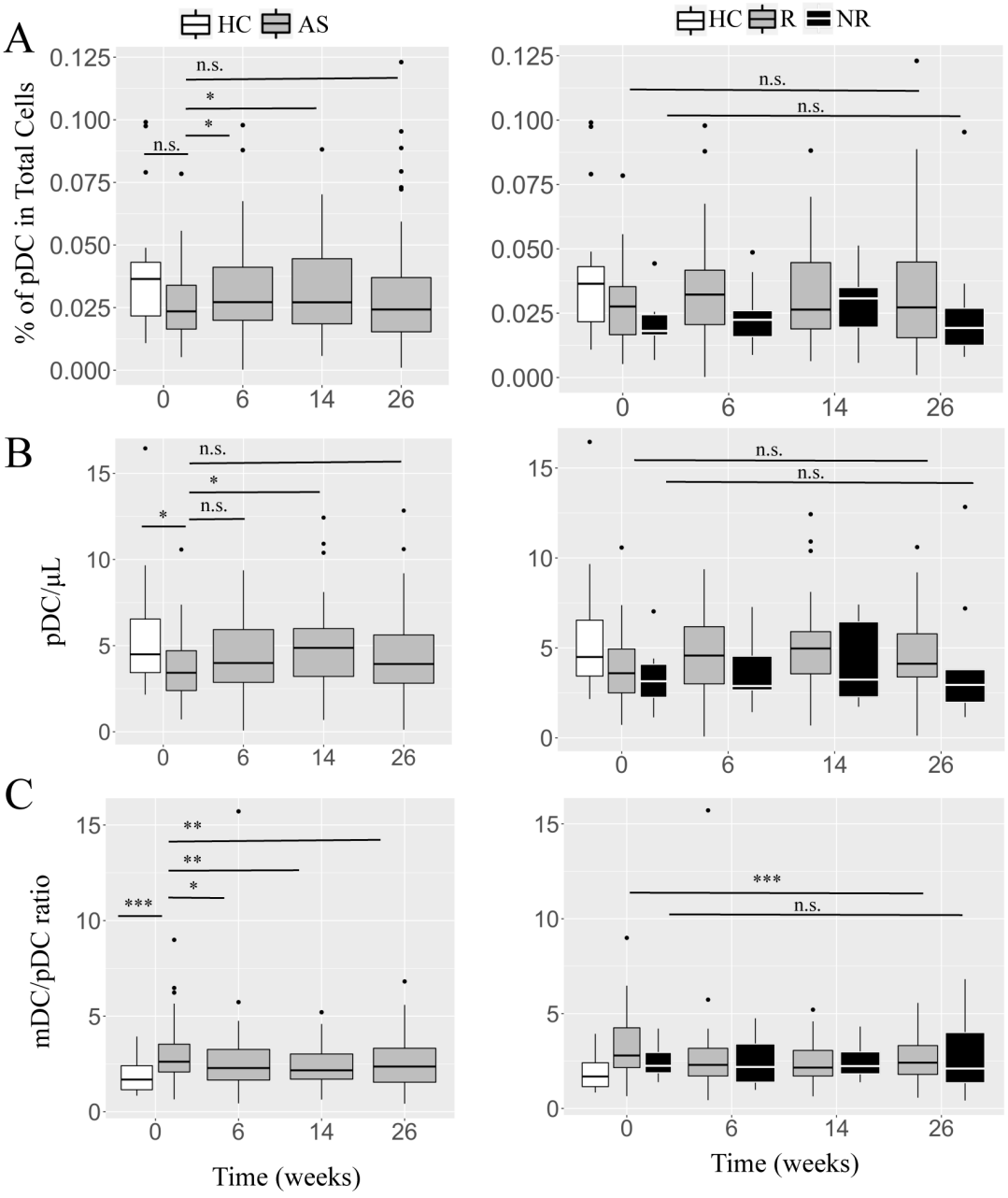


Figure 4.3. Asthma patients have an aberrant mDC/pDC ratio that is altered by anti-IgE treatment.

(A) Average percentage of pDCs in whole blood and (B) average number of pDCs per μL. (C) Average ratio of the percentage of mDC to pDC. The left panels indicate all patients over the time course of anti-IgE treatment and the right panels indicate anti-IgE responders and non-responders over time. **P*<0.05, ***P*<0.01, ****P*<0.001, and n.s.: not significant. Error bars indicate SD.

patient blood on week 6 (percentage in Fig. 4.3A, left) and week 14 (percentage in Fig. 4.3A and number in Fig. 4.3B, left panels). However, such changes were not observed on week 26. The percentage (Fig. 4.3A, right) and number (Fig. 4.3A, right) of pDCs in responders and non-responders were further analyzed. Although non-responders showed a slightly decreased average frequency of blood pDCs compared to responders, neither percentage nor number was significantly different between the two groups.

Since the mDC/pDC ratio has been shown to be altered in both atopic dermatitis (135) and chronic obstructive pulmonary disease (COPD) (136), we also compared this ratio between patient and healthy controls, before and with anti-IgE treatment. As shown in Figure 4.3C, there was a greater mDC/pDC ratio in patients than in healthy controls at baseline. With anti-IgE treatment, this ratio decreased, starting at week 6. Interestingly, there was no difference in the mDC/pDC ratio in non-responders between baseline and week 26; however, there was a decrease in mDC/pDC ratio in responders at week 26 compared with baseline.

Taken together, we concluded that moderate-to-severe adult asthma patients have decreased numbers of blood pDCs compared to healthy controls. In addition, the average number of blood pDCs was slightly lower in non-responders than responders, although this was not significant. However, anti-IgE treatment significantly increased the blood mDC/pDC ratio, and this was most significant in responders.

Anti-IgE Treatment Decreases Surface CCR7, HLA-DR, and Costimulatory Molecule Expression on Blood pDCs

pDCs are known to express FcεR1α (137). We further found that blood pDCs in asthma patients expressed greater levels of FcεR1α than those of healthy controls at

baseline (Fig. 4.4A, left). Anti-IgE treatment resulted in significant decreases of FcεR1α on pDCs from week 6 to week 26. In addition, such decreases of FcεR1α were similarly observed in both responders and non-responders (Fig. 4.4A, right). Consistant with the decreased expression of FcεR1α, CCR7 expression levels were also significantly lower from week 6 until week 26 when comparing all patients (Fig. 4.4B, left). Interestingly, however, when patients were divided into responders and non-responders, this decrease was significant only in responders (Fig. 4.4B, right). Similarly, anti-IgE treatment also decreased HLA-DR expression levels in asthma patients (Fig. 4.4C, left), largely due to the decreases in responders (Fig. 4.4C, right). We further found that anti-IgE treatment significantly decreased CD86 (Fig. 4.4D), CD83 (Fig. 4.4E), and CD80 (Fig. 4.4F) over time. Interestingly, the decreases in the expression of these costimulatory molecules were not always significant in non-responders.

We thus concluded that anti-IgE treatment results in the decreased expression of FcεR1α followed by a decreased activation of blood pDCs in asthma patients. In addition, such decreases were more significant in responders than in non-responders, although there was no significant difference in FcεR1α between responders and non-responders.

Asthma Patients have Increased Number of $CD14^+$ Blood Monocytes that are not Altered by Anti-IgE Treatment

Monocytes are also known to participate in inflammatory responses by not only presenting antigens, but also by producing multiple pro-inflammatory cytokines and chemokines. However, the frequency and functions of monocytes in the context of asthma patients have not been well characterized.

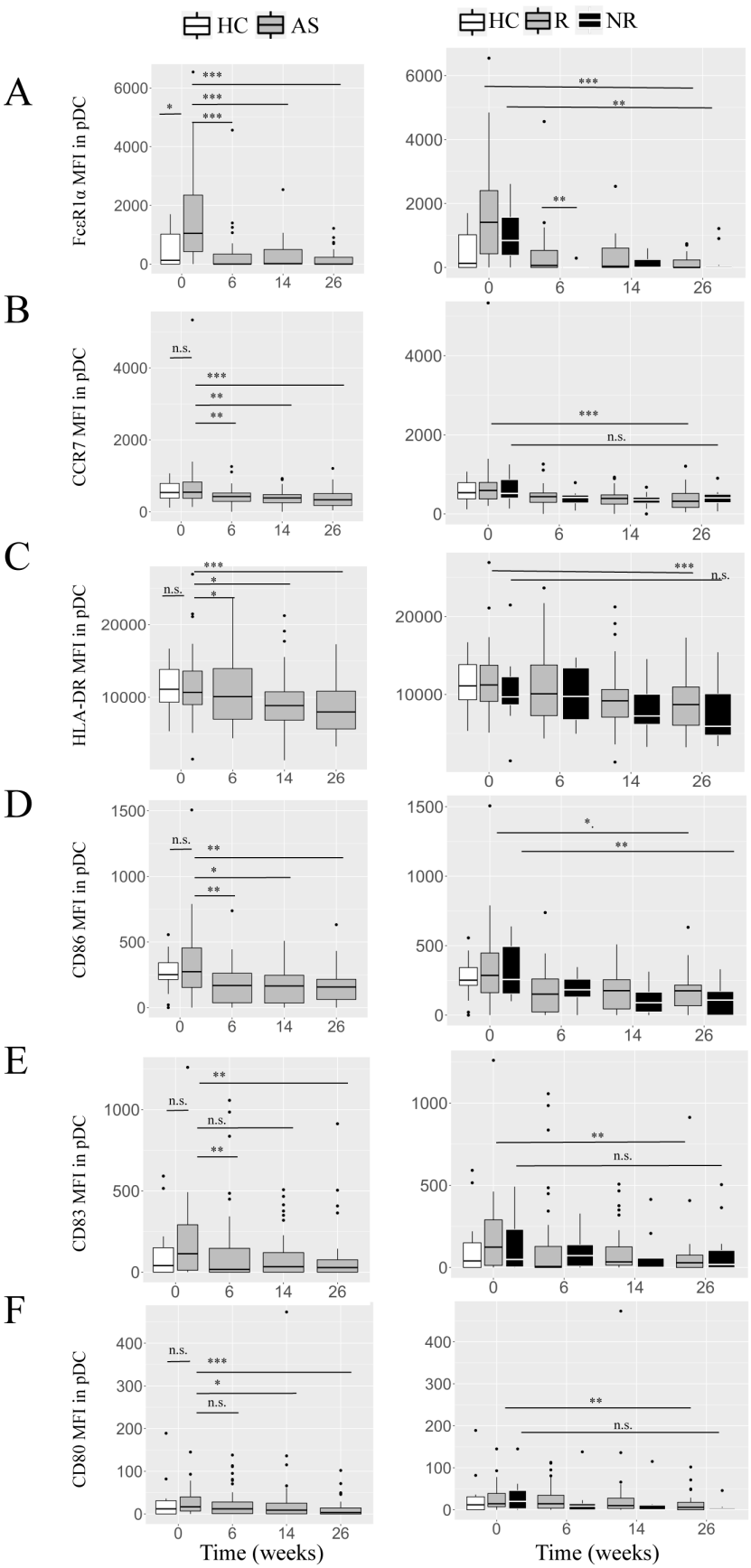


Figure 4.4. Anti-IgE treatment decreases CCR7, HLA-DR and co-stimulatory molecule expression in pDCs, particularly from anti-IgE responders. (A-F) Average marker expression, determined as mean fluorescent intensity (MFI) in pDCs. The left panels indicate all patients over the time course of anti-IgE treatment, and the right panels indicate anti-IgE responders and non-responders over time. **P*<0.05, ***P*<0.01, ****P*<0.001, and n.s.: not significant. Error bars indicate SD.

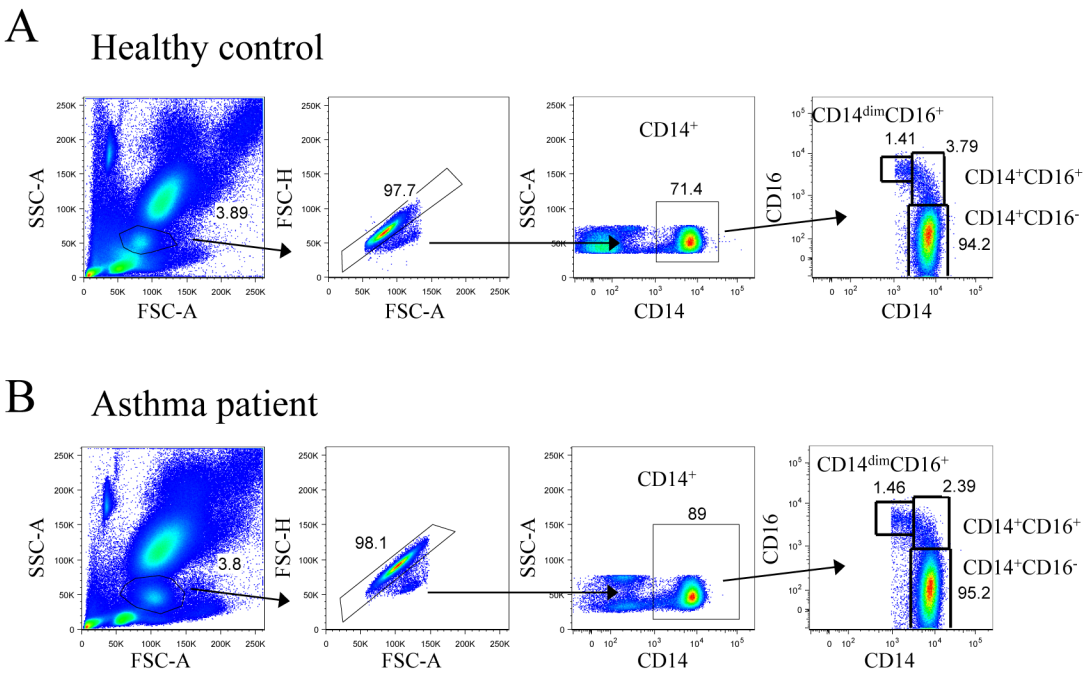


Figure 4.5. Gating strategy for blood monocyte subsets.
Whole blood myeloid cells were gated based on medium FSC and SSC, and singlets were removed using FSC-A vs. FSC-H. Total monocytes were identified as $CD14^+$ and than divided into three subtypes based on the expression levels of CD14 and CD16: 1) classical monocytes ($CD14^+CD16^-$), 2) intermediate monocytes ($CD14^+CD16^+$), and 3) non-classical monocytes ($CD14^{dim}CD16^+$).

Monocytes can be divided into three subtypes, depending on expression levels of CD14 and CD16 (Fig. 4.5): 1) classical monocytes ($CD14^+CD16^-$), 2) intermediate monocytes ($CD14^+CD16^+$), and 3) non-classical monocytes ($CD14^{dim}CD16^+$). $CD14^+CD16^-$ monocytes, the major fraction of blood monocytes, showed significantly increased percentage (Fig. 4.6A, left) and number (Fig. 4.6B, left) in patients compared with healthy controls. The $CD14^+CD16^+$ monocytes only showed an increase in their percentage (Fig. 4.6C, left) in patients, but no change in the number (Fig. 4.6D, left). There was no significant difference between patient and healthy controls in percentage or

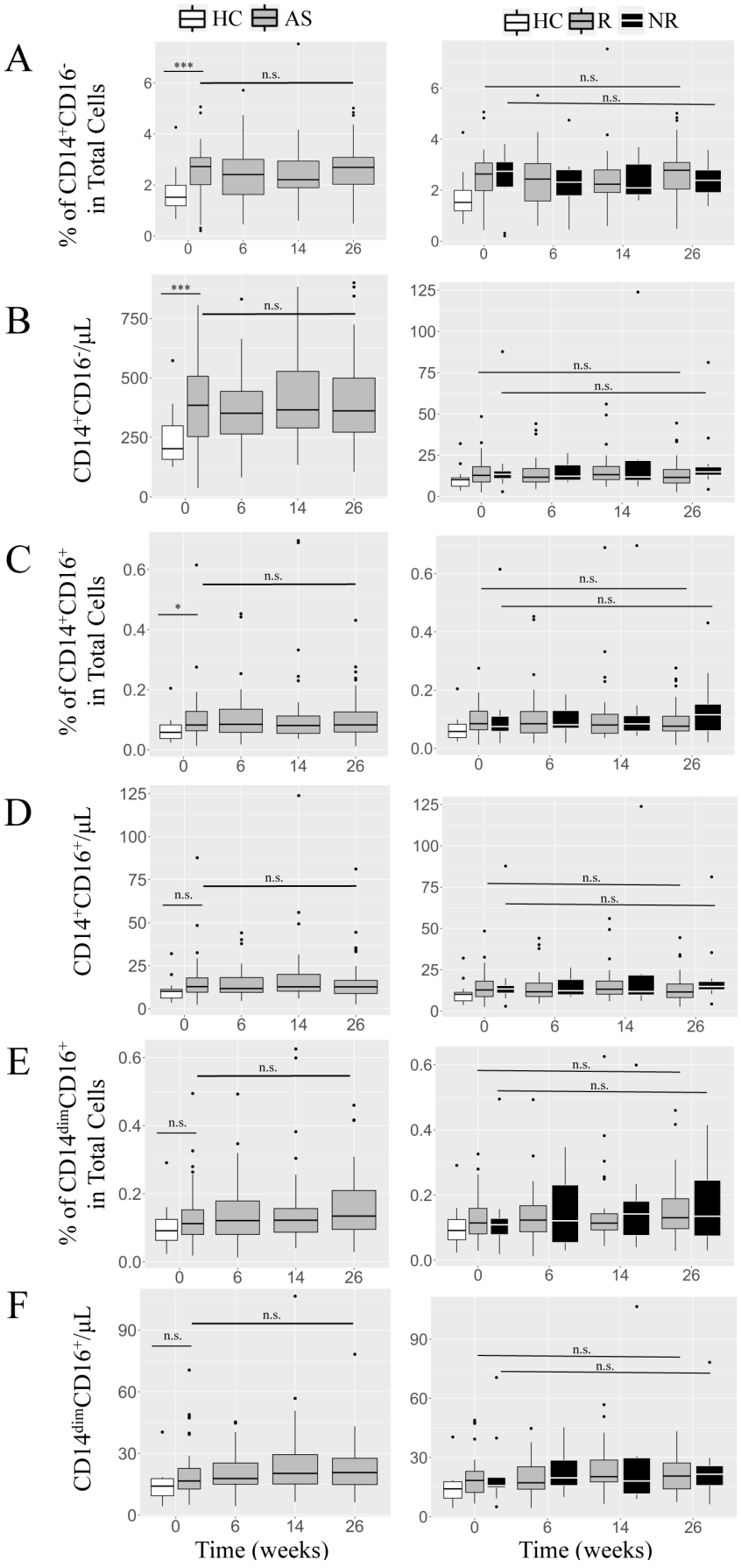


Figure 4.6. Asthma patients have increased peripheral blood monocytes and this alteration is not affected by anti-IgE treatment. (A) Average percentage and (B) number per μL of $CD14^{+}CD16^{-}$ monocytes in whole blood. (C) Average percentage and (D) number per μL of $CD14^{+}CD16^{+}$ monocytes in whole blood. (E) Average percentage and (F) number per μL of $CD14^{dim}CD16^{+}$ monocytes in whole blood. The left panels indicate all patients over the time course of anti-IgE treatment, and the right panels indicate anti-IgE responders and non-responders over time. *P<0.05, ***P<0.001, and n.s.: not significant. Error bars represent SD.

number of $CD14^{dim}CD16^{+}$ monocytes (Fig. 4.6E and 4.6F, left). No monocyte subset showed a change in population percentage or number with anti-IgE treatment (right panels, Fig. 4.6A-4.6F). There was also no difference between anti-IgE responders and

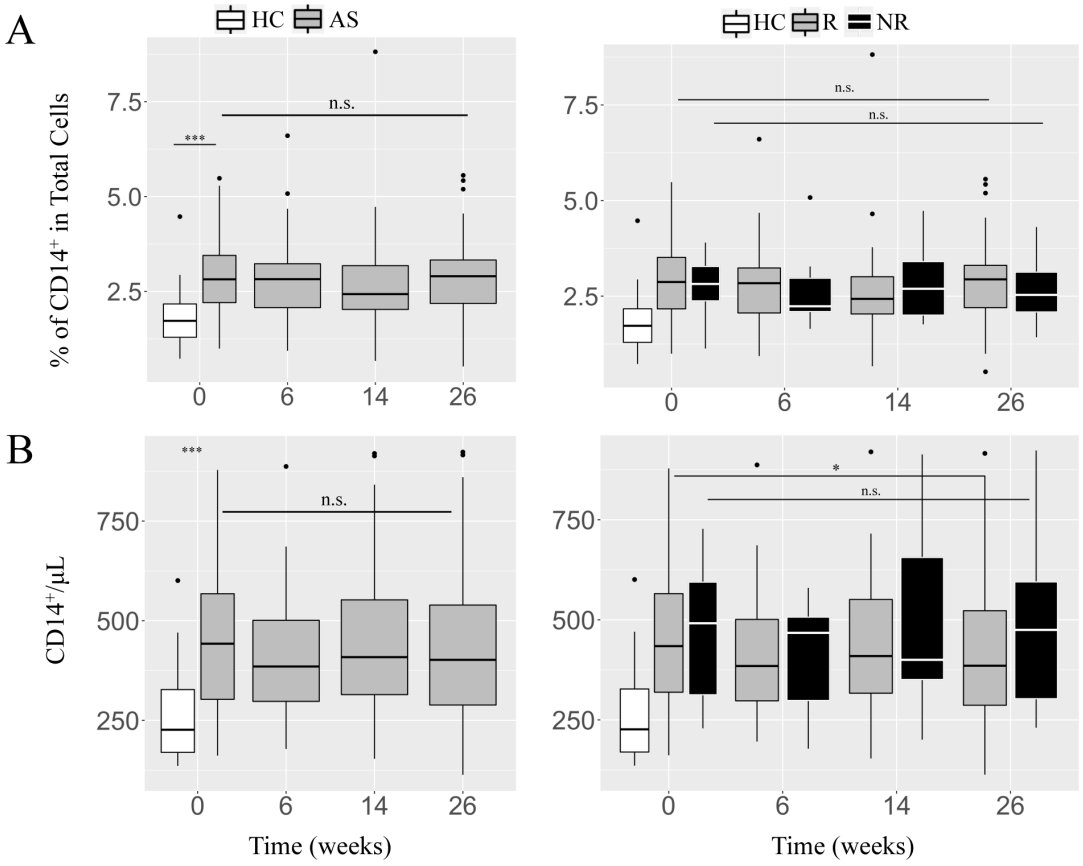


Figure 4.7. Asthmatic patients have increased frequency of total $CD14^{+}$ monocytes compared with healthy controls.
(A) Average percentage and (B) number per µL of $CD14^{+}$ monocytes in whole blood. *P<0.05, ***P<0.001, and n.s.: not significant. Error bars indicate SD.

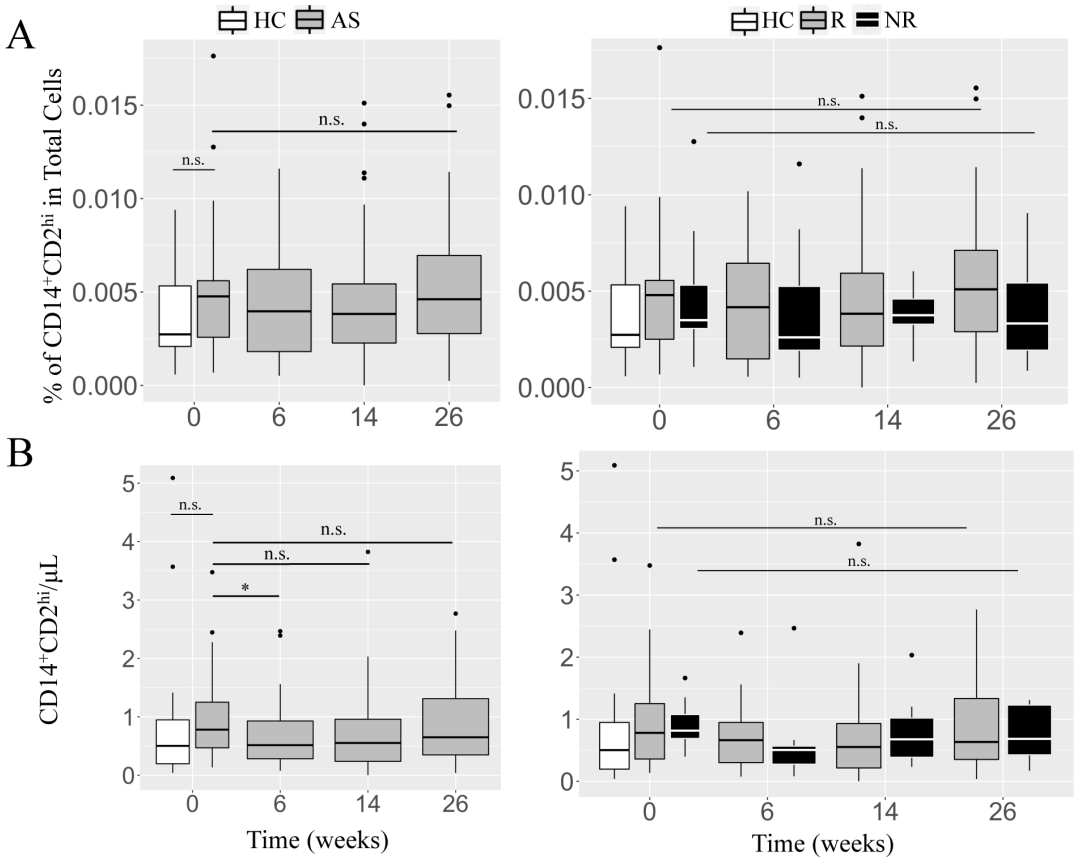


Figure 4.8. There is no difference in $CD14^{+}CD2^{high}$ monocytes between healthy controls and asthmatic patients.
(A) Average percentage and (B) number per μL of $CD14^{+}CD2^{high}$ monocytes in whole blood. **P*<0.05 and n.s.: not significant. Error bars indicate SD.

non-responders. We thus concluded that adult asthma patients have increased frequency of $CD14^{+}$ blood monocytes compared to healthy controls, as further demonstrated in Figure 4.7. However, there was no significant difference in the frequency of total and individual subsets of monocytes in the two groups of asthma patients, responders and non-responders. In addition, anti-IgE treatment did not significantly alter the frequency of

blood monocytes. Asthma patients (both responders and non-responders) and healthy controls also had similar frequencies of $CD14^{+}CD2^{high}$ monocytes in their blood (Fig. 4.8) that was not altered by anti-IgE treatment.

Anti-IgE Treatment Suppresses Blood Monocyte Activation in Asthma Patients

We next investigated the expression levels of FcεR1α, CCR7, HLA-DR, CD86, CD80, and the complement receptor C5a (CD88) on individual monocyte subsets at baseline and during anti-IgE treatment (Figs. 4.9-4.10).

The left panels in Figure 4.9A-4.9F show that $CD14^{+}CD16^{-}$ monocytes from asthma patients and healthy controls expressed similar levels of the surface molecules tested. By week 26, $CD14^{+}CD16^{-}$ monocytes (left panels, Fig. 4.9A-4.9F) showed significant reduction in the expression of FcεR1α, CCR7, HLA-DR, CD80, CD86, and CD88. Interestingly, however, when patients were broken up into responders and non-responders, only responders showed significant decreases in FcεR1α, CCR7, CD80 and CD88 expression in response to anti-IgE treatment (right panels, Fig. 4.9A, 4.9B, 4.9E, and 4.9F). Anti-IgE treatment decreased the expression levels of HLA-DR and CD86 in both asthma patients and healthy controls (right panels, Fig. 4.9C and 4.9D).

The left panels in Figure 4.10A-4.10F show that $CD14^{+}CD16^{+}$ monocytes from both asthma patients and healthy controls expressed similar levels of the surface molecules tested. However, anti-IgE treatment resulted in a significant reduction in the expression of CCR7, HLA-DR, CD86 and CD88 by week 26 (Fig. 4.10B, 4.10C, 4.10E and 4.10F, left panels). Anti-IgE treatment reduced surface FcεR1α expression only at week 6 (Fig. 4.10A, left), which could be due to the low levels of FcεR1α expressed on $CD14^{+}CD16^{+}$ monocytes prior to treatment. When we split patients into responders and

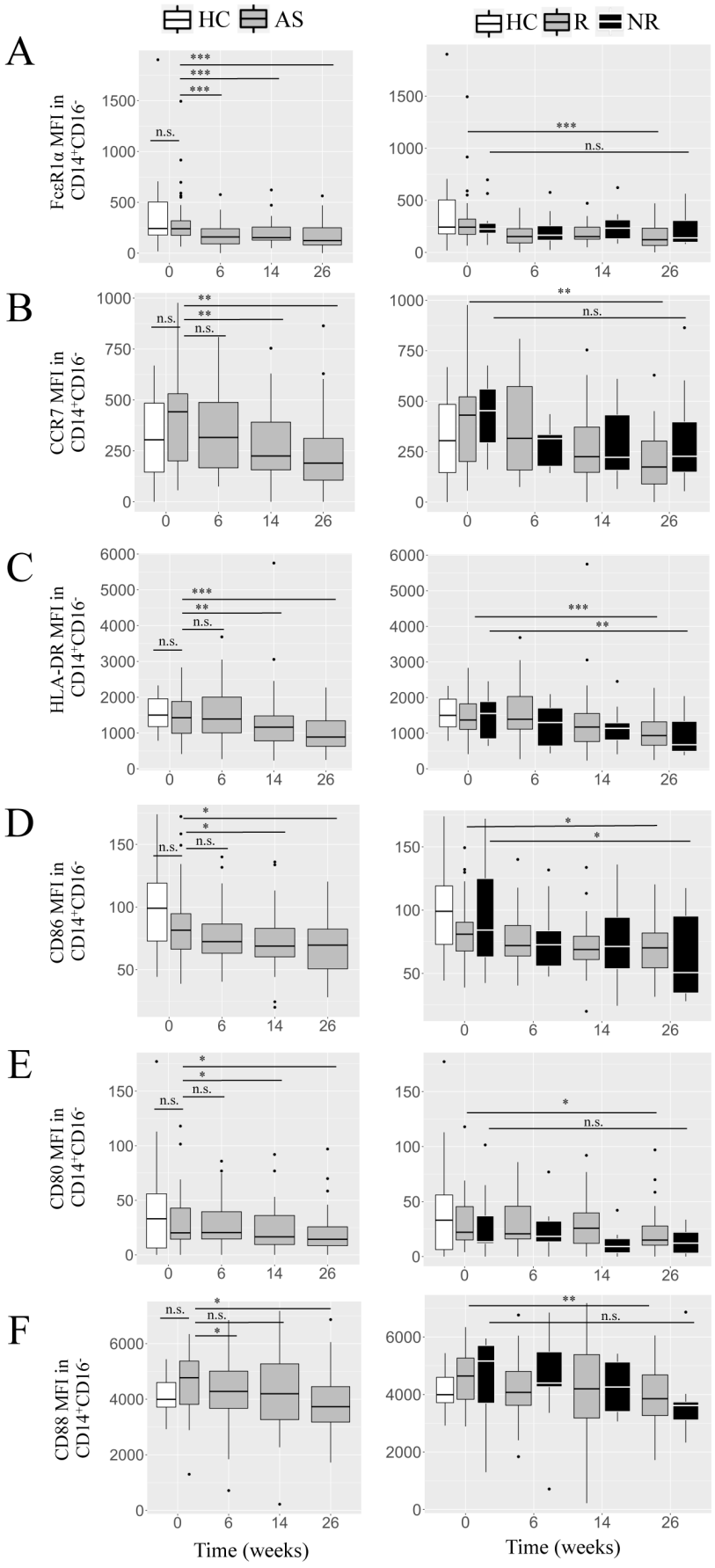


Figure 4.9. Anti-IgE treatment suppresses $CD14^{+}CD16^{-}$ blood monocyte activation in asthma patients. (A-F) Average marker expression, determined as mean fluorescent intensity (MFI) in $CD14^{+}CD16^{-}$ monocytes. The left panels indicate all patients over the time course of anti-IgE treatment, and the right panels indicate anti-IgE responders and non-responders over time. **P*<0.05, ***P*<0.01, ****P*<0.001, and n.s.: not significant. Error bars indicate SD.

non-responders, we observed that only responders exhibited a significant decrease in CCR7 (right, Fig. 4.10B) and CD88 (right, Fig. 4.10F). Both patient types showed a decrease in the expression of HLA-DR (right, Fig. 4.10C), however this decrease was more significant in responders than non-responder. Like $CD14^{+}CD16^{+}$ monocytes, $CD14^{dim}CD16^{+}$ monocytes showed a decrease in the expression of CCR7 and HLA-DR expression, however this was specific to responders (data not shown).

Taken together, we concluded that asthma patients have greater frequency of total monocytes ($CD14^{+}$) as well as certain subsets of monocytes ($CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$) in their blood than healthy donors. Anti-IgE treatment did not alter the frequency of any blood monocyte subsets. However, treatment did result in decreased activation of monocytes, as measured by the reduced expression of CCR7, HLA-DR, and CD88 (the C5a receptor).

Discussion

This study investigated characteristics of blood DC and monocyte subsets from moderate-to-severe adult asthma patients who received anti-IgE (omalizumab) treatment. We report for the first time that anti-IgE treatment results in significant decreases of CCR7 and HLA-DR expression, as well as decreased expression of key costimulatory molecules, on both DCs and monocytes. More importantly, the degree of such decreases was more significant in anti-IgE responders than non-responders. Interestingly, however, anti-IgE treatment did not significantly alter the frequency of the DC and monocyte subsets investigated, although patients and healthy controls do not always have similar frequencies of DC and monocyte subsets, particularly in pDCs and $CD14^{+}$ total

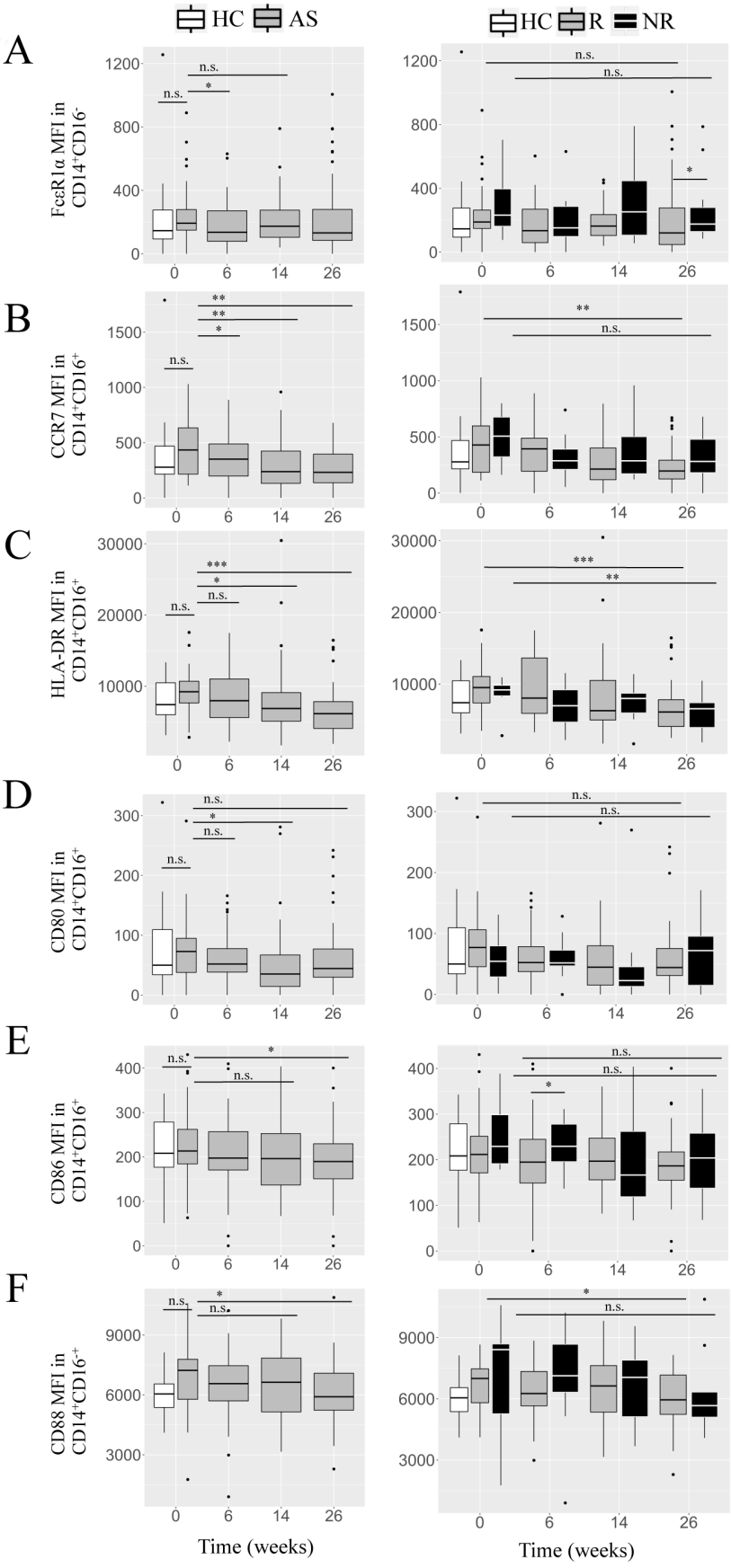


Figure 4.10. Anti-IgE treatment suppresses $CD14^{+}CD16^{+}$ blood monocyte activation in asthma patients. (A-F) Average marker expression, determined as mean fluorescent intensity (MFI) in $CD14^{+}CD16^{+}$ monocytes. The left panels indicate all patients over the time course of anti-IgE treatment, and the right panels indicate anti-IgE responders and non-responders over time. $*P<0.05$, $**P<0.01$, $***P<0.001$, and n.s.: not significant. Error bars indicate SD.

monocytes; patients and healthy controls also have different mDC/pDC ratios. This series of data strongly supports the idea that the reduction of APC (especially DC) functions followed by the decrease in allergic immune responses can be one of the major mechanisms of action of anti-IgE. Although DCs and monocytes in the periphery may not entirely be the same as those in the lower airway, they can reflect phenotypes and functions of the cells in the bronchial mucosa, owing to a "spillover" or circulation of these cells into the periphery (138-141).

The effects of anti-IgE treatment on the actions of APCs have been mainly explained by the decrease of surface FcεRI expression. This decrease is followed by the reduction of FcεR1-mediated internalization of IgE-bound allergens and thereby decreased allergen-specific T cell responses (124). Novak et al. (73) showed that APCs increase the efficiency of allergen presentation to T cells up to 1000-fold through an antigen focusing mechanism in which FcεRI-bound IgE on APCs selectively captures allergens (142). Alternatively, crosslinking of FcεRI on APCs may also induce expression of proinflammatory mediators and chemokines (73, 143). As previously described (123, 130), anti-IgE treatment resulted in a significant decrease of FcεR1α expression on both DCs (123) and $CD14^+$ monocytes (130) from asthma patients, although individual subsets of DCs and monocytes from asthma patients express different levels of surface FcεR1α. Most importantly, we found that anti-IgE treatment resulted in a greater decrease of CCR7 and HLA-DR expression on DCs from anti-IgE responders than non-responders, which were identified based on a number of clinical parameters, including ACT scores. However, because non-responders comprised of a small subset of all the asthmatic patients, their lack of change in receptor expression by anti-IgE is

hidden when the effects of anti-IgE are analzyed across all patients. Both CCR7 and HLA-DR can play critical roles in magnifying allergen-specific T cell responses by enhancing DC migration into the lymph nodes and by presenting allergen-derived peptide antigens to T cells, respectively. In line with this, APCs in anti-IgE responders were more sensitive than those in non-responders to anti-IgE treatment at the downregulation of surface expression of costimulatory molecules (CD86, CD83, and CD80). However, it is of note that responders and non-responders have similar expression levels of such molecules at baseline (before anti-IgE treatment). In addition, the expression levels of such molecules at baseline were not significantly different from those of healthy controls. Taken together, these data suggest that the intrinsic properties of DCs and monocytes in anti-IgE responders and non-responders may not be the same. This re-emphasizes the heterogeneity of the pathogenesis of human asthma and more studies for addressing this question are needed.

This study also reports that anti-IgE responders had a significantly increased frequency of blood mDCs than non-responders at baseline, while there was no difference when we compared the frequency of mDCs from all patients with that of healthy controls. This was confirmed in both percentage and number. The number of pDCs was lower in asthma patients than healthy controls at baseline, although a previous study showed that asthma patients have an increased frequency of blood pDCs than healthy subjects (144, 145). However, it is important to note that corticosteroids can significantly reduce circulating pDC numbers (146, 147), and the majority of asthma patients recruited in this study was being treated with corticosteroids at baseline. Anti-IgE treatment resulted in the increase of pDCs on week 14, but it did not last by week 26. We have also observed

that the average percentage and number of pDCs were lower in anti-IgE non-responders than in responders; however, these differences were not statistically significant. Future studies with increased numbers of patients might be necessary to confirm these data. In line with the decreased frequency of pDCs (number) in asthma patients, mDC/pDC ratios were higher in asthma patients than in healthy controls. This ratio was significantly reduced in responders only, which further supports the idea that the intrinsic functions of DCs in anti-IgE responders may not be the same to those of non-responders. In this study, we have also assessed the frequency of $CD141^+$ DCs, which were reported to be higher in asthma patients (145, 148) than healthy controls, although there has also been a report that their numbers are decreased in asthma subjects (149) and that they are also known to polarize Th2-type T cell responses (150). However, asthma patients (neither anti-IgE responders nor non-responders) did not have an altered frequency of $CD141^+$ DCs. Frequency and cell surface phenotypes of $CD141^+$ DCs measured in this study were not significantly altered by anti-IgE treatment (data not shown).

Alternatively, we found that asthma patients have a significantly increased frequency of $CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes, but not $CD14^{dim}CD16^+$ monocytes, when compared with healthy controls at baseline. Inflammatory conditions usually lead to an increase in $CD16^+$ monocytes (151). The lack of increase in $CD14^{dim}CD16^+$ monocytes in asthma patients could be due to corticosteroid treatment, which can deplete $CD16^+$ monocytes (152), as it does to pDCs. It is also important to note that anti-IgE treatment decreased CD88 expression levels on monocytes. CD88 is the receptor for the complement C5a and can act as a chemotactic in monocytes (153). The decrease in CD88 expression across all monocyte subtypes with anti-IgE treatment

may reflect a decrease in their capacity to migrate to sites of inflammation, similar to their decrease in CCR7, which limits their passage to the lymph nodes. While CD88 expression on monocytes has been shown to decrease with the addition of C5a (154), it is unlikely that anti-IgE treatment is increasing C5a levels. Thus, the reduction in CD88 expression may reflect a novel regulation of CD88 through the reduction of IgE. It has been shown in skin DCs that activation increases CD88 expression (155); therefore, the reduction in CD88 expression on monocytes may also represent a decrease in their activation status, similar to the reduction in CD80, CD86 and HLA-DR expression on monocytes.

