A Study of Eosinophils – From the Human Thymus to Eosinophilic Esophagitis

Sofie Albinsson Högberg

Department of Infectious Diseases

Institute of Biomedicine

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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sofie.albinsson@gu.se

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ABSTRACT

Eosinophils are cells of the innate immune system. They primarily reside within tissues and are most numerous in the gastrointestinal tract but are absent from the healthy esophagus. During the inflammatory disease eosinophilic esophagitis, eosinophils infiltrate the esophagus but their function is unknown. Eosinophils also reside within the human thymus, although their function within this organ dedicated to T cell development is unclear. This thesis is a study of eosinophils resident within the human thymus and of the disease eosinophilic esophagitis. The aim was to increase knowledge on the eosinophil granulocyte and to improve healthcare of patients afflicted with eosinophilic esophagitis. In paper I, molecular patterns of eosinophils in the blood of patients with eosinophilic esophagitis were combined with patient-reported outcomes to yield a multivariate model capable of separating patients who had responded to topical corticosteroid treatment from patients who did not respond to treatment. The use of such multivariate models based on less invasive methods than endoscopic procedures to evaluate success of therapy could improve patient care and patients' quality of life. Paper II was also focused on the advancement of patient care. The first Swedish instrument specifically developed for assessment of symptom severity of eosinophilic esophagitis was translated and validated in order to improve clinical assessment of the disease among Swedish adults. In paper III, esophageal eosinophils found in biopsies from patients with eosinophilic esophagitis were studied using fluorescent immunohistochemistry. These analyses revealed associations between eosinophils and CD4+ T cells, the presence of CD16+ "suppressive" eosinophils, and massive release of extracellular galectin-10 within the esophageal mucosa. Tissue resident eosinophils were further studied in human thymus of thymectomized children in papers IV and V. In paper IV, eosinophils were studied using fluorescent immunohistochemistry to investigate their maturity, distribution, and cellular interactions within the thymus. Immature and proliferating eosinophils were found, as well as mature cells, and the primary location of thymic eosinophils was in the corticomedullary junction. In paper V,

thymic eosinophils were phenotypically characterized using cytometry by time-of-flight and their interactions with thymocytes were studied by co-culture experiments of fluorescence-activated cell sorted thymic eosinophils and thymocytes of the double-positive (CD4+CD8+), CD4 single-positive, and CD8 single-positive stages. The thymic eosinophils displayed a distinct phenotype compared to blood eosinophils and the thymic eosinophils were capable of directing thymocytes toward the CD4 single-positive phenotype. In this thesis, T cell suppression is presented as a possible function of eosinophils in eosinophilic esophagitis and thymic eosinophils are shown to be specialized cells that can affect the thymocyte development process.

Keywords: eosinophil, eosinophilic esophagitis, galectin-10, human, patient-reported outcome, T cell, thymocyte, thymus

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SAMMANFATTNING PÅ SVENSKA

Eosinofiler är en del av vårt immunförsvar och är mest kända för att försvara oss mot parasiter. Denna typ av vita blodcell finns att hitta i de flesta delarna av den mänskliga kroppen, främst av allt finns de i mag-tarmkanalen, men inte i en frisk matstrupe. På grund av just detta karaktäriseras sjukdomen eosinofil esofagit av ökad infiltration av eosinofiler till matstrupen. Eosinofil esofagit, även kallat allergisk matstrupe, är en sjukdom som initieras av en allergisk reaktion mot födoämnen eller luftburna ämnen och som leder till en inflammation i matstrupen. Att eosinofiler ökar till antal är känt, men funktionen hos eosinofiler i eosinofil esofagit är ännu okänd. I den friska kroppen finns eosinofiler även i thymus (brässen) och även här är deras funktion oklar. Målet med denna avhandling var således att studera och öka kunskapen om eosinofiler i dessa två miljöer: i en frisk thymus och i matstrupen hos patienter med eosinofil esofagit. Ytterligare ett syfte med avhandlingen var att underlätta och förbättra vården av patienter med eosinofil esofagit. I det första arbetet i denna avhandling studerades möjligheten att använda en statistisk modell baserad på biomarkörer i blodet och enkätsvar för att utvärdera om patienter med eosinofil esofagit har svarat på behandling. Den framtagna modellen kunde skilja individer som svarat på behandlingen från individer som inte svarat och öppnar därmed upp för att i framtiden kunna utvärdera behandlingssvar utan upprepade endoskopier. Det andra arbetet var fokuserat på att förbättra den kliniska bedömningen av sjukdomen eosinofil esofagit hos individuella patienter. Ett frågeformulär som är utvecklat speciellt för att utvärdera symtom hos patienter med eosinofil esofagit översattes till svenska och validerades. Detta frågeformulär är den första svenska enkäten som är riktad till patienter med eosinofil esofagit. Det tredje arbetet innefattar en studie av eosinofiler i vävnad från patienter med eosinofil esofagit. Genom att färga in celler i vävnaden kunde ett samband mellan eosinofiler och CD4+ T-celler påvisas. Eosinofiler som uttryckte receptorn CD16 upptäcktes i vävnaden och proteinet galectin-10 hittades spritt utanför cellerna. I de fjärde och femte delarbetena studerades eosinofiler i thymus från barn som genomgått korrigerande hjärtoperationer. Vid dessa operationer plockas delar av (och ibland hela) thymus bort för att ge åtkomst till hjärtat. Genom att färga in tunna bitar av vävnaden kunde eosinofilernas mognadsgrad, fördelning och interaktioner i thymus studeras. Eosinofiler från thymus jämfördes också med eosinofiler från blod och resultaten visade att det fanns en tydlig skillnad på dessa celler. Eosinofilers påverkan på thymocyter (omogna T-celler) undersöktes genom att odla dessa celler tillsammans. Dessa odlingar tydde på att eosinofiler kan driva thymocyter till en speciell subgrupp som uttrycker CD4. Sammantaget ger denna avhandling en inblick om eosinofiler och deras funktion i den mänskliga kroppen.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Lingblom C, **Albinsson S**, Johansson L, Larsson H, Wennerås C. Patient-Reported Outcomes and Blood-Based Parameters Identify Response to Treatment in Eosinophilic Esophagitis. Dig Dis Sci. 2021 May;66(5):1556-1564.
doi: 10.1007/s10620-020-06368-2

II. **Albinsson S**, Tuomi L, Wennerås C, Larsson H. Patient-Reported Dysphagia in Adults with Eosinophilic Esophagitis: Translation and Validation of the Swedish Eosinophilic Esophagitis Activity Index. Dysphagia. 2021 Mar 8.
doi: 10.1007/s00455-021-10277-5

III. **Albinsson S**, Lingblom C, Johansson L, Larsson H, Wennerås C. Eosinophils release galectin-10 and co-localize with T cells in eosinophilic esophagitis (submitted manuscript).

IV. **Albinsson S***, Lingblom C*, Lundqvist C, Telemo E, Ekwall O, Wennerås C. Eosinophils interact with thymocytes and proliferate in the human thymus. Eur J Immunol. 2021 Jun; 51(6):1539-1541.
doi: 10.1002/eji.202049080
*The authors contributed equally

V. **Albinsson S***, Lingblom C*, Lundqvist C, Hennings V, Telemo E, Ekwall O, Wennerås C. Phenotypic and functional studies of eosinophils in the human thymus (in manuscript).
*The authors contributed equally

PUBLICATIONS NOT INCLUDED IN THE THESIS

Mohammad G, Rustom F, **Albinsson S**, Wennerås C, Akyürek LM. Isolated Eosinophilic Myometritis: A Case Report of an Extremely Rare Phenomenon. Int J Gynecol Pathol. 2021 May 3.

Hennings V*, Thörn K*, **Albinsson S**, Lingblom C, Andersson K, Andersson C, Järbur K, Pullerits R, Idorn M, Paludan SR, Eriksson K*, Wennerås C*. The presence of serum anti-SARS-CoV-2 IgA appears to protect primary health care workers from COVID-19. Eur J Immunol. 2022 Feb 7
*The authors contributed equally

Albinsson S, Tuomi L, Wennerås C, Larsson H. Validation of the Swedish Watson Dysphagia Scale for adult patients with Eosinophilic Esophagitis. Dis Esophagus. 2022 Jan 22

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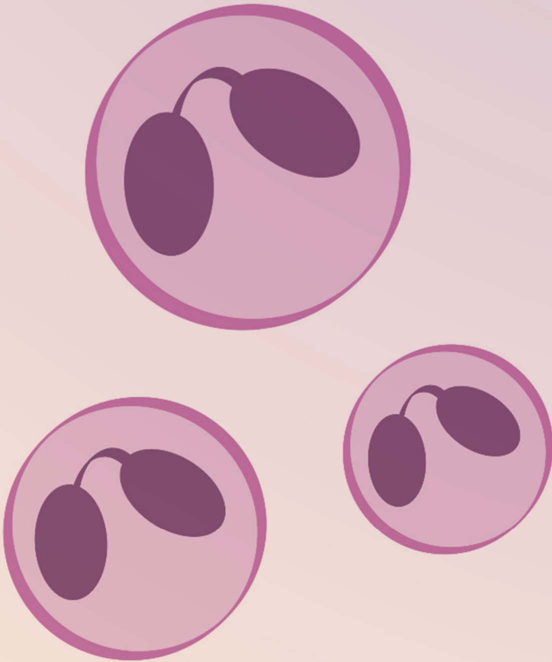
ABBREVIATIONS

APC	Antigen-presenting cell
CCL	Chemokine (C-C motif) ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CyTOF	Cytometry by time-of-flight
DN	Double-negative
DP	Double-positive
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetic acid
EDN	Eosinophil-derived neurotoxin
EEsAI	Eosinophilic Esophagitis Activity Index
EET	Eosinophil extracellular trap
EMR1	Epidermal growth factor-like module containing mucin-like hormone receptor 1
EoE	Eosinophilic esophagitis
EORTC QLQ- OES18	European Organization for Research and Treatment of Cancer Quality of Life Questionnaire-Oesophageal Module 18
EPX	Eosinophil peroxidase
EV	Extracellular vesicle
FACS	Fluorescence-activated cell sorting
FOXP3	Forkhead box P3
hEoP	Human eosinophil lineage-committed progenitor
HPF	High-power field
ICC	Intraclass correlation coefficient
Ig	Immunoglobulin
IL	Interleukin

mAb	Monoclonal antibody
MBP	Major basic protein
MHC	Major histocompatibility complex
OCT	Optimal cutting temperature
OPLS	Orthogonal projection to latent structures
pAb	Polyclonal antibody
PBS	Phosphate-buffered saline
PPI	Proton pump inhibitor
PRO	Patient-reported outcome
RT	Room temperature
S-EEsAI	Swedish Eosinophilic Esophagitis Activity Index
S-WDS	Swedish Watson dysphagia scale
SF-36	36-Item Short Form Health Survey
Siglec-8	Sialic acid-binding immunoglobulin-like lectin 8
SP	Single-positive
TBS	Tris-buffered saline
TBSS	Tris-buffered saline + 0.1% saponin
TCR	T cell receptor
Th cell	T helper cell
Treg	T regulatory cell
WDS	Watson dysphagia scale



INTRODUCTION

Eosinophils are cells of the immune system that perform a variety of functions. In the past, eosinophils were considered superfluous and harmful but recent advances in research have revealed the opposite. The eosinophil (Figure 1) has the capacity to defend its host against viruses, bacteria, and parasites and it can regulate lymphocyte functions.

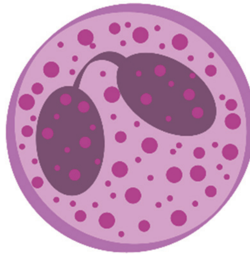


Figure 1. Illustration of an eosinophil.

THE IMMUNE SYSTEM

Our immune system protects us from pathogens and tissue damage via a network of proteins and cells. In humans, the immune system is divided into innate immunity and adaptive immunity. The innate immune response is constant and can readily be employed to combat pathogens and hinder infections at an early stage. Pathogens that cross the initial defense of the epithelial barriers such as the skin or the lining of the gastrointestinal tract, will encounter soluble molecules and white blood cells that recognize and eliminate common classes of pathogens. Cells of the innate immune system recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) via pattern recognition receptors.[1] Recognition of pathogens or injured cells via the pattern recognition receptors triggers cellular responses including secretion of cytokines, phagocytosis of the pathogen, or release of cytotoxic content. The cells associated with innate immunity are dendritic cells, macrophages, monocytes, mast cells, natural killer cells, neutrophils, basophils, and eosinophils.[2]

The adaptive immune response is initiated when pathogens have escaped the defenses of the innate immune response. Two classes of lymphocytes are responsible for the adaptive immunity: T lymphocytes (T cells) and B lymphocytes (B cells).[3] The B cells and T cells recognize pathogens via receptors specific for antigens of the invading pathogen. Once a pathogen is encountered,

only the cells with receptors that recognize its specific antigens will proliferate and differentiate into effector lymphocytes, i.e., plasma cells, helper T cells (Th cells), or cytotoxic T cells. T cell responses are initiated via antigen-presenting cells (APCs) that present pathogen-derived peptides using glycoproteins termed major histocompatibility complexes (MHCs). Antigens from intracellular pathogens are presented by MHC class I and favor the response of CD8 cytotoxic T cells, antigens from extracellular pathogens are presented via MHC class II and promote a CD4 helper T cell response (Figure 2).[4]

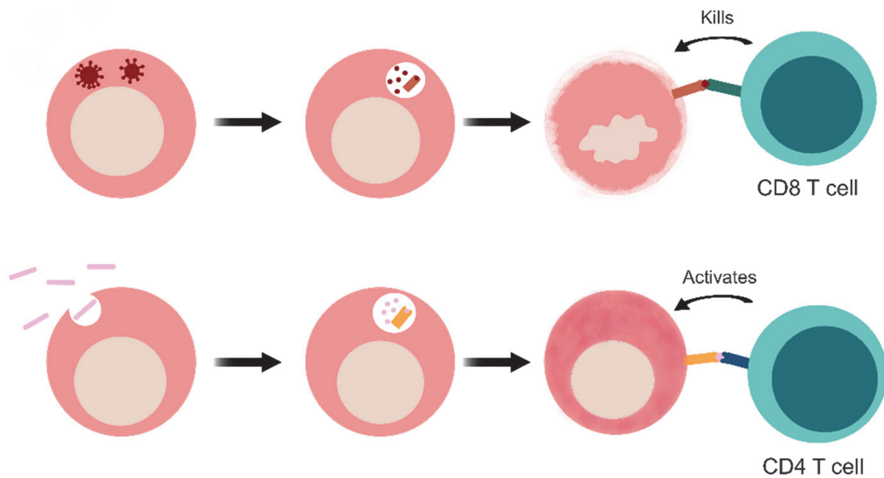


Figure 2. Illustration of T cell responses to intracellular pathogens (upper panel) and extracellular pathogens (lower panel). In the upper panel, MCH I/peptide complexes are presented to CD8+ T cells, which kills infected cells. In the lower panel, MHC II/peptide complexes are presented to CD4+ T cells, which enhances the response of other immune cells.

Th cells can be divided into subtypes, the most common being Th1 and Th2 cells. The immune response associated with Th1 cells is referred to as type 1 immunity and is characterized by the cytokines IL-2, tumor necrosis factor-α/β, lymphotoxin-α, and interferon-γ. These cytokines stimulate proliferation of T cells and activate macrophages and act as a defense against intracellular pathogens.[5] A type 2 immune response is associated with Th2 cells that produce IL-4, IL-5, IL-9, and IL-13 for defense against extracellular parasites and helminths but is also linked to allergic inflammation.[6] Increased antibody production, degranulation of mast cells, and eosinophil tissue infiltration are characteristics of a type 2 immune response.[7]

Unlike the innate immunity, the adaptive immunity develops a memory of the encountered pathogen, enabling swifter response upon re-infection. When the recognized antigen is a self-antigen or a harmless environmental antigen autoimmune diseases and allergies can arise.

IMMUNE TOLERANCE

Immune tolerance is the capacity of the immune system to be unresponsive to an antigen, such as tissues and cells of our own bodies. Without immune tolerance, autoimmune diseases arise where lymphocytes attack the body's own constituents. There are two types of immune tolerance, central tolerance and peripheral tolerance. Central tolerance refers to the elimination of self-reactive B cells and T cells during lymphocyte development in the primary lymphoid organs (bone marrow and thymus).[8] The process of removing self-recognizing lymphocytes is also called negative selection. This process is not 100% effective and self-reactive lymphocytes are released into the periphery.[9] Suppression of these autoreactive lymphocytes in the periphery is called peripheral tolerance. Several tolerance checkpoints are involved in peripheral tolerance of T cells including ignorance, quiescence, anergy, senescence, exhaustion, and death. Some of these act directly on the autoreactive T cells and others are dependent on additional cells including regulatory T cells (Tregs) and dendritic cells.[10] Tregs are a subset of T cells with the capacity to mediate T cell suppression and they are central to upholding peripheral tolerance and homeostasis. Tregs express forkhead box P3 (FOXP3), which is a master regulator of Treg function and development and is important for transcription regulation, TCR signaling, and cell communication.[11]

THE EOSINOPHIL

Eosinophils are granular leukocytes of the myeloid lineage that constitute approximately 1-5% of the white blood cells in the blood.[12] The eosinophil has a distinct polylobed nucleus and is packed with granules. The eosinophilic granules contain cationic proteins and cytokines that can be released upon stimulation. Eosinophils in the blood are few, <400/mm^3,[13] and have a short life span with a half-life of approximately 18 hours.[14] Instead, most eosinophils reside in tissues where they can survive for up to 2 weeks.[15, 16]

HISTORY AND BACKGROUND

Back in 1879, Paul Ehrlich discovered a type of cell that was stained bright pink with the dye eosin.[17] Based on this feature, he named this newfound cell "the eosinophil".

