Human eosinophils and their

activation by allergens via danger signal receptors

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Cover illustration photo: Kerstin Andersson (Eosinophils)

Abstract

Human eosinophilic granulocytes are polymorphonuclear cells with a powerful arsenal of cytotoxic substances in their granules, which are mainly found in the gastrointestinal mucosa, and the respiratory and genitourinary tracts. Their physiological role is incompletely understood, although it is likely they protect the mucosal surfaces, perhaps by recognizing danger signals present on microorganisms or released from damaged tissue.

We have earlier shown that eosinophils can recognize and become directly activated by aeroallergens such as house dust mite (HDM) and birch pollen. Eosinophils exposed to (HDM) release both of the cytotoxic granule proteins eosinophil peroxidase (EPO) and major basic protein, whereas birch pollen extract only triggers EPO release.

Here we further investigate which receptors on eosinophils are used to signal the presence of HDM and birch pollen. Recognition was found to be mediated by the formyl peptide receptors (FPRs) FPR1 and FPR2. We also characterized the expression of this family of receptors in human eosinophils and found that they express FPR1 and FPR2, but not FPR3, similar to neutrophilic granulocytes. We also discovered that signaling through FPR1 can desensitize the eotaxin-1 receptor CCR3 rendering the cells anergic with respect to chemotaxis in response to eotaxin-1, but not regarding respiratory burst. Hence, there is cross-talk between these two receptors regarding one important effector function of eosinophils.

Eosinophilic reactivity *in vitro* to the aeroallergens HDM, birch pollen, timothy grass pollen and cat dander did not differ between individuals with allergy and healthy individuals. Hence, eosinophilic degranulation and low grade cytokine release was seen in cells derived from both allergic and non-allergic study persons. However, both allergic and healthy individuals showed decreased TNF production from eosinophils during the birch pollen season.

We have also shown, for the first time, that human eosinophils can become directly activated by the food allergens cod fish and cow's milk. Whereas cod fish evoked eosinophilic chemotaxis, milk triggered EPO degranulation. Moreover, substances resembling prostaglandin D_2 appeared to be the bioactive substances in cod recognized by eosinophils. The receptor mediating this recognition seems to be the prostaglandin D_2 receptor DP2. Our studies may increase the understanding of the complex interaction between the innate and acquired immune system in allergy.

Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV):

I. Lena Svensson, Elin Redvall, Camilla Björn, Jennie Karlsson, Ann-Marie Bergin, Marie-Josèphe Rabiet, Claes Dahlgren and Christine Wennerås. House dust mite allergen activates human eosinophils via formyl peptide receptor and formyl peptide receptor-like 1. *Eur. J. Immunol. 2007 Jul;37(7):1966-77.*

II. Lena Svensson, Elin Redvall, Marianne Johnsson, Anna-Lena Stenfeldt, Claes Dahlgren and Christine Wennerås. Interplay between signaling *via* the formyl peptide receptor (FPR) and chemokine receptor 3 (CCR3) in human eosinophils. *J Leukoc Biol. 2009 Aug;86(2):327-36.*

III. Responsiveness of eosinophils to aeroallergens may be independent of atopic status. Elin Redvall, Ulf Bengtsson and Christine Wennerås. *Scand J Immunol. 2008 Apr;67(4):377-84.*

IV. Human eosinophils are differentially activated by food extracts derived from cod fish and milk. Elin Redvall, Kerstin Andersson, Åsa Brunnström, Said Elsayed and Christine Wennerås. *In manuscript.*

Table of contents

Abbreviations

AHR	airway hyperresponsiveness
ANOVA	analysis of variance
APC	antigen presenting cell
BSA	bovine serum albumin
C5a	complement factor 5a
CCR3	CC chemokine receptor 3
CD	cluster of differentiation
cDNA	complementary DNA
CRTH2	chemoattractant receptor homologous molecule expressed on Th2 cells
CsH	cyclosporine H
DAMP	damage-associated molecular pattern
DMSO	dimethylsulfoxide
DP1	PGD_2 receptor 1
DP2	PGD_2 receptor 2
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
EDTA	ethylene diamine tetraacetic acid
EPO	eosinophil peroxidase
FACS	fluorescence-activated cell sorting
fMLF	*N*-formyl-methionyl-leucyl-phenylalanine
FPR	formyl peptide receptor
GDP	guanosine diphosphate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
G-protein	guanine-nucleotide-binding protein
GTP	guanosine triphosphate
HDM	house dust mite
HES	hypereosinophilic syndrome
H_2O_2	hydrogen peroxide
ICAM	inter-cellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
IFN-γ	interferon gamma
KRG	Krebs-Ringer glucose buffer
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
LTC4	leukotriene C_4
mab	monoclonal antibody
MAPK	mitogen-activated protein kinase
MBP	major basic protein
MCPM	10^6 counts per minute
MFI	mean fluorescence intensity
medianFI	median fluorescence intensity
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
$O_2^{-}\cdot$	superoxide
OD	optical density
OPD	*o*-phenylendiamine
PAF	platelet-activating factor
PAMP	pathogen associated molecular pattern
PALM	pollen-associated lipid mediator
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PKC	protein kinase C
PMA	phorbol myristate acetate
PMD	piecemeal degranulation
PRR	pathogen recognition receptor
PTX	pertussis toxin
RANTES	regulated upon activation, normal T cell expressed and secreted
SNAP	*N*-ethylmaleimide-sensitive attachment protein
SNARE	SNAP-receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
WKYMVM	tryptophan-lysine-tyrosine-valine-methionine
WRW^4	hexapeptide tryptophan-arginine-tryptophan-tryptophan-tryptophan-tryptophan

Introduction

The immune system is a vital part in keeping us protected against danger such as invasive bacteria, parasites and viruses. However, sometimes this system becomes too vigilant and starts reacting to innocuous substances such as pollen and common food stuffs, giving rise to allergies.

Innate immunity

The immune system can be divided into two parts, innate and adaptive immunity. Innate immunity is responsible for the initial response towards microbes and is often successful in stopping potential infection without the aid of adaptive immunity. The cells of the innate immune system are dendritic cells, mast cells, macrophages, granulocytes (neutrophils, eosinophils and basophils) and natural killer cells. These cells rely on germline-encoded pathogen recognition receptors (PRRs) to identify pathogen-associated molecular patterns (PAMPs) such as single and double-stranded viral DNA, bacterial cell wall components like lipopolysaccharide (LPS), lipotechoic acid, peptidoglycan and formylated peptides, such as *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe or fMLF). Examples of eosinophilic PRRs are the family of Toll-like receptors (TLRs) and the formyl peptide receptors (FPRs). The signaling cascades initiated by these receptors often act to trigger the cells of the adaptive immunity and thus bridge the two parts of the immune system.

The eosinophil

In 1879, Paul Ehrlich discovered a leukocyte whose granules were stained pink by the acidic dye eosin. He named the cell eosinophil [1]. Due to the fact that the eosinophil has been difficult to separate from neutrophils by the use of density centrifugation only, it has remained a poorly studied and understood cell. The possibility to routinely obtain eosinophils of 99% purity from peripheral blood using magnetic microbeads came during the 1990s [2-3] and since then the field of eosinophil research has expanded greatly.

Morphology, progenitors and migration to tissues

The eosinophil is a bi-lobed granulocyte approximately 8 μm in diameter, making up 1-5% of the circulating leukocytes in a healthy human. Eosinophilopoiesis, the formation and differentiation of eosinophils, occurs in the bone marrow of the trabecular bones. The CD34+ hematopoietic stem cell is the progenitor cell for all leukocytes including eosinophils. As can be seen in **Fig. 1**, the closest relative of the eosinophil is not the neutrophil, but the basophil. However, in mice it appears as if the eosinophil stems from a single progenitor, and basophils and mast cells instead share a common lineage [4]. The differentiation from a CD34+ hematopoietic stem cell into an eosinophil is governed mostly by the transcription factors GATA binding protein 1 (GATA-1) [5], PU.1 [6] and CCAAT enhancer binding protein (c/EBP) [7-8] and the cytokines interleukin (IL) -3, IL-5 and GM-CSF. The eosinophil leaves the bone marrow after maturation to take up residence in the gastrointestinal tract under physiologic conditions. The major eosinophil-specific chemoattractant responsible for migration to the tissues is CCL11/eotaxin-1 [9], which signals through CCR3 [10].