In summary, this study highlights that anti-IgE (omalizumab) treatment can result in a significant decrease of APC (DC and monocyte) functions, particularly by the downregulation of CCR7 and HLA-DR, as well as decreases in the expression of costimulatory molecules. Such decreases are more significant in anti-IgE responders than non-responders, indicating that APCs in the two groups of patients have distinct intrinsic functions that need to be further studied in the future. In addition, the decreased frequency of blood mDCs in non-responders compared to responders at baseline may be a biomarker that could eventually allow us to predict responders and non-responders before the initiation of anti-IgE treatment, although more biomarkers are needed to enhance the accuracy of response prediction.

CHAPTER FIVE

Increased $CD23^+IgG1^+$ Memory B Cells and Plasmablasts and Decreased $CD5^+$ Transitional B Cells are Hallmarks of Peripheral B Cells in Asthmatic Adults

Abstract

The traditional role of B cells in asthma is to produce IgE, which is a prerequisite for allergic inflammation in the lung. However, recent evidence suggests that different subsets of B cells may have distinct functions in the pathogenesis of asthma. To investigate whether the compositions and phenotypes of B cell subsets in asthmatic patients are associated with the pathogenesis of asthma, we analyzed B cell subsets in the blood of moderate-to-severe adult asthmatic patients (N=45) and than compared them with those of healthy controls (N=21). We further investigated whether any alterations of B cell subsets in patients was associated with clinical variables of disease severity. Both patients and healthy subjects have a similar frequency of the majority of B cell subsets investigated (including naïve, memory, transitional, marginal zone-like, IgD^-CD27^-, B1, B10, plasmablasts with surface immunoglobulins, and plasma cells). However, asthmatic patients showed significant increases (>2-fold on average) of $CD23^+$ B cells, including memory B cells and plasmablasts, that express surface IgG1. Such alterations of $CD23^+$ B cells in patients strongly correlated with serum IgE levels and clinical variables of disease severity (ACT score, β-agonist usage per week, nights woken by asthma per week, and FVC% of predicted). Although there was no significant difference in total transitional B cells ($CD27^{high}CD38^{high}$), asthmatic patients had a significant decrease in transitional B cells co-expressing CD5; however this did not correlate with any clinical

variables. Asthmatic patient have increased $CD23^{+}IgG1^{+}$ plasmablasts and memory B cells and decreased $CD5^{+}$ transitional B cells. In addition, B cells expression CD23 are highly correlated with disease severity. We also observed effects of anti-IgE treatment on patient B cell populations, both over time and between anti-IgE responders and non-responders. We noted a reduction of memory B cells and increase in transitional B cells by anti-IgE. However, there were few differences between anti-IgE responders and non-responders, either before treatment or at week 26 of anti-IgE treatment.

Introduction

B cells play a central role in the pathogenesis of asthma by producing IgE in response to inhaled allergens. Allergen-specific B cells undergo isotype-switching to produce IgE in the presence of Th2-type cytokines, particularly IL-4 and IL-13(156). The affinity of IgE to its antigen is critical in determining the fate of cells that express IgE receptors. It has been shown in the murine model that the generation of a high affinity IgE is required for indirect sequential class switching from IgM to IgG1 to IgE. Upon subsequent antigen encounter, IgE class-switching can be achieved by IgG1-expressing memory B cells, as well as IgM-expressing memory B cells (71). Crosslinking of receptor-bound IgE on mast cells and basophils, where the high affinity IgE receptor (FcεRI) are present, induces the activation and degranulation of these cells, leading to the release of pro-inflammatory mediators (72).

B cells ($CD19^{+}$) develop in the bone marrow and enter the peripheral blood as immature/transitional cells, defined as $CD24^{high}CD38^{high}$. A large fraction of immature B cells express the B cell progenitor marker CD10, which is downregulated as they mature.

Although these immature/transitional B cells may have the capacity to act in a regulatory manner in several murine models of chronic inflammation (157), much is still unknown about this particular subset of B cells in humans. When B cells mature, they lose CD24 and begin to express IgD (158). As they encounter antigens, along with the presence of other activation signals, they can gain a memory phenotype and express CD27 in conjunction with additional antibody class-switching, although a fraction of memory B cells are $CD27^{-}$ (159) Through additional activation, these B cells can also differentiate into antibody secreting plasmablasts and plasma cells. Both of these subsets express CD38, but plasma cells will also express CD138. Apart from conventional B cells, B1 cells, considered innate or natural B cells, express CD27 and CD43 and constitutively produce natural IgM in the peritoneal cavity (160). Recently, specific subsets of B cells that exert immunosuppressive function by producing IL-10 have been identified. They are termed regulatory B cells (Bregs) or B10 cells (161) and are classified as $CD24^{high}CD27^{+}$ B cells (162). Although phenotypes and functions of human Bregs still need to be further characterized, it is generally agreed that fractions of human Bregs can also express CD24, CD27, CD38, CD1d and CD5 (163). While almost all human B cells have the capacity to produce IL-10, the "most efficient" IL-10–producing B cells are Bregs, and they are similar to $CD24^{high}CD38^{high}$ transitional B cells (157, 164). Associations between $CD24^{high}CD38^{high}$ B cells and immune regulation were noted in the favorable clinical outcome of patients with chronic inflammatory and autoimmune diseases (165). However, the roles of such B cell subsets in the pathogenesis of human asthma still remain controversial (166-169). To this end, we hypothesized that the compositions and phenotypes of B cell subsets in asthma patients could be either

positively or negatively associated with the pathogenesis of asthma. We also hypothesized that the neutralization of free IgE through anti-IgE (omalizumab) treatment may alter B cell populations, possible to become more similar to healthy donors. To test this hypothesis, we characterized subsets of B cells in the blood of asthma patients and compared them with those of healthy subjects. We investigated whether any altered compositions of B cell subsets was associated with clinical variables of disease severity in asthmatic patients. We also compared B cell populations before and at week 26 of anti-IgE treatment and between anti-IgE responders and non-responders in these asthma patients.

Methods

Study Subjects

Described in methods section of Chapter Three.

Study Design

Described in methods section of Chapter Three.

Determination of Responders versus Non-Responders

Described in methods section of Chapter Three.

Whole Blood B Cell Staining

Described in methods section of Chapter Four. Detailed information for antibodies used in this study is summarized in Table 5.1.

Table 5.1. Antibodies used for B cell analysis

Marker	Clone	Company
CD19	HIB19	BD
CD20	2H7	BioLegend
CD24	ML5	BD
CD27	O323	BioLegend
CD38	HIT2	BD
CD138	MI15	BD
CD1d	51.1	BioLegend
CD5	UCHT12	BD
CD23	M-L233	BD
CD10	HI10a	BioLegend
CD11b	ICRF44	BD
CD43	1G10	BD
CD86	IT2.2	BioLegend
IgA	N/A (polyclonal)	Southern Biotech
IgD	IA6-2	BD
IgE	MB10-5C4	Miltenyi BioTec
IgG	G18-145	BD
IgG1	HP6001	Southern Biotech
IgM	G20-127	BD
live/dead	N/A	Invitrogen

PBMC Isolation and Staining

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Plaque PLUS (GE Healthcare, Pennsylvania). For surface immunoglobulin staining, $1x10^6$ PBMCs were incubated with fluorochrome-labeled antibodies for 30 min at 4°C. All stained cells were then fixed and stored in 200 μL of PBS (Life Technologies) with 1% paraformaldehyde (Sigma, Missouri) before running on an LSR Fortessa flow cytometer (BD).

Statistical Analysis

Described in methods section of Chapter Four. The sample correlations between the flow variables and the patient clinical outcomes were calculated using Spearman's rho. All correlations were processed through JMP Genomics software (SAS, North Carolina).

Results

No Significant Alteration of Naïve, Memory, or Activated B Cells, or of Plasmablasts or Plasma Cells in Asthmatic Patients

To investigate whether asthma patients have altered compositions of peripheral B cell subsets, we measured both percentages and numbers of a variety of B cell subsets defined based on the expression of known surface markers. Both asthma patients and healthy subjects had a similar proportion and number of $CD19^+$ total B cells in the blood as well as in PBMCs (Table 5.2). Depending on IgD and CD27 expression, B cells were divided into four subsets: (1) IgD^+CD27^-; naïve, (2) IgD^-CD27^+; memory, (3) IgD^+CD27^+; marginal zone-like and IgD memory, and (4) IgD^-CD27^-; double negative and switched memory. Both asthma patients and healthy subjects showed similar proportions and numbers of each of these four subsets of B cells. There was also no significant alteration in either plasmablasts ($CD20^-CD38^+$) or plasma cells ($CD138^+$) in patients when compared to healthy subjects. In line with these observations, the two groups of subjects had similar proportions and numbers of activated B cells, as assessed by staining with anti-CD20, anti-CD86, anti-CD38, and anti-CD27 antibodies. We thus concluded that asthmatic patients do not have significantly altered B cells, in terms of frequencies of $CD19^+$ total B cells, B cell subsets divided based on IgD and CD27 expression, plasmablasts, or plasma cells.

Table 5.2. Analysis of the frequencies and numbers of naïve and memory B cells, plasmablasts, and plasma cells in the blood of adult asthma patients and healthy subjects

Variables	Frequency			Cells/μL		
	Healthy	Patients	*p*-value	Healthy	Patients	*p*-value
$CD19^+$ total B cells **in Whole Blood**	0.987 (±0.52)	1.185 (±0.85)	0.3074	161.1 (±78.34)	189.6 (±137.1)	0.38
$CD19^+$ total B cells **in PBMCs**	2.636 (±1.61)	3.297 (±2.25)	0.2217	134.4 (±91.92)	151.9 (±135.7)	0.6183
In total B cells						
Naïve, memory, and other B cells						
IgD^+CD27^-	61.68 (±12.21)	59.07 (±18.45)	0.5483	84.43 (±64.56)	95.61 (±116.4)	0.7037
IgD^-CD27^+	14.78 (±8.41)	17.62 (±11.15)	0.2948	19.06 (±14.21)	23.89 (±18.99)	0.3375
IgD^+CD27^+	18.45 (±6.47)	18.54 (±8.95)	0.9657	23.75 (±17.51)	25.32 (±20.44)	0.7767
IgD^-CD27^-	5.085 (±4.12)	4.724 (±2.42)	0.6516	7.127 (±7.62)	7.109 (±8.13)	0.9935
Plasmablasts						
$CD38^+CD20^-$	1.501 (±1.30)	1.863 (±2.81)	0.5612	1.683 (±1.11)	2.948 (±4.11)	0.172
Plasma cells						
$CD138^+$	1.202 (±0.91)	1.091 (±1.28)	0.7128	1.954 (±1.80)	1.651 (±1.43)	0.4677
Activated B cells						
$CD20^-$	3.573 (±4.27)	3.218 (±3.81)	0.7302	3.425 (±4.30)	2.796 (±2.39)	0.4786
$CD86^+$	32.93 (±19.65)	31.94 (±20.30)	0.8721	44.05 (±30.76)	66.36 (±101.1)	0.4258
$CD27^+$	26.36 (±9.57)	28.23 (±15.93)	0.6082	43.54 (±29.39)	47.25 (±36.71)	0.6884
$CD27^+CD38^+CD86^+$	1.856 (±1.63)	1.263 (±1.14)	0.1493	2.288 (±2.74)	2.256 (±3.09)	0.9742

Values represent mean with standard deviations

Alterations in Subsets of Transitional/Immature B Cells in Asthmatic Adults

We next investigated the frequencies and numbers of transitional B cells ($CD24^{high}CD38^{high}$), which also contain Bregs and B10 cells. As shown in Table 5.3, there was no significant alteration in transitional/immature B cells in asthma patients when compared to those of healthy subjects. In addition, both asthma patients and healthy subjects had a similar range of $CD24^{high}CD38^{high}CD27^+$ B cells. Transitional/immature B cells were further divided into five groups based on CD5, CD1d, co-expression of CD5 and CD1d, CD10, and co-expression of CD5 and CD10. We found that asthma patients had a decreased frequency of $CD24^{high}CD38^{high}$ transitional B cells that express CD5.

However this was not the case for the frequency of transitional B cells expressing either CD1d or CD10. In addition, the frequency of transitional B cells co-expressing both CD5 and CD1d were also lower in patients, while the frequency of total B cells expressing CD5 and/or CD1d were similar in the two groups of subjects. Decreased frequencies of $CD24^{high}CD38^{high}CD5^{+}CD10^{+}$ and $CD5^{+}CD10^{+}$ B cells in asthma patients were also observed, although both asthma patients and healthy subjects showed similar frequencies of transitional B cells that co-expressed CD10, a B cell progenitor marker (170).

Further analysis of transitional B cells revealed that asthma patients had a decreased frequency of $CD5^{+}$ cells within the transitional B cell population. Interestingly, however, they had an increased frequency of $CD27^{+}$ cells within the transitional B cell subsets when compared to healthy subjects. There was no significant alteration in either B10/Bregs ($CD19^{+}CD24^{high}CD27^{+}$) or B1 ($CD19^{+}CD20^{+}CD27^{+}CD43^{+}$) B cells in asthma patients. We thus concluded that adult asthmatic patients have a decreased frequency of transitional/immature B cells that co-express CD5, and an increased frequency of $CD27^{+}$ B cells within transitional/immature B cells when compared to those of healthy subjects.

Asthmatic Patients show Increases of $CD23^{+}$ B Cells Expressing Surface IgG1 and IgE

To assess the frequencies and numbers of B cells expressing different Igs on their surface, B cells in PBMCs were stained with anti-IgD, anti-IgM, anti-IgG, anti-IgA, anti-IgG1 and anti-IgE antibodies. As shown in Table 5.4, both asthma patients and healthy subjects had similar frequencies and numbers of B cells that expressed individual Igs on their surface. This applied to total $CD19^{+}$ B cells and memory B cells as well as to plasmablasts. B cells do not express the high-affinity IgE receptor (FceRI), but fractions

Table 5.3. Analysis of the frequencies and numbers of transitional/immature B cells in the blood of adult asthma patients and healthy subjects

Variables	Frequency			Cells/μL		
	Healthy	**Patients**	***p*-value**	**Healthy**	**Patients**	***p*-value**
In total $CD19^+$ B cells						
Transitional/immature B cells						
$CD24^{hi}CD38^{hi}$	3.592 (±1.69)	3.791 (±2.88)	0.7607	5.931 (±3.91)	8.265 (±13.42)	0.4397
$CD24^{hi}CD38^{hi}CD27^+$	0.2848 (±0.16)	0.3636 (±0.27)	0.2061	0.4619 (±0.33)	0.6638 (±0.64)	0.1817
$CD24^{hi}CD38^{hi}CD5^+$	**1.700 (±0.98)**	**1.116 (±0.87)**	***0.0159**	2.189 (±1.61)	1.950 (±2.23)	0.6909
$CD5^+$	18.67 (±7.85)	17.23 (±9.70)	0.5414	29.60 (±20.30)	37.25 (±55.50)	0.5448
$CD24^{hi}CD38^{hi}CD1d^+$	2.35 (±1.02)	1.95 (±1.19)	0.1826	2.92 (±2.20)	3.36 (±2.96)	0.5862
$CD1d^+$	65.74 (±17.36)	64.42 (±18.57)	0.7808	104.8 (±55.55)	125.1 (±95.89)	0.3737
$CD24^{hi}CD38^{hi}CD5^+CD1d^+$	**1.47 (±0.81)**	**1.00 (±1=0.80)**	***0.0279**	1.83 (±1.49)	1.90 (±2.17)	0.9088
$CD5^+CD1d^+$	10.73 (±4.97)	10.26 (±6.72)	0.7676	17.24 (±13.64)	25.63 (±22.94)	0.1281
$CD24^{hi}CD38^{hi}CD10^+$	0.1922 (±0.204)	0.2428 (±0.427)	0.6437	0.1922 (±0.2041)	0.2428 (±0.4268)	0.6437
$CD10^+$	2.103 (±1.02)	1.930 (±0.79)	0.4477	2.852 (±2.33)	2.821 (±2.33)	0.9634
$CD24^{hi}CD38^{hi}CD5^+CD10^+$	**0.0702 (±0.055)**	**0.0466 (±0.040)**	***0.0498**	0.0923 (±0.086)	0.0746 (±0.082)	0.4657
$CD5^+CD10^+$	**0.9043 (±0.48)**	**0.6656 (±0.37)**	***0.0282**	1.242 (±0.93)	0.9769 (±0.82)	0.2861
In Transitional B Cells						
$CD5^+$	**58.65 (±17.71)**	**45.01 (±21.43)**	***0.0118**	NA	NA	
$CD1d^+$	84.66 (±8.09)	83.57 (±7.83)	0.5964	NA	NA	
$CD10^+$	4.88 (±3.83)	8.66 (±8.90)	0.062	NA	NA	
$CD27^+$	**22.32 (±15.42)**	**35.31 (±24.39)**	***0.0259**	NA	NA	
In total $CD19^+$ B cells						
B10 cells						
$CD24^{high}CD27^+$	19.04 (±7.91)	20.70 (±14.17)	0.6052	32.14 (±23.72)	34.96 (±30.80)	0.7137
In B10 cells						
$CD5^+$	7.131 (±3.92)	8.621 (±4.47)	0.1871	2.690 (±2.10)	3.844 (±3.82)	0.2479
$CD1d^+$	90.19 (±6.05)	88.67 (±6.67)	0.371	28.49 (±16.34)	41.63 (±41.72)	0.216
$CD10^+$	1.84 (±1.34)	2.00 (±1.13)	0.6252	0.5408 (±0.3514)	0.8318 (±0.9849)	0.2428
In total $CD19^+$ B cells						
B1 B cells						
$CD20^+CD27^+CD43^+$	6.56 (±5.25)	6.54 (±7.92)	0.9921	10.08 (±15.34)	7.50 (±5.98)	0.3615
$CD20^+CD27^+$	26.36 (±9.57)	28.23 (±15.93)	0.6082	43.54 (±29.39)	47.25 (±36.71)	0.6884
$CD20^+CD43^+$	11.98 (±16.50)	9.67 (±9.56)	0.4709	22.52 (±52.39)	17.48 (±29.66)	0.6458

Values represent mean with standard deviations. *P<0.05
NA: not assessable

of them do express the low-affinity IgE receptor (CD23), which may be linked to the pathogenesis of asthma (171-173).

We thus investigated the frequencies and numbers of B cells expressing CD23. As shown in Table 5.5, asthma patients had significantly more $CD23^+$ B cells per μL when compared to healthy subjects. A similar trend was also observed in memory B cells, although the difference was not significant. More importantly, we also found that the numbers of those B cells expressing both IgG1 and CD23 were significantly greater in asthma patients than healthy subjects, in total $CD19^+$ B cells, memory B cells and plasmablasts. Asthma patients also displayed a significant increase in the number of total B cells expressing IgE and CD23, although there was no significant difference in memory B cells or plasmablasts co-expressing these markers. We thus concluded that adult asthmatic patients have significant increases of $IgG1^+$ and IgE^+ B cells, but only in the $CD23^+$ B cell subset.

Correlations between Serum IgE Levels and B Cell Subsets in Asthma Patients

We next assessed correlations between the subsets of B cells (studied in Tables 5.2-5.5) in the blood of asthma patients and patient serum IgE levels. Both percentages and numbers of individual subsets of B cells were examined. A raw *p*-value cutoff of less than 0.05 and a Spearman's rho of greater than 0.4 were considered significant. All correlations meeting the above criteria are summarized in Table 5.6. The percentages of $CD23^+IgG1^+$ and $CD23^+$ plasmablasts, and $CD23^+$ memory B cells correlated highly with serum IgE levels. In line with this, both the percentage and number of total plasmablasts

($CD19^+CD20^-CD38^+$) as well as the number of activated B cells ($CD19^+CD27^+CD38^+CD86^+$) correlated with serum IgE levels. We also found that there

Table 5.4. Analysis of B cells expressing surface immunoglobulins in the blood of adult asthma patients and healthy subjects

Variables	Frequency			Cells/μL		
	Healthy	Patients	*p*-value	Healthy	Patients	*p*-value
In total $CD19^+$ B cells						
IgD^+	76.67 (±7.50)	76.39 (±10.44)	0.9107	103.9 (±73.56)	119.3 (±121.5)	0.6209
IgM^+	13.04 (±3.67)	13.55 (±6.09)	0.7147	18.60 (±14.10)	18.73 (±14.32)	0.9758
IgG^+	10.12 (±3.91)	10.06 (±5.31)	0.9616	12.64 (±10.00)	14.69 (±13.18)	0.5587
IgA^+	10.92 (±4.14)	12.25 (±6.16)	0.3616	14.20 (±11.76)	15.96 (±12.92)	0.6222
$IgG1^+$	7.436 (±2.88)	8.132 (±4.20)	0.4857	6.449 (±3.21)	10.33 (±7.95)	0.0573
IgE^+	0.6682 (±0.43)	0.8776 (±0.87)	0.2889	0.6403 (±0.32)	0.9903 (±1.13)	0.2177
In Memory B Cells ($CD19^+CD27^+CD38^-CD20^+$)						
IgD^+	43.85 (±14.83)	48.35 (±13.46)	0.2158	18.31 (±13.83)	24.70 (±22.85)	0.2758
IgM^+	22.04 (±4.82)	22.83 (±9.00)	0.6994	9.006 (±6.53)	10.51 (±10.61)	0.579
IgG^+	25.10 (±10.22)	21.76 (±7.64)	0.1358	8.521 (±5.96)	9.928 (±8.57)	0.5293
IgA^+	26.19 (±7.56)	27.59 (±9.39)	0.5442	10.27 (±8.80)	11.50 (±10.21)	0.6599
$IgG1^+$	16.95 (±5.98)	16.11 (±6.12)	0.5927	4.615 (±2.38)	6.961 (±5.75)	0.111
IgE^+	0.5900 (±0.34)	0.7480 (±0.55)	0.221	0.2048 (±0.16)	0.3171 (±0.38)	0.25
In Plasmablasts ($CD20^-CD38^+CD19^+$)						
IgD^+	7.940 (±7.43)	12.81 (±12.21)	0.1467	0.1088 (±0.089)	0.1049 (±0.098)	0.8835
IgM^+	4.829 (±7.88)	6.897 (±7.61)	0.375	0.06145 (±0.092)	0.04037 (±0.042)	0.2266
IgG^+	8.074 (±4.96)	9.882 (±7.69)	0.3932	0.1040 (±0.078)	0.1246 (±0.161)	0.6069
IgA^+	61.64 (±11.59)	60.22 (±12.69)	0.7047	0.7906 (±0.54)	0.8858 (±1.29)	0.7646
$IgG1^+$	1.685 (±1.78)	2.703 (±3.90)	0.2481	0.03509 (±0.037)	0.8858 (±1.29)	0.4248
IgE^+	0.8129 (±0.80)	2.164 (±3.48)	0.0775	0.0236 (±0.0252)	0.0745 (±0.2272)	0.3631

Values represent mean with standard deviations.

Whole blood staining was performed, but PBMCs were used for staining surface Igs.

Table 5.5. Analysis of the frequencies and numbers of $CD23^+$ B cell subsets in the blood of adult asthma patients and healthy subjects

Variables	Frequency			Cells/μL		
	Healthy	Patients	*p*-value	Healthy	Patients	*p*-value
In total $CD19^+$ B cells						
$CD23^+$	66.08 (±7.76)	61.18 (±13.10)	0.2968	**49.57 (±23.75)**	**104.2 (±64.10)**	***0.0420**
$IgG1^+CD23^+$	2.615 (±1.43)	3.866 (±2.30)	0.1378	**2.050 (±1.90)**	**5.618 (±3.59)**	***0.0226**
IgE^+CD23^+	0.1883 (±0.08)	0.2233 (±0.10)	0.3547	**0.1303 (±0.072)**	**0.3728 (±0.238)**	***0.0164**
In Memory B Cells						
($CD19^+CD27^+CD38^-CD20^+$)						
$CD23^+$	28.99 (±9.66)	28.38 (±8.70)	0.8692	7.18 (±6.11)	15.37 (±9.88)	0.0568
$IgG1^+CD23^+$	5.11 (±2.39)	6.43 (±3.50)	0.308	**1.42 (±1.53)**	**3.69 (±2.55)**	***0.0414**
IgE^+CD23^+	0.119 (±0.078)	0.137 (±0.094)	0.6074	0.0404 (±0.0395)	0.1015 (±0.1020)	0.1437
In Plasmablasts						
($CD20^-CD38^+CD19^+$)						
$CD23^+$	14.13 (±7.42)	17.66 (±8.48)	0.3086	0.163 (±0.178)	0.210 (±0.219)	0.6215
$IgG1^+CD23^+$	0.375 (±0.496)	1.504 (±3.463)	0.3452	**0.0014 (±0.0034)**	**0.0130 (±0.0123)**	***0.0368**
IgE^+CD23^+	0.270 (±0.576)	1.463 (±3.544)	0.3305	0.0 (±0.0)	0.0070 (±0.0102)	N/A

Values represent mean with standard deviations. *$P<0.05$

Whole blood staining was performed, but PBMCs were used for staining surface Igs.

NA: not assessable

were significant correlations between other B cell subsets (including the percentage of IgA^+ plasmablasts, IgG^+ memory B cells, and IgG^+ B cells in total $CD19^+$ B cells) and serum IgE levels. In addition, the percentage of $IgG1^+CD20^+CD38^-CD27^+$ (memory) in total $CD19^+$ B cells correlated with serum IgE levels, although not to as great a degree, with a Spearman's rho of 0.3948 and a *p*-value of 0.00799 (not presented in Table 5.6). Taken together, these data indicate that the frequencies of $CD23^+$ plasmablasts and memory B cells, along with the percentage of $IgG1^+CD23^+$ B cells, correlated with serum IgE levels in adult asthmatic patients.

Table 5.6. Subsets of B cells in the blood of asthma patients correlate with serum IgE levels

Variables	Frequency		Cells/μL	
	Spearman's rho	***p*-value**	**Spearman's rho**	***p*-value**
In $CD19^+$				
$IgG1^+CD23^+$	**0.7088**	****0.0021**	0.4118	0.1130
$CD20^-CD38^+CD23^+$	**0.5471**	***0.0283**	0.4559	0.0759
$CD20^+CD27^+CD23^+$	**0.5592**	***0.0243**	0.2088	0.4377
$CD20^-CD38^+$	**0.4816**	*****0.0009**	**0.5147**	*****0.0006**
$CD27^+CD38^+CD86^+$	0.396	*0.0274	**0.4505**	***0.0161**
$CD20^-CD38^+IgA^+$	**0.4523**	****0.0021**	0.2646	0.0990
$CD20^+CD27^+CD38^-IgG^+$	**0.4469**	****0.0024**	0.2468	0.1247
IgG^+	**0.4125**	****0.0054**	0.2464	0.1254

Values represent mean with standard deviations. **P*<0.05, ***P*<0.01, ****P*<0.001

Correlations between $CD23^+$ B Cell Subsets and Clinical Variables of Disease Severity

Serum IgE levels do not necessarily correlate with disease severity of asthma (174). In line with this, there was no significant correlation between serum IgE levels and the ACT scores of asthma patients who participated in this study (data not shown). The ACT score is one of the most reliable parameters that allow us to assess the level of asthma control (175). In certain instances, poor asthma control may also indicate greater asthma severity (176). Asthma severity can also be indicated by the levels of patient impairment, as measured by the frequency of asthma symptoms, amount of nighttime awakenings, the use of controller medication, and the capacity of lung function (177). Thus, it is important to know whether such B cell subsets, particularly $CD23^+$ plasmablasts and memory B cells and $CD23^+IgG1^+$ B cells, are associated with clinical variables of disease severity and control.

As shown in Table 5.7, the numbers of $CD23^+$ memory B cells strongly inversely correlated with the ACT score. Interestingly, the numbers of $CD23^+$ B cells expressing surface $IgG1^+$ were also inversely correlated with the ACT score. The number of $CD23^+$ plasmablasts did not show a significant association with ACT score but correlated with the frequency of β-agonist usage per week. The percentage of $CD23^+$ memory B cells also correlated with the number of nights woken per week, although the numbers of $CD23^+$ memory B cells did not. The quality of patient pulmonary function, as measured by FVC % of predicted, was used to correlate lung function with B cell subsets. We found that the numbers of $CD23^+$ memory B cells and $CD23^+IgG1^+$ B cells were inversely correlated with FVC % of predicted. In addition to $CD23^+$ B cells, the numbers of $CD24^{high}CD27^+$ B10 cells inversely correlated, although to a weaker extent, with the ACT score (r=-0.3148, p=0.0479, data not shown), even though there was no significant difference in the numbers of B10 cells between patients and healthy subjects (Table 5.3). It is also notable that the numbers of $CD23^+IgE^+$ B cells inversely correlated with an additional measure of lung function, FEV_1 % of predicted (r=-0.5052, p=0.0459, data not shown).

Collectively, we conclude that increased numbers of $CD23^+$ B cells, plasmablasts, memory B cells, and $IgG1^+$ B cells are associated with increased serum IgE levels and the clinical variables of disease severity and asthma control.

Certain Subpopulations of B Cells are Sensitive to the Blocking of IgE

In order to understand the effects of IgE blocking on B cells, we compared B cell populations before treatment (baseline) with week 26 (W26) of treatment, as shown in Table 5.8. There were changes within prominent B cell types, including memory, B10,

transitional/immature B cells, plasmablasts (PB), $CD5^+$ B cells, activated B cells, and IgA^+ B cells. Within the memory ($CD27^+$) B cell compartment, there was a decrease in the overall percentage of $CD27^+$ B cells, $CD27^+CD38^-$ B cells and B10 ($CD24^{high}CD27^+$). There was also a decrease in the number of $CD10^+$ B10 cells. Within the transitional/immature B cells ($CD24^{high}CD38^{high}$), there was an increase in both the percentage and number of $CD5^+$ transitional B cells, as well as an increase in the percentage of $CD5^+$ in transitional B cells and $CD5^+CD1d^+$ transitional B cells. There

Table 5.7. Correlations between $CD23^+$ B cell subsets and clinical variables of disease severity and control

Variable	Spearman's rho	*p*-value
vs ACT		
$CD19^+CD20^-CD38^+CD23^+$	-0.2393	0.3721
$CD19^+CD20^+CD27^+CD23^+$	**-0.712**	****0.0020**
$CD19^+IgG1^+CD23^+$	**-0.6529**	****0.0061**
vs β-agonist usage/week		
$CD19^+CD20^-CD38^+CD23^+$	**0.5224**	***0.0457**
$CD19^+CD20^+CD27^+CD23^+$	0.1875	0.5034
$CD19^+IgG1^+CD23^+$	0.2348	0.3996
vs nights woken/week		
$CD19^+CD20^-CD38^+CD23^+$	-0.1933	0.5078
$CD19^+CD20^+CD27^+CD23^+$	**0.4378 (%, 0.528)**	0.1174 (%, *0.0287)
$CD19^+IgG1^+CD23^+$	0.2533	0.3821
vs FVC % of pred		
$CD19^+CD20^-CD38^+CD23^+$	0.1325	0.6249
$CD19^+CD20^+CD27^+CD23^+$	-0.5121	0.0425
$CD19^+IgG1^+CD23^+$	**-0.5107**	***0.0432**

Values represent cell numbers per μL, unless otherwise specified, as mean with standard deviations. **P*<0.05, ***P*<0.01, ****P*<0.001

ACT: asthma control test, FVC: forced vital capacity

was a decrease in the number of $CD27^+$ transitional B cells, as well as a decrease in the percentage of $CD27^+$ within transitional B cells. Within the PB ($CD20^-CD38^+$) there was an increase in the percentage of overall $CD38^+$ B cells, but a decrease in the percentage of $CD20^-CD27^+CD38^+$, IgA^+ and IgG^+ PB, and a decrease in the percentage of IgE^+ within PB. There was a strong increase in the percentage and number of total $CD5^+$ B cells, as well as total $CD5^+CD1d^+$ B cells. There was a strong increase in B cell activation, with treatment yielding a significantly greater frequency, both percentage and number, of $CD86^+$ B cells, an increase in the intensity of CD86 expression (as monitored by mean fluorescent intensity [MFI]), and an increase in the percentage and number of $CD27^-CD86^+$ B cells. Finally, there was a decrease in the percentage of IgA^+ B cells, $CD27^+IgD^+IgA^+$ B cells and a reduction in the proportion of IgA^+ within memory ($CD27^+CD38^-CD20^+$) B cells. These results indicate that anti-IgE treatment can increase the levels of transitional and activated B cells, while decreasing the levels of memory B cells and this may be a result of decreased sensitivity to allergen stimulation.

There are more Variations between Responder and Non-Responder B Cells at Baseline than at Week 26 of Anti-IgE Treatment

In order to determine whether B cell populations can define anti-IgE response, we compared B cell populations between anti-IgE treatment responders and non-responders both before treatment and at week 26 of treatment. Response was gauged by the improvement, or lack thereof, in their asthma symptoms with treatment (see Chapter Three). As shown in Table 5.9**,** similar to the changes in B cells with anti-IgE treatment, there was a significant decrease in the percentage and number of $CD27^+IgD^+IgA^+$ B cells in non-responders compared with responders at baseline. Also similar to the changes

Table 5.8: Comparisons of B cell population before and after anti-IgE treatment

Variables in $CD19^+$ B Cells	Baseline	Week 26	*p*-value
% of $CD27^+$	27.64 (±16.03)	25.46 (±16.18)	***0.0001
# of $CD27^+$	47.12 (±37.16)	43.48 (±39.66)	0.1389
% of $CD27^+CD38^-$	24.71 (±14.89)	23.42 (±14.81)	**0.0079
# of $CD27^+CD38^-$	42.49 (±34.11)	39.87 (±34.44)	0.3145
% of $CD24^{high}CD27^+$	20.03 (±14.14)	18.92 (±13.65)	**0.0089
# of $CD24^{high}CD27^+$	34.62 (±31.10)	32.32 (±31.07)	0.3033
% of $CD38^+$	7.15 (±4.54)	8.03 (±3.41)	*0.0465
# of $CD38^+$	13.77 (±17.43)	14.51 (±11.12)	0.1281
% of $CD20^-CD27^+CD38^+$	1.34 (±1.74)	0.74 (±0.95)	**0.0057
# of $CD20^-CD27^+CD38^+$	1.24 (±1.60)	0.78 (±1.15)	0.0958
% of $CD20^-CD38^+IgA^+$	0.851 (±1.21)	0.501 (±0.94)	*0.0106
# of $CD20^-CD38^+IgA^+$	0.904 (±1.302)	0.556 (±1.034)	0.1044
% of $CD20^-CD38^+IgG^+$	0.148 (±0.224)	0.075 (±0.105)	*0.0333
# of $CD20^-CD38^+IgG^+$	0.126 (±0.162)	0.090 (±0.151)	0.3402
% of $CD20^-CD38^+IgE^+$	0.070 (±0.162)	0.022 (±0.056)	0.0665
# of $CD20^-CD38^+IgE^+$	0.074 (±0.227)	0.020 (±0.053)	0.1134
% of IgE^+ in $CD20^-CD38^+$	2.20 (±3.58)	0.94 (±1.99)	*0.0190
% of $CD24^{high}CD38^{high}CD5^+$	1.09 (±0.87)	1.86 (±1.66)	**0.0031
% of $CD5^+$ in $CD24^{high}CD38^{high}$	44.16 (±21.13)	56.14 (±19.93)	**0.0074
# of $CD24^{high}CD38^{high}CD5^+$	1.85 (±2.21)	3.58 (±4.82)	*0.0239
% of $CD24^{high}CD38^{high}CD27^+$	0.378 (±0.312)	0.307 (±0.269)	0.0539
% of $CD27^+$ in $CD24^{high}CD38^{high}$	35.25 (±24.11)	26.12 (±23.05)	**0.0059
# of $CD24^{high}CD38^{high}CD27^+$	0.645 (±0.652)	0.436 (±0.395)	*0.0203
% of $CD5^+$	15.44 (±7.77)	19.61 (±11.15)	**0.0041
# of $CD5^+$	27.02 (±32.79)	32.16 (±40.05)	*0.0162
% of $CD5^+CD1d^+$	13.09 (±7.02)	16.33 (±8.66)	***0.0005
# of $CD5^+CD1d^+$	23.12 (±28.02)	27.53 (±38.12)	**0.0042
% of $CD86^+$	15.40 (±6.30)	28.46 (±17.55)	*0.0464
# of $CD86^+$	33.28 (±31.38)	61.64 (±53.59)	*0.0129
CD86 MFI	246.7 (±121.9)	528.3 (±430.0)	*0.0150
% of $CD27^-CD86^+$	10.85 (±7.20)	22.13 (±16.68)	*0.0485
# of $CD27^-CD86^+$	25.86 (±31.60)	56.50 (±61.98)	**0.0042
% of IgA^+	12.33 (±6.20)	11.06 (±5.67)	*0.0223
# of IgA^+	16.05 (±13.07)	14.72 (±11.58)	0.6267
% of $CD27^+IgD^+IgA^+$	2.12 (±2.23)	1.24 (±1.28)	*0.0103
# of $CD27^+IgD^+IgA^+$	2.25 (±2.15)	1.79 (±2.27)	0.1616
% of $CD27^+CD38^-CD20^+IgA^+$	8.72 (±5.39)	8.12 (±4.31)	0.3660
% of IgA^+ in $CD27^+CD38^-CD20^+$	27.89 (±9.26)	25.82 (±8.66)	*0.0218
# of $CD27^+CD38^-CD20^+IgA^+$	11.61 (±10.31)	10.84 (±8.49)	0.9025

Values represent mean with SD. MFI: mean fluorescent intensity. *P<0.05, **P<0.01, ***P<0.001.

over time with treatment, non-responders has an increased number of $CD38^+$ B cells, and an increase in the percentage of $CD23^+$ PB ($CD20^-CD38^+$). Responders showed an increased percentage of IgM^+ PB, and an increase in the proportion of $IgG1^+$ and IgA^+ cells within the PB population. Responders also showed an increase in the percentage of $CD27^+CD43^-CD11b^-$ (non-B1) B cells and in the percentage of overall IgM^+ with a decrease in the percentage of $CD1d^+$ within transitional B cells. Interestingly, the only population that is significantly different between responders and non-responders at both baseline and at week 26 of treatment is the percentage of IgM^+ B cells. At week 26 we also observed a difference in the percentage of $IgD^+CD27^+IgA^+$ and the number of $CD23^+CD20^-CD38^+$ B cells between responders and non-responders, with non-responders having less of both. These results indicate that the make-up of B cells in non-responders compared with responders before treatment more closely mirrors that of B cells after anti-IgE treatment, which may be why non-responders are not sensitive to IgE blocking.

Discussion

The major focus of B cells in asthma has been on their IgE production. However, recent progresses made in our understanding of the roles of B cells in inflammatory diseases suggest that certain subsets of B cells might play distinctive roles in the development and/or progression of asthmatic symptoms in patients. To this end, we have extensively analyzed subsets of B cells in adult asthmatic patients who have moderate-to-severe disease symptoms. We found that they have a significant alteration of the $CD23^+$

Table 5.9. Comparison of B cell population between anti-IgE responders and non-responders at baseline and at week 26 of anti-IgE treatment

Variables in $CD19^+$ B Cells	Baseline			Week 26		
	Responder	**Non-Responder**	***p*-value**	**Responder**	**Non-Responder**	***p*-value**
% of $IgD^+CD27^+IgA^+$	2.47 (±2.45)	1.03 (±0.61)	**0.0081	1.20 (±1.23)	1.35 (±1.47)	0.8444
# of $IgD^+CD27^+IgA^+$	2.58 (±2.36)	1.23 (±0.74)	*0.0234	1.99 (±2.49)	1.16 (±1.23)	0.3174
% of $IgD^+CD27^+IgM^+$	7.04 (±5.82)	4.31 (±4.09)	0.0785	6.32 (±4.45)	3.53 (±2.48)	*0.0467
# of $IgD^+CD27^+IgM^+$	9.26 (±10.04)	5.21 (±4.65)	0.1301	8.87 (±9.48)	4.35 ± (4.17)	0.0671
% of $CD27^+CD43^-CD11b^-$	26.61 (±15.34)	15.10 (±8.14)	*0.0291	25.58 (±14.68)	22.64 (±21.26)	0.3849
# of $CD27^+CD43^-CD11b^-$	37.59 (±32.22)	29.37 (±22.88)	0.4755	30.32 (±21.13)	27.37 (±26.39)	0.5017
% of $CD38^+$	7.23 (±4.67)	6.55 (±4.19)	0.6071	8.31 (±3.21)	7.19 (±4.00)	0.2934
# of $CD38^+$	13.00 (±15.67)	16.25 (±23.02)	**0.0075	14.02 (±9.59)	16.13 (±15.70)	0.7313
% of $IgM^+CD20^-CD38^+$	0.080 (±0.148)	0.023 (±0.020)	*0.0308	0.036 (±0.073)	0.038 (±0.052)	0.8671
% of IgM^+ in $CD20^-CD38^+$	6.64 (±7.42)	5.77 (±6.62)	0.582	4.03 (±4.06)	4.87 (±11.92)	0.2849
# of $IgM^+CD20^-CD38^+$	0.039 (±0.042)	0.043 (±0.049)	0.8496	0.052 (±0.124)	0.033 (±0.042)	0.5169
% of $IgG1^+CD20^-CD38^+$	0.058 (±0.076)	0.037 (±0.042)	0.2731	0.038 (±0.069)	0.059 (±0.079)	0.4668
% of $IgG1^+$ in $CD20^-CD38^+$	3.19 (±4.39)	1.32 (±1.31)	*0.0428	1.48 (±2.14)	2.48 (±3.13)	0.4301
# of $IgG1^+CD20^-CD38^+$	0.042 (±0.044)	0.053 (±0.066)	0.6611	0.047 (±0.126)	0.026 (±0.031)	0.453
% of $IgA^+CD20^-CD38^+$	0.717 (±0.753)	1.265 (±2.077)	0.4833	0.534 (±1.073)	0.398 (±0.288)	0.8398
% of IgA^+ in $CD20^-CD38^+$	57.66 (±12.00)	67.97 (±12.83)	*0.0434	51.86 (±20.20)	57.55 (±11.64)	0.2357
# of $IgA^+CD20^-CD38^+$	0.681 (±0.888)	1.595 (±2.049)	0.2260	0.597 (±1.180)	0.429 (±0.298)	0.8306
% of $CD24^{high}CD38^{high}CD1d^+$	2.10 (±1.26)	1.49 ± (0.85)	0.1197	2.63 (±2.19)	2.51 (±1.84)	0.9312
% of $CD1d^+$ in $CD24^{high}CD38^{high}$	82.48 (±8.37)	86.94 (±4.70)	*0.0282	84.20 (±8.96)	87.21 (±11.56)	0.5405
# of $CD24^{high}CD38^{high}CD1d^+$	3.05 (±2.45)	4.26 (±4.14)	0.6414	4.50 (±4.73)	5.67 (±10.89)	0.7678
% of IgM^+	14.49 (±6.58)	10.58 (±3.36)	*0.0358	13.57 (±4.65)	10.32 (±4.15)	*0.0479
# of IgM^+	19.15 (±14.55)	17.18 (±14.98)	0.6054	19.84 (±15.29)	14.82 (±11.26)	0.311
% of $CD23^+CD20^-CD38^+$	0.133 (±0.112)	0.463 (±0.118)	*0.0131	0.167 (±0.233)	0.208 (±0.351)	0.8231
# of $CD23^+CD20^-CD38^+$	0.122 (±0.109)	0.631 (±0.272)	0.0565	0.141 (±0.105)	0.069 (±0.039)	*0.0107

Values represent mean with SD. #: number of cells per μL, %: percent of cells in $CD19^+$, unless otherwise specified.
*P<0.05, **P<0.01.