For a long time, eosinophils were considered mainly as cytotoxic, harmful cells that were superfluous to human existence, despite no known cases of humans born without eosinophils and adverse effects in individuals with eosinophil deficiency.[18] The described features of individuals with eosinophil deficiency include thymomas (tumors of the thymus), agammaglobulinemia, dysgammaglobulinemia (immune deficiencies resulting in low blood antibody concentration), and recurrent infections.[18, 19]

Early in the study of eosinophils, their presence in parasite and helminth infections was established and eosinophils were believed to be niched effector cells focused on their elimination. Eosinophils were also associated with tissue damage in disorders characterized by eosinophilia.[20] Over the years, more and more evidence of eosinophil immunomodulatory and homeostatic properties have appeared. The LIAR hypothesis proposes the eosinophil's principal roles to be Local Immunity And/or Remodeling/Repair in health and disease.[21]

DEVELOPMENT

Eosinophils develop from multipotent hematopoietic stem cells in the bone marrow and are of the myeloid lineage (Figure 3). The cytokines IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor are central for the development of eosinophils.[22, 23] Eosinophils from humans and mice develop via different precursor stages. In mice, eosinophils develop via common myeloid progenitors into granulocyte/macrophage progenitors that have the capacity to differentiate into either eosinophils, neutrophils, monocytes, basophils, or mast cells.[24] In contrast, human eosinophil lineage-committed progenitors (hEoP) develop directly from common myeloid progenitors and not from granulocyte/macrophage progenitors.[25] The hEoPs express CD34, CD38, CD123, and CD125 and have upregulated gene expression for the eosinophil proteins galectin-10 and major basic protein (MBP).[25]

Following lineage commitment, structural changes occur during maturation of human eosinophils. The eosinophil precursors first develop into large myeloblasts then into promyelocytes with a rounded nucleus and primary granules, followed by the myelocyte stage where the size of the cells start to reduce following condensation and segmentation of the nucleus, shrinking of organelles, and size reduction of specific granules. Finally, metamyelocytes that can no longer undergo cell division develop into mature eosinophils with a distinct polylobed nucleus, which are subsequently released into the blood stream.[26, 27]

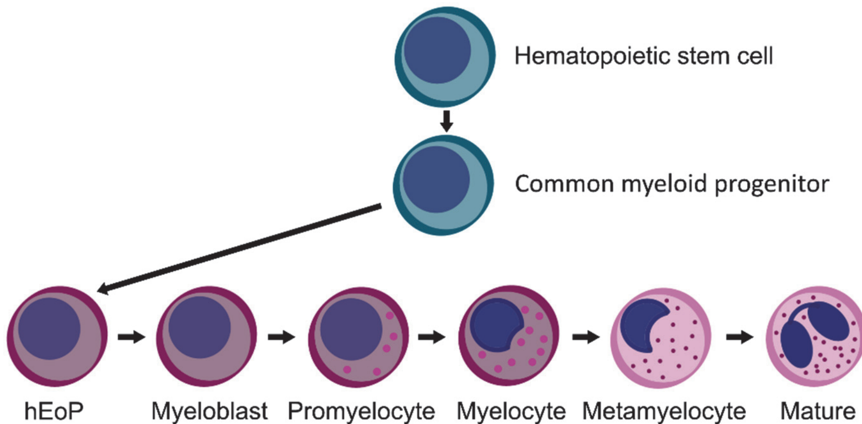


Figure 3. Eosinophil developmental stages in humans. Human eosinophils develop from hematopoietic stem cells via common myeloid progenitors into eosinophil lineage-committed progenitors (hEoP) committed to the eosinophil lineage. The lineage committed cells then develop into fully mature eosinophils.

PHENOTYPE

Eosinophils, like other cells of the immune system, express a battery of cell surface receptors important for e.g., adhesion, intracellular signaling, and survival. Eosinophil-associated receptors include CD193/CCR3,[28] sialic acid-binding immunoglobulin-like lectin 8 (Siglec-8),[29, 30] and CD294,[31] although these receptors are not exclusively expressed by eosinophils. CCR3, i.e., C-C chemokine receptor type 3, is a receptor that binds a variety of chemokines including CCL5/RANTES, CCL11/eotaxin-1, and CCL26/eotaxin-3, which induce eosinophil chemotaxis.[32] Siglec-8 is a surface lectin, blockage of Siglec-8 using monoclonal antibodies (mAbs) induces apoptosis in eosinophils.[33] The CD294 receptor binds the chemoattractant prostaglandin D2, which activates and recruits eosinophils.[34] The epidermal growth factor-like module containing mucin-like hormone receptor 1 (EMR1) is a surface receptor uniquely expressed by eosinophils.[35] It is the ortholog of the murine receptor F4/80 and the expression of EMR1 is induced late in eosinophil development.[36]

Eosinophils can act as APCs,[37, 38] they can express MHC class II[39] and co-stimulatory molecules CD28, CD40, and CD86.[40, 41]

Upon activation, eosinophils can express CD16, the low-affinity Fc receptor for IgG.[42] Little is known regarding the function of CD16 in eosinophils, but in neutrophils and NK cells, the receptor can bind the Fc part of cell-bound IgG antibodies and induce degranulation and lysis of the target cell.[43, 44] The CD16-

positive eosinophils have been shown to be better at suppressing T cell proliferation compared to CD16-negative eosinophils *in vitro* and are called "suppressive eosinophils".[45] The suppressive eosinophils have an increased expression of CD4, CD40, CD44, CD54, CD66c, CD183, CD194, CD199, CD274, thymic stromal lymphopoietin receptor, and formyl peptide receptor 1 and lower expression of CD9, CD11a, CD45, CD49d, CD66b, CD71, CD294, and Siglec-8.[45]

GRANULE PROTEINS

The membrane-enveloped specific granules of the eosinophils have a crystalloid core and contain cationic proteins, cytokines, chemokines, and growth factors that can readily be secreted upon stimulation. The cationic proteins include major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPX) (Figure 4).[46] MBP is stored as inert nanocrystals with amyloid-like structures and are localized to the crystalloid core.[47] Once secreted, MBP monomers become aggregated into toxic components.[48] MBP can damage cells and bacteria by disrupting cell membranes.[49] EDN is a potent ribonuclease effective against single-stranded RNA viruses.[50] ECP is another ribonuclease that is toxic to helminths, bacteria, and single-stranded viruses.[51, 52] EPX creates highly reactive oxidizing species that have the capacity to induce apoptosis and necrosis of cells and kill parasites.[53-55] The cytotoxic nature of the granule content can cause tissue damage and remodeling in the host during prolonged exposure, such as in asthma and eosinophilic esophagitis (EoE).

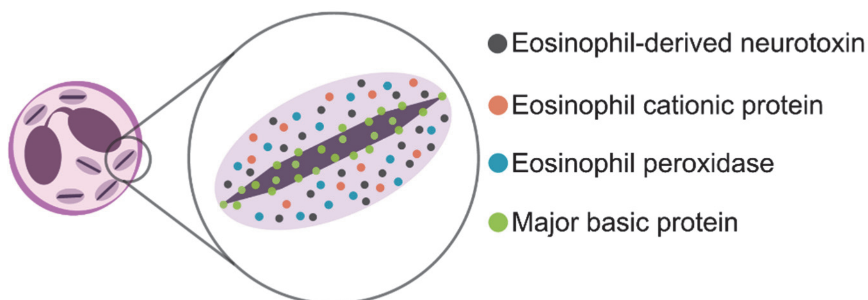


Figure 4. Eosinophil specific granule content includes the proteins eosinophil-derived neurotoxin, eosinophil cationic protein, and eosinophil peroxidase, which are located in the matrix of the specific granules, and major basic protein localized to the core of the granules.

GALECTIN-10

Galectin-10 is a hydrophobic protein that constitutes approximately 7-10% of the eosinophil's protein content.[56] Galectin-10 is mainly localized in the cytoplasm of the eosinophils but also in so called "primary granules" that have recently been redefined as an immature stage of the specific granules.[57, 58] It is a member of the galectin superfamily, but unlike most other galectins, it cannot bind β-galactoside-containing oligosaccharides and only has 7 of the 12 conserved amino acid residues in the carbohydrate recognition domain.[59] Galectin-10 lacks a signal peptide sequence and is thus not secreted via classical secretion. Instead, galectin-10 has been shown to be released following cytolysis in free form, in eosinophil extracellular traps (EETs), in membrane bound extracellular vesicles (EVs), and crystallized as Charcot-Leyden crystals.[60] Our group has demonstrated that eosinophils co-cultured with proliferating T cells released galectin-10 in a sequential process. First, galectin-10-containing immune synapses with T cells formed, secondly, galectin-10 was accumulated in "cap-like" formations, and finally, galectin-10 was released in EETs.[61]

Knowledge regarding the function of galectin-10 is incomplete, but the protein mediates T cell suppression in both Tregs and eosinophils.[45, 61, 62] Extracellular galectin-10 has the capacity to partly suppress T cell proliferation as has been shown using *in vitro* cultures of activated T cells and recombinant galectin-10, and also by administrating galectin-10-neutralizing antibodies to co-cultures of proliferating T cells and eosinophils.[45] In addition, CD16-positive suppressive eosinophils with high levels of galectin-10 are better suppressors of T cell proliferation than conventional eosinophils with lower levels of galectin-10.[45]

Even though the gene for galectin-10 is one of the most highly expressed in human eosinophils, it is absent in mice.[63-65] This is a prominent difference between mice and humans and a caveat to interpret results based on mouse models with caution.

PROTEIN SECRETION

Secretion of eosinophilic granule content occurs via four mechanisms. Classical exocytosis is a process of eosinophil degranulation where the content of single granules is released following fusion of granule and plasma membranes.[66] In compound exocytosis several granules fuse before the contents are released through a single fusion pore at the plasma membrane.[67] Piecemeal degranulation is a regulated release of granule proteins where parts of the granule content are captured in secretory vesicles, also named sombrero vesicles, and are transported to the plasma membrane.[68] Cytolysis is a non-apoptotic form of cell death and in this process whole intact granules are released into the extracellular space.[69]

Cytoplasmic and granule proteins can be secreted in EETs, i.e., released extracellular DNA strands of mitochondrial or nuclear origin, and have been proposed to be a defense against invading bacteria.[70-72] Mitochondrial EETs can be released by live eosinophils whereas chromatin EETs can be released during cytolysis where the plasma membrane and nucleus become disintegrated.[73]

HOMEOSTATIC FUNCTIONS

During homeostasis, eosinophils are mainly located in the non-esophageal portions of the gastrointestinal tract, thymus, bone marrow, lymph node, and spleen.[74, 75] Eosinophils can be found in lower numbers in other tissues including the lung, pancreas, adipose tissue, and uterus.[74, 75] These tissue-resident eosinophils display important homeostatic functions. Experiments have shown that in eosinophil-deficient mice, the intestinal mucus layer between the gut lumen and epithelial cells was reduced, despite no alteration in mucus-producing goblet cells, and that the composition of the microbiota of the gut lumen was altered.[76] The eosinophils were also necessary for the development and maintenance of IgA-expressing plasma cells in the gut and promoted IgA isotype switch[76]; secretory IgA has been shown to be central for removal of pathogenic microorganisms in the intestinal lumen.[77] In the mouse small intestine, these homeostatic functions of the eosinophil are believed to be mediated via IL-1β.[78] In the bone marrow of mice, eosinophils produce a proliferation-inducing ligand (APRIL) and IL-6 to support the survival of long-lived plasma cells.[79] Eosinophils in adipose tissue of mice produce IL-4 that sustain alternatively activated macrophages, which in turn maintain glucose homeostasis and prevent insulin resistance.[80]

T CELL REGULATION

Increasing amounts of evidence demonstrate the different ways eosinophils can regulate T cells. Their capacity to act as APCs has been demonstrated in mice where antigen-challenged eosinophils were able to induce antigen-specific CD4+ T cell proliferation within lymph nodes.[37, 81]

Multiple mouse studies have demonstrated the eosinophil's capacity to influence and downregulate T cell subsets. Small intestine eosinophils have been shown capable of downregulating Th17 cells via IL-1Ra production.[82] During nematode infections, eosinophils can suppress Th2 responses by downregulating IL-4.[83] In the lamina propria, eosinophils can induce differentiation of naïve T cells into Tregs in a process dependent on transforming growth factor-β1 or a rate-limiting step during synthesis of all-trans retinoic acid.[84] Recent data suggest that eosinophils contribute to the protection from experimental cerebral malaria by limiting CD8+ T cell infiltration of the brain.[85] Lung transplanted mice exhibited

T cell suppression via PD-L1/PD1-mediated synapse formation of T cells with eosinophils and suppression of T cell responses via inhibition of the T cell receptor (TCR)/CD3 subunit association.[86]

In humans, eosinophils have the capacity to suppress the proliferation of CD4+ and CD8+ T cells,[87] which is partly mediated via galectin-10.[45, 87] Galectin-10-mediated T cell suppression has been demonstrated to occur via synapse formation and by the release of galectin-10 containing EETs.[45, 61]

EOSINOPHILIC ESOPHAGITIS

Eosinophilic esophagitis (EoE) is a chronic inflammatory disease of the esophagus most commonly triggered by antigens in the food and by aeroallergens.[88, 89] The disease is mediated via Th2 responses,[90-92] and cannot develop in T cell deficient mice,[93] but is hallmarked by the substantial eosinophil infiltration of the esophagus (Figure 5). There is no cure for EoE and prolonged inflammation can lead to tissue fibrosis and consequently a narrowing of the esophagus, making swallowing and eating difficult.[94]

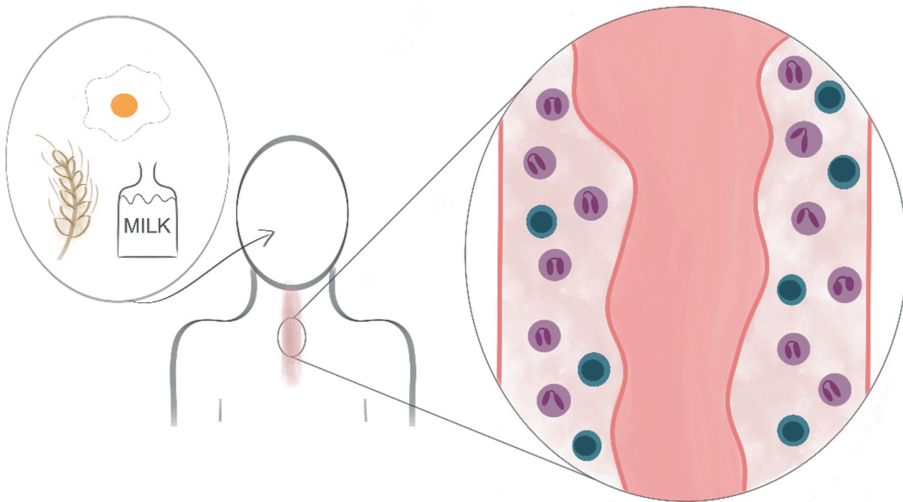


Figure 5. Overview of eosinophilic esophagitis. Antigens from food or the environment induce Th2 immune responses, which results in the recruitment and infiltration of eosinophils in the esophageal mucosa. Prolonged inflammation can result in fibrosis and narrowing of the esophagus.

EoE is more common in men than in women, 75% of the affected individuals are male,[95] and is manifested in both children and adults. The state of the disease fluctuates over time and symptoms can be managed by various treatment options including topical corticosteroids, proton pump inhibitors (PPIs), and dietary restrictions.[96, 97] Although EoE can lead to structural changes of the esophagus,

the disease is not associated with esophageal cancer.[98] The most common symptoms in adults include swallowing difficulties, food impaction, chest pain, and experiencing that food passes slowly through the esophagus.[99] Concomitant atopic disorders such as asthma, rhinitis, and eczema are generally more common in patients with EoE than the rest of the populace.[99] Individuals with EoE commonly adapt their eating behavior by eating slowly, drinking lots of water with a meal, cutting food into tiny pieces, or avoiding foods that are difficult to swallow.[100]

EoE is a relatively new disease with the first case reported in 1978,[101] and was described as a disease entity in 1993 and 1994.[102, 103] The prevalence of EoE in adults in Europe, North America, and Australia is 0.04%[104] but in Sweden it has been estimated to 0.4%.[105]

DIAGNOSIS

To set the diagnosis of EoE, both symptoms of esophageal dysfunction (commonly dysphagia in adults) and an eosinophilic inflammation of the esophageal mucosa are required. Endoscopic procedures are conducted to collect at least six biopsies from the proximal and distal parts of the esophagus, which are evaluated for eosinophil presence.[99, 106] A minimum of 15 eosinophils per high-power microscopic field (HPF) in one of the biopsies are required for diagnosis.[107] Histological assessment of the esophageal biopsies is routinely performed using hematoxylin-eosin stainings.[99] Although eosinophil infiltration of the esophagus is the requirement for diagnosis, other histologic findings are common. An increased presence of T cells,[90, 91] B cells,[108] basophils,[109] and mast cells[110] is associated with EoE and structural abnormalities of the tissue including basal layer hyperplasia, dilated intracellular spaces, and papillary elongations are common.[111, 112]

During endoscopy, manifestations of EoE-related inflammation, remodeling, and fibrosis in the esophagus may be found including furrows, strictures, rings, white plaques, and edema (Figure 6).[113-115] Following treatment of EoE, endoscopic procedures and histological evaluation of eosinophil infiltration of the esophageal mucosa are needed to evaluate the outcome of therapy.

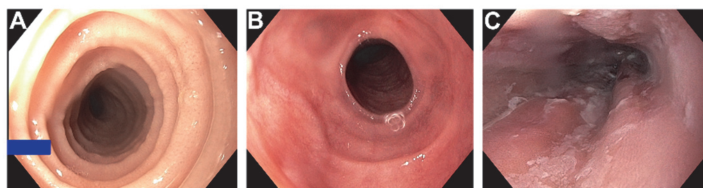


Figure 6. Endoscopic features of eosinophilic esophagitis include esophageal rings (A), strictures (B), and white plaques (C).

