The Search for Severity Predictive Biomarkers in Food Allergy: From Literature Review to Experimental Setup

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Plain language summary

Food allergies are a growing problem worldwide, affecting millions of people. Researchers think that food allergies occur when the immune system mistakenly identifies certain parts of food as harmful. This results in the activation of the immune system and leads to a range of symptoms that can affect various parts of the body, like the skin, digestive, and respiratory systems. Understanding the mechanisms underlying food allergy is important for developing better diagnostic tests. A specific type of cell is believed to dominate this immune response in food allergy. This cell is called the Th2 cell. These Th2 cells release substances that can amplify the immune response. These substances also enhance the production of other substances that cause the allergic reaction to specific foods.

This report aims to search for new markers in the body that can indicate the presence and severity of food allergies. Diagnosing food allergies can be challenging, as traditional tests may not always provide precise results or are intensive for patients and healthcare providers. Therefore, researchers are exploring new approaches to diagnosing food allergies. Scientists have been studying different kinds of cell types (like Th2) that seem to be only present in people who are allergic. These cells can be characterized by their expression of specific characteristic markers. These markers can be measured with a laser, emitting light that can be detected.

Another thing that was researched was how cells can move through the body. This process of moving between places in the body is regulated. We studied how cells can move toward the digestive, respiratory, and skin systems. This was done by setting up a panel with markers that, like markers for cells, can emit light when a laser is set on the cells. Research on these markers showed that stimulating cells with stronger and longer stimulation increased their expression.

The basophil activation test is another key aspect in the search for biomarkers that predict the severity of food allergy. Basophils are a type of cell that releases all sorts of substances that cause the symptoms we see in food allergies. The test measures how basophils respond to the food that is believed to cause the allergy. We looked at how well the test predicted a similar outcome as the food challenge. The results showed that in 82% of the cases, the test could correctly predict the outcome.

Abstract

In food allergy, the immune system mistakenly identifies specific food proteins as harmful, initiating an immune response. A skewed Th2 profile characterizes these immune responses. Th2 cells are a major player in the cytokine secretion of IL-4, IL-5 and IL-13. These cytokines increase

the differentiation of naïve T cells to Th2 cells and stimulate B cells to produce IgE towards the allergens. TH2A cells are an allergen-specific Th2 subset that has increased cytokine secretion and are thought to be only present in atopic individuals. In addition, Tfh13, a Tfh cell subset, has increased IL-13 secretion and is believed to be responsible for producing high-affinity anaphylactic IgE against allergens. For these cells, a flow cytometric panel was designed to identify these cells in PBMCs. An essential aspect of the immune system is the chemotaxis of immune cells through the body. We developed a panel for a flow cytometric analysis of T cells homing toward skin, lung, and gut tissue. The designed panels were tested on healthy donor PBMCs. The activation of the PBMCs in the homing panel tests showed that more prolonged stimulation (as well as stimulation with a higher concentration of stimuli) showed increased homing phenotypes in lung and gut homing tissues. In addition, the BAT was correlated to its ability to correctly predict the outcome of an oral food challenge (OFC). This study showed that BAT is better at predicting a positive outcome of the OFC for patients with more severe symptoms (82%). More false results were given with less severe or tolerant clinical outcomes (9% false positive and 9% false negative).

1. Introduction

Food allergy can be seen as an abnormal immune response triggered by exposure to food proteins. The immune system mistakenly sees these proteins as harmful.[1] While food allergies should not be correlated with food intolerance, both can cause similar symptoms. Still, food allergy triggers various symptoms across multiple organ systems, while food intolerance only affects the digestive system.[2] While the exact reason for food allergy is unknown, much research has been conducted to explore the differences between food-allergic individuals and healthy controls. Most food allergies are characterized by a Type I antibody-mediated immune response, whereas only a small portion is characterized by a Type IVb (T2) cell-mediated immune response. It should be noted that a part of the Type I response depends on T2 signals that regulate the production of allergen-specific IgE.[3] Therefore, this report will focus on the Type I antibody-related immune response and how cells mediate IgE production.

The antibody-mediated immune response is divided into 2 phases: the sensitization and effector phases. The production of IgE characterizes the sensitization phase. The production of IgE to allergens is an intricate cooperation between the innate and adaptive immune systems. Exposure to allergens in the skin, gut- and lung mucosa leads to antigen internalization by antigen-presenting cells (APCs), mainly dendritic cells (DCs), or through transcellular migration. APCs process the allergen and present parts of the allergen linked to major histocompatibility complex (MHC) class II to naïve T cells in lymphoid tissues. Together with other surface molecules and environmental factors on the APC (cytokines and metabolites), it promotes the activation and differentiation of naïve T cells to differentiate in helper type 2 T cells (Th2).[4]

Besides the internalization of the allergen by APCs, allergens can also activate the epithelial cells in the skin and endothelial cells in gut and lung tissue. Activation stimulates the production and secretion of IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). These cytokines are also called alarmins. The secretion of alarmins activates innate lymphoid cells (ILCs) type 2 (ILC2) and creates an inflammatory environment where APCs process allergens. ILC2 activation stimulates the secretion and production of typical Th2-promoting cytokines (IL-4, IL-5, and IL-13). Mainly, IL-5 and IL-13 play a role in the environment of antigen processing by DCs.[1,4]

After the DCs have processed the allergen, they upregulate the expression of CCR7, which enables them to move (home) towards lymphoid tissues. In the lymphoid tissue, DCs will activate

naïve T cells and differentiate the naïve T cells into subsets of T cells. The naïve T cells in food allergy will primarily differentiate into Tfh or Th2 cells due to the IL-4 produced by ILC2s, mast cells, and basophils.[3] The Th2 cells are significant players in the secretion of IL-4, IL-5, and IL-13 (similar to ILC2). These cytokines can activate mast cells and basophils (but cannot degranulate them). IL-4 is essential for this, but IL-13 can also activate them.[3,5]

The cytokines IL-4 and IL-13 promote the class-switching of B cells, enabling the B cells to produce specific IgE to the allergen. IL-4 and IL-13 also increase tissue migration of Th2 cells. Besides the effects of cytokines from Th2 cells on B cells, a different subset of Th cells, Tfh cells, provide stimulatory signals to B cells in the germinal centers of the lymph nodes through cytokines and costimulatory molecules.[4]

After Tfh cells activate B cells and B cells differentiate into IgE plasma cells, IgE for the specific allergens (sIgE) is secreted. This sIgE will be bound by the FcεRI receptor on basophils and mast cells. The FcεRI is a high-affinity IgE receptor. Basophils and mast cells with bound IgE are "sensitized" and ready to react following an allergen encounter.

The effector phase is triggered by re-ingesting the offending food. Allergen exposure will lead to the crosslinking of IgE bound on basophils and mast cells with the allergen, resulting in cell activation. Activation of mast cells and basophils through IgE degranulates the cell, and degranulation releases pre-stored mediators (histamine, heparin, proteases, and cytokines), together with newly formed prostaglandins, leukotrienes, and adenosine nucleotides.[4] The mediators released by basophils and mast cells cause food allergy symptoms, such as vasodilation, smooth muscle contraction, and mucus production. IgE binding also upregulates FcεRI, leading to more sensitivity to allergens.[4]

For a long time, it has been a consensus that long-lived memory B cells were the primary source of higher IgE titers in food allergy. To keep the sIgE levels high enough, these cells need continued allergen exposure and CD4+ T cell interaction. On the other hand, recent research showed that depletion of these memory B cells did not lead to lower levels of sIgE. This suggests that another B cell subset could be responsible for the high sIgE titer in food allergies. Although research on it is still lacking, a long-lived IgE+ plasma cell is thought to be responsible for the high sIgE levels.[1]

Unfortunately, only theories exist on how this altered immune response develops. One of these theories is the hygiene hypothesis, which suggests that decreasing exposure to microbes during early life affects the development of the immune system.[6] Lower microbial exposure can be due to higher hygiene standards and cesarean section delivery.[7] This less-developed immune system cannot initiate sufficient immunoregulatory responses to food allergens for tolerance but instead initiates an adverse immune response. A different theory is the modern diet hypothesis, which suggests that the modern diet, which is rich in (highly) processed foods, contributes to an inflammatory immune state and thus increases susceptibility to food allergies.[6] Nevertheless, food allergy's actual cause(s) are still unknown, and only hypotheses exist. This emphasizes the need for even more research on food allergies.

Food allergy prevalences have increased over the last few decades, particularly in developed countries. While exact global numbers are unknown, Bartha et al. (2024) estimated that food allergies affect approximately 8-10% of individuals in developed countries, with lower numbers in less-developed countries.[4,8] This regional difference in prevalence suggests that environmental and lifestyle factors (e.g., nutrition) might play a role in the development of food allergies.

The increasing prevalence and the social burden of food allergy emphasize the need for proper diagnosis of food allergy. In 2023, The European Academy of Allergy and Clinical Immunology (EAACI) released updated guidelines for diagnosing food allergy. The first step in the revised guidelines is an allergy-focused clinical history analysis. Diagnostic tests for IgE sensitization follow the history analysis in the form of a skin prick test or blood testing.[4]

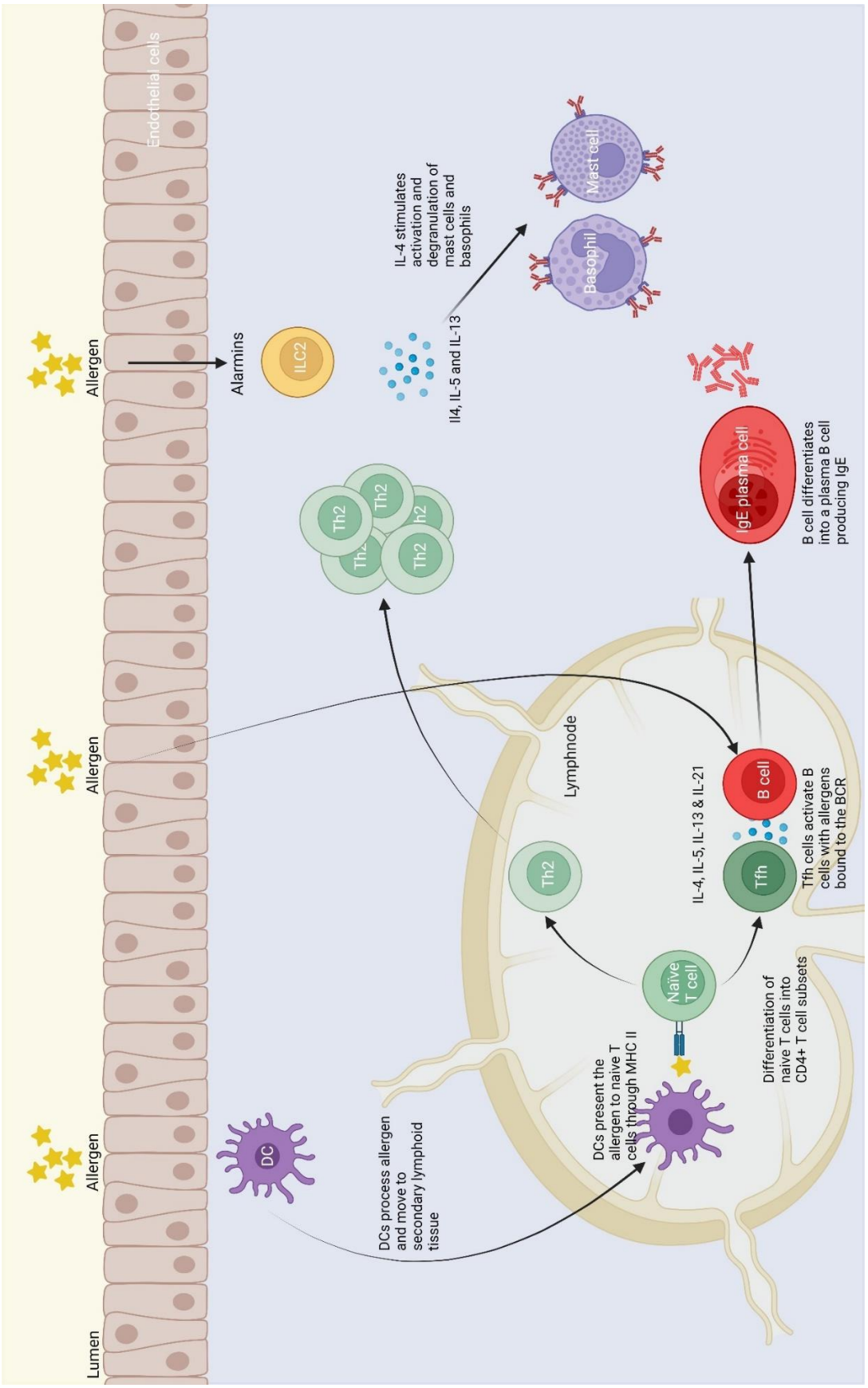

Figure 1. Mechanism of IgE-mediated food-allergic immune response.
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IgE sensitization tests come in multiple sorts: the skin prick test (SPT), IgE measurement, and the Basophil Activation Test (BAT). The SPT is a common test in which mast cells and basophils are activated in the skin with allergens that are "pricked" in the skin.[9] The SPT has limitations, like variable accuracy (especially for peanut and hazelnut allergens). IgE-sensitization with these allergens does not always correlate with clinical manifestations of food allergy. When the conventional cut-off value of >3 mm is used, inconclusive results are yielded.[10]

Measuring IgE can be done by measuring total or specific IgE (sIgE).[4] IgE testing also has limitations. Higher levels of IgE do not always correlate with more severe reactions, and false positive results may occur. Even more importantly, high levels of IgE might correlate with chronic allergies instead of an acute allergic reaction. Similarly, low levels of IgE do not correlate with clinical outcomes; patients can still be at risk for anaphylaxis. Serum IgE testing only tests free IgE in blood but does not measure bound IgE on basophils or mast cells.[1]