B cell subset, particularly memory B cells, plasmablasts, and B cells expressing surface IgG1. More importantly, such alterations were strongly associated with serum IgE levels as well as with clinical variables of disease severity and control in patients. We also report that adult asthmatic patients have decreased percentages, but not numbers, of transitional B cells ($CD24^{high}CD38^{+}$) co-expressing CD5. There was no such alteration in the total transitional B cell population. In addition, we characterized B cell subsets in allergic asthma patients both before and at W26 of their treatment with anti- IgE. We were also able to compare B cell profiles between anti-IgE responders and non-responders in order to better determine whether these cells play a role in determining the response to IgE blocking. The effects of anti-IgE on B cells induced a reduction of memory B cells, as defined by CD27 expression, and particularly reduced IgA-producing memory B cells. There was also a reduction in plasmablasts by anti-IgE treatment. However, there was an increase in transitional/immature B cells, particularly those expressing CD5, along with an increase in activated B cells. We moreover observed that patients unresponsive to anti-IgE treatment where characterized by a decrease in IgA^{+} and IgM^{+} plasmablasts before the start of treatment, and by W26 of treatment, still retained their reduction of surface IgM, but in addition, showed a reduced amount of activated B cells. These findings are a fundamental base for us to further explore additional roles of such B cell subsets in asthma and for the mechanisms of anti-IgE, which may eventually lead us to discover novel and effective therapeutic targets for asthma in the future and to identify better biomarkers of response to anti-IgE treatment.

CD23 (FcεRII) is the low-affinity receptor for IgE. CD23 monomers display relatively low-affinity ($Ka \approx 10^{6}–10^{7}$) for IgE, but membrane-bound CD23 can form a

trimer allowing it to bind to IgE with high affinity (Ka≈10^8–10^9) (79, 173, 178). CD23 is expressed by multiple cell types, including subsets of B cells, activated T cells, eosinophils, monocytes, platelets, DCs, including Langerhans cells (173, 178) and airway structure cells (predominantly epithelial cells) (179, 180). The CD23 expression on epithelial structural cells was reported to be necessary and sufficient for the initiation and perpetuation of allergic inflammatory responses in airways (180). In line with this, blocking CD23 is considered a therapeutic strategy for allergic immune disorders, including asthma (180-182). However, CD23 can also serve as a negative feedback regulator of IgE production. For example, mice over-expressing CD23 show reduced IgE production (183), while CD23-deficient mice have increased IgE production (184). Nonetheless, the roles of CD23 expressed on individual cell types in allergic immune responses are still elusive. Furthermore, CD23 expressed on different cell types might have distinct functions in allergic immune responses. In our findings, the increase of $CD23^+$ plasmablasts and memory B cells and those expressing IgG1 in asthma patients strongly suggest that CD23 on such B cells might act as a positive feedback regulator for the pathogenesis of asthma. This is further supported by significant associations between $CD23^+$ B cell populations and serum IgE levels, in addition to multiple clinical variables of disease activity.

The role of CD23 on B cells as a positive feedback regulator is further supported by data from previous studies: cross-linking of B cell receptor and CD23 by IgE-antigen complexes significantly increases B cell activation (185, 186) followed by enhanced antigen presentation to helper T cells, thus further promoting B cell responses (187). However, crosslinking CD23 with surface IgG has been shown to inhibit mouse B cell

proliferation (188). The direct influence of CD23 on B cell activation and differentiation may also occur through multiple mechanisms, including ligation of membrane CD23, the presence of soluble CD23 and crosslinking of CD23 by multiple ligands. CD23 does not belong to the super Ig family but is a type II membrane protein with a calcium-dependent lectin domain in the C-terminal end of its extracellular region (189). In line with this, not only IgE, but also CD21 and some integrins (178, 189) can bind to CD23, although there could be more natural ligands of CD23. CD23 also plays an important role in inflammation through the promotion of the release of pro-inflammatory mediators and the enhancement of cell adhesion activity (182, 190). It is thus possible that the activation of CD23 and its promotion of B cell growth and differentiation may be another mechanism by which IgE synthesis is increased. Collectively, data from this and previous studies indicate that the increase of $CD23^+$ B cells is highly relevant to the pathogenesis of asthma. However, it is important to note that genetic polymorphisms of CD23, as well as their expression levels (171), could also be important contributors to allergic immune responses (172, 191). Murine CD23 showed a more restricted cellular expression than human CD23, and this also needs to be carefully considered in the study of CD23's roles in asthma.

One recent study also showed that $CD23^+$ transitional B cells proliferated more vigorously and were rescued from BCR-induced apoptosis to a greater degree by T cell help signals than $CD23^-$ transitional B cells (192). In human B cells, low-level expression of CD23 is a constitutive feature of IgM^+IgD^+ B cells in the periphery, whereas immature B cells in bone marrow do not express CD23. B cells lose CD23 expression after undergoing isotype switch to γ-, α-, or ε-bearing cells. Importantly,

however, we found that asthmatic patients have significantly higher frequencies of class-switched IgG1-expressing plasmablasts and memory B cells, and to some extent have increased IgE^+ B cells. This suggests that the increase of $CD23^+$ B cells in asthmatic patients is not due simply to the increase of certain subsets of transitional B cell ($CD24^{high}CD38^{high)}$ populations.

Transitional B cells represent an important target for negative selection to self antigens *in vivo* and play an important role in maintaining tolerance to peripheral antigens not present in the bone marrow. They represent a heterogeneous population of B cells with phenotypic variability that can be further subdivided by multiple surface proteins. This population of B cells is particularly relevant to inflammatory diseases since B cells producing IL-10 (termed regulatory B cells, Bregs) are consistently found within $CD24^{high}CD38^{high}$ and $CD24^{high}CD27^+$ B cell subsets (157, 193). We thus expected that asthmatic patients may have an alteration in such B cell subsets, as previously described (194, 195). Interestingly, however, there was no significant difference between the subsets of transitional B cells (based on CD27, CD1d, and CD10 expression) tested from asthmatic patients and healthy subjects, except for transitional B cells that express CD5. Although patients had decreased percentages of transitional B cells expressing CD5, both patients and healthy subjects had similar numbers (cells per μL). However, biological functions of these individual subsets of transitional B cells in patients may not be the same to those in healthy subjects and needs to be further tested in the future.

It has been shown that anti-IgE treatment can reduce the number of B cells in the bronchial submucosa (96), and we show here that the percentage of B cells in PBMCs is slightly but significantly reduced by W26. With the neutralization of IgE, we observed a

reduction in memory ($CD27^+$) and B10 ($CD27^+CD24^{high}$) B cells. The decrease in memory B cells is likely a result of a decrease in antigen stimulation and T cell activation. It has been shown *in vitro* that B10 cells can be induced by pro-inflammatory signaling (193). Thus the decrease in B10 cells may also reflect a decrease in activation signals. $CD24^{high}CD38^{high}$ immature B cells can also be considered regulatory B cells (Bregs), and may in fact be a greater producer of IL-10 than the $CD24^{high}CD27^+$ B10 cells (196). While we observe a decrease in B10 cells with anti-IgE, we see an increase in Breg cells co-expressing CD5. $CD5^+$ Breg cells have been shown to induce regulatory T cells (Tregs) (197). Indeed, we also obverse an increase in Tregs and IL-10-producing T cells with the treatment of anti-IgE (see Chapter Six). The increase in Bregs may be inducing the increase in Tregs, both of which may be a result of a decreased pro-inflammatory environment initiated by the neutralization of IgE. In addition to the increase in $CD5^+$ Bregs, there was a strong increase in the percentage and number of total $CD5^+$ B cells and $CD5^+CD1d^+$ B cells. CD5 is usually a characterizing feature of B1a B cells, capable of producing broadly reactive natural IgM that acts as innate immunity (198). Since these B1 cells generally appear earlier than the memory B2 B cells, this may represent a wave of naïve B cells filling the spots left behind by the lack of memory B cells.

The increase of $CD86^+$ B cells, while normally associated with an increase in their activation status, may also indicate their expansion, particularly of recently matured B cells (199). Like the reduction in memory B cells, anti-IgE treatment also reduced PB, particularly those producing IgA, IgG and IgE. There was an increase in the overall expression of $CD38^+$ B cells, but this is probably reflecting the increase in transitional B

cells, which also express CD38. There was also a strong overall decrease in IgA^+ B cells, including total IgA^+ B cells, IgA^+ memory B cells and IgA^+ PB. IgA is most associated with immune protection in the mucosa, particularly the gut mucosa. The particular reduction of IgA with anti-IgE treatment may reflect an alteration in T cell TGF-β production or CD40L expression, however, if CD40L expression is not altered, it may also be from a lack of IL-5, which has been noted to decrease in the serum with treatment (see Chapter Seven) and is capable of enhancing the production of endogenous TGF-β (200).

With the treatment of IgE, two classes of patients are revealed: those that respond and those that do not respond to the neutralization of IgE. To better understand these disparities, we compared patient B cells both before and at W26 of treatment. However, many of the observations of responders compared with non-responders are difficult to explain and do not seem to follow any specific pattern. Overall, this indicates that the variations in B cells between responders and non-responders before treatment is highly subtle, and not dominated by any one subtype of B cell. Somewhat similar results are observed at 26 weeks of treatment. Non-responders consistently show a reduction of IgM expression compared with responders, however, no other observations are conserved over time. There are some indications that non-responder B cells are less activated at W26 compared with responders, shown by a decrease in the percentage of $CD86^+CD27^+CD38^+$ B cells. As $IgM^+IgD^+CD27^+$ B cells have shown to have been involved in germinal center (GC) B cell differentiation and T cell-dependent affinity maturation (201), their decrease in non-responders at W26 indicates a possible lack of T cell responsiveness in their B cells and possible disruptions in their GC formation.

Data from this study implicates CD23 expression on B cells and $CD23^+$ B cells in the pathogenesis of human asthma. More detailed and in-depth analysis of the roles of CD23 expressed on B cells in the context of allergic immune responses will be important. These results also indicate a complicated role of B cells, both in responding to anti-IgE treatment and in the differentiation between anti-IgE responders and non-responders. There are a number of variations between anti-IgE responder and non-responder B cells before the initiation of treatment. These variations may aid in the understanding of both IgE-dependent and independent asthma pathogenesis. Further study is required, but these discrepancies may yield a patient phenotype to discern responders from non-responders and aid in a more targeted treatment.

CHAPTER SIX

Peripheral $CD4^+$ and $CD8^+$ T Cells of Adult Asthma Patients Support Chronic Inflammation in the Airway

Abstract

Asthma is a chronic inflammatory disease of the airway in which T cells play an important role in its pathogenesis. However, the underlying mechanisms for chronic inflammation in patients remain poorly understood. To discover the characteristics of $CD4^+$ and $CD8^+$ T cells that support chronic inflammation in the lungs of asthma patients, phenotypes of $CD4^+$ and $CD8^+$ T cells in fresh whole blood of moderate-to-severe adult asthmatic patients (N=45) were assessed by flow cytometry and than compared them with those of healthy controls (N=21). Asthma patients have significantly increased numbers of T cells that display a central memory phenotype ($CD45RA^-CD45RO^+CCR7^+$), but not effector memory ($CD45RA^-CD45RO^+CCR7^-$). Accordingly, both CCR4 and CRTH2 were highly expressed on central memory $CD4^+$ T cells in asthma patients, while there was no significant difference in the expression of CCR5, CCR6, CXCR3, or CXCR5 on $CD4^+$ T cells in the two groups. Interestingly, however, asthma patients have increased frequency of both $CD45RO^+CCR7^+$ and $CD45RO^+CCR7^-$ $CD8^+$ T cells. More importantly, asthma patients had a significant decrease in the number of $\beta7^+CD8^+$ T cells, but an increase in $\beta7^+CD8^+$ T cells co-expressing $\alpha4$ and CD11a, which contribute to their migration into the lung. A large fraction (>50-60%) of $\beta7^+\alpha4^+CD11a^+CD8^+$ T cells also co-express CCR5 and CD62L. Furthermore, $CD8^+$ T cells in asthma patients showed increased expression of granzyme B and perforin. Increases of central memory $CD4^+$ and

both central and effector memory $CD8^+$ T cells expressing inflammatory markers, support chronic inflammation in asthma patients who are intermittently exposed to allergens. We also studied T cells from these asthma patients over the course of anti-IgE (omalizumab) treatment to identify potential markers of response. By comparing T cell composition and activation status between anti-IgE responders and non-responders, a profile for non-response may be created.

Introduction

Asthma is a highly heterogeneous chronic inflammatory disease of the airway that can be influenced by multiple factors, including genetic, environmental, and immunological factors (202-204). Disease pathology is characterized by airway inflammation that is classically eosinophilic in nature and concomitant with changes in lung function – typically airway hyper-responsiveness – although severe asthma is dominated by polymorphic white blood cells. Structural changes within the lung, termed airway remodeling, are also present. Airway obstruction runs a variable course with symptom-free periods interrupted by periods of exacerbated symptoms, often caused by microbial infections (202, 205). The normal response to a harmless airborne allergen is tolerance, but asthmatic patients respond with an acute inflammatory response that either resolves or becomes chronic. Allergic sensitization to inhaled allergens is often found in children with asthma; this is due to atopy, which is a predisposition to the development of allergic hypersensitivity reactions and to the production of IgE in response to allergens. Not all allergic patients develop asthma, but there is a great tendency for the development of asthma in allergy patients (120, 206). Pathogenesis of asthma has been classically

explained as Th2-type inflammatory responses in the airway. $CD4^+$ T cells, particularly Th2-type $CD4^+$ T cells, mast cells, eosinophils, and basophils, have been thought to be the major driving forces for either allergy progressing to asthma or for exacerbating asthma in patients (207). Cytokines, including IL-4, IL-5, and IL-13, secreted from such Th2-type T cells can recruit and/or activate granulocytes in addition to promoting IgE responses. They can thus play various important roles in asthma pathogenesis. However, the high level of clinical heterogeneity of asthma suggests that the pathogenesis of asthma may not be solely driven by Th2-type immune responses. In line with this notion, recent studies have shown that other Th subsets, including Th1, Th9, Th17, T follicular helpers (Tfh), and Th22, could also be linked to the pathogenesis of asthma (208) Similarly, in almost all patients with asthma, one can also find a counter-regulatory population of allergen-specific regulatory T cells (Tregs) that are capable of suppressing allergic inflammation (209, 210). In almost all patients with asthma, a counter-regulatory population of allergen-specific Tregs is found (209, 210). Furthermore, $CD8^+$ T cells could also participate in the etiopathology of asthmatic inflammation (211, 212). Information from previous studies has greatly expanded our knowledge on the pathogenesis of asthma. However, there is much that still remains poorly understood, particularly in the context of asthma being a chronic rather than acute inflammatory disease of the lower airway. By studying in-depth the characteristics of T cells in the blood of asthma patients, we anticipate a better understanding of how these cells may perpetuate chronic inflammation in the lower airway.

To this end, we hypothesized that T cells in the peripheral blood of asthma patients can display unique phenotypes and functions that could support chronic

inflammation in the lower airway. Additionally, these T cells may be altered by anti-IgE treatment to become more similar to healthy controls over time. In this study, we therefore investigated the cellular composition of T cell subsets ($CD4^+$ and $CD8^+$, naive and central and effector memory, and Tregs) as well as the expression levels of chemokine receptors (CXCR3, CCR4, CCR5, CCR6, CCR7, and CXCR5), adhesion molecules (α4, β7, CD11a, and CD62L), and chemoattractant receptor-homologous molecule expressed on Th2 (CRTH2) using whole blood from patients with moderate-to-severe asthma, both before anti-IgE treatment and after 26 weeks of treatment. We also compared T cell profiles between anti-IgE responders and non-responders to determine whether these cells can act as a biomarker for response, in addition to uncovering mechanisms of response to anti-IgE. $CD8^+$ T cell functions were further assessed by measuring their IFNγ expression, as well as perforin and granzyme B expression. Asthma patient data were compared with those of age-, race-, and sex-matched healthy subjects. This comprehensive analysis will extend our knowledge of the pathophysiology of asthma, particularly in the context of a chronic inflammatory disease, and potentially contribute to new therapeutic approaches for asthma and biomarkers for determining response to anti-IgE in the future.

Methods

Study Subjects

Described in methods section of Chapter Three.

Study Design

Described in methods section of Chapter Three.

Determination of Responders versus Non-Responders

Described in methods section of Chapter Three.

Table 6.1. Antibodies used for T cell analysis

Marker	Clone	Company
CD3	UCHT1	BD
CD4	RPA-T4	Tonbo
CD8	RPA-T8	Tonbo/BD
CD45RA	HI100	BD
CD45RO	UCHL1	BioLegend
CXCR3	G025H7	BioLegend
CCR4	TG6/CCR4	BioLegend
CCR5	HEK/1/85a	BioLegend
CXCR5	51505	R&D Systems
CCR6	R6H1	eBioscience
CCR7	G043H7	BioLegend
CRTH2	BM16	BD
CD28	28.2	eBioscience
CD57	HCD57	BioLegend
CD11a	MEM-25	ExBio
CD62L	DREG-56	BD
α4	9F10	BioLegend
β7	FIB504	BD
CD25	M-A251	BD
CTLA-4	14D3	eBioscience
IL-10	JES3-9D7	eBioscience
Foxp3	PCH101	eBioscience
IFNγ	4S.B3	BD
Granzyme B	GB11	Invitrogen
Perforin	dG9	BioLegend
Live/Dead		Invitrogen
mIgG2a	G155-178	BD
mIgG1	P3.6.2.8.1	eBioscience
mIgG2b	MPC-11	BIoLegend
mIgG1	MOPC-173	BD
mIgG2a	MOPC-173	BioLegend
rIgG2a	R35-95	BD
mIgG1	MOPC-173	BD
rIgG2a	R35-95	BD

Whole Blood T Cell Staining

Described in methods section of Chapter Four. Detailed information for antibodies used in this study is summarized in Table 6.1.

PBMC Isolation, Intracellular Staining, and Measurement of T Cell Cytokines

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Plaque PLUS (GE Healthcare, Pennsylvania). For intracellular staining, cells were plated at $5x10^5$ cells/100 μL in 96-well U-bottom plates in complete RPMI 1640 (cRPMI) (Invitrogen, California) supplemented with HEPES (Invitrogen), 1% non-essential amino acids, 2 mM L-glutamate (Sigma-Aldrich, Missouri), 50 units/mL penicillin, and 50 μg/mL streptomycin (cRPMI). Cells were stimulated with anti-CD3/anti-CD28 human dynabeads (Life Technologies) at a 1:1, bead:cell, ratio for 5-6 hours, with Golgiplug (BD) added 1-2 hours after stimulation. After surface staining, cells were permeabilized and fixed with 200 μL BD Perm/Fix. Intracellular IFNγ and granzyme B were stained in the presence of BD Perm/Wash buffer. All stained cells were stored in PBS (Life Technologies) before running on the cytometer.

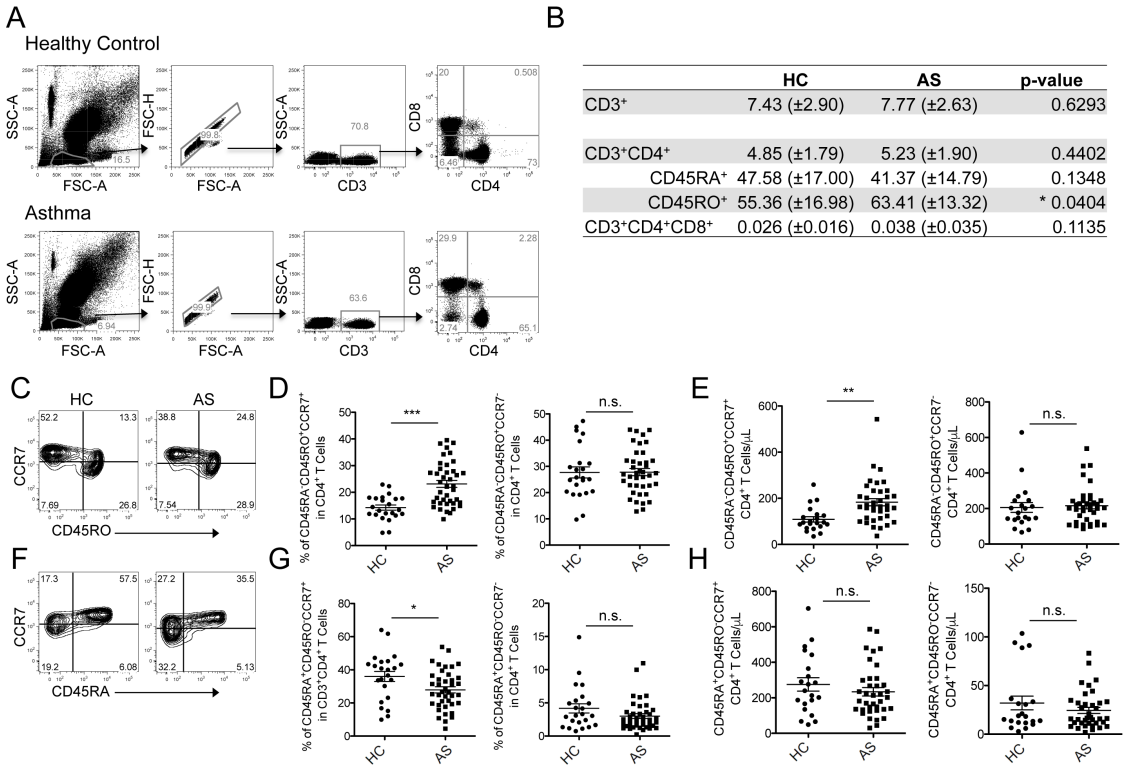
Statistical Analysis

Described in methods section of Chapter Four.

Results

Increased Frequency of CD45RO$^+$CCR7$^+$, but not CD45RO$^+$CCR7$^-$, CD4$^+$ T Cells in Asthma Patients

We first investigated CD4$^+$ T cell subsets in asthma patients (N=45) by staining whole blood with antibodies specific for surface molecules (Table 6.1) and than compared them with those of healthy control subjects (N=21). The gating strategy is



	HC	AS	p-value
CD3$^+$	7.43 (±2.90)	7.77 (±2.63)	0.6293
CD3$^+$CD4$^+$	4.85 (±1.79)	5.23 (±1.90)	0.4402
CD45RA$^+$	47.58 (±17.00)	41.37 (±14.79)	0.1348
CD45RO$^+$	55.36 (±16.98)	63.41 (±13.32)	* 0.0404
CD3$^+$CD4$^+$CD8$^+$	0.026 (±0.016)	0.038 (±0.035)	0.1135

Figure 6.1. Altered distribution of central memory CD4$^+$ T cells in patients with asthma.
(A) Whole blood lymphocytes were gated on low FSC and SSC, and singlets were removed using FSC-A vs. FSC-H. T cells were identified by CD3 expression and than differentiated based on CD4 and CD8 expression. (B) Average percentages with standard deviation of T lymphocytes. CD45RA$^+$ and RO$^+$ are quantified as a percent of CD3$^+$CD4$^+$ T lymphocytes. (C) and (F) Representative FACS plots of whole blood gated as indicated in (A). CD4$^+$ cells were further divided by either CD45RA$^+$ or CD45RO$^+$ and than analyzed for CCR7 staining. (D), (E), (G), and (H) The four subpopulations of T cells are: i) CD45RA$^-$CD45RO$^+$CCR7$^+$: central memory, ii) CD45RA$^-$CD45RO$^+$CCR7$^-$: effector memory, iii) CD45RA$^+$CD45RO$^-$CCR7$^+$: naïve, and iv) CD45RA$^+$CD45RO$^-$CCR7$^-$: effector. (D) and (G) show percentages of CD4$^+$ T cells, while (E) and (H) show the number of cells/μL of whole blood. *P<0.05, **P<0.01, ***P<0.001. Error bars indicate SD.

summarized in Figure 6.1A. As summarized in Figure 6.1B, asthma patients and healthy subjects have similar percentages of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD4^+CD45RA^+$, and $CD3^+CD4^+CD8^+$ T cells in their blood. However, asthma patients have a greater percentage of $CD45RO^+$ memory $CD4^+$ T cells than healthy subjects, as previously described (213). Both $CD45RA^+$ and $CD45RO^+$ $CD4^+$ T cells were further investigated based on their CCR7 expression. Representative flow cytometry data in Figure 6.1C show that asthma patients have a greater percentage of $CD45RO^+CCR7^+$ $CD4^+$ T cells (24.8%) (right panel) than healthy subjects (13.3%) (left panel), while asthma patients and healthy subjects have a similar percentage of $CD45RO^+CCR7^-$ $CD4^+$ T cells (~26-29%). Summarized data generated with 45 asthma patients and 21 healthy subjects (Fig. 6.1D) further support the observation made in Figure 6.1C. The numbers of $CD45RO^+CCR7^+$ (Fig. 6.1E, left) and $CD45RO^+CCR7^-$ $CD4^+$ T cells (Fig. 6.1E, right) per microliter of blood also supported the data in Figure 6.1C and 6.1D. Although there was a tendency for a decrease in the number of $CD45RA^+$ $CD4^+$ T cells in asthma patients (Fig. 6.1B), the difference was not significant. However, this difference was more significant when combined with CCR7 expression. Representative flow cytometry data (Fig. 6.1F) show that asthma patients have a lower percentage (35.5%) of $CD45RA^+CCR7^+$ $CD4^+$ T cells than a healthy subject (57.5%). Summarized data in Figure 6.1G further demonstrated that asthma patients had a significantly lower percentage of $CD45RA^+CCR7^+$ naïve $CD4^+$ T cells (left panel), but not $CD45RA^+CCR7^-$ $CD4^+$ T cells (right panel). This difference was not observed when we counted the numbers of these two $CD4^+$ T cell subsets (Fig. 6.1H). Therefore, we concluded that moderate-to-severe adult asthma patients have a greater frequency of

$CD4^+CD45RO^+CCR7^+$, but not $CD4^+CD45RO^+CCR7^-$, T cells than healthy subjects. Asthma patients have a lower frequency of $CD45RA^+CCR7^+$ naive $CD4^+$ T cells in their blood than healthy subjects.

Asthma Patients have Increased Frequency of both $CD45RO^+CCR7^+$ and $CD45RO^+CCR7^-$ $CD8^+$ T Cells

$CD8^+$ T cells can also contribute to the pathogenesis of asthma (214-216). We thus investigated the subsets of $CD8^+$ T cells as we did for $CD4^+$ T cells in Figure 6.1. Summarized data in Figure 6.2A shows that asthma patients and healthy subjects have similar percentages of $CD3^+CD8^+$ T cells in their blood. However, asthma patients have a

A

	HC	AS	p-value
$CD3^+CD8^+$	1.90 (±0.95)	1.90 (±1.02)	0.9862
$CD45RA^+$	55.40 (±14.41)	44.94 (±17.60)	* 0.017
$CD45RO^+$	39.66 (±13.95)	49.19 (±16.88)	* 0.0234
CD4/CD8 ratio	2.99 (±1.29)	3.40 (±1.79)	0.345

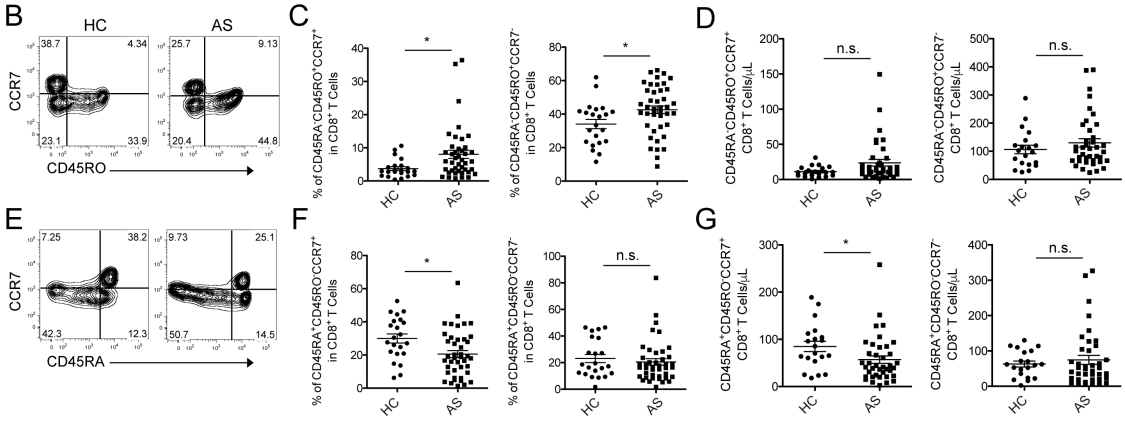


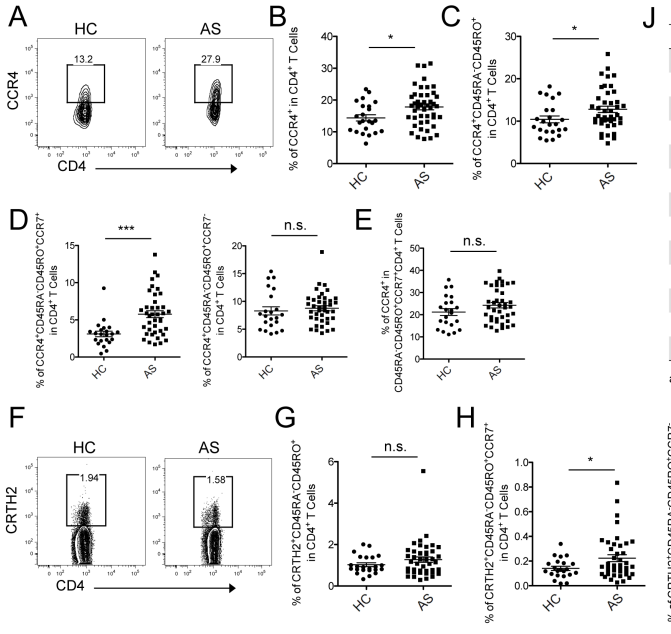
Figure 6.2. Asthma patient $CD8^+$ T cells show an altered distribution of memory and naïve populations.
Cells were gated as shown in **Fig. 6.1A**. (A) Average percentages with standard deviation for total $CD8^+$ T cells and their CD45RA and CD45RO populations. CD4/CD8 represents the ratio of $CD4^+$ to $CD8^+$ T cells. (B) and (E) Representative FACS plots of $CD3^+CD8^+$ T lymphocytes divided by either CD45RO (B), CD45RA (E) or CCR7. (C) and (F) show percentages of CD8+ T cells. (D) and (G) show the number of cells/μL of whole blood. **P*<0.05. Error bars indicate SD.

higher percentage of $CD45RO^+CD8^+$ T cells and a lower percentage of $CD45RA^+CD8^+$ T cells than healthy subjects, as previously described (217). There was no significant difference in the ratio of $CD4^+$ to $CD8^+$ T cells (CD4/CD8) between the two groups. We further investigated $CD45RO^+$ and $CD45RA^+$ $CD8^+$ T cells based on their CCR7 expression. Representative flow cytometry data (Fig. 6.2B) show that asthma patients have a greater percentage of $CD45RO^+CCR7^+$ (9.13%) and $CD45RO^+CCR7^-$ (44.8%) than healthy subjects (4.34 and 33.9%, respectively). Summarized data (Fig. 6.2C) from 45 asthma patients and 21 healthy donors further support the data in Figure 6.2B, although this was not the case when the numbers of the two different subsets of $CD8^+$ T cells were counted (Fig. 6.2D). We also found that asthma patients have a significantly decreased percentage (Fig. 6.2E and 6.2F) as well as the number of cells per μL (Fig. 6.2G) of $CD45RA^+CCR7^+$ naïve $CD8^+$ T cells, when compared to healthy subjects. No such difference was observed when we compared the percentage of the $CD45RA^+CCR7^-$ $CD4^+$ T cell population. Therefore, we concluded that asthma patients have increased percentages of both $CD45RO^+CCR7^+$ and $CD45RO^+CCR7^-$ $CD8^+$ T cells, while they have significantly decreased numbers of $CD45RA^+CCR7^+$ naïve $CD8^+$ T cells, but not $CD45RA^+CCR7^-$ $CD8^+$ T cells.

Increase in the Occurrence of $CCR4^+$, but not $CRTH2^+$, $CD4^+$ T Cells in Asthma Patients

Chemokine receptors expressed on T cells contribute to T cell migration into local tissues as well as into lymph nodes. They are also indicative markers of T cell subsets – CXCR3 for Th1, CCR4 for Th2, CCR6 for Th17, and CXCR5 for follicular helper T cells (Tfh) (218). We thus investigated $CD4^+$ T cells for their expression of such chemokine receptors, along with CRTH2, which is mainly expressed on Th2-type memory $CD4^+$ T

cells (219) and is also associated with the pathogenesis of asthma (220, 221). Representative flow cytometry data (Fig. 6.3A) show that asthma patients have an increase in the percentage of $CCR4^+CD4^+$ T cells. Compiled data further demonstrate the increases of $CCR4^+CD4^+$ (Fig. 6.3B) and $CCR4^+CD45RO^+$ $CD4^+$ T cells (Fig. 6.3C). The difference between asthma patients and healthy subjects was even greater when the percentage of $CCR4^+CD45RO^+CCR7^+$ $CD4^+$ T cells was compared (Fig. 6.3D, left). However, there was no significant difference in the proportion of $CCR4^+CD45RO^+CCR7^-$ $CD4^+$ T cells between the two groups (Fig. 6.3D, right), which



J	HC	AS	*p*-value
In $CD3^+CD4^+$			
$CXCR3^+$	30.24 (±13)	35.85 (±11.6)	0.0761
$CCR5^+$	15.11 (±11)	14.62 (±8)	0.836
$CXCR5^+$	12.38 (±4.2)	14.29 (±4.9)	0.1214
$CCR6^+$	18.22 (±9.1)	16.63 (±6.2)	0.3951
In $CD3^+CD8^+$			
$CXCR3^+$	40.87 (±16.7)	46.72 (±16.8)	0.1778
$CCR5^+$	38.23 (±8.3)	43.26 (±16.4)	0.1717
$CCR6^+$	7.02 (±6.5)	2.58 (±2.7)	***0.0002
$CCR4^+$	4.72 (±2.7)	6.57 (±5.4)	0.1327
[a]$CRTH2^+$	0.92 (±0.7)	2.9 (±4.3)	*0.0381
[a]$CXCR5^+$	2.38 (±1.4)	2.31 (±1.5)	0.8491

[a]Data is compiled from $CD3^+CD4^-$

Figure 6.3. Asthma patient $CD4^+$ T cells show a significant increase in the expression of CCR4 but not CRTH2.
Representative FACS staining of (A) CCR4 and (F) CRTH2 in $CD3^+CD4^+$ T lymphocytes. Percentages of (B) total $CCR4^+$ $CD4^+$ T cells, (C) memory $CCR4^+$ T cells, (D) central and effector memory $CCR4^+$ T cells, and (E) CCR4 expression in central memory $CD4^+$ T cells. Percentages of (G) memory $CRTH2^+$ $CD4^+$ T cells, (H) central and effector memory $CRTH2^+$ T cells and (I) CRTH2 expression in central memory $CD4^+$ T cells. (J) Additional marker expression levels in both $CD4^+$ and $CD8^+$ T cells. Data represent average percentages with SD.*P<0.05, **P<0.01, ***P<0.001. Error bars indicate SD.

was consistent with the data in Figure 6.1. No significant difference was observed when the percentage of $CCR4^+$ cells in $CD45RO^+CCR7^+$ $CD4^+$ T cells was compared (Fig. 6.3E). This suggests that the increased percentage of $CCR4^+$ $CD4^+$ T cells in Figure 6.3A-6.3D was mainly due to the increase of the percentage of $CD45RO^+$ T cells, particularly $CD45RO^+CCR7^+$ $CD4^+$ T cells, in asthma patients. We also analyzed the proportions of $CCR4^+$ $CD8^+$ T cell subsets ($CD45RA^+$, $CD45RO^+$, $CD45RA^+CCR7^+$, $CD45RA^+CCR7^-$, $CD45RO^+CCR7^+$, and $CD45RO^+CCR7^-$), but there was no significant difference between asthma patients and healthy subjects (data not shown).

There have been conflicting reports on the frequency of circulating $CRTH2^+$ T cells in asthma patients, with some reporting an increase in $CRTH2^+$ cells (222), and others reporting no difference (220, 221). We observed that asthma patients and healthy subjects have similar percentages of $CRTH2^+$ $CD4^+$ T cells (data not shown); additionally, there was no difference in the proportions of $CRTH2^+CD45RO^+$ $CD4^+$ T cells (Fig. 6.3F and 6.3G). Interestingly, however, asthma patients had an increase of $CRTH2^+CD45RO^+CCR7^+$ $CD4^+$ T cells (Fig. 6.3H, left) without an increase of $CRTH2^+CD45RO^+CCR7^-$ $CD4^+$ T cells (Fig. 6.3H, right). Figure 6.3I shows that there was no significant difference in the percentage of $CRTH2^+$ cells in $CD45RO^+CCR7^+$ $CD4^+$ T cells, suggesting that the increase of $CRTH2^+CD45RO^+CCR7^+$ $CD4^+$ T cells in asthma patients was due to the increase of $CD45RO^+CCR7^+$ $CD4^+$ T cells. No significant difference was seen in $CXCR5^+$, $CXCR3^+$, $CCR5^+$, or $CCR6^+$ $CD4^+$ T cells in the two groups (Fig. 6.3J). Aside from the decrease of $CCR6^+$ $CD8^+$ T cells in asthma patients, there was no significant difference of $CXCR3^+$, $CXCR5^+$, $CCR4^+$, or $CCR5^+$ $CD8^+$ T

cells in the two groups. However, $CD8^+$ T cells from asthma patients did show a significant increase in $CRTH2^+$ cells (Fig. 6.3J).

We thus concluded that asthma patients have increased $CCR4^+$ $CD4^+$ T cells compared to healthy subjects and that this difference is mainly due to the increase of $CCR4^+CD45RO^+CCR7^+$ $CD4^+$ T cells. Asthma patients and healthy donors had a similar percentage of $CRTH2^+$ memory $CD4^+$ T cells, although asthma patients had an increased percentage of $CRTH2^+CD45RO^+CCR7^+$, but not $CRTH2^+CD45RO^+CCR7^-$, $CD4^+$ T cells. In contrast to $CD4^+$ T cells, asthma patients did have an increase in the frequency of $CRTH2^+$ $CD8^+$ T cells.

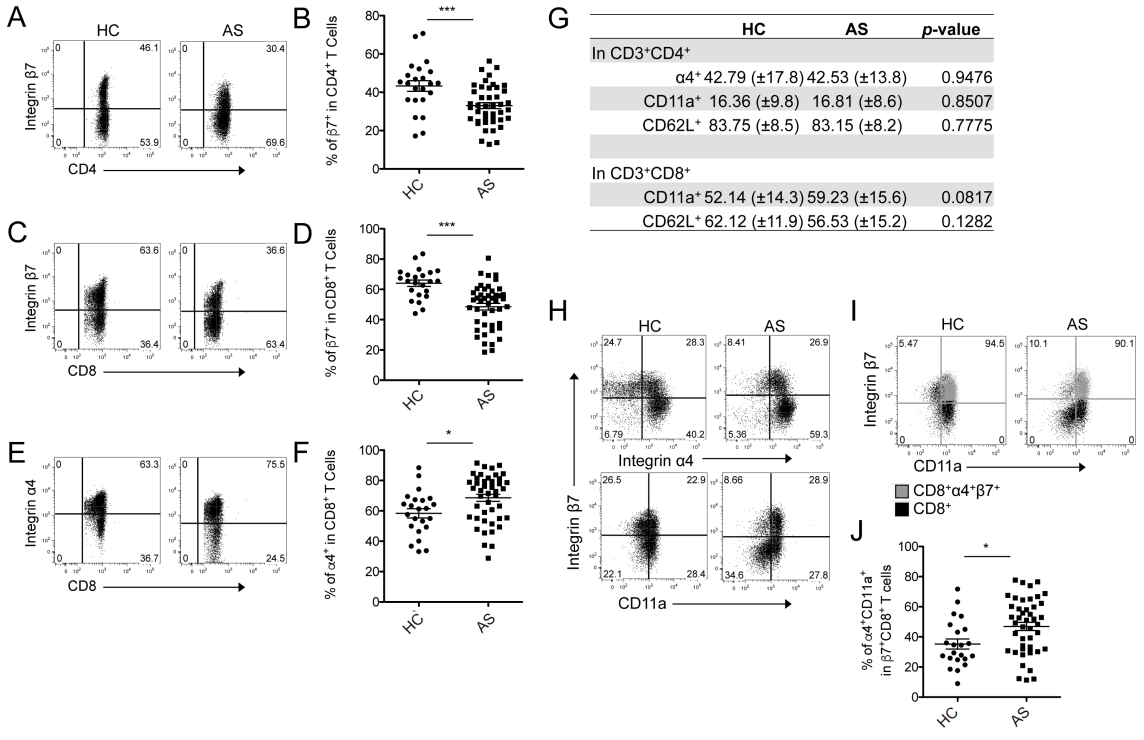


Figure 6.4. Both $CD4^+$ and $CD8^+$ T cells in asthma patients show altered integrin expression. Representative whole blood staining of (A) $\beta7^+$ $CD4^+$ T cells, (C) $\beta7^+$ and (E) $\alpha4^+$ $CD8^+$ T cells. Compiled percentages of (B) $\beta7^+$ in $CD4^+$ T cells and (D) $\beta7^+$ and (F) $\alpha4^+$ in $CD8^+$ T cells. (G) Represents average percentages with SD for additional markers in both $CD4^+$ and $CD8^+$ T cells. (H) Whole blood staining of β7, α4 and CD11a in $CD8^+$ T lymphocytes. (I) Whole blood staining showing that $\beta7^+\alpha4^+$ $CD8^+$ T cells are mostly (>90%) $CD11a^+$. (J) Compiled data of $\alpha4^+CD11a^+$ frequency in $\beta7^+$ $CD8^+$ T cells. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars indicate SD.