TREATMENT

EoE is chronic and there is no cure for the disease. However, anti-inflammatory treatment is used to alleviate symptoms and to dampen the eosinophil inflammation that can lead to fibrosis, tissue remodeling, and narrowing of the esophagus. The first-line treatment options include topical corticosteroids, PPIs, and dietary restrictions.[99] Cessation of treatment will most commonly result in relapse of inflammation and symptoms.[99, 116]

Topical corticosteroid treatment has been shown to alleviate symptoms, improve quality of life, reduce endoscopic findings, and reduce the eosinophil infiltration in the esophagus of patients with EoE.[116-120] A meta-analysis of placebo-controlled randomized clinical trials showed that histological remission to <15 peak eosinophils/HPF or a mean of 20 eosinophils/HPF was achieved in approximately 82% of the EoE patients treated with corticosteroids.[121] The treatment is given as swallowed topical corticosteroids including mometasone, budesonide, or fluticasone delivered either as orally disintegrating tablets, viscous slurry, suspension, or as spray.[99]

PPIs reduce the production of acid and has been demonstrated to downregulate the Th2 inflammation of the esophagus and reduce fibrosis. [122] [123] PPIs induce histological remission of eosinophil numbers in approximately 50% of the treated patients and improve the symptom burden in approximately 60% of patients with EoE.[124]

Since EoE is driven by the ingestion of allergens, diet alterations can be used as a treatment option. However, exclusion of specific foods based on skin allergy testing has low efficacy.[125] A six-food elimination diet where wheat, egg, cow's milk protein, peanut/tree nuts, soy, and seafood are excluded has been shown to induce histologic remission in 71% of adult patients.[125] Four-food and two-food elimination diets are two less restrictive alternatives, beginning with the elimination of the two most common causative allergens, wheat and milk.[126] Elemental diets based on amino acid formulas are the most efficient diets for inducing histologic remission (72% in adults[127] and 90% in children[125]) but are difficult to adhere to.[127] If a reasonable diet results in clinical and histological improvement, the patient is recommended to continue with the altered diet.

PATIENT-REPORTED OUTCOMES

Clinical symptoms of EoE can be evaluated using patient-reported outcome (PRO) instruments, i.e., questionnaires. The Eosinophilic Esophagitis Activity Index (EEsAI) is a PRO instrument developed for assessment of symptom severity in adult patients with EoE and is focused on dysphagia, eating habits, and

behavioral adaptations related to the disease.[128] The Swedish Watson dysphagia scale (S-WDS) is a PRO instrument validated for patients with esophageal cancer[129] that has been used for assessment of dysphagia in patients with EoE in Swedish research and care.[117, 130-133]

EOSINOPHILS IN EOSINOPHILIC ESOPHAGITIS

Increased blood eosinophil counts and prominent endoscopic alterations have been associated with more severe dysphagia in patients with EoE.[134] Eosinophils in the esophageal mucosa of patients with EoE can cause fibrosis development via cytokines including IL-4, IL-13, and transforming growth factor-β.[135-137] Reduced number of eosinophils in the esophageal mucosa following anti-IL-5 treatment with the drug mepolizumab resulted in reduced levels of transforming growth factor-β[138] and a double-blind randomized study of swallowed corticosteroids showed reduced remodeling in treated patients compared to placebo.[139] Tissue-resident eosinophils of patients with EoE have also been shown to produce EETs, their numbers increasing with increased eosinophil infiltration of the esophagus.[140]

Blood eosinophils of patients with EoE have an altered phenotype in relation to healthy controls with increased expression of CD11c, CD23, CD54, and CD294 and reduced expression of CD44 and CD193.[141] Additionally, a higher fraction of eosinophils expressing FOXP3 has been shown in patients with EoE compared to healthy controls.[142] Eosinophils in patients with EoE have been demonstrated to be inferior at suppressing T cell proliferation compared to healthy controls and a decreased capacity of their immunoregulatory function has been suggested.[142]

THE HUMAN THYMUS

The thymus is a primary lymphoid organ situated in the upper anterior part of the chest, in front of the heart.[143] The thymus is the center for T cell development and maturation. The thymus has two lobes, each containing two types of areas important for T cell development: medulla and cortex (Figure 7).[144] The cortex is a denser region mostly consisting of thymocytes, i.e., immature T cells, and cortical thymic epithelial cells.[144, 145] The medulla is less dense, comprising fewer thymocytes and medullary thymic epithelial cells.[144, 145] Other leukocytes are present in the thymus as well including macrophages,[146] dendritic cells,[147] B cells,[148] and eosinophils.[149, 150] Thymocyte developmental stages can be divided based on the expression of the co-receptors CD4 and CD8.

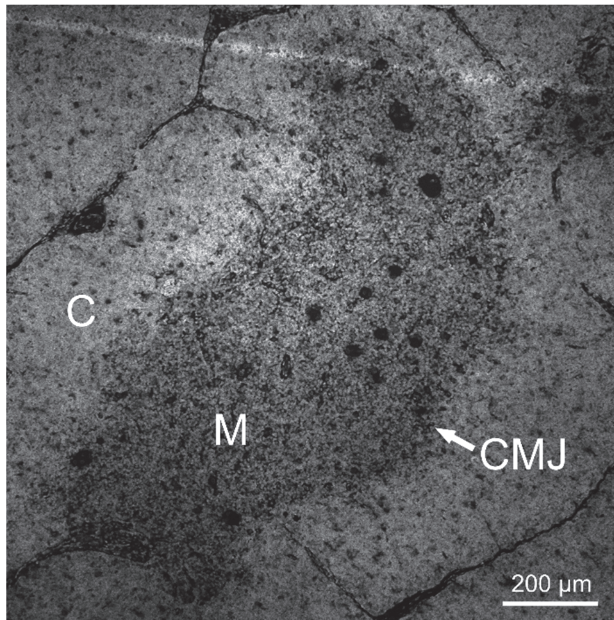


Figure 7. Confocal microscopy image of the human thymus showing the dense cortex (C), less dense medulla (M), and the region between the cortex and the medulla: the corticomedullary junction (CMJ).

THYMOCYTE DEVELOPMENT

Hematopoietic stem cells migrate from the bone marrow to the thymus where they enter into the region separating the cortex and medulla, the corticomedullary junction. Exposure to the thymic milieu initiates the Notch signaling pathway, which causes T cell lineage commitment.[151] The early CD4/CD8 double-negative (DN) thymocytes migrate to the cortex where they initiate construction of the T cell receptor (TCR). A pre-TCR is initially constructed following gene rearrangements of the β, γ, and δ genes and signaling through the pre-TCR initiates CD4 and CD8 expression, which results in CD4/CD8 double-positive (DP) thymocytes.[152] The α and β chains of the fully developed TCR are highly variable, theoretically, up to 10^{20} variants of the TCR are possible.[153]

The thymocytes then undergo a process called positive selection where thymocytes with TCRs that recognize and bind to self-peptides in complex with MHC molecules on cortical epithelial cells are provided with survival signals, whereas the thymocytes with receptors that don't recognize MHCs die by neglect.[154] The duration of the TCR and co-receptor binding to the peptide/MHC complex determines the CD4 or CD8 lineage commitment of the thymocytes (Figure 8).[155] During positive selection, the expression of the co-receptor CD8 is downregulated.[156] In thymocytes with TCRs recognizing MHC class I this will lead to cessation of the TCR/MHC engagement as CD8 no longer provides the

necessary stimulatory signals. As a result, the thymocytes will differentiate into CD8 single-positive (CD8 SP) thymocytes.[155] Signaling in thymocytes with TCRs specific for MHC class II on the other hand, will not be disrupted by the downregulation of CD8 as the CD4 binding to the MHC will remain intact. This will subsequently lead to the differentiation into CD4 single-positive (CD4 SP) thymocytes.[155]

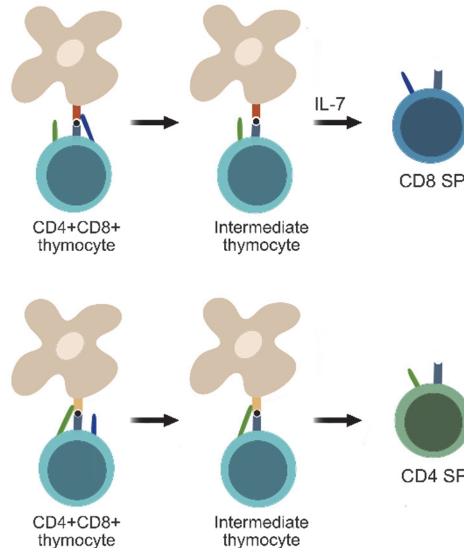


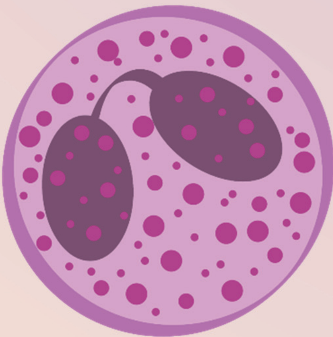
Figure 8. Schematic overview of CD4 and CD8 lineage commitment. The T cell receptor of the double positive thymocyte bind to the MHC/self-peptide complex. Signaling through T cell receptors specific for MHC class I is dependent on CD8 expression and will consequently cease when expression of CD8 is reduced. The cytokine IL-7 can induce co-receptor reversal (silencing of CD4 expression and re-expression of CD8) and these cells will consequently become CD8 single-positive cells (upper panel). For T cell receptors specific for MHC class II, the signaling is not dependent on CD8 and the signal will persist when CD8 expression is diminished. These cells will commit to the CD4 single-positive lineage (lower panel).

The single-positive thymocytes then migrate into the medulla where they go through a process called negative selection whereby potentially autoreactive thymocytes are removed by inducing apoptosis in thymocytes with high affinity for self-peptides presented by MHC molecules.[157] A subset of CD4 SP thymocytes with affinity for self-peptides develop into Tregs.[158, 159] Medullary thymic epithelial cells have the capacity to regulate negative selection via the expression of a vast array of self-peptides, so called tissue restricted antigens, due to a transcription factor named the autoimmune regulator (AIRE).[160, 161] Positively and negatively selected thymocytes are then released into the circulation as mature, naïve T cells. Of all the thymocytes, only 3-5% develop into mature T cells.[162, 163]

EOSINOPHILS IN THYMUS

Eosinophils are primarily located in the corticomedullary junctions and the medulla of the thymus and are occasionally found in the cortex.[149, 150] Approximately 30-50% of the eosinophils in the thymus are immature eosinophils of different developmental stages.[164] The physiologic role of the eosinophil in the thymus is not known but studies in mice suggest that eosinophils have a immunomodulatory role. In a MHC class I-restricted mouse model of negative selection, eosinophil numbers doubled following cognate peptide injection and the eosinophils were associated with clusters of apoptotic cells, this led the authors to interpret thymic eosinophils as immunomodulatory cells.[165] In eosinophil-deficient mice, apoptotic cell clearance in the thymus was reduced following experimentally induced cell death.[166]

In the human thymus, eosinophils have been shown to express indoleamine 2,3-dioxygenase and this expression was found to decrease with increased age.[167] The enzyme indoleamine 2,3-dioxygenase depletes tryptophan and initiates production of kynurenines, which induces T cell suppression and apoptosis, and favors suppression of Th1 cells over that of Th2 cells.[168] The eosinophil numbers in the thymus were found to correlate with thymic levels of IL-4, IL-5, and IL-13, which are cytokines associated with type 2 immune response.[167]



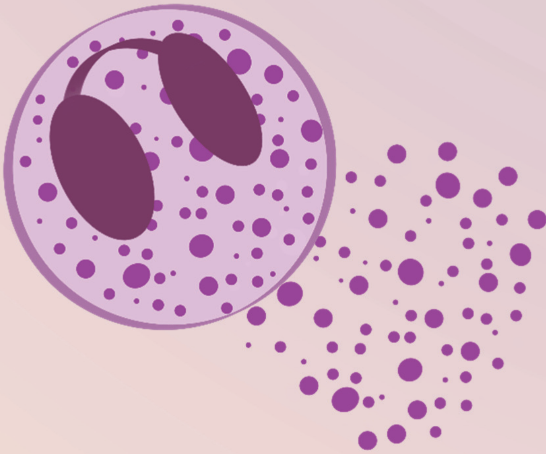
AIM

The aims of this thesis were to explore the role of eosinophils in relation to T cells within the healthy thymus and in the diseased esophagus of patients with EoE and to advance health care of patients with EoE in Sweden. To do so, the following hypotheses were stated:

- Blood-based immune parameters combined with patient-reported outcomes can be used to assess treatment outcome in patients with EoE.
- A Swedish translated version of the EEsAI instrument can be used to assess symptom severity in adult patients with EoE.
- Eosinophils suppress T cells in the esophageal mucosa of patients with EoE.
- Eosinophils in the thymus are involved in the thymocyte selection process.
- Eosinophils in the thymus are specialized cells with a different molecular expression pattern compared to eosinophils in blood.

The specific aims to test the hypotheses were:

- Develop an algorithm to monitor response to treatment in patients with EoE using blood-based biomarkers and patient-reported outcomes.
- Translate and validate the first Swedish PRO instrument developed for assessment of symptom severity in patients with EoE.
- Investigate eosinophil interactions with T cells, presence of suppressive eosinophils, and release of galectin-10 in the esophageal mucosa of patients with EoE.
- Describe the localization, cellular interactions, and possible function of eosinophils in the human thymus.
- Determine the phenotype and assess the maturity of thymic eosinophils.



PATIENTS AND METHODS

EOSINOPHILIC ESOPHAGITIS STUDIES

STUDY SUBJECTS AND SAMPLE TYPES

Adult patients with EoE from the NU Hospital Group, Trollhättan, Sweden and Skaraborg Hospital, Skövde, Sweden were studied in this thesis. Twenty patients completed a 2-month course of topical corticosteroid treatment (4 doses of 200 μg orally sprayed mometasone furoate per day). EDTA-blood (10 ml) and six esophageal biopsies from the distal and proximal esophagus taken during endoscopic procedures were donated before and after treatment. At least 15 eosinophils/HPF (0.229 mm^2) in one of the biopsies and symptoms of esophageal dysfunction were required for inclusion in the study based on the current diagnostic criteria.[99]

Ninety-seven of the 199 eligible adult patients with confirmed EoE at the NU hospital group and 97 age- and sex-matched controls with no history of esophageal disease were included for validation of the Swedish EEsAI (S-EEsAI).

All study participants provided informed consent and the studies were approved by the Regional Ethical Review Board of Gothenburg, Sweden (dnr 137-09, T664-11, 2019-00602/1116-18, and 2020-00668).

FLOW CYTOMETRY

Blood samples were analyzed by flow cytometry to investigate extracellular molecules on eosinophils, and to enumerate eosinophils and granulocytes, suppressive eosinophils, and CD4+ and CD8+ T cells.

Within 24 hours of donation, the erythrocytes in the blood were depleted using repeated hypotonic lysis with water. Samples were washed using Ca^{2+}-free Krebs-Ringer glucose (120 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 1.7 mM KH_2PO_4, 10.5 mM Na_2HPO_4, 10 mM glucose), incubated with Vivaglobin (1 mg/ml, CLS Behring, King of Prussia, PA, USA), 15 min dark at 4°C, and then incubated with fluorochrome-conjugated mAbs listed in Table 1 for 20 min, dark at 4°C. Cells were washed and resuspended in MACS-buffer (Ca^{2+}-free Krebs-Ringer glucose, 2.5 mM EDTA, 0.5% BSA, pH 7.3) before analysis using a FACSCanto II Flow Cytometer with FACSDiva Software 6.0 (BD Biosciences, Franklin Lakes, NJ, USA). The "Fluorescence minus one" concept,[169] and an isotype control (Table 1) were used to monitor for background staining. The cells were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA) and eosinophils were

identified based on high forward- and side-scatter (granulocytes), and high CD193 expression combined with higher side-scatter than CD16+ neutrophils.[45] T cells were gated based on low side-scatter and high CD3 levels, CD4+ and CD8+ T cells were then identified among the CD3+ T cells. Flow cytometry data are expressed as percent cells expressing a certain marker or as median fluorescence intensity.

FLUORESCENT IMMUNOHISTOCHEMISTRY

Fluorescent immunohistochemistry was used to analyze esophageal biopsies. Formalin-fixed paraffin-embedded tissue sections of 4 μm were deparaffinized in a Tissue-Tek Linearstain II instrument (Sakura, Alphen aan den Rijn, the Netherlands) using Histo-Clear. Antigen retrieval was predominantly performed using heat-induced epitope retrieval in a pressure cooker with EDTA Decloaker and Hot Rinse solution (Biocare Medical, Pacheco, CA, USA) and a wash with distilled water. However, for the analysis of galectin-10, proteolytic-induced epitope retrieval was utilized: the sections were washed with Tris-Buffered Saline (TBS) and incubated with a pronase solution (TBS, pronase [2 mg/ml, Sigma-Aldrich, St. Louis, MO, USA], 10 mM $C_2H_3NaO_2$, 5 mM $CaCl_2$), 20 min, room temperature (RT). The tissue sections were sequentially washed with TBS and TBS + 0.1% saponin (TBSS), incubated with blocking buffer (TBSS with 5% donkey serum [Sigma-Aldrich]) for 20 min, RT, incubated with primary antibodies listed in Table 1, (1 h, RT), and washed using TBSS. Incubation with secondary antibodies (Table 1) and Hoechst 34580 (Thermo Fisher Scientific, Waltham, MA, USA) was performed for 45 min, dark at RT. Consecutive washes with TBSS and TBS were done and slides were mounted with ProLong Diamond Antifade Mountant (Invitrogen, Carlsbad, CA, USA). Analyses of the biopsy sections were done using a Leica TCS SP5 confocal microscope and the Leica Application Suite X software (Leica Microsystems, Wetzlar, Germany) or with a LSM700 confocal microscope combined with ZEN software (Carl Zeiss AG, Oberkochen, Germany). Negative controls were included in the analysis in the form of isotype controls (Table 1) and exclusion of primary antibodies.

