Functional and Phenotypic Studies of Eosinophilic Granulocytes in Patients with Eosinophilic Esophagitis

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Cover illustration: *Upper right:* Eosinophilic extracellular traps containing galectin-10. *Upper left:* Transfer of galectin-10 from an eosinophil to a T cell. *Lower right:* Flow cytometry plot of CD16^{hi} and CD16^{neg} eosinophilic granulocytes. *Lower left:* Image flow cytometry picture of an eosinophil, with a DAPI-stained lobulated nucleus, expressing FOXP3.

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ABSTRACT

Eosinophilic Esophagitis (EoE) is a chronic inflammatory disorder of unknown etiology, in which the esophagus is infiltrated by eosinophils and T cells. Topical corticosteroids are one of the treatment options for patients with EoE. The function of eosinophils in EoE is unknown, here we hypothesize that eosinophils serve as immunoregulatory cells. The eosinophils in the blood of untreated adult patients with EoE have a distinct phenotype. The aims of this thesis were to explore whether the eosinophilic phenotype of untreated patients with EoE can be reverted to the healthy phenotype by topical corticosteroid treatment, and to examine whether blood eosinophils from children with EoE have a distinct phenotype, different from that of healthy children. Moreover, we tested the hypothesis that eosinophils, similar to regulatory T cells, can diminish T cell proliferation and express FOXP3. The role of the eosinophilic protein galectin-10 in mediating immunosuppression was also investigated. This thesis demonstrates that the EoE phenotype of blood eosinophils is not restored by topical corticosteroid treatment, except with respect to CD18. We also show that eosinophils from patients with EoE have an immunoregulatory phenotype, i.e., increased levels of FOXP3 and galectin-10. Moreover, eosinophils from healthy subjects and patients with EoE are able to suppress T cell proliferation *in vitro*, in part *via* galectin-10. We show that eosinophils exposed to activated T cells release galectin-10 *via* DNA nets and appear to transfer this protein to T cells through synapses. Two subsets of eosinophils emerge after co-culturing. Finally, we demonstrate that the blood eosinophils of children with EoE have a distinct phenotype, different from that of healthy children and that of adults with EoE. Importantly, we reveal marked age-related differences regarding the molecular patterns displayed by the blood eosinophils of healthy donors. Our finding that eosinophils from patients with EoE have upregulated immunoregulatory molecules could indicate that the function of eosinophils in EoE is to reduce a T cell-mediated inflammation in the esophagus.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Eosinofil esofagit är en sjukdom som drabbar ungefär 1 procent av Sveriges befolkning. Symtomen är sväljningssvårigheter, illamående, kräkningar och barn kan drabbas av dålig viktuppgång. Om sjukdomen inte behandlas kan den orsaka ärrbildning i matstrupen. Det finns många studier som föreslår att eosinofil esofagit är en allergisk reaktion som utlöses av födoämnen eller luftvägsburna allergen t.ex pollen. Majoriteten av patienterna med eosinofil esofagit har någon typ av allergi. Diagnosen ställs genom att analysera vävnadsprover från matstrupen. Hittar man vita blodceller vid namn eosinofila granulocyter i vävnaden så anses man ha eosinofil esofagit. Orsaken till att dessa celler befinner sig i matstrupen är okänd. Den eosinofila granulocyten vistas normalt sett i mag- och tarmkanalen, benmärg, thymus, mjälte, lymfkörtlar samt blodet. Man vet sedan tidigare att eosinofilen är delaktig i allergiska reaktioner och försvarar oss även mot parasitinfektioner.

Målet med denna avhandling var att undersöka vilken funktion den eosinofila granulocyten har i matstrupen hos eosinofil esofagit-patienter. Vi vill också studera hur den blir rekryterad till den inflammerade vävnaden och hur den interagerar med andra celler. Hypotesen är att den tar sig till matstrupen för att dämpa inflammationen som drivs av en annan vit blodkropp som kallas T-lymfocyt.

Vi har med hjälp av spektrometriska och molekylärbiologiska metoder samt cellodlingar undersökt den eosinofila granulocyten i blodet hos vuxna och barn med eosinofil esofagit. Vi har även studerat eosinofilen hos friska åldersmatchade försökspersoner för jämförelse.

I denna avhandling föreslår vi att eosinofilen har en annan typ av funktion i kroppen mot vad man tidigare har trott, vi tror att den kan reglera och dämpa immunförsvaret. Med vår forskning kan förhoppningsvis nya förbättrade diagnosmetoder och behandlingsätt bli verklighet.

LIST OF PUBLICATIONS

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

I. **Topical corticosteroids do not revert the activated phenotype of eosinophils in eosinophilic esophagitis but decrease surface levels of CD18 resulting in diminished adherence to ICAM-1, ICAM-2 and endothelial cells.**
    Christine Lingblom, Henrik Bergquist, Marianne Johnsson, Patrik Sundström, Marianne Quiding-Järbrink, Mogens Bove & Christine Wennerås.
    Inflammation 2014; 6: 1932-1944.

II. **Eosinophils from eosinophilic esophagitis patients express FOXP3 and use galectin-10 to suppress T cells.**
    Christine Lingblom, Madeleine Ingelsten, Jennie Andersson, Mogens Bove, Henrik Bergquist & Christine Wennerås.
    Submitted

III. **Galectin-10 is secreted *via* eosinophilic extracellular traps and a subset of eosinophilic granulocytes is a strong T cell suppressor**
    Christine Lingblom, Madeleine Ingelsten, Jennie Andersson, Timo Käppi, Robert Saalman, Amanda Welin & Christine Wennerås.
    In manuscript

IV. **Differences in eosinophilic molecular profiles between children and adults with eosinophilic esophagitis**
    Christine Lingblom, Timo Käppi, Henrik Bergquist, Mogens Bove, Richard Arkel, Robert Saalman & Christine Wennerås.
    Submitted

Publications not included in this thesis.

Eosinophils from Hematopoietic Stem Cell Recipients Suppress Allogenic T Cell Proliferation
Jennie Andersson, Julia Cromvik, Madeleine Ingelsten, Christine Lingblom, Kerstin Andersson, Jan Erik Johansson & Christine Wennerås.
Biol Blood Marrow Transplant., 2014; 20: 1891-1898.

Exploring a Cascade Heck-Suzuki reaction based route to kinase inhibitors using Design of Experiment
Andreas Ekebergh, Christine Lingblom, Peter Sandin, Christine Wennerås & Jerker Mårtensson
Organic and Biomolecular Chemistry, 2015; 13: 3382-3392.

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ABBREVIATIONS

APC   antigen-presenting cell
cAMP   cyclic adenosine monophosphate
CCR3   C-C chemokine receptor type 3
CCL   chemokine (C-C motif) ligand
CD   cluster of differentiation
CD40L   CD40 ligand
CLC   Charcot Leyden crystal
CRTH2   chemoattractant receptor-homologous molecule expressed on Th2 cells
CTL   cytotoxic T lymphocyte
DC   dendritic cell
EDN   eosinophil-derived neurotoxin
EDTA   ethylenediaminetetraacetic acid
EETs   eosinophilic extracellular traps
EoE   eosinophilic esophagitis
EPX/EPO   eosinophilic peroxidase
FACS   fluorescence activated cell sorter
FBS   fetal bovine serum
FMO   fluorescence minus one
FOXP3   forkhead box P3
FPR   formyl peptide receptor
GERD   gastroesophageal reflux disease
GM-CSF   granulocyte-macrophage colony-stimulating factor
HLA   human leukocyte antigen
HRP   horseradish peroxidase
HUVEC   human umbilical vein endothelial cells
ICAM   intercellular adhesion molecule
IFN   interferon
Ig   immunoglobulin
IL   interleukin
KRG   Kreb´s ringer glucose
LAF   leukocyte function-associated antigen-1
LPS   lipopolysaccharide
mAb   monoclonal antibody
MBP   major basic protein
mFI   median fluorescence intensity

MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
mRNA	messenger RNA
OPLS-DA	orthogonal partial least squares-discriminant analysis
PAF	platelet-activating factor
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PG	prostaglandin
PI	propidium iodide
RNase	ribonuclease
PRR	pattern recognition receptor
PVDF	polyvinylidene fluoride
RANTES	regulation on activation, normal T cell-expressed and-secreted
RNA	ribonucleic acid
ROS	reactive oxygen species
SFED	six food elimination diet
Siglec-8	sialic acid-binding Ig-like lectin 8
siRNA	small interfering RNA
TBST	Tris-buffered saline and Tween-20
Th cell	T helper cell
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
VCAM	vascular cell adhesion molecule

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The human immune system is divided into two arms; the innate and the adaptive, which consist of extremely complicated networks that comprise numerous cellular and chemical processes. The mission of the immune systems is to defend us against danger, whether that danger is foreign pathogens (non-self) or injured tissue. It should also be able to distinguish these factors from our own healthy cells (self). The key players in this defense system are the white blood cells, which are also called leukocytes. All leukocytes are produced from the hematopoietic stem cell which is a multipotent cell in the bone marrow. Upon exposure to different stimuli, the multipotent cells mature to cells with distinct appearances and functions, i.e., monocytes, lymphocytes, neutrophils, basophils, or to the leading character in this thesis, the eosinophil (Figure 1).

Innate immunity is so named because it is present at birth and does not have to be educated through exposure to an invader. Therefore, the innate immune system provides an immediate response to foreign antigens. A number of identifying antigens, which are common to many different invaders or danger elements, are recognized. The innate immune system is triggered through pattern recognition receptors (PPRs)[1] that recognize so-called pathogen-associated

molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), with the latter originating from the fragments of injured or stressed cells.[2] Innate immunity, unlike adaptive immunity, has no memory of encounters with these molecules. The white blood cells involved in innate immunity are dendritic cells, monocytes, neutrophils, eosinophils, basophils, and natural killer T cells.[3]
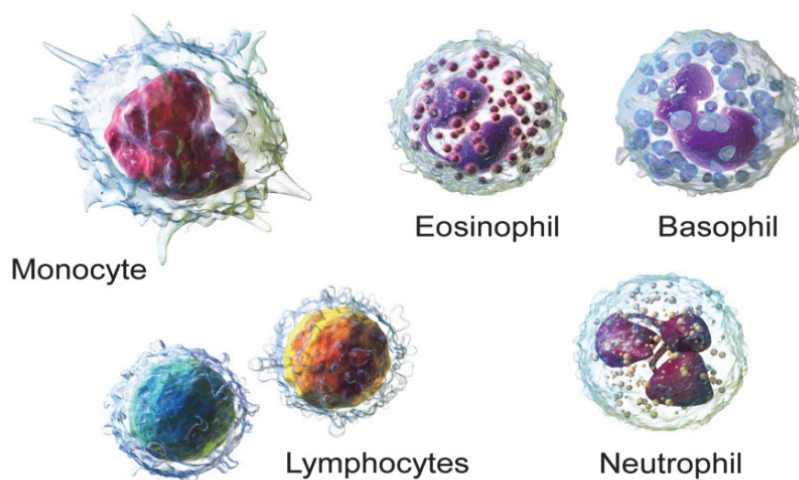
Figure 1. White blood cells. Reproduced with permission from Blausen.com staff. "Blausen gallery 2014".

Adaptive immunity is not preformed at birth. It is memorized. As a person's immune system encounters foreign antigens, the components of adaptive immunity begin to develop a memory for that antigen. Adaptive immunity is called specific immunity because it adapts its attack to a specific antigen that it has previously encountered. Adaptive immunity needs 1-2 weeks to develop after first exposure to a new antigen. If the same antigen is encountered a second time, the response to that antigen is quicker and more effective than the response upon first exposure. The white blood cells responsible for adaptive immunity are the T lymphocytes (T cells) and B lymphocytes (B cells).[4] All foreign antigens are of course not harmful to the body. Therefore, the innate immune system alerts the adaptive immune system when it recognizes molecules that are typical of harmful invading pathogens. The innate immune system can distinguish between different pathogens and can recruit the most effective cells from the adaptive immune system for that specific intruder. Sometimes, the immune system mistakenly attacks harmless antigens. Unfortunately, this is what happens in persons with allergies and other autoimmune diseases.

1.1.1 ACTIVATION AND IMMUNE REGULATION

Initially, antigen-presenting cells (APCs) bring together antigens and major histocompatibility complexes. These can be recognized by naïve T cells from the thymus, which are stimulated by secreting cytokines to become cytotoxic $CD8^+$ T cells (CTL) or helper $CD4^+$ T cells. $CD4^+$ T cells can subsequently differentiate into other subtypes, e.g., T_H1, T_H2 and Tregs (Figure 2). APCs determine which one of these subtypes that is required for a particular immune response. To prevent excessive and potentially harmful escalation of the immune response, regulation is essential. This is where the regulatory T cells (Tregs) play an important role by suppressing the activated T cells. Tregs can also suppress NK cells, B cells, and APCs both *in vitro* and *in vivo*.[5] Tregs express FOXP3 (forkhead box P3), which is a member of the FOX protein family. It is a transcription factor that appears to function as a master regulator for Tregs.[6] In human Tregs, high and stable FOXP3 expression is required for suppressive actions and decreased levels of FOXP3 reduce the capabilities of Tregs to suppress T cell functions.[7, 8] FOXP3 can also be expressed by activated $CD4^+$ effector cells, which do not have suppressive capabilities.[9]
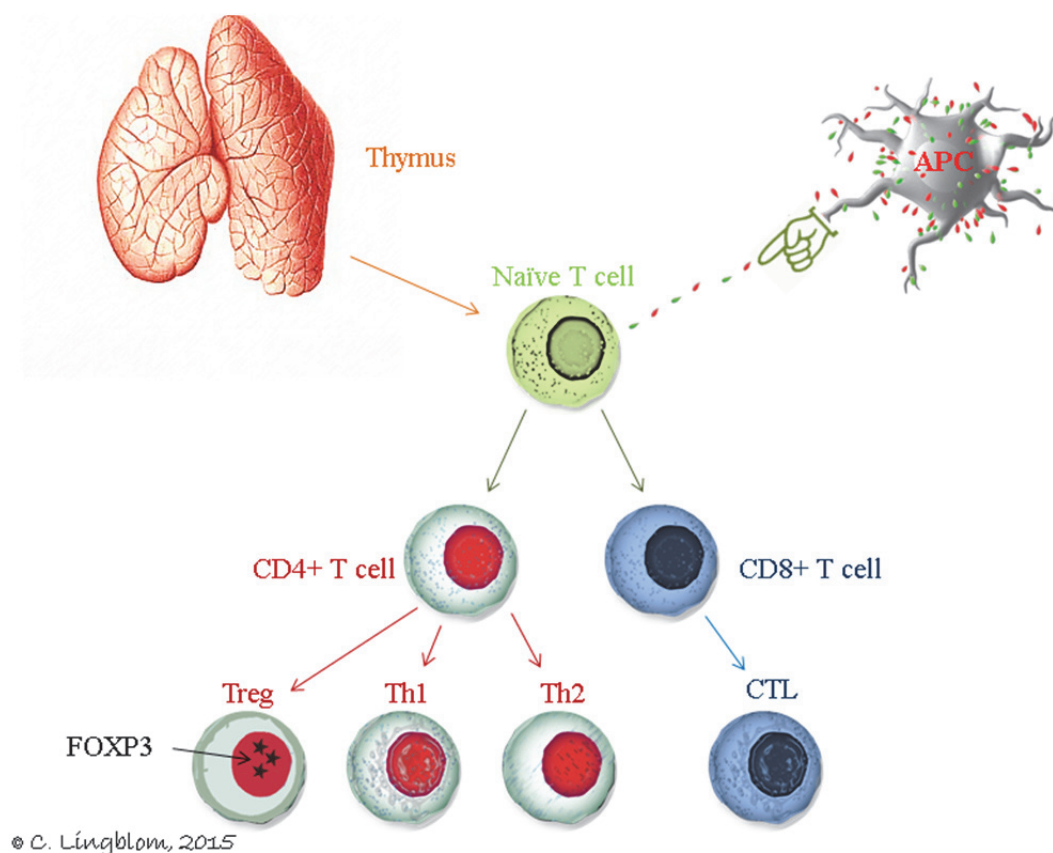

Figure 2. T cell development.

Treg-mediated suppression is thought to be contact-dependent or due to soluble factors or to act *via* a passive mechanism where Tregs take up important growth factors and consequently depriving effector T cells of these factors.[10] Recently, it has been reported that Tregs require galectin-10 to be able to suppress T cells.[11] However, it was unclear how galectin-10 exerted this inhibitory function. Another report has suggested that cyclic adenosine monophosphate (cAMP) is transferred through the gap junctions between Tregs and conventional T cells to inhibit interleukin (IL)-2 and subsequently inhibit T cell proliferation.[12]

1.2 EOSINOPHILIC GRANULOCYTE

In 1879, the eosinophilic granulocyte, more commonly called the eosinophil, was first discovered by the German scientist Paul Ehrlich.[13] He gave it the name 'eosinophil' because he found that the acidic dye eosin stained the granules bright pink inside the eosinophil. Eosinophils mature in the bone marrow and migrate *via* the blood to tissues. Eosinophil migration from the bone marrow into the circulation is primarily regulated by IL-5, IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF).[14]

Eosinophils are normally present in the intestinal mucosa (with the exception of the esophagus), bone marrow, thymus, lymph nodes and spleen but they are of low abundance in the blood. The eosinophil belongs to the polymorphonuclear leukocyte family and can easily be recognized based on its lobulated nucleus. Eosinophils are also a member of the granulocytes, together with neutrophils and basophils, due to their large amounts of cytoplasmic granules. In healthy individuals, blood eosinophils represent 1-4% of all the white blood cells and they have a diameter of 12–17 µm. Eosinophils spend only a short time in the circulation (~18hours),[15] whereas they can survive up to 12 weeks in tissues.[16]

1.2.1 FUNCTIONS OF THE EOSINOPHIL

Eosinophils, as mentioned, are part of the innate immune system, and although they were discovered over 100 years ago, their roles in the immune system are far from fully understood. We know that they are responsible for defending us against infections by worms and helminthic parasites and that they take part in allergic reactions, although it remains uncertain as to what their precise roles are in these conditions.