Altered Expression of Integrins on $CD4^+$ and $CD8^+$ T Cells in Asthma Patients

In addition to chemokine receptors, cell surface integrins are also linked to the migratory capacity of T lymphocytes. We thus investigated whether T cells in asthma patients have altered expression levels of the integrins β7, α4, and CD11a and with CD62L, a cell adhesion molecule. Representative flow cytometry data (Fig. 6.4A) shows the decrease of $\beta7^+$ $CD4^+$ T cells in asthma patients, and this is further supported by the summarized data in Figure 6.4B. No significant difference was observed for $\alpha4^+$, $CD11a^+$ or $CD62L^+$ $CD4^+$ T cells (Fig. 6.4G). Asthma patients also have a decreased percentage of $\beta7^+$ $CD8^+$ T cells, as shown in Figure 6.4C (representative flow cytometry data) and Figure 6.4D (summarized data). Interestingly, however, asthma patients had an increase of $\alpha4^+$ $CD8^+$ T cells (Fig. 6.4E and 6.4F). There were no significant differences in $CD11a^+$ or $CD62L^+$ $CD8^+$ T cells (Fig. 6.4G). However, we found that a large fraction of $\beta7^+$ $CD8^+$ T cells in asthma patients express α4 and CD11a (Fig. 6.4H). In addition, Figure 6.4I shows that the majority of $\beta7^+\alpha4^+$ CD8+ T cells also express $CD11a^+$. Approximately 60% and 50% of $\beta7^+\alpha4^+CD11a^+$ $CD8^+$ T cells also express CCR5 and CXCR3, respectively (data not shown). Further analysis of the frequency of $\beta7^+$ $CD8^+$ T cells revealed that asthma patients have significantly increased percentage of $\beta7^+$ CD8+ T cells co-expressing both α4 and CD11a (Fig. 6.4J), although the proportion of total $\beta7^+$ CD8+ T cells is lower in asthma patients than healthy subjects (Fig. 6.4C and 6.4D).

We thus concluded that asthma patients have a significantly increased percentage of $\alpha4^+CD11a^+$ in their $\beta7^+$ $CD8^+$ T cells, while they have decreased percentage of $\beta7^+$ $CD4^+$ and $\beta7^+$ $CD8^+$ T cells compared to healthy subjects.

An Increased Percentage of Tregs in Asthma Patients

Although Tregs in asthma patients have been reported to show certain levels of functional deficiency (223, 224), Tregs can also suppress allergic inflammatory responses (209, 210). We thus assessed the frequency of Tregs in asthma patients and than compare those of healthy subjects. As shown in Figure 6.5A (representative flow cytometry data

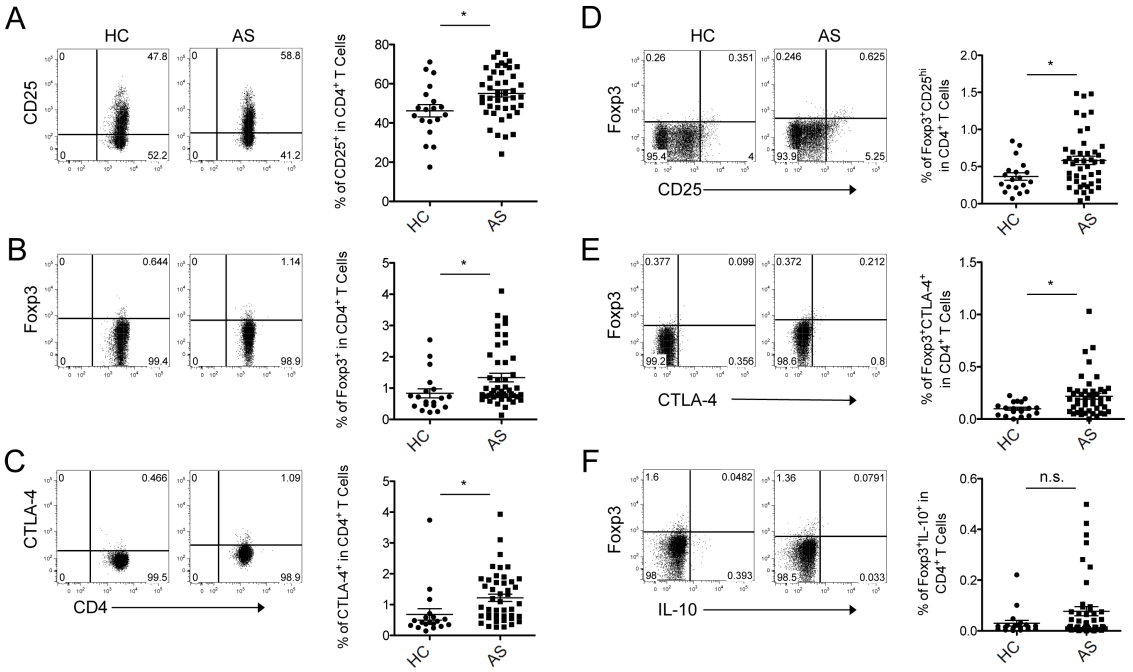


Figure 6.5. $CD4^+$ T cells in asthma patients show an increase in regulatory T cell markers. PBMCs were isolated from whole blood and cultured for 4-5 hours with golgiplug (A-E) or stimulated with αCD3/αCD28 beads at a 1:1 bead:cell ratio for 5-6 hours with the addition of golgiplug to view cytokine expression (F). Representative FACS staining and summarized data for the frequency of (A) $CD25^+$, (B) $CTLA\text{-}4^+$ and (C) $Foxp3^+$ in live $CD4^+$ T cells. Staining and summarized frequencies of (D) $Foxp3^+CD25^{hi}$ and (E) $Foxp3^+CTLA\text{-}4^+$ in live $CD4^+$ T cells. (F) Staining and summarized percentage of $Foxp3^+IL\text{-}10^+$ in αCD3/αCD28 stimulated $CD4^+$ T cells. *P<0.05. Errors bars indicate SD.

on the left and summarized data generated on the right), asthma patients have significantly more $CD25^+$ $CD4^+$ T cells in the periphery than healthy donors. In addition,

significant increases of Foxp3$^+$ (Fig. 6.5B) and CTLA-4$^+$ CD4$^+$ T cells (Fig. 6.5C) in asthma patients were also observed. When we further analyzed double-positive Foxp3 populations, we found that there were greater proportions of both Foxp3$^+$CD25^{hi} (Fig. 6.5D) and Foxp3$^+$CTLA-4$^+$ (Fig. 6.5E). We also examined IL-10 expression by Foxp3$^+$ CD4$^+$ T cells stimulated with αCD3/αCD28 beads. While some patients had a large percentage of Foxp3$^+$IL-10$^+$ CD4$^+$ T cells, there was no significant difference between patient and healthy (Fig. 6.5F). We therefore concluded that asthma patients have an increase in Tregs that express CTLA-4 and IL-10.

CD8$^+$ T Cells in Asthma Patients show an Increase in Granzyme B Production

CD8$^+$ T cells in asthma patients were further investigated by assessing granzyme B, perforin, and IFNγ expression and comparing them with those of healthy subjects. Figure 6.6A shows that asthma patients have an increased percentage of CD8$^+$ T cells

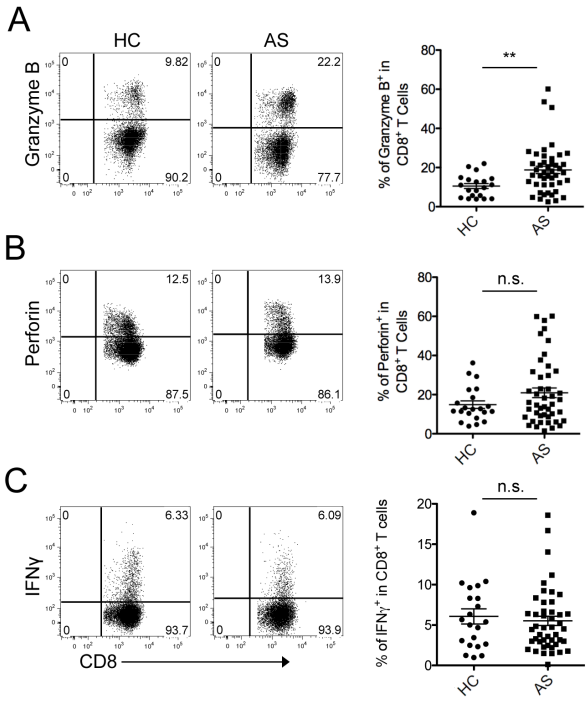


Figure 6.6. Asthma patients show an increase in cytotoxic CD8$^+$ T cells. PBMCs were isolated from whole blood and stimulated for 6 hours with anti-CD3/anti-CD28 beads given at a 1:1 bead:cell ratio in the presence of golgiplug before staining for intracellular proteins. Staining and compiled percentage of (A) granzyme B, (B) perforin and (C) IFNγ in live CD8$^+$ T cells. ***P*<0.01. Error bars indicate SD.

expressing granzyme B. This was unique to granzyme B, as there was no significant difference for the percentage of perforin$^+$ CD8$^+$ T cells (Fig. 6.6B) or IFNγ$^+$ CD8$^+$ T cells (Fig. 6.6C) in the two groups, although patients do show a trend for an increase of perforin expression. Granzyme B and perforin expression were not analyzed in CD4$^+$ T cells, as their expression levels were very low. We can infer that asthma patients have an increase in the cytotoxicity of CD8$^+$ T cells in the periphery.

Table 6.2. Alterations of CD4$^+$ T cell variables with anti-IgE treatment

Variables in CD4$^+$ T cells	Baseline	Week 26	*p*-value
% of CCR6$^+$	16.63 (±6.24)	15.29 (±7.18)	*0.0268
# of CCR6$^+$	128.7 (±66.01)	106.5 (±56.21)	*0.0197
% of CCR4$^+$	17.83 (5.89)	18.98 (7.41)	0.9113
% of CRTH2$^+$	1.59 (0.96)	1.59 (0.95)	0.8126
% of CCR4$^+$ in CRTH2$^+$	52.95 (±16.71)	60.64 (±15.36)	***0.0005
% of memory CRTH2$^+$CCR4$^+$	0.799 (0.574)	0.854 (0.539)	0.1012
# of memory CRTH2$^+$CCR4$^+$	5.578 (±3.89)	6.251 (±3.80)	*0.0392
% of EM in CRTH2$^+$	71.09 (±12.74)	77.67 (±9.72)	**0.0058
% of CM in CRTH2$^+$	9.59 (5.85)	7.92 (5.29)	*0.0321
% of CXCR5$^+$	14.47 (4.80)	14.53 (4.51)	0.8324
% of memory CXCR5$^+$	10.30 (±3.76)	11.02 (±4.23)	*0.036
% of memory CXCR5$^+$CCR4$^+$	1.407 (±0.69)	1.820 (±1.23)	*0.0465
% of α4$^+$β7$^+$	8.943 (±3.54)	8.453 (±3.71)	*0.0356
# of α4$^+$β7$^+$	70.52 (±35.86)	56.62 (±28.60)	*0.0273
% of CD11a$^+$	16.78 (8.72)	15.45 (8.03)	0.0903
# of CD11a$^+$	129.2 (±77.54)	101.7 (±55.40)	*0.0335
% of α4$^+$CD11a$^+$	15.05 (6.72)	14.28 (7.71)	0.0984
# of α4$^+$CD11a$^+$	118.9 (±62.88)	92.41 (±47.55)	*0.0284
% of IFNγ$^+$	1.55 (0.63)	1.28 (0.81)	*0.0197
% of IFNγ single positive	1.427 (±0.62)	1.180 (±0.78)	*0.0245
% of IL-4$^+$	0.2192 (±0.203)	0.3680 (±0.533)	*0.0453
% of IL-13$^+$	0.216 (0.592)	0.161 (0.152)	0.5679
% of Foxp3$^+$CD25$^+$	0.5785 (±0.377)	0.9033 (±0.710)	**0.0093
% of IL-10$^+$	0.1402 (±0.321)	0.3552 (±0.622)	*0.0498

Data represents mean with SD. *P<0.05, **P<0.01, ***P<0.001. IFNγ levels are from unstimualted cells. CM: central memory, EM: effector memory

Changes in T Cell Populations by the Blockade of IgE

Since peripheral blood T cells do not express any IgE receptors and only a limited population of tonsil T cells express the low affinity IgE receptor (CD23) (76), the effects of IgE blocking by anti-IgE occur indirectly. In order to determine the effects of anti-IgE treatment on T cells, we compared T cells before treatment with week 26 of treatment. $CD4^+$ T cells showed a greater degree of changes than $CD8^+$ T cells, however both still exhibited a number of variances. Interestingly, in $CD4^+$ T cells, treatment with anti-IgE induces a significant increase in Treg percentage, including an increase in IL-10 production. This was followed with a decrease in the expression levels of IFNγ, CCR6, α4, β7 and CD11a. Conversely, there was an increase in memory $CRTH2^+CCR4^+$ and the percentage of $CCR4^+$ cells in $CRTH2^+$, along with an increase in memory $CXCR5^+$ and IL-4 production (Table 6.2).In $CD8^+$ T cells, there were far less changes in populations with anti-IgE treatment (Table 6.3).

Table 6.3: Alterations of $CD8^+$ T cell variables with anti-IgE treatment

Variables in $CD8^+$ T cells	Baseline	Week 26	*p*-value
% of CM	8.039 (±7.94)	6.610 (±7.08)	*0.0163
# of CM	23.86 (29.77)	20.45 (29.59)	0.2096
% of $IFN\gamma^+$	1.72 (0.84)	1.37 (1.01)	*0.0182
% of IFNγ single positive	3.219 (±2.03)	2.698 (±2.17)	*0.0274
% of $IL\text{-}4^+$	0.2029 (±0.592)	0.1586 (±0.234)	*0.0253
% of $IL\text{-}21^+$	0.0775 (±0.067)	0.3254 (±0.878)	*0.0418

Data represents mean with SD. *P<0.05. IFNγ levels are from unstimulated cells. CM: central memory, EM: effector memory

Similarly to $CD4^+$ T cells (Table 6.2), $CD8^+$ T cells showed a reduction in IFNγ and IL-4 expression, with an increase in IL-21 production, with the treatment of anti-IgE. $CD8^+$ T cells also showed a decrease in the percentage of central memory cells

(CD45RA$^-$CD45RO$^+$CCR7). Unlike CD4$^+$ T cells, CD8$^+$ T cells did not show any alterations in integrin expression.

Comparisons between Responders and Non-Responders before Treatment

In order to discover potential biomarkers for anti-IgE response, we compared greater percentage of memory CXCR5$^+$ and CCR4$^+$. In addition, responders also showed

Table 6.4: Comparison of CD4$^+$ T cell populations between anti-IgE responders and non-responders at baseline and at week 26 of anti-IgE treatment

	Baseline			Week 26		
Variables in CD4$^+$ T cells	**Responder**	**Non-Responder**	***p*-value**	**Responder**	**Non-Responder**	***p*-value**
% of memory CRTH2$^+$	1.40 (0.92)	0.96 (0.53)	0.0754	1.45 (±0.91)	0.91 (±0.33)	*0.0182
# of memory CRTH2$^+$	9.75 (5.52)	7.30 (5.05)	0.2375	10.25 (6.28)	7.52 (3.60)	0.3
% of memory CRTH2$^+$CCR4$^-$	0.456 (±0.338)	0.250 (±0.121)	**0.0056	0.45 (±0.35)	0.27 (±0.14)	*0.0239
# of memory CRTH2$^+$CCR4$^-$	3.336 (±2.115)	1.858 (±1.101)	*0.0341	3.19 (2.37)	2.11 (1.30)	0.182
% of CM in CRTH2$^+$	0.240 (0.185)	0.179 (0.159)	0.3382	0.211 (0.175)	0.191 (0.141)	0.7676
% of EM in CRTH2$^+$	1.108 (±0.825)	0.681 (±0.383)	*0.0424	1.19 (±0.81)	0.76 (±0.27)	*0.0385
% of memory CXCR5$^+$	11.15 (±3.69)	8.14 (±2.63)	*0.0308	11.44 (4.12)	9.72 (4.49)	0.2269
# of memory CXCR5$^+$	83.34 (31.18)	61.07 (27.82)	0.1107	78.70 (35.86)	73.47 (22.45)	0.9028
% of memory CXCR5$^+$CCR4$^-$	9.731 (±3.215)	6.600 (±1.988)	**0.0072	9.54 (3.26)	7.81 (3.61)	0.1551
# of memory CXCR5$^+$CCR4$^-$	73.83 (±27.94)	49.28 (±20.27)	*0.0438	66.54 (31.37)	57.79 (18.24)	0.5767
% of CXCR5$^+$CCR4$^-$ in memory	18.10 (±5.28)	14.85 (±2.81)	*0.0341	17.84 (4.75)	15.28 (3.63)	0.098
% of CD11a$^+$	15.75 (7.25)	19.89 (12.05)	0.2889	14.48 (7.46)	18.43 (9.34)	0.197
# of CD11a$^+$	130.19 (84.36)	128.83 (84.97)	0.6331	90.93 (±50.61)	135.08 (±55.69)	*0.0309
% of IFNγ$^+$	1.66 (0.62)	1.20 (0.54)	*0.0478	1.28 (0.79)	1.29 (0.92)	0.8352
% of IFNγ single positive	1.55 (0.62)	1.06 (0.50)	*0.0218	1.18 (0.77)	1.18 (0.87)	0.2174

Data represents mean with SD. *P<0.05, **P<0.01. IFNγ levels are from unstimulated cells. CM: central memory, EM: effector memory

responder and non-responder T cells before the start of treatment (Tables 6.4 and 6.5). Within the $CD4^+$ T cell compartment (Table 6.4), there were a number of differences, particularly in CRTH2 and CXCR5 expression. Responders at baseline showed a greater number and percentage of memory $CRTH2^+CCR4^-$ and memory $CXCR5^+CCR4^-$, and a a greater percentage of IFNγ single positive (granzyme B^-perforin$^-$) cells. However, many of the alterations between responders and non-responders at baseline are lost at week 26. Similar to differences at baseline, at week 26 responders have a significantly increased percentage of memory $CRTH2^+$ and memory $CRTH2^+CCR4^-$ $CD4^+$ T cells as well as the percentage of effector memory cells within $CRTH2^+$ $CD4^+$ T cells.

Interestingly, non-responders showed a significant increase in the number of $CD11a^+$ $CD4^+$ T cells only at week 26. It is also apparent that responders are becoming more similar to non-responders at week 26 compared with baseline; while the frequency of $IFN\gamma^+$ cells is significantly different between responders and non-responder at baseline, this significance is lost at week 26, where responders have a similar percentage to non-responders. The alteration between responder and non-responder CXCR5 expression is also lost at week 26.

In addition to alterations in $CD4^+$ T cells, there were many differences between responders and non-responders in $CD8^+$ T cells (Table 6.5). Like $CD4^+$ T cells, responder $CD8^+$ T cells had a greater percentage of IFNγ expression, effector memory cells ($CD45RA^-CD45RO^+CCR7^-$) and a greater percentage and number of memory ($CD45RA^-CD45RO^+$) and $CD57^-CD28^+$ effector memory cells. Non-responders also showed an increase in the percentage of naïve $CD8^+$ T cells, which balances the strong reduction of memory $CD8^+$ T cells. At week 26 of anti-IgE treatment, responders still

exhibit an increased in the percentage and number of $CD57^{-}CD28^{+}$ effector memory cells, and in the number of memory cells compared with non-responders, however the alterations in naïve cells is lost. In addition, at week 26 non-responder exhibit significantly more granzyme B^{+} $CD8^{+}$ T cells, which was not observed at baseline.

Table 6.5: Comparison of $CD8^{+}$ T cell populations between anti-IgE responders and non-responders at baseline and at week 26 of anti-IgE treatment

	Baseline			Week 26		
Variables in $CD8^{+}$ T cells	**Responder**	**Non-Responder**	***p*-value**	**Responder**	**Non-Responder**	***p*-value**
% of naïve	40.82 (±14.02)	60.73 (±22.38)	*0.0336	42.83 (16.05)	55.63 (23.38)	0.221
# of naïve	141.38 (104.88)	185.95 (140.29)	0.4272	134.26 (87.48)	224.96 (172.03)	0.2451
% of memory	52.69 (±14.17)	35.50 (±20.92)	0.0497	52.49 (15.60)	38.64 (20.05)	0.0607
# of memory	168.74 (±108.41)	82.91 (±42.08)	*0.0128	159.81 (±91.96)	96.25 (±32.92)	*0.0102
% of EM	45.27 (±12.70)	32.61 (±18.61)	**0.0041	46.15 (15.26)	36.44 (18.78)	0.1391
# of EM	142.30 (94.41)	91.44 (90.94)	0.0746	144.05 (91.13)	101.75 (75.77)	0.1262
% of $CD57^{-}CD28^{+}$ EM	29.98 (±10.54)	20.71 (±13.78)	*0.0395	30.60 (±11.51)	21.12 (±14.02)	*0.0366
# of $CD57^{-}CD28^{+}$ EM	92.29 (±57.22)	48.09 (±20.66)	**0.0077	89.37 (±41.22)	53.14 (±19.27)	**0.0029
% of $IFN\gamma^{+}$	1.86 (0.87)	1.27 (0.58)	*0.0296	1.36 (0.92)	1.38 (1.33)	0.8352
% of IFNγ single positive	1.318 (±0.935)	0.606 (±0.319)	**0.0011	0.996 (0.585)	0.677 (0.602)	0.2174
% of Granzyme B^{+}	31.51 (17.43)	47.04 (25.48)	0.0775	34.42 (19.23)	50.05 (21.50)	*0.0472

Data represents mean with SD. *P<0.05, **P<0.01. IFNγ levels are from unstimulated cells. EM: effector memory

Discussion

To better understand the underlying mechanisms of the chronic inflammation in asthma patients, we characterized T cells in moderate-to-severe adult asthma patients using fresh whole blood. We than compared them with those of healthy subjects.

Although T cells in the periphery may not entirely be the same as those in the lower airway, they can reflect phenotypes and functions of T cells in the bronchial mucosa, owing to a "spillover" or circulation of these cells into the periphery (138-141). This study reports several important characteristics of T cells in asthma patients that extend our current knowledge of the underlying mechanisms of chronic inflammation in the lung.

Persistent inflammation requires an abundant presence of readily primed memory T cells (225). In both murine models and asthma patients, $CD4^+$ memory T cells are thought to be involved in recurrent episodes of inflammation (226). Accordingly, we found that asthma patients have a significant increase of $CD45RO^+$ memory $CD4^+$ T cells compared to healthy subjects, as previously reported (217, 227). Such an increase in the memory T cells in lung or peripheral blood could be evidence of chronic inflammation (217). In this study, however, we further found that asthma patients have a significant increase in central memory, but not effector memory, $CD4^+$ T cells. This was surprising, since one would expect to observe increased effector memory rather than central memory in asthma patients, as effector memory T cells can provide pro-inflammatory signals that contribute to the persistence of airway inflammation (212, 226, 228). Instead, cells expressing the central memory marker CCR7 can migrate to the lymph nodes where they can quickly respond to infiltrating APCs and as such are considered reactive memory cells (229, 230). Through TCR stimulation, such central memory T cells are able to acquire an effector-like phenotype and produce a greater amount of cytokines and effector memory T cells (231). It is therefore possible that such central memory $CD4^+$ T cells could be responsible, at least in part, for the chronic inflammation in the lower

airway. They can be readily activated by APCs, including dendritic cells (DCs), sensitized by a variety of allergens that are intermittently available year-round, as asthma patients usually show allergic reaction to multiple allergens.

It may be that these central memory T cells, once stimulated by allergen-presenting APCs in the lymph nodes, acquire effector function and migrate to the lung, leading to the overabundance of T cells found in the asthmatic lung. Additionally, central memory T cells efficiently respond to stimulation by proliferating (232). Therefore, in asthma patients, these cells could have a very high degree of replication that would alter their frequency compared with normal donors. In future studies, it would be important to examine the effectiveness of inhaled corticosteroid therapy on effector versus central memory T cells. There is evidence for differential effectiveness of corticosteroids on different subsets of T cells (233, 234). As moderate-to-severe asthma, patients in this study were also being treated with inhaled corticosteroid.

In line with the increase of central memory $CD4^+$ T cells, asthma patients have an increase of CRTH2, but only in the central memory $CD4^+$ T cell compartment. This increase was not observed when we analyze the frequency of $CRTH2^+$ cells in total or in $CD45RO^+$ memory $CD4^+$ T cells. This could explain inconsistent results from previous studies of the frequency of $CRTH2^+$ cells in asthma patients (221, 222). We also observed that asthma patients have significantly more $CRTH2^+$ $CD8^+$ T cells than healthy subjects. An increase in $CRTH2^+$ $CD8^+$ T cells has been demonstrated in atopic dermatitis (235); however, not much has been done to characterize $CRTH2^+$ $CD8^+$ T cells in asthma.

Consistent with the results from a previous study (213), asthma patients had an increase of $CCR4^+$ $CD4^+$ T cells in the periphery. However, the difference in the percentage of $CCR4^+$ $CD4^+$ T cells between asthma patients and healthy subjects were greater when we compare their percentage in central memory $CD4^+$ T cell subsets. CCR4 acts as a Th2 chemoattractant to the airways via TARC, which is produced by bronchial epithelial cells (236). Similarly, CRTH2 responds to prostaglandin $(PG)D_2$, which is released by activated mast cells, to migrate to the lung (237). $CCR4^+$ central memory T cells has also been considered as pre-Th2 cells (238) and upon antigenic stimulation in the lymph nodes may be primed to become effector memory Th2 cells capable of homing to the lung. Other chemokine receptors tested in this study were similarly expressed on $CD4^+$ T cells from asthma patients and healthy subjects. We have also shown that asthma patients have an increased frequency of $CD4^+$ Tregs ($Foxp3^+CD25^{hi}$), compared to healthy subjects. Although functional activity of these Tregs may need to be further investigated in the future (239, 240), the percentages of $Foxp3^+IL\text{-}10^+$ $CD4^+$ T cells in the two groups of subjects were similar.

Another important finding in this study is that asthma patients have a decreased frequency of $\beta7^+$ $CD8^+$ T cells, but they have a significantly increased frequency of $\beta7^+$ $CD8^+$ T cells that co-express both CD11a and $\alpha4$, which are known to play important roles in migration into the airway mucosa (241-244). Furthermore, a large fraction of $\beta7^+\alpha4^+CD11a^+$ $CD8^+$ T cells also co-express CCR5 and CD62L, which can also contribute to migration into lung. Not only do $CD8^+$ T cells in asthma patients show a decrease in $\beta7$ and increase in $\alpha4$, they also show a similar change in the density of these markers on the surface (data not shown). This further influences their migratory capacity,

as the density of the integrin on the cell surface can control cell adhesion and migration (245).

It was interesting to note that asthma patients have decreased frequency of $CD8^+$ T cells, but not $CD4^+$ T cells, expressing CCR6. CCR6 is known to be expressed on multiple cell types, including DCs, epithelial cells, and lymphocytes, In T cell population, memory T cells, including most $\alpha_4\beta_7$ memory T cells, are the major subsets of T cells expressing CCR6 (246). Interestingly, while most other chemokine receptors bind to multiple chemokines, CCR6 has only one chemokine ligand, CCL20, and non-chemokine human β-defensin-1 and -2 (246). It has also been known that CCL20 expression is strongly upregulated by pro-inflammatory cytokines TNFα and IL-1β as well as by pro-allergic cytokines IL-4 and IL-13. It is therefore postulated that $CD8^+$ memory T cells expressing CCR6 could be constantly being recruited to the lung where CCL20 is being expressed by allergic tissue microenvironment in the lower airway. Increased expression of granzyme B by $CD8^+$ T cells from asthma patients further indicates that these lung-migrating $CD8^+$ T cells could be pathogenic and may be a source of tissue damage in the lung (247). These data, along with the increased proportion of both central and effector memory $CD8^+$ T cells in asthma patients, strongly support a growing role of $CD8^+$ T cells in the pathogenesis of moderate-to-severe asthmatic inflammation in asthma patients (212, 217, 247, 248).

While T cells are not directly influenced by IgE, they frequently come in contact with those that are and thus can be affected. In comparing T cell composition and activation status between responders and non-responders, we may be able to better understand mechanisms behind non-response in addition to generating a profile of a non-

responder. We found that non-responders have a global T cell profile of decreased memory Th2 cells, decreased memory $CD8^+$ T cells, particularly those with little antigenic stimulation, and decreased IFNγ expression in both $CD4^+$ and $CD8^+$ T cells.

Blocking IgE will have the greatest impact on cells expressing the high affinity IgE receptor (FcεR1), such as mast cells, basophils and dendritic cells (DCs). However, each of these cell types, particularly DCs, can directly affect T cells. To study these effects in T cells, we compared their population composition before and at week 26 of treatment. Unexpectedly, anti-IgE treatment yielded more memory Th2 and T follicular helper (Tfh), as determined by the increase in $CCR4^+$/$CRTH2^+$ and $CXCR5^+$, respectively. Although we did not observe any changes in IL-21 production (data not shown), its production by Tfh cells is known to prohibit B cell IgE class-switching (249), there are no indications that they can otherwise play a role in allergic immune response (250). The increase in Tfh cells may be corresponding to a change in DCs, as they have the capacity to polarize naïve T cells into various T cell subtypes. The increase in Tfh may also have consequences in B cell development, since they are critical in driving B cell proliferation and differentiation.

It was quite unexpected that anti-IgE treatment yielded a greater Th2 population, since these are the cell type most commonly associated with allergic responses. However, this could also indicate a lack of migration of these cells to the lung, which would cause their peripheral population to be increased. To further support the lack of migration, $CD4^+$ T cells also show a decrease in integrin expression with treatment, particularly in α4 and CD11a. CCR6, a marker for Th17 cells (251), was decreased, along with IFNγ. While IFNγ may be used as an indicator of activation, it has also been

shown to be positively correlated with asthma severity (252). As IFNγ is decrease in both $CD4^+$ and $CD8^+$ T cells, this could indicate a decrease in their overall activation status, in addition to a decrease in asthma severity. An encouraging change in T cells with treatment was the increase in the percentage of regulatory T cells (Treg), and a corresponding increase in IL-10, which may aid in perpetuating a decreased allergic response.

$CD8^+$ T cells showed far less changes by anti-IgE than $CD4^+$ T cells and all the significant alterations only show a slight significance (P=0.05-0.01). $CD8^+$ T cells exhibited a reduction in central memory cells, which may indicate a decreased response occurring in the lymphoid system. Similar to $CD4^+$ T cells, there was an increase in $IFN\gamma^+$ $CD8^+$ T cells. These cytotoxic $CD8^+$ T cells could be acting in an immune-suppressive capacity (253), which would aid in the reduction of allergic responses. It is interesting that $CD4^+$ T cells were much more susceptible to anti-IgE treatment than $CD8^+$ T cells. This may be a result of the greater interaction between $CD4^+$ T cells and APCs, many of which do express FcεR1 and would be highly impacted by anti-IgE treatment.

In addition to changes over time with treatment, we also compared T cell profiles between responders and non-responders, both before and over the course of treatment. Interestingly, the variations between responders and non-responders before treatment closely mirrors the differences in T cells before and at 26 weeks after treatment. This indicates an IgE-independent mechanism in the non-responders, since they are more similar to responder T cells after treatment even before treatment is started. Since all the differences between responders and non-responders in $CD4^+$ T cells showed a reduced

population in the non-responders, this may indicate that non-responders have a depressed $CD4^+$ T cell response, although it may indicate an increase in a population that we neglected to include markers for. This was also reflected in the $CD8^+$ T cells, where the only population that was increased in non-responders were naïve cells. Many of the differences between responder and non-responder T cells were maintained at week 26, although the overall number of differences was decreased. This may indicate that responders and becoming more similar to non-responders with treatment. Similar to baseline, non-responders had less memory Th2 cells at week 26, however they had a greater number of $CD11a^+$ $CD4^+$ T cells, which may indicate a greater capacity to migrate to sites of inflammation. The inclusion of an increase of granzyme B expression in non-responder $CD8^+$ T cells at week 26 signifies that non-responders may have greater amount of cytotoxic T lymphocytes (CTLs), which may also indicate increased inflammation in non-responders, as expected if their asthma is still uncontrolled.

In summary, this study found an increase in central memory $CD4^+$ T cells as well as an increase in lung-targeting pathogenic $CD8^+$ T cells in the peripheral blood of asthma patients. Additionally, we found that anti-IgE treatment can increase the levels of regulatory T cells while decreasing T cell migratory capacity. Anti-IgE non-responders displayed a decreased level of Th2 T cells and memory $CD8^+$ T cells before treatment that was not changed over time with treatment. The insights that we can glean from the changes in T cells, both over time with treatment, between responders and non-responders before treatment and with treatment, may reveal novel mechanisms in allergic asthma and chronic inflammation pathogenesis. It is particularly important to analyze the differences between anti-IgE responders and non-responders. Even though there is no

significant difference in the levels of serum IgE between both patient groups, non-responders do not show any improvement in their asthma symptoms after the blockade of IgE. Further, the T cell profile of non-responders before treatment may more closely resemble the responder profile with treatment, again indicating that the non-responder inflammatory environment is independent of IgE.

CHAPTER SEVEN

Analysis of Cytokines from T Cells and Serum in Response to Anti-IgE Antibody (Omalizumab) Treatment

Abstract

Asthma is a chronic inflammatory disease of the airway. Asthma driven by allergen sensitivity, termed allergic asthma, is characterized by the prevalence of Th2-type cytokines such as IL-4, IL-5 and IL-13. T cells isolated from asthmatic patients' blood show a greater capacity to produce these types of cytokines. In addition, these cytokines can also be found in the serum, and they reflect the chronic inflammatory state. Here, we assessed cytokine levels from allergic asthma patients and compared them with cytokine levels from healthy control subjects. We analyzed cytokine expression from both isolated PBMCs and sera. Additionally, we were able to analyze the effects of anti-IgE treatment on allergic asthma patient cytokine and chemokine production and compare their profiles between anti-IgE responders and non-responders. We found a high degree of change in patient sera before and at week 26 of anti-IgE treatment, representing a decrease in inflammation. There were very few differences between anti-IgE responders and non-responders, indicating that clinical response to IgE neutralization is not reflected by the pattern of cytokine and chemokine expression. These results identify the global effect of anti-IgE on serum cytokine and chemokine levels. However, these results are not observed in isolated PBMCs.

Introduction

Asthma is characterized by airway inflammation associated with changes in lung function, such as airway hyper-responsiveness, airway remodeling, airway obstruction and mucus over-secretion. Asthma driven by the generation of allergen-specific IgE is termed allergic asthma. In allergic asthma, the asthmatic response is triggered by the presence of allergens, although it can also be exacerbated by microbial and viral infections (202, 205). Cytokines and chemokines play a critical role in directing the immune response. They are involved in influencing cellular migration, differentiation, activation and maturation. In allergic asthma, activation of lung epithelium by allergens can induce the release of various cytokines and chemokines, further attracting additional immune cells. In addition to lung epithelial cells, there are other structural cells that make up the lung tissue, such as fibroblasts and endothelial cells. These structural cells are capable of reacting to allergens or inflammatory signals, such as from respiratory viruses, and releasing cytokines and chemokines. Cytokines and chemokines can recruit various leukocytes. They can also act directly on their surroundings, influencing the integrity of the local tissue and leading to the classic features of chronic asthma, such as airway remodeling (254). These cytokines and chemokines can be found not only in the lung microenvironment, but also circulating in the periphery. Thus, cytokines and chemokines found in the blood may offer a reflection of the status of the lung without invasive procedures.

In order to understand the role of cytokines and chemokines in allergic asthma, we investigated the cytokine and chemokine profile of allergic asthma peripheral blood and compared that with the profile of healthy control subjects. To gain a further

understanding of the pathogenesis of allergic asthma, we also observed the alterations in cytokine and chemokine expression induced by the neutralization of IgE by studying allergic asthma patients receiving anti-IgE (omalizumab) treatment, both before the start of treatment and at 26 weeks after the initiation of treatment. Further, we were able to compare cytokine and chemokine profiles between anti-IgE treatment responders and non-responders, enabling additional observation of both IgE-dependent and independent asthma pathogenesis. We observed differences between asthmatic patients and healthy controls similar to what has already been published, including an increase in patient RANTES, MIP-1β, IL-5 and IL-13 (57, 255-258). As expected (259-262), the neutralization of IgE in allergic asthma patients lowered the levels of a wide range of cytokines and chemokines involved in the pro-inflammatory response, likely reflecting the similar decrease in asthma occurrence and increase in asthma control observed with anti-IgE treatment. Interestingly, patients unresponsive to anti-IgE treatment showed lower IL-13 expression, both before treatment and at week 26. Before treatment, non-responders also showed an increase in IP-10, which may indicate that they have a non-Th2 asthmatic phenotype. This comprehensive analysis will improve our understanding of asthma pathology, particularly in regard to the role of IgE in pathogenesis.

Methods

Study Subjects

Described in methods section of Chapter Three.

Study Design

Described in methods section of Chapter Three.

Determination of Responders versus Non-Responders

Described in methods section of Chapter Three.

Serum Collection

Whole blood was spun at 916g for 5 min. Plasma was drawn from the top, above the layer of blood in the tube. The plasma was spun again at 916g for 5 min to remove any residual debris. To isolate serum, the plasma was treated with 10% thrombin (King Pharmaceuticals, Tennessee) for 30 min at 37°C. The resulting mass was removed and the remaining sera aliquoted and stored at -80°C until further use.

PBMC Isolation and Stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation using Ficoll-Plaque PLUS (GE Healthcare, Pennsylvania). PBMCs were then plated in triplicate at $2x10^5$ cells/100 μL in 96-well U-bottom plates in complete RPMI – which contains RPMI 1640 (Invitrogen, California) supplemented with HEPES (Invitrogen), 1% non-essential amino acids, 2 mM L-glutamate (Sigma-Aldrich, Missouri), 50 units/mL penicillin, and 50 μg/mL streptomycin – with 10% human AB serum. Cells were stimulated with αCD3/αCD28 human dynabeads (Life Technologies, California) at a 1:1, bead:cell, ratio. Cell supernatants were harvested after 48 hours.

Cytokine and Chemokine Analysis

Cytokines and chemokines in the serum were quantified using a Millipore array for 20 cytokines (EMD Millipore, Darmstadt, Germany) according to the manufacturer's instructions. They are epidermal growth factor (EGF), fibroblast growth factor (FGF)-2, eotaxin (CCL11), granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-

regulated oncogene (GRO) (CXCL1), macrophage-derived cytokine (MDC) (CCL22), soluble CD40 ligand (sCD40L), interleukin (IL)-17A, IL-5, IL-7, interferon gamma-induced protein (IP)-10 (CXCL10), monocyte chemotactic protein (MCP)-1 (CCL2), macrophage inflammatory protein (MIP)-1β (CCL4), regulated-on-activation normal T-cell expressed and secreted (RANTES) (CCL5), IL-13, MCP-2 (CCL8), B cell attracting (BCA)-1 (CXCL13), MCP-4 (CCL14), thymus and activation-regulated chemokine (TARC) (CCL17), epithelial cell-derived neutrophil activating peptide-78 (ENA-78) (CXCL5), IL-3 and interferon (IFN)-α. Cytokines in stimulated PBMC supernatants that were quantified using in-house protocols are IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, IL-21, IL-22, IFNγ, and tumor necrosis factor (TNF)-α.

Statistical Analysis

Described in methods section of Chapter Four.

Results

Asthmatic Patients show an Increase in Th2-Type Cytokines and Chemokines Compared with Healthy Controls

Cytokines and chemokines from both sera and anti-CD3/anti-CD28 stimulated PBMCs were compared between healthy control subjects and asthma patients (Table 7.1). Compared to healthy controls, asthma patients had increased concentrations of RANTES, FGF2, MIP-1β, GM-CSF, BCA-1 and IL-3, but decreased concentrations of eotaxin and MCP-4 in the serum. In the stimulated PBMC supernatants, asthmatic patients showed increased concentrations of IL-5 and IL-13. There was a trend for an increase in IL-4 from patient PBMCs; however, this difference was not significant. There were no

alterations between patients and healthy controls in serum concentrations of EGF-2, GRO, MDC, sCD40L, IP-10, IL-5, IL-7, IL-13, IL-17A, MCP-1, MCP-2, TARC, ENA-78 or IFNα (data not shown). Additionally, there were no alterations in stimulated PBMC concentrations of IL-2, IL-4, IL-10, IL-17, IL-21, IL-22, IFNγ or TNFα (data not shown).

Effects of Anti-IgE Treatment on Cytokine and Chemokine Expression

In order to observe the effects of anti-IgE treatment on cytokine and chemokine expression, we compared cytokine and chemokine expression levels from both serum and stimulated PBMC supernatants before treatment and at week 26 (W26) of treatment. As shown in Table 7.2, there was a decrease in the concentrations of BCA-1, EGF, GRO, TARC, MCP-1, ENA-78, MCP-1, RANTES, MCP-4, GM-CSF, sCD40L, eotaxin, IP-10 and IL-5 from patient serum with anti-IgE administration. The only serum cytokine or chemokine that showed an increased concentration with treatment was IL-7. In stimulated PBMCs, there was a decreased concentration of IL-22 at W26. There were no alterations with anti-IgE treatment in serum concentrations of FGF-2, MDC, IL-13, IL-17A, IFNα or IL-3 or in

Table 7.1. Significant differences in cytokine and chemokine concentration (pg/mL) between healthy controls subjects and asthma patients at baseline

Variables	HC	AS	*p*-value
In Serum			
RANTES	718 (±257)	30225 (±41327)	1.55E-06
Eotaxin	155 (±81)	116 (±63)	1.04E-03
FGF-2	66 (±55)	190 (±407)	1.83E-03
MIP-1β	31 (±19)	112 (±173)	2.85E-03
GM-CSF	10.19 (±19.64)	54.72 (±168)	6.38E-03
MCP-4	74.35 (±37.88)	55.05 (±27.20)	1.36E-02
BCA-1	14.92 (±9.40)	18.57 (±21.18)	4.92E-02
IL-3	0.699 (±1.764)	3.131 (±3.389)	3.84E-02
From PBMCs			
IL-5	208 (±139)	870 (±782)	1.72E-06
IL-13	525 (±186)	1485 (±1333)	3.78E-06

Data represent mean with standard deviation

stimulated PBMC concentrations of IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, IL-21, IFNγ or TNFα (data not shown).

Table 7.2. Significant differences in cytokine and chemokine concentration (pg/mL) before treatment and at week 26 of anti-IgE treatment

Variables	Baseline	W26	*p*-value
In Serum			
BCA-1	18.57 (±21.18)	16.46 (±21.66)	2.33E-09
EGF	128 (±125)	107 (±119)	2.44E-04
GRO	4498 (±4959)	2639 (±1807)	2.48E-04
TARC	74.07 (±58.05)	55.17 (±57.15)	5.14E-04
MCP-1	509 (±224)	465 (±203)	1.30E-03
ENA-78	1154 (±614)	916 (±586)	1.73E-03
MIP-1b	112 (±173)	103 (±165)	1.89E-03
MCP-2	65.69 (±150.7)	22.73 (±9.32)	4.50E-03
RANTES	30225 (±41327)	15950 (±27137)	1.03E-02
MCP-4	55.05 (±27.20)	48.67 (±29.48)	1.39E-02
GM-CSF	54.72 (±167.6)	37.64 (±115.0)	1.93E-02
sCD40L	1633 (±1303)	1401 (±1256)	2.23E-02
Eotaxin	116 (±63)	102 (±57)	2.76E-02
IP-10	248 (±119)	223 (±129)	3.87E-02
IL-5	62.19 (±105.2)	3.65 (±5.68)	3.60E-03
IL-7	27.65 (±61.36)	29.64 (±84.14)	4.77E-03
From PBMCs			
IL-22	62.44 (±110)	11.26 (±27.70)	7.22E-04

Data represent mean with standard deviation

Variations in Cytokine and Chemokine Expression between Anti-IgE Responders and Non-Responders

Finally, to determine whether anti-IgE responders differed from non-responders based on their cytokine and chemokine expression profile, we compared them both before treatment and at W26. All significant observations are presented in Table 7.3. At baseline, non-responders had significantly decreased concentration of serum IL-13, BCA-1 and MCP-2 and a significantly increased concentration of IP-10. After 26 weeks of

anti-IgE treatment, non-responders still had a significantly decreased level of serum IL-13 compared with responders. In addition, they had significantly increased levels of serum MCP-1. There was no difference in cytokine or chemokine secretion between responders and non-responders from the stimulated PBMCs.