Table 1. Antibodies used for fluorescence based analyses of adult patients with EoE.

Antibody target	Clone	Isotype	Conjugate	Manufacturer	Analysis
CD3	SK7	Mouse IgG_1,κ	FITC	eBioscience	FC
CD4	SK3	Mouse IgG_1,κ	APC-H7	BD Biosciences	FC
CD4	pAb	Goat IgG	None	R&D Systems	IHC
CD8	SK1	Mouse IgG_1,κ	PE	BD Biosciences	FC
CD8	C8/144B	Mouse IgG_1,κ	None	Dako	IHC
CD16	3G8	Mouse IgG_1,κ	FITC	BD Biosciences	FC
CD16	DJ130c	Mouse IgG_1	None	Bio-Rad	IHC
CD25	2A3	Mouse IgG_1,κ	APC	BD Biosciences	FC
CD44	G44-26	Mouse IgG_{2b},κ	PE	BD Biosciences	FC
CD49d	9F10	Mouse IgG_1,κ	PE	BD Biosciences	FC
CD54	HA58	Mouse IgG_1,κ	APC	BD Biosciences	FC
CD66c	KORSA3544	Mouse IgG_1,κ	PE	BD Biosciences	FC
CD193	5E8	Mouse IgG_{2b},κ	BV421	BD Biosciences	FC
CD193	5E8	Mouse IgG_{2b},κ	AF647	BD Biosciences	FC
CD199	L053E8	Mouse IgG_{2a},κ	PE	BD Biosciences	FC
CD274	29E.2A3	Mouse IgG_{2b},κ	APC	BD Biosciences	FC
CD294	BM16	Rat IgG_{2a},κ	APC	BD Biosciences	FC
Galectin-10	B-F42	Mouse IgG_1,κ	None	Diaclone	IHC
Isotype control	MOPC-21	Mouse IgG_1,κ	FITC	BD Biosciences	FC
Isotype control	P3.6.2.8.1	Mouse IgG_1,κ	None	Thermo Fisher Sci.	IHC
Isotype control	eBM2a	Mouse IgG_{2a},κ	None	Thermo Fisher Sci.	IHC
Isotype control	pAb	Goat IgG	None	Thermo Fisher Sci.	IHC
Isotype control	pAb	Rabbit IgG	None	Southern Biotech	IHC
MBP	pAb	Rabbit IgG	None	Atlas antibodies	IHC
Goat	pAb	Donkey IgG	AF555	Thermo Fisher Sci.	IHC
Mouse	pAb	Donkey IgG	AF647	Thermo Fisher Sci.	IHC
Rabbit	pAb	Donkey IgG	AF488	Thermo Fisher Sci.	IHC

FC, flow cytometry; IHC, immunohistochemistry; MBP, major basic protein; pAb, polyclonal antibody; Sci., Scientific

PATIENT-REPORTED OUTCOMES

INSTRUMENTS

Eosinophilic Esophagitis Activity Index

The EEsAI is a PRO instrument developed for the assessment of symptom severity in adult patients with EoE,[128] which was translated to Swedish and validated in paper II. The EEsAI includes questions focused on both dysphagia and behavioral adaptations related to EoE. The EEsAI consists of five domains: "visual dysphagia question" (VDQ, 1 item), "avoidance, modification and slow eating score" (AMS, 4 items), "frequency of trouble swallowing" (Frequency, 1 item), "duration of trouble swallowing" (Duration, 1 item), and "pain when swallowing" (Pain, 1 item). A PRO score based on the five domains is calculated based on a scoring manual and ranges from 0-100,[128] where 100 represents maximum symptom severity. Two extra items are included in the instrument, but not in the PRO score, to check for jaw injuries and possible discrepancies in the VDQ domain. The items of the EEsAI are answered based on self-assessment of a seven-day period. The VDQ and AMS domains are centered around eight food consistencies: solid meat, soft foods, boiled rice, ground meat, white bread,

porridge, raw fibrous foods (e.g., apple), and French fries. The VDQ domain concerns the difficulty of swallowing these eight foods and is scored from 0-3. In the AMS domain behavioral adaptations (food consumption, avoidance of foods, modification of foods, and slow eating) are evaluated on a yes/no format. In the Frequency domain the rate of recurrence of the swallowing difficulties are evaluated on a scale ranging from never (=0) to every day (=3). The Duration domain scores the length of an episode of swallowing difficulties from no trouble (=0) to >5 minutes (=4). The final domain, Pain, is a yes/no item that explores if swallowing is a painful experience.

Watson Dysphagia Scale

The Watson dysphagia scale (WDS) is PRO instrument developed for the assessment of dysphagia induced by the ingestion of nine liquids and foods: water, milk, yoghurt, jam/jelly, omelet/mashed potatoes, boiled vegetables/potatoes/fish, soft white bread/pasta, fresh fruit, and meat.[170] A Swedish version of the WDS (S-WDS) has been devised and validated for assessment of dysphagia in patients with esophageal cancer.[129] The nine items of the instrument are answered on a 3-point Likert scale, demonstrating how often the swallowing difficulties occur: 0 (never), 0.5 (sometimes), and 1 (always). The answer (0, 0.5, or 1) is multiplied by the item number, i.e., item 1 (water) is multiplied by 1 and item 9 (meat) is multiplied by 9, resulting in a summarized score that ranges from 0-45, where higher score indicates more severe dysphagia. The S-WDS was distributed to patients with EoE in paper I before and after treatment.

European Organization for Research and Treatment of Cancer Quality of Life Questionnaire-Oesophageal Module 18

The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire-Oesophageal Module 18 (EORTC QLQ-OES18) instrument has been developed for the assessment of quality of life in patients with esophageal cancer.[171] The instrument consists of 18 items divided in 10 domains: dysphagia, swallowing saliva, choking, eating, dry mouth, taste, cough, speech, reflux, and pain. The items are answered based on a 1-4 scale where 1 represents "not at all" and 4 "very much". The score of each domain is transformed to a scale of 0-100 and is evaluated separately; no summary score of the entire instrument is calculated. A low score represents a low level of discomfort and higher scores represent increased discomfort. For this thesis, the EORTC QLQ-OES18 instrument was distributed to patients with EoE before and after treatment in paper I and it was included for the validation of the S-EEsAI in paper II.

36-Item Short Form Health Survey

The 36-Item Short Form Health Survey (SF-36) is an instrument developed for the assessment of quality of life,[172] and was distributed to patients with EoE before and after treatment in paper I. The instrument comprises 36 items in eight domains: limitations in physical activities, limitations in social activities, limitations in usual roles due to physical problems, limitations in usual roles due to emotional problems, bodily pain, vitality, mental health, and general health. Two scores are summarized and calculated based on a scoring manual[173]: the "Physical Component Summary" and "Mental Component Summary". The two scores range from 0-100 where high scores indicate high levels of well-being.

TRANSLATION AND VALIDATION

In paper II, the EEsAI was translated to Swedish (S-EEsAI) and validated for the assessment of dysphagia in adult Swedish patients with EoE. Translation from English to Swedish was performed with the forward-backward translation method, based on international and World Health Organization recommendations.[174, 175] Autonomous Swedish translations were provided from two English-proficient, native Swedish speakers with competence in medical care and terminology. An expert panel of three individuals provided a consensus version based on the two translations, which was subsequently retranslated to English via a bilingual, English native speaker unacquainted with the instrument. The Swedish version was also adapted to suit Swedish food culture. The Swedish version was tested in a pilot study of 10 adult Swedish patients with EoE. The patients filled out the instrument and answered a semi-structured phone interview a week later. The semi-structured interview contained open questions about the phrasing, how easy the instrument was to interpret, and how relevant the items appeared. The S-EEsAI was then slightly modified based on comments and suggestions received during the pilot study. The final version of the S-EEsAI was validated by allowing 97 adult patients with EoE recruited via telephone and 97 esophagus healthy controls fill out the S-EEsAI, together with the EORTC QLQ-OES18 and 20 supplementary questions. Two weeks after the completion of the set of instruments, 29 randomly chosen patients were asked to fill out the instruments once more for a test-retest evaluation.[176] The provided answers were then used to evaluate the reliability, reproducibility, and validity of the S-EEsAI as described in the "Statistics" section.

THYMIC STUDIES

STUDY SUBJECTS AND SAMPLE TYPES

Removal of the thymus during corrective heart surgery of children with congenital heart defects is performed approximately 200 times/year in Sweden as the thymus blocks the access of the heart.[177] In this thesis, thymuses removed during cardiac surgery and 2 ml heparinized venous blood from a total of 34 children under the age of 5 at the Queen Silvia Children's Hospital, Sahlgrenska University Hospital, Gothenburg, Sweden were studied. The thymic tissue was either processed to yield single cell suspensions (shredded thymus injected with FACS buffer [PBS, 10 % heat-inactivated fetal calf serum, 2 mM EDTA, 10mM Hepes] was pressed through a 40-µm cell strainer), 4 µm formalin-fixed paraffin-embedded tissue sections, or 7 µm frozen optimal cutting temperature (OCT) compound-embedded tissue sections.

All study participants provided informed consent via their legal guardians and the studies were approved by the Regional Ethical Review Board of Gothenburg, Sweden (dnr 217-12).

FLUORESCENCE-ACTIVATED CELL SORTING

Single cell suspensions of thymic tissue were Ficoll-Paque (Cytvia/GE Healthcare Life Sciences, Marlborough, MA, USA) gradient-separated and the mononuclear and polymorphonuclear cell fractions were sequentially washed using PBS and MACS-buffer. The samples were stained using Fixable Viability Dye eFluor 780 (eBioscience, San Diego, CA, USA) for 30 min in the dark, 4°C, and washed with MACS buffer. The samples were incubated with antibodies listed in Table 2 for 40 min, dark at 4°C. The samples were washed, resuspended in MACS-buffer and subjected to cell sorting using a Sony SH800 cell sorter (Sony Biotechnology, San Jose, CA, USA) or a BD FACSAria Fusion (BD Biosciences). Populations of DP, CD4 SP, and CD8 SP thymocytes and eosinophils were collected.

FLOW CYTOMETRY

Eosinophils in thymic cell suspensions and venous blood were analyzed by flow cytometry (n=9), as were sorted CD3/CD28-stimulated DP, CD4 SP, and CD8 SP thymocytes cultured with or without sorted thymic eosinophils (10:1 ratio, n=2).

Eosinophils in venous blood and thymic cell suspensions were enriched via Ficoll-Paque gradient separation, the blood samples were lysed using water, the samples were then washed with PBS, and incubated with Fixable Viability Dye eFluor 780

for 30 min, dark at 4°C. The samples were washed with MACS buffer, incubated with Vivaglobin (1 mg/ml), 15 min dark at 4°C, then stained for extracellular markers using antibodies in Table 2 (20 min, dark at 4°C), and washed with MACS buffer. Permeabilization and fixation were done with Foxp3/Transcription Factor Staining buffer (eBioscience), 25 min dark at 4°C. The samples were subsequently incubated with mAbs for staining of intracellular molecules (Table 2), 30 min dark at 4°C, washed with permeabilization buffer, and resuspended in MACS buffer. A FACSCanto II Flow Cytometer with FACSDiva Software 6.0 was used to analyze the samples and gating was done in FlowJo version 10.7.1. Eosinophils were gated based on high forward- and side-scatter and high MBP expression. The voltages of the side- and forward-scatter were decreased to identify thymic eosinophils. Data are presented as median fluorescence intensity.

Sorted thymic cells were resuspended in x-vivo buffer (Lonza, Basel, Switzerland) supplemented with gentamicin (50 μg/ml, Gibco/Thermo Fisher Scientific), transferred to an anti-CD3-coated (1μg/ml, Table 2) 96-well culture plate, anti-CD28 (8 μg/ml, Table 2) was added, and the cells were incubated for 36 h, 37°C before staining for flow cytometry analysis as described in the paragraph above. Lymphocytes were identified in FlowJo based on side- and forward-scatter and thymocyte populations were subsequently gated based on CD4 and CD8 expression. Data are presented as percent of thymocytes.

IMAGING FLOW CYTOMETRY

Thymic cell suspensions and the polymorphonuclear cell fractions of Ficoll-Paque-separated thymic cell suspensions were analyzed with imaging flow cytometry (n=8). The cells were stained according to the flow cytometry procedure presented in the "Thymic studies" section using antibodies listed in Table 2. Analyses were performed using an ImageStream X Mk II imaging flow cytometer (Amnis, Seattle, WA, USA) and IDEAS software v. 6.0., where single cells and doublets expressing galectin-10+ were gated.

CONFOCAL MICROSCOPY

Single cell suspensions of thymus (n=3) and sorted thymic eosinophils (n=2) were separately investigated using a LSM700 confocal microscope and ZEN software.

Single cell suspensions of thymic tissue were seeded onto x-well cell culture chambers (Sarstedt, Nümbrecht, Germany). The culture chambers were either uncoated or coated with anti-CD3. Anti-CD28 was added to initiate CD3/CD28 stimulation and the slides were incubated for 90 min at 37°C to allow for synapse formation. The excess liquid was removed and Cytofix/Cytoperm (BD Biosciences) was added for 20 min, RT. The cells were incubated with antibodies

listed in Table 2, Hoechst 34580, and phalloidin (Invitrogen) for 30 min, dark at RT. The slides were mounted using ProLong Diamond Antifade Mountant and subsequently analyzed.

X-well cell culture chambers containing sorted thymic eosinophils resuspended in x-vivo with gentamicin (50 μg/ml) were incubated overnight at 37°C. The cells were fixed and stained as stated in the paragraph above with antibodies listed in Table 2.

FLUORESCENT IMMUNOHISTOCHEMISTRY

Thymic tissue sections were analyzed using fluorescent immunohistochemistry (n=14). Formalin-fixed paraffin-embedded thymic tissues were analyzed according to the procedure described in the EoE studies section above. Frozen OCT compound-embedded thymic sections were analyzed in the same manner, not including deparaffinization and antigen retrieval, which were substituted with 10 min rehydration in TBSS. The tissue sections were stained with Hoechst 34580 and antibodies presented in Table 2.

Table 2. Antibodies used for fluorescence-based analyses of thymus tissue.

Antibody target	Clone	Isotype	Conjugate	Manufacturer	Analysis
CD3	OKT3	Mouse IgG_{2a},ϰ	None	eBioscience	CM, IHC, stim
CD3	UCHT1	Mouse IgG_1,ϰ	PE	BioLegend	ISX
CD4	pAb	Goat IgG	None	R&D Systems	IHC
CD4	RPA-T4	Mouse IgG_1,ϰ	FITC	BD Biosciences	FACS, FC
CD8	C8/144B	Mouse IgG_1,ϰ	None	Dako	IHC
CD8	RPA-T8	Mouse IgG_1,ϰ	PerCP-Cy5.5	BD Biosciences	FACS, FC
CD28	CD28.2	Mouse IgG_1,ϰ	None	Invitrogen	stim
CD34	pAb	Sheep IgG	None	R&D Systems	IHC
CD34	8G12	Mouse IgG_1,ϰ	PerCP-Cy5.5	BD Biosciences	FC, ISX
CD86	BU63	Mouse IgG_1	None	Abcam	IHC
CD193	5E8	Mouse IgG_{2b},ϰ	BV421	BioLegend	FACS, FC
EMR1	A10	Hamster IgG	APC	Bio-Rad Laboratories	FC
Foxp3	PCH101	Rat IgG_{2a},ϰ	None	Thermo Fisher Sci.	IHC
Galectin-10	B-F42	Mouse IgG_1,ϰ	APC[b]	Diaclone	CM, FC, ISX
Goat	pAb	Donkey IgG	AF555	Thermo Fisher Sci.	IHC
Isotype control	P3.6.2.8.1	Mouse IgG_1,ϰ	None	Thermo Fisher Sci.	IHC
Isotype control	eBM2a	Mouse IgG_{2a},ϰ	None	Thermo Fisher Sci.	IHC
Isotype control	pAb	Rabbit IgG	None	Southern Biotech	IHC
Isotype control	eBR2a	Rat IgG_{2a},ϰ	None	Thermo Fisher Sci.	IHC
Isotype control	pAb	Sheep IgG	None	Thermo Fisher Sci.	IHC
Isotype control	pAb	Goat IgG	None	Thermo Fisher Sci.	IHC
Ki-67	B56	Mouse IgG_1,ϰ	None	BD Biosciences	IHC
Ki-67	20Raj1	Mouse IgG_1,ϰ	PE	Invitrogen	FC
MBP	pAb	Rabbit IgG	None	Atlas antibodies	FC, IHC, ISX
MBP	pAb	Mouse pAb	FITC	LSBio	FC, ISX
Mouse	pAb	Donkey IgG	AF647	Thermo Fisher Sci.	IHC
Rabbit	pAb	Donkey IgG	AF488	Thermo Fisher Sci.	IHC
Rabbit	pAb	Donkey IgG	BV421	BioLegend	FC, ISX
Rat	pAb	Donkey IgG	AF647	Abcam	IHC
Sheep	pAb	Donkey IgG	AF546	Thermo Fisher Sci.	IHC
Siglec-8	7C9	Mouse IgG_1,ϰ	APC	BioLegend	FACS, FC

CM, confocal microscopy of cell suspension; FACS, fluorescence-activated cell sorting; FC, flow cytometry; IHC, immunohistochemistry; ISX, imaging flow cytometry, MBP, major basic protein; pAb, polyclonal antibody; Sci., Scientific; Stim, CD3/CD28 stimulation.
[a]APC conjugation performed in-house, unconjugated by vendor.