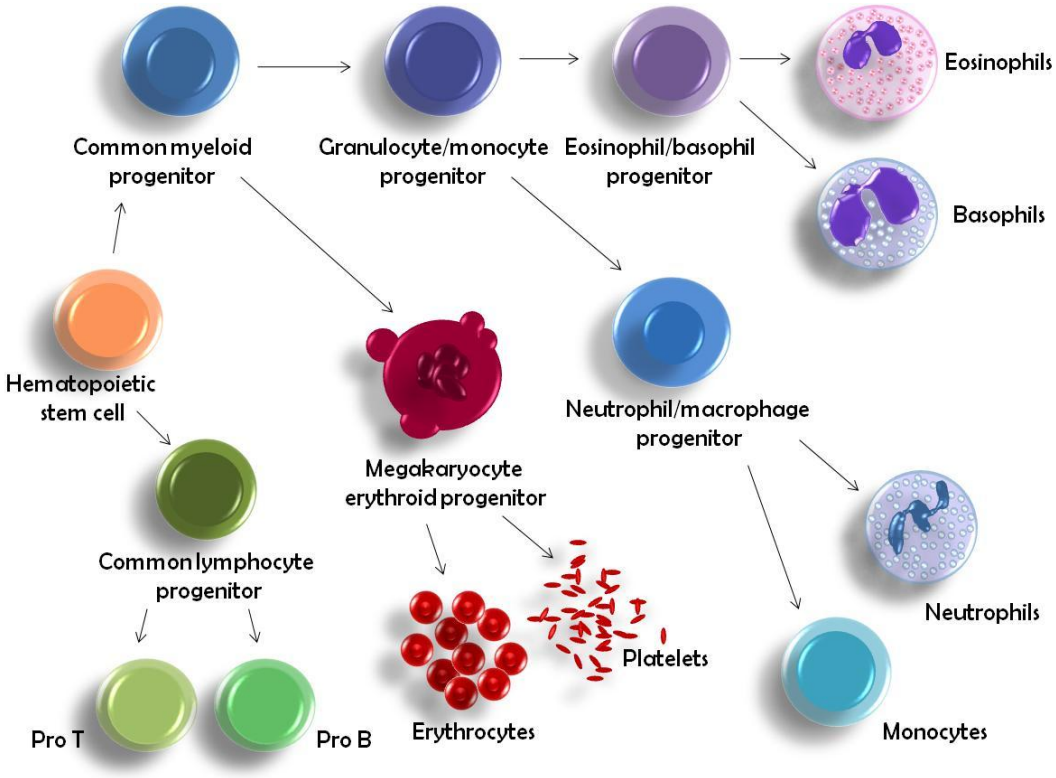

Figure 1. Eosinophilopoiesis

Granule proteins

The granules of the eosinophil can be divided into four different types, the primary and the small granules, the secretory vesicles and the secondary granules, the latter also known as crystalloid granules. Stored in these granules is an array of cytotoxic substances, interleukins, leukotrienes and lipid mediators. A list of the proteins and mediators secreted by eosinophils is seen in **Table 1**. The most abundant proteins can be found in the secondary granules which consist of two parts, the matrix-containing eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN), and eosinophil cationic protein (ECP) and the major basic protein (MBP) filled core. Those four are highly basic, cationic proteins with a pI above 9, with potent cytotoxic properties and it is those which bind eosin and stain the secondary granules their characteristic pink color.

Table 1. Eosinophilic granular proteins and cytokines

		Location
Granular proteins	EPO	Secondary granules (matrix)
	MBP	Secondary granules (core)
	ECP	Secondary granules (matrix)
	EDN	Secondary granules (matrix)
Interleukins (IL)	IL-1α	?
	IL-2	Secondary granules (matrix)
	IL-3	?
	IL-4	Secondary granules (matrix)
	IL-5	Secondary granules (?)
	IL-6	Secondary granules (matrix)
	IL-8	?
	IL-9	?
	IL-10	?
	IL-12	?
	IL-13	?
	IL-16	?
	IL-17	?
	IL-18	?
Interferons and others	GM-CSF	Secondary granules (core)
	TNF	Secondary granules (matrix)
	INF-γ	?
Chemokines	Eotaxin-1 (CCL11)	Secondary granules
	RANTES	Secondary granules (matrix) and small secretory granules
	MIP-1α	?
Lipid mediators	Leukotrienes	?
	Platelet activating factor	?
Growth factors	TGF-α	Secondary granules (matrix) and small secretory vesicles
	TGF-β1	?
	Nerve growth factor	?
	Stem cell factor	Membrane, cytoplasm

Adapted from Hogan *et al.*, Clin Exp Al, 2008 and Rothenberg & Hogan, Ann rev immunol, 2006

EPO is a haloperoxidase, oxidizing halides i.e. bromide, chloride, and iodide, and the pseudohalide thiocyanate, in the presence of hydrogen peroxide produced by oxidative burst. The oxidative burst in eosinophils can produce as much as 10 times as many superoxide anions as neutrophils [11]. Activation of the NADPH-oxidase facilitates electron transfer from NADPH to oxygen molecules.

$$NADPH + 2\ O_2 \rightarrow NADP^+ + H^+ + 2\ O_2^{-}\cdot$$

The resulting superoxide anion is further catalyzed into hydrogen peroxide, which then facilitates the halide oxidation by EPO. The hypohalous acids formed in this way are bactericidal, but they are toxic to mammalian epithelial cells as well and can degrade connective tissue [12].

EDN, sometimes referred to as eosinophil protein X [13] is less basic compared to ECP and MBP, and thus it is not as cytotoxic [14]. It is a member of the RNase A multifamily, and not exclusively expressed by eosinophils, but expressed in neutrophils [15] and mononuclear cells [16] as well. EDN has been shown to possess both antiviral properties [17] and to act as a chemoattractant to dendritic cells [18].

ECP is another RNase, but about 100-fold weaker than EDN [19]. It has bactericidal activities [20], is toxic to helminths [21] and mammalian epithelial cells [14] and promotes degranulation of mast cells [22].

MBP is expressed as two homologues, MBP1 and MBP2, the former also expressed to a lesser extent in basophils [23]. The capacity of the eosinophil to synthesize MBP is lost early on in eosinophilopoiesis, and thus mature eosinophils carry a finite amount of MBP [24]. It is extremely basic and thus toxic to bacteria [20], helminthic parasites [25] and airway epithelium [14]. MBP is also involved in signaling by causing release of mediators from mast

cells, neutrophils, and basophils [22, 26] and can also increase smooth muscle contractions by affecting the function of muscarinic receptors M2 and M3 [27].

The four granular proteins mentioned above share traits which make them powerful weapons against infection, but they also have great capacity for host damage and their secretion needs to be tightly regulated. However, as can be seen in **Table 1**, there is a great number of substances which can be secreted by the eosinophil to respond to and/or alter its environment. Common for most of those cytokines is that they are pre-synthesized and stored, making rapid release (within 60 minutes) possible [28].

LTC_4, PGD_2, PGE_2 and their receptors

Leukotrienes and prostaglandins belong to the eicosanoids, and are signaling molecules derived from arachidonic acid. They are also referred to as lipid mediators and are important effectors in immunity and inflammation [29]. Because of their short half-life, they do not circulate [30] but act in the immediate vicinity of their release.

Leukotriene C_4 (LTC_4) exerts a bronchoconstrictor effect on airway smooth muscle [31] and promotes airway inflammation [32-33]. The immune cells mostly responsible for the production of LTC_4 are mast cells [34] and macrophages [35], but eosinophils are also able to release LTC_4 and expresses both cysteinyl leukotriene receptors, CysLT1 and CysLT2 [16].