Eosinophils were previously considered to be harmful cells, a belief that emanated from the following observations: their capacity to damage epithelial

and mucosal cells as well as nerves; and their capabilities to induce bronchoconstriction and excessive mucus production by releasing granule proteins, lipid mediators, and reactive oxygen species.[17] However, there is today increasing evidence that the eosinophils also have healing functions, e.g., they can help to heal damaged tissues by producing growth factors.[18] Unfortunately, this may lead to scarring and impaired organ function.[18]

In more recent times, several reports have suggested that eosinophils possess immunoregulatory capabilities. For example, the eosinophilic granule proteins, eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) suppress T cell proliferation *in vitro;* this effect is irreversible and is not due to cytotoxic damage.[19] Eosinophils can also regulate T cell subset selection by expressing indoleamine 2,3-dioxygenase (IDO).[20] IDO is an IFN-γ-inducible enzyme that acts as a catalyst during the production of kynurenines (KYN) from tryptophan. KYN has been reported to induce the apoptosis and inhibition of proliferating T cells,[21] whereby it appears that Th1 cells are more affected by the outcome of KYN than are Th2 cells.[22, 23] We have recently demonstrated that eosinophils from hematopoietic stem cell transplant recipients and healthy individuals require cell-cell contact to suppress T cell proliferation.[24]

Eosinophils are also able to produce T cell-regulatory cytokines dose-dependently, in stimulated T cell cultures, including IFN-γ, which is a Th1 cytokine, as well as IL-5 and IL-13, which are Th2 cytokines.[25]

Furthermore, eosinophils have been reported to act as professional APCs. They can traffic to lymph nodes after exposure to an antigen and present antigens to T cells.[26] Eosinophils can express MHC class II, which is required for the presentation of antigens[27, 28] and co-stimulatory molecules such as CD28, CD40, CD80 and CD86.[29-32] It has also been demonstrated that eosinophils lose their ability to act as APCs when CD80 and CD86 are blocked.[33]

In addition, it has been demonstrated that eosinophils ensure the survival of long-lived plasma cells in the bone marrow of mice by secreting APRIL and IL-6[34] and for the production of IgA in the gastrointestinal tract.[35] Moreover, murine eosinophils have been reported to prime B cell responses.[36] Alum, which is used as an adjuvant in human vaccines, acts *via* Gr1^{+}, IL-4-expressing eosinophils to stimulate B cells to produce IgM. After alum administration, the number of eosinophils is increased in both the bone marrow and spleen.[36]

Human subjects who totally lack eosinophils in the blood and bone marrow display dysregulated immunity and autoimmune disorders.[37] This implies that eosinophils regulate the function and development of lymphocytes. Tulic et al. have suggested that eosinophils play an essential role in the selection of T cells in the thymus during the creation of the adaptive immune system.[38]

1.2.2   GRANULAR PROTEINS AND LIPID MEDIATORS

The eosinophilic cytoplasm contains several cationic components that are stored in eosinophilic granules, primary granules, and secondary granules. Primary granules are unicompartmental structures and secondary granules are bicompartmental structures. The proteins stored in the granules include major basic protein-1 and -2 (MBP-1, MBP-2), eosinophil peroxidase (EPX/EPO), ECP, and EDN. These are all basic and cytotoxic proteins. Galectin-10 is also stored in these granules, although it is not cationic; instead it is a hydrophobic autocrystallizing protein that constitute a large fraction of the total eosinophilic protein content.[39]

Primary granules contain galectin-10 and EPX and secondary granules contain MBP, ECP, EDN and EPX. MBP is localized to the crystalloid core of the secondary granule and it can damage bacteria and cells by disrupting cell membranes.[40] EPX is situated in both the primary and secondary granules and can form reactive oxygen species, leading to apoptosis and necrosis in the target cells.[41] EPX is able to kill parasites[42] and also contributes to both anti-inflammatory[43] and pro-inflammatory[44] actions.

ECP, which belongs to the ribonuclease (RNase) A superfamily, is toxic for helminthic parasites, bacteria, single-stranded RNA viruses, and host tissues,[45] and it can also create pores in the membrane of other cells to allow the entry of various molecules.[46] EDN, which also belongs to the RNase A superfamily, has antiviral activities and is particularly effective against single-stranded RNA viruses.[47] As mentioned above, both ECP and EDN inhibit T cell proliferation.[19] EPX and MBP-2 are not found in cells other than eosinophils, whereas the other proteins are expressed by other cells, albeit not at the levels found in eosinophils.[48] Following activation, eosinophils release their granule proteins. It has been reported that eosinophils degranulate in the esophageal tissue of patients with EoE, 98% of the eosinophils infiltrating the esophagus in patients with EoE had morphologic changes and 70% of the images in this report demonstrated extracellular granules.[49]

Eosinophils also contain lipid bodies, which are the sites where synthesis of cysteinyl leukotrienes (C4, D4, E4), thromboxane B2, prostaglandins E1 and E2 and platelet-activating factor takes place.[50] These lipid mediators are signaling molecules that play important roles in inflammatory and immune responses and they are also essential for the regulation of cell proliferation.[51] An electron micrograph showing the nucleus, cytoplasmic granules and lipid bodies in a mature eosinophil is presented in Figure 3.
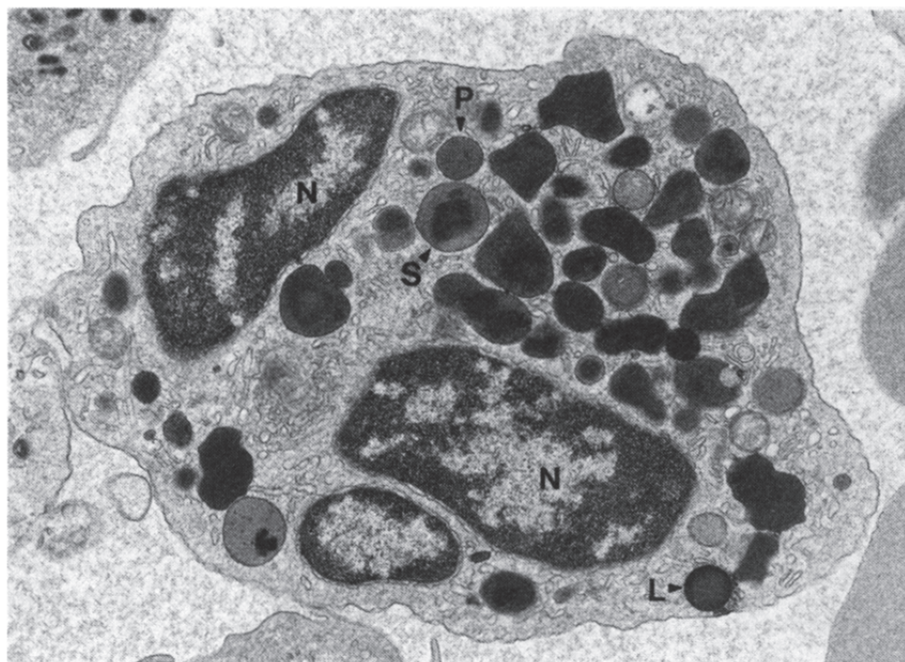

Figure 3. A mature human eosinophil has a tabulated nucleus (N) and cytoplasmic structures that include: specific granules (S), with their distinctive electron-dense crystalloid core which contains major basic proteins; primary granules (P); and non-membrane-bound lipid bodies (L), cytoplasmic inclusions that become more numerous in cells that are engaged in inflammatory responses. Reproduced with permission from the Massachusetts Medical Society.[52]

1.2.3   GALECTIN-10

Galectin-10, which is also called Charcot Leyden Crystal protein, belongs to the galectin family of proteins that specifically bind to beta-galactoside sugars, although galectin-10 primarily binds to mannose.[53] Galectin-10 is a 16.5 kDa polypeptide with 142 amino acids.[54] In eosinophils, about 10% of the total protein mass is composed of galectin-10.[39] Galectin-10 has been found to form hexagonal bipyramidal crystals when the eosinophils have participated in inflammatory reactions (Figure 4).[55] These crystals were identified more than 150 years ago, first by J.M. Charcot in 1853, who detected the crystals in the

spleen of a patient with leukemia[56] and subsequently by Leyden in 1872, who noted these crystals in the sputum samples collected from asthmatics.[57] Even so, we still do not know much about these crystals. It is not known how galectin-10 is secreted from eosinophils; it does not seem to be secreted by vesicular transport.[58] We know that, with the exceptions of eosinophils and basophils,[59] CD4$^+$CD25$^+$ Tregs express three isoforms of galectin-10, which is essential for the maintenance of their suppressive capacity.[11] Of course that report led many to wonder as did Helene F. Rosenberg who wrote in Blood 2007 "Might human eosinophils, *via* abundant expression of galectin-10/CLC, also inhibit the proliferation and function of CD4$^+$ T cells?".[60] Galectin-10 has been reported to be upregulated in several eosinophilic disorders, such as EoE,[61] celiac disease,[62] and asthma.[63]
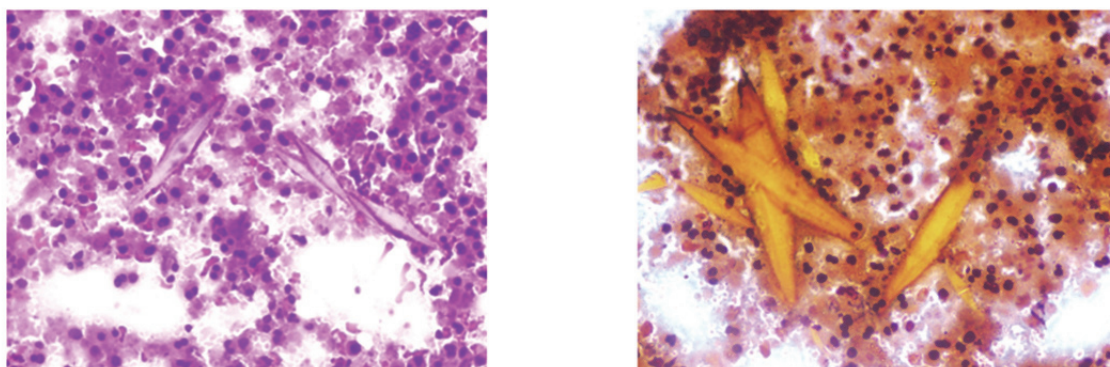
Figure 4. Galectin-10 crystals in a human liver during parasite infection. Reproduced with permission from Wiley & Sons, Diagnostic Cytopathology, 2014.[64]

1.2.4   SECRETORY MECHANISMS

When eosinophils are exposed to stimulation they may secrete their granular proteins. To date, three mechanisms for this have been identified: 1) exocytosis, in which cytoplasmic granules merge their way through the cell membrane and secrete the granular proteins to the extracellular environment; 2) piecemeal degranulation, a process characterized by vesicular transport that enables regulated release of granular proteins;[58, 65] and 3) cytolysis, where the eosinophils burst and release their intact granules.[66] The exact mechanism of cytolysis remains unclear, although it is believed to be different from that of necrosis.[67]

One mechanism by which eosinophils can release their cytoplasmic contents is through the release of "eosinophilic extracellular traps" (EETs).[68] These

structures have been reported to be composed of mitochondrial DNA nets that contain granular proteins.[68] It has been suggested that this is a strategy for capturing and killing bacteria.[68] The EETs contain eosinophilic granular proteins and form sticky extracellular structures.[69] Release of EETs is also seen in patients with allergic diseases,[68, 70, 71] Crohn's disease,[68] and several skin diseases, both infectious and noninfectious.[71] It has been demonstrated that when eosinophils are stimulated with the cytokine thymic stromal lymphopoietin (TSLP) they release EETs that contain ECP (Figure 5).[72] This phenomenon is also seen when eosinophils are stimulated with LPS, complement factor C5a or eotaxin.[68] More recently, it has been demonstrated that eosinophils, upon exposure to different stimuli, such as eotaxin-1, undergo extracellular DNA trap cell death (ETosis). Initially, nuclear lobular formation is lost and some granules are released, and thereafter, nuclear chromatolysis occurs and DNA nets are released together with free intact granules.[73]
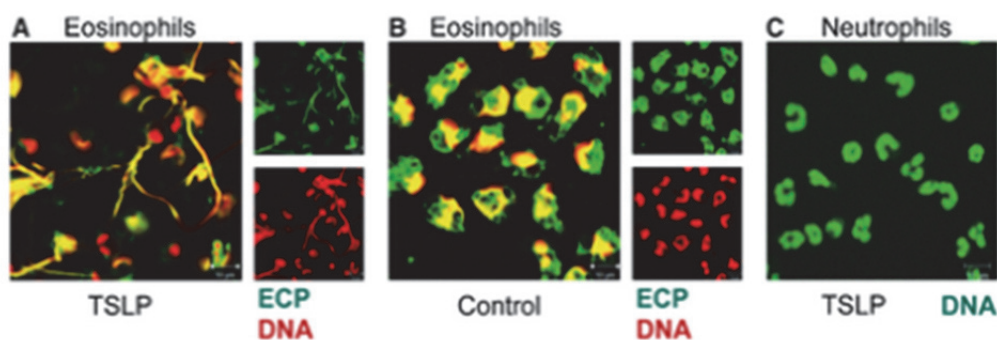

Figure 5. Eosinophil extracellular traps contain DNA and granule proteins. (A) After stimulation with thymic stromal lymphopoietin (TSLP) (20 ng/ml for 30 min), the eosinophils release DNA (stained with PI, red) together with eosinophilic cationic protein (ECP) (green). (B) Unstimulated eosinophils retain both DNA and ECP in the cell (yellow overlay). (C) Neutrophils do not release DNA upon TSLP stimulation (DNA stained with SYTO 13, green). Reproduced with permission from Wiley & Sons. Allergy, 2012.[72]

1.2.5   RECEPTORS

Integrin beta-2

Integrin beta-2, which is also called CD18, is the subunit to four members of the leukocyte integrin subfamily. Integrins are heterodimeric adhesion molecules that mediate important cell-cell and cell-extracellular matrix interactions.[74] The four members of the leukocyte integrin subfamily, all of which share the common β_2 subunit (CD18), have distinct α subunits, i.e., α_L (CD11a), α_M (CD11b), α_X (CD11c) and α_D (CD11d) these complexes are called LFA-1, Mac-1, p150,95, and integrin $\alpha_D\beta_2$ respectively.[75] LFA-1 is expressed on all

leukocytes and is the receptor for intracellular adhesion molecules -1, -2, and -3 (ICAM-1, -2, and -3).[75] Mac-1 is expressed by monocytes, neutrophils and eosinophils and is the receptor for ICAM-1.[75] When eosinophils are stimulated with IL-5, eosinophilic adherence to endothelial cells is enhanced and part of the underlying mechanism is Mac-1- and LFA-1-dependent.[76] Integrins are responsible for many of the adhesive interactions that are crucial for various immune and inflammatory responses.[77] During inflammatory responses, eosinophils, as well as other leukocytes, are recruited from the circulation to the site of inflammation. The recruitment of circulating leukocytes is based on a carefully coordinated interplay between chemokines, adhesion molecules, and adhesion receptors on the endothelium.[78] The recruitment of leukocytes to the inflamed tissue occurs in five steps: 1) priming; 2) rolling and tethering along endothelial cells; 3) firm adhesion to the endothelium; 4) transendothelial diapedesis; and 5) chemotaxis to the inflammatory site (Figure 6).[78]
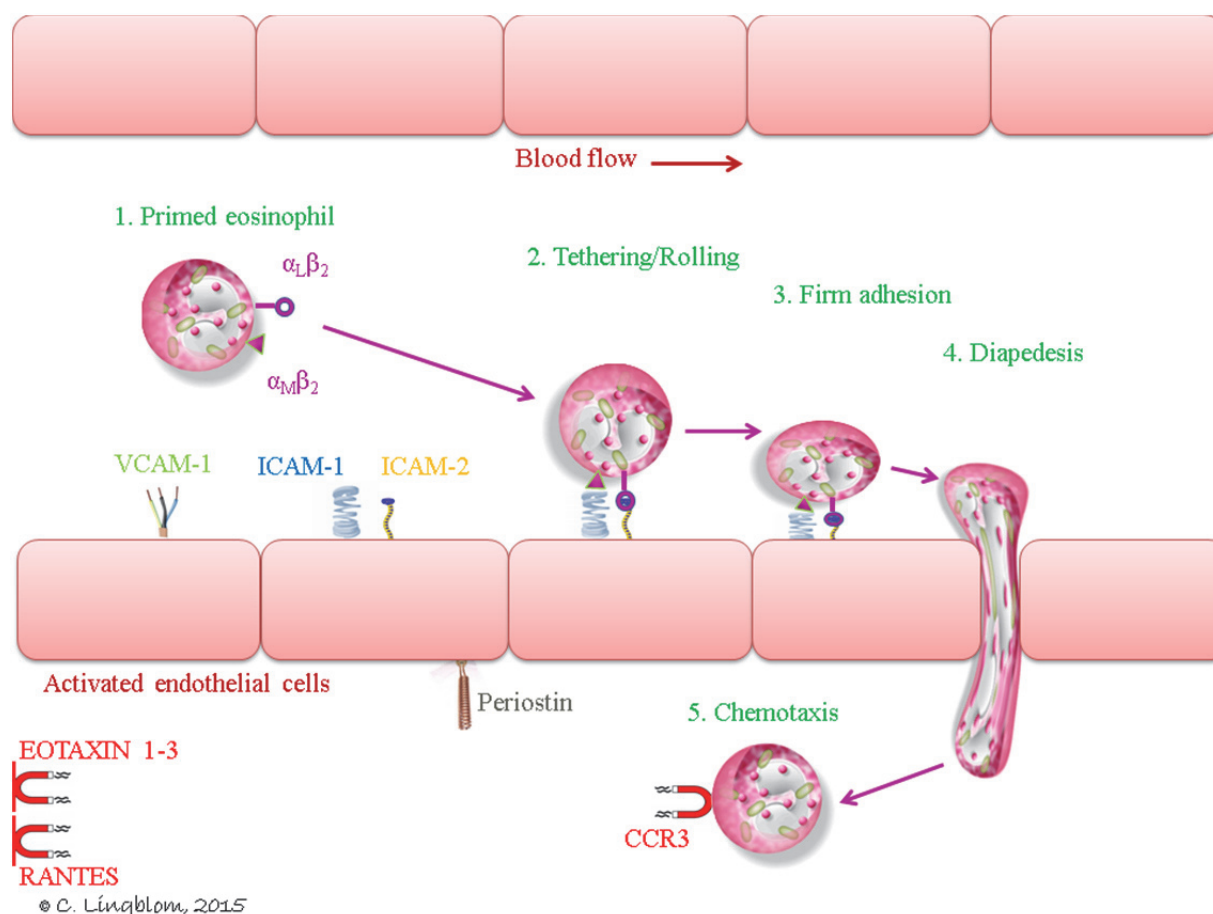

Figure 6. Multistep process of eosinophil trafficking.