CYTOMETRY BY TIME-OF-FLIGHT

Phenotyping and in-depth analyses of thymic eosinophils were done by cytometry by time-of-flight (CyTOF) analysis of peripheral blood leukocytes and thymic cell suspensions (n=7) within 24 hours of donation. The blood samples were lysed with ammonium chloride, 10 min, RT. Both sample types were washed with PBS and Maxpar PBS (Fluidigm, South San Fransisco, CA, USA) and 5×10^6 thymic cells and 4×10^6 blood cells were incubated with 10 μM Cell ID Cisplatin (Fluidigm) for 5 min, RT. Two consecutive washes (300g, 5 min) with Maxpar Cell Staining buffer (CSB, Fluidigm) were performed and the samples were incubated with an antibody cocktail (Table 3) for 30 min, RT: samples were vortexed after 15 min. Two washes with CSB, fixation using 1.6% formaldehyde (10 min, RT), centrifugation (800g, 5 min), and permeabilization with Foxp3/Transcription Factor Staining buffer (1 h, RT) were done. The samples

were sequentially washed with CSB and permeabilization buffer (eBioscience), incubated with an antibody cocktail against intracellular molecules (1.5 h, RT, Table 3), and washed with permeabilization buffer and CSB. The samples were incubated for 45 min, RT with 125 nM intercalation solution (Maxpar Fix and Perm Buffer, Cell-ID Intercalator-Ir [125 μM], Fluidigm), washed twice with CSB, and stored overnight at 4°C in Maxpar PBS. Centrifugation of the samples and resuspension at a concentration of 1×10^6 cells/ml (blood) or 2×10^6 cells/ml (thymus) in Maxpar Cell Acquisition Solution (CAS, Fluidigm) were performed. 0.1X EQ Four Element Calibration Beads (Fluidigm) were added and samples were analyzed using a Helios CyTOF instrument complemented with CyTOF Software v7.0. (Fluidigm). Gating of eosinophils was performed in FlowJo: DNA-expressing cells were separated from beads, intact cells were singled out, events with event length >35 were removed, live cells were identified, granulocytes (CD45+, CD66b+) were separated from lymphocytes, and eosinophils were identified by high expression of CD294 and Siglec-8.

Sorted CD3/CD28-stimulated DP, CD4 SP, and CD8 SP thymocytes cultured with or without sorted thymic eosinophils (10:1 ratio) were also analyzed using CyTOF (n=3). These cells were stained according to the same procedure as stated above, with antibodies listed in Table 3. Thymocytes were gated in a similar manner to eosinophils but instead of granulocytes, lymphocytes were located (CD45+, CD66b-) and thymocyte populations were identified based on CD4 and CD8 expression.

Table 3. Antibodies used for CyTOF experiments and clustering analyses.

Metal label	Antibody	Clone	Source
89Y	CD45[a,b]	HI30	Fluidigm
106Cd	IgG[c,d]	G18-145	In house
110Cd	RANKL	#685857	In house
111Cd	CD69	FN50	In house
112Cd	CD31	WM59	In house
113Cd	CD1a	HI149	In house
114Cd	CD5	UCHT2	In house
116Cd	IgA[c,d]	HP6123	In house
141Pr	CD196/CCR6	G034E3	Fluidigm
142Nd	CD19	HIB19	Fluidigm
143Nd	CD127/IL-7Ra	A019D5	Fluidigm
144Nd	CD38	HIT2	Fluidigm
145Nd	IL-5R	26815	In house
146Nd	IgD[c,d]	IA6-2	Fluidigm
146Nd	CD8[e]	RPA-T8	Fluidigm
147Sm	CD11c	Bu15	Fluidigm
148Nd	CD274	29E.2A3	Fluidigm
149Sm	CD34	581	Fluidigm
150Nd	CD44	IM7	Fluidigm
151Eu	CD123/IL-3R	6H6	Fluidigm
152Sm	TCRgd	11F2	Fluidigm
153Eu	CD185/CXCR5	RF8B2	Fluidigm
154Sm	CD3	UCHT1	Fluidigm
155Gd	CD45RA	HI100	Fluidigm
156Gd	CD14	HCD14	Fluidigm
158Gd	CD27	L128	Fluidigm
159Tb	FOXP3	PCH101	Fluidigm
160Gd	CD28	CD28.2	Fluidigm
161Gd	CD294[a]	BM16	In house
162Dy	CD66b[a,b]	80H3	Fluidigm
163Dy	CD183/CXCR3	G025H7	Fluidigm
164Dy	Siglec-8[a]	7C9	Fluidigm
165Ho	CD45RO	UCHL1	Fluidigm
166Er	CD24	ML5	Fluidigm
167Er	CD197/CCR7	G043H7	Fluidigm
168Er	CD8[c,f]	SK1	Fluidigm
168Er	CD199[e]	L053E8	Fluidigm
169Tm	CD25	2A3	Fluidigm
170Er	Galectin-10	B-F42	In house
171Yb	CD20[f]	2H7	Fluidigm
172Yb	IgM[c,d]	MHM-88	Fluidigm
172Yb	Ki-67[e]	B56	Fluidigm
173Yb	HLA-DR	L243	Fluidigm
174Yb	CD4	SK3	Fluidigm
175Lu	CD193	5E8	Fluidigm
176Yb	CD56	NCAM16.2	Fluidigm
Ir191	DNA1[a,b]	-	Fluidigm
Ir193	DNA2[a,b]	-	Fluidigm
Pt195	Cell-ID Cisplatin[a,b]	-	Fluidigm
209Bi	CD16	3G8	Fluidigm

[a]Used for gating eosinophils, excluded from viSNE and clustering
[b]Used for gating thymocytes, excluded from viSNE and clustering
[c]Only used for analysis of blood and thymic eosinophils
[d]Excluded from all data analyses
[e]Only used for analysis of cultivated thymocytes
[f]Excluded from viSNE and clustering of eosinophils

STATISTICS

CELL COUNTS

Enumeration of eosinophils and T cells in fluorescently stained tissue sections was performed using macros and the Cell Counter plugin in the Fiji ImageJ software,[178] and by using ZEN software (Carl Zeiss AG).

Cell numbers and cell-cell interactions in cell suspensions analyzed by confocal microscopy were counted using ZEN software.

Cell interactions visualized using imaging flow cytometry were enumerated using IDEAS software (version 6.0). The interactions of the galectin-10+ eosinophils with CD3+ thymocytes were examined.

UNIVARIATE AND BIVARIATE ANALYSES

For comparisons of two groups, the Wilcoxon matched-pairs signed rank test (paired groups), Student's t-test (paired groups), and Mann-Whitney *U* test (unpaired groups) were used. For unpaired categorical data, the Fisher´s exact test, Chi-square test, and Mantel–Haenszel test were used. In paper I and III, correlations between data sets were determined using Spearman correlation. *P*-values <0.05 were considered as statistically significant. The GraphPad Prism software (versions 7.0, 8.4.2, 9.1.2, and 9.2.0, GraphPad, San Diego, CA, USA) and IBM SPSS Statistics software (version 25, IBM, Armonk, NY, USA) were used for the statistical computations.

RELIABILITY, REPRODUCIBILITY, AND VALIDITY OF THE S-EESAI

For the validation of the S-EEsAI in paper II, the reliability, reproducibility, and validity of the instrument were evaluated. Reliability and reproducibility were evaluated based on a Cronbach's alpha coefficient, which reflects the internal consistency of the instrument, Pearson correlation between items and domains, and based on intraclass correlation coefficients (ICCs) derived from test-retest results using the two-way mixed-effects model and absolute agreement.[179] Construct validity was investigated to evaluate if the instrument measured the intended construct,[176] i.e., in the case of S-EEsAI, symptom severity in patients with EoE. This was done using Spearman correlations between the S-EEsAI domain/PRO scores and the domain scores of the EORTC QLQ-OES18. The validity of the S-EEsAI was also evaluated using Spearman correlation with a self-assessment score of disease severity and by determining ceiling and floor effects of the items, i.e., the fraction of responses which were of the maximum and minimum scoring options, respectively.

Cut-off values for the different methods were set as follows: Cronbach's alpha coefficients >0.7 were considered to indicate satisfactory internal consistency[176]; Pearson correlation coefficients ≥0.6 were considered strong, 0.4–0.59 moderate, and ≤0.39 weak correlations[180]; ICCs >0.75 were considered to indicate excellent reliability and 0.4–0.75 good reliability[179]; Spearman correlation coefficients >0.7 were considered strong, 0.3–0.7 moderate, and <0.3 weak correlations.[181]

MULTIVARIATE ANALYSES

ORTHOGONAL PROJECTION TO LATENT STRUCTURES

Orthogonal Projection to Latent Structures (OPLS) is a multivariate analysis of pattern recognition.[182] In OPLS, the relationship between a Y-variable and multiple X-variables is examined. In paper I, the outcome variable Y was set to "histologic response to therapy" of the study subjects and X-variables included questionnaire scores, clinical data, and immune parameters. The resulting model from an OPLS analysis is described by R^2Y and Q^2Y values. R^2Y is an estimation of the variance in Y explained by the X-variables and is referred to as the explanatory power of the model. A high R^2Y value indicates that the generated model is capable of explaining differences between groups. Q^2Y is the measurement of robustness of the model and is determined by repeated calculations of the model's predictive capacity to separate the groups when one study subject at a time is excluded. A high Q^2Y value shows that the model is stable, independent of which subject that is excluded from the model. The Variable Importance Parameter (VIP) module was used to assess the contribution of each X-variable to the model and to enable the exclusion of low-impact parameters. The program also generates a loading plot that graphically demonstrates the impact of each X-variable on the model. The OLPS analyses were done using the SIMCA-P statistical package version 13.03 (MKS Data Analytics Solutions, Malmö, Sweden).

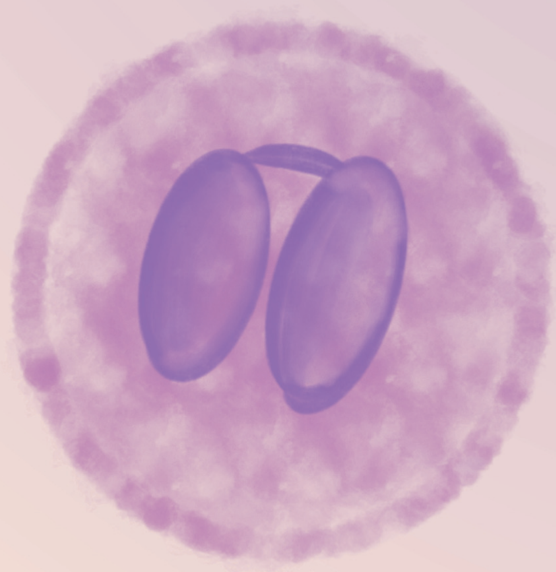
VISNE

viSNE is a tool developed to map high-dimensional single-cell data in two dimensions.[183] It is based on the t-Distributed Stochastic Neighbor Embedding (t-SNE) algorithm and results in a map where phenotypically similar cells are clustered together in so called "islands". In paper V, pre-gated eosinophils and thymocytes were subjected to viSNE dimensionality reduction, respectively. Proportional event sampling (due to small and dissimilar sample sizes) and default advanced settings (1000 iterations, 30 perplexity, and 0.5 theta) on the Cytobank platform were used.[184] Channels used for the mapping are presented in Table 3.

X-SHIFT

X-shift is a clustering algorithm that identifies and quantifies the cell subsets within a population.[185] X-shift is based on weighted k-nearest-neighbor density estimation.[186] The density estimate for each data point is calculated and the local density maxima in a nearest-neighbor graph is identified and converted to cluster centroids. Density-ascending paths in the graph then connect the residual data points to the centroids, which results in clusters. To ensure that the clusters are unique density-separated populations, the algorithm checks for density minima between the neighboring centroids and, if necessary, merges them. Different numbers of nearest neighbors are tested to yield the optimal number of cell clusters. The results can be visualized using different methods, including minimum spanning trees and module maps. Within each cluster, it is possible to identify each individual contributing cell.

In paper V, pre-gated eosinophils and thymocytes, respectively, were analyzed using the X-shift clustering algorithm of the VorteX software. Recommended settings were used,[187] and the import max was set to 200 with "num. neighbors for density estimate (K)" as 150 to 5, 30 steps. Channels used for clustering are listed in Table 3.



RESULTS

PAPER I

In paper I, blood parameters and PRO scores from 20 adult patients with EoE were investigated to evaluate if these data could be used to assess treatment outcome following 2 months of topical corticosteroid therapy.

A MULTIVARIATE MODEL FOR THE SEPARATION OF RESPONDERS AND NON-RESPONDERS TO TREATMENT IN EOSINOPHILIC ESOPHAGITIS

We investigated if a multivariate model based on a combination of blood-based immune parameters, clinical data, and PRO results had the capability to separate responders to treatment from non-responders to treatment. A model including 26 parameters with an explanatory power of 50% and a robustness of 27% was able to separate responders from non-responders (Figure 9).

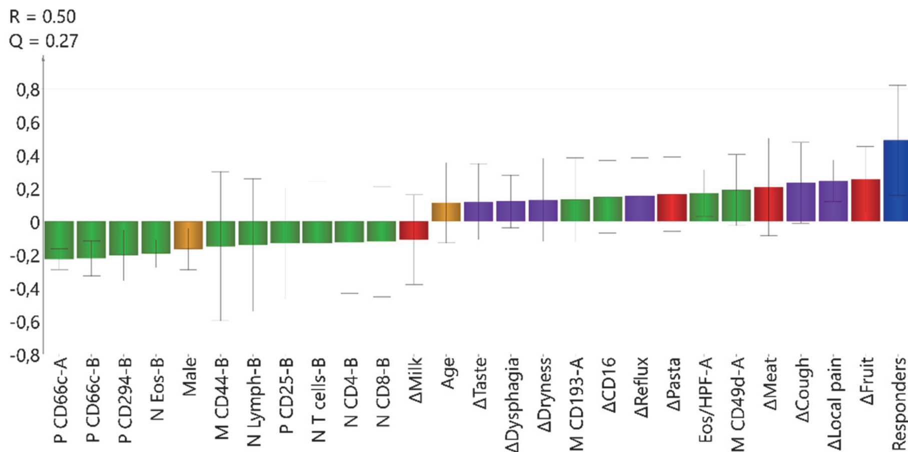


Figure 9. Loading plot for the OPLS multivariate model. The Y-variable, i.e., histological response to topical corticosteroid treatment, is presented as a blue bar to the far right. X-variables are shown as red (Watson dysphagia scale items), purple (EORTC QLQ-OES18 domains), green (blood-based immune parameters), and orange (clinical data) bars. Bars in the same direction as the Y-variable were positively associated with being a responder to treatment and bars in the opposite direction were negatively associated. Abbreviations include "A": before treatment, "B": after treatment, "P": percent of blood eosinophils, "M": median fluorescence intensity, "⊿": difference in values before and after treatment. The figure is reprinted from the published article "Patient-Reported Outcomes and Blood-Based Parameters Identify Response to Treatment in Eosinophilic Esophagitis" by Lingblom et al., i.e., paper I of this thesis, which is licensed under CC BY-NC 4.0.

PARAMETERS ASSOCIATED WITH RESPONDERS AND NON-RESPONDERS

The responders were associated with increased median expression of CD49d and CD193 on blood eosinophils before initiation of treatment, higher numbers of eosinophils/HPF before treatment, and a reduced fraction of CD16+ eosinophils post-treatment. In addition, improvement regarding nine PRO scores was associated with response to treatment. The stronger associations were for decreased scores of the pain, cough, and reflux domains of the EORTC QLQ-OES18 instrument and of item 8 (fruit) and item 9 (meat) of the S-WDS.

Non-responders were associated with high absolute numbers of lymphocytes, T cells, CD4+ T cells, CD8+ T cells, and eosinophils in the blood post-treatment. Furthermore, the fractions of CD66c positive eosinophils were higher for the non-responders pre- and post-treatment, the post-treatment fractions of CD25+ and CD294+ eosinophils in the blood were higher, and so was the median eosinophil expression of CD44. Male sex was also associated with non-response to treatment.

SUPPRESSIVE BLOOD EOSINOPHILS

Before initiation of treatment, the fractions of CD16-expressing eosinophils in the blood correlated with the absolute numbers of blood CD3+ T cells (r=0.47, *P*=0.036) and CD4+ T cells (r=0.53, *P*=0.016).

PAPER II

In paper II, we translated, culturally adapted, and validated a Swedish version of the EEsAI. The goal was to validate the first Swedish PRO instrument for the assessment of symptom severity in adult patients with EoE and consequently improve the monitoring and assessment of symptoms in Swedish care.

TRANSLATION AND CULTURAL ADAPTATION OF THE EOSINOPHILIC ESOPHAGITIS ACTIVITY INDEX

The S-EEsAI was adapted from the original EEsAI to better suit Swedish food culture. The cultural adaptations included the exchange of "grits" to "porridge/oatmeal", "dry rice (grains don't stick) or stocky Asian rice" to "boiled rice", and "pudding, jelly, and apple sauce" to "pudding, omelet and mash". For further clarity, the pictures displaying two of these food categories in the instrument were updated (Figure 10).

Figure 10. New images included in the S-EEsAI as a part of the cultural adaptation of the instrument. The foods show an omelet instead of the original pudding (left) and oatmeal instead of grits (right).

VALIDATION OF THE SWEDISH EOSINOPHILIC ESOPHAGITIS ACTIVITY INDEX

The initial pilot study with 10 respondents showed that the definition of "mild difficulties" in the VDQ domain needed to be clarified and the definition was thus elaborated to contain "slow passage of food when swallowing".

In the final validation with 97 patients and 97 esophagus healthy controls, the reliability, reproducibility, and validity of the instrument were evaluated. Statistical analyses related to reliability and reproducibility resulted in Cronbach's alpha values of 0.83 and 0.85 for the VDQ and AMS domains respectively, an ICC of 0.89 for the total PRO score, and predominantly medium and strong Pearson correlations between items and their corresponding domain (7/8 for the VDQ

domain and 20/24 for the AMS domain). Analysis of construct validity using Spearman correlation between the S-EEsAI domain/PRO scores and EORTC QLQ-OES18 domain scores resulted in moderate and weak correlations. The moderate correlations were found for the S-EEsAI domain/PRO scores and the dysphagia, choking, eating, and pain domain scores of the EORTC QLQ-OES18 and weaker correlations were dominant for the swallowing saliva, dry mouth, taste, cough, speech, and reflux domain scores of the EORTC QLQ-OES18. Additionally, Spearman correlation between the S-EEsAI PRO score and a self-assessment score of disease severity was of moderate strength ($r=0.68$, $P<0.001$).