Prostaglandin D_2 (PGD_2) is the major prostaglandin produced by mast cells and has been implicated in promoting allergic asthma. However, it appears as if the role of PGD_2 in allergic inflammation has been neglected as basic research about this is scarce [36]. Eosinophils express the PGD_2 receptors DP1 and DP2/CRTH2 (chemoattractant receptor homologous molecule expressed on Th2 cells) [16].

Prostaglandin E_2 (PGE_2) is predominantly a pro-inflammatory prostanoid by enhancing leukocyte infiltration via promotion of blood flow [30] and increase of vascular permeability [37]. PGE_2 has previously been found to inhibit migration of eosinophils towards eotaxin, PGD_2 and C5a [38]. The PGE_2 receptors is believed to have up to eight splice variants [30], eosinophils express EP1 and EP4, but prefers signaling through EP4 [39].

Receptors and other surface molecules

The eosinophil expresses a vast number of surface molecules and research continuously identifies new structures, previously believed to be expressed by other cell types only [40]. **Table 2** gives an overview of some of the surface molecules expressed by eosinophils. However, none of the molecules on the list are exclusively expressed by eosinophils and thus separation by fluorescence-assisted cell separation (FACS) has not yet become a standardized way to isolate eosinophils. In 2007 Hamann *et al.* reported that the EGF-like module containing mucin-like hormone receptor (EMR) 1 was eosinophil-specific and co-expressed with CCR3 and Siglec-8 [41]. Our research group has not been able to use this molecule for positive selection of human blood-derived eosinophils because of its weak expression (data not shown).

The most commonly used separation protocol today for purifying eosinophils from blood is based on the fact that resting eosinophils do not express surface CD16 (FcγRIII, a low affinity IgG receptor), and thus can be separated from neutrophils which do express surface CD16.

Table 2. Eosinophil surface markers

Ig receptors and members of the Ig superfamily			
Cluster of differentiation (CD)	Common synonym	Cluster of differentiation (CD)	Common synonym
CD4	T4; Leu-3; L3T4	CD58	LFA-3
CD16[1]	FcγRIII	CD66	BGP-1
CD32	FcγRII	CD89	FcαR
CD48	BCM1; Blast-1	-	HLA-class I
CD50	ICAM-3	-	HLA-DR
CD54	ICAM-1	-	FcεRI
Cytokine receptors			
Cluster of differentiation (CD)	Common synonym	Cluster of differentiation (CD)	Common synonym
CD25	IL-2R α chain; p55	CD125	IL-5R α chain
CD116	GM-CSFR α chain	CD131	Common β subunit of CD116, CD123 and CD125
CD117	c-Kit	CD213	IL-13Rα
CD119	IFN-γR	-	IL9-R
CD120	TNFR	-	IL-13Rα1
CD123	IL-3R α chain	-	TGFβR
CD124	IL-4R α chain		
Chemokine, complement, and other chemotactic receptors			
Cluster of differentiation (CD)	Common synonym	Cluster of differentiation (CD)	Common synonym
CD35	CR1; C3bR	-	PAFR
CD88	C5aR	-	LTB4R
CD183	CXCR3; GPR9	-	CystLT1R
CD191	CCR1; RANTESR	-	CystLT2R
CD193	CCR3; eotaxin receptor	-	FPR1
-	Histamine 4R	-	CRTH2

Apoptosis, signaling and others			
Cluster of differentiation (CD)	Common synonym	Cluster of differentiation (CD)	Common synonym
CD39	ENTPD1	CD98	4F2; FRP-1
CD43	Sioalophorin; leukosialin	CD99	E2; MIC2
CD48	BCM1;Blast-1	CD137	4-1BB; ILA
CD53	OX44	CD139	-
CD63	Granulophysin	CD148	HPTP-η
CD65	VIM-2	CD47R	IAP; CDw149
CD69	AIM	CD151	PETA-3; SFA-1
CD71	T9; transferrinR	CD153	CD30L
CD92	CTL1	CD161	NKR-P1A; KLRB1
CD95	Fas antigen; APO-1	-	Siglec-8
-	Toll-like receptor 7	-	Siglec-10
-	Toll-like receptor 8		

Adapted from Hogan *et al.*, Clin Exp Al, 2008

However, eosinophils carry intracellular stores of CD16 and this becomes relocated to the surface after stimulation with PAF, C5a or IFNγ or when the eosinophils are activated in individuals with allergic asthma [42-44], IFNγ being capable of eliciting new synthesis and expression of CD16 [43].

Naturally, eosinophils express receptors for the three cytokines most important for their differentiation and maturation, IL-3 (CD123/IL-3Rα), IL-5 (CD125/IL-5Rα) and GM-CSF (CD116/GM-CSFRα). The corresponding cytokine receptors are all hetero-dimers which share the common β-chain CD131.

CD193/CCR3 and FPR1 will be discussed more thoroughly below, as will DP1 and DP2/CRTH2.

Release of granular proteins

As previously mentioned, eosinophils are able to release preformed effector molecules rapidly upon activation. This is performed by exocytosis, piecemeal degranulation (PMD) or cytolysis. In classical exocytosis, granules fuse with the cell membrane and release their entire contents into the surroundings. The mechanism of PMD is a bit more complex, as this enables differential release of specific granule proteins [45]. This is achieved by intermediary trafficking vesicles, either small round vesicles [46] or large vesiculotubular eosinophil, so called "sombrero" vesicles [47], emptying the granules gradually and selectively [46, 48] a process which can be visualized by electron microscopy, the granules appearing "lighter" [46]. The mechanisms for sorting and loading the vesicles are yet to be determined though it appears as if a combination of receptor-mediated recruitment and soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP)/ SNAP-receptor (SNARE) binding is involved [49]. SNAREs are small membrane-bound proteins which can vary much in structure and size, but they all share a common SNARE motif which is responsible for the membrane fusing capacities of SNAREs. They can simplistically be grouped into vesicle SNAREs (v-SNAREs) located on the vesicles budding from the granules, and target SNAREs (t-SNAREs) located on the target membrane, in this case the cell membrane. Docking and fusing of vesicle to membrane occurs by the formation of a SNARE-complex [50]. PMD appears to be the mechanism of choice for eosinophilic granule release as exocytosis is almost exclusively seen in close proximity to helminths [51].

The third mechanism of degranulation, cytolysis, has recently gained new attention. Disintegration of cell integrity does not appear to release granular proteins in an uncontrolled manner. There have been indications of the presence of membrane-bound, intact granules in tissue samples and sputum [52-53], but recent studies have shown those cell free granules to

be extracellular secretion competent organelles [54] with the capacity to become activated by surface receptors for IFN-γ, eotaxin and cysteinyl leukotrienes [54-55] capable of signaling release of granular contents. The release of cell-free granules has also been implicated as a means of activating eosinophils in the airway mucosa of allergic individuals with rhinitis during the pollen season [52].

The eosinophil in health and disease

Even though the eosinophil was identified and named as early as 1879 [1], very little is known about its role in homeostasis. After maturation, eosinophils preferentially home to the *lamina propria* of the gastrointestinal tract [56], save the esophagus which is devoid of eosinophils in a healthy individual [57]. This homing is mainly driven by a constitutive expression of CCL11/eotaxin-1 in the intestine, with the highest levels in the colon and the small bowel [58].

Eotaxin-1 is also constitutively expressed in the thymus in mice [59] and eosinophils have been shown to act as antigen presenting cells [60], expressing the MHC II protein human leukocyte antigen (HLA)-DR [61-62] and CD40 [63], proteins necessary for antigen presentation to and activation of $CD4^+$ T cells [64]. Also, the co-stimulator CD86 has been identified on eosinophils from allergic subjects [65], a molecule necessary for the activation of naïve T-cells as well as to promote proliferation of activated T-cells. However, it has been shown that eosinophils only possess the ability to stimulate already activated T cells, and not naïve ones [66]. The function for the thymic eosinophils may then be to expand the subsets of T cells already activated to a certain substance and modulate/enhance the immune response already in motion [16].