Table 7.3. Comparison of cytokine and chemokine expression (pg/mL) between anti-IgE responders and non-responders

Variables		**Responder**	**Non-Responder**	***p*-value**
ignificance at baseline				
n Serum				
	L-13	27.94 (±57.24)	1.45 (±3.85)	0.0124
	IP-10	231.90 (±128.02)	297.77 (±68.85)	0.0243
	BCA-1	19.39 (±17.38)	10.23 (±4.70)	0.0280
	MCP-2	30.59 (±12.20)	20.16 (±7.31)	0.0433
From PBMCs				
	NONE			
Significance at W26				
In Serum				
	IL-13	21.28 (±44.57)	1.54 (±2.97)	0.3120
	MCP-1	445.83 (±226.90)	523.38 (±83.38)	0.0422
From PBMCs				
	NONE			

Data represent mean with standard deviation

Discussion

In this study, we confirmed the increase in various pro-inflammatory cytokines and chemokines in asthmatic patient serum and stimulated PBMC cultures. We also showed the global effects of anti-IgE treatment on the reduction of a large number of pro-inflammatory cytokines and chemokines. The analysis of patient serum can give us a good idea of what is happening at the site of inflammation since cytokines and chemokines can easily leave the tissue and enter the blood stream. Thus, the usage of

serum is a viable alternative to collecting tissue biopsies or sputum. Our results show the effects of IgE neutralization as a systemic alteration. Interestingly, since some patients do not respond to anti-IgE treatment, this also allows us to observe differences in allergic asthma pathogenesis and perhaps reveals the mechanism behind anti-IgE non-responder IgE-independent allergic asthma.

Most of the cytokines and chemokines increased in patients compared with healthy controls, RANTES, MIP-1β, GM-CSF, BCA-1, IL-3, IL-5 and IL-13, have been well characterized in their involvement in allergic reactions and are known to be increased in patients (255-258, 263-265). We also showed an increase in FGF-2 expression in patients. While this has not been shown in allergic asthma, it has been shown in chronic obstructive pulmonary disease (COPD) (266). Interestingly, two additional chemokines known to be increased in asthmatic patients, eotaxin and MCP-4 (267, 268), were decreased in our patient serum. This may be a reflection of patient medication. Since all patients enrolled in our study have uncontrolled asthma, they have a higher degree of medication usage, particularly corticosteroids. It has been shown that oral corticosteroids can downregulate the expression of eotaxin and MCP-4 (269).

While all of the results comparing patients to healthy subjects match what has been reported previously, little work has been done to characterize the changes in cytokine and chemokine expression with IgE neutralization in human allergic asthma patients. Patients show a global trend for a decrease in the chemokines targeting CCR1, 2, 3 and 5, including MCP-1 (CCL2), MIP-1β (CCL4), MCP-2 (CCL8), RANTES (CCL5), MCP-4 (CCL13) and eotaxin (CCL11). In part, this reflects the highly promiscuous binding of chemokines to multiple receptors; each chemokine is capable of

binding to several receptors. However, this also indicates the decrease in inflammatory response and subsequent decrease in leukocyte recruitment, particularly monocytes, lymphocytes and eosinophils (254). Treatment also induced a reduction of TARC (CCL17), which is able to target CCR4 and CCR8, both of which are associated with aiding Th2 cell trafficking to the lung (270). This indicates that one way anti-IgE treatment works to improve asthma control is by specifically preventing the migration of pathogenic Th2 cells to the lung. The other CCL chemokine tested was MDC (CCL22), which, like TARC, binds to CCR4. However, there was no reduction of MDC with anti-IgE treatment. This may reflect the lack of change in B cells, as they are the only cell capable of producing MDC, but not TARC (271).

There is also a global trend for a decrease in chemokines targeting CXCR2, including GRO (CXCL1) and ENA-78 (CXCL5). Both chemokines are important in attracting neutrophils to the lung and can further support their activation and survival. CXCR2 plays such a large role in promoting the neutrophil response in asthma that a CXCR2 antagonist has been developed to target neutrophilic asthma (272). Interestingly, GRO is generally produced by Th17 signaling (273) and the reduction of GRO may reflect an alteration in T helper type responses. ENA-78 is largely produced by platelets (274), and its reduction may signal a decline of platelet activation by anti-IgE. The other CXC chemokines tested were IP-10 (CXCL10) and BCA-1 (CXCL13), both of which also decreased with anti-IgE treatment. Lung endothelial cells can produce both of these chemokines. While treatment did not improve lung function (Table 3.2), it likely decreased the pro-inflammatory response — decreasing activation of surrounding lung tissues, including lung epithelial, endothelial, and smooth muscle cells. Similar to this,

we also found a reduction in EGF, which may again reflect a decrease to lung epidermal injury (275).

Two chemokines not within the CC or CXC families that showed a significant reduction in their concentrations with the usage of anti-IgE were GM-CSF and sCD40L. GM-CSF is produced by a wide variety of cells, including both hematopoietic and non-hematopoietic cells. It is quickly induced by immune stimuli, inducing bacterial products and pro-inflammatory cytokines and chemokines (276). GM-CSF is critical for the activation of T cells and for the development and maturation of DCs (276), thus, the decrease of GM-CSF by anti-IgE may further aid in the resolution of asthma by preventing T and DC activation, two cell types that are critical to the maintenance of allergic asthma (9). CD40L is critical for the development of antigen-specific immune responses and is required for B cell isotype switching from IgM to IgE and IgG (277). Many of the functions of CD40L can also be performed by sCD40L (278). One of the largest producers of sCD40L are activated platelets (279). Platelets have been shown to have an underappreciated role in the pathogenesis of allergic asthma (280) and the decrease of sCD40L may reflect a similar decrease in platelet activation with anti-IgE.

The decrease of serum IL-5 with anti-IgE is unsurprising, considering the well-established role of IL-5 as a Th2-type cytokine and in promoting eosinophilic inflammation (281). Perhaps more intriguing is the reduction of IL-22 by stimulated PBMCs with anti-IgE. IL-22 is generally considered to be a Th17-type cytokine, along with IL-17, although it has been suggested that there is another T helper subtype capable of expressing IL-22 (termed Th22) (282). IL-22 is unique in that it only targets non-hematopoietic cells and thus is an important part of the immune-tissue interface. IL-22 is

primarily involved in mucosal immunity and plays a large role in promoting tissue repair (283). It is largely produced through the stimulation of innate immune receptors (283), and the decrease of IL-22 production by stimulated PBMCs indicates an overall decrease in immune activation with anti-IgE. While the cytokines IL-5 and IL-22 were decreased by anti-IgE, the concentration of IL-7 was increased. IL-7 is critically important for T cell development and survival (284) and is mainly produced by epithelial cells (285). It is unclear why other epithelial-derived cytokines and chemokines would be decreased, while IL-7 would be increased.

There is a need for a biomarker to predict response to anti-IgE treatment. We thus compared cytokine and chemokine concentrations between anti-IgE responders and non-responders. We found no alterations in cytokine or chemokine expression from stimulated PBMCs, either before or at W26 of treatment. Since PBMCs were stimulated by anti-CD3/anti-CD28, we expect that the response should be coming from T cells. The lack of difference in T cell cytokine expression indicates that non-responders do not have a significant alteration in their T cell helper types, and this alteration does not change with treatment. However, non-responders did show differences in their serum cytokine and chemokine concentrations compared with responders, particularly before treatment. One cytokine that was consistently downregulated in non-responders was IL-13. IL-13 is largely produced by $CD4^+$ T cells, but can also be expressed by basophils, mast cells and innate lymphoid cells (286). It is critical for mucus secretion, airway hyperresponsiveness (57) and B cell IgE production (156). Since there is no alteration of IL-13 produced by PBMCs, the variation of IL-13 in the serum may reflect alterations between responders and non-responders in lung inflammation. Severe, uncontrollable asthma tends to be

more neutrophilic (2), and this may be reflected in the lack of IL-13 in our non-responders. Alternatively, non-responders showed an increased concentration of IP-10. IP-10 is secreted in response to interferons (IFNs) and binds to CXCR3. Thus IP-10 is associated with Th1 responses (287). Similar to the reduction of IL-13 in non-responders, the increase in IP-10 may indicate a lack of Th2 responses in the site of inflammation. Before treatment, non-responders also showed reduced concentrations of BCA-1 and MCP-2. BCA-1 is known to promote the recruitment of B and T cells to the site of lung inflammation in allergic asthma (288). The lack of BCA-1 in non-responders points to a possible decreased involvement of B cells. Since these patients do not have an improvement in their allergic asthma even with the neutralization of IgE, this points to an IgE-independent pathway. MCP-2 can also aid in the migration of Th2 cells and eosinophils to the lung, particularly through its interaction with CCR3. Thus, its reduction in non-responders also points to a non-eosinophilic asthma phenotype in these patients. At W26, the only alteration in non-responders compared with responders, aside from IL-13, was an increased concentration of serum MCP-1 in non-responders. MCP-1 can act on airway fibrocytes and possibly promote airway remodeling and bronchial wall remodeling (289). The increase in serum MCP-1, therefore, may represent the continued pathogenesis of asthma in the non-responders.

Overall, these results show the importance of IgE on indicators of inflammation and in the migration of leukocytes from the periphery to the lung. Patients that show a lack of response to anti-IgE treatment, termed non-responders, must have an IgE-independent mechanism to further the pathogenesis of allergic asthma. We can see hints of this when comparing serum cytokine and chemokine expression between responders

and non-responders, particularly before anti-IgE treatment. Since cytokines and chemokines secreted by cells in the lung can circulate in the periphery, they may be more useful to use as an insight into the status of inflammation in the lung rather than analyzing cellular profiles in the blood. Based on our observations, non-responders may suffer from more Th1 and neutrophilic asthma, which is known to be very resistant to treatment. This highlights the highly heterogeneous nature of asthma, as these patients also exhibit the classic features of Th2 allergic responses, including increased secretion of IL-5 and IL-13 by their T cells. It may be beneficial to compare cytokine and chemokine expression for patient sputum between anti-IgE responders and non-responders, since this may give an even better picture of the inflammatory processes in the lung.

CHAPTER EIGHT

Alterations in Innate Lymphoid Populations in Allergic Asthma Patients Treated with Anti-IgE

Abstract

Asthma is a highly heterogeneous disorder characterized by the involvement of many cell types. One such cell type that may be involved are innate lymphoid cells (ILCs), such as natural killer (NK) and NKT cells (type 1 ILCs) and type 2 ILCs (ILC-2). In order to observe the role of ILCs in the pathogenesis of allergic asthma, we characterized their frequency and activation status in the whole blood of patients with allergic asthma, and compared them with healthy donor controls. We were also able to follow the asthmatic patients over the course of anti-IgE treatment in order to better understand the mechanism of action of anti-IgE. We found that allergic asthma patients had less population of NK cells, particularly those expressing dim levels of CD56, than their healthy controls and this was not altered by anti-IgE. No significant difference was seen between healthy and patient NKT cells. We observed that anti-IgE induced a significant decrease in the activation, as measured by CD69 expression, in NK $CD56^{dim}$ cells. While no difference was observed in ILC-2 between patient and healthy, their frequency, percentage and number, was decreased by anti-IgE. There were no variations between anti-IgE responders and non-responders. Data from this study will aid in understanding the role of innate lymphoid cells in allergic asthma, as well as the mechanism of action of anti-IgE therapy in these asthmatic patients.

Introduction

Innate lymphoid cells (ILCs) are similar to T cells in that they produce cytokines in varieties that parallel the various T helper subsets. However, ILCs, as their name suggests, act in the innate arm of the immune system and can respond quickly to stimuli. There are three main types of ILCs: type-1 ILCs, which includes natural killer (NK) cells, type-2 ILCs, known for their production of Th2-type cytokines, and type-3 ILCs, known for their production of Th17- and Th22-type cytokines (290). NK cells are capable of lysing their target cells through the release of cytotoxic mediators. They are defined by their expression of CD56 and lack of CD3. They can be further subdivided based on the intensity of CD56 expression: $CD56^{hi}$ and $CD56^{dim}$. The majority of NK cells in the periphery are $CD56^{dim}$, while $CD56^{hi}$ NK cells are mostly found in the lymphoid tissue. In addition to their differing levels of CD56 expression, these two subsets also express differing levels of the Fcγ receptor III (CD16). $CD56^{hi}$ cells express lower levels of CD16, while the majority of $CD56^{dim}$ cells are $CD16^{+}$. CD56, in conjunction with CD3, can be used to define natural killer T (NKT) cells. Since these cells express the T cell receptor (TCR), they are able to recognize antigens and thus function in the gap between innate and adaptive immunity. Both NK and NKT cells have been implicated in the pathogenesis of asthma. NK cells can respond to IgE through CD16, and thus can contribute to IgE-dependent allergic mechanisms, where they may be able to promote Th2-type responses through the production of Th2 cytokines (81), in addition to IL-17, which may promote neutrophilic, steroid-resistant asthma (291). Similarly to NK cells, NKT cells can also produce Th2-type cytokines, particularly IL-4 and IL-13 (292). As NKT cells are able to respond to lipid antigens, allergens containing lipids, such as fungal

glycosphinoglipid (293), they may also directly contribute to allergen sensitization. As much as NK and NKT cells have been shown to have the capacity to promote asthmatic responses, they have also been shown to protect against allergic airway responses, mainly through the production of IFNγ and the promotion of Th1-type responses (81).

Type-2 ILCs, also known as ILC-2, are classified based on their production of IL-5 and IL-13 and can be defined based on their expression of CRTH2, CD161, CD127 and lack of CD3, CD19 and CD16 (83). Because of their production of Th2 cytokines, ILC-2 have been associated with allergic diseases and can initiate many of the hallmark features of asthma (1). ILC-2 are found at only very low levels in peripheral blood, with their main residence being in the gut and the lungs (83).

In order to generate a global picture of ILC involvement in allergic asthma, we characterized NK (ILC-1), NKT cells and ILC-2 from the whole blood of moderate-to-severe allergic asthma patients and compared them to healthy controls. Our results indicated that, while NKT cells show very little differences between patients and healthy, patients have significantly less frequency of NK cells, particularly NK $CD56^{dim}$. These cells were also sensitive to anti-IgE treatment, and we observed a significant reduction in their activation over time by anti-IgE. We did not find any difference in ILC-2 levels between healthy and patient, although their population was decreased by anti-IgE treatment. Due to the extremely low frequency of these cells in the periphery (<0.01% of total cells), we were unable to assess their activation status. NKT cells do no appear to play much, if any, of a role in asthma pathogenesis.

Methods

Study Subjects

Described in methods section of Chapter Three.

Study Design

Described in methods section of Chapter Three.

Determination of Responders versus Non-Responders

Described in methods section of Chapter Three.

Table 8.1. Antibodies used for innate lymphocyte analysis

Marker	Clone	Company
CD56	HCD56	BioLegend
CD3	UCHT1	BD
CD16	3G8	BD
CRTH2 (CD294)	BM16	BD
CD127	HIL-7R-M21	BD
CD161	HP-3G10	BioLegend
NKG (CD314)	1D11	BioLegend
NKp30 (CD337)	P30-15	BioLegend
CD57	HCD57	BioLegend
CD69	CH/4	Invitrogen
IFNγ	4S.B3	BD
Granzyme B	GB11	Invitrogen
Perforin	dG9	BioLegend
Live/Dead		Invitrogen

Antibodies, Reagents and Flow Cytometry

Described in methods section of Chapter Four. Antibody information is provided in Table 8.1.

PBMC Isolation and Intracellular Staining

Described in methods section of Chapter Five.

Statistical Analysis

Described in methods section of Chapter Three.

Results

Asthmatic Patients show a Significant Decrease in NK Percentages and Numbers Compared with Healthy Donors, but are not Altered by Anti-IgE treatment

We first compared the differences in NK ($CD3^{-}CD56^{+}$) and NKT ($CD3^{+}CD56^{+}$) populations between patient and healthy controls (Fig. 8.1). If we further analyze NK cells based on $CD56^{high}$ and $CD56^{dim}$, we see that both population percentages are

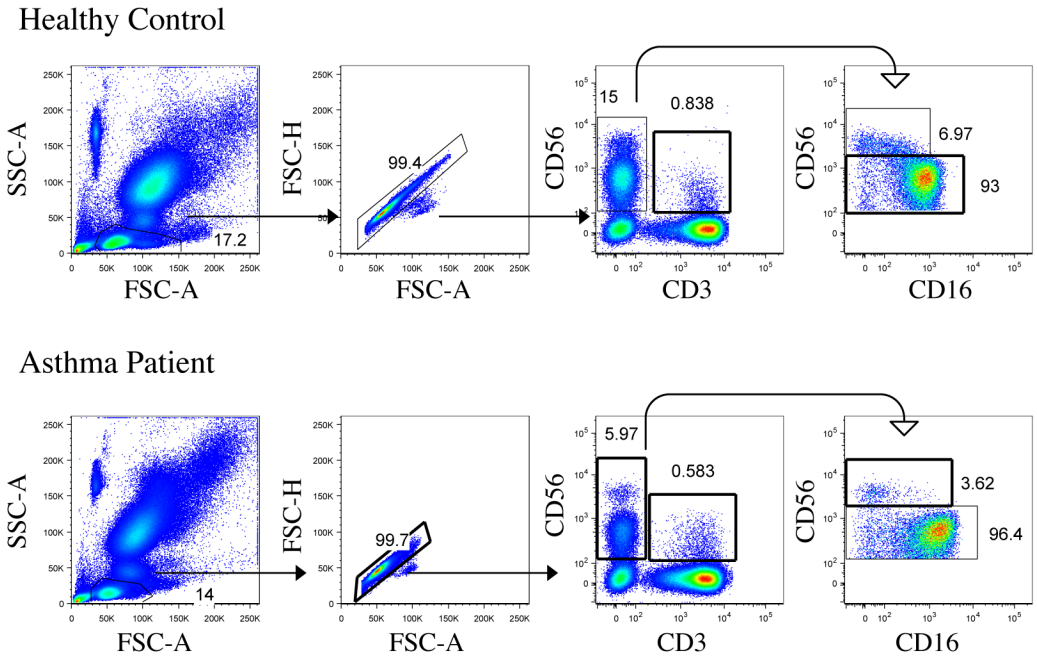


Figure 8.1. Gating strategy for NK, both $CD56^{hi}$ and $CD56^{dim}$, and NKT cells.
Total whole blood lymphocytes were first gated based on low FSC and SSC. NK cells were identified as $CD3^{-}CD56^{+}$ and than further divided into $CD56^{hi}$ and $CD56^{dim}$. NKT cells were identified as $CD3^{+}CD56^{+}$.

depressed in patients (Fig. 8.2A and 8.2C, left), but $CD56^{dim}$ additionally show a decrease in their number (Fig. 8.2D, left). NKT cells do not show a difference between healthy and patient, in either frequency or number (Fig. 8.2E and 8.2F, left). Additionally, there were no alterations in their population with anti-IgE treatment.

Treatment with Anti-IgE Decreases the Activation Status of NK^{high} Cells

NK and NKT cells were further analyzed for their levels of activation and phenotype marker expression. There were no difference between patient and healthy NK and NKT activation markers (data not shown). However, NK^{high} cells showed a strong reduction in activation with the treatment of anti-IgE, as shown by a significant decrease in the percentage of cells expressing IFNγ and co-expressing IFNγ with granzyme B (Table 8.2). In addition, NK^{high} cells also showed an increase in the percentage of cells expressing NKp30. However, this was offset by a similar decrease in the percentage of cells expressing both CD57 and NKp30. NK^{dim} cells showed a strong reduction in activation status by anti-IgE, as measured by the decrease in CD69 expression, including a decrease in the percentage of

Table 8.2: Changes in NK and NKT cells with anti-IgE treatment

Variables	Baseline	Week 26	*p*-value
In NK^{hi}			
% of $IFN\gamma^+$	6.63 (±6.01)	4.20 (±3.93)	0.0091
% of $IFN\gamma^+$Granzyme B^+	3.76 (±3.51)	2.65 (±3.16)	0.0281
% of $CD57^+NKp30^+$	2.55 (±2.05)	2.01 (±1.66)	0.0147
% of $NKp30^+$	88.28 (±7.08)	91.48 (±6.00)	0.0250
In NK^{dim}			
% of $CD69^+$	5.09 (±5.65)	3.67 (±2.58)	0.0354
CD69 MFI	199.1 (±84.21)	173.3 (±54.23)	0.0248
% of $CD57^+CD69^+$	2.28 (±1.99)	1.65 (±1.52)	0.0147
# of $CD57^+CD69^+$	3.41 (±3.42)	2.11 (±1.65)	0.0030
In NKT			
% of $CD57^+CD161^+$	2.01 (±1.62)	1.71 (±1.18)	0.0162

Values represent mean with standard deviation
MFI: mean fluorescent intensity

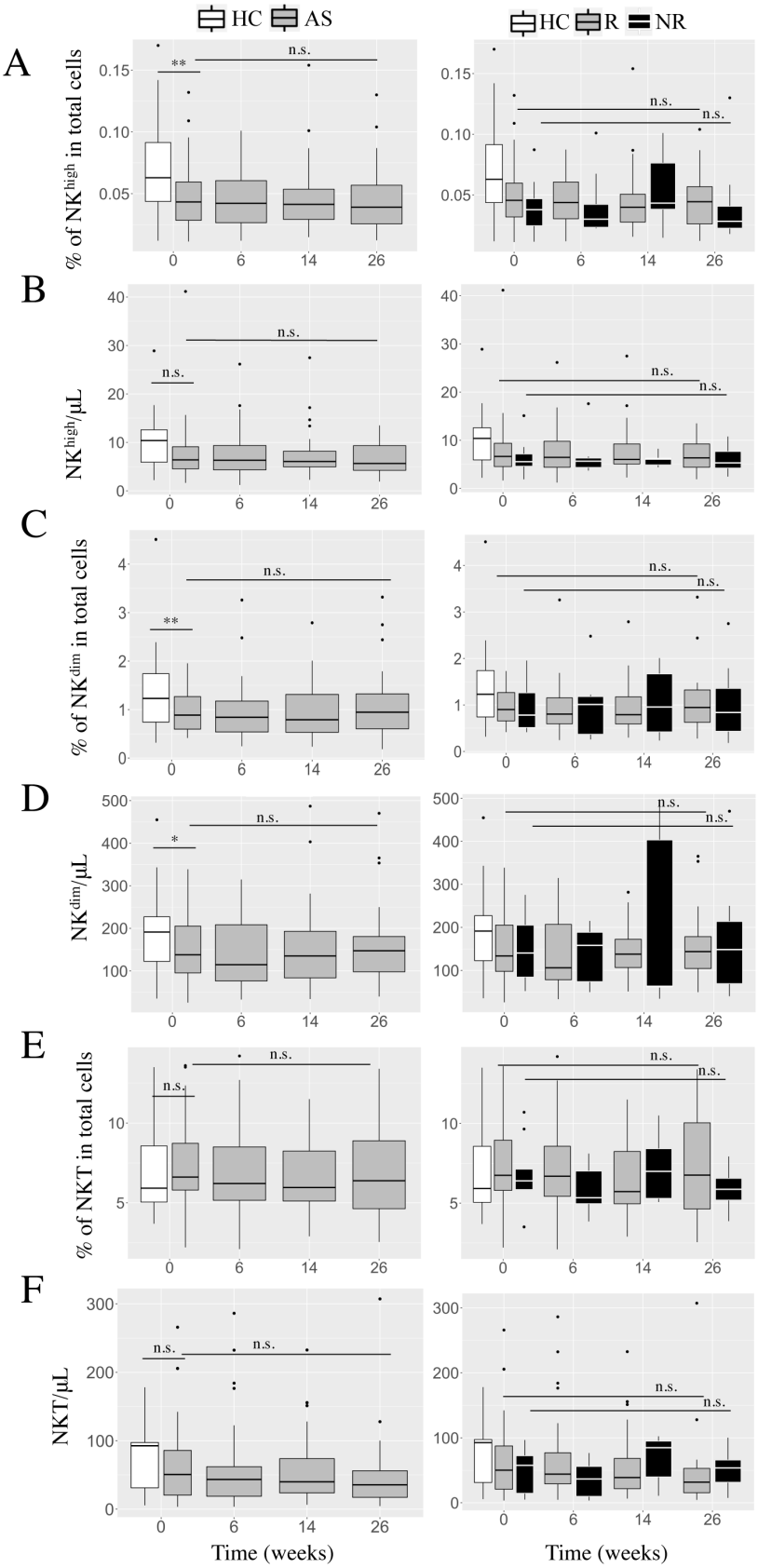


Figure 8.2. Anti-IgE treatment does not affect NK or NKT populations. (A) Average percentage and (B) number per µL of NK CD56^{hi} (NK^{hi}) cells in whole blood. (C) Average percentage and (D) number per µL of NK CD56^{dim} (NK^{dim}) cells in whole blood. (E) Average percentage and (F) number per µL of NKT cells in whole blood. **P*<0.05, ***P*<0.01, and n.s.: not significant. Error bars represent SD.

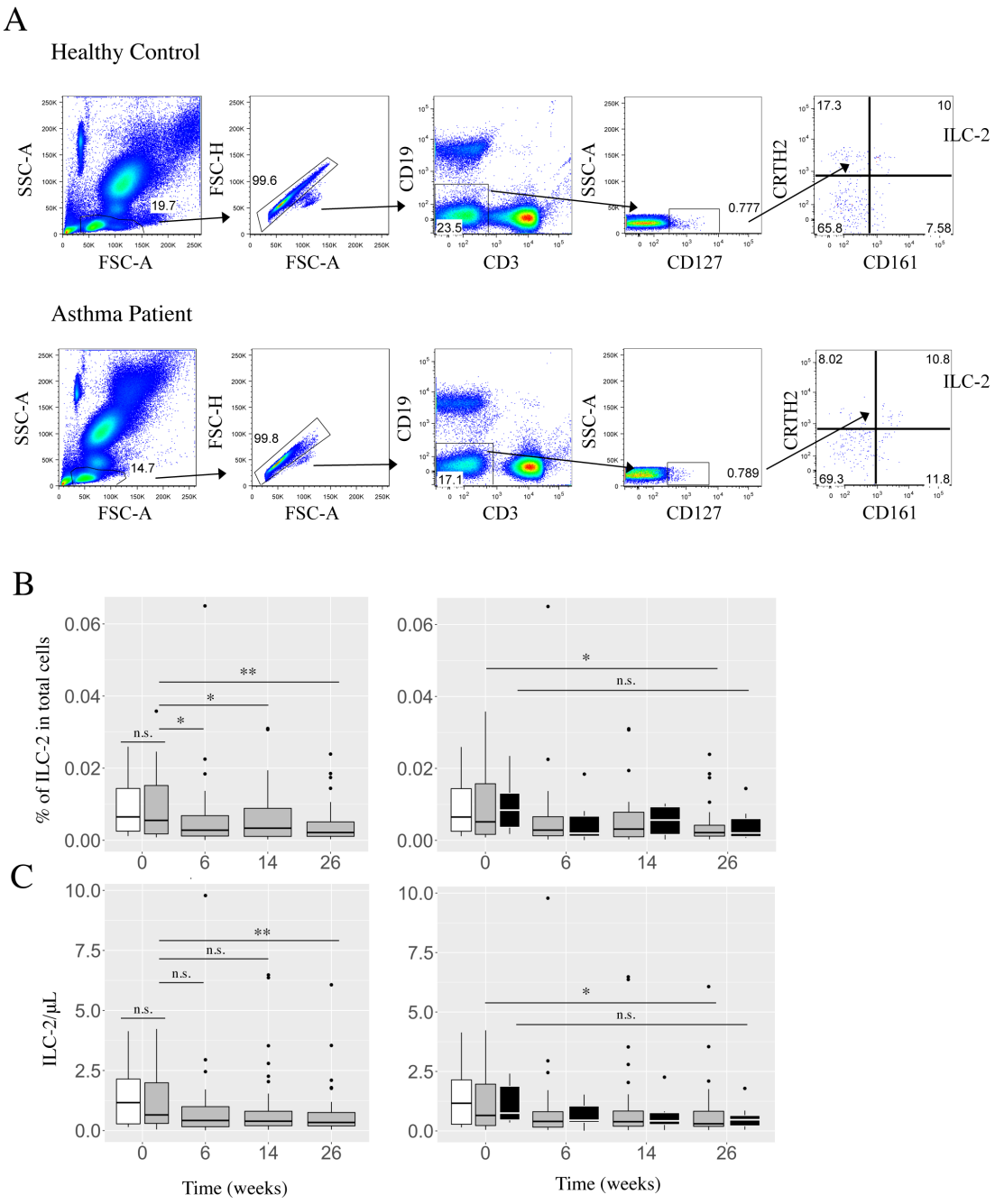


Figure 8.3. ILC-2 population frequency is decreased by anti-IgE treatment.
(A) Total whole blood lymphocytes were first gated based on low FSC and SSC. ILC-2 cells were identified as $CD3^{-}CD19^{-}CD127^{+}CD161^{+}CRTH2^{+}$. (B) Average percentage and (C) number per μL of ILC-2 cells in whole blood. **P*<0.05, ***P*<0.01, and n.s.: not significant. Error bars represent SD.

$CD69^{+}$ cells, the intensity of CD69 expression (measured by fluorescent intensity [MFI]), and the percentage and number of $CD57^{+}CD69^{+}$ cells (Table 8.2). Compared with NK

cells, NKT cells had very little change with treatment. The only alteration significant was a decrease in the percentage of $CD57^{+}CD161^{+}$ NKT with treatment. These results indicate that NK and NKT cells show little effect by anti-IgE treatment, although NK cell activation status seems to be reduced. Although we were unable to observe any activation markers in ILC-2 due to their low frequency in peripheral blood, we did find that their population, both percentage and number, decreased significantly with treatment (Fig 8.3), and this was most evident in anti-IgE responders.

Discussion

To better understand the pathogenesis of allergic asthma, we characterized ILCs in the periphery of moderate-to-severe allergic asthma patients using fresh whole blood and compared them with healthy controls. Research of ILCs in asthma is generally focused on the main site of inflammation, the lungs. However, much can be learned from the analysis of these cells in peripheral blood and how they may be impacting other cell types. This study uncovered several as yet unidentified alterations in allergic asthma NK cells. We also observed a decreased number of T2M cells (data not shown), which may be capable of exacerbating the asthmatic response. This study may aid in the understanding of chronic inflammation in the context of allergic asthma.

NK cells are capable of producing large quantities of both Th1- and Th2-type cytokines (294), while NKT cells have been shown to produce Th1-, Th2- and Th17-type cytokines (291). In addition, both NK and NKT cells can act as cytotoxic lymphocytes and lyse target cells through the production of perforin and granzymes (295, 296). There are conflicting reports on the changes in NK/NKT population frequency in inflammatory

conditions (295, 297, 298), although most agree that there are large numbers of NK cells in the lung of asthmatic patients (81). In this study, we found that asthma patients have a significant decrease in the percentage and number of NK cells, particularly in the $CD56^{dim}$ subtype. As has been suggested (299), these cells may be migrating to the lung after antigen challenge, and thus have a decreased presence in the periphery. We also showed that patient NK $CD56^{hi}$ cells had a greater degree of activation, in addition to greater cytotoxicity, similar to data shown by Jira et al. (300).

In contrast to NK cells, NKT cells showed no significant difference between patients and healthy controls in any of the markers studied. Like with NK cells, there are multiple conflicting reports on the role of NKT cells in asthma, however the majority of this work has been done using either lung biopsies or bronchoalveolar lavage fluid (80). Since NKT cells can recognize antigens through their T cell receptor (TCR), they can be directed by APCs. NKT cells have a restricted TCR that only allows for recognition of antigen presentation by CD1d (296). Dendritic cells (DCs) are potent APCs and constitutively express CD1d, thus they are primed for directing NKT activation (296, 297). As such, NKT cells may be directly activated through allergen-presenting DCs. Since these allergen-specific DCs would be found in the lung or lung-draining lymph node, NKT cells in these locations would be much more susceptible to DC influence. This may support our findings of normal numbers and phenotype of NKT cells in the blood of asthmatic patients.

Based on their low frequency in peripheral blood, ILC-2 have been difficult to characterize. While it has been shown that ILC-2 percentage and number is increased in allergic asthma patient PBMCs compared with both healthy controls and allergic rhinitis

patients (301), in our study, we did not observe any difference in ILC-2 between allergic asthma patients and healthy donors. One large discrepancy between our study and that done by Bartemes et al. (301) is that they first isolated PBMCs from blood before ILC-2 characterization. Thus, their frequency in PBMCs is likely increased due to the removal of granulocytes and platelets and this may amplify the differences between patients and healthy. This study therefore may serve to emphasize the usage of PBMCs, rather than whole blood, to study ILC-2.

With anti-IgE treatment, NK^{hi} cells showed a reduction in IFNγ and NKp30 expression. It has been shown that NK^{hi} cells respond to maturing dendritic cells (DCs) through the production of IFNγ, and this may help promote Th1-type responses (302, 303). Their reduction of IFNγ expression may be a result in the reduction of DC activation status that is also seen with anti-IgE treatment (see below). The decrease of NK^{hi} cells expressing IFNγ and granzyme B may indicate a decreased cytotoxic capacity of these cells. NKp30 is a type of natural cytotoxicity receptor and is also involved in cytotoxicity. One of the isoforms of NKp30, NKp30c, is associated with immunostimulatory properties, including the downregulation of IFNγ production (304). While our staining antibody cannot discern the difference in NKp30 isoforms, the increase of NKp30 expressing cells, along with the decrease in IFNγ expression, may indicate a change of NK^{hi} towards immune modulation with anti-IgE treatment. Conversely, treatment yields a reduction in the percentage of NK^{hi} cells expressing both CD57 and NKp30. CD57 expression in NK cells can identify maturity, in addition to increased cytotoxicity (305). The decrease in the frequency of $CD57^{+}NKp30^{+}$ NK^{hi} cells may again indicate a reduction of NK cytotoxicity with the blocking of IgE. Because of

flow cytometry panel limitations, we were unable to include CD57 and NKp30 in the same panel as IFNγ, but it is likely that NK cells expressing NKp30 also express IFNγ.

In comparison to NK^{hi} cells, NK^{dim} cells are known for their propensity for cytotoxicity. Since the majority of NK^{dim} cells also express the Fcγ receptor CD16, they are capable of mediating antibody dependent cell-mediated cytotoxicity (ADCC). We show that blocking IgE leads to the reduction of CD69 expression on NK^{dim} cells. This includes a reduction in both the percentage and number of CD69, the intensity of CD69 expression, and the percentage and number of $CD57^{+}CD69^{+}$ NK^{dim} cells. CD69 is a well characterized marker of activation and, in NK cells, it can also mediate cytolysis (306). The decrease of CD69, particularly in conjunction with CD57, in NK^{dim} cells with anti-IgE treatment indicates a decrease in their activation and cytolytic capacity, similar to that seen in NK^{hi} cells with treatment.

Like NK cells, NKT cells express CD56, although at a level similar to NK^{dim} cells. In addition, like T cells, they express CD3, and are capable of expressing an invariant T cell receptor recognizing glycolipids. The only change in NKT cells with anti-IgE treatment was a reduction in the percentage of $CD57^{+}CD161^{+}$ expressing cells. CD161 was originally considered only a NK cell marker, but has since known to be expressed on both NKT and T cells. While there is still much that is unknown about the function of CD161, in both T cells and NK/NKT cells (307), it has been generally associated with T cells expressing IL-17 (Th17), . It has also been shown that CD161 can act as a costimulatory receptor (308). Thus, the reduction of NKT cells expression the maturation marker CD57 and the costimulatory receptor CD161 signifies that these cells may have a more naïve phenotype.

The frequency of ILC-2 in peripheral blood is very low, and without any prior enrichment, we were only able to quantify their percentage and number. ILC-2 are capable of replacing Th2 responses in allergic asthma through their production of large quantities of the type-2 cytokines IL-5 and IL-13 in response to IL-25 and IL-33 produced by stimulated lung epithelial cells (309, 310). The decrease in ILC-2 percentage and number reflects the similar decrease in type-2 cytokines that has been observed by the usage of anti-IgE (259, 261). Because IL-25 and IL-33 can influence ILC-2 recruitment, expansion and function (290), a reduction in these cytokines, which may be mediated through the neutralization of IgE, would thus impact ILC-2 population frequency. We also compared the population of T2M cells, noted by Petersen et al. (311) to potentially promote chronic allergic asthma, however, we did not observe that anti-IgE treatment had any effect on either their frequency or number (data not shown).

When comparing ILC composition between anti-IgE responders and non-responders, there were only minor differences between the two groups at baseline. Before treatment, anti-IgE non-responders exhibited a decrease in the percentage of total NK cells expressing IFNγ, but displayed an increase in CD69 expression intensity in NK^{hi} cells. There were no alterations in ILC-2 populations between responders and non-responders. The reduction of IFNγ expression in non-responder NK cells may reflect a difference in APC activation or maturation status, but could also reflect a decrease in cytotoxicity, although there were no alterations in granzyme B or perforin expression. The increase in CD69 expression intensity in non-responder NK^{hi} cells indicates that they have greater levels of activation. It is interesting that this is specific to NK^{hi} cells, as it is not significant in either NK^{dim} or NKT. It has been shown that NK^{hi} cells are the type of

NK cell preferentially present in sites of peripheral inflammation, where they demonstrate an activated phenotype, based on an increase in CD69 expression (312). It may be that anti-IgE non-responders have increased peripheral inflammation, which may be too embedded to be disrupted by the blocking of IgE. In other words, there is such a degree of pro-inflammatory signaling that the inflammation is sustained even without IgE stimulation. It is unsurprising that the ILC-2 population was not varied between responders and non-responders, as these two types of patients seem to have more differences in their cellular activation status than in any overall population frequency.

Overall, these results suggest that the reduction of free IgE by anti-IgE leads to the decrease in activation and cytotoxicity of NK cells, the decrease of mature NKT cells, and a decrease in ILC-2. While ILCs do not express or express very little IgE receptors (313, 314), they are likely more sensitive to the status of APCs, particularly DCs, than to changes in free IgE levels. Their reduction in activation and cytotoxicity is likely a result of a decreased activation status of APCs. Similarly, the decreased maturity of NKT cells may reflect a decrease in antigen presentation by APCs. They may also be reflecting the decrease in pro-inflammatory conditions resulting in anti-IgE treatment. Since the majority of patients receiving anti-IgE are able to reduce or stop the usage of rescue and control medications, this indicates that anti-IgE can reduce the inflammatory milieu, indirectly affecting ILCs. In addition, allergic asthma patients that do not respond to anti-IgE treatment may have increased peripheral inflammation, as indicated by their increase in CD69 expression on NK^{hi} cells, and thus would not need signaling from IgE to continue the promotion of inflammation. A better determination of the status of

peripheral inflammation in allergic asthma patients may aid in the selection of anti-IgE responders.

NK cells can act in either a pathogenic or regulatory fashion (294). While our results indicate a decrease in NK cells in allergic asthma patients, the NK cells that are present are more activated. Without functional activity data, it is difficult to determine whether these patient NK cells are helpful or harmful for the pathogenesis of asthma and the maintenance of chronic inflammation. Interestingly, we did not observe an increase amount of ILC-2 in allergic asthma peripheral blood. This may reflect the difficulty in isolating these cells in whole blood, as their frequency is typically less than 0.01% in whole blood. Since no alterations were seen in NKT cells, further studies on allergic asthma peripheral blood may want to focus more on NK cells. In particular, analysis of chemokine and integrin expression on NK cells may indicate the capacity of these cells to migrate to the lung.

CHAPTER NINE

Alteration in Granulocyte Populations in Allergic Asthma Patients with Anti-IgE Treatment

Abstract

The pathogenesis of allergic asthma is characterized by a large involvement of granulocytes, particularly by eosinophils, mast cells and basophils. There is also growing evidence that neutrophils can play a critical role, especially in the pathogenesis of corticosteroid-resistant asthma. The introduction of anti-IgE (omalizumab) as an add-on therapy for inadequately controlled persistent moderate-to-severe or severe allergic asthma provided a valuable new treatment option for these patients. However, the effects of anti-IgE treatment on granulocytes and its mechanisms of action of anti-IgE are not well understood. Thus, we investigated the frequency and activation status of blood granulocytes during anti-IgE treatment in adult asthma patients. We found a global signature of reduced granulocyte activation status by 26 weeks of treatment. This reduction in activation was more significant in patients who responded to anti-IgE treatment than in non-responders. While overall granulocyte populations were, for the most part, not affected, we did observe a rapid increase in the frequency of circulating mast cell precursors with anti-IgE therapy. We also observed that anti-IgE treatment reduced the frequency of blood neutrophils. Data from this study will help us to understand the effects of anti-IgE and reveal alterations in granulocyte profiles between responders and non-responders.

Introduction

Asthma is a chronic inflammatory disease of the airways that is mainly triggered by allergic reactions and is characterized by the presence of allergen-specific IgE (1). IgE can exert the effects of allergic asthma through cell types that express IgE receptors, particularly the high-affinity IgE receptor (FcεR1). While each type of granulocyte (basophils, mast cells, eosinophils and neutrophils) are capable to expressing this receptor, basophils and mast cells express the greatest levels and contain an additional subunit in their FcεR1 that serves to enhance signaling (117). Upon binding and cross-linking of IgE with FcεR1, basophils and mast cells can be activated and induced to release their pro-inflammatory mediators, such as histamine and tryptase, which further exacerbate the inflammatory response (72, 118). Based on the critical importance of IgE in the pathogenesis of asthma, an IgE-blocking antibody, omalizumab (anti-IgE), was generated (78). While most patients receiving anti-IgE exhibit a significant reduction in inhaled steroid usage and see an improvement in their quality of life (96, 104), the mechanism of anti-IgE action is incompletely understood.

The numbers of all types of granulocytes are increased in the lungs of asthmatic patients (6, 45, 315, 316), and each has a role in maintaining airway inflammation. While basophils and mast cells are the predominant mediators of allergic asthma, based on their sensitivity to IgE, eosinophils and neutrophils also play a critical part. Eosinophils are predominately associated with the Th2-type allergic response, and their migration to the lungs is driven by the Th2 cytokine, IL-5 (317). Similar to the activation of basophils and mast cells, eosinophil activation can lead to the release of pro-inflammatory mediators, such as granule-stored cationic proteins, that further damage the lung

epithelium (317). Furthermore, the pro-inflammatory mediators released by eosinophils can also contribute to hallmark features of asthma, including airway remodeling, hyperreactivity (AHR) and mucus over-production (117). Neutrophils have a more complicated role in allergic asthma. Neutrophilic asthma is associated with obesity and smoking, and it presents with a highly reduced lung function (2). Since neutrophils are also resistant to corticosteroid treatment (48), neutrophilic asthma is usually more severe since patients do not respond to inhaled corticosteroid (ICS) medication. Neutrophilic asthma is also associated with Th17-type responses. While much about the role of IL-17 in allergic asthma is unknown or contradictory findings have been reported, IL-17 can promote lung remodeling and bronchial cell contraction, and it can resist suppression of inflammation by regulatory T cells (1).