Validity was further investigated by examining floor and ceiling effects: item minimum scores were more common than maximum scores. The score distribution of the S-EEsAI PRO score for the EoE patient cohort is presented in Figure 11A. The median PRO score was 30/100. The S-EEsAI domain and PRO scores of the patients were compared to the scores obtained by the esophagus healthy control group. Statistically significant differences between the two groups were found for all scores ($P=0.013$ for the Duration domain and $P<0.001$ for the remaining domains and the PRO score). Pervading higher scores were recorded for the patients with EoE and the median PRO score of the esophagus healthy control group was 0. The score distribution of the S-EEsAI PRO score for the esophagus healthy control group is presented in Figure 11B.

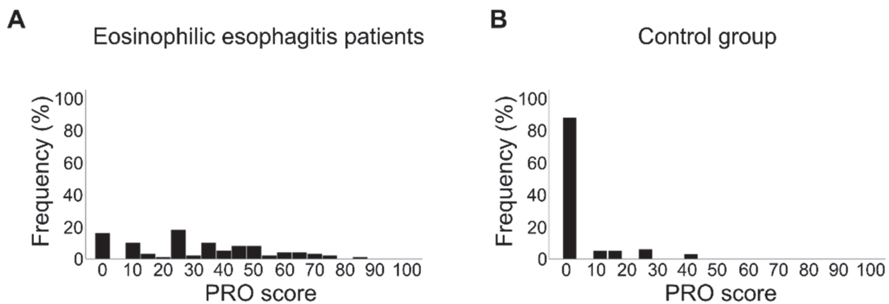


Figure 11. S-EEsAI PRO score distribution for (A) patients with EoE (n=97) and (B) the esophagus healthy control group (n=97).

PAPER III

In paper III, esophageal biopsies from 20 adult patients with EoE before and after treatment were analyzed using fluorescent immunohistochemistry to investigate the relationship of eosinophils with T cells and their possible suppression of T cells within the esophageal mucosa.

EOSINOPHILS AND T CELLS IN THE ESOPHAGEAL MUCOSA OF PATIENTS WITH EOE

Quantification of CD4+ T cells, CD8+ T cells, and eosinophils revealed a significant threefold decrease in numbers of CD4+ T cells (P=0.030) and a twofold decrease of CD8+ T cells (non-significant) after successful treatment. Eosinophil numbers in the esophageal mucosa of the group of responders to treatment also correlated with the number of CD4+ and CD8+ T cells before and after treatment (r=0.49, P=0.0068 and r=0.43, P=0.019, respectively).

Less than a tenth of the eosinophils in the esophageal mucosa of untreated patients were found to be in direct contact with either CD4+ or CD8+ T cells (Figure 12). However, CD4+ T cell numbers were found to be 1.2-fold higher (P=0.032) in the most eosinophil dense regions compared to the eosinophil deprived regions of the investigated tissue section. A similar but non-significant increase in cell numbers was seen for the CD8+ T cells.

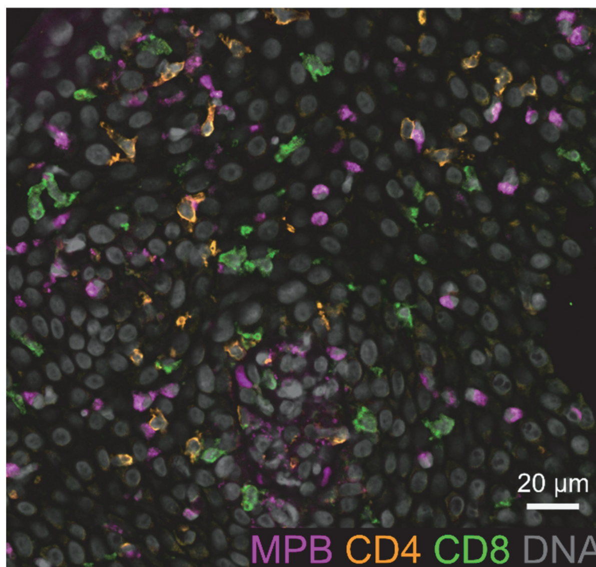


Figure 12. Dispersion of eosinophils (MBP, magenta), CD4+ T cells (orange), and CD8+ T cells (green) in in the esophageal mucosa of an unsuccessfully treated patient with EoE.

SUPPRESSIVE EOSINOPHILS

Studies of CD16 expression showed that suppressive eosinophils were located in the esophageal mucosa of the patients. The median fraction of CD16+ eosinophils before treatment was 28% (25-75 percentile: 12-47%). The fraction of CD16+ eosinophils was reduced for the responders but not for the non-responders after treatment. In addition, the fractions of CD16+ eosinophils were lower in the esophageal mucosa than in the blood of the responders after treatment.

RELEASED GALECTIN-10

Extracellular galectin-10 was a common finding in the esophageal biopsies taken before treatment (Figure 13). The galectin-10 was found in extracellular vesicles (EVs) and in net-like structures, i.e., trails of galetin-10 originating from the cells similar in appearance to EETs but without DNA. The galectin-10-containing EVs were large (median diameter: 2.2 µm, 25-75 percentile: 1.6-2.6 µm) and frequent in the esophageal mucosa (median: 109 EVs/mm^2, 25-75 percentile: 48-176 EVs/mm^2). The net-like structures were less common: 19 structures/mm^2 (median, 25-75 percentile: 11-26 structures/mm^2). After treatment, the levels of free galectin-10 in the esophageal mucosa of the responders were vastly reduced with a median of 0 EVs/mm^2 (25-75 percentile: 0-24 EVs/mm^2) and 0 net-like structures/mm^2 (25-75 percentile: 0-0 structures/mm^2). Correlations between the number of eosinophils/mm^2 and both the EVs/mm^2 and net-like structures/mm^2 were found ($r=0.65$, $P<0.0001$ and $r=0.62$, $P<0.0001$, respectively).

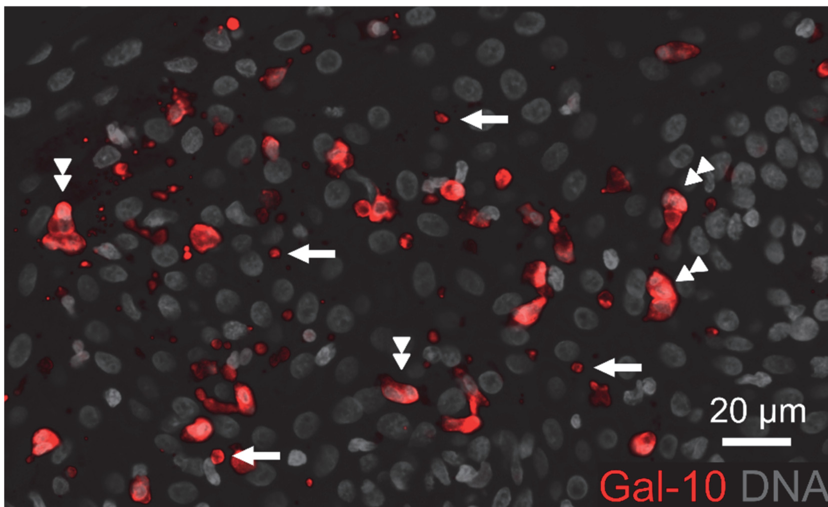


Figure 13. Esophageal tissue stained for galectin-10 (gal-10, red) shows cell-bound and extracellular galectin-10. Arrows indicate extracellular vesicles and double arrowheads cell-bound galectin-10.

PAPER IV

The T cell regulatory properties of peripheral eosinophils made us want to study eosinophils within the human thymus and to investigate possible immunomodulatory functions of thymic eosinophils.

DETERMINATION OF EOSINOPHIL LOCALIZATION

Using fluorescent immunohistochemistry stains of thymic tissues (n=7) we could characterize the anatomical distribution of eosinophils within the human thymus. The majority of the eosinophils were located in the corticomedullary junctions, followed by the medulla, cortex, and interlobular septa (Figure 14).

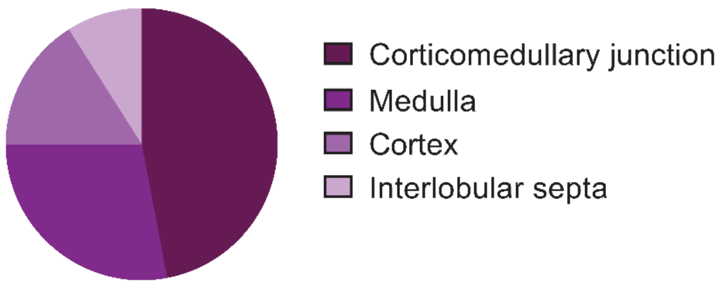


Figure 14. Pie chart illustrating eosinophil anatomic distribution in the human thymus.

INITIAL EOSINOPHIL CHARACTERIZATION

A preliminary phenotypic study of the eosinophils in the thymus was conducted to investigate possible differences compared to their blood counterpart. Initially, the eosinophil population within the human thymus was noted to consist of different maturational stages, in contrast to blood where eosinophils are of a phenotypically mature state.[13] Confocal microscopy studies of thymic cell suspensions (n=3) revealed that a third of the eosinophils were immature cells of different maturational stages (Figure 15).

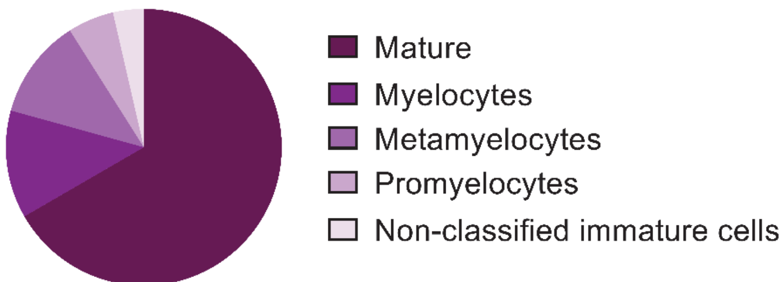


Figure 15. Pie chart displaying the fractions of eosinophils of different maturational stages in the human thymus.

CD34+ early eosinophil precursors were also found in the thymus: 8% (median, 25-75 percentile: 3-11%) of the eosinophils in the thymic tissue sections expressed the CD34 molecule and were primarily found in the interlobular septa (n=6). Flow cytometry analyses (n=9) further showed that the median expression levels of the maturation markers Siglec-8 and EMR1 were reduced in the thymus compared to blood (P=0.0078 and P=0.039, respectively).

Since there were immature eosinophils in the thymus, we wanted to investigate if the eosinophils were capable of proliferation within the human thymus. Using immunohistochemistry, we found that a median of 25% (25-75 percentile: 24-29%) of the eosinophils in the thymus expressed the proliferation marker Ki-67.

Because the thymus is dedicated for T cell development, we also wanted to see if eosinophils in the thymus expressed the T cell co-stimulatory marker CD86. In thymic tissue sections (n=5), a median of 42% (25-75 percentile: 27-56%) of the eosinophils expressed the marker CD86 and two thirds of these CD86+ eosinophils were found within the medulla of the thymus.

CELLULAR INTERACTIONS OF EOSINOPHILS AND THYMIC CELLS

As peripheral eosinophils have the capacity to interact with and modulate the function of effector T cells and Tregs we sought to investigate the co-localization and interaction of eosinophils with these cells in the human thymus. Co-localization was studied using immunohistochemistry (Figure 16) where we found that 81% of the studied eosinophils were located next to CD3+ thymocytes (n=5) and 7% of the eosinophils were located in direct proximity to FOXP3+ Tregs (n=5). In addition, the eosinophils appeared in a neat line in the border between the CD4 SP and CD8 SP thymocytes in the medulla and the DP thymocytes of the cortex.

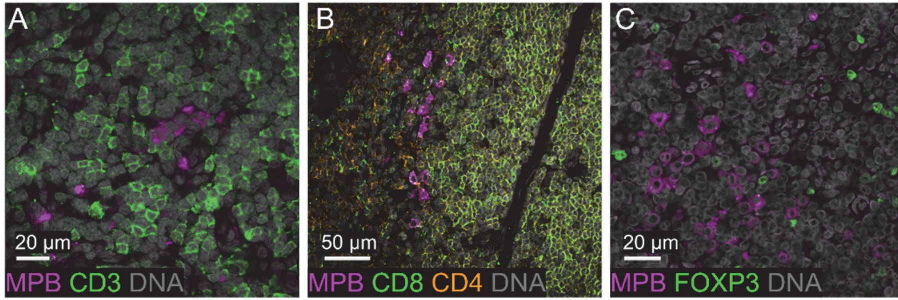


Figure 16. Fluorescent immunohistochemistry stains of thymic tissue sections showing eosinophil co-localization with CD3+ thymocytes (A), CD4+/CD8+ thymocytes (B), and FOXP3+ regulatory T cells (C).

The ability of thymic eosinophils to form synapses with mononuclear cells was studied using confocal microscopy of thymic cell suspensions (n=5). The eosinophils were found to form actin-containing synapses with small mononuclear cells (Figure 17). For unstimulated cells a median of 1.2 actin synapses/eosinophil (25-75 percentile: 0.4-2.3 synapses/eosinophil) were found and for CD3/CD28-stimulated cultures there were 1.8 actin synapses/eosinophil (25-75 percentile: 1.1-2.8 synapses/eosinophil).

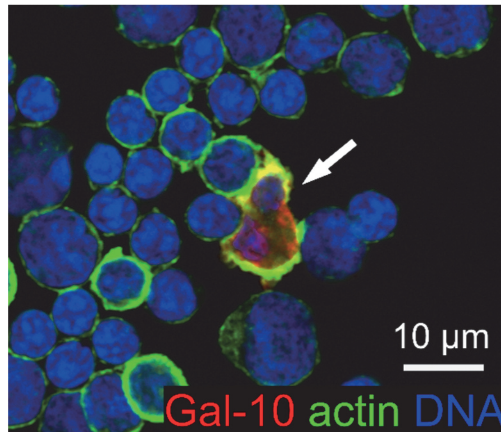


Figure 17. Eosinophils form synapses with mononuclear thymic cells in single cell suspension. A confocal image of an eosinophil (indicated with an arrow, red: galectin-10, blue: DNA) forming synapses (green: actin) with three mononuclear cells.

Eosinophil interactions with CD3+ thymocytes (n=3) were further studied using imaging flow cytometry of thymic cell suspensions. Eosinophil and thymocyte interactions were frequent, 74% of the eosinophil interactions were with CD3+ thymocytes.

PAPER V

In paper V, the hypotheses that thymic eosinophils differ from blood eosinophils and that thymic eosinophils have a role in the thymocyte selection process were tested further.

PHENOTYPING THYMIC EOSINOPHILS

In paper IV we saw that eosinophils in the thymus appeared to have a different phenotype compared to eosinophils in the blood. We therefore set out to conduct an in-depth phenotyping analysis of thymic eosinophils using CyTOF (n=7) and compare them to blood eosinophils to investigate possible differences. Univariate analysis showed that nine surface molecules were expressed by either a higher or lower fraction of thymic eosinophils compared to their blood counterpart (Figure 18).

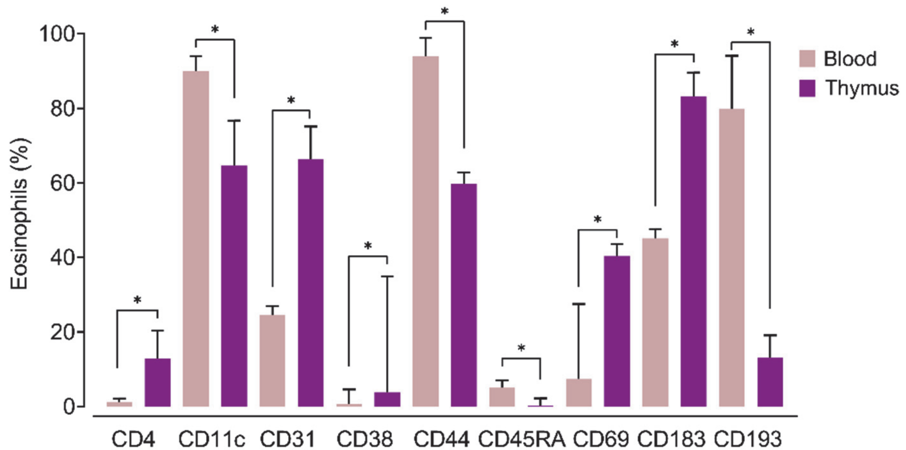


*Figure 18. Bar graphs of fractions of eosinophils from seven thymectomized children expressing the markers CD4, CD11c, CD31, CD38, CD44, CD45RA, CD69, CD183, and CD193 in blood and in thymus. Bar graphs represent medians with interquartile range. * represents P-values <0.05.*

The CyTOF data were subjected to viSNE dimensionality reduction and clustering analysis using the X-shift algorithm. In the resulting viSNE map, blood eosinophils from the donors were located in different islands, whereas the majority of thymic eosinophils were localized in one island, indicating a conserved phenotype of thymic eosinophils for all study participants. X-shift clustering analysis revealed seven eosinophil sub-populations within the blood and thymus samples. Three of these populations were more frequent in thymus samples and two were more frequent in blood. The most prominent of the blood-specific populations made up 58% of the blood eosinophils but only 1% of the thymic

eosinophils. Conversely, the most prominent thymus-specific population made up 57% of the thymic eosinophils and 24% of the blood eosinophils. The most common thymus-specific eosinophil population expressed higher levels of CD24, CD25, CD38, CD44, CD69, CD197, and of galectin-10 and lower levels of CD11c, CD193, and Siglec-8 than the most common blood-specific eosinophil population (Figure 19).