Helminth infection

The eosinophilic granulocyte has long been described as being the effector cell in the defence against parasites which are too big to be phagocytosed, i.e. helminths. Parasite infection elicits a Th2 response resulting in IgE being released and attaching to the invading parasites, and also the secretion of IL-4 and IL-5 from Th2 T-cells [64]. IL-5, together with eotaxin-1 and RANTES, promotes the activation and recruitment of eosinophils to the site of infection, tissue and blood eosinophilia being one of the hallmarks of parasite infection. As the eosinophil's FcεRI, the high affinity IgE receptor, binds the helminth-attached IgE, degranulation of the cytotoxic granular proteins is evoked. *In vitro* studies support this by confirming that cytotoxic granular proteins cause damage to helminths [67-68].

Lately, this theory has been debated as the results from *in vivo* studies have shown varying results in animal models. Moreover, it appears as if the species of parasite determines whether the eosinophils are capable of expelling the invading helminth [69]. Interestingly, a recent study has demonstrated that the helminth nematode *Thrichinella spiralis* may in fact be dependent on the eosinophil for its survival. Infected eosinophil-depleted mice showed a marked resistance to the parasite as compared to the wild type [70]. Similarly, neither anti-IL-5 therapy appears to cause a decrease in immunity [71-72], nor do hyper-eosinophilic transgenic IL-5 mice exhibit increased resistance to infection [73-74]. It appears as if the eosinophil might have a slightly different role to play with regard to helminths than previously postulated. Instead of being a destructive effector cell, it appears as if eosinophils instead act as immunosuppressant cells by recruiting Th2-skewed lymphocytes to the site of infection, thus protecting the invading organism [70].

Hypereosinophilic syndromes

While eosinophilia in association with helminth infection can be viewed as a "healthy" response, at least in those instances where there appears to be an effect in lessening infection, there also exists disease-associated eosinophilia. "Hypereosinophilic syndrome" (HES) is a diagnosis based on an eosinophil count of 1.5 x 10^9/L for at least 6 months without apparent cause (such as a parasite infection) [75], though many of those cases may in fact be one of several rare clonal hematologic malignancies such as undiagnosed platelet-derived growth factor receptor α (PDGFRA)-associated chronic eosinophilic leukemia [76]. Further, it is likely that patients with HES, who respond to the tyrosine kinase-inhibitor imatinib, in fact suffer from chronic myeloid leukemia or chronic eosinophilic leukemia [77-78].

Eosinophil-associated gastrointestinal disorders

There are a number of disorders in the gastrointestinal tract accompanied by eosinophilia. A few of them are briefly mentioned below in order to highlight the diversity of diseases eosinophils are associated with.

Eosinophil-associated gastrointestinal disorders (EGID) include eosinophilic esophagitis, eosinophilic gastritis, and eosinophilic gastroenteritis. Those disorders are not accompanied by peripheral blood eosinophilia [79]. There appears to be an association between atopy and eosinophil-associated gastrointestinal disorders and eosinophils can often be found in inflamed segments of the gastrointestinal tract in relation to adverse reactions to food [80]. Eosinophilic colitis is a disease where eosinophils accumulate in the colon, with or without accompanying blood eosinophilia [81-82], during the first months of life and cause frequent bloody diarrhea [83-84]. This is believed to be caused by cow's milk proteins as elimination of dairy products from the diet attenuates those symptoms [83, 85]. Individuals with either

type of inflammatory bowel disease (Crohn's disease and ulcerative colitis) have gastrointestinal eosinophilia [86-88] but it appears as if eosinophils are differently activated in the two conditions, reflecting differing expression of Th1/Th2 cytokines in the bowels [89].

Though not as common as the models of asthma, mouse models of gastrointestinal eosinophil-associated allergies have been developed [90-91]. Hopefully, those models will provide insights into the mechanisms of this group of heterogeneous diseases.

Eosinophils and allergy

Eosinophils are also involved in allergic inflammation of the airways, i.e. asthma, rhinitis, and rhinosinusitis. The capacity of the cell to cause damage to epithelial and mucosal cells, and nerves, induce bronchoconstriction and excessive mucus production by releasing granule proteins, lipid mediators and reactive oxygen species [16] has put the cell in focus as an effector cell. Eosinophil survival is prolonged in this environment [92] and blood eosinophils isolated from asthmatics show an upregulation of adhesion molecules [93]. Also, eosinophil infiltration into tissues and release of granule proteins is increasingly thought of as the guilty party concerning the "airway remodeling" seen in chronic asthmatics [94].

Two mouse models exist in which eosinophils are ablated [95-96]. Lee et al. chose a transgenic construct in which an EPO promoter was coupled to expression of diphtheria toxin A. In effect, this causes myeloid cells attempting to produce EPO to die. This mouse strain was named PHIL [95]. Using a different approach, Yu et al. deleted the high-affinity GATA-binding site in the GATA-1 promoter, effectively blocking eosinophil differentiation in those ΔdblGATA mice [96]. In a model of asthma, PHIL mice were protected from airway hyperresponsiveness (AHR) [95], while ΔdblGATA were not [94]. Instead they exhibited decreased airway remodeling [94].

Danger signals

The concept of danger signals was proposed by Polly Matzinger in 1994 [97] in opposition to the then current paradigm that the immune system acted based on the discrimination between self/non-self. The self-nonself (SNS) theory was proposed in 1959 by Burnet [98] and hypothesized that the immune system was activated by foreign matter and that self-reactive cells were deleted in infancy. However, as understanding of immunity increased, modifications to this theory had to be made. Altogether, three new postulates have been added, introducing the concepts of helper-cells, co-stimulation and the discrimination of antigen presenting cells (APCs) between infectious-nonself and noninfectious-self.

The danger model does not focus on self vs. non-self. Instead, this new theory proposed that innate immunity instead reacts to molecules signaling danger. This danger can be perceived either in the sense of invading microbes by detection of e.g. LPS, or as host damage represented by, e.g. eukaryotic DNA from a necrotic cell. Irrespective of the origin of these signals, they are recognized by PRRs on resting APCs [99] causing those cells to become activated, thereby initiating an immune response in the host.

Eosinophils possess the capacity to recognize a host of danger signals, both foreign ones, i.e. bacteria [100] and allergens [101] and signals derived from self [102-103].

Allergy

Classical allergy and hypersensitivity

"Allergy" has become a rather wide term, encompassing adverse reactions to metals, food, smell and airborne substances. In its strictest meaning, allergy is an IgE-mediated reaction requiring both sensitization and later a challenge, in order for a reaction to occur. The

pathways and cells involved in IgE-mediated immediate hypersensitivity reaction are depicted in **Fig. 2.** The immediate reaction mostly affects the smooth muscles and the vascular system, the late phase is characterized by inflammation and recruitment of eosinophils and Th2 cells.