The BAT is an in vitro test to measure IgE sensitization. The BAT measures the expression of activation markers on the surface of blood basophils. By incubating basophils with allergens, crosslinking the sIgE (bound on the basophils) with the allergens occurs, activating the basophil. When basophils are activated after crosslinking, they increase the expression of their activation marker, CD63. With flow cytometric analysis, the percentage of basophils that express CD63 is measured. When the number of basophils that express CD63 is above a threshold, it can be seen as a positive BAT showing IgE sensitization.[11,12]

The BAT also has limitations. One is the time between blood collection and the test's performance. A more extended period between these two correlates with a decrease in reactivity. Also, medication is a limitation for the BAT: oral steroids should be avoided for three weeks before the test. Materials used in the BAT can also affect the test's results.[12]

When the earlier-mentioned tests fail to give a conclusive diagnosis for a patient, EAACI guidelines recommend the Oral Food Challenge (OFC). During the OFC, patients with a suspected food allergy are challenged by the alleged allergen in increasing amounts. If a patient shows a reaction, food allergy is diagnosed. Patients are thought to be tolerant if no reaction occurs during the provocation. Similarly, as with the other diagnostic tests, the OFC also has limitations. It is a troublesome test that requires trained professionals and should only be performed in appropriate settings due to potential anaphylactic reactions. This is, of course, a very intensive test, both for the healthcare providers and patients.[4]

The unknown reasons for developing food allergy, in combination with the shortcomings of the diagnostic tests, show the need for new biomarkers in food allergy. Fortunately, much research has been conducted and is still being done on the immunological differences in food allergies. Recent research on T cell subsets has identified allergen-specific T cells in allergic individuals, which are practically absent in healthy individuals.[13] Other research has also identified a Tfh subset responsible for producing high-affinity IgE to allergens that correlate with anaphylaxis.[14]

This report aims to identify potential biomarkers correlating with the severity of food allergies. To achieve this, a comprehensive literature study will be performed to explore previously reported biomarkers and their relevance in severity prediction. Building on the foundation of the literature study, a flow cytometric panel will be designed and optimized through antibody titration to target the biomarkers effectively. The functionality of the panels will be tested to evaluate their reliability and applicability in future studies. Additionally, the basophil activation tests will be performed to investigate the potential of this test to predict the severity of food allergies.

2. Methods

2.1 Literature review

A systematic literature study was conducted to identify potential novel biomarkers for food allergies. Searches on electronic databases, including but not limited to PubMed and Google Scholar, were performed. The search strategy was tailored to include, but not limited to, combinations of the following keywords: “food allergy,” “biomarkers,” "diagnosis," "immunological markers," "allergic sensitization," "serological markers," "genomic markers," and "metabolomic markers." Boolean operators (AND, OR) were used to refine the search. The search focused on studies published from 2019 until today, but older papers were not disregarded, and no restrictions were placed on the type of food allergen. Studies that concentrated on animals were avoided whenever possible.

2.2 Peripheral Blood Mononuclear Cell collection

2.2.1 Peripheral Blood Mononuclear Cell (PBMC) collection

PBMCs were obtained from healthy volunteers of the Minidonordienst (MDD) of the University Medical Centre Utrecht to use as healthy controls. Informed consent from these donors was part of their participation in the MDD. PBMC isolation occurred through a density gradient solution with Ficoll Paque. In short, blood was diluted and pipetted on top of the density gradient. After centrifugation, the PBMC layer was pipetted and washed twice before cryopreservation.

2.3 Panel design

2.3.1 Antibodies

Antibodies that were used to measure the chemotaxis of T cells were FITC-conjugated anti-CD69 (clone FN50, ThermoFisher Scientific), NovaFluor Blue 660-120s-conjugated anti-CD3 (clone SK7, ThermoFisher Scientific), PE-conjugated anti-CCR4 (clone D8SEE, ThermoFisher Scientific), NovaFluor Yellow 730-conjugated CD4 (clone SK3, ThermoFisher Scientific), Alexa Fluor 700-conjugated anti-CCR10 (*314305*, R&D Systems), Alexa Fluor 647-conjugated anti-CD29 (clone TS2/16, BD Biosciences), PE-Cy5-conjugated anti-CD25 (clone M-A251, BD Biosciences), PE-Cy5-conjugated anti-CD134 (ACT35, BD Biosciences), Brilliant Violet 421-conjugated anti-Ki-67 (clone B56, BD Biosciences), Brilliant Violet 510-conjugated anti-CCR3 (clone 5E8, BD Biosciences), Brilliant Violet 605-conjugated anti-CLA (clone HECA-452, BD Biosciences), Brilliant Violet 650-conjugated anti-CXCR4 (clone 12G5, BD Biosciences), Brilliant Violet 786-conjugated anti-Integrin Beta 7 (clone FIB504, BD Biosciences), PE-Dazzle 594-conjugated anti-CCR9 (clone L053E8, BioLegend) and PE-Cy7-conjugated anti-CD49d (clone 9F10, BioLegend).

Antibodies that were used to measure Th2A cells and Tfh cells were FITC-conjugated anti-CD69 (clone FN50, ThermoFisher Scientific), NovaFluor Blue 660-120s-conjugated anti-CD3 (clone SK7, ThermoFisher Scientific), Alexa Fluor 700-conjugated anti-CD27 (clone O323, ThermoFisher Scientific), eFluor 780-conjugated viability dye, PE-conjugated anti-CD161 (clone HP-3G10, ThermoFisher Scientific), PE-Cy5-conjugated anti-CRTH2 (clone BM16, ThermoFisher Scientific), NovaFluor Yellow 730-conjugated anti-CXCR5 (clone MU5UBEE, ThermoFisher Scientific), PE-Cy7-conjugated anti-CD49d (clone 9F10, BioLegend), Brilliant Violet 421-conjugated anti-PD-1, eFluor 506-conjugated anti-CD4, Super Bright 600-conjugated anti-CCR6, Super Bright 645-conjugated anti-CD45RO, PE-Cy7-conjugated anti-CD49 (BioLegend). The antibodies were titrated on frozen PBMCs.

The antibodies for CD69 and CD274 (PD-1) were titrated on stimulated cells with anti-CD3/anti-CD28 beads in a bead-to-cell ratio of 1:5 for 24 hours. PBMCs were seeded at 300.000 cells per

well. The CD25, OX40, and Ki-67 antibodies were titrated on stimulated cells using soluble anti-CD3/anti-CD28. A 96-well plate (round-bottom) was coated with 1 ug/mL anti-CD3, and cells were added with 4 ug/mL anti-CD28. Stimulation was done for 48 hours. All other antibodies were titrated on non-stimulated cells.

2.3.2 Flow cytometry

Cryopreserved PBMCs were thawed and washed twice before blocking with human serum. Cells were stained with extracellular antibodies at 37°C (5% CO2) for 60 minutes. Cells were washed twice before staining with Fixable viability stain eFluor 780 (ThermoFisher Scientific). Cells were then fixed before permeabilization with saponine. After permeabilization, cells were stained with Ki-67 and washed. Stained cells were measured on a BD Fortessa, and further analysis was conducted using FlowJo V10.8.1.

Cells were gated according to their homing potential to the skin (CD29+, CD49d+, CLA+, CCR4+, and CCR10+), gut mucosa (CD29+, CD49d+, CCR9+, CXCR4+, and Integrin B7+), or lung mucosa (CD29+, CD49d+, and CCR3+).

A gating strategy was derived from the Wambre et al. (2017) paper for the Th2A cells.[13] Th2A cells were defined as CD3+CD4+CD45RO+CD27-CRTh2+CD161+CD49d+. Tfh cells were defined as CD3+CD4+CCR7-CXCR5+PD-1+.

2.4 Chemokine receptor expression

Frozen PBMCs were thawed and seeded at a concentration of 250.000 cells/well to be stimulated with anti-CD3 and anti-CD28 in multiple concentrations (0.5 ug/mL anti-CD3 with 2 ug/mL anti-CD28 or 1 ug/mL anti-CD3 with 4 ug/mL anti-CD28) or rested for 24 or 48 hours. 500.000 cells were stained as described above and measured. A compensation matrix was calculated with FlowJo V10.8.1.

2.5 Allergen-specific T cells and follicular helper T cells after stimulation

Frozen PBMCs were thawed and stimulated with 1 ug/mL anti-CD3 and 4 ug/mL anti-CD28 for 24 hours in an anti-CD3-coated round bottom 96 well-plate. After 24 hours, cells were stained and measured as described above. Analysis was done with FlowJo V10.8.1.

2.6 Basophil Activation Test

Whole blood was collected in sodium heparin tubes from patients with a suspected peanut or hazelnut allergy from the UMCU Department of Dermatology/Allergology who underwent an OFC. Cells were incubated with corresponding allergens or controls. Cells were stained with PerCP-Cy5.5-conjugated anti-CD193 (clone 5E8), PE-conjugated anti-CD63 (clone MEM-259), APC-conjugated anti-CD203c (clone NP4D6) and FITC-conjugated CD123 (clone 6H6). Cells were lysed and washed before measuring on a BD Canto. Analysis was performed with FlowJo V10.8.1.

3. Literature study

3.1 Chemotaxis of immune cells

Chemotaxis is the process by which cells move throughout the body. This plays a central role in immune surveillance and response. This movement relies on signaling molecules known as chemokines, which interact with specific chemokine receptors on cell surfaces. The binding of chemokines to their corresponding G protein-coupled receptors (GPCRs) triggers intracellular signaling cascades through G-proteins, which lead to cytoskeletal reorganization, forming a "front" and "back" to orient cell movement along the chemokine gradient. This cytoskeletal rearrangement enables the cell membrane to extend toward the chemokine source. Table 1 shows the chemokine receptors and their chemokine ligands. Table 1 also indicates which cells express the chemokine receptor.[15,16]

During migration toward the chemokine gradient, immune cells form temporary adhesions with integrins on endothelial cells, which allows them to "roll" along the vessel walls. The activation of chemokine receptors also activates integrins on the cell surface, inducing conformational changes that strengthen adhesion and enable cells to stop rolling, promoting their subsequent transmigration across the endothelium into tissues.[17]

Most lymphocytes, including T cells, reside in peripheral tissues. Evidence from animal models has indicated that the site of initial immune activation influences tissue-specific homing in memory T cells. Unlike short-lived effector T cells, which can migrate through multiple tissues, memory T cells persist in peripheral tissues, offering long-term protection at sites of previous immune activation. This means most T cells will reside within tissue and not migrate through the body.[18]

DC activation mainly sets this site of initial immune activation. The local environment regulates their contribution to tissue-specific T-cell homing. Cues in different tissues imprint DCs to activate naïve T cells that will express certain tissue-homing markers. DCs in the gut-associated lymphoid tissue (GALT) can convert vitamin A to retinoic acid, which induces the gut-homing receptors α4β7 and CCR9 on T cells.[17] Conversely, skin-resident DCs metabolize vitamin D3 to its active form, 1,25-dihydroxyvitamin D3, promoting CCR10 expression and T cells' skin migration while suppressing gut-homing receptor expression.[17,18]

Different chemokine receptors and integrins mediate T-cell migration to specific tissues. CCR9 (not for colon homing), CXCR4, VLA-4 (integrin a4 and integrin b1), LFA-1 (integrin αL and integrin ß2), CCR6 and α4β7 are crucial for the homing of T cells to the intestine and colon. For skin-homing, CCR10, VLA-4, CCR4, LFA-1, and Cutaneous Lymphocyte Antigen (CLA) facilitate migration to the skin. For lung-homing, LFA-1, CCR3, CCR4, CXCR4, and VLA-4 are mainly responsible for the homing of cells (see Fig 2).[17] In addition to these receptors/integrins, more exist that are thought to play a role in cell migration. For example, CCR8 is found on T cells in healthy skin, leading to the thought that it is a marker for skin-homing.[18] CD43E is also believed to be a homing marker for skin-homing, and selectin ligands are also considered homing markers for gut and lung homing.[17]

While CCR10 is induced by Vitamin D, conditions that induce CCR8 and CLA fail to induce CCR10 expression. This supports the idea that CCR8 may regulate the localization of resident memory T cells (TRM) in the skin. At the same time, CCR10 mediates the migration of active effector T cells during inflammation.[18]

In food allergies, immune cells exhibit different homing properties depending on their phenotype. For instance, peanut-allergic patients have been observed to possess peanut-specific T effector cells with either an allergen-specific (CRTH2+) or a Th17-associated (CCR6+) phenotype. In a study by Calise et al., these two subsets exhibited contrasting homing potentials: allergen-specific cells were associated with systemic type 2 inflammatory responses and showed minimal expression of skin-homing receptors like CLA. In contrast, CCR6+ Th17 cells displayed elevated CLA expression, suggesting a greater potential for skin-homing.[19]

In contrast, similar research in peanut-allergic infants indicates that peanut-specific T effector cells show a higher prevalence of skin-homing markers like CLA, whereas, in non-allergic children, T-cell homing markers are more balanced between skin and gut homing.[20]

Chemokine receptors are one part of the whole process of homing, but chemokines (chemoattractant cytokines) are a different part of the process. Chemokines are secreted by different cells and bind to their receptors, which are expressed in all types of cells (see Table 1). Chemokines are important in recruiting immune cells towards the site of inflammation. Still, they are also involved in the development of lymphoid organs and, of course, the migration of DCs to lymphoid tissues (CCL17 and CCL22).[5]