Intercellular adhesion molecules

ICAM-1, which is also called CD54, and ICAM-2, also called CD102, are both members of the superfamily of intercellular adhesion molecules. ICAM-1 and ICAM-2 are typically expressed on endothelial cells and leukocytes and upon cytokine stimulation, their levels on the leukocyte surface increase. ICAM-1 has been shown to facilitate the interaction of eosinophils with T cells.[79]

Vascular cell adhesion molecule-1

Vascular cell adhesion molecule-1 (VCAM-1), which is also called CD106, is expressed by endothelial cells. During inflammation, endothelial cells use the expression of VCAM-1 to recruit selectively mononuclear leukocytes.[80] It has also been demonstrated that VCAM-1 plays an indirect role in the recruitment of eosinophils[81] and that it is upregulated in esophageal tissue of patients with EoE.[82]

Periostin

Periostin is an extracellular matrix protein that can induce the migration and adhesion of cytokine-stimulated eosinophils.[83] This adhesion process has been shown to be $\alpha_M\beta_2$-dependent (CD11b/CD18, Mac-1).[83] It has previously been revealed that periostin is overexpressed in human EoE[61] and that it is involved in the recruitment of eosinophils to the esophagus in a murine model of EoE.[84]

CD44

CD44 is involved in cell-cell interactions, cell adhesion and migration;[85] it is sometimes referred to as HCAM (homing cell adhesion molecule). CD44 is the receptor for hyaluronic acid, which is distributed in the extracellular matrix.[85] Eosinophils most likely adjust their levels of CD44 to regulate their adherence and migration. It has been reported that the levels of CD44 on blood eosinophils are lower in patients with poorly controlled asthma, as compared with patients who have well-controlled asthma.[86] It has also been demonstrated that eosinophils stimulated with IL-5 increase their surface levels of CD44.[87]

CD40

CD40 is a member of the TNF receptor superfamily and is expressed by antigen-presenting cells e.g., B cells, macrophages, monocytes, dendritic cells, endothelial cells, and epithelial cells, and it is necessary for the activation of these cell types. CD40 is also expressed by eosinophils.[88] CD40 is present either as a membrane-bound protein or as a freely soluble protein. The CD40 ligand (CD40L, CD154) is expressed on many cell types, such as activated T cells,

dendritic cells, NK-cells, monocytes, mast cells and eosinophils. When CD40 and CD40L interact, the APC becomes activated. It has been reported that ligation of CD40 enhances the survival of human eosinophils *in vitro via* the secretion of GM-CSF[30] and induction of the expression of cellular inhibitor of apoptosis protein 2.[89]

Sialic acid-binding immunoglobulin-like lectin 8
Sialic acid-binding immunoglobulin-like lectin 8 (Siglec-8) is expressed by eosinophils, mast cells and basophils.[90, 91] Blocking of Siglec-8 with mAbs results in the apoptosis of eosinophils, and this has been suggested as a treatment for eosinophilic disorders.[92]

C-C chemokine receptor type 3
C-C chemokine receptor type 3 (CCR3 also known as CD193) is highly expressed on eosinophils,[93] basophils,[94] monocyte-derived dendritic cells,[95] and a subset of Th2 lymphocytes.[96] CCR3 is the receptor for the chemokines CCL11/eotaxin-1, CCL24/eotaxin-2, CCL26/eotaxin-3, CCL5/RANTES, CCL7/MCP-3 and CCL13/MCP-4. CCR3 also plays a significant role in the accumulation of eosinophils and T cells during allergic inflammation, as in cases of asthma and atopic dermatitis.[97]

CRTH2
CRTH2 also called G protein-coupled receptor 44 or CD294 is expressed by Th2 cells,[98] basophils and eosinophils[99] and is the receptor for the potent eosinophilic chemoattractant prostaglandin D2, the level of which is increased during mast cell-mediated allergic inflammation.[99] CRTH2 on blood eosinophils has been reported to be upregulated in patients with EoE as compared with healthy individuals.[100]

Formyl peptide receptor
Formyl peptide receptor (FPR) belongs to the G protein-coupled receptors that are involved in chemotaxis. Humans have three FPRs (FPR1, FPR2 and FPR3). FPR is the receptor for fMLP. It has been reported that house dust mite allergens activate human eosinophils *via* FPR1 and FPR2.[101] Signaling through FPR1 can desensitize eotaxin-1 receptor CCR3 leading to decreased chemotaxis towards eotaxin-1.[102]

CD23

CD23, is the low-affinity receptor for IgE. CD23 participates in several immune responses, either as a membrane-bound glycoprotein or as a freely soluble protein.[103] The two isoforms **a** and **b** of CD23 are expressed by eosinophils.[104] CD23 is also expressed by T and B cells,[105] neutrophils,[106] monocytes,[107] follicular dendritic cells,[108] intestinal epithelial cells[109] and bone marrow stromal cells.[110] CD23 can also function as an adhesion molecule by pairing with CD21.[111]

CD16

CD16, also known as FcεRIII, is the low-affinity Fc receptor for IgG. CD16 is found on the surfaces of NK cells and neutrophils. Activated eosinophils can also express CD16 in several eosinophilic disorders.[112]

Thymic stromal lymphopoietin

Thymic stromal lymphopoietin (TSLP) is primarily produced by keratinocytes, epithelial cells, smooth muscle cells, allergen-activated basophils and IgE-primed mast cells.[113-119] TSLP is an immunostimulatory factor that is linked to asthma, allergy, and inflammatory diseases. The TSLP receptor (TSLPR), which binds specifically to TSLP, is expressed by $CD11c^+$ dendritic cells, mast cells, and preactivated $CD4^+$ and $CD8^+$ T cells.[120-123] TSLP has been reported to induce the proliferation and differentiation of $CD4^+CD25^+FOXP3^+$ Tregs.[124] Eosinophils also express TSLPR, and when TSLP binds to this receptor it can significantly delay eosinophil apoptosis, upregulate cell surface expression of CD18 and ICAM-1, downregulate L-selectin, and enhance eosinophil adhesion to fibronectin.[125] It has been reported that the TSLP gene is upregulated among patients with EoE.[126]

1.3   EOSINOPHILIC ESOPHAGITIS

Eosinophilic esophagitis (EoE) was unknown until 1978 when it was first described in a case report of an adult man[127] and thereafter for the first time in children in 1995.[128] What is troubling is that the incidence of EoE is increasing.[129] Its geographic distribution is wide, with reported cases not only in the US and northern Europe but also Switzerland, Italy, Spain, Japan and Australia.[130, 131] In a large study conducted in northern Sweden, it was revealed that 0.4-1% of the adult population had the disease.[132] EoE afflicts both children and adults and is somewhat more common among males.[133, 134] Since EoE is

more common among men it has been suggested to have a genetic linkage. As mentioned, the TSLP gene is upregulated among patients with EoE[126] and interestingly, its receptor is situated on the Y chromosome,[135] which may explain the male predominance. However, a recent report suggested that environmental factors play a more important role in this disease than the genetic factors.[136] EoE is now the second leading cause of chronic esophagitis, after gastroesophageal reflux disorder (GERD).[137] EoE and GERD are sometimes confused due to similar symptoms. However, in contrast to GERD, EoE is associated with normal pH levels in the esophagus.[138]
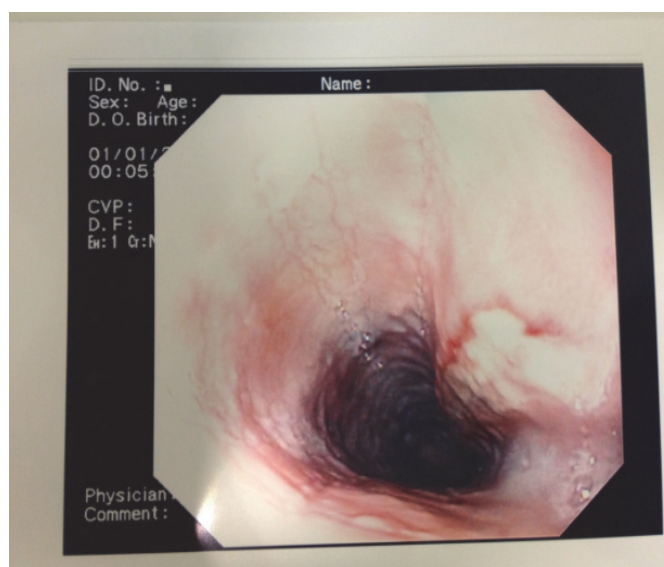

Figure 7. The esophagus from one of our adult patients with EoE. Courtesy of Dr. Mogens Bove, Department of Ear, Nose and Throat, Head and Neck Surgery, NÄL Hospital, Trollhättan, Sweden.

1.3.1   ETIOLOGY

EoE is an antigen-driven inflammatory disease in which eosinophils invade the mucosa of the esophagus. Whereas the esophagus of healthy individuals is devoid of eosinophils,[139] the esophagus of patients with EoE have infiltrated eosinophils and this is used as one of the diagnostic markers. Even though the type of antigen that drives the inflammation in EoE remains unknown, food and inhalant allergens have been implicated.[140] It has been suggested that T cells are responsible for the etiology of the disease, since T cell co-stimulatory molecules seem to play an important role in the immunopathogenesis of EoE.[141] Another report has demonstrated that T cell-deficient mice cannot develop EoE.[142] It has been suggested that EoE is both an IgE-mediated[143] and non-IgE-mediated[144] inflammatory process in which Th2 cytokines, such as IL-4, IL-13, IL-5, IL-6,[145]

and TSLP,[126] and eosinophilic chemokines i.e., eotaxin 1-3[146] and RANTES,[145] are key mediators.

It has also been demonstrated that patients with EoE have an eosinophilic phenotype in the blood that is distinct from that of healthy individuals, in that CD23, CD54, CRTH2 and CD11c are upregulated and CCR3 and CD44 are downregulated.[100] Recently, EETs were found in the tissues taken from patients with EoE and the authors proposed that this was due to an bacterial infection leading to the development of EoE (Figure 8).[147]
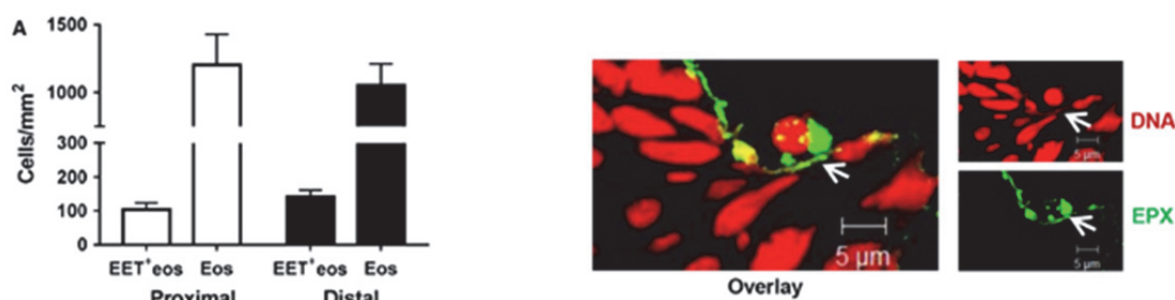

Figure 8. EETs are found in EoE specimens. (A) The number of eosinophils infiltrating the esophageal epithelium and the fraction forming EETs. Right-hand panel: Representative image of an EET (arrows). Reproduced with permission of Wiley and Sons, 2015.[147]

1.3.2 CLINICAL PICTURE

The symptoms of EoE may differ between affected children and adults. Young children with EoE often suffer from feeding problems, vomiting, nausea, and abdominal pain, whereas adults with EoE tend to have dysphagia, food impaction, and retrosternal pain. Many patients also develop altered eating and drinking habits. Infants can be the most difficult age group to diagnose, since they are not able to describe their symptoms in detail. Repeated vomiting is the most common symptom among infants, while other symptoms may include a chronic cough and a refusal to eat. EoE can arise at any age, beginning from infancy through adolescence to adulthood, although the symptomatology of EoE often varies with age. Since the symptoms can be mistaken for GERD symptoms, EoE has been under-recognized for a long time. The disease is chronic with repeated flares.[148] EoE has been reported to significantly affect the quality of life of those affected.[149]

1.3.3 DIAGNOSTICS

The diagnosis of EoE requires a combination of typical symptoms and an endoscopic examination in which typical EoE inflammation is characterized by the presence of furrows, exudates, corrugated rings and/or strictures in the esophagus (Figure 9). Biopsies from different parts of the esophagus are also examined. If the patient has a high number of mucosal eosinophils, i.e., more than 15 cells per high-power field under the microscope, the patient is declared to have EoE.[150] Other typical findings in the esophageal biopsies are increased numbers of T cells, the majority of which are $CD8^+$ T cells, as well as increased numbers of B cells and mast cells[145, 151] and local production of IgE.[152] Patients with EoE usually also display blood eosinophilia. Before the diagnosis is confirmed, other disorders associated with similar clinical, histologic, or endoscopic features, especially GERD, must be excluded. One clue that the patient's symptoms are due to EoE rather than GERD is that he/she normally does not respond to anti-reflux medications, such as proton pump inhibitors (e.g., omeprazole).
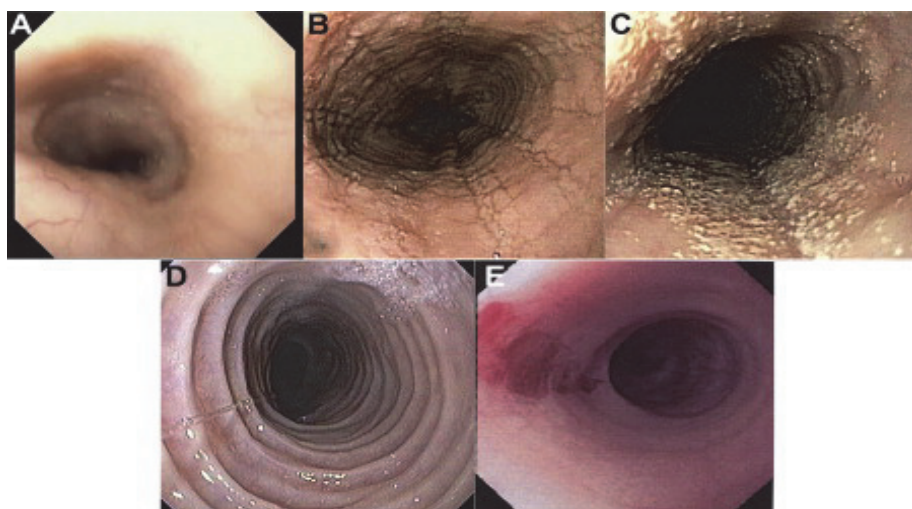
Figure 9. Endoscopic features of eosinophilic esophagitis. (A) Normal esophagus. (B) Esophageal furrowing. (C) White mucosal plaques. (D) Esophageal ring trachealization. (E) Small-caliber esophagus with mucosal tearing after endoscopy. Reproduced with permission of the American Gastroenterological Association.[153]