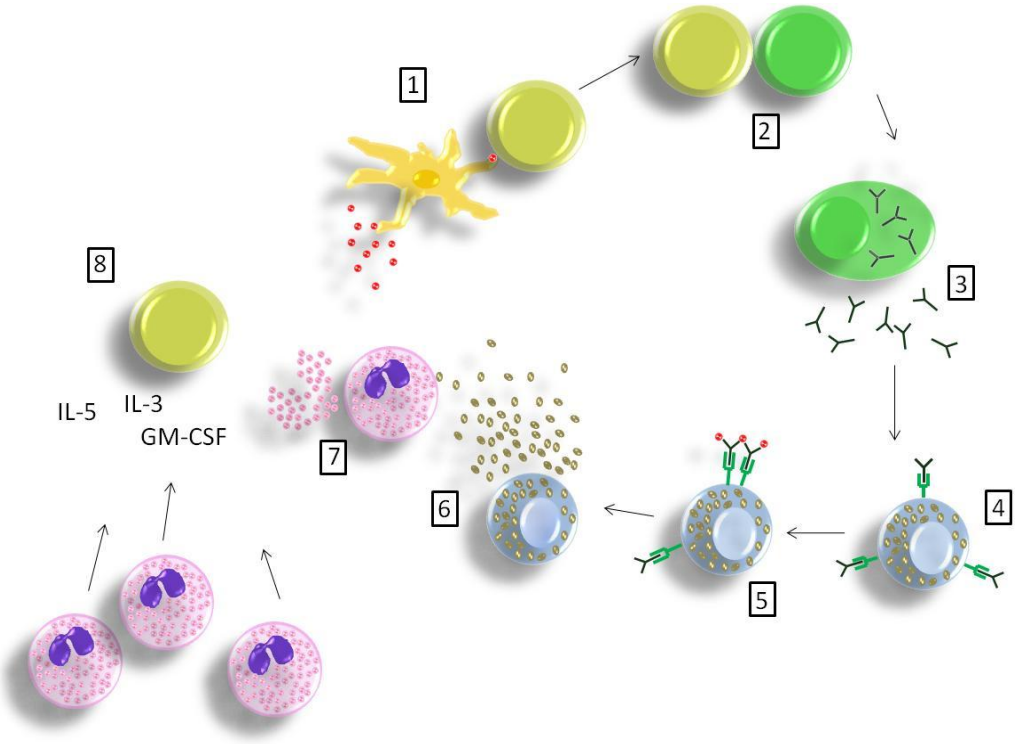

Figure 2. Sensitization and the immediate hyper- sensitivity reaction

1) Allergen is detected, captured and processed by an antigen-presenting cell, in this case a dendritic cell. Allergen epitopes are then presented to T cells which switch to the Th2 subtype **2)** Th2 cells activate B cells and by secretion of IL-4 induce isotype switching and production of IgE **3)** The activated B cell has become an IgE secreting plasma cell **4)** Circulating IgE bind to the high-affinity IgE receptor (FcεRI) on mast cells **5)** Upon re-exposure to the specific allergen, Fc-receptor bound IgE on mast cells bind the allergen and signal activation **6)** Mast cell activation results in the release of granule proteins (histamine), and the synthesis and release of lipid mediators (PGD_2 and LTC_4) and cytokines (IL-3,IL-4, IL-5, IL-6 and TNF) **7)** Activation of tissue eosinophils and release of granular proteins **8)** The late phase is initiated by recruitment of eosinophils into tissue by cytokines secreted by Th2 cells, mast cells and epithelial cells (eotaxin-1). Adapted from Cellular and Molecular Immunology, 5^{th} edition, Abbas and Lichtman, 2003

The development of allergies is sometimes described as “the atopic march”. This refers to the progression from the earliest allergic symptoms such as eczema in infants and toddlers to the subsequent development of allergic rhinitis and, in the end, asthma in older toddlers and children [104]. A recent cohort study reports a steady increase in the number of children who are sensitized to one or more allergens between 1996 and 2006 [105]. However, clinical symptoms of airways allergy have not increased correspondingly, probably due to a decrease in both respiratory infections and exposure to tobacco smoke [105].

Prevalence

Even though many population-based studies regarding the prevalence of allergy have been performed, it is difficult to estimate the true number of allergic individuals due to a combination of factors. For one, self-perceived sensitivity to a substance is in many cases not IgE-mediated allergy [106-108]. It is also difficult to compare questionnaire-based studies as there is great uncertainty about the vocabulary used. Used in this text, atopy refers to a genetic disposition to develop classic, IgE-mediated allergic disease. Allergy is the IgE-mediated reaction to an innocuous substance, and an atopic individual does not have to become allergic. One in four Scandinavian blood donors had immunoreactive IgE to at least one of fourteen common aero- and food allergens [109], and if those figures were to be transferred to the overall Swedish population, 2.25 million individuals would be sensitized to at least one allergen. It is estimated that 4-8% of children, and 4% of adults, suffer from food allergies, adding another 400 000 individuals to the tally. There are clearly benefits to finding effective treatment strategies for allergic diseases, both for the individual and to society.

Airway allergies

Airway allergies include rhinitis and rhinosinusitis, affecting the upper airways. In the context of eosinophil involvement of airway allergy, the most studied disease is asthma and there are several mouse models of asthma [110] but those models are often acute and short-term, while asthma in humans is a chronic disease [111]. This is also a heterogeneous disease with symptoms ranging from wheezy breathlessness, occurring in episodes, to a chronic narrowing of the airways [112]. The hallmarks of asthma are inflammation in the airways, airway hyperresponsiveness (AHR), excessive airway mucus production and increased thickness of the airway wall. This thickening is usually referred to as "airway remodeling" and is believed to be caused by a too extensive repair process of damaged cells, leading to increased deposition of collagen and airway smooth muscle mass [113]. Although it has often been claimed that allergic asthma is associated with a Th2 response, a meta-analysis of 82 studies measuring the increase in cytokine expression after allergen challenge of atopic individuals, showed a mixed Th1/Th2 response [114].

The sensitization of Swedish children to aeroallergens usually starts with cat dander, followed by sensitization to dog and pollen from birch and timothy grass [105, 115].

Food allergies

Most food allergies are IgE-mediated, and the most common culprits are "the big eight" which account for 90% of those allergies. This group consists of cow's milk, egg, wheat, soy bean, peanut, tree nuts, shellfish and fish [116]. There also exist non-IgE mediated allergic reactions where the cellular and/or humoral immune factors involved are incompletely understood [117] and also the "non"-IgE-mediated allergy where allergen-specific IgE may be undetectable in blood and skin, but present locally in the gastrointestinal mucosa [118]. Even

though the adaptive immune system is mostly implicated in food allergies, there is beginning to emerge evidence that innate immune cells may also recognize allergen, exemplified by the recognition of peanut allergen by human dendritic cells [119].

Food allergies exhibit varied symptoms ranging from nausea, stomach cramps, diarrhea, respiratory symptoms and eczema to anaphylaxis [116].

MAPKs and intracellular signaling

GPCRs

The family of G-protein coupled receptors (GPCRs) all signal through a guanine-nucleotide binding (G) –protein. It is present in all eukaryotes and thousands of different receptors have been discovered. Also, one receptor can affect several responses and several receptors can synergistically signal the same response, forming a complex signaling network. The receptor itself consists of seven membrane-spanning α-helices with the N-terminus located on the extracellular side of the membrane, see **Fig. 3**. The ligand-binding site is located between the sixth and seventh α-helix and the G-protein interaction takes place with portions of the cytosolic loop stretching between helices five and six. Signaling specificity is achieved by the sequence of the cytosolic loop being unique for a particular G-protein [120]. There are actually two types of G-proteins, the first type are the small G-proteins which consist of only one unit and those will not be discussed further in this text.

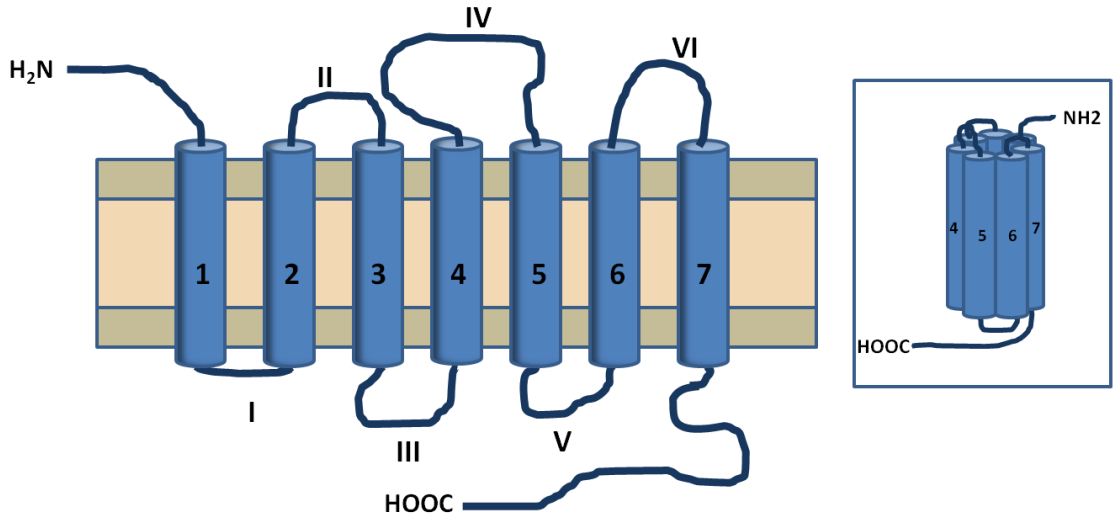

Figure 3. G-protein coupled receptor

The receptor consists of seven trans-membrane α-helices (1-7). The ligand-binding site is located between the sixth and seventh α-helix (VI) and the G-protein interaction takes place with portions of the cytosolic loop stretching between helices five and six (V). The inserted picture is a representation of how the α-helices are believed to be arranged three-dimensionally in the membrane.