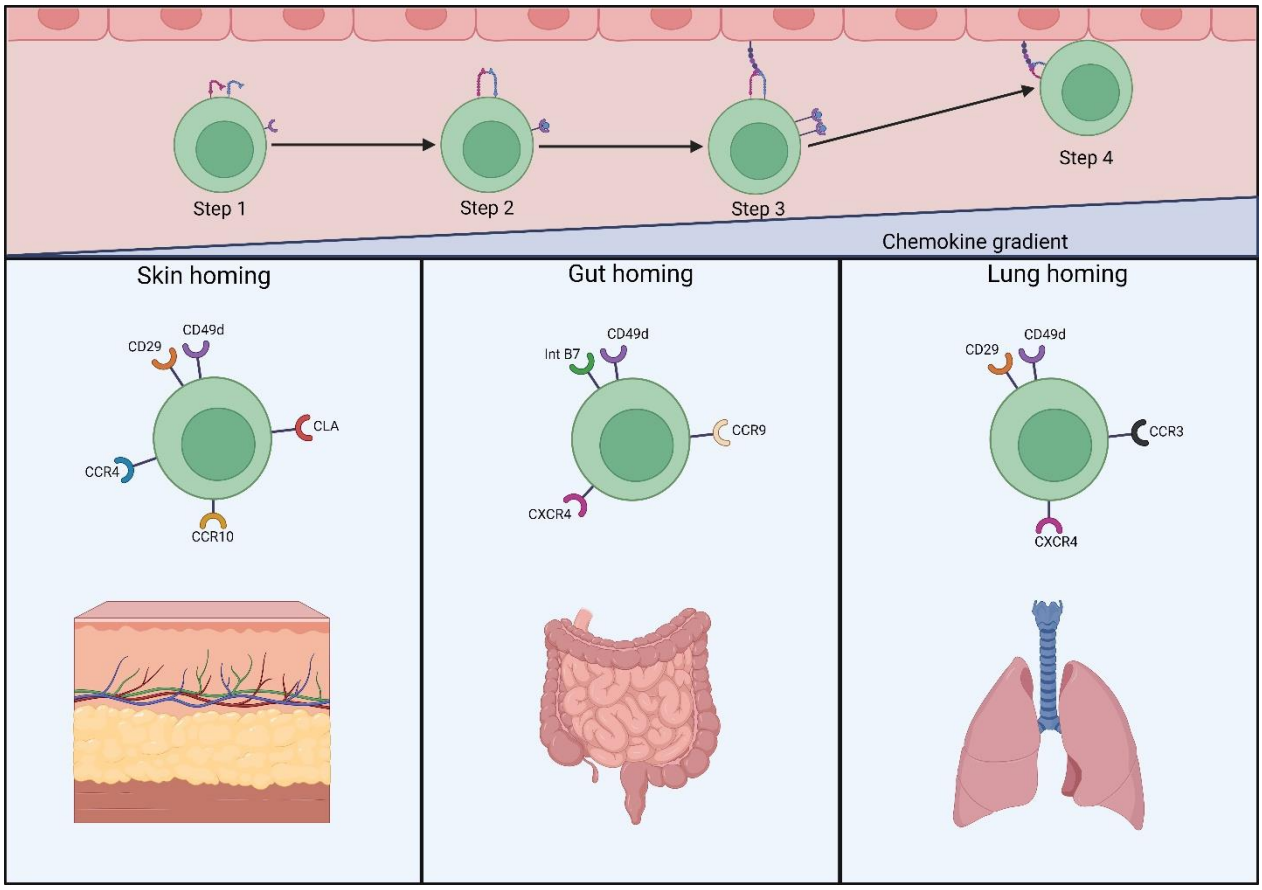

Figure 2. Process of chemotaxis.
Step 1: Cells flow/roll through blood vessels and express low levels of chemokine receptors and integrins. Following the chemokine gradient, cells bind chemokines to their corresponding receptors. Step 2: The integrins become activated and transformed after binding a chemokine on the chemokine receptor. Step 3: the integrins bind to their ligands bound to the endothelial cell wall. Step 4: Cells arrest on the endothelial cell wall and transmigrate into tissues. The expression of specific markers enables a cell to home towards either skin, gut, or lung tissue.

Table 1. Chemokine receptors and their chemokine ligands.[16,21–23]

Chemokine receptor	Expressed by	Chemokine ligands
CCR1	B cells, basophils, mast cells & DCs	CCL1
CCR2	B cells, memory CD4+ T cells, basophils & DCs	CCL2, CCL3, CCL3L1, CCL4, CCL7, CCL8 & CCL13
CCR3	Memory CD4+ T cells, basophils & mast cells	CCL3, CCL3L1CCL4
CCR4	Memory CD4+ T cell, ILC2 & mast cells	CCL5, CCL7, CCL8, CCL13 & CCL16
CCR5	B cells, memory CD4+ T cells, basophils, mast cells & DCs	CCL5
CCR6	B cells, memory CD4+ T cells, basophils & mast cells	CCL11, CCL14, CCL15, CCL17, CCL20, CCL22 & CCL24
CCR7	B cells, naive CD4+ T cells, memory CD4+ T cells, ILC2 & DCs	CCL17 & CCL22
CCR8	Memory CD4+ T cell & ILC2	CCL19
CCR9	B cells, ILC2 & DCs	CCL25
CCR10	Memory CD4+ T cells	CCL26, CCL27 & CCL28
CXCR1	Basophils & mast cells	CXCL1, CXCL2, CXCL6 & CXCL8
CXCR2	B cells, basophils & mast cells	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 & CXCL8
CXCR3	B cells, memory CD4+ T cells, mast cells & DCs	CXC9, CXCL10 & CXCL11
CXCR4	B cells, naive CD4+ T cells, memory CD4+ T cells, ILC2, basophils, mast cells & DCs	CXCL12
CXCR5	B cells and memory CD4+ T cells	CXCL13
CXCR6	Memory CD4+ T cell & ILC2	

3.2 T cells in immune response

T cells are a major cell component of the adaptive immune system and are responsible for cell-based adaptive immune responses. T cells can recognize antigens on MHC molecules that APCs present. The antigen presentation by APCs enables the T cell receptor (TCR) to bind to the allergen and, together with costimulatory molecules and cytokines, activate the naïve T cell. Activation of the T cell leads to clonal expansion and differentiation so the T cells can exert their effector functions (like killing infected cells, producing cytokines, and regulating the immune response. Besides CD4+ and CD8+ T cells, T cells can be divided into regulatory T cells (Tregs)

and natural killer T (NKT) cells.[5,24] While the CD4+ helper cells play a major role in the food allergy immune response, CD4+ T cells can also be divided into several subsets based on the expression of certain markers, transcription factors, and secretion of cytokines and chemokines (see Table 2).

Table 2. CD4+ T cell subsets[5,21,24]

Name	Defining markers	Cytokines and chemokines	Transcription factors
Th1	CCR1, CCR5, CXCR3	IFN-y, IL-2, TNF-a, TNF-b	STAT1, STAT4, T-bet
Th2	CCR3, CCR4, CCR8, CXCR4	IL-4, IL-5 and IL13	GATA-3, STAT-5, STAT-6, IRF4
Th9	CCR3, CCR6, CXCR3	IL-9, CCL17, CCL22	IRF4, PU.1
Th17	CCR4, CCR6, CCR10	IL-17, IL-21, IL-22, IL-26, CCL20	Batf, IRF4, STAT3, RORa, RORg
Th22	CCR4, CCR6	IL-10, IL-13, IL-21, IL-22, TNF-a	AHR, Batf, STAT3
Treg	CD5, CD25, CTLA4	IL-10, IL-35, TGF-b	FoxP3, Helios, STAT5
Tfh	CXCR4, CXCR5, ICOS, PD-1	IL-4, IL-10, IL-17, IL-21	Bcl-6, c-Maf, STAT3

3.3 Allergen-specific T cells

As already mentioned in the introduction, Th2 cells play a major role in the immune response to food allergy.[3] Food allergy is known to be associated with a skewed cytokine profile in favor of Th2 cytokines IL-4, IL-5, and IL-13. In 2012, Wambre et al. studied the characterization of CD4+ T cell subsets in allergy and mentioned the existence of allergen-specific T cells both in healthy and atopic individuals.[25] In 2017, Wambre et al. continued to study allergen-specific T cells and characterized them through ex-vivo pMHCII tetramer-based T cell profiling. According to the results of this study, allergen-specific T cells are highly differentiated T cells that express CRTh2 (CD294), CD161, and a differentiation marker (either CD45RB or CD27). They named these allergen-specific T cells Th2A cells (Fig 3A).[13]

They also studied the cytokine secretion of these Th2A cells compared with other CD4+ T cell subsets (Th2 and Th1/Th17) and showed that, after ex vivo stimulation, Th2A cells had increased IL-5 and IL-9 expression. Th2A cells were also found to be more polyfunctional, producing more Th2 effector cytokines when compared to normal Th2 cells.[13] In contrast, a study by Makiya et al. in 2023 showed that pathogenic effector cells (CD3+CD4+CD27-CD49d+CRTh2+CD161+) have different cytokine excretion patterns. In food-allergic patients, the TH2A cells showed elevated IFN-y or IL-17A production, while in eosinophilic gastrointestinal disorder patients, the same characterized TH2A cells showed increased IL-5 and IL-13 production.[26] The reason for this discrepancy in cytokine production is still unknown.

Besides the characterization proposed by Wambre et al., TH2A cells can also be characterized by their expression of other surface molecules. The IL-33 receptor (ST2), the IL-25 receptor, and the TSLP receptor were all highly expressed on Th2A cells. These are receptors for the alarmins that are secreted after endothelial cells are activated by allergens. In addition, Th2A cells also seem to express genes that are involved in arachidonic acid signaling.[13] Arachidonic acid can be released from the phospholipid layer by phospholipase A2 (PLA2) and can be converted to PGH2

through cyclooxygenases 1 and 2. PGH2 is converted to prostaglandin 2 (PGD2) through HPGDS, and PGD2 is able to bind to and stimulate CRTh2. CRTh2 stimulation leads to the secretion of Th2 canonical cytokines IL-4, IL-5, and IL-13.[27] Interestingly enough, mast cells can release PGD2 after degranulation and thus stimulate TH2A cells. Note that TH2A cells produce IL-4, which stimulates basophil and mast cell degranulation.[28]

Calise et al. also found that the TH2A cells were mainly CD161+CD27-. They also studied the expression of ST2 and IL-17RB and found that both markers were upregulated only in TH2A cells. Calise et al. also analyzed the expression of PD-1 on TH2A cells and found that PD-1 is significantly higher expressed in TH2A cells. The PD-1 receptor shows T-cell exhaustion and poor survival rate after multiple antigenic stimulation. Cytokine analysis of the TH2A subset showed that these cells can immediately produce Th2 cytokines after short allergen stimulation. In addition, they showed that TH2A-high peanut-allergic patients had higher sIgE titers. This correlation can be connected by cytokine secretion that stimulates B cell activation and differentiation to plasma cells, producing sIgE.[19]

These results were in accordance with earlier research by Calise et al., which showed that the expression of ST2 was attributed to allergic individuals and restricted to TH2A cells. They also showed that the exposure of TH2A cells to IL-33 (alarmin) enhanced the secretion of IL-4 and IL-5.[29]

Research by Ruiter et al. (2022) showed that a positive outcome of oral immunotherapy against peanuts correlated with a decrease in Th2 signatures in TH2A-like cells.[30] Similarly, Wambre (2017) also found that a positive outcome of oral immunotherapy was correlated with decreased numbers of Th2A cells.[13] Although the numbers of TH2A cells decrease over time, they will persist, suggesting that TH2A cells arise from some sort of a precursor with a unique differentiation pathway. [25]

In summary, TH2A cells are a major player in food allergy and show enhanced secretion of Th2 cytokines. They are also practically absent in healthy individuals, showing their potential as a biomarker for food allergy. A downside of the TH2A cells is that they are allergen-specific T cells, meaning that, without allergen-specific stimulation, it is unknown if a patient is allergic to only one allergen.

3.3 Follicular helper T cells

The follicular helper T (Tfh) cell is a CD4+ T cell subset essential in forming germinal centers (GCs), the sites within secondary lymphoid organs where B cells undergo class-switching recombination to produce high-affinity antibodies. Tfh cells are also essential for promoting B cell differentiation into plasma cells and memory B cells. Tfh cells function through the secretion of cytokines like IL-4, IL-13, IL-17, IL-21, and IFN-y and interactions with co-stimulatory molecules like ICOS and CD40L.[31] For a long time, Th2 cells were thought to be the main drivers in IgE production since the deletion of Th2 lineage-defining transcription factors (such as STAT6 or GATA3) or IL-4 reduced the amount of IgE. However, evidence on the role of Tfh cells as the primary regulators of B cell differentiation has shifted the focus from Th2 cells to Tfh cells.[32]

Although IL-4 is a key cytokine and stimulatory factor for IgE production, it is insufficient to produce the high-affinity IgE needed for anaphylactic responses. Gowthaman et al. proposed that Tfh subsets are responsible for inducing different affinities of IgE. They hypothesized that IL-4 could induce switching of B cells to low-affinity IgE after type 2 immune responses but that a

different Tfh subset is needed to produce high-affinity IgE. Their research on mice found that a Tfh subset, Tfh13, produced IL-13 and IL-4 while simultaneously downregulating IL-21. Deletion of these cells or IL-13 led to the abrogation of high-affinity IgE production to allergens. They also researched their findings in a human cohort of peanut-allergic patients. They found that the allergic patients had increased sIgE to peanuts compared to healthy controls. In addition, they found that Tfh13 cells were identified (as CXCR5+CD40L+CD4+ IL4+IL-13+) in peanut-allergic patients but not in the healthy controls. [32]

More research on Tfh13 cells was conducted by Olle et al. They studied the role of the Tfh13 subset in patients with food allergies. They compared Tfh13 levels in food-allergic patients with a history of anaphylaxis to those with tolerance. The results demonstrated that anaphylactic patients had more Tfh13 cells identified as CD3+CD4+CD45RA-CXCR5+IL-4+IL-13+ (Fig 3B). In addition to the increased number of Tfh13 cells, they also found that the sera of anaphylactic patients yielded greater degranulating mast cells than the sera of the tolerant patients. They observed a correlation between the degranulation of mast cells (CD63+) and Tfh13 presence, suggesting a central role for Tfh13 cells in high-affinity production.[14]

In summary, the role of Tfh cells has become clearer over the last few years. Their role in the production of IgE, and especially the role of Tfh13 in high-affinity anaphylactic sIgE, shows that they are an interesting subset within the food allergic immune response.