1.3.4 TREATMENT OPTIONS

Dietary treatment

The majority of patients with EoE (50%–80%) show an atopic constitution based on the coexistence of atopic dermatitis, allergic rhinitis, asthma or the presence of allergic antigen sensitization, determined based on skin prick testing or measurement of serum antigen-specific IgE.[154]As exclusion of the allergen

often improves the symptoms; a dietary elimination regimen is the first-line of treatment for pediatric patients with EoE.[155] The dietary restrictions are divided into three classes: 1) an elemental diet completely lacking in proteins (amino acid-based); 2) a six-food elimination diet (SFED), which involves the exclusion of cow's milk, soy, wheat, eggs, seafood and tree nuts; and 3) the elimination of a specific food allergen identified by allergy testing (either skin prick test or allergen-specific IgE in the serum).[155] The allergen revealed by the allergy test does not always have to be the one that once it is removed eases the symptoms of EoE. Although the elemental diet has been shown to be the most effective approach,[156] the downside of this regimen is that the patients have to live the remainder of their lives on this constrained diet. However, elimination of food allergens from the diet shows that a majority of the children respond well (Figure 10).[128] Conflicting reports indicate that adult patients with EoE may improve,[157] or may not improve[158] when allergenic food is removed from the diet.
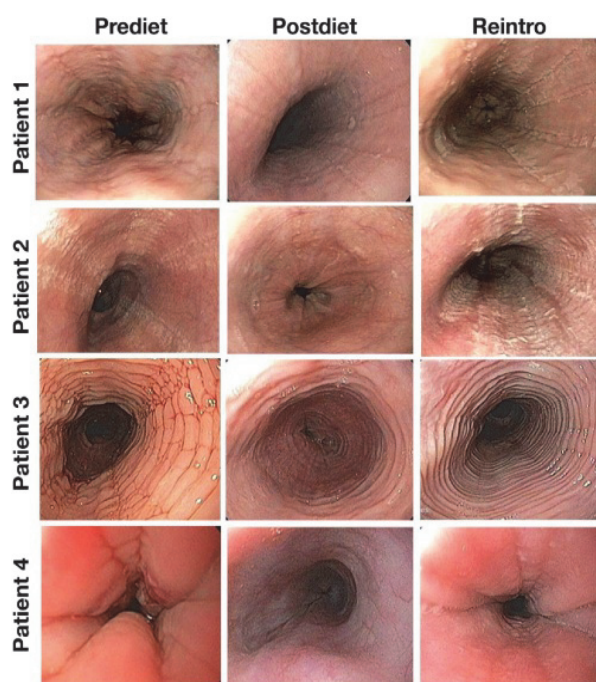
Figure 10. Characteristic endoscopic changes at baseline (left) improve after introduction of the six-food elimination diet (SFED) (middle), and recur upon reintroduction of the trigger food (right). In Patient 1, prominent furrows are noted at baseline, which improve with the SFED and recur upon the reintroduction of wheat. In Patient 2, prominent exudates and subtle rings at baseline improve after the SFED and recur after the reintroduction of wheat. In Patient 3, prominent rings, furrows, and edema are noted, which improve after the SFED and recur with the reintroduction of milk. In Patient 4, prominent furrowing and edema are noted at baseline, which resolve after the SFED and recur with the reintroduction of wheat. Reproduced with permission of the American Gastroenterological Association.[159]

Corticosteroid treatment

In adults with EoE, the treatment of choice is usually topical corticosteroids.[137] As mentioned above, patients with EoE typically do not respond to proton pump inhibitors, such as omeprazole. When this treatment option has been ruled out, topical treatment with a corticosteroid (fluticasone or mometasone furoate) for a period of 2 months is the next choice. Topical corticosteroid treatment has proven to decrease efficiently the number of eosinophils in the esophagus (Figure 11),[137] improve the macroscopic picture,[160] diminish the symptoms[154] and improve the quality of life of the patients with EoE.[161] Unfortunately, many of these patients relapse after a period of time.[162] It has been reported that removal of the eosinophils from patients with EoE, achieved by administration of anti-IL-5 mAbs, results in minimal improvement of symptoms.[163] If patients with EoE are left untreated, esophagitis can lead to fibrosis with structural changes and narrowing of the esophagus, which can lead to a need for mechanical dilatation.[82, 164]
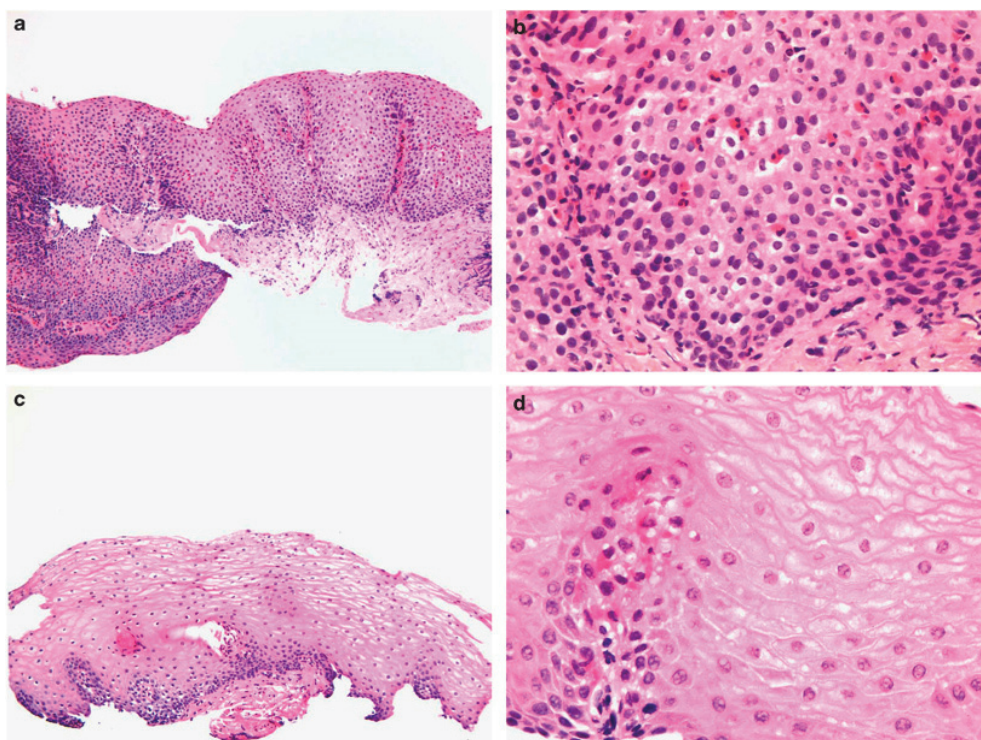
Figure 11. Histopathologic features of eosinophilic esophagitis biopsies before and after successful treatment with corticosteroids. (A,B) Biopsies of eosinophilic esophagitis before therapy. (A) Low-power view shows basal cell hyperplasia, papillary elongation and numerous intraepithelial eosinophils. (B) High-power view shows epithelial cells with reactive nuclear changes and marked eosinophilic infiltration. (C,D) Biopsies of eosinophilic esophagitis after therapy. (C) Low-power view shows normalized mucosa with normal epithelial maturation and resting basal cell layer. (D) High-power view highlights absence of intraepithelial eosinophils and epithelial cells with small pyknotic nuclei and normal maturation of cytoplasms. Biopsies were stained with hematoxylin and eosin. Reproduced with permission of the Nature Publishing Group. Modern Pathology, 2013.[165]

1.3.5 DIFFERENCES BETWEEN PEDIATRIC AND ADULT EOSINOPHILIC ESOPHAGITIS

There are some differences between pediatric and adult cases of EoE, which raises the question as to whether they are separate disease entities.[166] Aside from the symptoms mentioned above, children have more macroscopic signs of active inflammation, such as furrows and exudates of the esophageal mucosa, while adults seem to show signs of chronic inflammation, such as corrugated rings and strictures.[167] There also appear to be histopathologic differences in the esophageal mucosa between children and adults with EoE. For example, there are higher numbers of T cells[168, 169] and mast cells[170, 171] in the adults than in the children, and there are lower number of FOXP3$^+$ regulatory T cells in adults[169] compared with children with EoE.[168, 172] Food allergy appears to be more strongly linked to pediatric EoE than to adult EoE.[173, 174]

2   HYPOTHESES AND AIMS

The purpose of this thesis was to broaden our knowledge of EoE, which in the long run could lead to the development of less invasive diagnostic procedures and new strategies for treatment. The thesis is based on the following hypotheses:

- Topical corticosteroid treatment restores the eosinophilic phenotype in the blood of adult patients with EoE to a healthy phenotype.
- Eosinophils have an immunoregulatory phenotype.
- Eosinophils can, similar to Tregs, suppress T cells by using galectin-10.
- Eosinophils are recruited to the esophagus to dampen the T cell-mediated inflammatory process of EoE.
- Eosinophils isolated from the blood of patients with EoE are more potent T cell suppressors than eosinophils from healthy individuals.
- Blood eosinophils from children with EoE will have a distinct molecular pattern compared with healthy children.
- The eosinophilic molecular pattern of children with EoE differs from that of adults with EoE.

To test these hypotheses, the thesis has the following specific aims:

- Investigation of eosinophilic markers associated with adult EoE before and after topical corticosteroid treatment.
- Study of the intracellular immunoregulatory markers galectin-10 and FOXP3 in blood eosinophils from patients with EoE and healthy individuals using flow cytometry and qPCR.
- Perform *in vitro* co-cultures with eosinophils, from patients with EoE and healthy donors, and polyclonally activated T cells, to examine the capacities of eosinophils to act as suppressor cells.
- Blockage of galectin-10 with monoclonal antibodies, to investigate whether T cell proliferation can be restored.
- Comparison of the molecular patterns of blood eosinophils in children with EoE with those in healthy children using flow cytometry.
- Comparison of the eosinophilic molecular patterns in children with EoE and adults with EoE
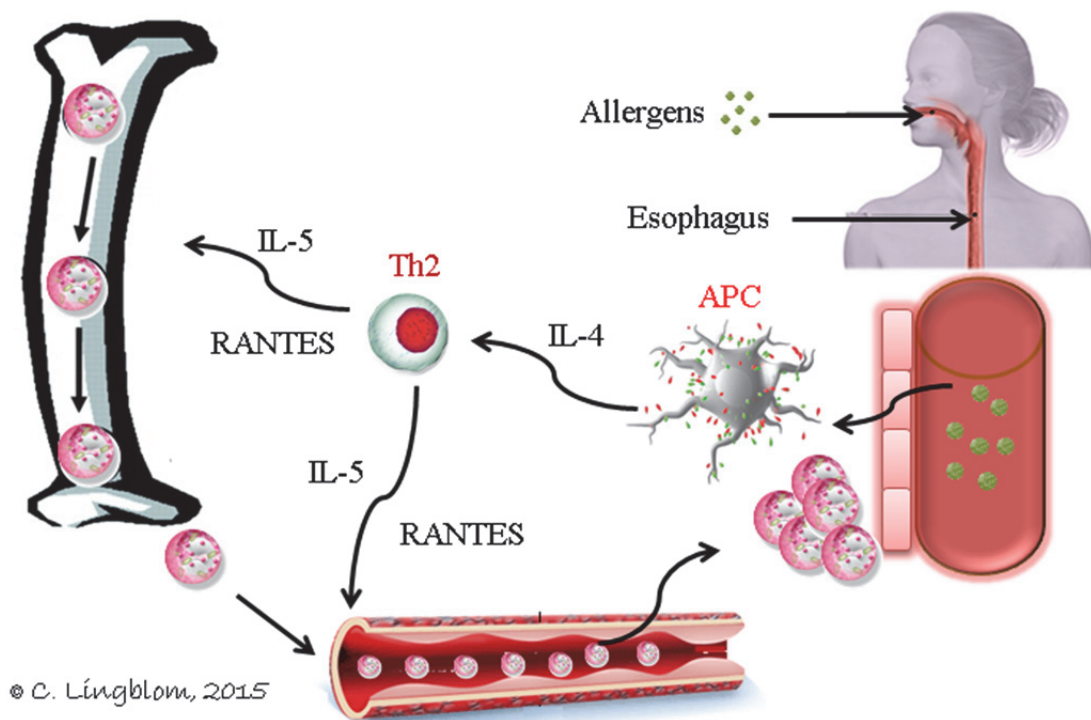

Figure 12. Suggested model of eosinophil recruitment during EoE inflammation. Allergens activate APCs in the tissue, which produces Th2 cytokines, such as IL-4. Successively, Th2 cells produce IL-5 and RANTES, which induce the recruitment of eosinophils from the bone marrow to the blood and eventually to the inflamed esophagus.

3   PATIENTS AND METHODS

3.1   STUDY SUBJECTS

In total, 191 study subjects (108 healthy adults, 20 healthy children, 52 adults with EoE, and 11 children with EoE) donated EDTA-anticoagulated venous blood for flow cytometry analyses and heparin-anticoagulated venous blood for the purification of eosinophils. The maximal time between drawing of blood and flow cytometric analyses was 24 h. The diagnostic criteria for the patients with EoE are taken from the latest EoE guidelines.[150]
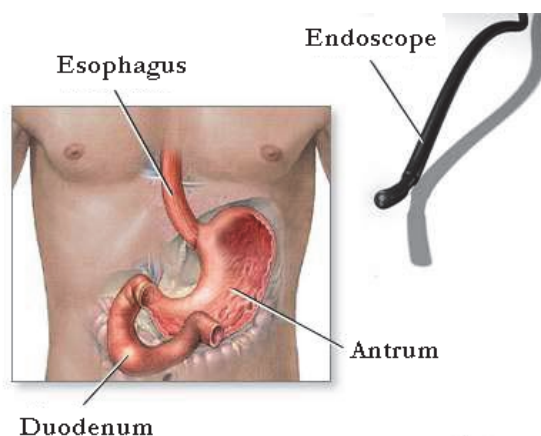

Figure 13. Schematic indicating the sites from which biopsies were taken. Revised version of figure from ADAM Health Solutions

An absolute requirement for EoE diagnosis was the presence of ≥ 15 peak count eosinophils per high-power field in one of the biopsies collected with an endoscope from the proximal and distal parts of the esophagus. Biopsies from the duodenum and gastric antrum were also examined, to ensure that patients with alternative gastrointestinal disorders were not included.

3.2 FLOW CYTOMETRY

Erythrocytes in EDTA-anticoagulated venous blood were removed by repeated hypotonic lysis. The remaining leukocytes were washed once in Krebs-Ringer-glucose buffer (KRG) without calcium.[175] The unfractionated leukocytes were incubated with 1 mg/ml Vivaglobin (CLS Behring, King of Prussia, PA, USA) to hinder unspecific binding of mAbs.

Unfixed, leukocytes were incubated with antibodies directed against extracellular markers for 20 min and then washed with MACS buffer (with KRG without Ca^{2+}). For intracellular staining, the cells were fixed and permeabilized with the FOXP3 Staining Buffer Set (eBioscience, San Diego, CA, USA) and labeled with anti-FOXP3 mAb or unconjugated anti-galectin-10 mAb followed by PE-conjugated secondary antibody (Table 1).

Measurements of the surface and intracellular expression levels of various molecules were performed using a FACSCanto II^{TM} Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with Diva 6 software; cells were analyzed using the software Flow Jo (Tree Star Inc., Ashland, OR, USA). Eosinophils were gated based on high side-scatter, low/no expression of CD16, and high levels of CCR3 in Paper I, II and IV. In Paper III the separation was based on high side-scatter, high expression of Siglec-8, and high levels of CCR3. Regulatory T cells were gated as CD4+, CD25^{pos}, and CD127$^{lo/neg}$. As a control for background staining for the surface markers "Fluorescence Minus One"-technique was employed, [176] and as a control for the intracellular markers an isotype mAb was used. Data are expressed as median fluorescence intensities (median-FI).

Table 1. Antibodies and probes used in the flow cytometric analyses

CD	ANTIGEN SPECIFICITY	CLONE	ISOTYPE	FLUOROCHROME
CD4	Co-receptor for MHC II	SK3	IgG$_1$	APC-H7
CD16	FcγRIIIb, IgG-R	3G8	IgG$_{1a, κ}$	FITC, PE
CD18	Integrin β2 chain	6.7	IgG$_{1a, κ}$	FITC
CD23	FcεRII, IgE-R	EBVCS-5	IgG$_{1, κ}$	APC
CD25	IL-2R α chain	2A3	IgG$_{1, κ}$	APC
CD40	Binds CD154 (CD40L)	5C3	IgG$_{1, κ}$	APC
CD44	Binds hyaluronic acid	G44-26	IgG$_{2b, κ}$	APC
CD54	ICAM-1	HA58	IgG$_1$	APC
CD127	IL-7 receptor α-chain	HIL-7R-M2	IgG$_{1, κ}$	FITC
CD193	CCR3	5E8	IgG$_{2b, κ}$	PE
CD294	CRTH2 (PGD$_2$ receptor 2, DP2)	BM16	IgG$_{2a}$	Alexa Fluor 647
	FPR2	304405	IgG$_{2b}$	APC
	FOXP3	259D/C7	IgG$_1$	PE
	Galectin-10	B-F42	IgG$_1$	APC
	Galectin-10	B-F42	IgG$_1$	Unconjugated
	Isotype control	MOPC-21	IgG$_1$	PE
	Secondary antibody	X56	IgG$_1$	PE
	Siglec-8	7C9	IgG$_1$	APC
	Phalloidin			Alexa Fluor 488
	Annexin V			PE
	7-Aminoactinomycin D (7AAD)			
	DAPI			

3.3   EOSINOPHIL PURIFICATION

Peripheral blood eosinophils were freshly isolated from heparinized venous blood. After removal of the majority of the erythrocytes by 20 min of dextran sedimentation (dextran:blood = 1:1) at room temperature, centrifugation (400 × *g* for 20 min at 4°C) on a Ficoll gradient was used to separate the granulocytes from the mononuclear cells (PBMC). The remaining erythrocytes were removed from the granulocyte fraction by 3 cycles of hypotonic lysis in 6 mL distilled water for 35–40 seconds and stopped by the addition of 2 mL of 3.4% NaCl.