The second type of G-protein consists of three subunits in its resting state, Gα, Gβ and Gγ, forming a heterotrimer [120]. The activation/inactivation of a typical heterotrimeric G-protein is shown in **Fig. 4**.

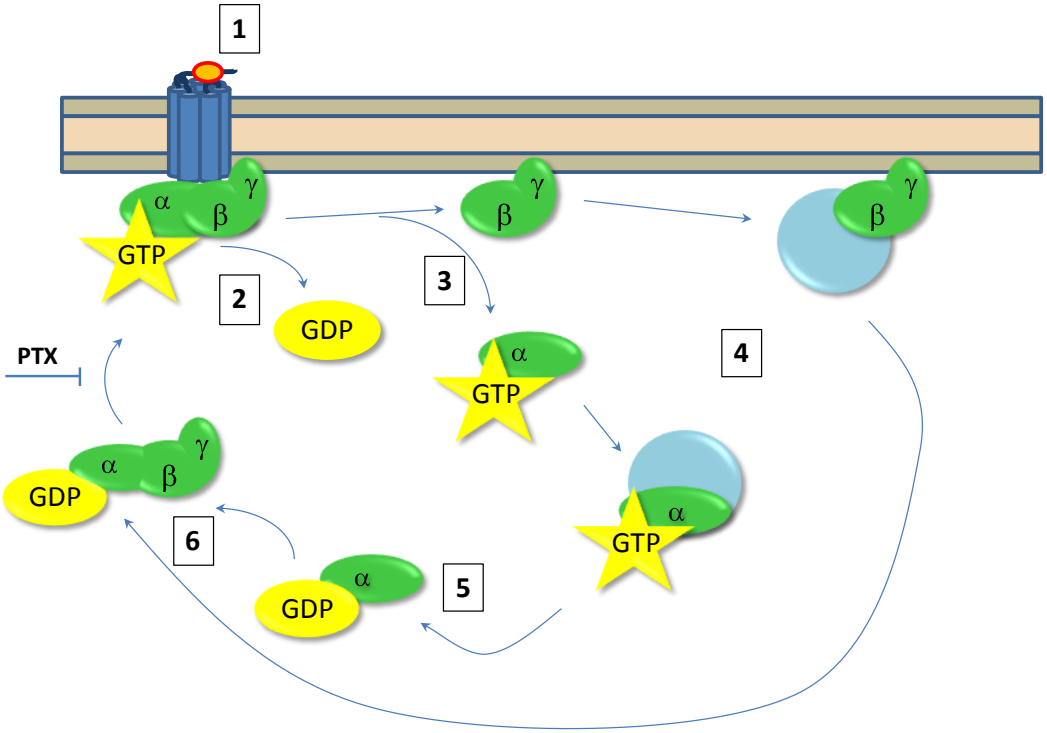

Figure 4. G-protein activation cycle

1) Ligand binds to the receptor **2)** Gα exchange GDP for GTP and the G protein becomes activated **3)** The subunits separate **4)** The G protein subunits interact with target proteins, either activating or inhibiting them **5)** The GTP is hydrolyzed by Gα into GDP, thus inactivating Gα **6)** The subunits recombine into an inactive G protein again. Pertussis toxin (PTX) blocks the conversion of GDP to GTP and stops signaling through the G protein coupled receptor

As long as the receptor's messenger-binding site is engaged and the cytosolic loop is in the activating conformation, the G-protein will continue cycling through activation/inactivation, but signaling will stop effectively as soon as the ligand disassociates from the receptor. One ligand-binding receptor can activate several G-proteins and thus amplifying the signal. Although there are a wide variety of G-proteins, the most common way of signal transduction is the activation of kinase-cascades, further amplifying the signal received on the receptor [120]. **Fig. 5** is a simplified chart of the pathways leading up to the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK) and p38.

One widely used inhibitor of GPCRs is pertussis toxin (PTX). This toxin is used to investigate signal transduction in live cells and uncouples G-proteins of the G_i type from their receptor and thus effectively blocks signaling [121].

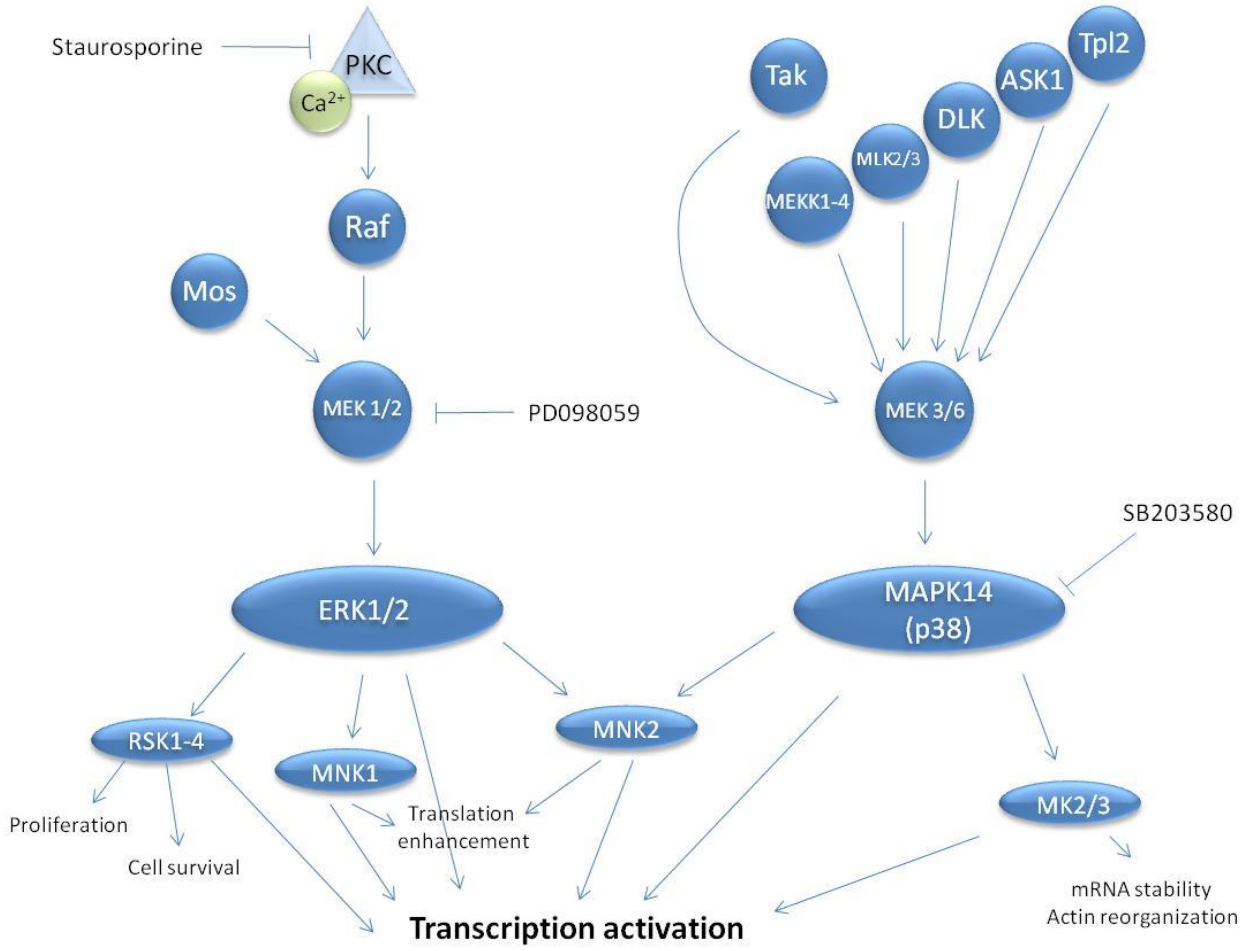

Figure 5. A simplified rendering of some of the intracellular signaling pathways of protein kinase C (PKC) and the mitogen activated protein kinase (MAPK) family.