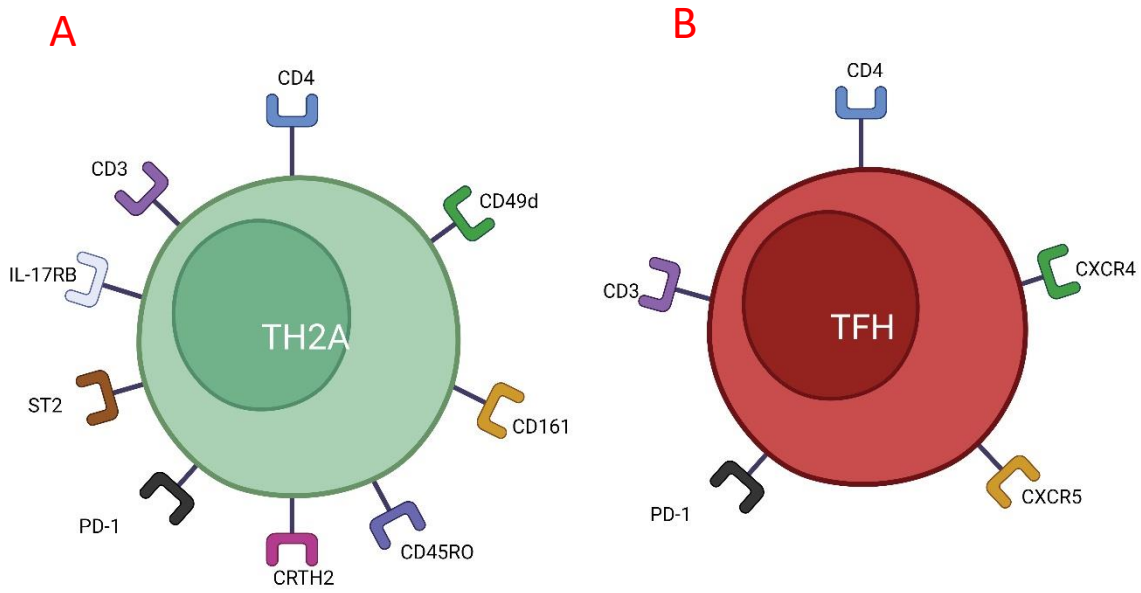

Figure 3. TH2A and Tfh cell identifying markers.[13,19,29]
Graphic representation of the different receptors identifying (A) TH2A and (B) TFH cells.

3.4 Marker choices

During the project, many markers were studied. Here, a short description of the marker's addition is shown.

CD3 is part of the T cell receptor (TCR) and, therefore, is a T cell-defining surface marker. With CD3 in the panel, the presence of T cells can be confirmed, enabling the identification of other T cell subsets.[5,24]

CD4 is a coreceptor expressed on T helper cells. As mentioned above, it is important in immune response, especially in the immune response seen in food allergy. CD4 is also needed to interact with naïve T cells and antigen-presenting cells. With CD4, it is possible to gate for subsets (in combination with other markers).[5]

CD25 (alpha chain of the IL-2 receptor) is an activation marker highly expressed on activated lymphocytes. It is a "long-term" activation marker, with optimal expression after 48 hours.[33] The goal of including this marker in the panel is to look at activated cells with allergen stimulation after a longer period. CD25 is a combinatory marker with OX40 (CD134) on the same fluorophore in the homing marker panel.

CD27 is a costimulatory receptor involved in activating lymphocytes, survival, and differentiation. CD27 expression can distinguish naïve T cells from memory T cells. Expression is lost after differentiation into effector cells. According to research by Wambre in 2017, CD27 is needed to identify TH2A cells. [13]

CD29 (Integrin ß1) is part of the VLA-4 complex and is important in the adhesion and migration of T cells. CD29 is used as a homing marker for both skin and lung homing.[17]

CD45RO is a marker for memory T cells. Like CD27, it can distinguish between naïve T cells and memory T cells. Expression of CD45RO is high after activation, while naïve T cells only express CD45RA. To identify TH2A cells, Wambre mentions the use of CD45RA, but the use of CD45RO is similar.[13]

CD49d, also known as integrin alpha 4, can form a complex with CD29 (Int ß1) to form the VLA-4 adhesion molecule for skin and lung homing. It can also form a complex with Integrin beta 7 to form the adhesion molecule α4ß7, which is responsible for the migration and adhesion of T cells to the gastrointestinal tract.[17]

CD69 is an activation marker and is expressed shortly after TCR activation. The expression of CD69 is highest around 24 hours after TCR activation. Using the marker can make a distinction between activated and non-activated cells.[33]

CD134(OX40) is, like CD25, OX40 is a "long activation" marker with optimal expression after 50 hours. OX40 is mainly associated with memory and effector T cells. According to Poloni et al., the combination of OX40 with CD25 is optimal for allergen-specific cell identification.[33]

CD161 is a Th17 marker. Nevertheless, multiple papers on allergen-specific T cells have shown that it is necessary to identify those cells.[13,19]

CD279 (PD-1) is an inhibitory receptor expressed on T cells after activation to prevent immune system overactivity. It is used to identify Tfh and TH2A cells from other CD4+ T cell subsets.[14,19,24]

CD294 (CRTh2) is a prostaglandin receptor highly expressed in allergen-specific T cells.[13,19,27]

CCR3 is a chemokine receptor associated with Th2 cells and can be used as a homing marker for T cell homing to lung mucosa.[34,35]

CCR4 is a chemokine receptor that attracts Th2 cells to sites of inflammation, particularly to skin tissues. High expression of CCR4 is also characteristic of Th2 cells.[17,21]

CCR6 is a chemokine receptor associated with Th17 cells and helps direct their migration to mucosal surfaces. It can be used as a negative selection marker for TH2A cells.[19]

CCR7 directs naive and central memory T cells to lymphoid tissues, where they encounter antigens and are activated. Including CCR7 in panels allows for differentiation between effector and central memory T cells. This is especially important when identifying Tfh cells.[24,32]

CCR9 is involved in the homing of T cells to the gut mucosa.[17]

CCR10 helps direct T cells to the skin. Its inclusion in a panel allows for the analysis of T cell trafficking to skin tissue, which is important for conditions like atopic dermatitis.[17]

CXCR3 is associated with Th1 cells and is relevant for tracking T-cell recruitment to inflamed tissues. Although mainly Th1-associated, CXCR3 can also be involved in modulating Th2 responses in allergic inflammation.

CXCR4 is a homing marker for T cell homing towards mucosal tissues (gut and lung).[17]

CXCR5 is essential for Tfh cells, directing them to follicles within lymphoid tissues. Including CXCR5 helps identify Tfh cells, which are key in supporting B cell activation and IgE production in allergies.[21]

Integrin B7 is a homing marker that, in combination with CD49d, forms the a4b7 complex that enables homing towards gut mucosal tissues. Expression is enhanced by Vitamin A on DCs.[17]

CLA is a homing receptor that guides T cells to the skin. Vitamin D on DCs can enhance the expression of CLA.[17]

Ki-67 is a nuclear marker of cell proliferation, indicating recently activated or dividing cells. Ki-67 helps measure T-cell activation and expansion in response to allergens.[36]

Viability dyes distinguish live cells from dead ones, essential for accurate flow cytometric analysis.

3.5 Receptor that did not make the panels

Many markers were not selected to be incorporated into the panel during the research. Below are some markers that could be included in other panels or that could replace other markers in the current panel if vendors produce the marker with a fitting fluorophore.

CCR8: CCR8 is a homing marker for skin tissue. T cells in atopic dermatitis have increased CCR8 expression.[37,38]

CD154: CD154 is an activation marker for short-term activation. It is also upregulated after peanut stimulation.[39]

ST2 (IL-33 receptor): ST2 is the IL-33 receptor that is highly expressed in TH2A cells. It can be used to identify TH2A cells but is hard to measure using flow cytometry.[13,19,27,40]

IL-17RB (IL-25 receptor): the IL-25 receptor is expressed on TH2A cells. [13,19,27,40]

CD45RB: CD45RB can be used similarly to CD45RO. It is a memory marker for TH2 cells. While CD45RO+ cells are memory cells, CD45RB+ cells are not memory markers.[13]

CCR2: CCR2 is a homing marker for the mucosa tissue in the lung. [34] It is also seen as a more general homing marker to sites of inflammation. [41]

Cytokines: cytokines can function as markers to identify certain subsets. For example, Th2 cells mainly secrete IL-4, IL-5, and IL-13. By analyzing these cytokines, one can distinguish Th2 cells from others. They have not been included in the panel due to insufficient space and the need for intracellular staining.

LFA-1: LFA-1 (a polymer of aLb2) can be a skin, gut, and lung-homing marker. It binds to ICAM-1. [17]

CD43E: CD43E can function as a homing marker for skin-homing. [17]

4. Results

4.1 Antibody titration

Antibodies were titrated to find the lowest concentration with the best separation between the positive and the negative population. All antibodies that were used in the designing of the panel were tested in 1:10, 1:20, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, and 1:5000 dilutions (with an exception for the Fixable Viability dye – eFluor 780, which was only done in a 1:500, 1:1000 and 1:2000 dilution based on previous experience in the department). The results of the titrations of the homing panel antibodies are shown in Fig 4 as concatenated figures. Fig 5 represents the concatenated figures of the antibodies' titrations in the TH2A/Tfh panel. The decision for a dilution was based on the concatenated files and the staining index.

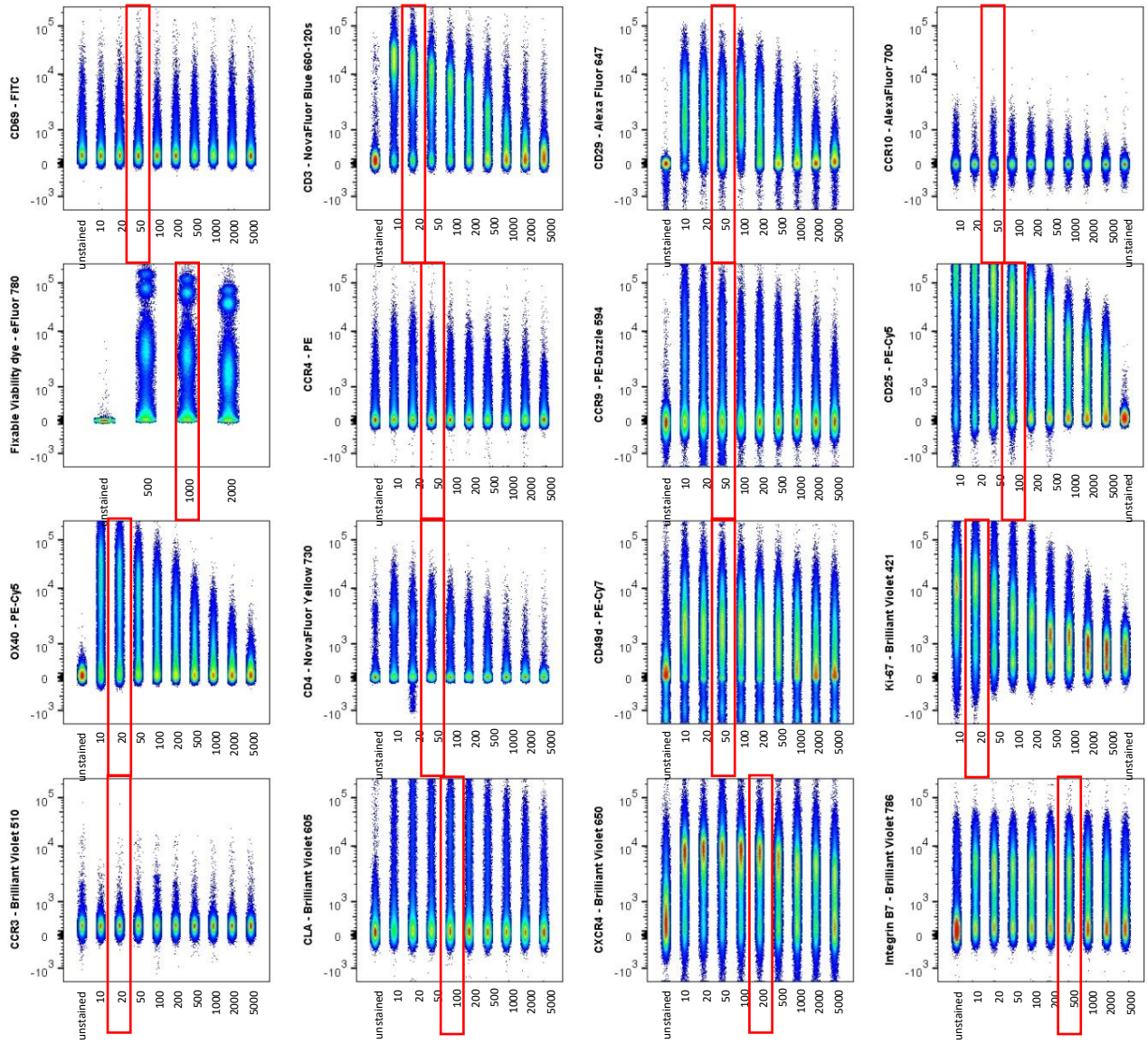

Figure 4. Antibody titrations for the homing panel.

Antibodies were titrated similarly as described in the material and method section. Cells for the following markers were stimulated with anti-CD3 and anti-CD28: CD69, CD25, Ki-67 and OX40.