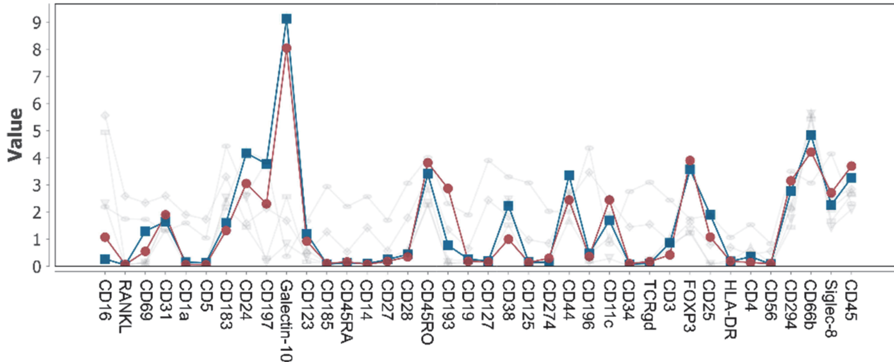


Figure 19. Expression profiles of the most frequent populations in thymus (blue) and blood (red).

Together, the three thymus-specific populations made up 93% of the thymic eosinophils, the corresponding fraction of these eosinophil populations in blood was 33%. The smallest thymus-specific population (5% of the thymic eosinophils) was almost absent in the blood (0.1%) and demonstrated increased expression of CD16 and less of FOXP3 and galectin-10 than the other two thymus-specific eosinophil populations (Figure 20).

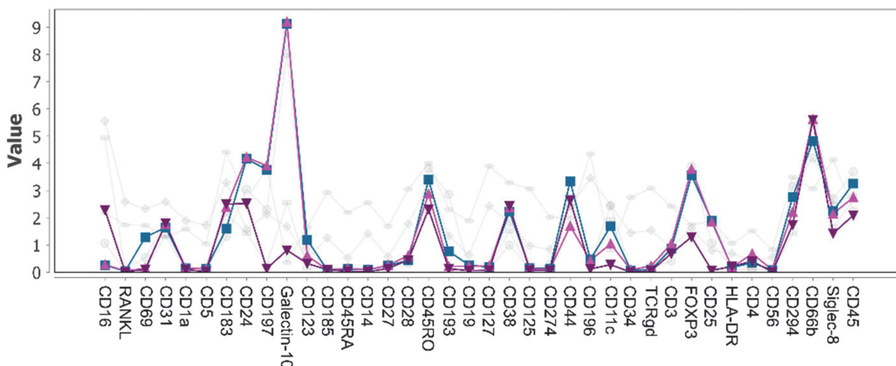


Figure 20. Expression profiles of the eosinophil populations specific for thymus. The blue and pink profiles correspond to the most common and second most frequent eosinophil populations, respectively. The purple line represents the smaller eosinophil population that was specific for thymus.

EOSINOPHIL INFLUENCE ON THYMOCYTES

Since thymic eosinophils mainly reside in the border between the DP and SP thymocytes, we were interested to study the influence of thymic eosinophils on thymocytes and to see if thymic eosinophils have the capacity to affect the expression of CD4 and CD8 on thymocytes. Therefore, we sorted thymic eosinophils, DP thymocytes, CD4 SP thymocytes, and CD8 SP thymocytes, cultivated the thymocytes in the presence or absence of eosinophils, and subsequently investigated the thymocyte distributions based on CD4 and CD8 expression using flow cytometry and CyTOF. X-shift clustering analysis of all cultivated thymocytes analyzed by CyTOF (DP, CD4 SP, and CD8 SP cultivated with and without eosinophils) showed that five thymocyte populations were present: CD4 SP, CD8 SP, DN, DP, and a DP dim population (Figure 21).

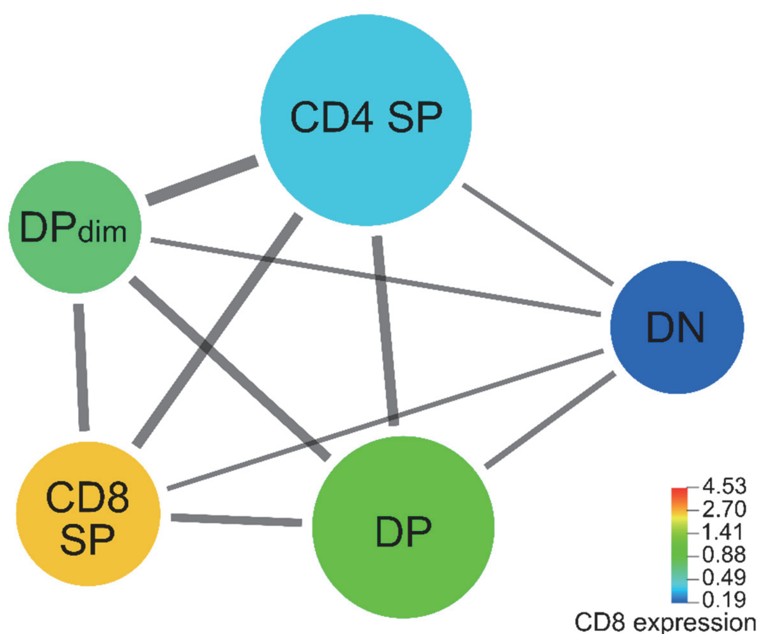


Figure 21. Module map showing the thymocyte populations in all cultivated samples analyzed by CyTOF. The size of the nodes reflect population density and the color shows the expression of CD8 in each of the five populations. The lines (edges) show how the different populations are linked and thicker edges correspond to more of links between the nodes.

Using a crude minimum spanning tree of only the CD4 SP and CD8 SP populations identified in the X-shift analysis, we could see that both the CD8 SP population size and the CD8 expression were diminished for the thymocytes cultivated with eosinophils whereas no change in CD4 expression levels and a slight increase in the CD4 SP population size were noticeable in the presence of eosinophils (Figure 22).

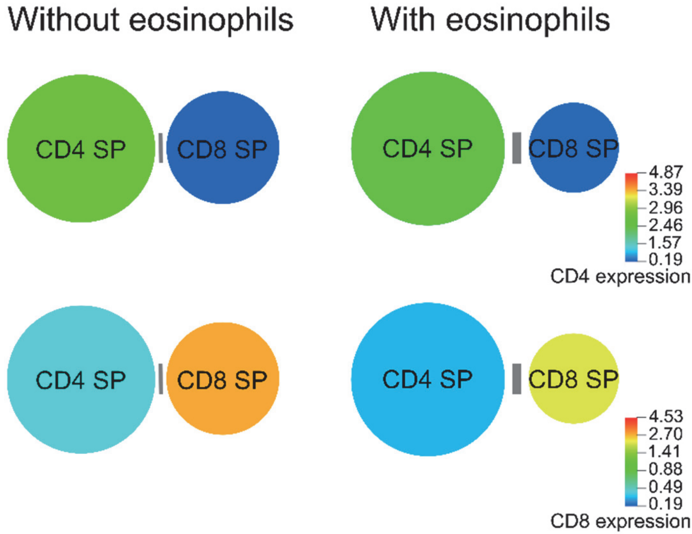


Figure 22. Crude minimum spanning tree with only the CD4 SP and CD8 SP populations derived from the X-shift clustering analysis. CD4 (upper panel) and CD8 (lower panel) expression levels are shown for thymocytes cultivated without or with eosinophils. The size of the clusters represents population size where a large size indicates a large thymocyte population and a smaller size a smaller population.

The thymocyte and eosinophil co-cultures were also analyzed by comparing the fractions of DN, DP, CD4 SP, and CD8 SP thymocytes present after cultivation as determined by flow cytometry and CyTOF (n=5). Differences between samples cultivated with eosinophils and those cultivated without were noted. For the sorted DP thymocytes, lower fractions of DN thymocytes (median 2% vs. 17%) and higher fractions of CD4 SP thymocytes (median 13% vs. 4%) were found in the presence of eosinophils. The sorted CD8 SP thymocyte cultures showed a reduced fraction of CD8 SP thymocytes when co-cultured with eosinophils (median 55% vs. 77%). The CyTOF data also showed a tendency for reduced fractions of DP thymocytes expressing the IL-7 receptor CD127 (Figure 23).

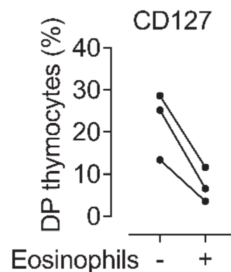
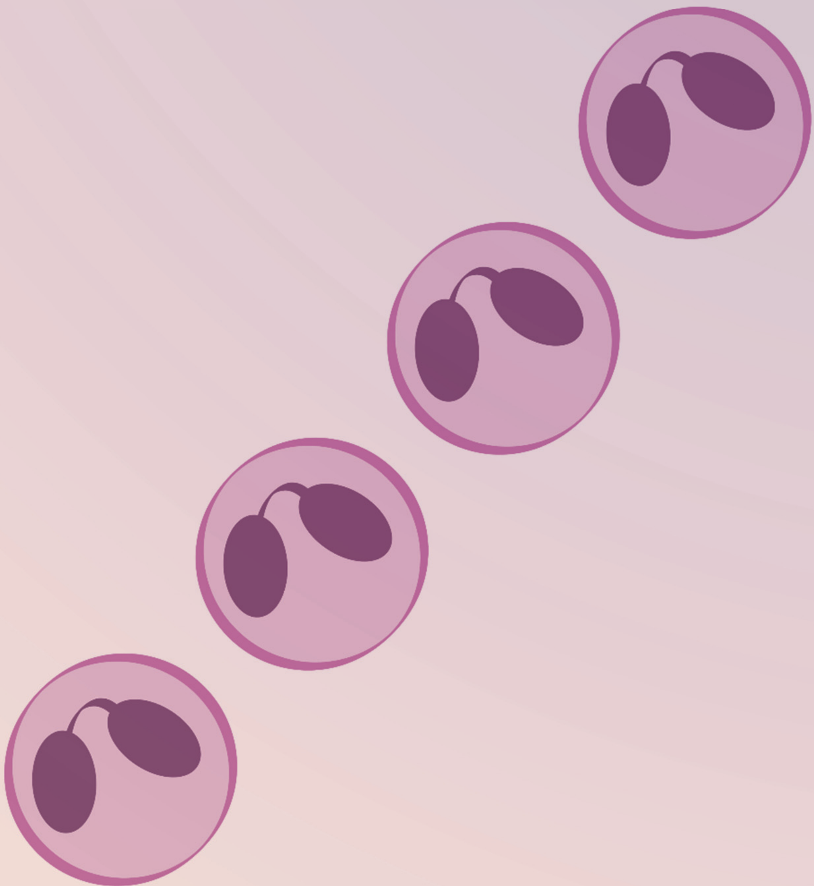


Figure 23. Graph of the fractions of sorted DP thymocytes expressing the molecular marker CD127 as analyzed by CyTOF after cultivation with or without eosinophils (n=3).



DISCUSSION

In this thesis, eosinophils present in the esophageal mucosa of patients with EoE and within the human thymus have been studied. The properties of the eosinophils have been investigated and characterized along with explorations on the relationship between eosinophils and T cells within these tissues. The aim was to increase knowledge on eosinophil function, specifically the T cell regulatory capacity of eosinophils, but also to improve treatment and patient care of patients with EoE.

The discovery of a blood-based biomarker panel for the detection of EoE could improve diagnosis and treatment follow-up but has eluded researchers for years.[188, 189] In paper I, we included blood-based immune parameters, clinical data, and PROs for the development of a multivariate model aimed to separate patients with histologic response to treatment from non-responders. The use of both pre- and post-treatment data was required for the generation of a sufficiently good model. That PRO scores were the most strongly associated parameters with response to topical corticosteroid treatment was initially surprising as PROs have been shown to not properly reflect histologic response.[190-192] However, the VIP module of the statistical program used for the computation of the model, removes parameters of low-impact and this could explain why we were able to identify certain PRO domain and item scores that were important for the separation of responders and non-responders. In addition, a previous study by our group using multivariate models showed that topical corticosteroid treatment did not result in the regress of the activated blood eosinophil phenotype associated with EoE and that the eosinophil phenotype of treated EoE patients could not be distinguished from that of untreated EoE patients.[193] In fact, this study showed that only CD18 was expressed in higher levels on the blood eosinophil surface of untreated patients.[193] In light of this, it is not surprising that only a few blood-based parameters and more questionnaire data were associated with responders in our model in paper I and that more frequent associations with blood-based parameters were found for the non-responders. It should be emphasized that corticosteroid treatment does not cure EoE nor abolish the underlying stimulatory trigger of this allergic condition.

We had hypothesized that alterations in the level of CD16+ suppressive eosinophils in the blood of EoE patients could be indicative of response to treatment in paper I. Indeed, a change in fraction of suppressive eosinophils was one of the three blood based parameters associated with response to treatment. The decreased fractions of suppressive eosinophils in the blood following

successful treatment could indicate a reduced "need" for these cells, which have been shown to have an increased capacity for T cell suppression.[45]

The difference in scores before and after treatment of individual items from the S-WDS were a part of the model in paper I. Even though we transformed the answers to each item into scores and tested them in our multivariate models, it is the S-WDS final score that is intended to be used for assessment of dysphagia and not individual item scores. By looking at individual item scores, however, we were able to see that it might be valuable for clinicians to ask patients pointed questions regarding specific foods when evaluating the severity of EoE and response to treatment.

The key steps for diagnosing EoE involves both high eosinophil infiltration of the esophageal mucosa and symptoms of esophageal dysfunction. This is why PRO instruments were included in the generation of models in paper I. However, the R^2Y and Q^2Y values of the model could possibly be increased by the use of PRO instruments specifically focused on EoE. No scores from the SF-36 instrument contributed to the model, four out of nine items from the S-WDS did, and the EORTC OES-QLQ18 domains that contributed were the dysphagia, pain, reflux, cough, dryness, and taste domains. This indicates that more specific PRO instruments would improve models of treatment response in EoE as meat and fresh fruit are often difficult for patients to swallow and dysphagia, pain, and reflux are known symptoms of EoE.[194] Indeed, in the validation of the S-EEsAI in paper II we saw that both the dysphagia and pain domain scores demonstrated moderate correlations to several S-EEsAI domain and PRO scores, indicating that these are good for EoE symptom assessment. The S-EEsAI instrument has been specifically developed for EoE and employing a PRO instrument validated for the disease in question would be beneficial and could result in even more stable and reliable models. In future studies, the inclusion of the S-EEsAI could thus result in refinement of the EoE treatment response models if domains regarding behavioral adaptations and the frequency and duration of swallowing difficulties were to be included.

In paper II, the calculations of construct validity showed that the strongest correlations, albeit of moderate strength, of the S-EEsAI domain/PRO scores were with the scores of the EORTC QLQ-OES18 domains most commonly associated with EoE. Weaker correlations were found for domain scores whose constructs are not as commonly associated with EoE,[195] the one exception being the reflux domain where we would have anticipated a stronger correlation. However, the S-EEsAI does not contain any domains that directly measure reflux. It is also important to bear in mind that the weak and moderate correlations most likely arose since the EORTC QLQ-OES18 is not an EoE instrument.

The reliability and repeatability of the S-EEsAI were deemed as acceptable with high Cronbach's alpha and ICC values. Only the Pearson correlation between softer foods of item 2 (porridge), 4 (porridge), and 6 (boiled rice, white bread, soft foods) of the S-EEsAI showed weak correlations to their corresponding domains. However, because these food consistencies are soft and un-textured and since bulky textured foods, such as meat, are generally more difficult for patients with EoE to consume,[196] high domain scores can still be obtained by scoring high for high-textured foods and low for soft textured foods which could explain these results.

A drawback of the S-EEsAI is the ambiguous layout of the AMS domain, which can be interpreted differently by different individuals and made the results harder to interpret and sometimes led to missing data. We provided an electronic version of the S-EEsAI where such ambiguity was reduced. The esophagus healthy control group used the electronic version to a larger extent than the patient group and this resulted in shorter answering times and no missing data.

The translation from English to Swedish and the few cultural adaptations of the S-EEsAI made in paper II are not believed to have had a negative impact on the accuracy of the instrument. This is reflected by the results of the validation that showed that the S-EEsAI is an appropriate instrument for the use of assessing symptom severity in patients with EoE.

There are several advantages of having a Swedish instrument for the assessment of symptom severity in adult patients with EoE. We believe that an instrument specifically designed for EoE symptom assessment will provide more accurate interpretation of the symptoms compared to instruments that have not been developed nor validated for EoE. This could be beneficial for diagnosing new patients, for evaluation of treatment response, and for the follow up of previously diagnosed patients with EoE. In research, the use of the S-EEsAI could provide more reliable results, better interpretation of the disease state, and results that can be compared across studies.