It has been shown that anti-IgE can reduce FcεR1 expression on basophils and mast cells (98, 318). There is evidence that this reduced FcεR1 expression can lead to reduced activity, particularly in basophils (99, 319). In addition, anti-IgE can decrease circulating eosinophil (320, 321) and neutrophil (322) frequencies. However, there is still much that is unknown about the effects of anti-IgE in granulocytes, and further study may yield insights into the mechanism of action of anti-IgE. We therefore explored the effects of anti-IgE treatment on peripheral blood granulocytes in moderate-to-severe allergic asthma patients with uncontrolled allergic asthma. We followed allergic asthma patients longitudinally, out to week 26 of anti-IgE treatment. At several time points throughout treatment (weeks 6, 14 and 26), we characterized granulocyte frequency and activation status and compared the course of treatment to the baseline profile (before treatment).

In this study, we observed no alterations in the frequency of individual granulocyte populations with anti-IgE, aside from an increase in circulating mast cell precursors. We also observed a global reduction in activation status across all granulocyte types. However, this reduced activation was primarily observed in patients who responded to anti-IgE therapy, rather than non-responders.

Methods

Study Subjects

Described in methods section of Chapter Three.

Study Design

Described in methods section of Chapter Three.

Determination of Responders versus Non-Responders

Described in methods section of Chapter Three.

Whole Blood Staining

Described in methods section of Chapter Four. Detailed information for antibodies used in this study is summarized in Table 9.1.

Statistical Analysis

Described in methods section of Chapter Four. Correlation analysis described in the methods section of Chapter Five

Table 9.1. Antibodies used for granulocytes analysis

Marker	Clone	Company
CD123	9F5	BD
CD203c	NP4D6	BioLegend
FcεR1α	CRA1	BioLegend
HLA-DR	L243	BioLegend
CD117 (c-kit)	104D2	BioLegend
CD14	TÜK4	Invitrogen
CD15	W6D3	BD
CD16	3G8	BD
CD66b	G10F5	BD
Siglec-8	7C9	BioLegend
CD11a (LFA-1)	MEM-25	ExBio
CD11b	ICRF44	BD
CD13	WM15	BD
CD63	H5C6	BD
CD88	S5/1	BioLegend
CRTH2	BM16	BD
CD2	RPA-2.10	BD
CD9	M-L13	BD
CD33	WM53	BD
CD49d (α4)	9F10	BioLegend
CD10	HI10a	BioLegend
CD62L	DREG-56	BioLegend

Results

Anti-IgE Treatment does not Alter the Frequency of Basophils in Patient Blood

We first assessed the frequency of basophils in the blood of healthy subjects and asthma patients. Figure 9.1A shows the gating strategy of basophils from the blood of healthy subjects and asthma patients. As shown in Figure 9.1B, left panel, healthy subjects and asthma patients have similar percentages of basophils in their blood at baseline. Although patients had a higher average number than healthy subjects, the difference was not significant (Fig. 9.1C, left). In addition, the frequency (percentage and number) of basophils was not significantly altered by anti-IgE treatment (Fig. 9.1B and

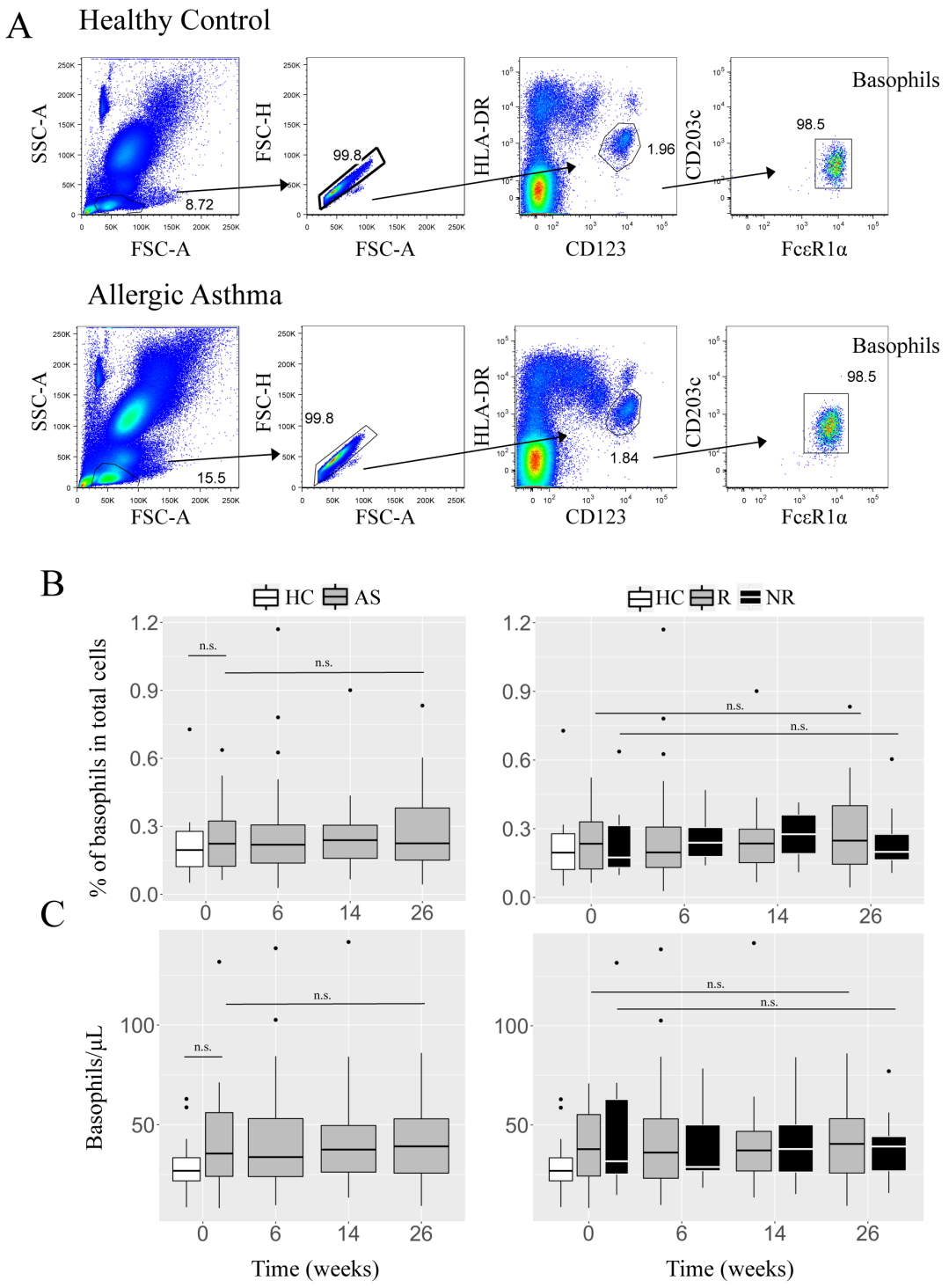


Figure 9.1. Basophil frequencies are not affected by anti-IgE treatment.
(A) Whole blood lymphocytes were gated on low FSC and SSC and singlets were removed using FSC-A vs FSC-H. Basophils were identified as $CD123^{+}HLA\text{-}DR^{low/med}\,Fc\varepsilon R1\alpha^{+}CD203c^{+}$. (B) Average percentage of basophils in total whole blood and (C) average number per μL. The left panels of (B) and (C) indicate all patients over the time course of anti-IgE treatment and the right panels indicate anti-IgE responders and non-responders over time. Error bars indicate SD.

9.1C, left panels). The right panels in Fig. 9.1B and 9.1C also show that the frequency of basophils in either responders or non-responders was not significantly altered by anti-IgE treatment.

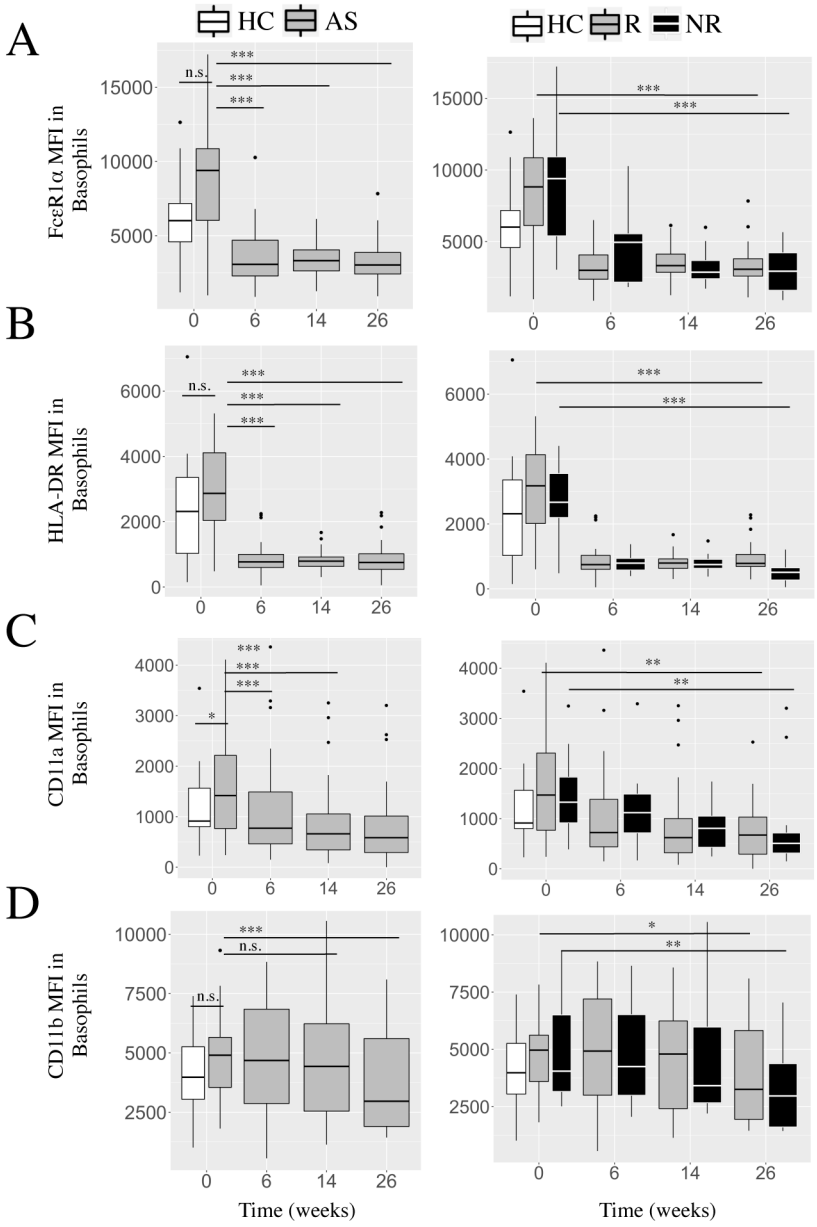


Figure 9.2. Anti-IgE treatment decreases basophil FcεR1a, HLA-DR, CD11a and CD11b expression, particularly from anti-IgE responders. (A-D) Average marker expression, determined as mean fluorescent intensity (MFI) in basophils. The left panels of indicate all patients over the time course of anti-IgE treatment and the right panels indicate anti-IgE responders and non-responders over time. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars indicate SD.

Anti-IgE Treatment Decreases FcεR1, HLA-DR, CD11a and CD11b Expression on Blood Basophils

Basophils express high levels of FcεR1, and the surface expression level of FcεR1 on basophils can be decreased by neutralizing IgE with anti-IgE antibody (98). In line with this, anti-IgE treatment resulted in significant decreases of FcεR1 expression on basophils, and this was observed from week 6 to week 26 (Fig. 9.2A, left). Similar to FcεR1, HLA-DR expression was also greatly reduced from week 6 to week 26 (Fig. 9.2B, left). In addition, we also observed that anti-IgE treatment reduced both CD11a (Fig. 9.2C, left) and CD11b (Fig. 9.2D, left) expression levels. However, such decreases were similarly observed in both responders and non-responders (Fig. 9.2A, 9.2B, 9.2C, and 9.2D, right). Basophils from patients express greater levels of both FcεR1 and HLA-DR than healthy subjects on average at baseline (week 0), but these differences were not significant (Fig. 9.2A and 9.2B, left). However, the CD11a expression level was greater on basophils from patients than healthy subjects (Fig. 9.2C).

Anti-IgE Treatment Increases CD13, CD88, CD203e, and CRTH2 Expression on Blood Basophils

We further assessed the expression levels of other activation and functional markers (CD13, CD63, CD88, CD203c, and CRTH2) expressed on the surface of basophils. The left panels in Figures 9.3A-9.3E show that basophils from both patients and healthy subjects expressed similar levels of these molecules, except for CRTH2 (Fig. 9.3E, left), for which basophils from healthy subjects expressed an increased level of CRTH2. However, we found that anti-IgE treatment resulted in increased expression of CD13, CD88, CD203c, and CRTH2. The only marker that did not show any alteration with treatment was CD63 (Fig. 9.3C). Interestingly, most of the markers exhibiting a

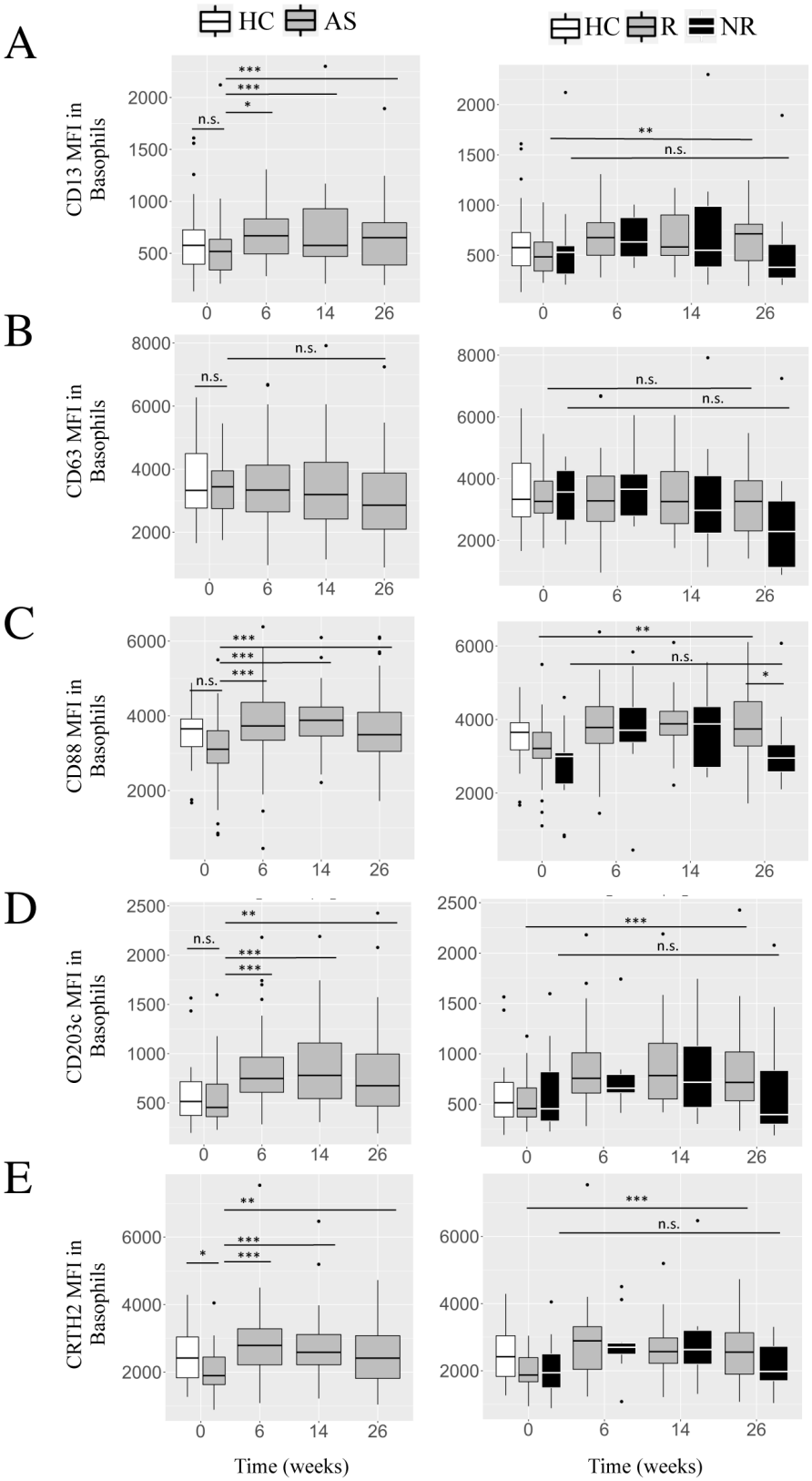


Figure 9.3. Anti-IgE treatment increases basophil CD13, CD88, CD203c and CRTH2 expression. (A-E) Average marker expression, determined as mean fluorescent intensity (MFI) in basophils. The left panels of indicate all patients over the time course of anti-IgE treatment and the right panels indicate anti-IgE responders and non-responders over time. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars indicate SD.

significant change with anti-IgE treatment were only observed in responders (Fig. 9.3, right panels). This included CD69, CD88, CD203c and CRTH2. These results show that there is a strong difference in how basophils from responders and non-responders change in response to anti-IgE treatment and may implicate them in determining the patient's response to anti-IgE treatment.

Blood Mast Cell Precursors are Increased by Anti-IgE but Display a Reduced Activation Status

Mast cells also express FcεR1. Its expression level on mast cells is also highly affected by anti-IgE treatment, similar to its expression on basophils (100, 318). As mast cells mature in the tissue, only progenitors/precursors are found in peripheral blood. Thus, we were only able to observe mast cell precursors in the blood, as described by Dahlin et al. (323) ($CD16^{-}HLA\text{-}DR^{-/dim}CD117^{+}Fc\varepsilon R1\alpha^{+}CD203c^{+}$) (Fig. 9.4A). Anti-IgE treatment induced a greater percentage (Fig. 9.4B, left) and number (Fig. 9.4C, left) of mast cell precursors, starting by week 6 of treatment. Interestingly, this increase was more significant in anti-IgE responders than non-responders (Fig. 9.4B and 9.4C, right).

Similar to basophils, mast cell precursors were highly affected by anti-IgE treatment, indicated by the rapid and significant reduction in the expression of FcεR1α, HLA-DR and CD33 (Fig 9.5A, 9.5B and 9.5C, left). Also like basophils, mast cell precursors displayed a significant increase in the expression of CD88 and CD203c (Fig. 9.5D and 9.5E, left) with treatment. There was a significant reduction of CD9 by week 14 and continuing at week 26 (Fig. 9.5G, left). There was no change in the expression of CD13 (Fig. 9.5H, left), and the expression of CD2 was only significantly altered at week 14 compared with baseline (Fig. 9.5F, left). In addition and consistent with basophils,

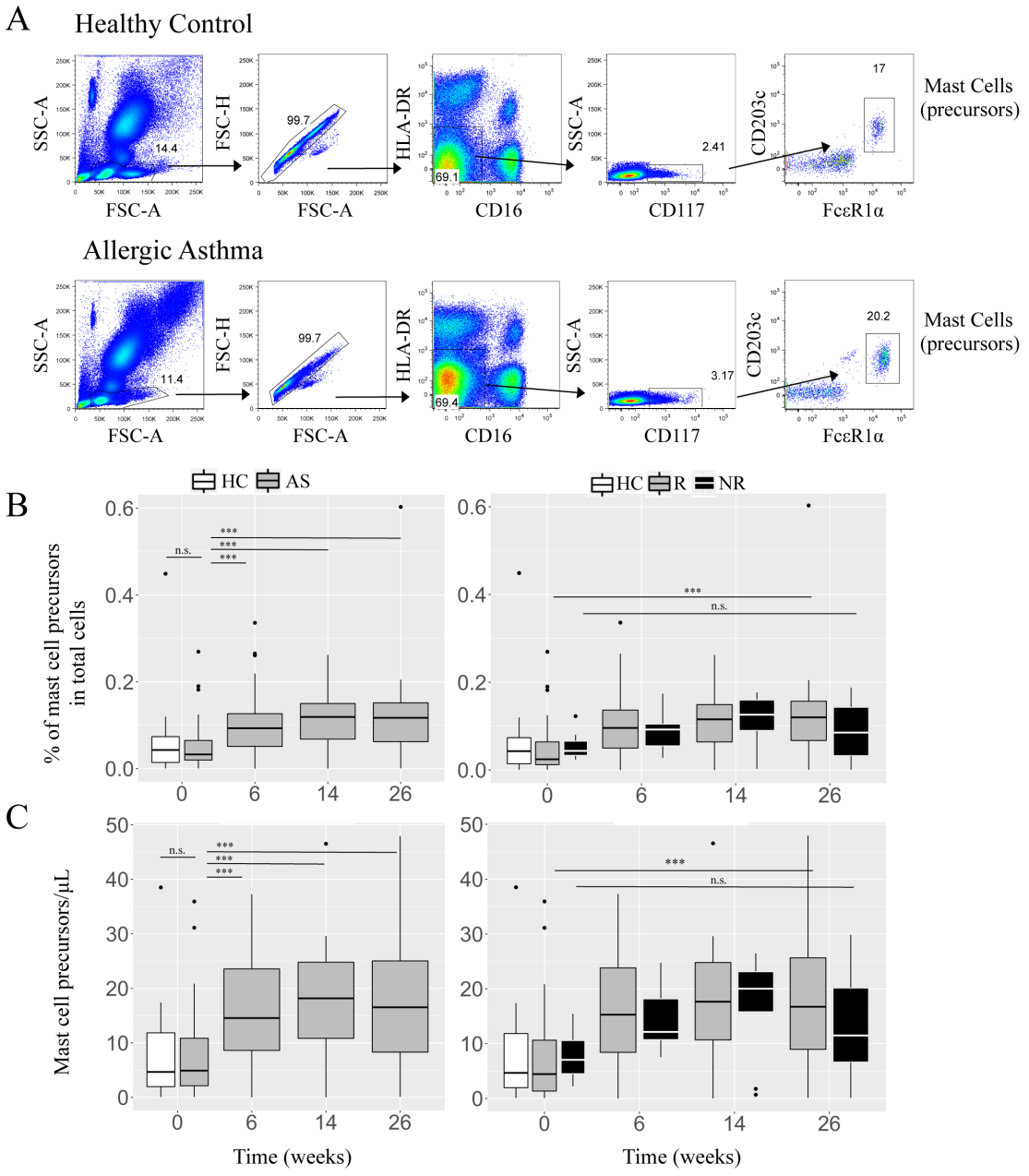
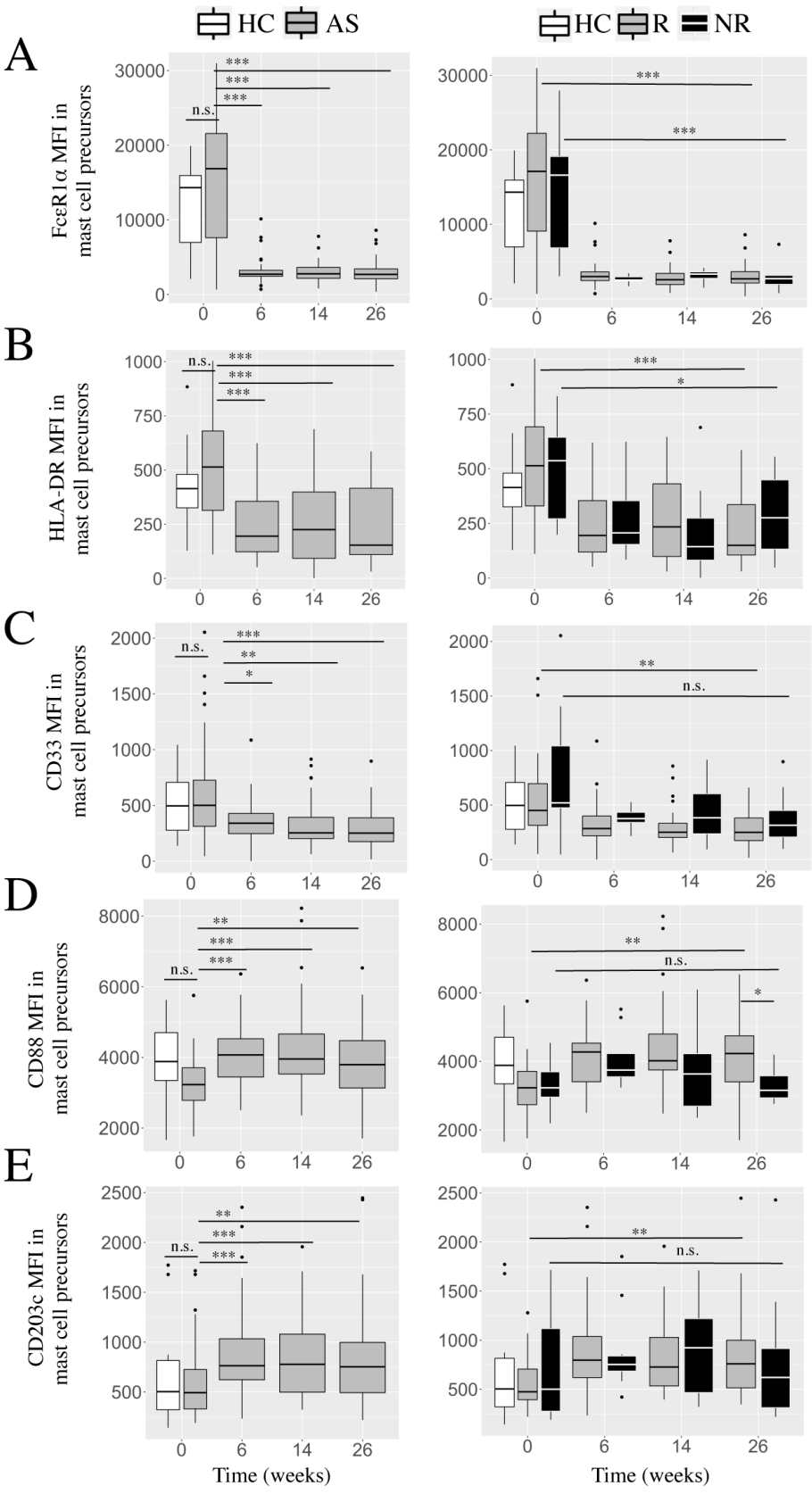


Figure 9.4. Anti-IgE treatment increases the frequency of peripheral blood mast cell precursors. (A) Whole blood lymphocytes were gated on low FSC and SSC and singlets were removed using FSC-A vs FSC-H. Mast cell precursors were identified as $CD16^{-}HLA\text{-}DR^{low/-}CD117^{+}Fc\varepsilon R1\alpha^{+}CD203c^{+}$. (B) Average percentage of mast cell precursors in total whole blood and (C) average number per μL. The left panels indicate all patients over the time course of anti-IgE treatment and the right panels indicate anti-IgE responders and non-responders over time. ***P<0.001. Error bars indicate SD.


HC
AS
HC
R
NR
A
FcεR1α MFI in mast cell precursors
B
HLA-DR MFI in mast cell precursors
C
CD33 MFI in mast cell precursors
D
CD88 MFI in mast cell precursors
E
CD203c MFI in mast cell precursors
n.s.
Time (weeks)
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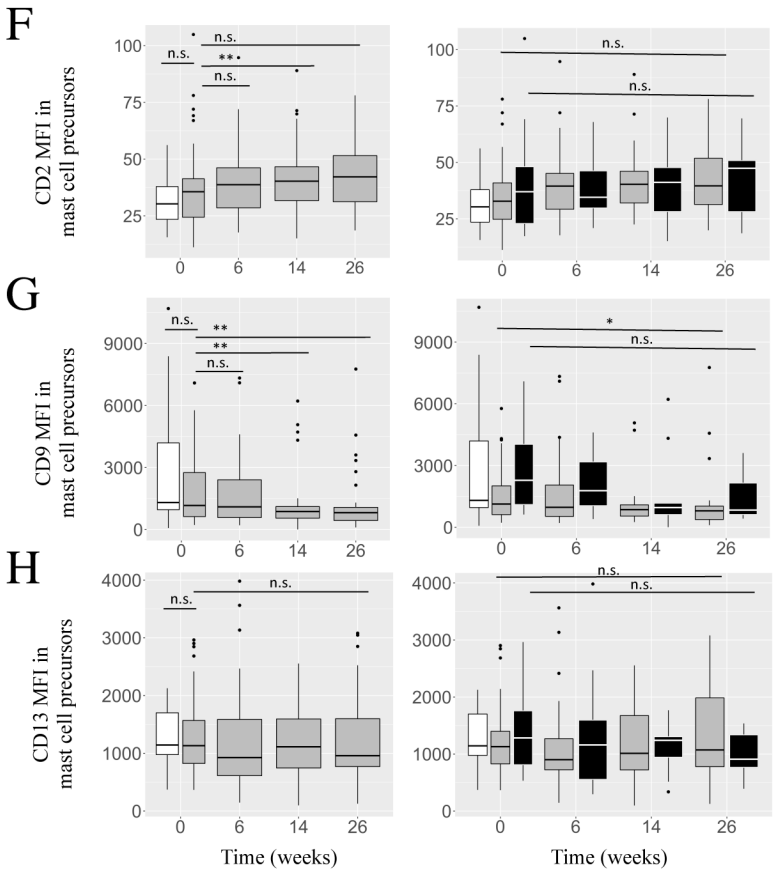


Figure 9.5. Anti-IgE treatment decreases blood mast cell precursor activation.
(A-H) Average marker expression, determined as mean fluorescent intensity (MFI) in mast cell precursors. The left panels of indicate all patients over the time course of anti-IgE treatment and the right panels indicate anti-IgE responders and non-responders over time. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars indicate SD.

only mast cell precursors from responders showed a significant change in the expression of CD33, CD88, CD203c, and CD9 with treatment (Fig. 9.5C-9.5E and 9.5G, right). Intriguingly, at week 26, non-responders exhibited significantly reduced expression of CD88 compared to responders (Fig. 9.5D, right). As these results closely mirror basophils, it also indicates that mast cells are implicit in the response to anti-IgE. However, the increase in blood mast cell precursors may signify an alteration in hematopoiesis by anti-IgE, particularly in anti-IgE responders.

While Anti-IgE Treatment does not Alter Eosinophil Frequency, it does Alter their Activation Status

Since eosinophils have been known to play a critical role in allergic asthma, we characterized their response to anti-IgE treatment. Figure 9.6A shows the gating strategy of blood eosinophils from healthy subjects and asthma patients. While there was a clear increase in eosinophil percentage and number compared with healthy subjects, there were no significant alterations by anti-IgE treatment (Fig. 9.6B and 9.6C, left). This applied to both anti-IgE responders and non-responders (Fig. 9.6B and 9.6C, right), except for the frequency difference observed on week 14 (Fig. 9.6C, right). Non-responders had significantly less eosinophils per microliter of whole blood.

We also analyzed eosinophil activation, maturation and migration markers (Fig. 9.7). By week 14 of treatment, there was a significant reduction in the expression of CD9 and CD13 (Fig. 9.7A and 9.7B, left). By week 26, eosinophils showed an additional reduction in the expression of CRTH2 (Fig. 9.7E, left) and an increase in the expression of HLA-DR (Fig. 9.7F, left). Additionally, all of the markers that showed a significant reduction with anti-IgE treatment (CD9, CD13, CRTH2 and HLA-DR) were significantly changed only in anti-IgE responders (Fig. 9.7A, 9.7B, 9.7E and 9.7F, right). At week 6, responders had significantly greater expression of CD13 (Fig. 9.7B, right). There were no alterations in the expression of CD49d or CD88 (Fig. 9.7C and 9.7D). Thus, while anti-IgE does not affect eosinophil frequency, it does affect their activation and migration capacity.

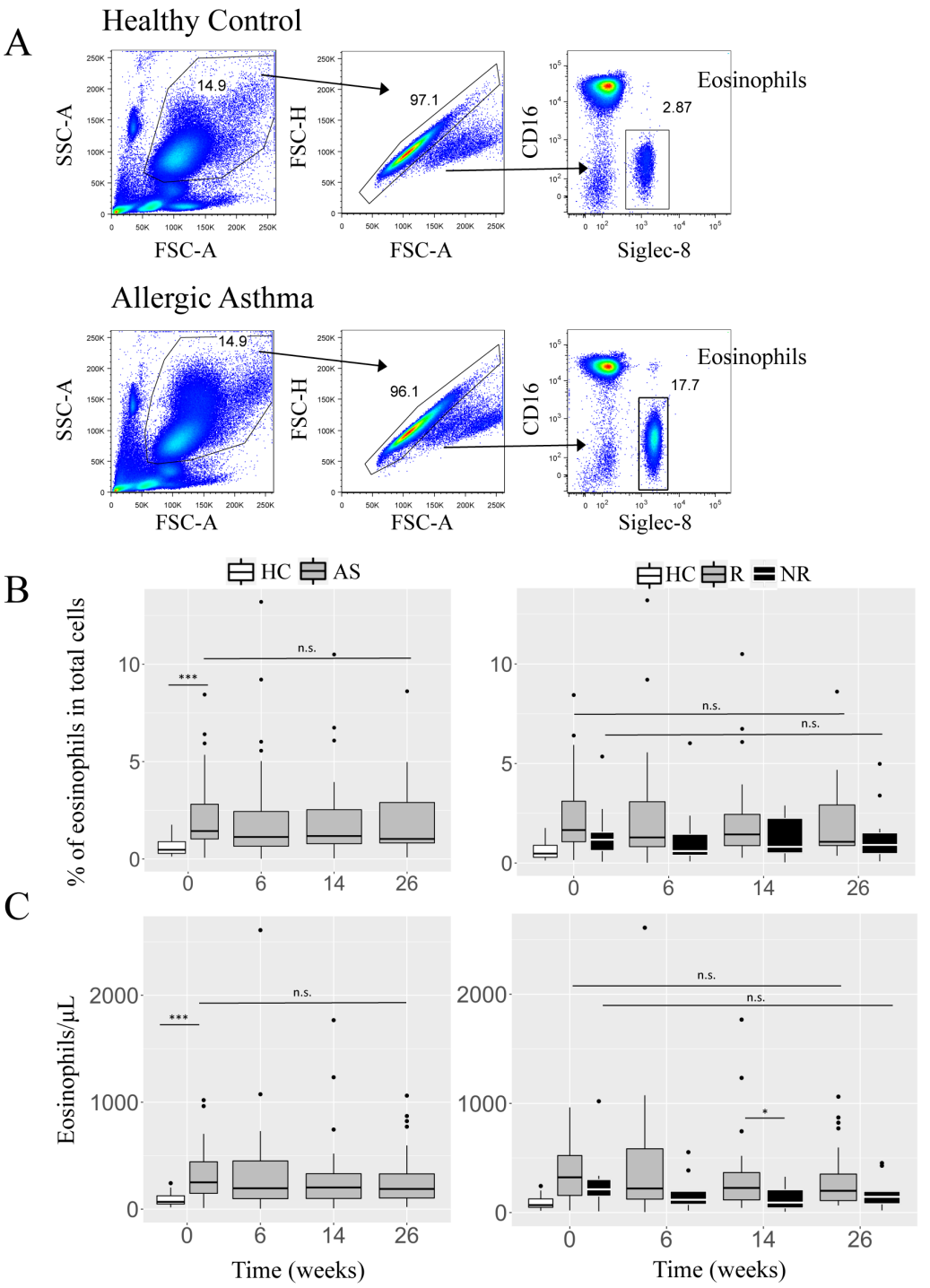


Figure 9.6. Asthmatic patients have an increase frequency of eosinophils that is not altered by anti-IgE treatment.
(A) Whole blood granulocytes were gated based on high FSC and SSC and singlets were removed using FSC-A vs FSC-H. Eosinophils were identified as $CD16^{-}$ and $siglec\text{-}8^{+}$. (B) Average percentage of eosinophils in total whole blood and (C) average number per µL. The left panels indicate all patients over the time course of anti-IgE treatment and the right panels indicate anti-IgE responders and non-responders over time. ***P<0.001. Error bars indicate SD.

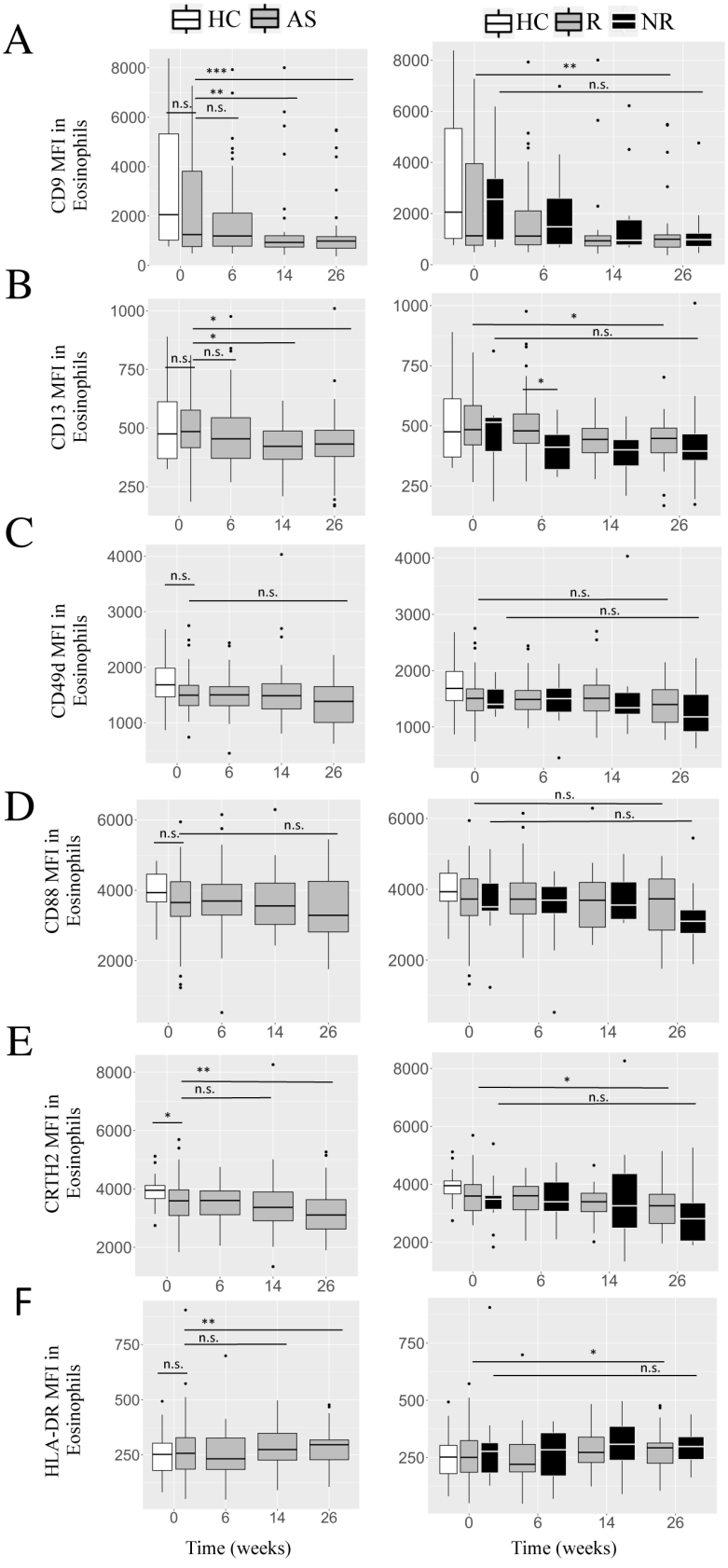


Figure 9.7. Anti-IgE treatment alters blood eosinophil activation status.
(A-E) Average marker expression, determined as mean fluorescent intensity (MFI) in eosinophils. The left panels of indicate all patients over the time course of anti-IgE treatment and the right panels indicate anti-IgE responders and non-responders over time. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars indicate SD.

Anti-IgE Decreases Blood Neutrophil Number and Activation Status

Neutrophils play an important role in the pathophysiology of asthma, particularly uncontrollable, steroid-resistant asthma (6, 47), but are not particularly associated with IgE responses (51). Thus, in order to determine the effects of IgE neutralization on neutrophils, we first compared their frequency (percentage and number) over time in response to anti-IgE administration. Figure 9.8A shows the gating strategy of blood neutrophils from healthy subjects and asthma patients. Compared to healthy subjects, asthma patients have an increased percentage and number of neutrophils in their blood at baseline (week 0) (Fig. 9.8B and 9.8C, left). There was no change in the percentage of neutrophils with treatment (Fig. 9.8B, left); however, the number of neutrophils was reduced starting by week 6 of treatment (Fig. 9.8C, left). Interestingly, this reduction in neutrophil number was only significant for anti-IgE responders (Fig. 9.8C, right).

We next analyzed neutrophil activation and migration markers (Fig. 9.9). There was a significant decrease in the expression of CD10 in week 26 compared with baseline (week 0) (Fig. 9.9A, left). There was also a decrease in the expression of CD11b at week 26 of anti-IgE treatment compared with baseline (Fig. 9.9B, left). However, only responders showed a significant decrease in the expression of CD62L (Fig. 9.9C, right). Both responders and non-responders showed a significant reduction in the expression intensity of CD10 (Fig. 9.9A, right). There were no alterations in the expressions of CD88 (Fig. 9.9D). These results indicate that, while anti-IgE can decrease neutrophil number, it has only a slight impact on neutrophil activation and maturation.