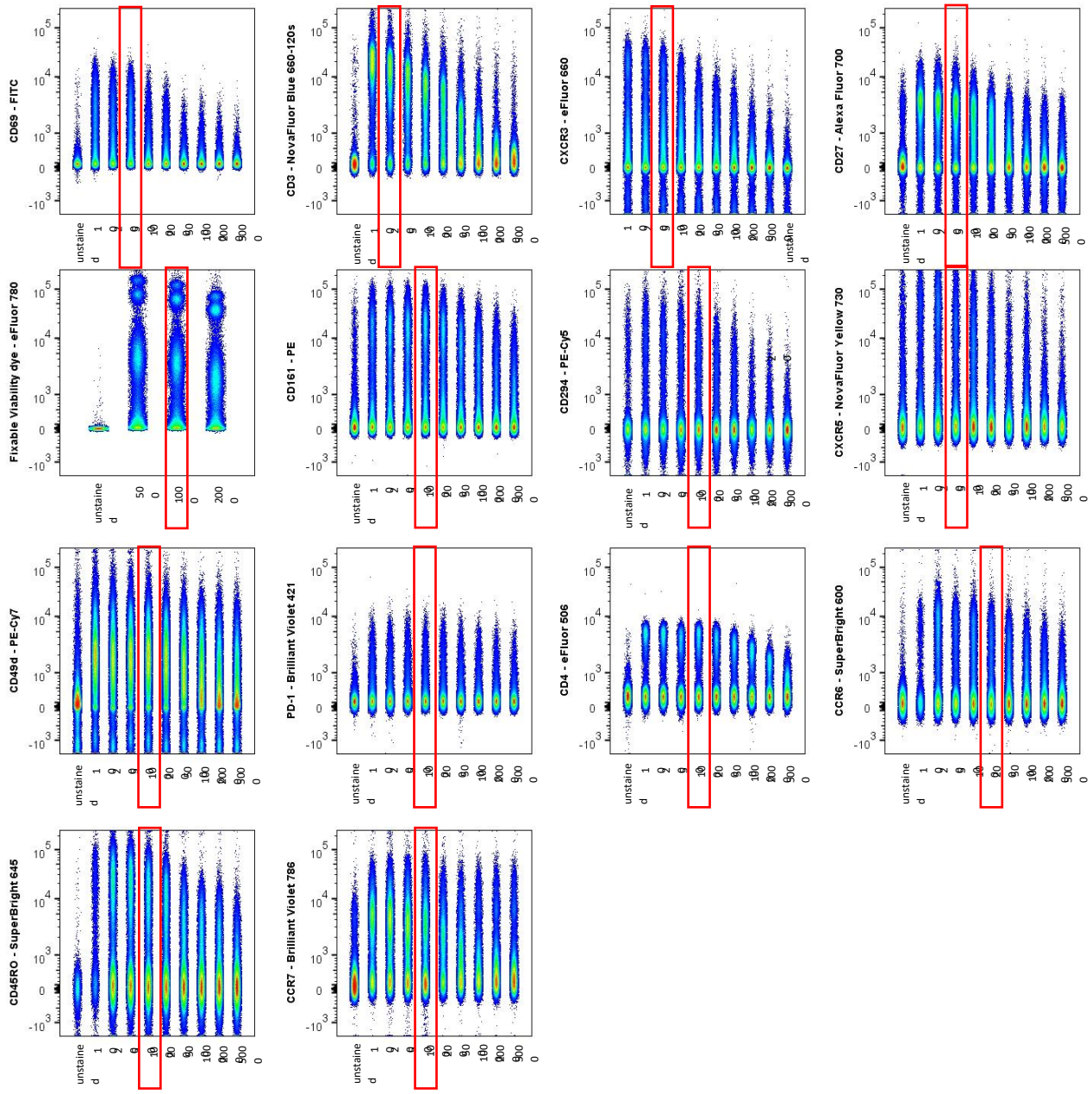

Figure 5. Antibody titrations for the TH2A/Tfh panel.

Antibodies were titrated similarly as described in the material and method section. Cells for the following markers were stimulated with anti-CD3 and anti-CD28: CD69.

Table S1 shows an overview of the antibodies used in the homing panel with the corresponding dilutions, clones, hosts, isotypes, vendors, and catalog numbers. Table S2 shows the complete overview of the TH2A/Tfh panel.

4.1.1 CCR3

CCR3 has been troublesome during the development of the homing panel. With the first titration, no clear or separate CCR3-positive population existed. During a second titration on PBMCs from a different donor, still, no population existed that expressed CCR3. This led to the thought of a non-working antibody or clone. An Optimized Multicolor Immunofluorescence Panel (OMIP) used the exact same antibody(clone) from the same vendor. The only difference was that in the OMIP, they used fresh blood, while we had only used previously frozen PBMCs. A small test with fresh

blood showed that the antibody is working, but apparently not on previously frozen PBMCs.[42] Fig S1A shows the expression of CCR3 on unstimulated frozen PBMCs (1:20 dilution). Fig S1B shows the CCR3 expression of anti-CD3/anti-CD28 (1 ug/mL and four ug/mL, respectively) stimulated frozen PBMCs (1:20 dilution). Figures S1C and S1D show the CCR3 expression of fresh blood and stimulated fresh blood, respectively. These results show that the antibody is working but that frozen cells cannot express CCR3.

4.2 Homing marker expression

To study the expression of homing markers on T cells, a flow cytometric panel was designed to target specific homing markers on T cells for skin, lung, and gut tissue. Gut homing of T cells was attributed to T cells with a CD49d+integrin B7+CCR9+CXCR4+ phenotype. Skin homing was attributed to CD49d+CD29+CLA+CCR4+CCR10+ T cells. Lung homing was attributed to CD49d+CD29+CXCR4+ phenotypic T cells. The expression of the homing markers was studied in three different conditions: 24-hour stimulation with 0,5 ug/mL anti-CD3 and 2 ug/mL anti-CD28, 24-hour stimulation with 1 ug/mL anti-CD3 and 4 ug/mL anti-CD28, and 48-hour stimulation with 1 ug/mL anti-CD3 and 4 ug/mL anti-CD28.

Flow cytometric analysis was done on all conditions using a stepwise gating strategy to identify the homing capacity of different tissues in T cells. Initial gating starts with the identification of lymphocytes through size exclusion (FCS-A vs SSC-A). Doublets are excluded by single cell identification (FSC-A vs FSC-W), and dead cells are excluded by viability dye. CD3+CD4+ T cells were gated (Fig 6A). Homing of T cells to gut mucosal tissue was done by gating on CD49d+Integrin B7+ cells, followed by gating of CCR9+CXCR4+ T cells. Skin homing was identified by gating on CD49d+CD29+ T cells, followed by CLA+ and CCR4+CCR10+ T cells. Lung homing T cells were gated by CD49d+CD29+ T cells followed by CXCR4+ T cells (Fig. 6B, C, and D). CCR3 was not included in the initial gating strategy; see section 4.1.1

To study the effects of different stimulating conditions on the expression of homing markers, we compared the percentage of activated T cells expressing gut, skin, and homing markers (Fig. 7). Fig 7A shows the difference between 0,5 ug/mL anti-CD3 and 2 ug/mL anti-CD28 stimulation in comparison to 1 ug/mL anti-CD3 and 4 ug/mL anti-CD28 stimulation for 24 hours. Gut-homing T cells increased from 1.23% of total CD69+ T cells to 1.48% with higher concentration stimuli. Skin homing T cells more than doubled (0.71% for low concentration vs 1.64% for high concentration stimulation). Lung homing T cells decreased from 61.56% to 56.32 with higher concentration stimuli.

In Fig 7B, the expression of homing markers was compared when stimulated with 1 ug/mL anti-CD3 and 4 ug/mL anti-CD28 for 24 or 48 hours. The percentage of CD69+ T cells that express gut homing markers increased from 1,48% of the total CD69+ T cells with short stimulation to 8.44% after long stimulation. Skin homing changed from 1.64% to 2.18%, while the percentage of activated T cells that express lung homing markers increased from 56.32% to 65.40%.

We also looked at the expression of homing markers between short-term and long-term activated T cells (CD69+ vs CD25/OX40+) after 48-hour stimulation with 1 ug/mL anti-CD3/anti-CD28 and compared them to cells that rested for 48 hours. Fig 7C shows that the expression of gut homing markers in short-term activated T cells is comparable to long-term activated T cells (8.44% vs 9.22%), while the expression of gut homing markers is low in resting cells (0.29%). Similar results are seen for lung homing (65.40% vs 73.73% vs 54.99%, respectively). Skin homing was lowest for the resting cells (0.43%) but highest in the CD69+ activated T cells (2.18%). 1.89% of the CD25/OX40+ T cells expressed skin-homing markers.

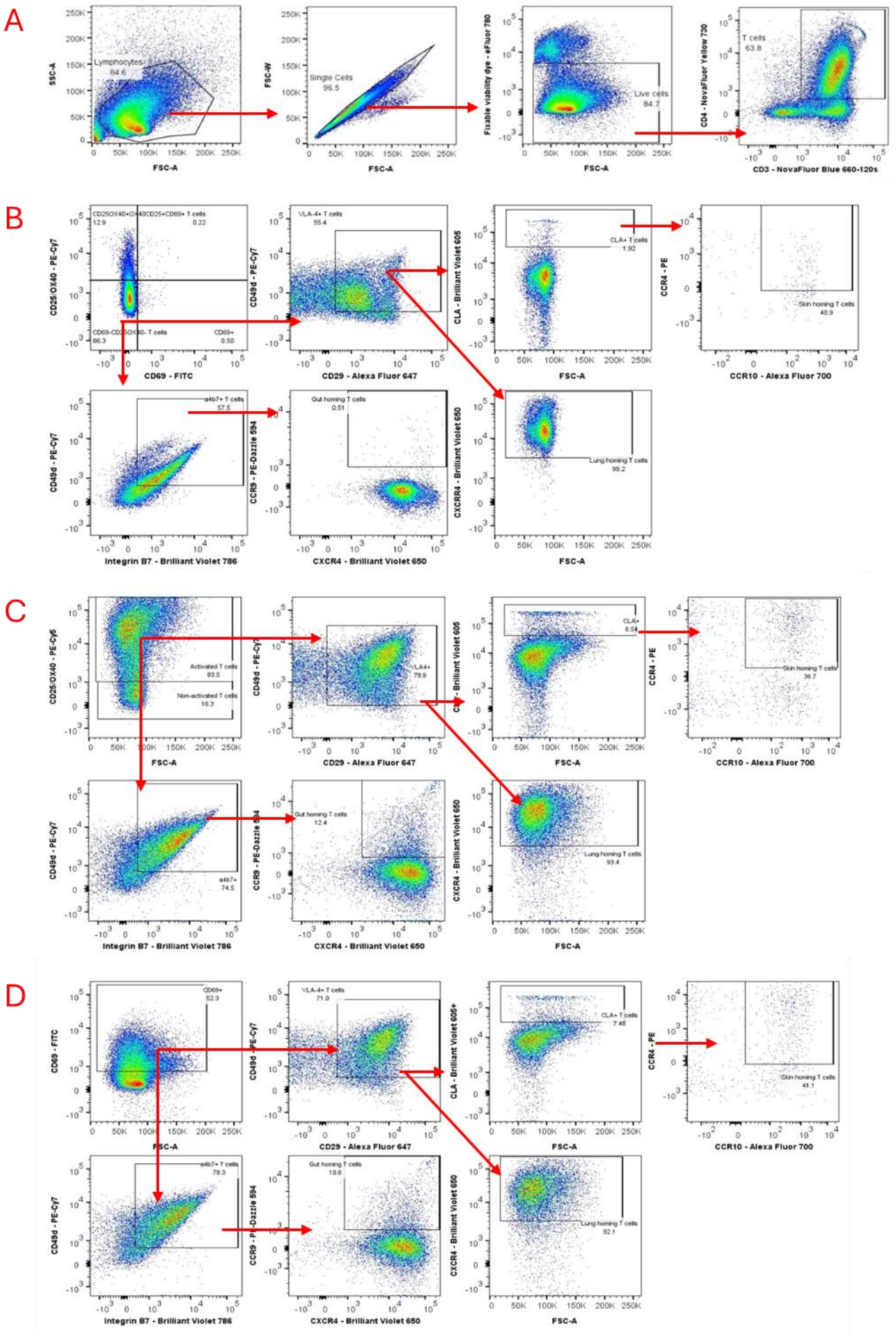
A
Lymphocytes
84.6
SSC-A
FSC-A
Single Cells
96.5
FSC-W
FSC-A
Fixable viability dye - eFluor 780
Live cells
84.7
FSC-A
T cells
63.8
CD4 - NovaFluor Yellow 730
CD3 - NovaFluor Blue 660-120s
B
CD25/OX40+OX40CD25+CD69+ T cells
12.9
0.22
CD69-CD25/OX40- T cells
86.3
CD69+
0.50
CD25/OX40 - PE-Cy7
CD69 - FITC
VLA-4+ T cells
55.4
CD49d - PE-Cy7
CD29 - Alexa Fluor 647
CLA+ T cells
1.92
CLA - Brilliant Violet 605
FSC-A
Skin homing T cells
40.9
CCR4 - PE
CCR10 - Alexa Fluor 700
a4b7+ T cells
57.5
CD49d - PE-Cy7
Integrin B7 - Brilliant Violet 786
Gut homing T cells
0.51
CCR9 - PE-Dazzle 594
CXCR4 - Brilliant Violet 650
Lung homing T cells
99.2
CXCR4 - Brilliant Violet 650
FSC-A
C
Activated T cells
83.5
Non-activated T cells
16.3
CD25/OX40 - PE-Cy5
FSC-A
VLA4+
78.9
CD49d - PE-Cy7
CD29 - Alexa Fluor 647
CLA+
6.54
CLA - Brilliant Violet 605
FSC-A
Skin homing T cells
36.7
CCR4 - PE
CCR10 - Alexa Fluor 700
a4b7+
74.5
CD49d - PE-Cy7
Integrin B7 - Brilliant Violet 786
Gut homing T cells
12.4
CCR9 - PE-Dazzle 594
CXCR4 - Brilliant Violet 650
Lung homing T cells
93.4
CXCR4 - Brilliant Violet 650
FSC-A
D
CD69+
52.3
CD69 - FITC
FSC-A
VLA-4+ T cells
71.0
CD49d - PE-Cy7
CD29 - Alexa Fluor 647
CLA+ T cells
7.48
CLA - Brilliant Violet 605+
FSC-A
Skin homing T cells
41.1
CCR4 - PE
CCR10 - Alexa Fluor 700
a4b7+ T cells
78.3
CD49d - PE-Cy7
Integrin B7 - Brilliant Violet 786
Gut homing T cells
10.8
CCR9 - PE-Dazzle 594
CXCR4 - Brilliant Violet 650
Lung homing T cells
92.1
CXCR4 - Brilliant Violet 650
FSC-A

Figure 6. Gating strategy of T cells expressing homing markers.
(A) Initial gating strategy of lymphocytes, single cells, live cells, and CD3+CD4+ T cells. (B) Homing gating strategy of cells that rested for 48 hours. (C) Homing gating strategy of T cells that are CD25/OX40+. (D) Homing gating strategy of T cells that are CD69+.