Arrows signify activation, blunt stops represent inhibition. ERK-extracellular signal-regulated kinase, MEK- ERK kinase, MEKK- MEK kinase, RSK- ribosomal S6 kinase, MNK- Menkes protein, Tak- Tat-associated kinase, MLK- mixed lineage kinase, DLK- death-associated protein kinase-like kinase, ASK- activator of S phase kinase, Tpl- MEK kinase 8, MK- MAPK-activated protein kinase. Adapted from Jeffrey *et al.* Nature Reviews Drug Discovery, 2007.

FPRs

In the mid-1970's, neutrophils were found to become activated by and migrate towards small bacterial formylated peptides [122], in particular *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe or fMLF). fMLF is referred to as fMLP in **papers I and II**. However, in the current one-letter amino acid code, P denotes proline, not phenylalanine [123], rendering the use of fMLP out-of-date. The receptor responsible for recognition of fMLF was not identified until 1990 when the formyl peptide receptor (FPR), a GPCR, was sequenced [124]. Soon, two homologues were added to the family, formyl peptide receptor-like 1 (FPRL1) and FPRL2 [125]. As the name implies, these receptors are preferentially activated by small proteins initiated by an *N*-formylated methionine, a property unique for proteins synthesized by prokaryotes or in the mitochondria of eukaryotes. Hence, these peptides are mainly released by metabolically active bacteria and damaged eukaryotic cells. The FPR-family belongs to the group of GPCRs which are pertussis toxin-sensitive.

In 2009, a name change was proposed for the family of FPR and FPR-like receptors. As FPRL-1 and -2 were names referring to the structural similarities to FPR and not to the ligand binding properties of these receptors, the names where changed accordingly; FPR to FPR1, FPRL-1 to FPR2 and FPRL-2 to FPR3 [126] and those are henceforth the names which will be used in this thesis. However, as this name change had not taken place when **studies I-III** were carried out, the receptors are referred to by their old names in those papers.

FPR1 was the first sequenced formyl peptide receptor [124], though it was first found through functional characterization of rabbit and human neutrophils [127-129]. FPR1 has been found to be expressed by neutrophils and monocytes [126]and has also been identified in cells of non-myeloid origin such as hepatocytes, astrocytes and microglial cells [130-131] suggesting there exist additional functions than those currently assigned to this receptor. The most

commonly used agonist for FPR1 is the *E. coli*-derived formylated tripeptide fMLF, which is also the smallest formyl peptide exhibiting full agonist properties [126]. By replacing the N-formyl group with a t-butyloxycarbonyl (t-Boc) group the peptide becomes an antagonist, boc-MLF [132]. Another antagonist of FPR1 is cyclosporine H (CsH), which is at least ten times as potent as boc-MLF [133]. It appears as if CsH blocks fMLF binding and acts as an inverse agonist, which suppresses the constitutive activity of FPR1 [134]. The biological significance of this action remains to be determined.

FPR2 exhibit a 69 % amino acid similarity with FPR1. Still, it shows much less affinity for fMLF than does FPR1 [125, 135-136], and it appears as if mitochondria-derived formyl peptides are the preferred ligands [137]. FPR2 is expressed in neutrophils and monocytes [126]. The eicosanoid lipoxin A4 (LXA4) has also been identified as a ligand [138] and thus FPR2 is sometimes referred to as FPR2/ALX. Additional ligands such as the hexapeptide tryptophan-lysine-tyrosine-methionine-valine-methionine (Trp-Lys-Tyr-Met-Val-Met or WKYMVM) has been identified using peptide libraries [139]. FPR2 can be inhibited using the hexapeptide tryptophan-arginine-tryptophan-tryptophan-tryptophan-tryptophan (Trp-Arg-Trp-Trp-Trp-Trp or WRWWWW (WRW^4)) [140].

FPR3 shares 56% of its amino acid sequence with FPR1, and though it does not bind fMLF [141], it can become activated by the mitochondrion-derived protein N-formyl-methionine-methionine-tyrosine-alanine-leucine-phenylalanine (fMet-Met-Tyr-Ala-Leu-Phe or fMMYALF). F2L, a naturally occurring peptide derived from heme-binding protein has also been implicated as a ligand for FPR3 [137].

CCR3

The G-protein coupled receptor CC chemokine receptor 3 (CCR3) was discovered as the receptor for eotaxin-1 [10, 142-143] and also for "regulated on activation, normal T cell expressed" (RANTES) [142-143]. Later, eotaxin-2 [144] and eotaxin-3 [145] were cloned and found to be ligands of CCR3. The receptor is expressed by eosinophils, mast cells, basophils and Th2 cells [146], though eotaxin-1 is considered an eosinophil-specific chemoattractant [147]. Murine models have shown that neutralization of eotaxin-1 decreases airway inflammation and airway hyperresponsiveness (AHR) [148-149] and decreases tissue eosinophilia after allergen challenge in sensitized animals [150]. An initial knock-out mouse model did exhibit a decrease in tissue eosinophils, but an increase in AHR. This increase was later demonstrated to be caused by mast cells [146], and using the CCR3 knock-out mouse in a model of allergic skin inflammation showed marked decrease of skin and lung eosinophilia and decreased AHR [151]. A more recent study with α-CCR3 mab inhibits eosinophilic airway inflammation and mucus overproduction [152] and a CCR3 antagonist has also been demonstrated to inhibit airway remodeling [153]. Both those studies were performed in mice, but as α-CCR3 mab appears to be well tolerated, trials with human subjects should soon be underway.

Desensitization and receptor hierarchies

One specific characteristic of G protein coupled receptors (GPCRs) is their ability to become desensitized. After ligand binding the response soon declines and re-stimulation with the same ligand will not generate a new response, this is called homologous desensitization and results from the agonist-bound receptor being phosphorylated by a GPCR kinase (GRK) [154]. An example of this can be seen in **Paper II, Fig. 4A**, where pre-incubation with

eotaxin-1 renders eosinophils unable to migrate towards the same ligand. Heterologous desensitization occurs when stimulation of one receptor renders the cell unresponsive to another ligand/receptor pair, as can be seen in **Paper II, Fig 4A**, where migration towards eotaxin-1is inhibited by pre-incubation with fMLF. This phenomenon is believed to stem from phosphorylation of free receptors by second messenger-activated kinases, after which the receptor is inactivated. Protein kinase C has been implicated in this type of process [155]. The biological function of desensitization may be to single out the most "important" chemokine in an environment with mixed chemoattractants [156].

Another attribute of desensitization is the fact that related receptors can become desensitized by each other's agonists. Those receptors are said to belong to the same receptor class [157]. There is also a hierarchy between receptors, i.e. fMLF can desensitize calcium mobilization in HEK293 cells in response to both C5a and IL-8, while C5a can only block the response to IL-8, not fMLF. fMLF is thus considered to be an "end-target chemoattractant" [158]. It is possible to bypass desensitization by using a higher concentration of stimuli than the first dose given [159]. Using a higher (at least ten-fold higher) concentration for desensitization than that used for later stimulation makes it more likely to correctly assess the desensitizing properties of a substance.