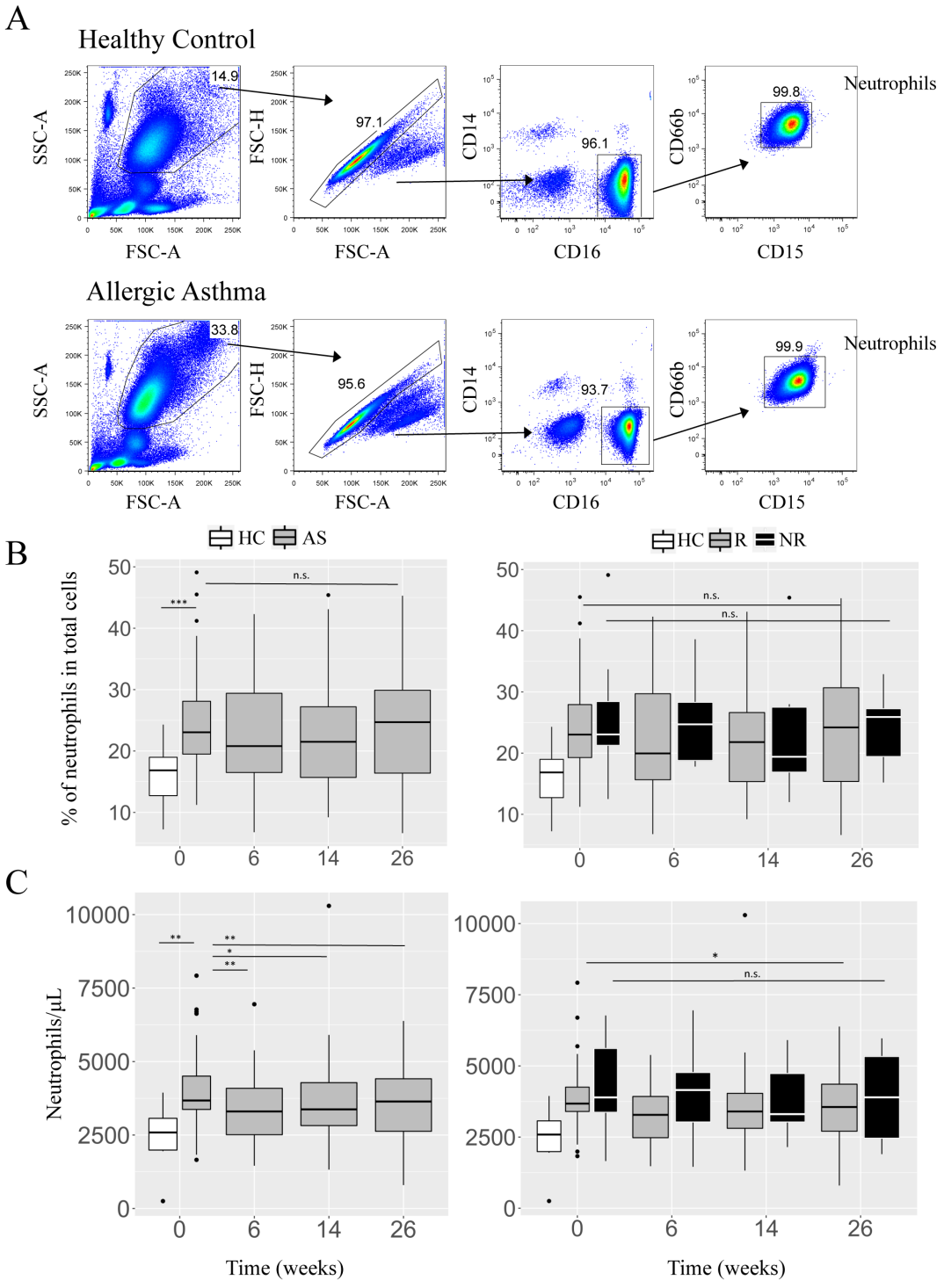


Figure 9.8. Neutrophil numbers are decreased by anti-IgE treatment.
(A) Whole blood granulocytes were gated based on high FSC and SSC and singlets were removed using FSC-A vs FSC-H. Neutrophils were identified as $CD14^{-}CD16^{+}$ than $CD15^{+}CD66b^{+}$. (B) Average percentage of neutrophils in total whole blood and (C) average number per µL. The left panels indicate all patients over the time course of anti-IgE treatment and the right panels indicate anti-IgE responders and non-responders over time. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars indicate SD.

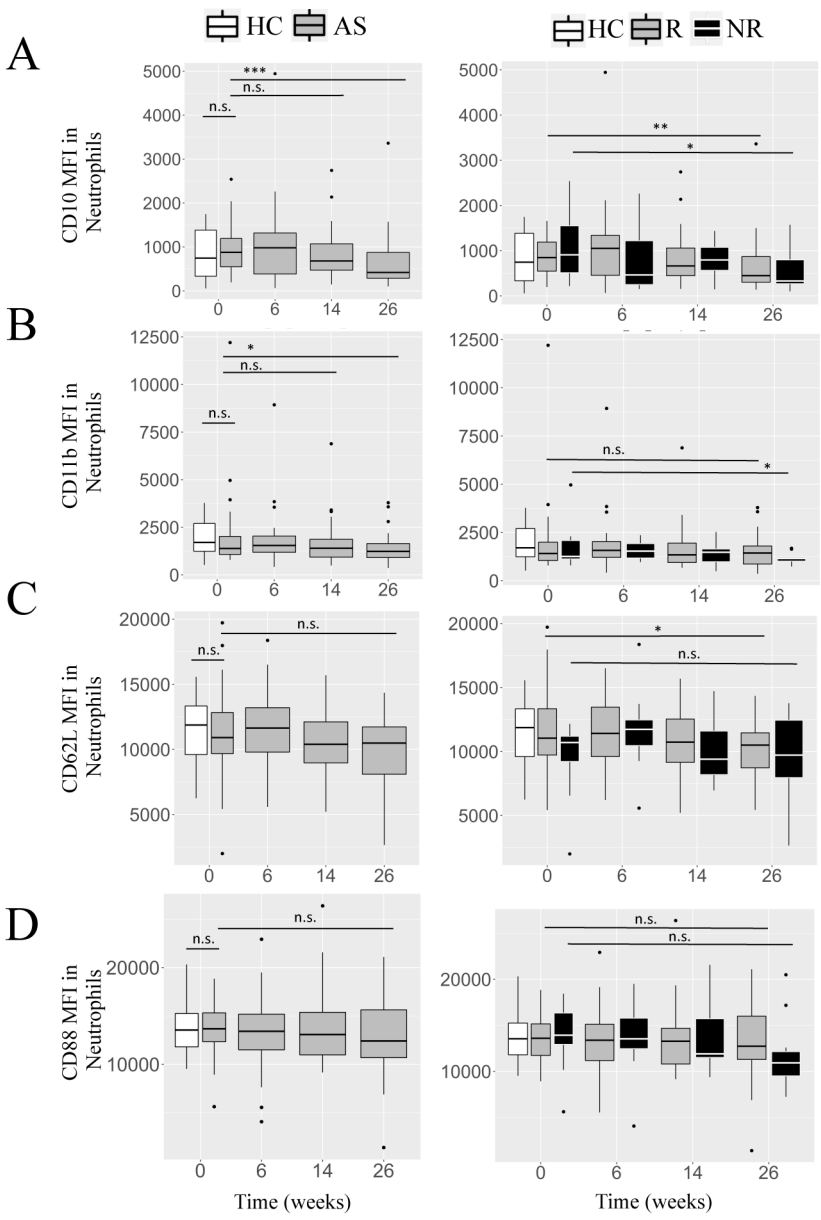


Figure 9.9. Anti-IgE treatment alters blood neutrophil activation status.
(A-D) Average marker expression, determined as mean fluorescent intensity (MFI) in eosinophils. The left panels of indicate all patients over the time course of anti-IgE treatment and the right panels indicate anti-IgE responders and non-responders over time. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars indicate SD.

Correlations between Granulocytes and Patient Clinical Data

Several patient characteristics are known to be correlated with clinical information, such as serum IgE levels and IgE receptor expression (131). We therefore compared all granulocyte flow information with the collected patient clinical information, which included their level of asthma control as measured by the asthma control test (ACT) score as well as asthma symptom frequency, β-agonist usage and nights woken by asthma during the previous week. Clinical information collected also included the level of lung function impairment as measured by FEV_1, FVC % of predicted, and FEV_1/FVC. Significance was set using a raw *p*-value cutoff of less than 0.05 and a Spearman's rho of greater than 0.4. All significant correlations are summarized in Table 9.2. As expected, FcεR1α expression was highly correlated with serum IgE in both basophils and mast cell precursors. There was also a positive correlation between serum IgE and basophil expression of HLA-DR, indicating that when more serum IgE is present, the basophils are more highly activated. The percentage and number of eosinophils also highly correlated with serum IgE. In addition to the correlations between basophils and serum IgE, the expression of CRTH2 on

Table 9.2: Correlations of granulocytes phenotypes with patient data

Variable	Spearman's rho	*p*-value
vs. asthma symptoms/week		
CRTH2 MFI in basophils	0.4512	0.0027
vs. β-agonist usage/week		
% of HLA-DR$^+$ eosinophils	0.4274	0.0047
vs. serum IgE		
FcεR1α MFI in basophils	0.6719	5.92E-07
HLA-DR MFI in basophils	0.5611	9.09E-05
CD69 MFI in basophils	0.4741	1.52E-03
FcεR1α MFI in pMC	0.4860	1.70E-03
% of eosinophils	0.4300	3.58E-03
# of eosinophils	0.4425	4.25E-03

MFI: mean fluorescent intensity, pMC: precursor mast cell

basophils was positively correlated with the number of asthma symptoms. Similar results were also observed for the percentage of HLA-DR$^+$ eosinophils and rescue inhaler usage (β-agonist). No significant correlations were seen with granulocyte populations and patient lung function. In addition, there were no significant correlations between patient information and neutrophils.

Discussion

While both neutrophils and eosinophils are capable of expressing the high-affinity IgE receptor, FcεR1, and thus are sensitive to anti-IgE treatment, basophils and mast cells have the greatest expression of FcεR1 and contain an additional subunit to the receptor that heightens the signal strength. This is evident when comparing granulocyte markers before and after anti-IgE treatment. There is a clear decrease in the activation status of blood granulocytes, particularly in basophils and mast cell precursors, with the blocking of IgE. Intriguingly, the responses that we see occurring to anti-IgE treatment mainly reflect the changes in anti-IgE responders; non-responders often do not show any significant change in marker expression between baseline and 26 weeks of anti-IgE treatment. However, there are very few direct differences in granulocytes between responders and non-responders, with the only differences occurring after the initiation of treatment. These results indicate that anti-IgE non-responders, despite that the neutralization of free IgE by anti-IgE administration, do not exhibit many changes in their granulocyte phenotypes, even by week 26 of anti-IgE treatment. This may reveal more information on variations in the pathogenesis of asthma between anti-IgE non-responders and responders. Several studies have shown that anti-IgE treatment can decrease

eosinophil counts (320, 324); however, no such studies have focused on neutrophil or mast cell progenitor populations. Here, we show that anti-IgE treatment decreased the number of neutrophils.

As expected (98-100, 325), basophils showed a large decrease in the expression of FcεR1α in response to anti-IgE administration. Basophils also had a large reduction in the expression of HLA-DR and several activation markers. Since these markers decreased in a similar fashion to FcεR1α, it indicates a close link between activation and FcεR1α. It is expected that anti-IgE treatment will reduce basophil activation. Indeed, it has been shown that anti-IgE reduces the production of pro-inflammatory cytokines and chemokines in basophils (99). Conversely, anti-IgE treatment induced an increase in the basophil activation markers CD13, CD88 and CD203c, all occurring early in the course of treatment (week 6). The increase in CD203c may reflect a similar increase in mast cell precursors, which are gated using many of the same markers as basophils and could be contaminating our basophil population. CD13 can also be considered a basophil activation marker (326). However, CD13 may also indicate maturation (327) and can mediate phagocytosis (328). It may be that the increase in CD13 is a coping mechanism to account for the decrease in FcεR1 expression. It has been shown that stimulation can reduce granulocyte CD88 expression (154), thus the increase in basophil CD88 expression may indicate a reduction in stimulation. Finally, basophils also increased CRTH2 expression with anti-IgE, unlike the eosinophil response in which CRTH2 expression decreased. This likely represents a differential regulation and activity of CRTH2 between basophils and eosinophils. While it appears that the changes in marker expression are happening simultaneously with the reduction of FcεR1 expression, it must

be noted that this observation was after 6 weeks of treatment; thus, it may not be a direct effect of anti-IgE, but may be in response to decreased FcεR1 signaling.

Because mast cells are not present in the blood in their mature state, it is difficult to study their role in allergy and asthma without resorting to the use of more invasive procedures to collect tissues. Using a similar gating strategy as in a recent paper by Dahlin et al. (323), we were able to characterize peripheral blood mast cell progenitors. Interestingly, the use of anti-IgE treatment increased both their percentage and number. This is a highly novel observation that needs to be more fully studied, particularly since very little is known about these cells in the periphery. However, it may indicate an alteration in hematopoiesis by IgE neutralization occurring very early in the course of treatment. Anti-IgE also induced the reduction of FcεR1α and HLA-DR expression on mast cells, as was seen in basophils. Mast cells also had a reduction of the activation marker CD9, similar to that seen on eosinophils, and a reduction of the maturation marker CD33(329, 330). Also similar to basophils, these progenitors had an increase in the expression of CD88 and CRTH2 with treatment, likely for similar reasons as basophils. These results indicate that, while the levels of mast cell progenitors are increasing, they are less activated.

In neutrophils, the most striking observations are the decrease in neutrophil counts per μL of whole blood and the reduction of CD10 expression. CD10 expression may be an indicator of neutrophil activation and maturation status (327). CD11b expression, which was also slightly decreased by anti-IgE, can also be associated with activation status (331). Thus, the decrease of both CD10 and CD11b expression in neutrophils signifies that anti-IgE treatment can decrease neutrophil activation. This is likely a result

of a decrease in overall inflammation, rather than a direct effect of anti-IgE on neutrophils, which is emphasized by the length of treatment time it takes for the effects to become significant; the decrease in neutrophil activation status is only significant at 26 weeks of treatment. While neutrophils are capable of expressing FcεR1 (54), little is known about its function in neutrophils, particularly in the pathogenesis of asthma, although it does not appear to influence neutrophil activation. It has recently been shown that anti-IgE treatment can reduce neutrophil percentage in patients with asthma-chronic obstructive pulmonary disease (COPD) overlap syndrome (ACOS) (322). However, this study did not examine neutrophil number and included patients exhibiting both asthma and COPD. COPD is known to have a greater neutrophil involvement (332, 333). Thus, the smaller significance we observe in neutrophil decrease may be affected by our patient type: allergic asthma.

Although anti-IgE has been shown to decrease eosinophils counts (320, 321, 324), in our study, we do not show a decline in eosinophil population with treatment. In one of the previous studies, patients continued steroid treatment while receiving anti-IgE (320). Steroid usage is known to decrease eosinophil survival (334); thus, the continued use of a steroid-regiment may be decreasing their numbers in the periphery. While some of our patients may continue steroid therapy for asthma control, many of them only use inhaled corticosteroids as a rescue medication, which they were able to decrease while using anti-IgE. The additional observation of decreased eosinophilia with anti-IgE (321) used a small sample set of only 13 patients. They also divided their patients based on their percentage of eosinophil decrease. While there are likely some patients in our cohort that saw a reduction in eosinophil counts, the average did not. Anti-IgE treatment did reduce

the expression of the activation markers CD9 and CD13 (327, 335). This reduction in activation is probably induced indirectly, since it is not observed until week 14 of treatment. It may be facilitated by either a change in cytokine/chemokine concentration or by reduced antigen-presenting cell activation. Anti-IgE treatment also resulted in a reduction in the expression of CRTH2, the prostaglandin D_2 (PGD_2) receptor. The reduction of CRTH2 on eosinophils may signify a reduction in their capacity to migrate to the lung. While PGD_2 and IL-5 have been shown to reduce CRTH2 expression on eosinophils (336), it may also decrease as eosinophils lose their activation status. Since blocking IgE does seem to decrease their activation, the decrease in CRTH2 likely reflects this. Indeed, the reduction of CRTH2 is not significant until after CD9 and CD13 have been reduced. Conversely, eosinophils showed a slight induction of HLA-DR expression, which can also be used as a marker for eosinophil activation (337). However, HLA-DR expression on eosinophils can also be regulated by specific cytokines (338). As the changes in HLA-DR expression are not significant until week 26 of anti-IgE treatment, this indicates that it may result from a change in the immune response that is initiated in response to the changes in cells directly affected by anti-IgE (as such, a tertiary response). As the timeline of changes to HLA-DR expression mirror those of CRTH2, it may signify a similar mechanism in the control of their expression, with an inverse correlation between them.

While each type of granulocyte showed some type of decrease in activation status with anti-IgE treatment, most of these changes occurred from the anti-IgE responder population. However, direct comparisons between responders and non-responders yielded very few significant differences. In fact, there was no difference between

responder and non-responder granulocytes before the start of treatment. However, there was a strong signature for a greater level of eosinophil activation in non-responders. This is likely a result of increased inflammation in non-responders compared with responders, since despite the reduction of IgE signaling, they still suffer from asthma. Both basophils and mast cell progenitors from non-responders show a decreased expression of CD88 compared with responders. In addition, non-responder basophils also show a decrease in HLA-DR and dectin-1 (data not shown) expression. Dectin-1 is a pattern recognition receptor that mainly recognizes fungal glucans (339), and its expression can be decreased following ligation of dectin-1 with receptive glucans (340). This may represent increased fungal allergies in the non-responders, which can present with more severe and corticosteroid-resistant asthma that may be more IgE-independent (341, 342). As mentioned above, in certain conditions, CD88 expression can be decreased by various stimuli (154). Thus, the decrease of CD88 in non-responders, like the increase in CD69 in non-responder eosinophils, may represent increased pro-inflammatory conditions specific to non-responders. It is unclear why non-responder basophils would express less HLA-DR than responders. In this instance, it may indicate a lack of capacity of these cells to control inflammation (343) rather than a decrease in activation status. Interestingly, non-responders exhibited a much lower reaction to anti-IgE treatment than responders, as indicated by the large number of markers that were significantly reduced by anti-IgE treatment only in responders. This explains part of the allergic asthma pathogenesis in the non-responders, despite the facts that they express similar levels of serum IgE as responders and the effects of IgE neutralization can clearly be seen by in non-responders by the reduction of surface IgE receptor expression. It is also evident that

the two granulocyte populations that express high levels of FcεR1, basophils and mast cell precursors, are the most affected by anti-IgE treatment. HLA-DR expression seems to be closely tied to FcεR1α expression, as both are always significantly reduced in both responders and non-responders. It is also evident in the number of significant alterations of additional markers in these cells; very few markers were significantly decreased in neutrophils and eosinophils, while almost all markers tested were reduced in basophils and mast cell precursors. Comparisons in the changes of marker expression by anti-IgE in responders and non-responders seem to indicate that non-responder granulocytes still exhibit a pro-inflammatory/activated phenotype, which is maintained even by the reduction of IgE responsiveness.

In summary, this study found a strong response to anti-IgE treatment, particularly in granulocytes that express large amounts of the high affinity IgE receptor. However, the reaction to anti-IgE treatment was mostly limited to anti-IgE responders. This reveals an IgE-independent activation pathway in non-responder granulocytes and their resistance to immune modulation away from allergic responses.

CHAPTER TEN

Transcriptional Profiling of Allergic Asthma Patients Receiving Anti-IgE Therapy Reveals Variations between Responders and Non-Responders

Abstract

Anti-IgE (omalizumab) is a monoclonal antibody used for the treatment of moderate-to-severe allergic asthma that is not controlled by inhaled steroids. Despite its success, some patients do not observe adequate control of their asthma even while on anti-IgE therapy. These patients, termed non-responders, may present a unique insight into the pathogenesis of IgE-independent allergic asthma. In order to elucidate the molecular heterogeneity of allergic asthma in response to IgE neutralization and to better target patient selection for response to anti-IgE, we longitudinally profiled the blood transcriptome of moderate-to-severe allergic asthma patients over the course of 26 weeks of anti-IgE treatment. Using network analyses we found that there are a number of pre-existing differences between responders and non-responders before anti-IgE treatment. Eight clusters of genes were further defined, using genes significant (FDR<0.05) in at least one comparison of each patient response status and time point with healthy controls. Analysis of the activity of individual clusters over time revealed that responders steadily become more similar to the healthy controls, while non-responders tend to remain more similar to their pre-treatment baseline. Additionally, the application of statistical approaches combined with internal cross-validation identified 25 genes that may indicate treatment response prior to treatment. Future validation using expanded datasets may

prove these genes to be effective biomarkers for predicting patient responses to anti-IgE treatment.

Introduction

Asthma is defined by chronic inflammation within the airways and is accompanied by bronchial hyper-reactivity (BHR), mucus overproduction, airway narrowing, and airway remodeling. Allergic asthma represents a subset of asthma that is triggered by normally harmless allergens. Allergic asthma is characterized by an overabundance of IgE and is mainly associated with Th2-type immune responses — increased production of IL-4, IL-5 and IL-13 cytokines. IgE is critical in allergic asthma pathogenesis as it can cross-link to the high-affinity IgE receptors on the surface of mast cells and basophils, inducing their degranulation and the release of additional pro-inflammatory mediators such as histamine (32, 45). Treatment for asthma includes short-term or quick relief medicines and long-term control medications. Short-term medications, such as short-acting β-agonists or bronchodilators, act to relax the airway. Long-term control medications, such as inhaled corticosteroids, act to reduce inflammation and prevent the initiation of symptoms. There are additional long-term controllers that work to block pro-inflammatory mediators of asthma, such as leukotriene and histamine. In the case of allergic asthma, immunotherapy can also be used to desensitize patients to specific allergens.

Recent, advancements in the understanding of allergic asthma responses have led to the production of immunomodulators, which are capable of modifying the immune response. One of the most promising treatments for patients with moderate-to-severe

uncontrolled allergic asthma is anti-IgE (omalizumab). This blocks asthma pathogenesis by neutralizing IgE, thus preventing downstream signaling events and leading to asthma control. By reducing the levels of free IgE, anti-IgE is able to block the initiation as well as the progression of the allergic inflammatory response, thus leading to a reduction in downstream symptoms and oftentimes a diminished need for asthma controlling medications (95, 104, 108). However, there are a sizeable number of patients that continue to suffer from uncontrolled asthma, despite the neutralization of IgE and even though their asthma is allergy based. Anti-IgE treatment works best in patients with severe asthma and those with higher serum IgE levels (95). However, it has been noted that not all patients responds to anti-IgE treatment; the response rate can be anywhere from 50 to 90 percent (95, 107, 112). Comparing the immune profile of anti-IgE responders with non-responders may generate a further understanding of the role of IgE in the pathogenesis of asthma. Furthermore, the clinical use of IgE-blocking antibodies offers a unique opportunity to study the contribution of IgE in human asthma and clarify the effects of anti-IgE treatment on the immune system.

Here, we aimed to identify biomarkers of response to anti-IgE by transcriptionally profiling longitudinal blood samples from allergic asthma patients undergoing anti-IgE treatment. Using network and pathway analyses and transcript-level cross-validation, we uncovered variations between anti-IgE responders and non-responders both before the initiation of treatment and during the course of treatment.

Methods

Study Subjects

Described in methods section of Chapter Three.

Study Design

Described in methods section of Chapter Three.

Determination of Responders versus Non-Responders

Described in methods section of Chapter Three.

RNA Extraction and Processing

Total RNA was isolated from whole blood using the MagMax Nucleic Acid Isolation Kit (Ambion, Texas) according to the manufacturer's instructions. Globin mRNA was removed using the GLOBIN Clear kit (Ambion). 1500 ng of RNA from all samples passing quality control were amplified and labeled using the Illumina TotalPrep RNA Amplification kit (Illumina, California). Amplified labeled RNA (1500 ng) was hybridized overnight to Illumina HT-12 V4 beadchips (Illumina). Chips were scanned on an Illumina HiScan following the manufacturer's protocol.

Microarray Data Processing

Each sample was background subtracted and scaled to the average of all samples. Raw values less than 10 were set to 10 and the data was $\log_2$ transformed. Probes were than filtered to select transcripts that are present in at least one sample (PALO) using an alpha of 0.01 for the signal detection, thus eliminating background. Out of approximately 47,000 probes, approximately 28,000 passed this

criterion. The probes were than additionally filtered by removing probes with the lowest variability using a standard deviation of less than 0.344. This left approximately 20,000 probes for differential analysis. Principle variance component analysis was then conducted to assess and correct for chip-to-chip variation among samples using the batch correction method implement in JMP Genomics software(344).

Differential Gene Expression Analysis

Differential gene expression analysis was performed using Welch's *t*-test for comparisons between responder status groups at each time point in addition to each group versus healthy controls at each time point. Paired *t*-tests were used to test for transcriptional changes within each responder group over time after initiation of anti-IgE treatment. The Benjamini-Hochberg false discovery rate (FDR) procedure was applied to adjust for multiple testing.

Cluster Generation and Annotation

Clusters were generated by first considering only transcripts that were statistically significant (FDR<0.05) in at least one comparison between the responders or non-responders at any time point versus healthy controls. Hierarchical clustering was than performed on the mean expression values for each responder/time point group. The Cubic clustering criterion was than used to select an optimal number of clusters for annotation. This procedure was conducted using JMP Genomics software (SAS, North Carolina). Clusters were annotated based on a combination of Ingenuity pathway analysis (IPA) and a comparison with the correspondence of gene clusters relative to the Baylor Module annotations (345, 346).

Gene set analysis was conducted using the QuSAGE algorithm (347). QuSAGE tests whether the average $\log_2$ fold change of a gene set is different from 0 and takes into account the correlations of the genes by incorporating an estimate of the variance inflation factor of the gene set. QuSAGE was applied using both the Baylor-derived modules as well as the 8 derived clusters from this study.

Cross-Validation Analysis

Nested loop cross validation randomly partitions the data into training and test sets (50 times) with the training set having a balanced number of responders and non-responder samples. For each training set, probes were identified as good candidates for classification using Random Forrest (348) and by varying the total number of features to consider. The resulting set of candidate probes were than used to build classification rules using various methods such as support vector machine (SVM), Diagonal Linear Discriminant analysis (DLDA), Random Forrest, and Partitioning Around Medoids (PAM) (349). The classification rule was than applied to the test set, and an appropriate aggregate of the performance (misclassification rate) was recorded across the 50 cross-validation runs (348, 350, 351). The overall performance was than assessed in addition to investigating the optimal number of features to consider. Similar approaches have been conducted previously in a wide variety of gene expression studies including biomarker discovery within tuberculosis, cancer, and asthma (352-355).

Results

Asthma Patients Display Distinct Gene Signatures from those of Healthy Subjects

We first defined the global gene signature for asthma patients by comparing all patient samples to healthy controls. In order to identify differentially expressed genes between healthy control and the asthma patients prior to anti-IgE treatment, we conducted a Welch's t-test with multiple test correction FDR<0.05. This comparison yielded 3,867 differentially expressed probes and revealed a clear signature difference between healthy controls and asthma patients (Fig. 10.1A). To further explore differences between responders and non-responders, we performed unsupervised clustering. As observed in Figure 10.1B, there was no clear separation of responders, non-responders and healthy controls, although healthy controls were more likely to form their own clusters. However, it is clear that the differences between responders and non-responder are minute, indicating the need for further analysis to tease out the alterations between these patient groups.

We were also able to use this same approach to view patient profiles over time with anti-IgE treatment from baseline (week 0) to week 26. Figure 10.2 shows the expression levels of the differentially expressed probes between patients and healthy controls at baseline (N=3867) at each time point, both grouped by patient response and time (Fig. 10.2A) and with unsupervised clustering (Fig. 10.2B). It is evident by the unsupervised clustering that asthma patients with anti-IgE treatment, particularly by week 26, are clustering by healthy controls.

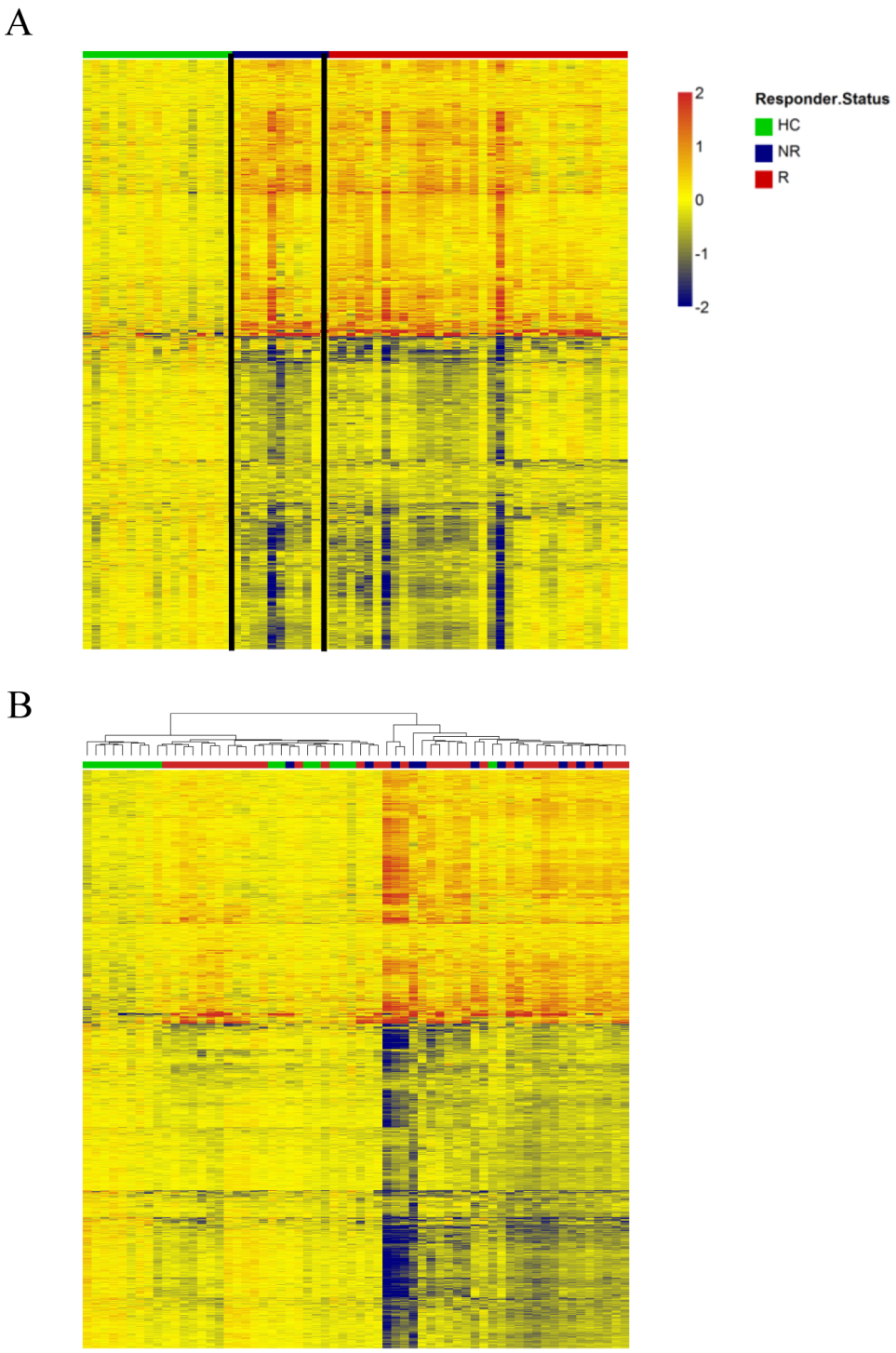


Figure 10.1. Allergic asthma blood transcriptional profiles.
(A) Hierarchical clustering and (B) unsupervised analysis of differentially expressed transcripts (N=3867) between patients and healthy controls at baseline (FDR<0.05). Samples are broken into healthy controls (green), anti-IgE non-responders (blue) and anti-IgE responders (red). Data areC represented as $\log_2$ expression normalized to healthy.

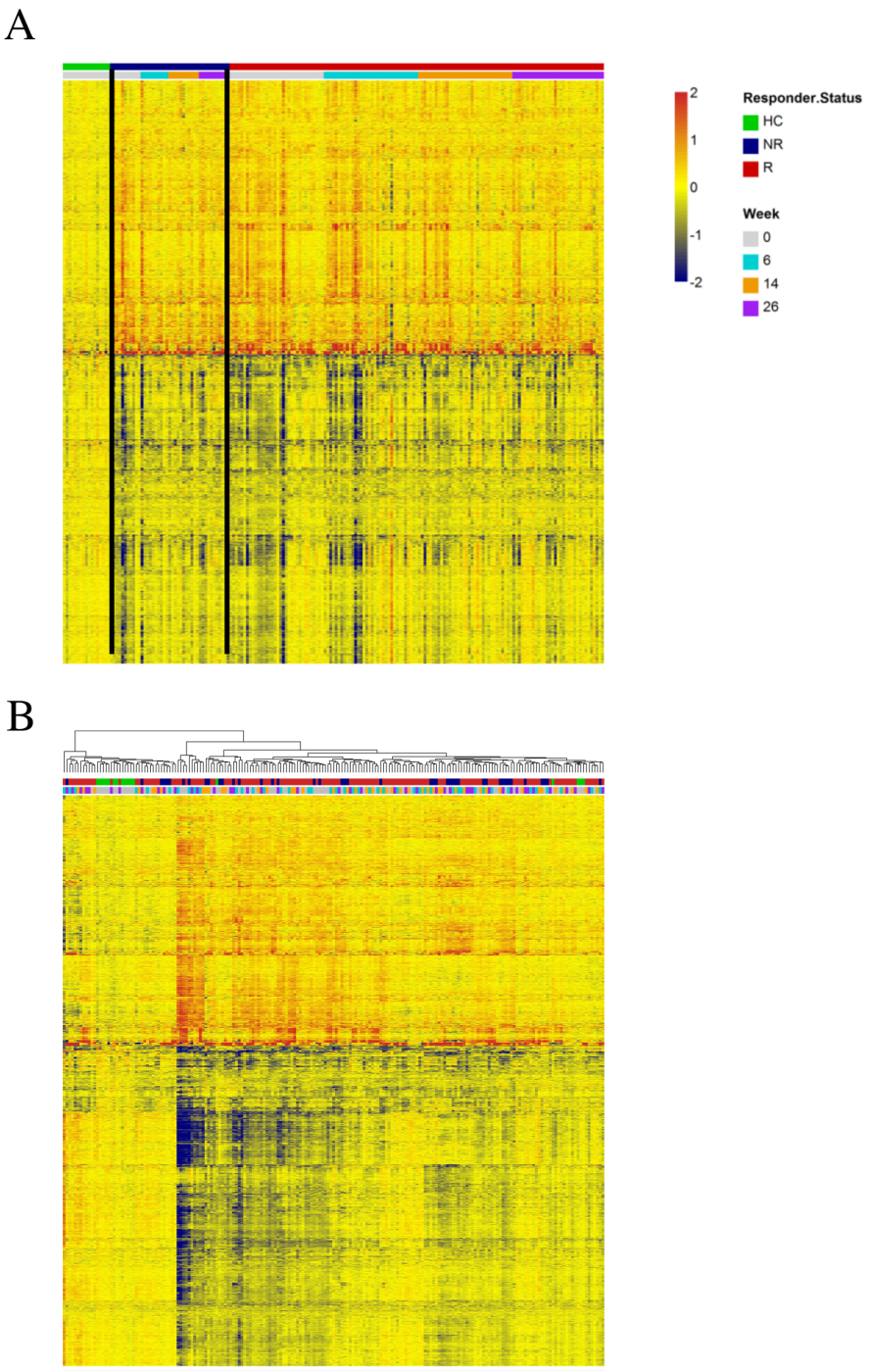


Figure 10.2. Asthma patient blood transcriptional profiles with anti-IgE treatment. (A) Hierarchical clustering and (B) unsupervised analysis of differentially expressed transcripts (N=3867) between patients and healthy controls at baseline (FDR<0.05) shown over the course of anti-IgE treatment. Samples are broken into healthy controls (green), anti-IgE non-responders (blue) and anti-IgE responders (red). Weeks of treatment are denoted as baseline/week 0 (gray), week 6 (aqua), week 14 (orange) and week 26 (purple). Data are represented as $\log_2$ expression normalized to healthy.

Comparison of Anti-IgE Responders and Non-Responders using Clustering Analysis

In order to differentiate between anti-IgE treatment responders and non-responders, we used hierarchical clustering (see method section) with complete linkage to stratify 8 similarly expressed transcript clusters, using genes significant (FDR<0.05) in at least one comparison of each patient response status and time point with healthy controls. Gene ontology and network enrichment analysis were used to annotate the 8 clusters (Fig. 10.3A and Table 10.1). Figure 10.3A shows gene expression with respect to healthy controls in both responders and non-responders over the course of treatment. By clustering similarly expressed genes, we were able to more easily view the changes in gene expression over time and compare this change between anti-IgE responders and non-responders. As shown in Table 10.1, cluster annotation was achieved by analyzing top canonical pathways and upstream regulators in each cluster, similar to that done in a previous study (356).

To assess longitudinal activity of each gene cluster, the average fold-change relative to healthy controls was determined (Fig. 10.3B and Fig. 10.4). Clusters 4 (cell cycle/proliferation) and 6 (monocytes) showed little difference before and during treatment between responders and non-responders. In some instances non-responders maintained more similarity to healthy than responders, such as in cluster 3 (hematopoiesis) and 7 (glucose metabolism). Clusters 1 (protein synthesis), 2 (T/NK/cytotoxicity) and 8 (granulocytes/inflammation) show that responders become more similar to healthy controls with treatment. In these clusters, the non-responders

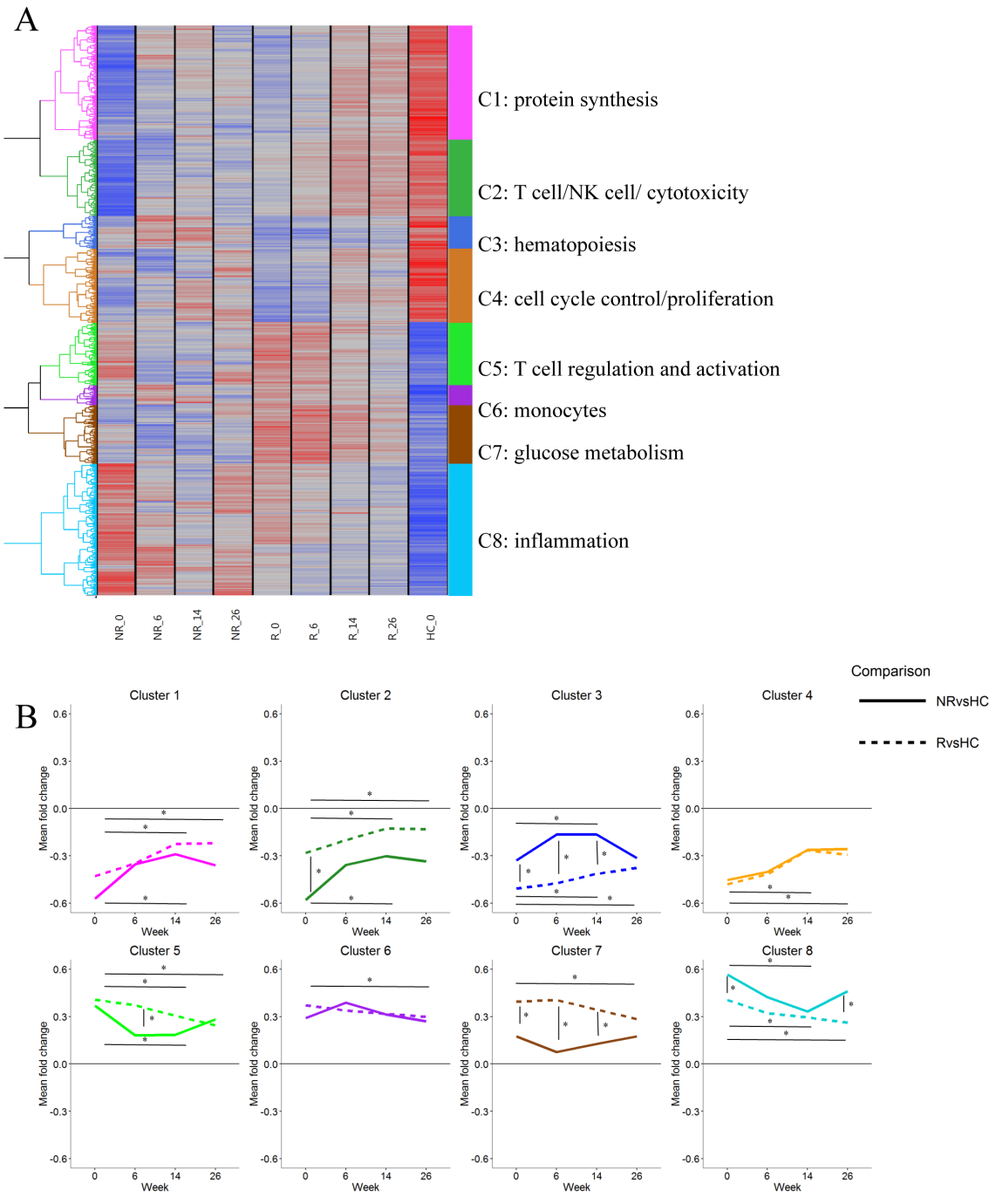


Figure 10.3. Clustering of mean expression profiles reveals variations to treatment between responders and non-responders.
(A) Genes with significant changes between patients and healthy controls were hierarchically clustered, and k-means (k=8) were used to group genes with similar expression profiles across the time points. IPA pathway analysis was used to annotate each cluster (Table 10.1). (B) Cluster average fold change compared with healthy controls was calculated at each time point. Color coding for each cluster matches that presented in the heat map in panel A. Responder/healthy control comparisons are denoted by a dashed line and non-responder/healthy control comparisons are denoted by a solid line. Significance for panel B was set to $p<0.05$.

initially change to become more similar to healthy controls but begin to deviate from the healthy baseline by week 14 or week 26 of treatment. By comparing the differences in fold change between the two groups, we were able to determine which clusters were significantly different ($p<0.05$). There was a significant difference at baseline (week 0) in cluster 2 (T cell/NK cell/cytotoxicity), 3 (hematopoiesis), 7 (glucose metabolism) and 8 (inflammation) between responders and non-responders. At week 6, there was still a significant difference between responders and non-responders in clusters 2 and 7 in addition to a lesser difference in cluster 5 (T cell regulation and activation). At week 14, only clusters 3 and 7 were significantly different; while at week 26, only cluster 8 was significantly different.

We were able to monitor the changes over time in each patient group. Interestingly, responders showed significant differences between baseline and week 26 in all clusters, while non-responders only had a significant change in cluster 8. Responders also had significant differences between baseline and week 14 in all clusters but 6 (monocytes) and 7. Non-responders showed significant differences in clusters 1 (protein synthesis), 2, 3, 5 and 8. There were no clusters in which patients had a significant change between baseline and week 6. These results are intriguing because non-responders appear to become closer to healthy controls by week 14, but this change is lost by week 26, at which time they seem to resemble the same patterns as before treatment (baseline). These longitudinal changes can be seen more clearly when the cluster activity of both responders and non-responders are normalized to healthy controls at each time point (Fig. 10.4). These results indicate differences between responders and non-responders, particularly in their changes over time with anti-IgE treatment.