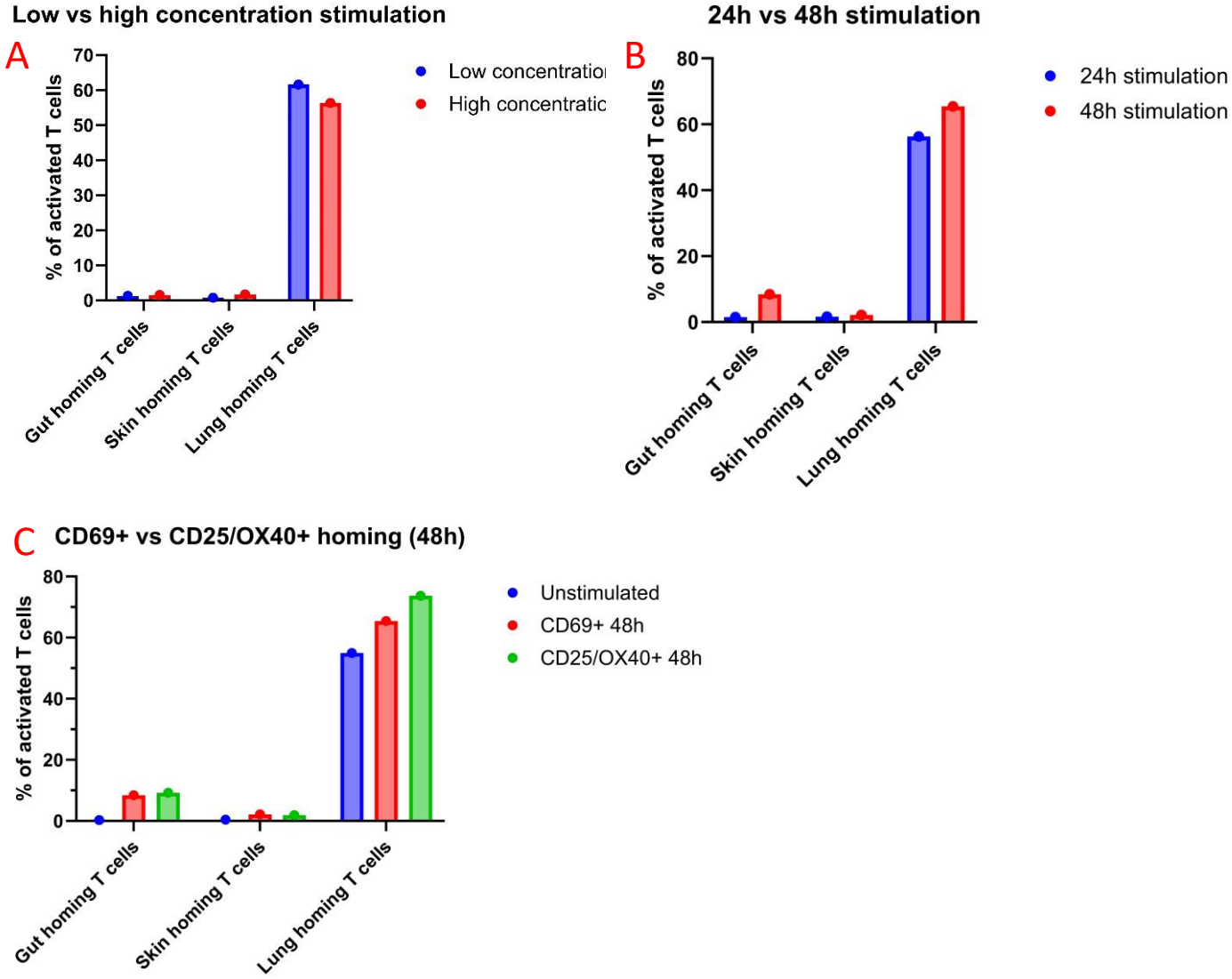

Figure 7. Homing marker expression in different stimulated T cells.
(A) % of CD69+ activated T cells that express homing markers with low vs high concentrations of anti-CD3/anti-CD28. (B) % of CD69+ activated T cells that express homing markers after 24 vs 48 hours with high concentration of anti-CD3/anti-CD28. (C) % of activated T cells expressing homing markers. Blue = non-stimulated T cells and % of activated T cells = % of non-activated T cells, red = % of CD69+ T cells, and green = % of CD25/OX40+ T cells.

4.3 TH2A and Tfh panel

To identify TH2A and Tfh cells, we designed a flow cytometry panel that includes markers that identify these cell subsets. Key markers are CD3, CD4, CRTh2, CD161, CXCR5, PD-1, CD27 and CD45RO. The panel was tested on 24-hour anti-CD3/anti-CD28 stimulated PBMCs from healthy controls to assess its ability to identify TH2A and Tfh cells from lymphocytes. We gated TH2A cells similarly to the gating strategy in Wambre (2017).[13] Cells are gated on size (FSC-A vs. SSC-A) and followed by single cell- (FSC-A vs. FSC-W) and live cell-gating (Fixable viability dye eFluor

780). CD3+CD4+ T cells were selected and were then gated on CD45RO expression (see Fig8A). To identify the allergen-specific T cells, cells were gated as CD49d+CD27- and CRTh2+CD161+. Since the panel was tested on healthy donor cells only, the presence of TH2A cells should be minimal. Tfh cells were gated similarly: lymphocytes, single cells, live cells, and CD3+CD4+ T cells. Cells were then gated on CCR7-CD45RO+ T cells and finally gated on CXCR5+PD-1+ cells (Fig 8B).

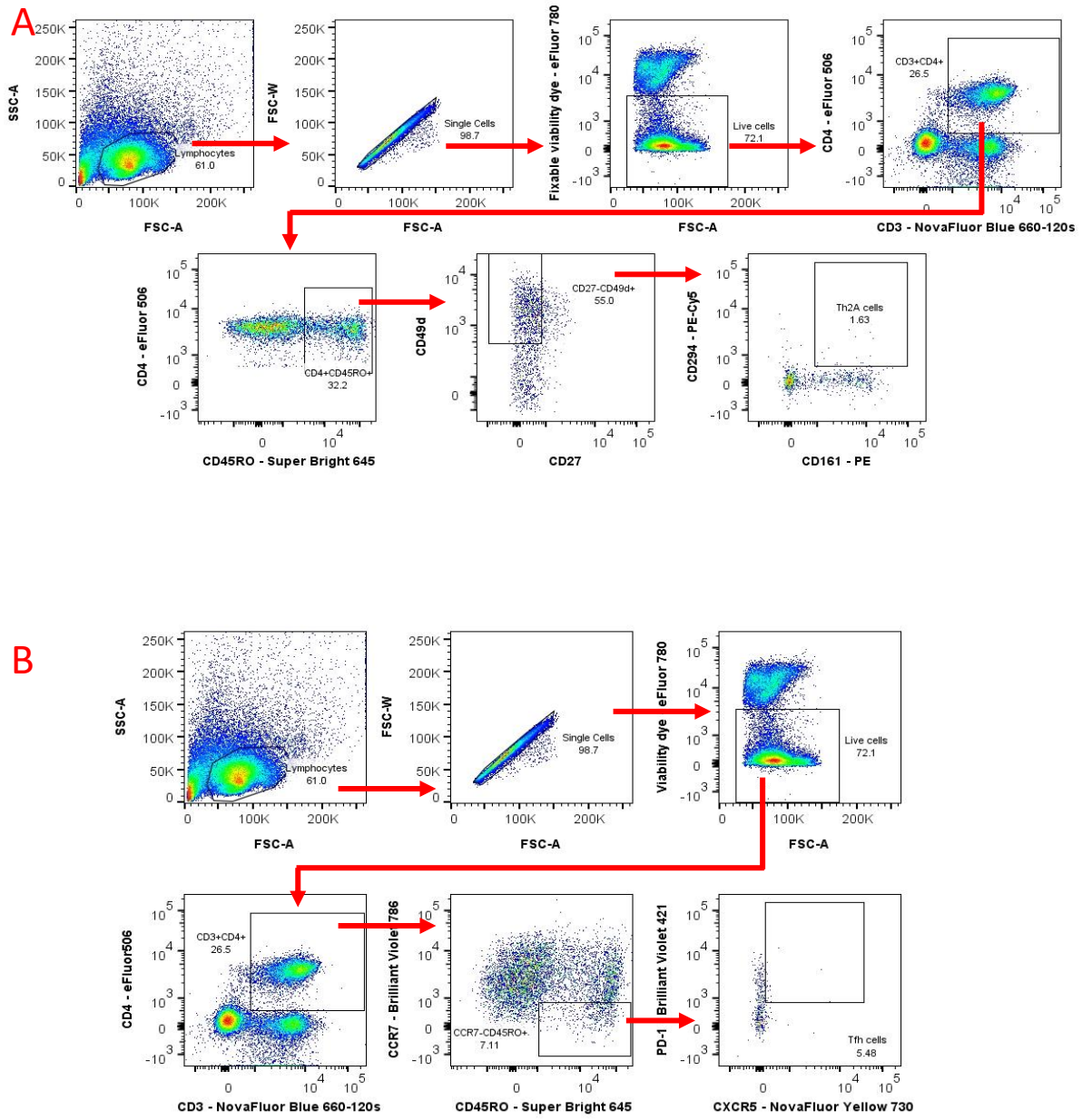

Figure 8. Gating strategy for TH2A and Tfh cells.
(A) Gating strategy for TH2A cells and (B) gating strategy for Tfh cells.

4.4 Basophil activation test

The basophil activation test (BAT) was used to evaluate dose-dependent basophil reactivity (measured as CD63+ population) to peanut and hazelnut allergens. For this report, the following requirements were set to pass a BAT: (1) the negative control (RPMI + IL-3) needed to be lower than the threshold, (2) the positive control (anti-IgE) needed to be higher than the threshold, (3) at least 200 basophils had to be recorded in each condition and (4) at least one concentration of the allergen extract had to be higher than the threshold. In this report, a 10% threshold was used. Lower thresholds led to more exclusions (5% = 27 exclusions, 7,5% = 20 exclusions).

We sometimes deviated from the requirements if this was deemed possible. In some cases, the negative control was higher than the 10% threshold. The CD63+% histogram was compared to the positive control (anti-IgE) for these patients. If there was a clear distinction between activation and background noise, the patient was included. 2 non-responders and 2 spontaneous releasers were excluded (excluding 5 BATs due to multiple days). 1 patient was excluded due to response to a placebo (excluding 2 BATs). 9 BATs were excluded due to technical problems with the test (not enough events or failed controls).

Basophils were gated on size (FSC-A vs. SSC-A), CD193+, CD123+CD203c+, and CD63+. Activated basophils were identified by CD63+. The gating strategy is depicted in Fig 9.

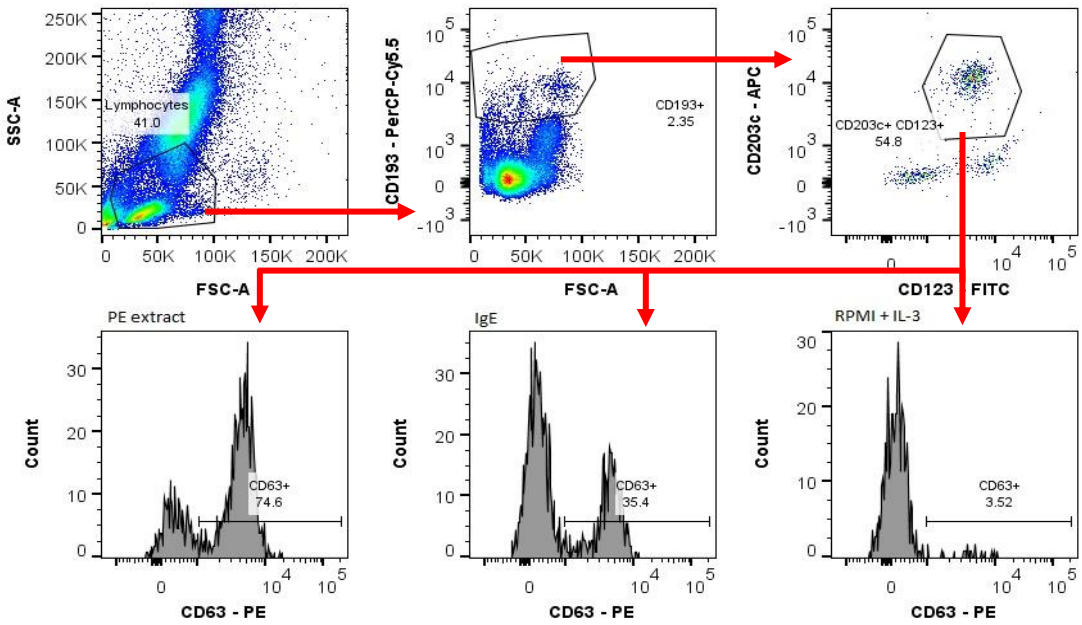

Figure 9. Basophil activation test gating strategy. Basophil activation test gating strategy. First, cells are gated on size (FSC-A vs. SSC-A), followed by gating on CD193+ cells. CD193+ cells are gated on CD123+CD203c+ cells to identify basophils. Basophils are gated on CD63+ to identify activated basophils.