The cells were then washed in 15 mL Ca^{2+}-free KRG (120 mM NaCl, 5 mM KCl, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, 10 mM glucose, 1.5 mM $MgCl_2$; pH 7.3). The eosinophils were isolated from the granulocyte fraction by negative immune depletion. The granulocytes were incubated with a mixture of magnetic beads coated with mAbs (MACS; Miltenyi Biotec Inc., Bergisch Gladbach, Germany) directed against CD3 (T cells), CD14 (monocytes), CD16 (neutrophils), and CD19 (B cells), and the cells that adhered to the beads were removed magnetically in a magnetic cell sorter VarioMACS, CS-column (MACS; Miltenyi Biotec). The eosinophils were collected and washed twice with KRG. The purity of eosinophils was routinely >98%, as determined by counting 200 Diff-Quik-stained (Dade Behring AG, Deerfield, IL, USA) cytospun cells (Cytospin; Shandon Scientific Co. Ltd., London, UK) under a light microscope (Figure 14).
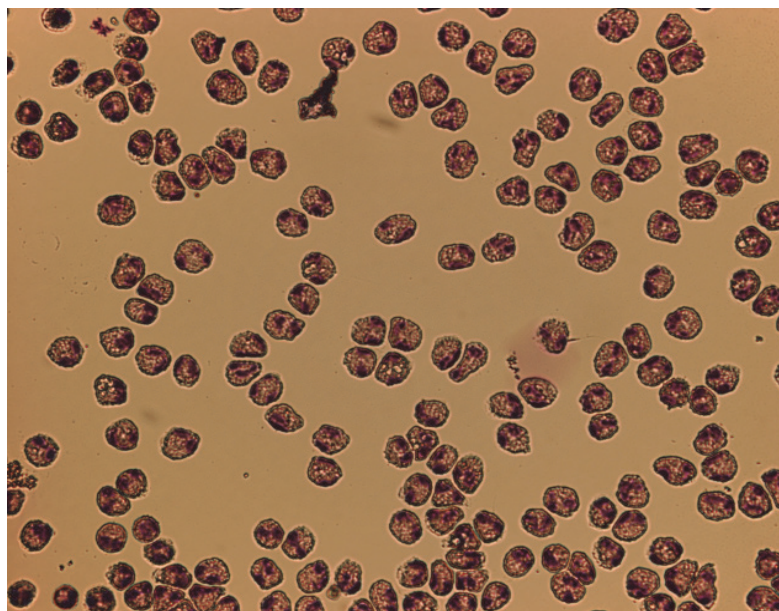

Figure 14. Micrograph of Diff-Quik-stained, cytospun eosinophils after purification.

3.4 *IN VITRO* TREATMENT OF EOSINOPHILS WITH CORTICOSTEROIDS

Purified eosinophils from healthy individuals were incubated with the same corticosteroid that the patients in the EoE study received. Eosinophils were incubated with mometasone furoate (Nasonex; Schering-Plough, Brussels, Belgium) at a final concentration of 50 (~0.1nM), 500 (~1nM) or 5000 (~10nM) pg/mL in X-Vivo buffer without phenol red (Lonza, Vievers, Belgium) for 1 h at 37°C in a thermostat that contained 5% CO_2. Eosinophil expression of CD18 was measured by flow cytometry. Eosinophils were stained with antibodies

against Annexin V (cat. no 556422; PE, BD Biosciences) and 7AAD (cat. no 559925; BD Biosciences) to determine the percentages of apoptotic and necrotic eosinophils. Annexin V+/7AAD- cells were defined as being apoptotic, Annexin V-/7AAD- cells were considered non-apoptotic, and Annexin V+/7AAD+ cells were designated as being dead.

3.5   ADHESION OF EOSINOPHILS TO ENDOTHELIAL CELLS

Human umbilical vein endothelial cells (HUVEC) were cultured for 2 days in M200 medium supplemented with the LSGS-kit (all from Cascade Biologics, Carlsbad, CA, USA) in 96-well plates (NUNC, Roskilde, Denmark). The endothelial cells were activated by treatment with 1 µg/mL of *Escherichia coli* LPS (Sigma-Aldrich St. Louis, MO, USA) for 4 h, and washed three times with M200 medium before purified eosinophils were added. Eosinophils were either preincubated for 10 min with anti-CD18 mAb (clone TS1/18, IgG$_1$,κ; BioLegend, San Diego, CA, USA) or an isotype control (clone HIT8a, IgG$_1$, κ; BD Biosciences), both at a final concentration of 3 µg/mL, or just M200 medium. Eosinophils (100,000 cells) were allowed to adhere for 30 min, and nonadherent cells were removed by washing three times with PBS. Adherent eosinophils were lysed with 1% Triton (Sigma Chemical Co., Steinheim, Germany) in PBS and quantified by calculation of eosinophil peroxidase activity, which was measured by the addition of 4 µL of 30% H$_2$O$_2$ and 10 mg *o*-phenylenediamine (Sigma Chemical Co.).[177] For calculation of adherent eosinophils the following formula was used: absorbance in wells that contained an unknown number of eosinophils / absorbance in wells that contained the maximum number of eosinophils (100,000).

3.6   ADHESION OF EOSINOPHILS TO ICAM-1, ICAM-2, VCAM-1 AND PERIOSTIN

96-well plates (catalog no. 351172; BD Labware, Franklin Lakes, NJ, USA) were coated with 1 µg ICAM-1 (CD54), ICAM-2 (CD102), VCAM-1 (CD106) or periostin (R&D Systems, Minneapolis, MN, USA) in 100 µl Tris-buffered saline (pH 8) for 2 h at 37°C, and thereafter blocked with 100 µL FBS. Control wells were coated with FBS alone. Eosinophils were either preincubated with 3 µg/mL of anti-CD18 mAb (clone TS1/18, IgG$_1$, κ; BioLegend), 3 µg/mL isotype control (clone HIT8a, IgG$_1$, κ; BD Biosciences) or resuspended in KRG that contained calcium. Eosinophils (10,000) were incubated for 15 min, non-

adherent cells were removed by washing with Tris-buffered saline three times, and adherent cells were quantified as described above. For the calculation of adherent eosinophils, the following algorithm was used: absorbance in wells that contained an unknown number of eosinophils / absorbance in wells that contained maximum number of eosinophils (10,000).

3.7 RNA EXTRACTION AND REAL-TIME PCR

Total RNA from eosinophils was extracted using the RNeasy Mini kit (Qiagen, Venlo, Netherlands). RNA quality was assessed using electrophoretic gel assessment of RNA degradation (Experion; Bio-Rad Laboratories, Berkeley, CA, USA) Total RNA (25 ng) was transcribed into cDNA in a 20-µl-reaction mixture that contained AMV reverse transcriptase, PCR-dNTP mix, RNase-inhibitor, and random primer pd(N)6 (all from Roche Applied Bioscience, Foster City, CA, USA). Reverse transcription reactions were performed under the following conditions: 20°C for 5 min; 42°C for 50 min; 70°C for 5 min; and storage at -80°C until further use. Transcript expression was evaluated using TaqMan gene expression assays that targeted *FOXP3* (accession no. Hs01085834_m1), galectin-10 (Hs00171342_m1), and the house-keeping gene hypoxanthine phosphoribosyltransferase 1, HPRT1 (Hs99999909_m1) (Applied Biosystems, Carlsbad, CA, USA). Analyses were performed using a 7500 Real-Time PCR system and the 7500 System SDS software (Applied Biosystems). The PCR instrument was programed as follows: one cycle of 2 min at 50°C; one cycle of 10 min at 95°C; followed by 40 cycles of 15 s at 95°C, and then 1 min at 60°C. The fold change in RNA level in each sample was estimated using the Pfaffl method:[178]

$$Fold\ change = \frac{\mathrm{E_{target}}^{\Delta Ct\ target(control-patient)}}{\mathrm{E_{reference}}^{\Delta Ct\ reference(control-patient)}} \qquad (Eq.\ 1)$$

$$E = 10^{\left(-\frac{1}{slope}\right)} \qquad (Eq.\ 2)$$

where E is the efficiency of each primer pair, Ct is the cycle threshold, and the slope is calculated from the standard curve. The results are presented as fold-changes in total RNA expression relative to the corresponding expression levels in healthy controls.

3.8 IMMUNOBLOTTING

Highly pure eosinophils (90,000–150,000 cells) were mixed with RIPA Buffer (cat no. 89900; Thermo Fisher Scientific, Rockford, IL, USA) with a protease and phosphatase inhibitor cocktail (cat no. 78440; Thermo Fisher Scientific), and stored at -20°C until immunoblotting was performed. Eosinophil lysates were separated on a 4%–15% SDS-PAGE gel, and electrophoretically transferred onto a PVDF membrane (Bio-Rad Laboratories). The membranes were blocked with 5% non-fat milk in TBS-Tween 20 (TBST) and incubated with anti-FOXP3 primary mAb (clone PCH101, diluted 1:200; eBioscience) at 4°C overnight, followed by incubation with HRP-conjugated secondary antibody (1:6000; eBioscience) in TBST at room temperature for 2 h. The blots were developed using the OPTI-4CN Substrate Kit (Bio-Rad Laboratories). Human FOXP3-transfected cell lysate (Imgenex, San Diego, CA, USA) and regulatory T cells were used as positive controls.

3.9 IMAGE FLOW CYTOMETRY

Image flow cytometry is a tool what combines quantitative *image* analysis and *flow cytometry*. This instrument has the advantage that one can obtain images of the cells, which makes it possible to study individual cells in greater detail, for instance co-localization between two cell types, internalization of different proteins and subcellular distribution of molecules of the nucleus *versus* the cytoplasm (Figure 15).

Unfixed, leukocytes were incubated with antibodies directed against extracellular antibodies for 20 min. and then washed with MACS buffer (with KRG without Ca^{2+}). For intracellular staining the cells were fixed and permeabilized with the FOXP3 Staining Buffer Set (eBioscience, San Diego, CA, USA) and labeled with PE-conjugated anti-FOXP3 mAb or APC-conjugated anti-galectin-10 mAb (Table 1). One thousand eosinophils were collected in the image flow cytometer (Image Stream X; Amnis, Seattle, WA, USA) and analyzed with the IDEAS software ver. 6.0. To determine the fractions of the investigated molecules in the nucleus and cytosol, an adapted nucleus mask was made, where the similarity intensities of staining with DAPI and the molecule in question, were calculated. The median distribution in the nucleus (MD) in 1000 eosinophils is calculated according to:

$$MD(\%) = \frac{\text{IT (Object (M01,DAPI,Tight),DAPI,50)}_{molecule}}{\text{IO(M03,molecule,Tight)}_{molecule}} \times 100 \qquad (Eq.\,3)$$

where IT is the intensity threshold, which means the overlap between the DAPI-stained nucleus and the intensity of the target molecule, and IO is the intensity object, which means the total intensity from the target molecule.
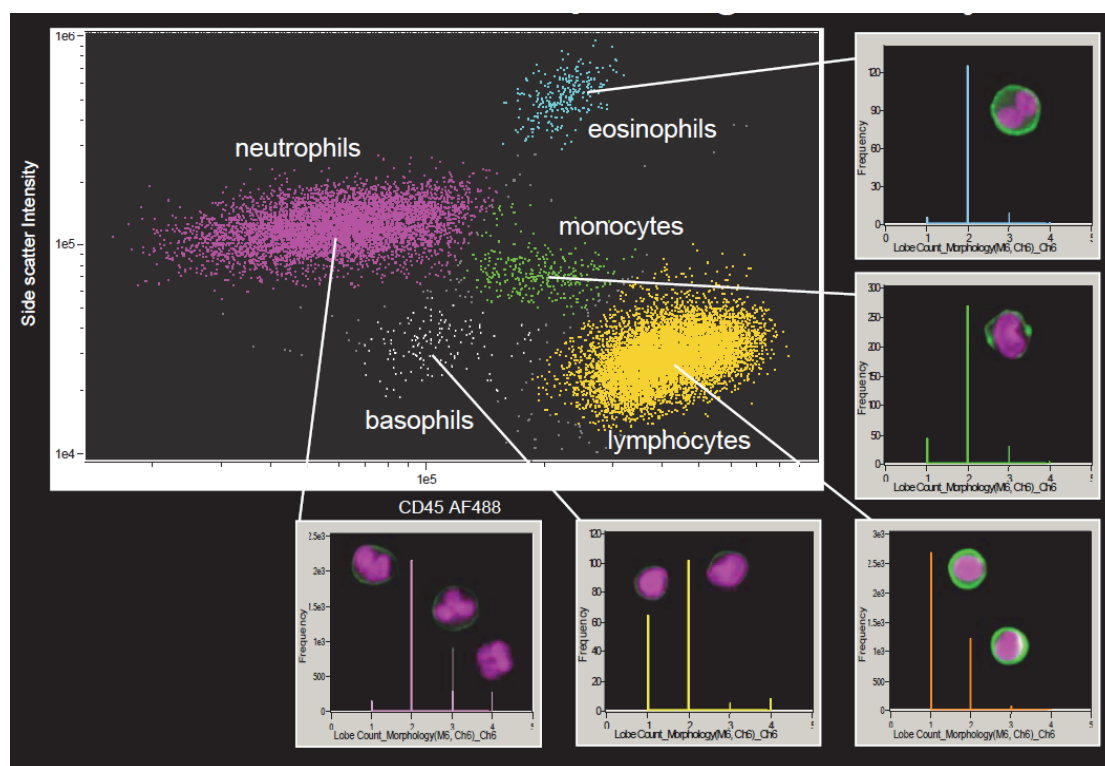

Figure 15. Human leukocytes separated according to size and expression of CD45. Visual confirmation with nuclear lobe count in individual cells. Expression of CD45 (green) and DAPI-stained nucleus (purple) are demonstrated.

3.10   VISUALIZING EETS WITH CONFOCAL MICROSCOPY

Cells from a 2-day eosinophil:PBMC co-culture were washed and incubated with an Alexa Fluor 488-conjugated mAbs against phalloidin (which binds to filamentous actin). MACS bead-purified eosinophils were stimulated with 20 ng/mL recombinant TSLP (R&D Systems) for 30 min, washed three times with MACS without calcium, and then incubated with APC-conjugated anti-galectin-10 mAb. The cells were mounted in Prolong antifade reagent with DAPI nuclear stain (Invitrogen) and air-dried on microscope slides. Confocal immunofluorescence microscopy (LSM700; Zeiss, Oberkochen, Germany) analysis was used to visualize the EETs.

3.11   MIXED LYMPHOCYTE REACTION

PBMC from a healthy blood donor (responder cells) were mixed with γ-irradiated (25 Gy; ^{60}Co source) pooled PBMC from 11 donors (trigger cells) in a

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96-well microplate (Techno Plastic Products AG, Trasadingen, Switzerland). Three hours later, eosinophils were added to the mixture of responder cells and trigger cells. In some cases mAb against galectin-10 (4µg/mL, clone B-F42) or an isotype control (mouse IgG1, clone 107.3; BD Biosciences) or 10 U of DNase (Roche Applied Bioscience) was added to the cultures. The mixed lymphocyte reaction (MLR) was incubated for 6 days at 37°C. Cellular proliferation was measured *via* the incorporation of ^{3}H-thymidine (20 Ci; 1 Curie/well; Perkin Elmer, Waltham, MA, USA) for 6 h, followed by cell harvesting. Cellular proliferation was quantified as counts per minutes (cpm) using a β-scintillation counter (1450 MicroBeta TriLux; Perkin Elmer). Cells in the MLR were stained with Annexin V (cat. No 556422, PE), anti-FOXP3 mAb (IgG1, clone 259D/C7; PE) and an isotype control antibody (clone MOPC-21, IgG$_1$, κ) (all from BD Biosciences), before and 2 and 6 days after the start of the co-culture. For the calculation of percentage inhibition of T cell proliferation with or without either galectin-10 or DNase, the following algorithm was used: (1-cpm with eosinophils/cpm without eosinophils)×100 compared with (1-cpm with eosinophils+galectin-10 or DNase/cpm without eosinophils+galectin-10 or DNase)×100.

3.12 EOSINOPHILS CO-CULTURED WITH CD3/CD28 ACTIVATED PBMC

PBMC from healthy blood donors were added to anti-CD3 (1µg/mL, eBioscience, San Diego, CA, USA) coated 96-well microplate (Techno Plastic Products AG, Trasadingen, Switzerland). Three hours later, eosinophils were added at a 1:1 ratio (10^5 cells) and lastly soluble anti-CD28 mAb (8µg/mL, eBioscience, San Diego, CA, USA) was added. The co-culture was incubated for 2 days at 37°C. Cellular proliferation was measured *via* the incorporation of ^{3}H-thymidine, as described above.

3.13 FLOW CYTOMETRY SORTING AND CO-CULTURING

Granulocytes and PBMC were freshly isolated from heparinized venous blood as described above. To isolate the neutrophils and the eosinophilic subgroups flow cytometry sorting was used. The eosinophils were incubated with 1 mg/ml Vivaglobin (CLS Behring, King of Prussia, PA, USA) to hinder unspecific binding of (mAbs), and then incubated with fluorochrome-conjugated mAbs against Siglec-8 (clone 7C9, IgG$_1$, APC, BioLegend), CD16 (clone 3G8, IgG$_1$, κ; PE, BD Biosciences) and CCR3 (CD193, clone 5E8, IgG2$_b$, κ; BV421; BD

Biosciences). A SY3200 flow cytometer sorter (Sony Biotech, Champaign, IL, USA) equipped with a 100µm-nozzle was used. To determine the purity levels of the eosinophilic subgroups the cells were stained with Diff-Quik. The PBMCs were added to 96-well microplates (Techno Plastic Products) that were coated with anti-CD3 mAb (1 µg/mL; eBioscience, San Diego, CA, USA). Three hours later, CD16^{neg}, CD16^{dim}, and CD16^{hi} eosinophils were added at a 1:10 ratio and finally, anti-CD28 mAb (8µg/mL, eBioscience) was added. The co-culture was incubated for 2 days at 37°C. Cellular proliferation was measured *via* the incorporation of ^{3}H-thymidine, as described above. The equation used for calculating the percentage inhibition of T cell proliferation was:

$$Inhib.\,(\%) = \frac{(1 - \text{ cpm for PBMC} + \text{eos})}{\text{cpm for PBMC}} \times 100 \qquad (Eq.\,5)$$

3.14 STATISTICAL ANALYSES

3.14.1 UNIVARIATE ANALYSIS

The ANOVA Kruskal-Wallis test was used to determine the statistical significance of the differences between three study groups. Dunn's multiple comparison test was employed to determine which of the three groups differed. Wilcoxon paired and Mann-Whitney non-paired tests were used for comparisons of two groups with datasets that included continuous parameters, and the Spearman test was used for determinations of correlations between datasets. The Fisher's exact test was used for comparisons of two groups with datasets with dichotomous parameters. A *P*-value <0.05 was considered statistically significant. For the statistical analyses, GraphPad Prism 5.0 was employed (GraphPad, San Diego, CA, USA).