The investigation of eosinophils and T cells within the esophageal mucosa of patients with EoE in paper III showed that eosinophils and especially CD4+ T cells appeared to be associated. For the patients who responded to treatment, the levels of CD4+ T cells were reduced after treatment and the CD4+ T cells were more numerous in eosinophil-dense regions compared to eosinophil-scarce regions. Actually, CD4+ effector Th2-like cells and Tregs have been found to be enriched in the esophagus of patients with EoE, using single-cell RNA sequencing, combined with an increased production of the Th2 cytokine IL-5.[91]

Additionally, CD4+ T cells have been shown to have a bigger impact than CD8+ T cells on the development of experimental EoE in mice.[93]

In paper I we saw that the fractions of CD16+ suppressive eosinophils in the blood of EoE patients decreased after successful treatment and that the CD16+ fractions in the blood correlated with CD3+ T cell and CD4+ T cell numbers. Because suppressive eosinophils have an increased capacity for T cell suppression,[45] the Th2 driven nature of the disease,[90, 91] and because of the association we found between eosinophils and CD4+ T cells, we wanted to investigate suppressive eosinophils in the esophagus of patients with EoE. The results from paper III showed that CD16+ eosinophils were present in the esophageal mucosa of patients with EoE and that the fractions were reduced in the esophageal mucosa of patients successfully treated with topical corticosteroids. Blood eosinophils from patients with EoE have previously been demonstrated to possess T cell suppressive capacity,[142] and it is possible that the CD16+ eosinophils found in the esophageal mucosa are cells that mediate T cell suppression in patients with EoE. *In vitro* studies of eosinophil-mediated T cell suppression have shown that CD16+ eosinophils release galectin-10-containing EETs when cultured with stimulated T cells.[61] In paper III we saw that, even though eosinophil and T cell numbers correlated, very little direct cell contact occurred. However, large amounts of released galectin were present in the tissues during active EoE and this release of free galectin-10 was subsequently vastly reduced in the esophageal mucosa of the responders after treatment. As free recombinant galectin-10 has been shown to mediate T cell suppression *in vitro*,[45] the released forms of galectin-10 could possibly be a way for eosinophils to mediate T cell suppression in the esophageal mucosa. On the other hand, *in vitro* studies have also shown that antibody-mediated neutralization of galectin-10 only partly reversed the eosinophil mediated T cell suppression.[45] However, the galectin-10 within the esophageal tissues was most commonly found in EVs and previous *in vitro* studies of galectin-10 release following experimentally induced cell death have shown that galectin-10 was released as either crystallized Charcot-Leyden crystals, soluble protein, in EETs, and as plasma enveloped EVs.[60] It is tempting to speculate that the EVs found in paper III also were enveloped by plasma membrane and that these could merge with T cells for direct intracellular deposition of the galectin-10 content.

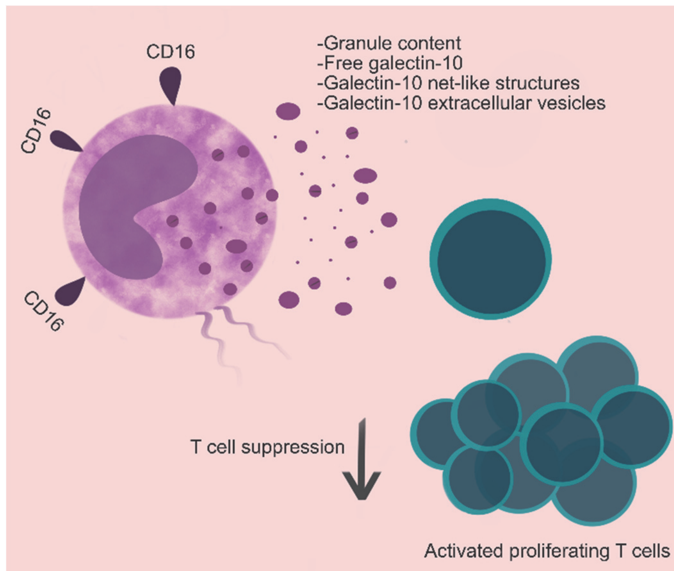


Figure 24. Illustration of the proposed suppressive function of eosinophils in the esophageal mucosa of patients with EoE. Suppressive CD16+ eosinophils and released eosinophil content including granule proteins, free galectin-10, galectin-10-containing extracellular vesicles, and galectin-10+ net-like structures suppress the proliferation of activated T cells.

The relationship between eosinophils and T cells was studied further by looking at eosinophils in the human thymus. In paper IV and V, we sought to pave the way towards an understanding of the functions of eosinophils in the human thymus by determining their anatomic location, phenotype, and preferential cellular interactions. In paper IV, we provided a more detailed description of eosinophil distribution in the thymus than what has been presented in earlier studies.[149, 150] We also confirmed the presence of immature eosinophils, initially demonstrated by Bhathal et al. and Lee et al.,[149, 164] while contributing new information on the fractions of eosinophil precursors of different maturational stages. This, combined with the large fraction of Ki-67+ positive eosinophils showed that eosinophils are capable of proliferation and differentiation from precursor cells within the thymus. We also found CD34+ eosinophils in the thymus, indicating that cells of the earliest eosinophil precursor stage are present in the thymus. Generally, eosinophils complete their differentiation and maturation process in the bone marrow before entering the blood stream and dissemination to the tissue. The high levels of eosinophil precursors found in the thymus are unique compared to other tissues (excluding the bone marrow). However, there is evidence for migration of eosinophil precursors into tissues followed by on site differentiation in states of allergic airway inflammation.[197-199] Similarly, early eosinophil precursors seem to migrate from the bone marrow via the blood stream in to the thymus, as the earliest CD34+ eosinophil precursors

were mainly found in the septa that contain the blood vessels that supply the thymus. Potentially, the eosinophils in the thymus require the thymic environment in order to develop into specialized thymic eosinophils. As demonstrated in paper V, the blood and thymic eosinophils differed from one another and it is possible that these specialized thymic eosinophils remain in the thymus rather than exiting the organ to seed other tissues of the body.

The phenotype of the thymic eosinophils was initially looked at in paper IV and then further studied in paper V. Initially, we saw that the expression of the surface molecules Siglec-8 and EMR1 were lower in eosinophils in thymus compared to blood. The expression of these two molecules are increased with maturity,[36, 200] supporting our data, and that of others, that eosinophils in the thymus are of a less mature state. The more detailed phenotyping in paper V showed that the phenotype of blood and thymic eosinophils differed. Striking differences in the frequency of different eosinophil populations were also found. The most common blood eosinophil population was barely found in the thymus, and equally, although eosinophils corresponding to the two most common thymic eosinophil populations were found in the blood they were not nearly as frequent. Overlap between the two sample types was expected, the extent of the differences on the other hand was rather surprising. By looking at univariate analysis of eosinophil molecular expression we got an overview of how the frequencies of eosinophils expressing these molecular markers differed between the two sample types. Clustering analysis on the other hand, provided more detailed information on the expression of different molecules within the different eosinophil populations found in blood and thymus. When comparing the most prominent thymus-specific eosinophil population with the most prominent blood-specific eosinophil population we saw that the thymic eosinophils demonstrated increased expression of surface markers that have been associated with eosinophil activation: CD25,[201] CD44,[202] and CD69.[203, 204] Thymic eosinophils also expressed CD38. Upregulation of CD38 has been linked to activation in T cells, B cells, and NK cells,[205] but little is known regarding the expression of CD38 by eosinophils except that CD38 is expressed by the eosinophil-lineage committed progenitor.[25] CD38 is an enzyme that can synthesize cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (NAADP), which in turn regulates intracellular calcium mobilization.[206] CD38 can also bind to CD31, which mediates adhesion to endothelial cells.[207] The functions of this molecule are likely similar in thymic eosinophils.

The thymic eosinophils also demonstrated distinctly lower expression of CD193 (CCR3) compared to blood, which was evident from both the univariate and X-shift clustering analyses. Eosinophil activation can result in decreased levels of CD193,[208, 209] but CD193 expression has also been shown to increase with

maturation and to be expressed in higher levels by blood eosinophils compared to mature eosinophils of the bone marrow.[210] The reason for the low CD193 levels of thymic eosinophils can be a result of either of these options, or a combination of them considering the low percentage that was present in the thymus. On the other hand, the thymus-specific eosinophils demonstrated an increased expression of CD197 (CCR7). In the thymus, CD197 and the ligands CCL19 and CCL21 are important for the migration patterns of thymocytes.[211, 212] Human eosinophils have been shown capable of expressing CD197 and chemotactically respond to the ligands CCL19 and CCL21 when activated by IL-5.[213] The migration of thymic eosinophils is thus also likely influenced by these ligands, which might be more essential for eosinophil migration within the thymus than the ligands of CD193.

Surprisingly, thymus-specific eosinophils demonstrated higher expression levels of galectin-10 compared to blood-specific eosinophils. Galectin-10 has been identified as an essential intracellular protein for the functional properties of Tregs and has been shown to be required for their T cell suppressive function.[62]As discussed previously, galectin-10 has been demonstrated to mediate T cell suppression in eosinophils and increased expression of galectin-10 has been found among the CD16+ "suppressive" eosinophils that possess an increased capacity for T cell suppression.[45] Although there was no increase in CD16 for the thymic eosinophils, one could speculate that increased levels of galectin-10 could signify an increased suppressive capacity of the thymic eosinophils as well.

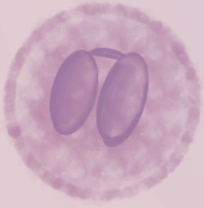
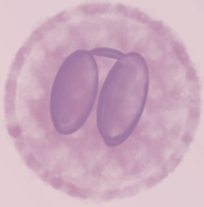
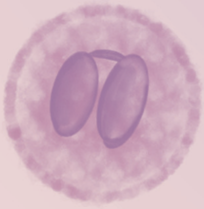
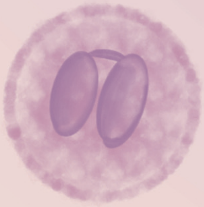
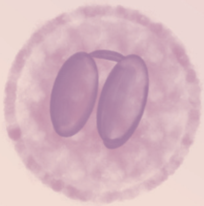
In paper IV we provided a detailed description on eosinophil localization in the thymus and found that the corticomedullary junction and medulla were the two primary locations for eosinophils. The corticomedullary junction is the entry site for early thymic progenitors arriving from the bone marrow via the blood.[214] Although the medulla is where the main part of negative selection happens, part of this process also occurs in the corticomedullary junction.[215, 216] Since the corticomedullary junction and medulla were the most eosinophil dense regions of the thymus, one possibility could be that eosinophils partake in the negative selection process, although this is highly speculative. A study on eosinophil involvement in negative selection has shown that the numbers of thymic eosinophils in mice increased in a model of acute negative selection.[165]

We were then interested to see if eosinophils could interact with thymocytes and possibly modulate their fate. In paper IV we found that eosinophils were able to form synapses with mononuclear cells, they were commonly found in direct contact with CD3+ thymocytes using imaging flow cytometry analyses, and close to half of the eosinophils in tissue sections were found to express the T cell co-stimulatory marker CD86. Previous *in vitro* studies of human peripheral blood

eosinophils have shown that CD4+ T cell proliferation can be induced following antigen presentation by CD86 expressing eosinophils.[217] The CD86+ eosinophils found in paper IV were most numerous in the medullary regions of the thymus, which is where negative selection most commonly occur. The very infrequent interactions found between thymic eosinophils and the Tregs mainly located in the medulla suggests that eosinophils probably do not have a role in directing thymocytes toward the Treg lineage, unlike what has been shown for eosinophils in the murine intestine.[84]

In order to shed light on the thymic eosinophil function, we sorted and co-cultured thymic eosinophils and DP, CD4 SP, and CD8 SP thymocytes. As the cultivations with eosinophils resulted in altered distribution of thymocyte populations we believe that thymic eosinophils can exert some form of immunomodulatory function. The extent and purpose of this, however, still remains to be sorted out. Positive selection causes DP thymocytes to terminate their CD8 gene expression which results in CD4+CD8- intermediate thymocytes. These intermediate thymocytes can then be committed to either the CD4 SP or the CD8 SP lineage depending on if the TCR signaling is maintained or not following CD8 downregulation.[218] In paper V, the cultivation of sorted DP thymocytes resulted in an increase of CD4 SP cells and a decrease in DN thymocytes. In addition, the fractions of DP thymocytes that expressed the IL-7 receptor (CD127) were reduced in the presence of eosinophils and IL-7 has been shown to cause co-receptor reversal and commitment to the CD8 SP lineage.[156] Lineage commitment is something that normally occurs during positive selection. The majority of positive selection within the thymus is however carried out by cortical thymic epithelial cells and whether eosinophils actually exert a similar function in the thymus is another matter. Furthermore, even though eosinophils were found in the cortex where positive selection occurs, the majority of the eosinophils were found at other anatomic locations. For the SP cultivations, CD4 SP cells remained unaltered whereas both a reduced expression of CD8 and a decrease in CD8 SP population size were seen following cultivation of the stimulated thymocytes with thymic eosinophils. The reduction in CD8 SP thymocytes could be a result of negative selection mediated via MHC class I. Further experiments on whether thymic eosinophils express MHC class I are however needed to investigate this. A possibility is that eosinophils are located in the thymus in order to present their own eosinophil-derived antigens to make thymocytes tolerant to eosinophils and prevent autoimmune reactions against eosinophils from developing. A limitation of these studies is the low sample number and using larger sample sizes in future studies could provide more insights on the roles of the thymic eosinophils.

The papers of this thesis present the idea that eosinophils and T cells interact in both EoE and within the human thymus. In the esophageal mucosa of patients with EoE, eosinophils released the T cell suppressive protein galectin-10, perhaps as a way to suppress the T cell-driven inflammation. Stronger associations between eosinophils and CD4+ T cells were found in the esophageal mucosa of patients with EoE. In the human thymus, eosinophils were capable of directing thymocytes towards a CD4 SP phenotype and reducing the population of CD8 SP thymocytes. Eosinophils developed within the thymus displayed an altered phenotype compared to its blood counterpart with increased expression of markers associated with eosinophil activation and increased levels of galectin-10, suggesting that eosinophil precursors subjected to the thymic milieu become specialized thymic eosinophils.

CONCLUSION

This thesis encompasses studies on eosinophils in two different settings: within the diseased esophageal mucosa of individuals with the T cell-driven allergic condition EoE and in the healthy thymus. Although the environment and disease state differs, the interactions of eosinophils with T cells connect them.

In paper I we showed that multivariate models based on analysis of non-invasive parameters could be used to separate patients who histologically responded to topical corticosteroid treatment from patients who had not responded to therapy. This pilot study showed that the inclusion of PRO scores and blood-based parameters could be used to separate these two patient groups. By using a larger study population and the now available S-EEsAI PRO instrument, it is possible that even more accurate and reliable models could be developed for assessing response to treatment in patients with EoE without the need for collection of esophageal biopsies.

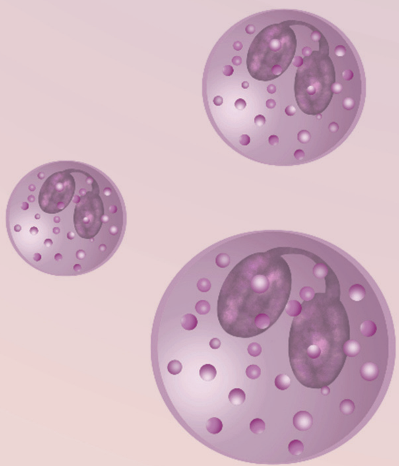
The validation of the S-EEsAI in paper II showed that this instrument is appropriate for assessment of symptom severity in adult Swedish patients with EoE. By using this instrument, specifically developed and validated for patients with EoE, a more precise interpretation of symptom severity of Swedish patients can be obtained.

In paper III we continued our studies on eosinophils in EoE that were initiated in paper I, by investigating eosinophils in esophageal tissue biopsies using immunohistochemistry. We identified a relationship between the esophageal eosinophils and T cells and found indicators of eosinophil-mediated T cell suppression via the presence of "suppressive" CD16+ eosinophils and extracellular galectin-10. Following therapy, the presence of CD4+ T cells, suppressive eosinophils, and the T cell suppressive protein galectin-10 were reduced in the esophageal mucosa of the patients who had responded to topical corticosteroid treatment.

In paper IV we studied eosinophils resident in the human thymus. We found that different subgroups of eosinophils exist within the human thymus, ranging from early CD34+ eosinophil progenitors to fully mature eosinophils. Importantly, we found that thymic eosinophils were capable of on-site proliferation and differentiation from precursor cells. This implies that thymic eosinophils require the specialized thymic environment to be able to execute their functions. Finally, thymic eosinophils seemed to interact directly with thymocytes, which suggests that they may be involved in the thymocyte selection process.

Lastly, in paper V we extended the studies on thymic eosinophils and were able to phenotype thymic eosinophils and see that they differed from blood eosinophils. *In vitro* studies of sorted thymic eosinophils and DP, CD4 SP, and CD8 SP thymocytes showed that thymic eosinophils directed the thymocyte populations towards the CD4 SP phenotype and reduced the population of CD8 SP thymocytes. We conclude that thymic eosinophils are specialized cells which might have a role in thymocyte selection.

Altogether, the studies on eosinophils in this thesis have provided information on tissue resident eosinophil localization and interactions, on cellular marker expression, and on ways to improve the care of patients with EoE. The studies presented herein have the capacity to improve basic knowledge of human homeostatic thymic eosinophils and the understanding of the disease EoE.



FUTURE PERSPECTIVES

The presented work in this thesis serves as a foundation on which further studies can be performed in order to finally elucidate the function of eosinophils within both EoE and in the human thymus.

In paper I, we showed that statistical models could be an alternative for assessment of response to treatment in the future, thus eliminating the necessity of repeated invasive endoscopies. The study population was small but further studies on this subject using the newly validated S-EEsAI and in-depth phenotyping with CyTOF could lead to even better predictive models.

The translation and validation of a Swedish PRO instrument specifically developed for the assessment of symptom severity of EoE will have a direct impact on Swedish health care where its implementation and use can lead to better understanding of the patients' situation, which consequently can improve the use of treatment options to better suit the patients' need. The implementation of the S-EEsAI in future studies on EoE can also lead to more coherent and comparable results.

The work on T cell suppression mediated by eosinophils has been advanced in this thesis but still more work remains. Further studies regarding the impact of galectin-10 on T cells are warranted to increase the knowledge of the role of galectin-10. For example, studies on the alteration in T cell gene expression in the presence of galectin-10 could provide more information on the effect this protein has on T cells.

For a long time, eosinophils were considered only to be end stage effector cells focused on the elimination of helminths and parasites. Newer insights have shown that eosinophils have important homeostatic functions as well, but still little of this is known. The thymic studies in this thesis have shown a distinct population of thymic eosinophils that likely have a homeostatic function, but exactly what this function is remains uncertain. Future studies regarding eosinophil and thymocyte interactions may reveal the actual function of thymic eosinophils. As little regarding the thymic eosinophil is known, the possibilities for future studies are vast. The extent and type of antigen presentation of thymic eosinophils, the degree of galectin-10 release, and studies focused on proliferation and viability of thymocytes cultivated with eosinophils could be conducted to investigate the possible involvement in the thymocyte selection process.

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