4.4.1 Patient characteristics

A total of 38 BATs have been performed on 34 unique patients. Of these 39 BATs, 22 were included in the study. The characteristics of patients who participated in the study are summarized in Table 3. Patients are divided into three groups according to their clinical response during the oral food

challenge (OFC). The tolerant group did not show any symptoms during the OFC; the mild group showed symptoms correlated with a Mueller score of 0 and 1, and the severe group showed symptoms correlated with a Mueller score of 2 or higher.

Table 3. Patient characteristics of the study

	Tolerant (n=2)	Mild (Mueller 0 & 1) (n=10)	Severe (Mueller 2, 3 & 4) (n=10)
Average age (in years)	26.5	33.9	28.7
Peanut allergies (%)	100%	20%	70%
Gender	100% female	50% female	90% female
Positive BAT	2 (100%)	8 (80%)	10 (100%)
Average total IgE (kU/L)	235.5	468.44*	410**

* 1 missing value for total IgE
** 3 missing values for total IgE

4.4.2 BAT vs OFC

The BATs included in the study were compared with a positive or negative OFC outcome, as seen in Table 4. Table 4 shows that in 81.8% of the cases, the BAT gives a correct diagnosis. Table 3 also shows how the positive BAT correlates with the severity of the clinical response. In the tolerant group, 100% of the BATs are positive and thus incorrect. In the mild group, 80% of the BATs predicted a correct outcome of the OFC, while in the severe group, 100% of the BATs correctly predicted a response to allergens. The tolerant group is the 9,1% of BAT+ OFC- and the 2 mild patients with a negative BAT are the 9,1% of BAT-OFC+.

Table 4. Basophil activation test vs. oral food challenge outcome

All BAT (n=22)	OFC +	OFC -
BAT +	18 (81,8%)	2 (9,1%)
BAT -	2 (9,1%)	0

4.4.3 BAT vs ImmunoCap

The predictive value of the BAT (correlated to the outcome of the OFC) was compared to the predictive value of the ImmunoCap test (CAP). A CAP test was positive if the value was higher than 0.35 kU/L. Fig 10A shows that in the tolerant group, the CAP is better at predicting food allergy with PE than the BAT. When the components of PE are compared, a similar trend is seen, with the CAP being a better predictor for tolerance (50% correct in PE and 100% 2 in Ara h 2 and Ara h 6). When the BAT is compared to the CAP in the mild group, Fig 10B shows that for peanut-allergic patients, both the BAT and CAP have similar predictive values (around 50% are correct compared to the OFC outcomes) for PE and the components. When the BAT and CAP are compared for hazelnut, they both have similar correct outcomes for HE (87.5% for BAT, 100% for CAP) and Cor a 1 (100% for BAT, 87.5% for CAP). For Cor a 9, Fig 10B shows that the BAT has a better prediction value than the CAP (50% vs. 12.5%, respectively). Cor a 14 has similar results (50% vs. 0%, respectively). Fig 10C shows that the severe group has similar results as the mild group. Both BAT and CAP are similar in PE (100% for both BAT and CAP), Ara h 2 (78.5% for BAT and CAP), and Ara h 6 (71.4% vs.

100%, respectively). In the hazelnut part of the severe group, the BAT and CAP are comparable to the mild hazelnut group: HE (100% for BAT and CAP), Cor a 1 (100% for BAT and CAP), Cor a 9 (33% vs. 0%, respectively) and Cor a 14 (0% for BAT and CAP).

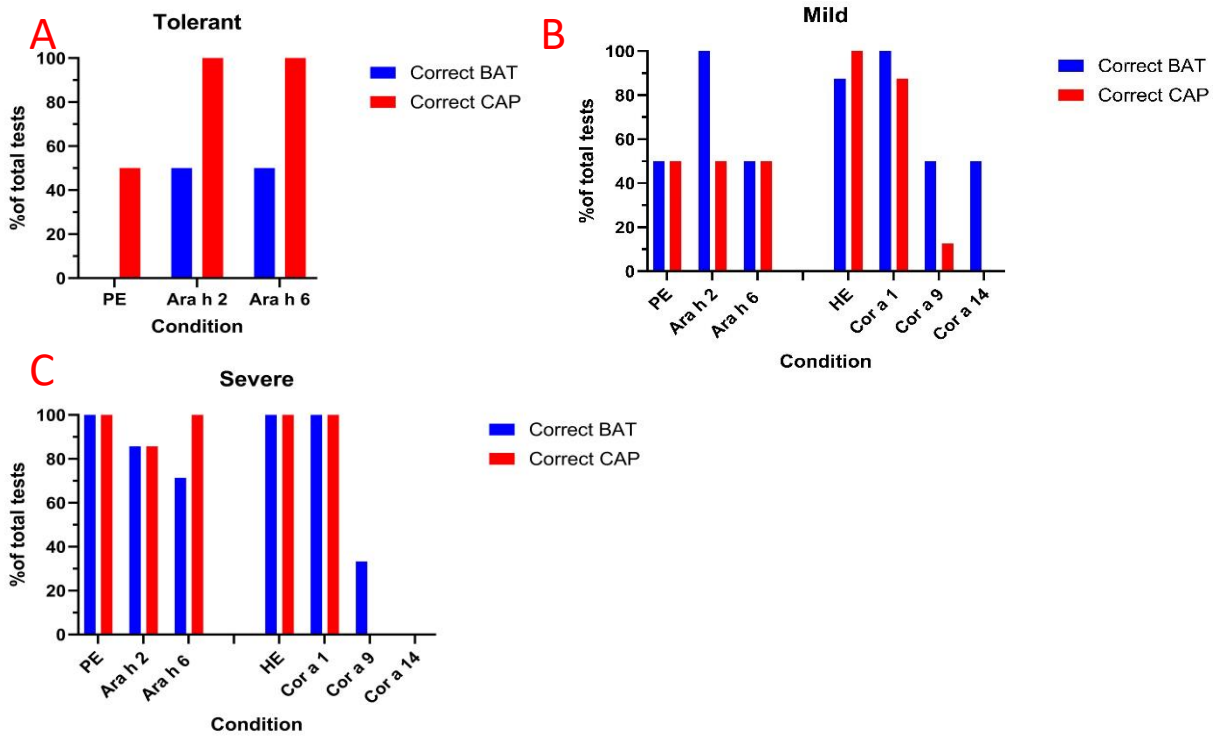

Figure 10. BAT vs CAP. Comparison of the basophil activation test against the ImmunoCAP as a predictor of the outcome of the OFC. An ImmunoCAP test was positive if it was above 0,35 kU/L. (A) Tolerant group (N=2). (B) Mild group (N=10, peanut (n=2), hazelnut (n=8). (C) Severe group (N=10, peanut (n=7), hazelnut (n=3)).

4.4.4 BAT dose-response curves

Dose-response curves were made to study the basophil activation response to peanut extract (PE) and hazelnut extract (HE). Figure 11A shows the results of the PE and HE dose-response curves. The PE dose-response curves follow a more general curve with an increase at 10 ng/mL. Within the HE dose-response curves, most patients only show a response at higher concentrations, with all of them only responding at 1000 ng/mL (with an exception for patients I0009 and I0014).

Fig 11B shows the dose-response curves of all patients grouped according to their clinical outcome (tolerant, mild, and severe). Tolerant patients show a minimal increase in %CD63+ basophils compared to the resting state (RPMI + IL-3). I007 shows a reaction at a high concentration (1000ng/mL) of PE extract. In the mild group (separated into peanut and hazelnut), the peanut group shows one patient (I0033) with minimal changes in %CD63+ basophils with higher PE concentration, while the other patient (I0037) shows an increase at 10 ng/mL PE. Again, a trend can be seen in the mild hazelnut patients where the response occurs at 1000 ng/mL HE, except for I0014. In the severe group, a trend can be recognized where peanut-allergic patients (squares) show more activation than their hazelnut counterparts (circles).

The dose-response curves for PE components were made for the three groups (Fig 11C). The components show that both Ara h 2 and Ara h 6 show close to no activation in the tolerant and

mild groups, except for patient I0007 (Ara h 6 tolerant) and I0033 (Ara h 6 mild group). These increases are due to low basophils in the conditions of the BAT. Ara h 2 and Ara h 6 show more activation in the severe groups. Fig 11D shows the dose-response curves of the HE components. The curves show that Cor a 1 is responsible for basophil activation and that both Cor a 9 and Cor a 14 show minimal basophil activation (only patient I0014 shows activation compared to the negative control).

Table 5 below shows the CAP values for the different extracts and components. Similar as the dose-response curves of the BAT, the CAP values show only a response to Cor a 1, while CAP values for Cor a 9 and Cor a 14 show no sensitization (<0,35 kU/L).

Table 5. CAP values

Patient ID (group)	HN IgE (kU/L)	Cor a 1 IgE (kU/L)	Cor a 9 IgE (kU/L)	Cor a 14 IgE (kU/L)
I0009 (severe)	>100	>100	0.03	0.02
I0014 (mild)	>100	>100	0.37	0.09
I0016 (severe)	20.4	26.2	0	0
I0020 (mild)	1.19	0.01	0	0
I0021 (mild)	12	17.3	0	0
I0024 (mild)	22.8	33.2	0.02	0
I0026 (mild)	41	77	0.02	0.2
I0027 (severe)	8.3	9.2	0	0
I0028 (severe)	9.6	13.3	0	0
I0029 (mild)	22.2	34	0.03	0.02
I0034 (mild)	>100	>100	0.04	0.06

In red are positive CAP values.

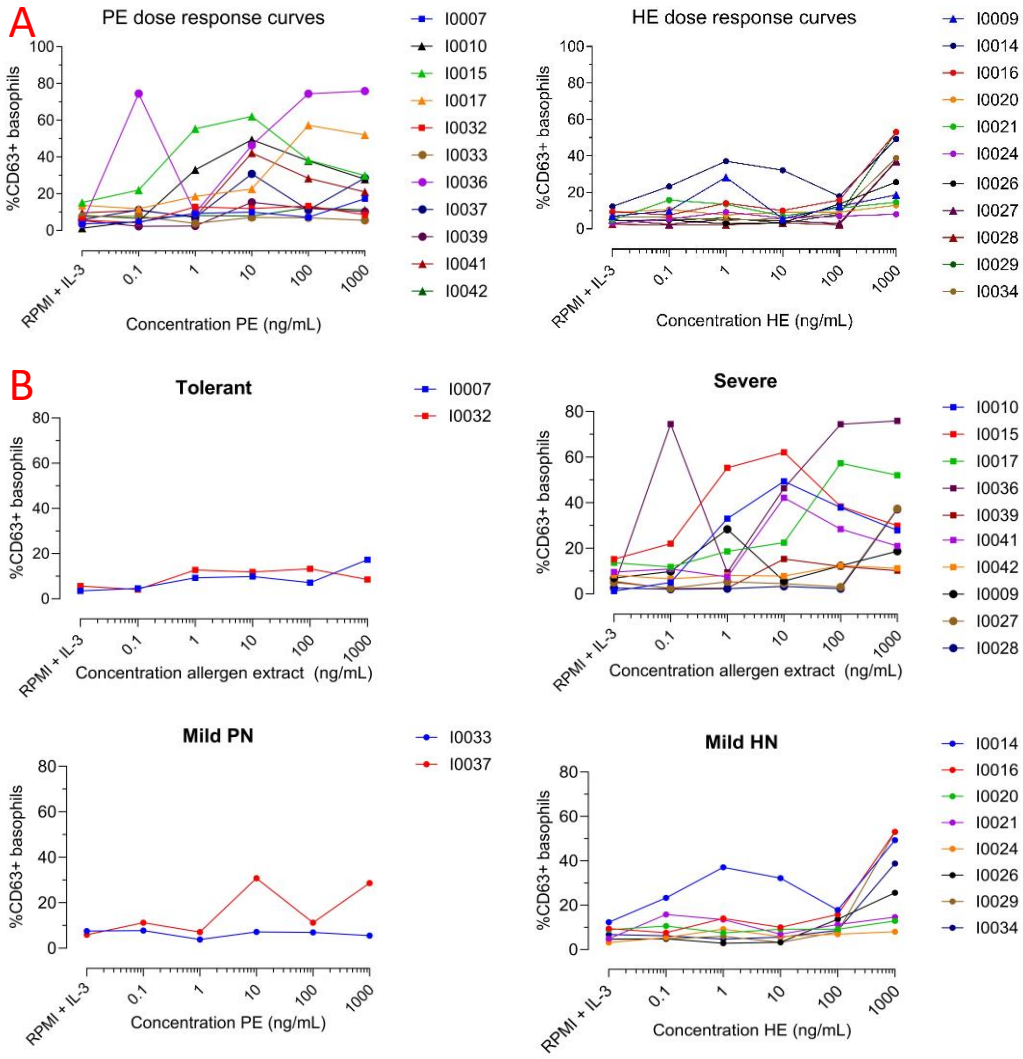
A
PE dose response curves
%CD63+ basophils
Concentration PE (ng/mL)
RPMI + IL-3
I0007
I0010
I0015
I0017
I0032
I0033
I0036
I0037
I0039
I0041
I0042
HE dose response curves
Concentration HE (ng/mL)
I0009
I0014
I0016
I0020
I0021
I0024
I0026
I0027
I0028
I0029
I0034
B
Tolerant
Concentration allergen extract (ng/mL)
Severe
Mild PN
Mild HN
C
Ara h 2 tolerant
Concentration Ara h 2 (ng/mL)
Ara h 6 tolerant
Concentration Ara h 6 (ng/mL)
Ara h 2 mild
Ara h 6 mild
Ara h 2 severe
Ara h 6 severe
D
Cor a 1 dose response curves
Concentration Cor a 1 (ng/mL)
I0025/1
I0031
Cor a 9 dose response curves
Concentration Cor a 9 (ng/mL)
Cor a 14 dose response curves
Concentration Cor a 14 (ng/mL)

Figure 11. Basophil activation test dose-response curves. (A). Dose-response curves to PE and HE. (■ = tolerant, ●= mild, ▲= severe). (B) Dose-response curves to PE and HE grouped into tolerant, mild (PE and HE), and severe (●= peanut, ■= hazelnut). (C) PE components are divided into tolerant, mild, and severe curves. (D) HE components (■ = tolerant, ●= mild, ▲= severe).