3.14.2 MULTIVARIATE ANALYSIS

Multivariate analyses of pattern recognition "Orthogonal Partial Least Squares-Discriminant Analysis" (OPLS-DA) and Orthogonal-Projection to Latent Structures (OPLS) were performed using the SIMCA-P (version 13.03) statistical package (Umetrics, Umeå, Sweden). OPLS-DA is an expansion of partial component analysis (PCA), but instead several Y-variables are introduced, and their relationship to X-variables examined.[179] In our studies, the study subjects were set as Y-variables and the levels of the different eosinophilic molecules were set as X-variables. Prior to all calculations, X-variables with >10-fold distribution were log-transformed using the SIMCA transformation

tool. Mean-centering and unit variance scaling were implemented to give all variables an equal chance of exerting the same influence on the model independently of data scale and distribution. Each model is defined by R^2Y, which estimates the amount of variance in Y that is explained by the X-variables. A high value indicates that the selected X-variables have generated a model that can explain differences that exist between the studied groups. A model is also given a Q^2Y value, which describes the validity of the model. This is determined with cross-validation, a procedure where one study subject is removed and the capacity of the remaining subjects to predict the separation between the groups is assessed. This procedure is repeated for all the subjects; a high value indicates that the model is stable no matter which subject is excluded. An additional test is performed to validate the model, i.e., a permutation test that evaluates the risk that the current model is spurious, i.e., the model just fits the current set well but does not adequately predict Y for new observations. The program also chooses the principal components (PC) that have the highest impacts on the model. If more than one PC has an impact on the model, a two-dimensional model is generated that shows the two most important components (PC1, PC2) for separation of the Y-variables. A loading plot shows the pq1 values for each X variable. This is the value upon which the principal components are based, and it explains the impact that the X-variables has on the model.[180]

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4 RESULTS

4.1 BLOOD EOSINOPHILS DOWNREGULATE CD18 IN PATIENTS WITH EoE AFTER TOPICAL CORTICOSTEROID TREATMENT (PAPER I)

In Paper I, the effect that topical corticosteroids have on the phenotype of blood eosinophils was studied. Blood eosinophils were analyzed by flow cytometry from 14 patients with EoE before treatment and from 9 of the patients with EoE after topical corticosteroid treatment. Since we hypothesized that the eosinophilic phenotype would revert to a healthy phenotype, the eosinophils from 10 healthy individuals were analyzed as controls. Comparing the eosinophils from patients with EoE before topical corticosteroid treatment with those of the healthy controls, we found that CD54 was upregulated, whereas CD44 and CCR3 were downregulated in the patients with EoE. We found that the eosinophilic phenotype did not change significantly after topical corticosteroid treatment, with the exception off one marker, CD18, the expression of which was significantly reduced. Thus, we conclude that the patients with EoE who received corticosteroids still had a "sick" phenotype and the only difference was that the ability of the eosinophils to enter the tissue had been decreased. To investigate this hypothesis, we blocked CD18 using a neutralizing antibody and examined how this influenced the capacities of eosinophils to adhere to endothelial cells and to ICAM-1, ICAM-2, VCAM-1,

and periostin. We discovered that the adherence of eosinophils to endothelial cells (38%, *P*=0.012, *n*=5), ICAM-1 (67%, *P*=0.0009, *n*=18), and ICAM-2 (56%, *P*=0.0008, *n*=18) decreased after neutralization of CD18. However, this was not the case for eosinophil adherence to VCAM-1 or periostin (Figure 16).
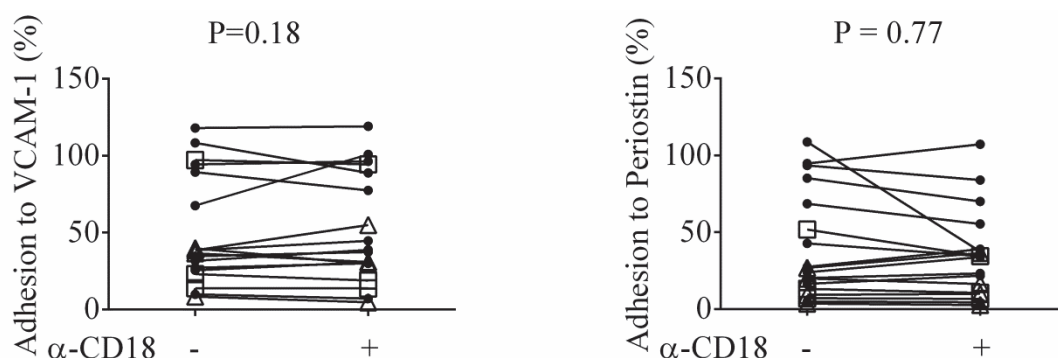

Figure 16. Adherence to immobilized VCAM-1 and periostin of eosinophils that were pretreated with and without anti-CD18 mAb. Three different groups were examined: healthy controls (dots, n=11); patients with eosinophilic esophagitis before corticosteroid treatment (squares, n=3) and patients with eosinophilic esophagitis with ongoing corticosteroid treatment (triangles, n=4).

We also saw that the expression of CD18 strongly correlated with the ability of eosinophils to adhere to ICAM-1 (r=0.71, *P*=0.0008, *n*=18) and ICAM-2 (r=0.68, *P*=0.0017, *n*=18). This was also true for VCAM-1 and periostin (Figure 17).
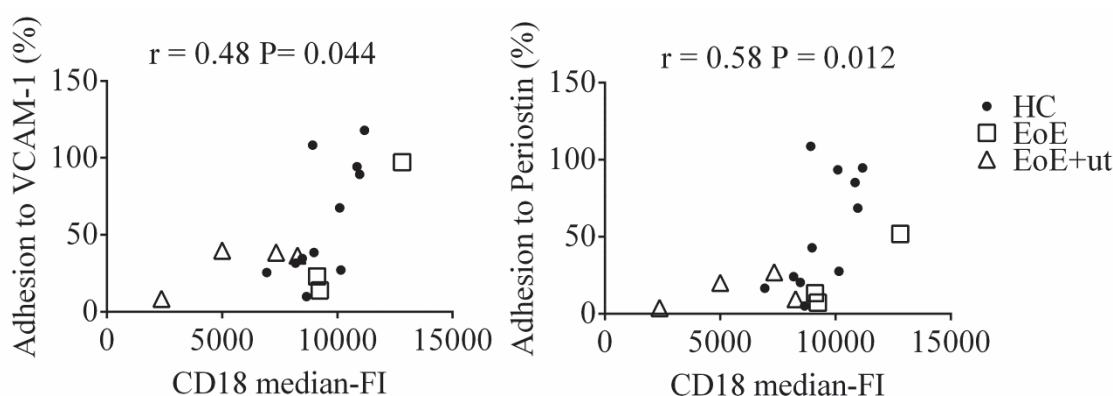

Figure 17. Correlation between eosinophil CD18 expression level and eosinophil adhesion to VCAM-1 and periostin. Three different groups were examined, healthy controls (HC, dots, n=11), patients with eosinophilic esophagitis before corticosteroid treatment (EoE, squares, n=3) and patients with eosinophilic esophagitis with ongoing corticosteroid treatment (EoE+ut, triangles, n=4).

As confirmation of the importance of CD18 for the entry of eosinophils into the esophagus, we found that the expression of CD18 on blood eosinophils was positively associated with the peak number of eosinophils/high-power field in

the esophageal biopsies collected from the patients with EoE (r=0.53, *P*=0.040, *n*=14).

The topical corticosteroids given to patients with EoE is believed to have <0.1% systemic effect, which can be translated into 40 pg/mL of mometasone furoate in the blood. The effect of this concentration of corticosteroids on eosinophilic CD18 expression was evaluated *in vitro*. CD18 levels on the eosinophil surface were not reduced when this low concentration of corticosteroid was added, but significantly depressed when 10 and 100 times higher concentrations of corticosteroid were administered.

Additionally, we uncovered an inverse correlation between the expression of CD40 (r=0.70, *P*=0.0050, *n*=14) as well as CCR3 (r=-0.57, *P*=0.032, *n*=14) and the number of eosinophils/high-power field in the esophageal biopsies collected from the patients with EoE.

4.2   EOSINOPHILS HAVE AN IMMUNOREGULATORY PHENOTYPE AND FUNCTION (PAPER II)

Paper II is based on the hypothesis that eosinophils are recruited to the inflamed esophagus to dampen the activated T cells in the T cell-mediated disorder of EoE. Initially, we investigated whether eosinophils from patients with EoE expressed higher levels of the immunoregulatory protein galectin-10 than healthy individuals, based on the hypotheses that eosinophils, in similarity to Tregs, use galectin-10 to suppress T cells. We found that the blood eosinophils of patients with EoE had more than two-fold higher galectin-10 protein levels and almost five-fold higher levels of mRNA for galectin-10 than eosinophils of the healthy controls.

Next, we wanted to investigate whether or not the eosinophils from patients with EoE could suppress T cells. We discovered that eosinophils from both patients with EoE and healthy individuals inhibited T cell proliferation. Eosinophils from patients with EoE inhibited T cell proliferation by a median of 51% whereas eosinophils from healthy donors inhibited T cell proliferation by a median of 90%; contrary to our expectations, the healthy eosinophils were more potent suppressors than those of the EoE patients. This made us wonder if eosinophils could also express FOXP3, which is a characteristic of Tregs. We demonstrated with flow cytometry, qPCR, immunoblotting, and image flow cytometry that eosinophils certainly express FOXP3, with the majority of this expression being

localized to the cytosol, and not to the nucleus as in Tregs. We also found that eosinophils from patients with EoE expressed two-fold higher levels of FOXP3 protein and more than three-fold higher levels of mRNA for FOXP3 than eosinophils from healthy donors. It is noteworthy that eosinophilic expression of FOXP3 decreased after 2 and 6 days of co-culturing of eosinophils with activated T cells (Figure 18) and that the initial expression of FOXP3 correlated positively with the number of viable eosinophils after 6 days of co-culture (r=0.76, *P*=0.015, *n*=10).
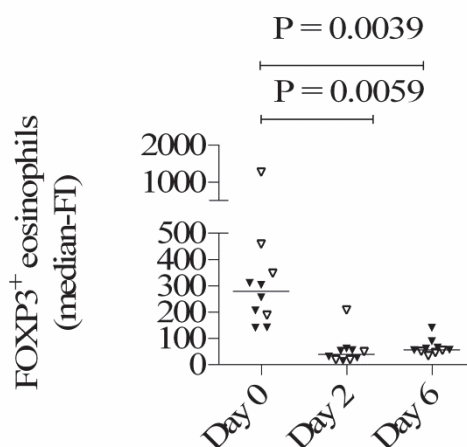

Figure 18. Expression levels of FOXP3 before and after 2o and 6 days of co-culture with activated T cells. Eosinophils from healthy donors (filled triangles, n=6) and from patients with EoE (open triangles, n=4) were used.

4.3   EOSINOPHILS USE GALECTIN-10 TO SUPPRESS T CELLS (PAPER II & III)

To investigate further the mechanism underlying eosinophil-mediated T cell suppression we introduced an anti-galectin-10 mAb to this reaction. The anti-galectin-10 antibody partially inhibited the suppressive capacity of the eosinophils. Approximately, 30% of the T cell proliferation was restored by blocking galectin-10. We continue this investigation into the function of galectin-10 in Paper III.

Preliminary data from confocal microscopy demonstrated that EETs are produced during co-culture of eosinophils with CD3/CD28-stimulated PBMC (*n*=1). DNA nets were observed to be attached to the eosinophils. Additional experiments using an image flow cytometer revealed that the EETs contained evenly distributed galectin-10 (*n*=2). No DNA nets were released when the

eosinophils were incubated with unstimulated PBMC. EETs that contained galectin-10 was also generated when eosinophils were stimulated with TSLP (n=1). We attempted to disrupt the galectin-10-containing EETs by adding DNase to the cell culture (n=1). The first experiment resulted in 20% restoration of the T cell proliferation (Figure 19). Additional studies to ascertain these findings are needed.
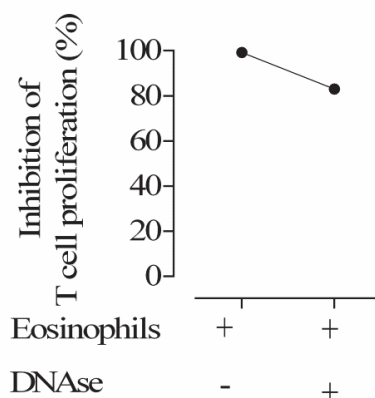

Figure 19. Eosinophils with and without DNase was added to the co-culture with stimulated T cells.

Using image flow cytometry, we observed that the eosinophils were attached to CD4$^+$ and CD25$^+$ T cells after 2 days of co-culturing, in what appeared to be a synaptic connection (n=2). We discovered that galectin-10 is apparently transferred from the eosinophils to the T cells during co-culturing. Galectin-10-containing protrusions were identified in the synaptic area between the cells (Figure 20). In several images, it appears to be completed transfer of galectin-10 from the eosinophil to the T cell (Figure 21).
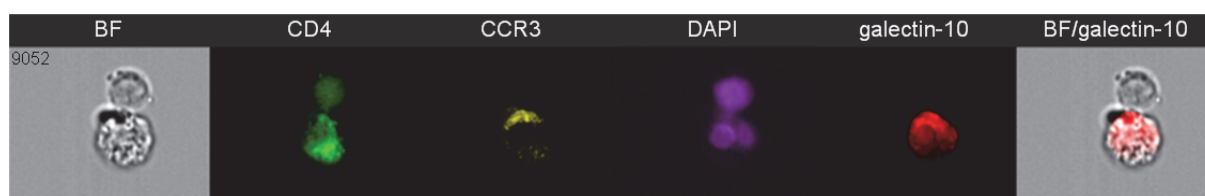

Figure 20. ImageStream picture of galectin-10 accumulation at the synaptic area between a CCR3+ eosinophil and a CD4+ T cell. Eosinophils can upregulate CD4 upon stimulation.[181]
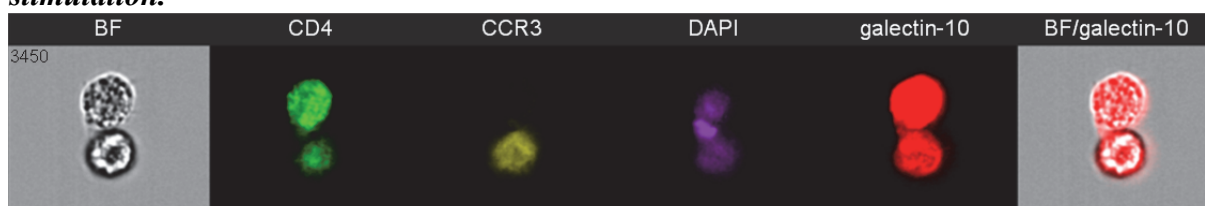

Figure 21. ImageStream picture of a transfer of galectin-10 from a CCR3+ eosinophil (bottom cell) to a CD4+ T cell (top cell). Activated eosinophils can upregulate CD4.[181]

4.4   EOSINOPHILIC SUBGROUP IS A SUPERIOR T CELL SUPPRESSOR (PAPER III)

During the isolation of eosinophils from the blood, the eosinophils are traditionally separated from the neutrophils based on the eosinophils supposed lack of CD16. Our preliminary data demonstrated that after 2 days of co-culture with CD3/CD28-stimulated PBMC, a subpopulation of the CD16^{neg} eosinophils became CD16^{hi}. In addition, the intracellular levels of galectin-10 decreased in this population (n=1). This subpopulation was not detected when eosinophils were co-cultured with unstimulated non-proliferating T cells (n=1). We then investigated the presence of CD16^{hi} eosinophils in blood samples. Healthy children had a larger fraction of CD16^{hi} eosinophils than adults: with a median of 12% (25%/75%=5%–24%) in children and a median of 5% (25%/75%=3%–7%) in adults (P=0.036). Contrary to the CD16^{hi} eosinophils that developed after co-culture with proliferating T cells, these CD16^{hi} eosinophils in the blood expressed <u>higher</u> levels of galectin-10 and FOXP3 (Figure 22), both in children (n=10) and in adults (n=9).
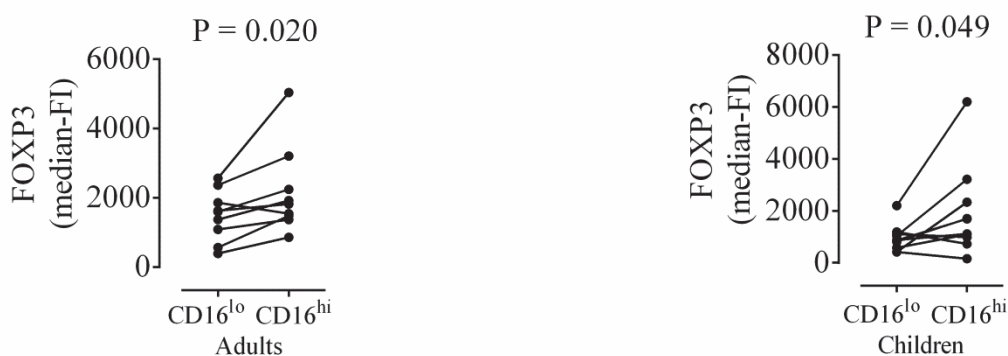

Figure 22. CD16^{hi} eosinophils express higher levels of FOXP3 in both adults and children.