Table 10.1. Summary of pathway analysis to identify networks enriched in each cluster

	Canonical Pathways	score -log(p-value)	Upstream Regulators	score -log(p-value)
Cluster 1: Protein Synthesis (n=678)	EIF2 Signaling	13.90	MYCN	9.68
	Regulation of eIF4 and p70S6K Signaling	6.02	HNF4A	4.20
	mTOR Signaling	4.18	FUBP1	4.05
	Estrogen-mediated S-phase Entry	3.09	DOT1L	3.04
	Diphthamide Biosynthesis	3.02	FOXO3	2.92
	Natural Killer Cell Signaling	2.79	RBL1	2.91
	Telomerase Signaling	2.41	CBFB	2.82
	Cell Cycle: G1/S Checkpoint Regulation	2.24	ZBED1	2.78
	Pyrimidine Deoxyribonucleotides De Novo Biosynthesis I	2.11	RFX3	2.74
			SOX11	2.73
Cluster 2: T cell/NK cell/ cytotoxicity (n=451)	EIF2 Signaling	5.60	E2F1	3.64
	T Cell Receptor Signaling	2.44	MYCN	3.46
	Natural Killer Cell Signaling	2.20	HNF4A	2.70
	Purine Nucleotides De Novo Biosynthesis II	2.03	TWIST1	2.59
			KMT2D	2.14
Cluster 3: Hematopoiesis (n=195)	Glutamine Biosynthesis I	2.31	HOXA10	3.69
	Polyamine Regulation in Colon Cancer	2.28	TP73	3.49
			ATN1	3.37
			HDAC1	3.10
			ID3	2.82
			TRIM28	2.80
			NKX3-1	2.75
			MYC	2.56
			CHD4	2.45
			WBSCR22	2.33
Cluster 4: Cell cycle control/ proliferation (n=439)	Protein Ubiquitination Pathway	4.09	HNF4A	5.08
	EIF2 Signaling	2.71	STAT5A	4.46
	Hypoxia Signaling in the Cardiovascular System	2.63	MYCN	3.17
	Triacylglycerol Biosynthesis	2.45	SIRT6	3.09
	Natural Killer Cell Signaling	2.09	MEN1	2.70
			NPAT	2.42
			TBX2	2.42
			E2F1	2.40
			FOXO3	2.24
			ERG	2.23
Cluster 5: T cell regulation and activation (n=368)	GNRH Signaling	3.16	HNF4A	3.42
	Nur77 Signaling in T Lymphocytes	2.81	TFDP2	2.74
	Calcium Signaling	2.28	SATB1	2.73
	B Cell Receptor Signaling	2.18	PPARGC1B	2.32
	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.17	ONECUT1	2.30
	Neuropathic Pain Signaling In Dorsal Horn Neurons	2.15	CUX1	2.26
	Cell Cycle Control of Chromosomal Replication	2.14	FOXP3	2.19
	IL-15 Production	2.14	CTNNB1	2.18
	RAR Activation	2.12	PLAG1	2.17
	NGF Signaling	2.09	SNAI1	2.14
Cluster 6: Monocytes (n=120)	Isoleucine Degradation I	2.67	CBX5	3.68
	Semaphorin Signaling in Neurons	2.63	STAT5A	2.74
	Valine Degradation I	2.45	SPIB	2.26
	Histamine Degradation	2.40	PRMT5	2.05
	Inflammasome pathway	2.31		
Cluster 7: Glucose metabolism (n=345)	GDP-mannose Biosynthesis	2.55	HDAC5	3.23
	Glycolysis I	2.30	BCOR	3.07
	UDP-N-acetyl-D-galactosamine Biosynthesis II	2.00	NUPR1	3.06
			SIN3B	2.69
			MYC	2.55
			ATF6	2.29
			MSGN1	2.25
Cluster 8: Inflammation (n=785)	Acute Phase Response Signaling	5.12	ESR1	6.28
	TREM1 Signaling	4.27	TP53	6.24
	IL-8 Signaling	4.26	CEBPA	6.15
	Role of JAK family kinases in IL-6-type Cytokine Signaling	4.22	SPI1	5.82
	Phosphatidylglycerol Biosynthesis II (Non-plastidic)	4.12	ECSIT	5.45
	Germ Cell-Sertoli Cell Junction Signaling	3.85	HIF1A	4.86
	Role of JAK1 and JAK3 in γc Cytokine Signaling	3.73	NKX2-3	4.81
	STAT3 Pathway	3.64	PRDM1	4.75
	Acute Myeloid Leukemia Signaling	3.56	STAT1	4.65
	IL-6 Signaling	3.53	GFI1	4.49

[a] For each cluster, the top 10 canonical pathways and upstream regulators were identified. The size of each gene list is displayed under the total. Based on an interpretation of significant pathways, each cluster is annotated where appropriate.

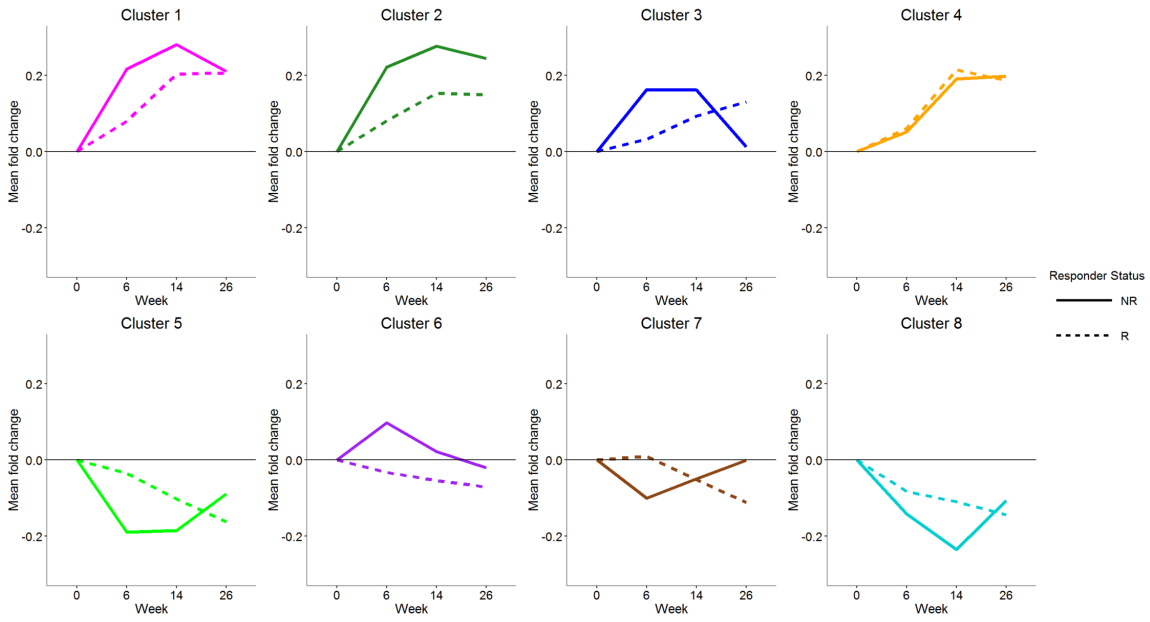


Figure 10.4. Clustering of mean expression profiles shows changes over time with respect to baseline are different in responders and non-responders.
Cluster average fold change from baseline/week 0 was calculated at each time point. Color-coding for each cluster matches that presented in the heat map in Figure 10.3. Responder/healthy control comparisons are denoted by a dashed line and non-responder/healthy control comparisons are denoted by a solid line.

Module-Based Analysis of Transcriptional Profiles of Responders and Non-Responders

To further identify differences between responders and non-responders, we conducted a module-based analysis (357). Briefly, we used a framework of 260 modules of transcripts co-expressed in blood across various immunological conditions. This was done to reduce data dimensionality while increasing interpretability of results. These modules were previously annotated using knowledge-based and data-driven approaches (345, 346). To assess changes in module activity across groups over time, we applied our modular analysis across all time points and compared variations in module activity patterns. Differential module expression was set using a raw p-value <0.05 and a fold change greater than ±0.4.

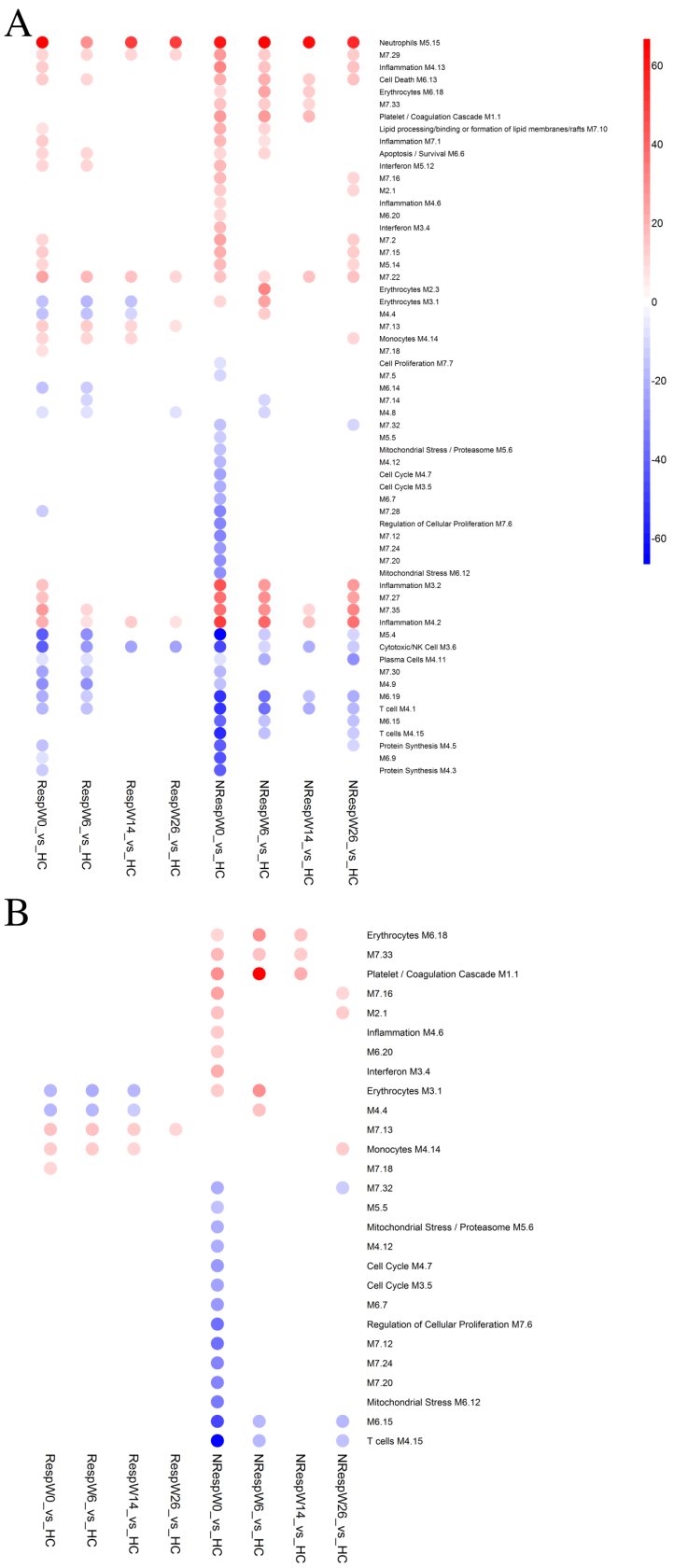


Figure 10.5. Modular analysis indicates non-responders are more different from healthy controls than responders. (A) Blood modular fingerprint of responders and non-responders compared with healthy controls over time. (B) Modules with difference in expression between responders and non-responders.

As shown in Figure 10.5A, we were able to compare responders and non-responders with respect to healthy controls and chart their changes over time with anti-IgE treatment. The largest number of variations between asthma patients and healthy controls occurs before treatment, and this is slowly decreased with treatment except for module M5.15, which is a neutrophil module. However, non-responders tend to exhibit a greater number of differentially expressed modules compared with healthy controls than responders do, even at week 26 of treatment. There are some modules that are similarly expressed by both responders and non-responders. One of the strongest signatures exhibited by both patient groups is M5.15, corresponding to neutrophils, and this is maintained in both groups even with anti-IgE treatment. We also observed that both groups of asthma patients exhibit increased inflammation modules, such as M4.2 and M4.13, compared with healthy controls at baseline. However, with treatment, this signature tends to be decreased in responders but remain unchanged in non-responders. Responders and non-responders also similarly have a decreased cytotoxic/NK cell and T cell signature compared with healthy controls, as shown by modules M3.6 and M4.15, respectively. Interestingly, the decreased T cell signature observed in both patient groups before treatment is only maintained through week 26 by non-responders.

To further uncover variations between anti-IgE responders and non-responders, we isolated modules that differed between non-responders and healthy, but not responder and healthy or vice versa. As shown in Figure 10.5B, there are a large number of modules only present in non-responders versus healthy controls before treatment. The majority of these modules are under-expressed in non-responders compared with healthy controls. There are also a smaller number of modules that are only present in responder

versus healthy. One of these modules, M3.1, is under-expressed in responders compared with healthy, but overexpressed in non-responders. Many of the modules overexpressed in non-responders are involved in erythrocytes. In addition, many of the modules underexpressed in non-responders are involved in cell cycle/proliferation and T cells, which may correspond to cluster 2 (Fig. 10.5).

In order to ensure that the variation in module expression between responders and non-responders was not due to a discrepancy in cell counts, we compared cell numbers acquired by complete blood counts performed at each blood draw. Since non-responders displayed a strong erythrocyte and platelet signature and a weak T cell signature, we

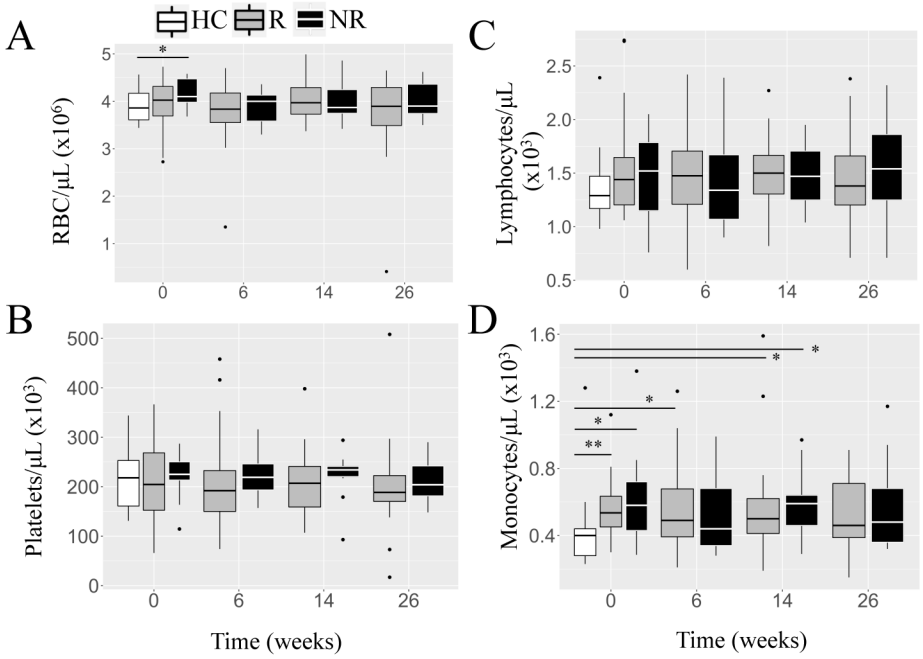


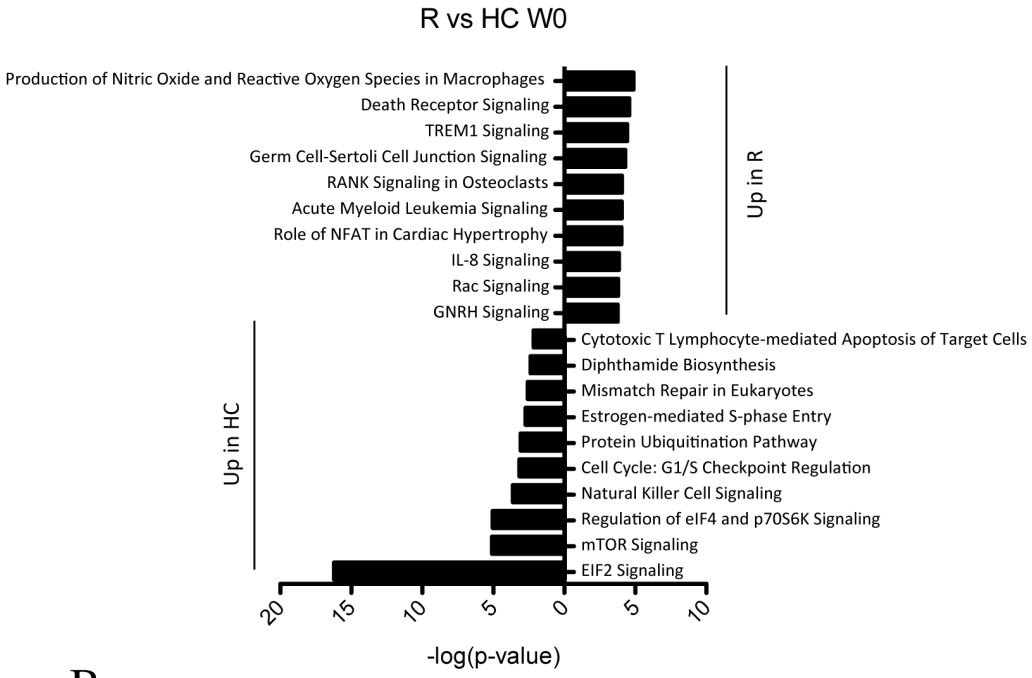
Figure 10.6. Increase in modular expression of non-responders compared with responders is not caused by an increase in cell counts.
Cell counts acquired by CBC for (A) red blood cells (RBC), (B) platelets, (C) lymphocytes and (D) monocytes as cells per μL of whole blood. **P*<0.05, ***P*<0.01. Error bars represent SD.

analyzed the numbers of red blood cells (RBCs, erythrocytes), platelets, lymphocytes and monocytes. As shown in Figure 10.6, there are no significant differences between responders and non-responders in any of these cell types, although both patient types exhibit an increased number of monocytes compared with healthy controls. These results reinforce many of the observations from Figure 10.3, and emphasize the differences between anti-IgE responders and non-responders, both before they begin anti-IgE treatment and as they react to IgE neutralization.

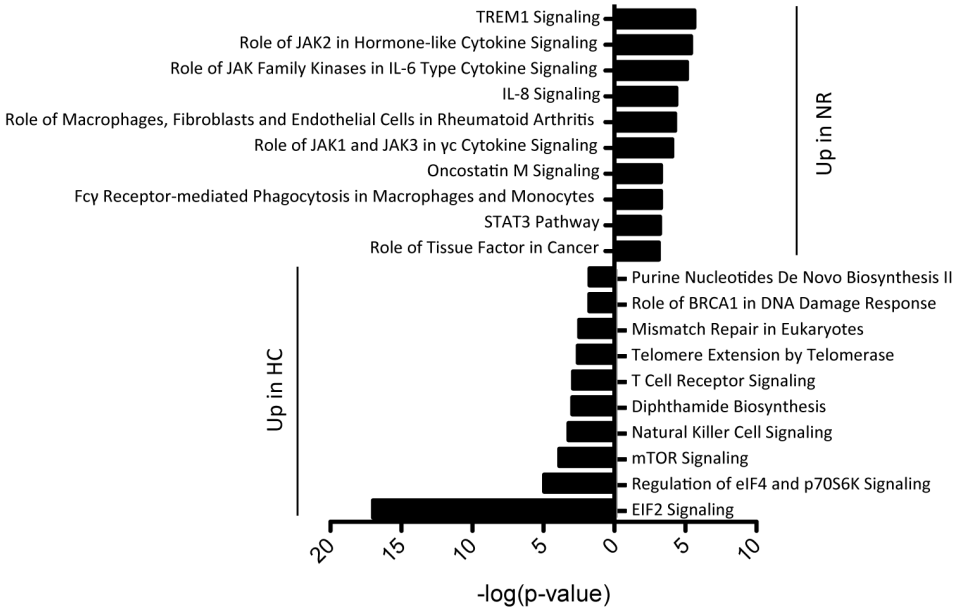
Pathway Analysis Comparisons between Responders and Non-Responders

To identify networks and pathways that contribute to the outcomes of anti-IgE treatment, we compared IPA canonical pathway and gene ontology biological process enrichment analysis between responders and non-responders before treatment. As shown in Figure 10.7A and 10.7B, both patient groups shared many of the same enriched pathways, particularly downregulated pathways compared with healthy (including EIF2 signaling, mTOR signaling (358), regulation of eIF4 and p70S6K singling (359, 360), natural killer cell signaling, diphthamide biosynthesis and mismatch repair in eukaryotes). Most of these pathways correspond to cluster 1 (protein synthesis) in Figure 10.3. There are fewer pathways that are similarly overexpressed in non-responders compared to healthy. These included TREM1 signaling and IL-8 signaling, and these correspond to cluster 8 (inflammation) in Figure 10.3. TREM1 is known to be associated with allergic lung inflammation (361, 362). Pathways that are uniquely upregulated in responders include the production of nitric oxide and reactive oxygen species in macrophages, death receptor signaling, germ cell-Sertoli cell junction signaling, RANK signaling in osteoclasts, acute myeloid leukemia signaling, the role of NFAT in cardiac

A


R vs HC W0
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages
Death Receptor Signaling
TREM1 Signaling
Germ Cell-Sertoli Cell Junction Signaling
RANK Signaling in Osteoclasts
Acute Myeloid Leukemia Signaling
Role of NFAT in Cardiac Hypertrophy
IL-8 Signaling
Rac Signaling
GNRH Signaling
Up in R
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells
Diphthamide Biosynthesis
Mismatch Repair in Eukaryotes
Estrogen-mediated S-phase Entry
Protein Ubiquitination Pathway
Cell Cycle: G1/S Checkpoint Regulation
Natural Killer Cell Signaling
Regulation of eIF4 and p70S6K Signaling
mTOR Signaling
EIF2 Signaling
Up in HC
20
15
10
5
0
5
10
-log(p-value)

B


NR vs HC: W0
TREM1 Signaling
Role of JAK2 in Hormone-like Cytokine Signaling
Role of JAK Family Kinases in IL-6 Type Cytokine Signaling
IL-8 Signaling
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis
Role of JAK1 and JAK3 in γc Cytokine Signaling
Oncostatin M Signaling
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes
STAT3 Pathway
Role of Tissue Factor in Cancer
Up in NR
Purine Nucleotides De Novo Biosynthesis II
Role of BRCA1 in DNA Damage Response
Mismatch Repair in Eukaryotes
Telomere Extension by Telomerase
T Cell Receptor Signaling
Diphthamide Biosynthesis
Natural Killer Cell Signaling
mTOR Signaling
Regulation of eIF4 and p70S6K Signaling
EIF2 Signaling
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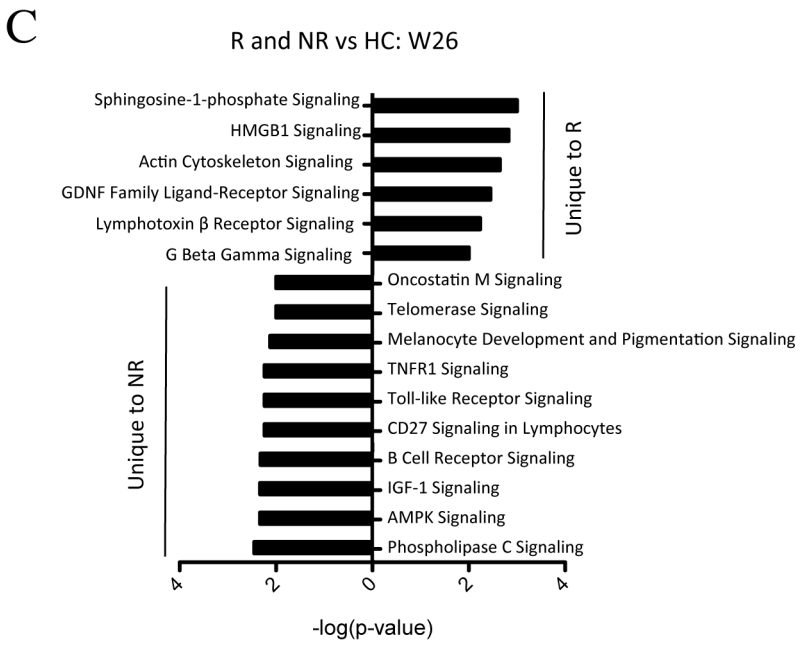

Figure 10.7. Pathway analysis at baseline differentiates responders and non-responders. Bar charts representing the IPA canonical pathway enrichment in (A) responders and (B) non-responders with healthy controls. (C) Unique canonical pathways in responders and non-responders at week 26.

hypertrophy, Rac signaling (363) and GNRH signaling. Many of these pathways are involved in cell adhesion and migration and NF-κB activation (364-368).

Non-responders show an upregulation in the role of JAK2 in hormone-like cytokine signaling, role of JAK family kinases in IL-6 type cytokine signaling, role of macrophage, fibroblasts and endothelial cells in rheumatoid arthritis, role of JAK1 and JAK3 in γc cytokine signaling, oncostatin M signaling, Fcγ receptor-mediated phagocytosis in macrophages and monocytes, STAT3 pathway and the role of tissue factor in cancer. These pathways are largely involved in cytokine signaling, inflammation and hematopoiesis (369-374). Overall, this highlights the differences in the underlying immune responses between responders and non-responders, with responders

showing increased cell motility and non-responders showing increased cytokine signaling and inflammation.

We also compared responders and non-responders to healthy controls at week 26 of treatment. However, the number of genes that were differentially regulated using an FDR<0.05 was too low to acquire statistically significant canonical pathways. This is unsurprising, as shown by the modular analysis (Fig. 10.7) that the number of differences between patients and healthy controls decreases with treatment. We were able to acquire a greater number of differently expressed transcripts if we increased our FDR cutoff to 0.2 (FDR<0.2), although the gene set for each comparison was still relatively low (Fig. 10.7C). By analyzing the top pathways unique to each patient group, we can observe that responders still have a strong cell migration capacity, as indicated by sphingosine-1-phosphate signaling, actin cytoskeleton signaling and GDNF family ligand-receptor signaling (375-377). Non-responders also showed a strong platelet signature, indicated by the upregulation of phospholipase C signaling and oncostatin M signaling (370, 378). Non-responders also still showed strong inflammatory signal and immune activation signals, as indicated by the upregulation of B cell receptor, TNFR1, toll-like receptor, CD27 and telomerase signaling (379-383). This indicates that there is much more inflammation still occurring at week 26 of anti-IgE treatment in non-responders than responders.

Assessment of Responder/Non-Responder Baseline Classification using Cross-Validation

Finally, in order to identify potential response-related biomarkers, we performed predictive modeling and cross-validation analysis on patients before treatment. We focused on pre-treatment since we were unable to detect direct differential changes

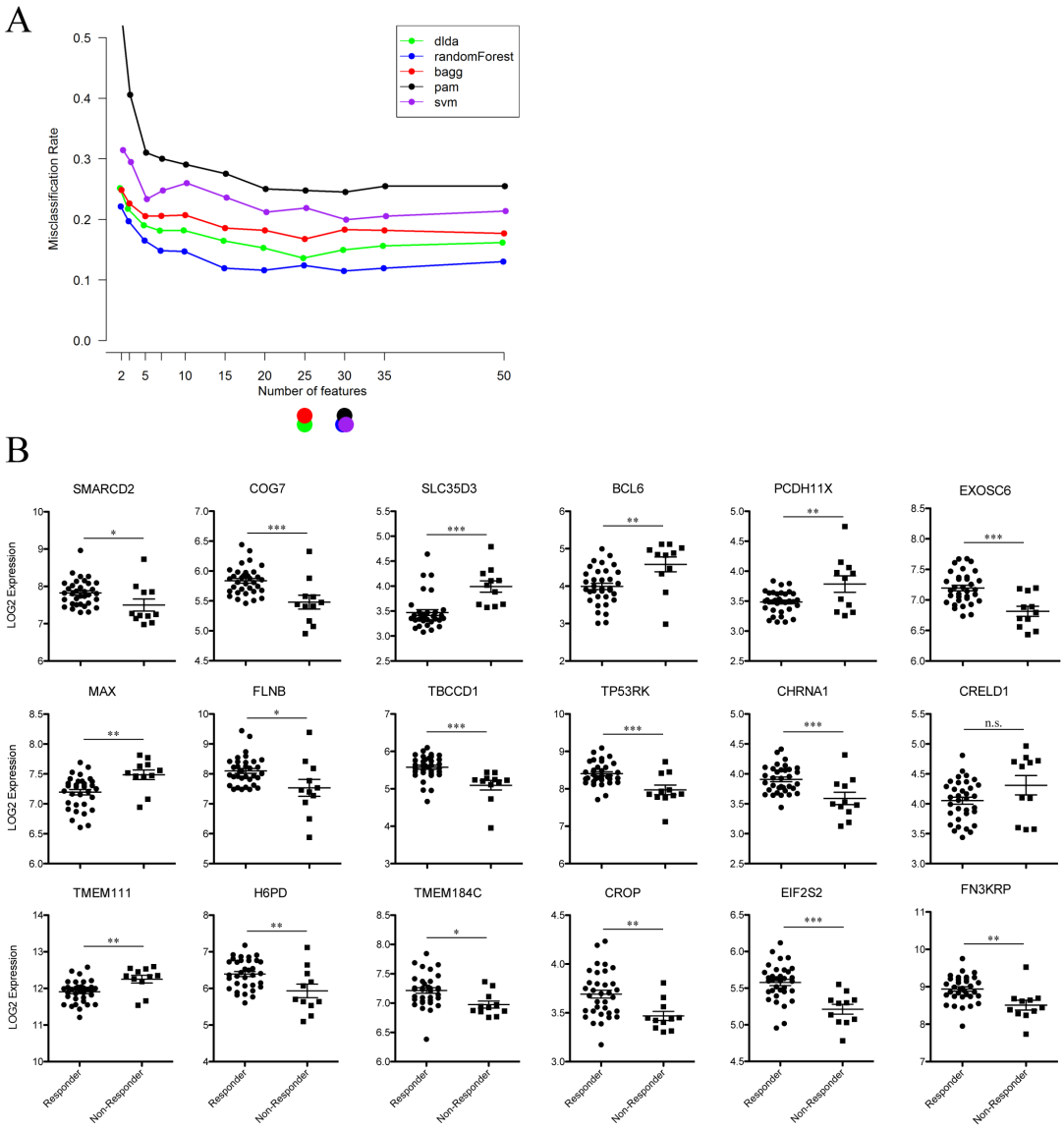

Figure 10.8. Cross-validation analysis enables the detection of biomarkers for response to anti-IgE treatment.
(A) Nested loop cross-validation. (B) Top annotated probes identified by cross-validation.

between responders and non-responders at baseline after FDR multiple test correction. This limitation was due to the small number of samples available. Using nested loop cross-validation (351), we assessed the performance of classifying responders and non-responders using multiple classification algorithms and a range of total number of probes

used in classification (Fig. 10.8). The performance varies between methods, with random Forrest and diagonal linear discriminant analysis (DLDA) performing the best (88% and 85% accuracy, respectively). We identified 25 probes as being ideal for responder/non-responder discrimination based on the lowest misclassification rate and agreement on the best number of features from each validation method. The overall results for nested loop cross-validation are shown in Figure 10.8A. Of the 25 candidate probes, only 18 were annotated. We further analyzed the annotated probes, as shown in Figure 10.8B. The genes that are significantly decreased in non-responders compared with responders are SMARCD2, COG7, EXOSC6, FLNB, TBCCD1, TP53RK, CHRNA1, H6PD, TMEM184C, CROP, EIF2S2 and FN3KRP. Non-responders exhibited significantly greater expression of SLC35D3, BCL6, PCDH11X, MAX, and TMEM111. Only one from the annotated probes, CRELD1, showed no difference in expression between responders and non-responders.

Discussion

To better understand allergic asthma pathogenesis and to determine transcriptional signatures of anti-IgE response, we profiled the blood transcriptomes of a longitudinal cohort of allergic asthma patients receiving anti-IgE treatment. We detected a clear difference between responders and non-responders, with responders showing a steady signature progression towards that of healthy controls, while non-responders showed an initial change towards healthy, but by week 26 of treatment, showed a pattern more similar to pre-anti-IgE treatment. We also identified differences between responders and non-responders before treatment, with responders exhibiting strong

upregulation in pathways involved in cell mobility and non-responders displaying a strong upregulation in pathways involved in cytokine signaling and inflammation. Finally, non-responders could be identified from responders before treatment using 25 markers generated by predictive modeling and nested-loop cross-validation.

To date, no studies have analyzed the effects of anti-IgE or the variations between responders and non-responders on whole blood gene expression. Several studies have tried to predict biomarkers for response to anti-IgE. Thus far, it has been shown that baseline total IgE (107), bronchial galectin-3 concentration (110), a history of severe asthma (109) and FE_{NO} (exhaled nitric oxide level), blood eosinophils and serum periostin (108) may help define patients that will respond best to anti-IgE treatment. However, the power of these biomarkers to determine response needs to be improved. The weakness of all of these studies was the limit in potential markers, with each group testing one or a handful of parameters. By using microarray analysis, the potential pool of biomarkers was much larger. As shown by predictive modeling and nested loop cross-validation analysis, the use of 25 markers could be ideal for distinguishing responders from non-responders.

It is unsurprising that it has been so difficult to isolate predictive biomarkers for response to anti-IgE. Based on the blood transcriptional profiles, there are very little differences between responders and non-responders. Analysis of heatmaps of differently expressed genes is not powerful enough to reveal such differences, and direct comparison of responders with non-responders yielded no transcripts that passed the FDR correction. In order to tease out the differences between these two groups, we analyzed the variances in the expressions of groups of genes, using cluster and module analysis. This not only

revealed differences before treatment, but also showed how responders and non-responders react differently over time with anti-IgE treatment. It is clear that responders adopt a signature similar to healthy controls over the course of treatment. It also appears that responders are more similar to healthy controls than non-responders even before the initiation of treatment. Non-responders show a much different response to anti-IgE by week 26 of treatment and tend to have expression patterns more similar to their baseline than to healthy. This is particularly evident in clusters 3, 5, 6, 7 and 8. It is also evident that non-responders tend to be more different from healthy controls at baseline than responders, which is shown in clusters 1, 2 and 8. These observations can also be seen in the modular analysis; non-responders have a very strong module signature at baseline, which persists even at week 26.

To further understand the underlying differences between responders and non-responders that may influence response to anti-IgE treatment, we compared pathway analysis at baseline between both groups of patients. Interestingly, responders and non-responders shared most of the downregulated pathways. EIF2 signaling and protein synthesis were particularly downregulated, and this may reflect corticosteroid administration as corticosteroid receptors can bind to transcription factors and prevent transcription (384). Therefore, the upregulated pathways may reflect alterations in asthma pathogenesis. In responders, there was a strong upregulation of pathways involved in cell migration and control of cellular activation. These pathways were largely absent from non-responders, which showed a strong upregulation of pathways involved in cytokine signaling and inflammation. Non-responders also had a strong signature of platelet activation, as shown by the upregulation of tissue factor signaling

and in the modules related to platelets. Platelets promote allergic asthma, partly by promoting Th2 responses, suppressing regulatory T cells (385) and airway remodeling (386). Platelet activation in anti-IgE responders compared with non-responders may warrant further investigation and potentially could be an additional biomarker for response. Although both responders and non-responders show an increase in inflammation, this signature is stronger in non-responders. While there is no difference in asthma control or severity between responders and non-responders before treatment, the increased inflammation in non-responders may indicate a greater degree of airway remodeling, which would not be susceptible to IgE neutralization. In addition, responders show a strong cell migration signature before treatment. This is likely more targeted by anti-IgE, since many of the cells involved in migrating to the lung could be directly sensitive to IgE (73, 96, 117, 119). Unfortunately, due to the low degree of differences between patients and healthy controls at week 26, we had to use a lower statistical cutoff to generate a gene list that could be used for pathway analysis. Despite this, there were still a relatively low number of genes differentially expressed between patients and healthy controls. However, by comparing only the pathways enriched in either responders or non-responders compared with healthy controls, we were able to observe some level of variation between our patient groups. Noticeably, non-responders displayed a greater number of uniquely upregulated pathways than responders, which alludes to the greater number of variations between non-responders and healthy controls at week 26 than responders and healthy controls. The pathways upregulated in non-responders indicate that these patients still have an inflammatory presence to a degree not observed in responders.

We also observed a variation in the kinetics of anti-IgE effects on responder and non-responder transcriptional profiles. While responders showed a significant difference between baseline/week 0 and week 26 for each gene set cluster, there were no clusters for which non-responders had a significant difference. However, in almost all clusters, non-responders were significantly different between baseline and week 14. There were also no clusters, either in responders or non-responders, which were significantly altered between baseline and week 6, which indicates that changes in gene signatures by anti-IgE take more than 6 weeks to become evident. The altered kinetics of signature change to anti-IgE in non-responders compared with responders highlights the variance in asthma pathogenesis between these two groups and may signify that an IgE-independent mechanism is being asserted, particularly between week 14 and week 26 of anti-IgE treatment. Responders and non-responders show a high degree of difference in cluster 7 (glucose metabolism). Glucose metabolism has recently come into the spotlight for its involvement in regulating T cell activation, differentiation and function (387). Non-responders tend to share a glucose metabolism signature more similar to healthy controls, which may indicate a lack of T cell involvement in their asthma pathogenesis. This is supported by their low T cell signature shown in cluster 2. There are also large differences between responders and non-responders in cluster 3, hematopoiesis. Like cluster 7, non-responders show more of a similarity with healthy controls than responders. This again indicates a variance in immune composition between responders and non-responders. It is quite clear, however, that non-responders exhibit a greater inflammatory signature than responders. Interestingly, this inflammatory signature is significantly different between responders and non-responders only at baseline and week

26. This shows that non-responders may initially exhibit signs of response to anti-IgE, but this response cannot be sustained.

Predicting response to anti-IgE is not only beneficial for patient quality of care, but it may reveal novel insights into asthma pathogenesis. Because we were unable to detect direct differential changes between responders and non-responders at baseline, we resorted to alternative methods. In particular, predictive modeling with internal nested loop cross-validation can assess the performance of classification accuracy of response and indicates a select number of probes that can be used for response classification. The result of the cross-validation analysis is encouraging given our relatively small sample size. However, this result does not imply that a select number of probes cannot provide reasonable classification accuracy of response. Based on the genes identified by cross validation, non-responders appear to have a depressed capacity for cell-cell interactions and cellular movement, including COG7, FLNB, TBCCD and EIF2S2 (388-391). This alteration in non-responder cellular movement was also identified by pathway analysis. However, these results need to be verified using a larger patient cohort, including a larger non-responder group.

In summary, this study provides new insights for the regulation of asthmatic inflammatory responses by omalizumab treatment. In addition, several candidate biomarkers discovered in this study will help us predict anti-IgE responders and nonresponders, although each of these candidates still need to be verified in future studies.

CHAPTER ELEVEN

Conclusions

- Patients with moderate-to-severe allergic asthma have an altered immune profile compared with healthy controls. This includes an altered mDC/pDC ratio, an increase in memory B cells and plasmablasts expressing the low affinity IgE receptor, a decrease in transitional B cells, an increase in central memory and regulatory $CD4^+$ T cells, cytotoxic and lung-migrating $CD8^+$ T ells, a decrease in NK cells, an increase in Th2-related cytokines from stimulated PBMCs, and an increase in eosinophils and neutrophils. This identifies the highly inflammatory environment present in allergic asthma patients.
- Anti-IgE therapy induces a global reduction of peripheral blood immune cell activation. This includes a reduction of DC and monocyte CCR7, HLA-DR and costimulatory markers, a reduction of NK activation and a reduction in granulocyte activation markers, particularly in basophils and blood mast cell precursors. This indicates that the reduction of free IgE can not only reduce FcεR1 expression in DCs, basophils and mast cell precursors, but it can also further decrease subsequent immune activation through these cells by the reduction of costimulatory markers and HLA-DR.
- Anti-IgE therapy induces asthma patients to become more similar to healthy controls, including an increase in the transitional B cell population and a decrease in T cell lung-migration capacity.

- Patients that do not respond to anti-IgE have a propensity for a comorbidity of obesity. This may be influencing response even though they are within the dosing range for weight and serum IgE.
- Patients that do not respond to anti-IgE exhibit less alterations in immune cell activation status than responders, indicating that neutralizing IgE in non-responders does not affect the immune response, even though cells expressing FcεR1 exhibit a decrease in this receptor expression. This is evident by the lack of significant change in costimulatory markers in DCs and in basophils and mast cell precursor activation markers in non-responders.
- While non-responders do not exhibit a significant neutrophilic or Th17 profile compared with responders, they do exhibit less Th2 cells. This is also evident in the variations in cytokine expression between responders and non-responders, with non-responders having less IL-13 and MCP-2 and more Th1-related cytokine IP-10.
- Microarray analysis of anti-IgE therapy reveals that the signature exhibited by patients before treatment is altered by anti-IgE to resemble healthy controls. The transcriptional profile of non-responders before treatment indicates they have elevated inflammation, but decreased T cell involvement, which is not caused by a decrease in lymphocyte count. They also exhibit a stronger platelet signature, which is also not caused by an elevated number of platelets, as responders and non-responders have a similar number.

- Cross-validation analysis to determine biomarkers for response identifies several transcripts down-regulated in non-responders compared to responders that are mainly involved in cell-cell interactions and cellular movement.

APPENDIX

List of Current and Future Publications

Publications Related to the Topics of Dissertation

Upchurch K, Cardenas J, Skinner J, Nattamai D, Lanier B, Millard M, Joo H, Turner J, Oh S. Transcriptional profiling of allergic asthma patients receiving anti-IgE therapy reveals variations between responders and non-responders. *Manuscript submitted.*

Upchurch K, Cardenas J, Ellis J, Xue Y, Lanier B, Millard M, Turner J, Joo H, Oh S. Longitudinal assessment of the frequency and activation status of granulocytes in adult asthma patients treated with anti-IgE antibody (omalizumab). *Manuscript submitted.*

Upchurch K, Cardenas J, Ellis J, Xue Y, Lanier B, Millard M, Turner J, Joo H, Oh S. Longitudinal analysis of blood dendritic cells and monocytes of asthma patients treated with anti-IgE antibody (omalizumab). *Manuscript submitted.*

Upchurch K, Cardenas J, Ellis J, Xue Y, Lanier B, Millard M, Turner J, Joo H, Oh S. Increased CD23+IgG1+ memory B cells and plasmablasts and decreased CD5+ transitional B cells are hallmarks of peripheral B cells in asthmatic adults. *Manuscript submitted.*

Upchurch K, Ellis J, Xue Y, Lanier B, Millard M, Joo H, Oh S. Peripheral CD4+ and CD8+ T cells of adult allergic asthma patients support chronic inflamation in the airway. *Manuscript submitted.*

Additional Publications

Upchurch K, Oh S, Joo H. Dectin-1 in the control of Th2-type T cell responses. Receptors Clin Investig 2016;3(1):e1094.

Upchurch KC, Boquin JR, Yin W, Xue Y, Joo H, Kane RR, et al. New TLR7 agonists with improved humoral and cellular immune responses. Immunol Lett 2015;168(1):89-97.

Yin W, Gorvel L, Zurawski S, Li D, Ni L, Duluc D, **Upchurch K**, Kim J, Gu C, Ouedraogo R, Wang Z, Xue Y, Joo H, Gorvel JP, Zurawski G, Oh S. Functional specialty of CD40 and dendritic cell surface lectins for exogenous antigen presentation to CD8(+) and CD4(+) T cells. EBioMedicine 2016;5:46-58.

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