5. Discussion

The results of this report provide insights into the complex immune mechanisms underlying food allergies and highlight the role of biomarkers in advancing our understanding of food allergy. At the same time, it highlights the potential of homing markers, TH2A cells, Tfh cells, and the BAT as markers that can be correlated with the severity of food allergic reactions. The homing of immune cells is a crucial process within the human body. The results of the homing marker expression profiles (section 4.2) show potential in advancing the flow cytometry panel since stimulation of T cells increases the expression of homing markers to the gut and lung tissue and, to a lesser extent, skin tissue. Suppose the expression of homing markers could be correlated to the severity or type of symptoms (i.e., symptoms per organ system). In that case, the panel even has the potential to predict the type of symptoms.

Unfortunately, the current homing panel requires further optimization. Correct compensation is difficult since almost no spaces are left in the panel (15 out of 16 filters of the BD Fortessa™ flow cytometer we use are occupied). Compensation beads could aid the compensation process but do not guarantee a perfect compensation matrix. This means that in some cases, the measured levels of specific markers are not reliable. Cell numbers are also a limitation of this panel. The total number of cells in the peripheral bloodstream that go home to tissues is generally minimal.[18] As mentioned, most T cells reside within the tissue, meaning no migration occurs. This means that measuring “homing” cells on PBMCs is difficult. A potential solution is measuring at least 200000 events (but preferably more). Still, even then, numbers could be very low, especially when looking at allergen-specific T cells (after stimulation with an allergen). Also, staining more cells aids in the process of measuring more cells (>1000000 per condition; we only measured 500.000/condition until now). Cell numbers also play a role in the controls for this panel. Fluorescence minus one (FMO) test and single stains “cost” many cells (at least 500000 cells per condition). This makes it more challenging to use this panel on limited allergic patient material or for small pilot studies.

At this point, the panel has only been tested on PBMCs of healthy controls, so it is unknown if the panel is viable on samples of allergic patients. It is also unknown if receptors are always expressed or if they become downregulated after a longer stimulation period. CCR3 has been shown to be not expressed on frozen T cells but could provide more information on lung homing of T cells. Results now suggest that most activated T cells have lung homing capacity. There is little data from our own lab or in the literature that states levels of cells homing towards the tissue. The difference between homing toward the lungs and homing toward both the gut and skin makes it difficult to assume the correctness of the results. A side-by-side comparison of CCR3 on fresh vs frozen blood could provide information on its potential to be kept in the current panel. If frozen PBMCs cannot express CCR3 (even after longer stimulation), it should be considered to remove CCR3 from the panel and include another lung-homing marker. This would aid in making conclusions on the lung homing capacity of T cells.

TH2A cells are key cells with multiple roles within the food-allergic immune response.[13,19,29] This shows how important this biomarker is. Multiple studies have already mentioned that the presence of TH2A cells and their disappearance with immunotherapy are correlated with each

other. In addition, research has already shown that TH2A cells have increased cytokine production.[13,19] Tfh cells are becoming more interesting with the identification of different subsets. Tfh13 especially seems to be an interesting biomarker in food allergy, especially in terms of its role in producing anaphylactic high-affinity sIgE.[14,32] Researching the presence of these cells/cell subsets could give more insights into their role in severity when correlated with clinical outcomes. Therefore, the presence of these cells should be studied in a bigger cohort of allergic patients and should be correlated with the severity.

The TH2A/Tfh panel also has limitations like the homing panel. Cell numbers are low, even with non-specific stimulation. The measurement of cytokine production aids in identifying both cell subsets. Unfortunately, this would mean splitting the panel into two separate ones to fit additional markers due to a lack of space. The separation will cost more cells, making using the panels together even less viable. The TH2A/Tfh panel has only been tested on the blood of healthy individuals. Further testing on an allergic cohort would give insight into its potential as a biomarker.

The BAT is a tool for assessing allergic reactions to allergens with in vitro testing. The test measures the activation of basophils through CD63+ in response to allergen exposure. In 2023, an EAACI task force for the BAT published a letter proposing a standardization protocol. In this letter, the task force proposes a gating strategy where the resting population is gated on 2,5%. Using that gate, the positivity of other conditions is analyzed (where conditions with more >5% CD63+ basophils are seen as positive).[43] We tried this gating strategy but concluded that it did not work with our samples. The proposed gating strategy led to incorrect gating and a loss of even more tests due to failures in the controls.

This study showed that in 82% of the cases, the BAT gave a correct result when compared with the outcomes of the OFCs. Previous research on the predictive properties of the BAT shows that the results of this study are lower. A meta-analysis showed that the BAT was highly specific for peanuts (90%), while a study in 2021 by Duan et al. showed an AUROC of 0.98 for peanut allergy and 0.92 for hazelnut.[44,45] A study in children showed an accuracy of 97% for the BAT and peanut allergy, with a positive prediction value of 95%, showing that the BAT is able to predict allergy in 95% of the cases correctly.[46] A different study by Santos et al. showed similar results of 96% diagnostic accuracy for the BAT.[47] When analyzing the false results of the BAT, all false results are seen in patients with less severe symptoms (tolerant and mild groups). The BAT seems comparable in predicting food allergy with the ImmunoCAP test. Especially in peanut-allergic patients, BAT and CAP were similar in predicting the correct outcomes of an OFC. The BAT and CAP predicted food allergy similarly, with HE and Cor a 1 for hazelnuts. Both performed worse in the prediction with Cor a 9 and Cor a 14, with a small favor for the BAT Cor a 9. No differences between severity were found in the peanut-allergic patients, except for severe hazelnut allergic patients, where both BAT and CAP could not predict food allergy with Cor a 9 and Cor a 14.

This study shows different patterns in basophil activation response to PE and HE. The dose-response curves show that PE generally induces basophil activation at lower concentrations while HE requires higher HE concentration (>100 ng/mL). This is especially seen in patients with mild symptoms. This would suggest different thresholds for basophil activation for peanut and hazelnut allergies. Nevertheless, most patients with a hazelnut allergy are pollen-food allergic. These patients show cross-reactivity for Cor a 1 with Bet v 1 since both proteins are very similar.[48,49] When compared by severity, tolerant patients had no significant basophil activation, although all patients had a positive BAT (with %CD63+ basophils > 10%). Mild patients in both the peanut and hazelnut groups also showed minimal activation. Activation was seen in severe

patients, with peanut patients more activated than hazelnut patients. When analyzing the components of HE and PE, Ara h 2 and 6 induce basophil activation in the severe group but not in the tolerant and mild groups. This suggests that other factors (like Ara h 1 or Ara h 8) might influence basophil activation in less severe patients. For hazelnuts, only Cor a 1 seems responsible for activating basophils in all groups. This is also seen in Table 5 of the IgE levels for Cor a 1. These findings emphasize that basophil activation patterns differ not only between peanut and hazelnut allergens but also across clinical severity levels and specific allergenic components.

Recently, a letter was published on how many basophils are needed for a proper BAT. Most studies enforce a minimum count of 200-1000 basophils. 500 is a broadly accepted lower limit. The letter studied the effects of low basophil counts on the rate of false positives. The letter shows that with a fixed cutoff, less than 450 basophils result in more false positives (especially with cutoffs below 10%).[50] When we look at our own BATs, we see that the tests done at the beginning of the study have lower basophil counts (<200). This may be attributed to a few reasons: (1) the protocol used in the BAT has not been updated in the last 7 years, (2) antibodies have changed during the study and have not been titrated properly in a long time, (3) multiple flow cytometers have been used during the study, leading to potential differences in measurements, (4) multiple people have done the tests and multiple fixation methods have been used (leaving cells in fixation buffer until measuring or washing the fixation buffer after 10 minutes) and (5) measuring 100.000 events at the beginning of the study. This may all contribute to lower cell numbers needed for a correct BAT. We changed the protocol during the study, measuring more events (200.000). Ultimately, we chose a minimum of 200 basophils as a requirement for a correct BAT. In some cases, less than 200 basophils were accepted if a clear distinction could be made.

The total number of BATs is a weakness for any conclusions made in this report. Only 22 patients/BATs were included in the report. These 22 patients were divided into three groups: tolerant (n=2), mild (n=10) and severe (n=10). With only peanut-allergic patients in the tolerant group, it is hard to draw conclusions about the predictive value of the BAT between peanuts and hazelnuts. Also, in the severe group, only 3 hazelnut patients are included, while in the mild group, only 2 peanut-allergic patients are included. In summary, with the results of this study, it is hard to draw any conclusions about the results of the BAT and it being a predictive biomarker.

This report focused on developing panels to identify TH2A, TFH, and homing markers. In the end, the panels that were developed seem to be viable, given the testing that has been done. Nevertheless, more testing (especially on samples from allergic patients) is needed to evaluate the viability of the panels in these samples with fewer cells (most likely). The incorporation of the BAT as a severity predictor is unlikely with the results of this study.

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For this project, I used Grammarly as a grammar control software while writing this report by providing feedback on my writing. Grammarly v1.2.115.1530. November 2024.

For this project, I used ChatGPT to generate ideas for title names and text structures to give form to this report. OpenAI Chat-GPT 3.5 November 2024.

Figures were made with the use of BioRender. This was done with a Premium account. No publication licenses were received for these figures.

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Supplemental information

Table S1. Homing panel antibodies

Fluorophore	Marker	Clone	Host	Isotype	Dilution	Manufacturer	Catalog number
FITC	CD69	FN50	Mouse	IgG1, k	1:50	ThermoFisher Scientific	11-0699-41
NFB 660-120s	CD3	SK7	Mouse	IgG1, k	1:20	ThermoFisher Scientific	H028T02B08-A
AF647	ITGB1	TS2/16	Mouse	IgG1, k	1:50	BD Biosciences	568992
AF700	CCR10	314305	Rat	IgG2a	1:50	R&D Systems	FAB3478N-100UG
Fixable viability stain 780	Viability				1:1000	ThermoFisher Scientific	
PE	CCR4	D8SEE	Mouse	IgG1, k	1:50	ThermoFisher Scientific	12-1949-42
PE-Dazzle594	CCR9	L053E8	Mouse	IgG2a, k	1:50	BioLegend	358917
PE-Cy5	CD25	M-A251	Mouse	IgG1, k	1:100	BD Biosciences	
PE-Cy5	CD134	ACT35	Mouse	IgG1, k	1:20	BD Biosciences	
NFY 730	CD4	SK3	Mouse	IgG1, k	1:50	ThermoFisher Scientific	H001T02Y07-A
PE-Cy7	CD49d	9F10	Mouse	IgG1, k	1:100	Biolegend	304314
BV421	Ki-67	B56	Mouse	IgG1, k	1:20	BD Biosciences	
BV510	CCR3	5E8	Mouse	IgG2b, k	1:20	BioLegend	310721
BV605	CLA	HECA-452	Rat	IgM, k	1:100	BD Biosciences	563960
BV650	CXCR4	12G5	Mouse	IgG2a, k	1:200	BD Biosciences	740599
BV786	ITGB7	FIB504	Rat	IgG2a, k	1:500	BD Biosciences	744013

Table S2. TH2A/Tfh panel antibodies

Fluorophore	Marker	Clone	Host	Isotype	Dilution	Manufacturer	Catalog number
FITC	CD69	FN50	Mouse	IgG1, k	1:50	ThermoFisher Scientific	11-0699-41
NFB 660-120s	CD3	SK7	Mouse	IgG1, k	1:20	ThermoFisher Scientific	H028T02B08-A
eFluor 660	CD183 (CXCR3)	CEW33D	Mouse	IgG1, k	1:50	ThermoFisher Scientific	50-1839-42
AF 700	CD27	O323	Mouse	IgG1, k	1:50	ThermoFisher Scientific	56-0279-42
Fixable viability stain 780	Viability				1:1000	ThermoFisher Scientific	
PE	CD161	HP-3G10	Mouse	IgG1, k	1:100	ThermoFisher Scientific	12-1619-42
PE-Cy5	CD294 (CRTh2)	BM16	Rat	IgG2a, k	1:100	ThermoFisher Scientific	15-2949-41
NovaFluor Yellow 730	CD185 (CXCR5)	MU5UBEE	Mouse	IgG2b, k	1:50	ThermoFisher Scientific	H037T02Y07-A
PE-Cy7	CD49d	9F10	Mouse	IgG1, k	1:100	Biolegend	304314
BV421	PD-1	J105	Mouse	IgG1, k	1:100	ThermoFisher Scientific	404-2799-41
eFluor 506	CD4	RPA-T4	Mouse	IgG1, k	1:100	ThermoFisher Scientific	69-0049-41
SB 600	CD196 (CCR6)	R6H1	Mouse	IgG1, k	1:200	ThermoFisher Scientific	63-1969-42
SB 645	CD45RO	UCHL1	Mouse	IgG2a, k	1:100	ThermoFisher Scientific	64-0457-41
BV 786	CD197 (CCR7)	3D12	Rat	IgG2a, k	1:100	BD Biosciences	563710

Figure S1. CCR3 expression on different cells