Next, we investigated if the T cell suppressive capacities of the eosinophilic subgroups identified in peripheral blood of healthy persons differed. CD16^{hi}, CD16^{dim}, and CD16^{neg} eosinophils were separated using a flow cytometry sorter. To avoid contamination with neutrophils, the eosinophils were separated based on high Siglec-8 levels. Eosin staining of sorted cells confirmed that the eosinophilic subpopulations were pure. The eosinophilic subgroups were co-cultured with PBMC at a ratio of 1:10 for 2 days. Eosinophils that expressed high levels of CD16 were more effective at suppressing T cells, as compared with eosinophils that expressed low/no CD16; the CD16^{hi} eosinophils inhibited

T cells by a median of 72%, $CD16^{dim}$ eosinophils by a median of 53% (P=0.016, n=7) and the $CD16^{neg}$ eosinophils by a median of 46% (P=0.016, n=7).

4.5 DIFFERENT EOSINOPHILIC MOLECULAR PATTERNS IN CHILDREN AND ADULTS WITH EoE COMPARED WITH HEALTHY INDIVIDUALS (PAPER IV)

Since we had found a distinct eosinophilic molecular pattern among adult patients with EoE compared with healthy adults we wanted to investigate the molecular pattern among pediatric patients with EoE compared with healthy children. We found that children with EoE had higher levels of CRTH2 and galectin-10 and lower levels of CD44 and FOXP3 than healthy age-matched controls. The eosinophilic molecular patterns in adults with EoE differed from those of children with EoE in terms of having higher levels of FOXP3 and CD54, while these two patient groups had in common increased levels of CRTH2 and galectin-10 and lower levels of CD44, as compared with healthy controls (Figure 23).
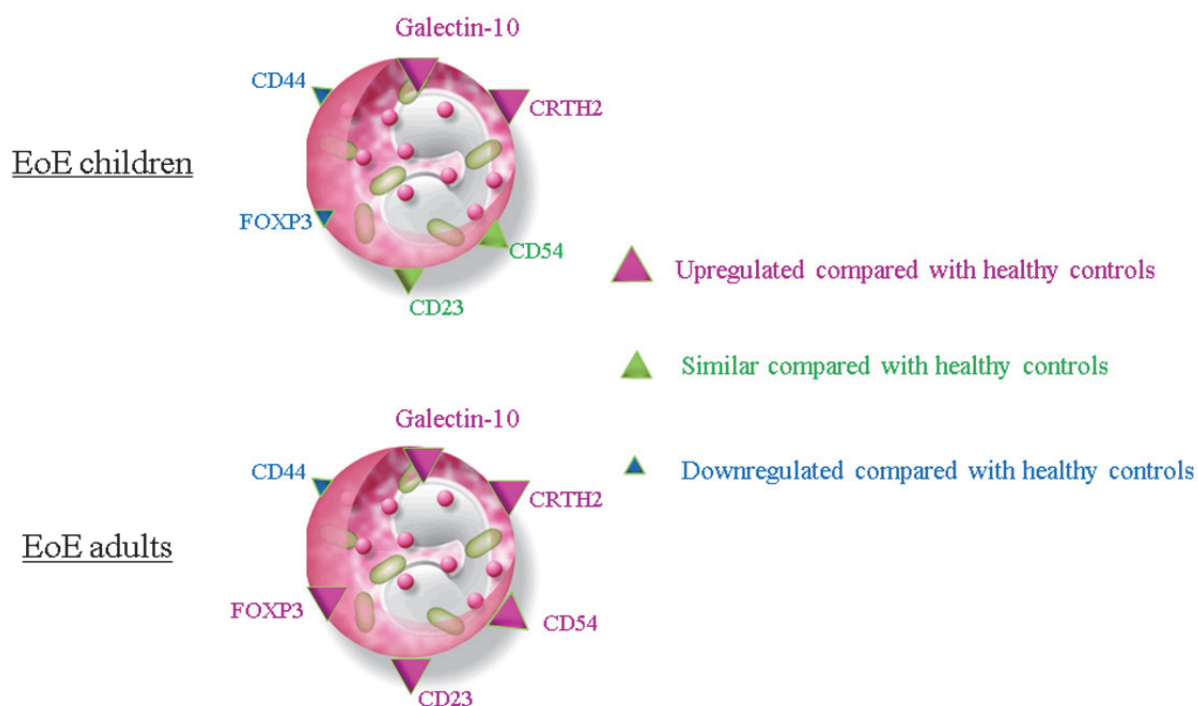
Figure 23. Upregulated and downregulated molecules in pediatric and adult EoE, as compared with healthy controls.

4.6 AGE-DEPENDENT DIFFERENCES IN THE LEVELS OF EOSINOPHILIC MOLECULES IN HEALTHY INDIVIDUALS (PAPER IV)

The divergent molecular patterns between children and adults with EoE led us to investigate if this could be due to age rather than different disease entities. We studied eosinophils from healthy children and adults between 1 to 75 years of age and made correlation analyses between age and the studied eosinophil markers.

Interestingly, we discovered age-dependent differences in the levels of several molecules on the blood eosinophils from healthy individuals. The levels of CD44 and CD23 increased with age, whereas the levels of CD54, CRTH2, and galectin-10 decreased with age. Moreover, a distinct increase in the levels of CD44 was noticed in individuals between the ages of 14 to 17.

5 DISCUSSION

This thesis examines the possibility that eosinophils are recruited to the esophagus in patients with EoE to dampen a T cell-mediated inflammatory condition that is evoked by some unknown antigen(s), possibly allergens. Several lines of evidence support the idea that EoE is a T cell-mediated disorder: 1) T cell-deficient mice are incapable of developing EoE;[142] 2) T cell co-stimulatory molecules, such as OX40, Light, and HVEM, which play important roles in T cell-mediated inflammation, are upregulated in the esophageal tissues of patients with EoE;[141] 3) increased numbers of T cells are found in the esophagus but not in the stomach and duodenum of patients with EoE;[182] and 4) in patients with EoE, the percentage of CD4^{+} T cells that express IL-5 correlates with the number of eosinophils in the esophagus.[183]

The results presented in Paper I suggest that blood eosinophils from untreated adult patients with EoE have a distinct phenotype that facilitates their adhesion, migration, and infiltration of the esophageal tissue. The adhesion molecule CD54 was upregulated on the surface of blood eosinophils of patients with EoE, as compared with healthy individuals, possibly facilitating adhesion to and migration into tissues. This is in agreement with a previous report, showing that CD54 is upregulated in esophageal tissues of patients with EoE.[82] TSLP has been reported to be upregulated in patients with EoE[126] and when TSLP binds to

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the TSLP receptor on eosinophils it has been demonstrated that the surface levels of CD54 on eosinophils increases. [125]

The levels of CD44 and CCR3 were downregulated in eosinophils of the patients with EoE, as compared with healthy individuals. CD44 is a receptor for hyaluronic acid.[85] When the eosinophil eventually enters the tissue, decreased levels of CD44 may reduce binding to hyaluronic acid in the extracellular matrix, thereby increasing eosinophil mobility and consequently, facilitating migration to the inflammatory site. This could be applied in another study in asthmatic patients.[86] The report demonstrates that the levels of CD44 on blood eosinophils were lower in patients with poorly controlled asthma compared with patients with well-controlled asthma.[86] It is possible that quicker recruitment of eosinophils to the airways is more essential for the patients with poorly controlled asthma, thus stronger binding to hyaluronic acid would slow down the migration.

CCR3 is a receptor for several chemoattractants such as eotaxins 1–3 as well as RANTES, the expression of which is increased in patients with EoE.[61, 100] $CD4^+$ T cells secrete RANTES upon stimulation.[184] It has been demonstrated that RANTES-stimulated eosinophils undergo a long-lasting internalization of CCR3.[185, 186] This may explain why we see lower levels of CCR3 on the surfaces of blood eosinophils. Additionally, we found that higher number of eosinophils in the esophagus correlated with lower levels of CCR3 on the surface of blood eosinophils. It is possible that stronger T cell activation produces higher levels of RANTES and requires more eosinophils that have RANTES-induced internalization of CCR3. In contrast, Bullock et al, found a positive correlation between esophageal eosinophils and levels of CCR3 among children with EoE.[183] There are many differences reported between children and adults with EoE,[166, 167] perhaps this is an additional one.

Unexpectedly, we found that the number of eosinophils in the esophagus correlated inversely with CD40 expression on the surfaces of blood eosinophils. It has been reported that CD40 on eosinophils and CD40L on T cells are increased in esophageal tissues from patients with EoE,[184] indicating that cell-cell contact may occur between these two cell types. Therefore, it is puzzling that we see this inverse correlation.

We hypothesized that topical corticosteroid treatment of patients with EoE would revert the "EoE" eosinophil phenotype to a healthy phenotype. The

majority of patients experience symptomatic relief after treatment.[161] To our surprise, the only marker that showed changes after corticosteroid treatment was the adhesion molecule CD18, which decreased for all the patients. In agreement, Dellon et al. recently demonstrated that several inflammatory factors known to be associated with EoE were not affected by topical corticosteroid treatment.[187]

It has been reported that the adhesion of eosinophils to endothelial cells is CD18-dependent,[188] therefore we investigated whether blocking CD18 might reduce the adhesion capacity of the eosinophils *in vitro*. We found that eosinophilic adhesion to endothelial cells, ICAM-1, and ICAM-2, but not VCAM-1 and periostin, was CD18-dependent. We also noted that CD18 levels on eosinophils correlate to the number of eosinophils in the tissue and that eosinophilic expression of CD18 correlated to the adhesion capacity to ICAM-1 and ICAM-2. Together, our findings suggest that higher levels of CD18 on the surface of eosinophils facilitates the infiltration to the esophagus, and that reduced levels of CD18 after corticosteroid treatment inhibit the recruitment of eosinophils to the tissue.

The fact that we were unable to reduce the adhesion of eosinophils to VCAM-1 and periostin by blocking CD18 was surprising as the expression of CD18 correlated to the adhesion capacity of VCAM-1 and periostin. It is possible that the levels of CD18 are correlated with other adhesion molecules, resulting in an indirect correlation to CD18 even though the adhesion in our study was not CD18-dependent. In contrast, a recent study shows that human eosinophils adhere to periostin in a CD11b/CD18-dependent manner.[83] An important difference between this study and ours is that we do not pretreat the eosinophils with IL-5.

Surprisingly, the CD18 levels of the healthy controls and untreated patients with EoE were similar. It is possible that this reflects steady-state expression, as CD18 is most likely important for the migration of eosinophils into tissues in non-pathologic conditions. However, in healthy individuals, migration of eosinophils is mainly into the gastrointestinal tract since these persons lack the alarm signals from the inflamed tissue of the esophagus that one sees in patients with EoE.

Stimulating eosinophils with corticosteroids *in vitro* did not affect the CD18 levels, indicating that the changes in CD18 levels observed in corticosteroid-treated patients with EoE are not due to a direct effect of corticosteroids on

eosinophils. It has been reported that T cell production of eosinophil-stimulatory cytokines (IL-5, IL-3, GM-CSF) is inhibited after corticosteroid exposure *in vitro*.[189] Our belief is that topical corticosteroids mainly exerted their effects on T cells in the esophageal mucosa, reducing their capacity to produce cytokines that acted as chemoattractants for eosinophils. In addition, we speculate that the reason for why eosinophils in blood of treated patients with EoE still have a "sick" phenotype is that EoE is not cured by topical corticosteroid therapy and that the underlying trigger still remains.

We conclude that, topical corticosteroids had minor effects on the eosinophilic phenotype of the patients with EoE. Reduction in the level of CD18 is most likely one of the mechanisms underlying the decreased number of eosinophils in the esophagus among the treated patients with EoE.

In Paper II, we investigated the function of the eosinophils in the blood of patients with EoE. This study is based on the hypothesis that eosinophils are recruited to the esophagus to inhibit activated T cells in the T cell-mediated disorder of EoE. We wanted to explore the possibility that eosinophils from patients with EoE can inhibit T cell proliferation. Kubach et al. demonstrated that galectin-10 is essential for the inhibitory capacity of Tregs.[11] Since about 10% of the total protein mass in eosinophils is composed of galectin-10,[39] we considered that galectin-10 might have the same function in eosinophils, and particularly in the eosinophils from patients with EoE. We found that blood eosinophils from patients with EoE expressed higher levels of galectin-10 mRNA and protein than the blood eosinophils of healthy individuals. It has previously been reported that patients with EoE have upregulated levels of galectin-10 mRNA in their esophageal tissues.[61]

In addition, we found that eosinophils from patients with EoE caused 50% inhibition of T cell proliferation in co-cultures. Surprisingly, the eosinophils from the patients with EoE were not better suppressors of T cells than the eosinophils from healthy donors. Instead, the opposite was seen, in that eosinophils from healthy individuals managed to suppress 90% of the T cell proliferation. It is possible that the eosinophils from the healthy donors became activated during co-culturing with proliferating T cells and developed an immunoregulatory phenotype similar to that of the eosinophils from the patients with EoE. Another possible explanation is that the eosinophils from the patients

with EoE are exhausted due to their pre-activated state. It has been reported that previously activated eosinophils can become anergic to re-stimulation.[102]

The eosinophil-mediated suppression of T cell proliferation was partially restored by blocking galectin-10 with monoclonal antibodies. This indicates that galectin-10 has an extracellular function. Kubach and co-workers reduced the galectin-10 levels in Tregs using siRNA, which resulted in diminished suppressive capacity. However, they were unable to block the suppressive function of galectin-10 in Tregs with mAbs. The reason why it is possible to achieve antibody-mediated neutralization of galectin-10 in eosinophils, but not in Tregs, may be that Tregs might not secrete galectin-10 extracellularly. A speculative idea is that Tregs suppress T cells *via* the transfer of galectin-10 through gap junctions, as has been demonstrated with cAMP.[12]

Based on the findings that eosinophils are able to suppress T cell proliferation we investigated further the immunoregulatory characteristics of eosinophils. We discovered that, similar to Tregs, eosinophils express the transcription factor FOXP3. We demonstrated by immunoblotting the two major bands that correspond to the two isoforms of human FOXP3.[190] The expression of FOXP3 in eosinophils was higher in patients with EoE than in healthy individuals. The levels of FOXP3 mRNA and protein were substantially upregulated in patients with EoE. We found no correlation between the FOXP3 levels displayed by eosinophils and their suppressive capacities. There are contradictory reports as to whether or not FOXP3 in human Tregs correlates with suppressive capacity. It has been reported that $CD4^+CD25^-$ effector T cells can upregulate FOXP3 upon activation,[190-192] whereas some reports state the opposite.[190] Similarly, some studies have reported a correlation between FOXP3 expression and regulatory function,[191, 192] whereas other studies indicate that there is no such association.[190, 193] We found a correlation between the expression of FOXP3 and the viability of the eosinophils. To sum up, additional studies of eosinophilic FOXP3 are needed before we can draw any firm conclusions about the function of this protein.

Image flow cytometry demonstrated that FOXP3 in eosinophils is mainly situated in the cytosol, in contrast to Tregs, where it is located in the nucleus. We also show that the expression of FOXP3 in eosinophils is diminished after 2 and 6 days of co-culturing with PBMC. Similarly, it has been reported that FOXP3 expression in Tregs is lost upon repetitive *in vitro* stimulation.[8]

Interestingly, increased numbers of FOXP3-positive cells, assumed to be Tregs, have been demonstrated in the esophagus of children with EoE.[151] Possibly, some of the FOXP3-positive cells in the mucosa of the above cited patients might have been eosinophils.

So at last we were able to answer Helene F. Rosenberg's question "Might human eosinophils, *via* abundant expression of galectin-10/CLC, also inhibit proliferation and function of CD4$^+$ T cells?".[60] In Paper III we continued the investigation of the mechanisms that underlie the T cell suppressive capacity of galectin-10. We have previously reported that cell-cell contact is necessary for the capacity of eosinophils to suppress T cell proliferation[24] and since we could partly restore T cell proliferation by adding anti-galectin-10 mAbs, we explored further the T cell-suppressive mechanism employed by eosinophils. Our preliminary data indicate that the eosinophils exposed to proliferating T cells respond by generating EETs that contained galectin-10. EETs that contain the eosinophilic granule protein EPX have recently been described in the esophageal tissues of patients with EoE.[147] Those authors hypothesized that EoE might develop in response to a bacterial infection.[147] We propose an alternative explanation: that these EETs are evoked by activated T cells in the esophageal mucosa (Figure 24).
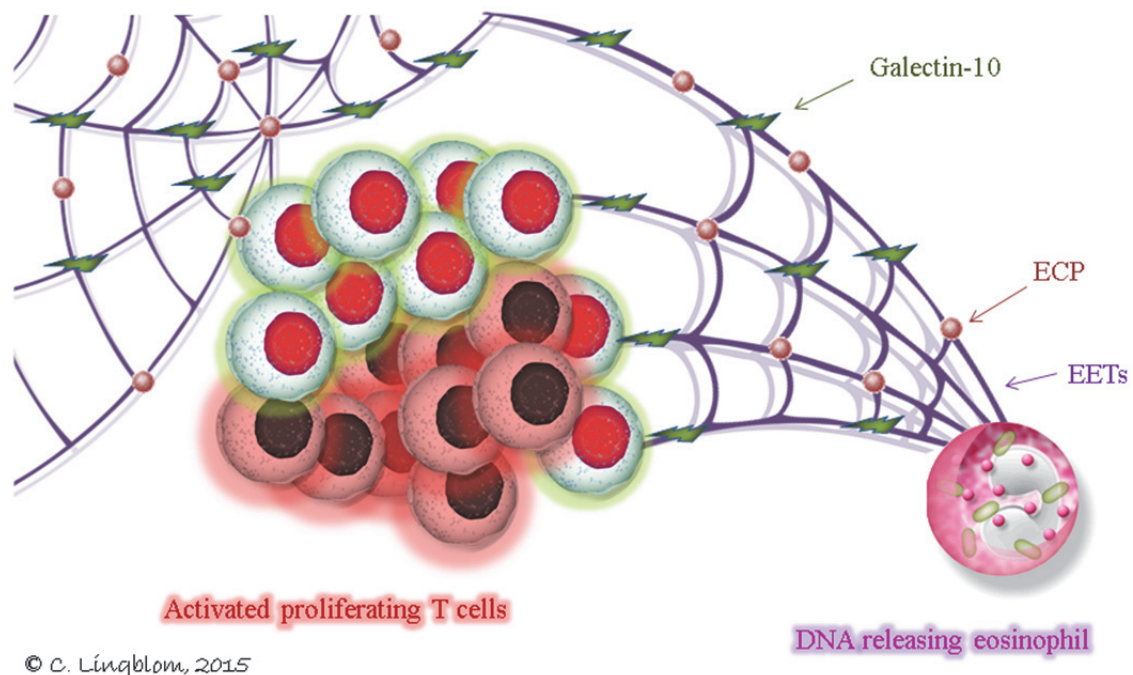

Figure 24. Proposed model for eosinophil-mediated T cell suppression by DNA nets that contain galectin-10 and ECP.

Another explanation is that TSLP, which is a signature cytokine of EoE,[126] has triggered the formation of these structures. EETs containing granular proteins, previously reported to inhibit T cell proliferation,[19] are released from TSLP-stimulated eosinophils.[72]

EETs have been identified in several T cell-mediated inflammatory diseases, such as Crohn's disease[68] and Wells syndrome.[71] Wells syndrome displays three-fold higher amounts of DNA nets as several other skin diseases caused by infection.[71] The blocking of extracellular galectin-10 that resulted in partial restoration of the T cell proliferation may be attributed to neutralization of galectin-10 in the DNA nets. In an attempt to block any extracellular communication *via* DNA nets, DNase was added to the cell culture, leading to partial restoration of the T cell-proliferative response. There are most likely additional cell bound and/or soluble molecules that contribute to the eosinophil-mediated suppression of T cells that should be investigated.

We also found that eosinophils became physically attached to CD4$^+$ T cells and CD25$^+$ T cells after co-culture with polyclonally activated T cells. Interestingly, we discovered galectin-10-containing protrusions in the synaptic area between individual eosinophils and CD4$^+$ T cells. This might be a novel secretory mechanism, that we tentatively denominate synaptic secretion. This observation may explain why cell-cell contact is needed for eosinophils to suppress T cell proliferation.[24] A strong correlation has been found between the number of CD25$^+$ T cells and the number of eosinophils in the proximal airways of patients with asthma, with the authors stating that eosinophilic accumulation is under the control of T cell products.[194] Perhaps also in this case the eosinophils are recruited to the airways to dampen the activated T cells.

In our search for a mechanism for galectin-10-mediated suppression of T cells, we discovered that eosinophils are divided into two subgroups after 2 days of co-culture. Before addition to the culture, all the eosinophils are CD16^{neg}, since CD16-based immunodepletion is the basis for our eosinophil isolation process. During co-culturing, 15% of the eosinophils acquired CD16 expression. These cells also expressed lower levels of galectin-10 than the CD16^{neg} cells. The reduction in galectin-10 levels could be due to an earlier release of EETs that contain galectin-10 or the transfer of galectin-10 to T cells during the 2-day co-culture. It has been reported that the fraction of CD16^{hi} eosinophils increases in patients with allergy and hypereosinophilia.[112]

Since we found that eosinophils acquired CD16 expression during co-culturing we wanted to examine more closely the CD16^{hi} blood eosinophils. Interestingly, we found that the CD16^{hi} eosinophils express higher levels of galectin-10 and FOXP3. This led us to investigate whether eosinophils that express high levels of CD16, galectin-10 and FOXP3 are better suppressors of T cells than eosinophils that do not express CD16. Indeed, compared with eosinophils that expressed no CD16 or lower levels of galectin-10 and FOXP3, the CD16^{hi} eosinophils were almost twice as potent at inhibiting T cell proliferation. The reason for this superior capacity to suppress T cells is most likely due to the CD16^{hi} eosinophils having higher levels of galectin-10. Interestingly, it has been reported that the release of DNA nets from neutrophils is CD16-dependent.[195] This could be another explanation as to why the CD16^{hi} eosinophils are more potent T cell suppressors. CD16 is consistently expressed intracellularly but rarely on the surfaces of nonactivated human eosinophils, whereas CD16 is translocated to the eosinophil surface upon stimulation.[196] It is possible that the CD16^{neg} eosinophils need to upregulate their expression of CD16 before they are able to suppress T cells *via* galectin-10 containing extracellular traps. It has also been reported that blocking CD16 on the surfaces of eosinophils inhibits degranulation.[197] However, we do not believe CD16 in itself mediates the T cell suppressive function of eosinophils, as we have shown that neutrophils are unable to suppress T cells *in vitro*.[24]

We found that a larger fraction of the eosinophils of healthy children are CD16^{hi} eosinophils compared to the eosinophils of adults. Interestingly, we also saw that the CD16^{hi} eosinophils of younger children contained higher levels of galectin-10 compared to the eosinophils of older children. This may indicate that the eosinophils in young children have a stronger immunoregulatory capacity. A recent study has suggested that eosinophils play an important role in the selection of T cells in the thymus during the creation of the adaptive immune system.[38] It is possible that children, who have an immature immune system, are in need of more immunoregulatory eosinophils. The very few case reports that exist of human subjects with true eosinophil deficiency indicate that a complete lack of eosinophils is associated with dysregulated immunity, e.g., thymoma, hypogammaglobulinemia, allergic and autoimmune disorders, such as asthma, urticaria, drug allergies, vitiligo, hemolytic anemia, and even lymphoma;[37] a possible explanation for this is that eosinophils regulate lymphocyte function and/or development.

In Paper IV, we first investigated whether blood eosinophils from children with EoE have a distinct molecular pattern compared with healthy children. Second, we evaluated if children with EoE express a different molecular pattern compared to adults with EoE. Our hypothesis was that the molecular patterns would differ in some aspects, since the clinical pictures for adults and children with EoE sometimes vary. We found that the levels of CD44 and FOXP3 were lower, and that the levels of CRTH2 and galectin-10 were higher in children with EoE than in healthy children. This is not the molecular pattern we see for adult patients with EoE. Instead, adults with EoE have higher levels of FOXP3 and CD54, and similar to children with EoE, they have increases levels of CRTH2 and galectin-10 and lower levels of CD44.

CRTH2 is the receptor for PGD2. PGD2 has a strong chemotactic effect on eosinophils[198] and is mainly produced by mast cells. It has been documented that there are increased numbers of mast cells in the esophageal tissues of patients with EoE.[199] It is possible that the level of CRTH2 is increased in the blood eosinophils of children and adults with EoE owing to the recruitment signals from mast cells in the inflamed tissue. Islam et al. have previously reported that mRNA levels of CRTH2 in esophageal biopsies are upregulated in individuals with active EoE.[200] The decreased levels of CD44 may be due to, as previously suggested, decreased binding to hyaluronic acid which might make the eosinophils more mobile.

We discovered that children and adults with EoE have higher levels of galectin-10 mRNA than healthy donors, although significantly higher galectin-10 protein levels were not seen in the children with EoE, as compared to healthy children. It is unclear why there is a lack of concordance between levels of galectin-10 mRNA and protein in children and not in adults. It is possible that children with EoE have a greater turn-over of galectin-10 than adults with EoE.

The most distinct difference between the children and adults with EoE was that the eosinophils from children with EoE had decreased levels of FOXP3 mRNA compared to healthy children, whereas the opposite was found for the adults. Although this is a surprising result, it perhaps reflects that other transcription factors are active in the eosinophils of children with EoE.

Due to the distinct differences in molecular patterns between children and adults with EoE we investigated if there were an association between age and the expression of the different molecules among healthy individuals. We discovered

that the levels of CD44 and CD23 increased with age, whereas those of CD54, CRTH2, and galectin-10 decreased with age. As suggested above, it is possible that young children need immune regulation back-up from the innate immune system due to the ongoing development of the adaptive immune system. We have shown that galectin-10 is important for the capacity of eosinophils to suppress T cells, and it has been reported that CD54 can contribute to interactions between eosinophils and T cells[79] and that increased levels of CRTH2 facilitate migration. All these findings indicate that the eosinophils in children have an increased capacity to communicate with T cells. This is in line with what we previously discussed, i.e., the larger CD16^{hi} population in children expresses higher levels of galectin-10 and is superior at T cell suppression, as well as with the suggestion of Tulic et al. that eosinophils play an essential role in the selection of T cells in the thymus during the creation of the adaptive immune system.[38]

The eosinophilic levels of CD44 and CD23 increase with age in healthy controls. We saw a distinct increase of CD44 between the ages of 14 and 17 years. Eosinophils may play an important role in the development of the mammary gland during puberty, eosinophils have been shown to gather around the terminal end-buds during the beginning of the pubertal burst of ductal growth of the mammary glands.[201] Binding to hyaluronic acid and subsequent binding to the extracellular matrix of the mammary glands are likely to be essential during this period. It has been reported that eosinophils treated with β-estradiol improve the eosinophilic adhesion to endothelial cells.[202] It has been reported that soluble CD23 (sCD23) decrease with age,[203] whereas we show that CD23 on the surface of eosinophils increase with age. It has also been demonstrated that sCD23 regulates the synthesis of IgE.[204] It is possible that sCD23 and regulation of IgE synthesis is more essential during childhood.

We are not aware of previous reports on physiological, age-dependent differences in the phenotype of polymorphonuclear granulocytes. The exception is immunosenescence,[205] where it has been described that eosinophils and neutrophils from old individuals have weakened functions, e.g. have reduced phagocytic capacity,[206] poorer respiratory burst and decreased degranulation.[207]

Our findings could indicate that pediatric and adult EoE represent different disease entities. However, we have identified marked age-related differences in eosinophilic molecular patterns that are physiological. Thus, we cannot at this

point determine if there are immunopathogenic differences between pediatric and adult EoE or if the age-related differences alone account for the dissimilarities between EoE in children and adults, respectively.

Several reports have proposed that certain biomarkers in the tissue and blood can be used to diagnose EoE. Eotaxins 1-3, IL-5, IL-13, EPX, and EDN have been suggested as biomarkers in tissues and serum.[208] Eotaxin-3 and IL-13 have emerged as the most promising biomarkers in terms of sensitivity and positive correlation to the disease process.[208] Still, more research is needed to be able to distinguish patients with EoE from healthy individuals on the basis of biomarker expression. If a simple blood sample could be used for diagnostic purposes and to monitor response to therapy, the patient's quality of life would improve substantially. Importantly, these patients would not need to undergo multiple invasive endoscopic procedures.

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6 SUMMARY AND CONCLUSIONS

The aim of the present study was to test the hypothesis that eosinophils in patients with EoE are recruited to the esophagus to dampen the T cell-mediated inflammation and also if there could be a difference in eosinophilic molecular pattern between children and adults with EoE. This thesis only scratches the surface of the mystery surrounding the role of the eosinophil and the disease trigger in EoE. Nevertheless, our work opens new avenues of research, particularly when it comes to the function of the eosinophil under inflammatory conditions.

In summary, we show that blood eosinophils have a distinct phenotype in patients with EoE, which explains in part the adhesion, infiltration, and migration of eosinophils towards the inflamed esophageal tissue. Topical corticosteroids had almost no effect on this activated phenotype, except for decreased expression of CD18. The reduced levels of CD18 could be one of the reasons behind the diminished number of eosinophils in the esophagus observed for patients with EoE who were treated with topical corticosteroids.

We also demonstrate a potential function for the recruited eosinophils during the chronic inflammation that characterizes EoE. We show that eosinophils, in similarity to Tregs, inhibit T cell proliferation by using galectin-10. Likewise,

eosinophils also express the transcription factor FOXP3. We demonstrate that during T cell suppression eosinophils release EETs that contain galectin-10. Furthermore, we found that eosinophils become attached to $CD4^+$ T cells during co-culture *via* a bridge of galectin-10-incorporating protrusions in the synaptic area between the cells.

Consistent with this, was the finding of a $CD16^{hi}$/galectin-10^{lo} eosinophilic subgroup after T cell suppression. We suggest that this is due to either the previous release of EETs that contain galectin-10 and/or the transfer of galectin-10 to T cells. Interestingly, when we investigated the $CD16^{hi}$ eosinophilic subgroup in the blood, we noticed that they expressed higher levels of galectin-10 than the conventional $CD16^{neg}$ eosinophils from both children and adults. Most importantly, the $CD16^{hi}$/galectin-10^{hi} eosinophils were more effective as T cell suppressors. Hopefully, in the future we will call them regulatory eosinophils (eos_{reg}).
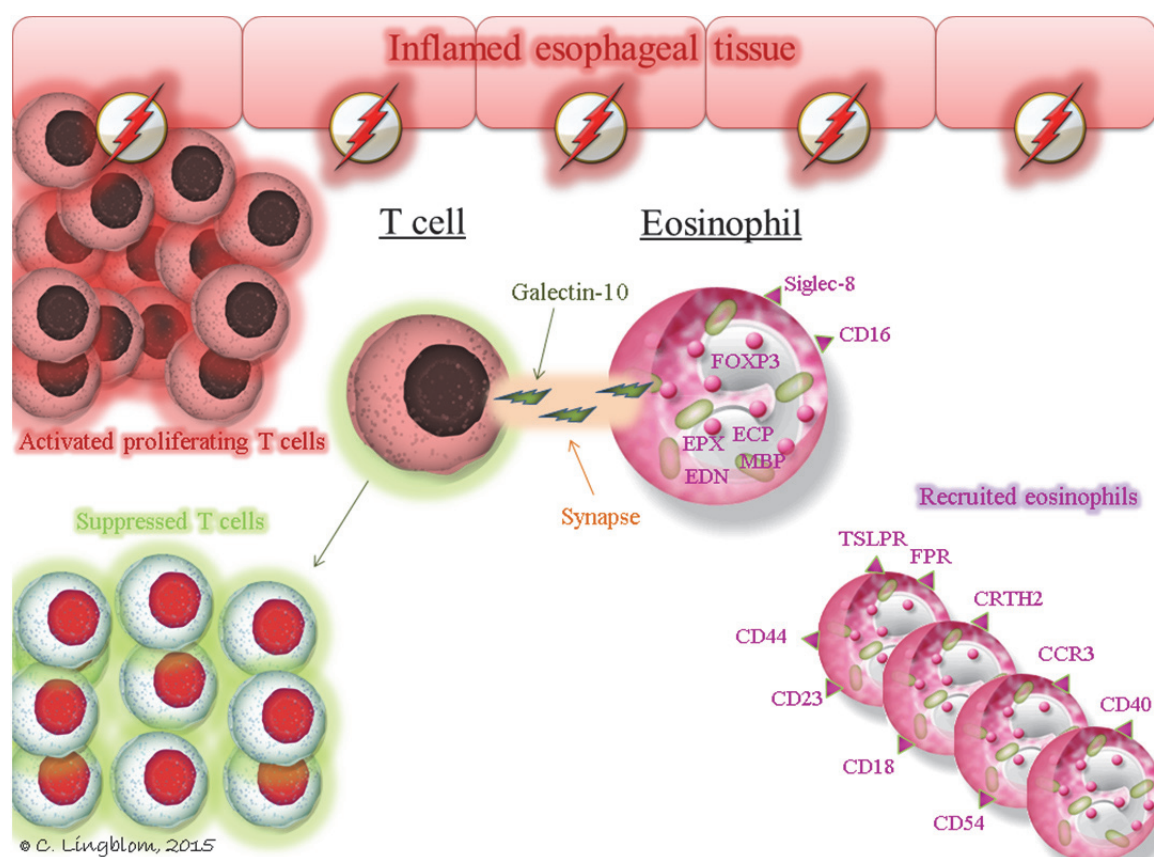

Figure 25. Model for galectin-10-mediated suppression of T cells during EoE.

We also demonstrate that the molecular patterns of eosinophils differ between children and adults with EoE, and we postulate that this reflects differences in both immunopathogenic mechanisms and capacities to respond to T cell-mediated inflammation. We also reveal that several eosinophilic molecules are age-related among healthy individuals, which we speculate may be due to the establishment of tolerance during childhood.

Finally, we propose a model for galectin-10-mediated T cell suppression that reflects the environment in the esophagus of a patient with EoE (Figure 25).

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8 FUTURE PERSPECTIVES

Further investigations into the ability of eosinophils to suppress T cells would elucidate the mechanisms underlying the functions of eosinophils, particularly in eosinophilic disorders, thereby aiding the design of better treatments. The following topics should be addressed:

- Defining the effects (necrosis, apoptosis or anergy) that eosinophils have on T cells.
- Silencing of galectin-10 expression in eosinophils using siRNA, to determine the feasibility of total inhibition of the suppressive capacity.
- Studies of the co-localization of T cells and eosinophils in the esophagus of patients with EoE to confirm the existence of galectin-10-containing synapses and EETs.
- Monitoring of the eosinophilic molecular pattern in response to diet-based treatment regimens for children with EoE.

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