Aims

I) To elucidate the role of formyl peptide receptors in alerting human eosinophils to the presence of the airborne allergens birch pollen and house dust mite

II) To establish if there is interplay between the eotaxin receptor CCR3 and the formyl peptide receptor FPR1 with respect to the triggering of chemotaxis and respiratory burst in human eosinophils

III) To investigate whether the atopic status of the donor affects the reactivity of eosinophils to airborne allergens *in vitro*, and whether this is affected by seasonal exposure to birch pollen

IV) To characterize the reactivity of human eosinophils to the food allergens cod and milk regarding activation patterns, receptor usage and the bioactive components of the allergens

Materials and methods

This section is intended as a more in-depth explanation of the methods used. For specifics regarding individual experiments, the reader is asked to look for this information in the corresponding **paper**.

Purification of leukocytes

Peripheral blood eosinophils were isolated from fresh buffy coats obtained from blood donors at Sahlgrenska University Hospital, Göteborg and Kungälv Hospital, Kungälv, or from heparinized blood derived from healthy volunteers. After removal of the majority of the erythrocytes by dextran sedimentation, centrifugation on a Ficoll gradient separated granulocytes from mononuclear cells. Those first two steps were performed at room temperature; the cell preparation was kept at 4º C for the remainder of the purification process. To further minimize the risk of activating the cells during the purification process, all solutes were Ca^{2+}-free. Remaining erythrocytes were removed from the granulocyte fraction by hypotonic lysis with distilled H_2O for 30-35 seconds after which the physiological salt balance was restored. The cells were washed and the lysis step was repeated 3-4 times. The next step removed neutrophils and contaminating mononuclear cells from the granulocyte fraction by positive selection using magnetic beads (MACS; Miltenyi Biotec Inc) coated with anti-CD16 (neutrophils), anti-CD3 (T cells), anti-CD14 (monocytes) and anti-CD19 (B cells) mabs. Neutrophils or monocytes were obtained by flushing out the CD16- or CD14-expressing cells, respectively, bound to the MACS column after passage of eosinophils. The cells were washed and resuspended in either Krebs-Ringer glucose buffer [120mM NaCl, 5

mM KCl, 1.7mM KH_2PO_4, 8.3 mM Na_2HPO_4, 10mM glucose, 1.5 mM $MgCl_2$; pH 7.3] (KRG) or X-vivo 15 buffer lacking phenol red. The purity of the eosinophils was determined by Diff-quik stain of an aliquot of cytospun cells and was routinely >95% when 200 cells were counted. The viability was assessed by Trypan Blue stain and the viability routinely >99% after purification.

The HL-60 cell-line

In order to study only one receptor at a time, without synergistic signaling from other receptors, we used HL-60 cells transfected with either FPR1 or FPR2. The HL-60 cell line was originally isolated from a patient with acute promyelocytic leukemia [160] and this cell line can be manipulated *in vitro* such that it differentiates into neutrophil-, monocyte-, macrophage, and eosinophil-like cells. Even though those differentiated cells cannot be considered to be true eosinophils they are invaluable as a granulocyte-like system. We also conducted experiments with purified eosinophils and neutrophils in order to see if our observations of the HL-60 cells could be transferred to native cells.

Stimulation of eosinophils

Eosinophils were resuspended in either X-vivo 15 buffer for 18 h incubations or KRG for incubations of 60 minutes or less. The cells were aliquoted in 96-well low-binding polystyrene plates and co-incubated with the substance of interest for the desired amount of time at 37°C, 5% CO_2. Eosinophil viability was routinely > 97% after stimulation, as determined by Trypan blue exclusion.

Release of Major Basic Protein, MBP (Paper I and III)

Eosinophils were fixated and permeabilized using the Cytofix/Cytoperm fixation and permeabilization solutions (BD Biosciences). This solution maintains the conformation of the cell while allowing antibodies to cross the membrane, making staining of intracellular substances possible. The cells were incubated with Cytofix for 20 minutes at 4º C. The Fc receptors were blocked with human IgG (Beriglobin) in room temperature for 15 min and stained with a mouse α-human-MBP mab or the isotype control anti-human CD22 for 15 min at 4º C, followed by PE-labeled rat α-mouse-IgG_1 for another 15 minutes at 4 º C. Between each step the eosinophils were washed with Permwash solution in order to remove unbound antibodies and still retain the permeability of the cellmembrane. The cells were kept dark during the incubation with the fluorochrome-labeled secondary antibody and up to the point of analysis, in order to preserve the intensity of the fluorochrome. The cells were then fixated in 3.7% paraformaldehyde-PBS. Mean fluorescence intensity (MFI) was analysed using a FACScan (Becton Dickinson). A change in intracellular MBP contents was determined using the formula: (MFI of medium-treated cells - MFI of allergen-stimulated cells) / MFI of medium-treated cells) x 100 = % MBP as compared to the medium-treated cells.

Quantification of eosinophil peroxidase, EPO

Eosinophil peroxidase activity in cell supernatants or lysates was measured enzymatically by the addition of H_2O_2 and *o*-phenylenediamine (OPD) dissolved in a lysis buffer [100mM sodium acetate, 20 mM ethylene diamine tetraacetic acid (EDTA), and 1% hexadecyl trimethyl ammonium bromide (HETAB), pH 4.5]. OPD acts as a substrate for EPO and the end-product is an orange-brown soluble which can be read at 490 nm. The intensity of the color correlates with the amount of EPO in the sample.

Chemotaxis of eosinophils

Eosinophilic migration was determined using 30 µL-volume 96-well microplate chemotaxis/cell migration chambers with hydrophobic filters and a pore size of 3 µm. The positive control eotaxin-1, the negative control diluent (KRG + 0.3% bovine serum albumin) and the chemoattractants of interest were added in triplicates to the wells in the lower chamber. Cell suspensions consisting of 30,000 eosinophils were added on top of the filter, the 30 µL-droplet contained by a hydrophobic ring of 5.7 mm Ø, and the cells were allowed to migrate to the lower wells for 90 minutes at 37°C. After removal of the filter, the eosinophils were incubated for 10 more minutes in order for newly migrated cells to settle. Transmigrated cells were then lysed by the addition of 1 % Triton-X 100 in PBS and peroxidase activity was measured as described above. To mimic maximal migration, 30,000 cells were lysed using 1 % Triton-PBS and the total peroxidase activity present in the lysate was determined. The percentage of transmigrated cells was determined using the following formula =

(Absorbance in wells containing unknown number of transmigrated eosinophils / Absorbance in wells containing maximum number (30,000) of cells) x 100 = % migrated cells.

For the inhibition and desensitization experiments, the substance used to pre-incubate the cells were also added to the lower wells in order to avoid creating an artificial concentration gradient.

Receptor inhibition and signal transduction blockade

The protocols for incubation with inhibitors vary somewhat between the papers regarding the temperature and time. This is mostly depending on the toxicity of the inhibitor and also on the

experimental system used to assay the response of the cells. Our attempt has been to achieve a good inhibitory effect without activating or killing the eosinophils in the process. In order to ascertain this we assessed the viability of treated cell with Trypan blue staining and, if possible, a positive control stimulus signaling through an unrelated receptor was used to make sure the cells were not rendered anergic by the treatment.

Table 3 lists the pairs of inhibitor and stimulus used for each experiment, and their concentrations.

Receptor desensitization (Paper I and II)

The mechanism behind this type of experiment has been described previously in the **Introduction**. The specifics of the desensitization experiments are listed in **Table 3**.

Table 3. Blocking of receptors and signal transduction, and desensitization experiments in Paper I, II and IV

Activation parameter	Pre-treatment	Conc.	time (min)	temp.	Stimulus	Conc.
EPO-release[1]	Pertussis toxin	200 ng/mL	120	37	aeroallergen[2]	100 µg/mL
EPO-release[1]	Pertussis toxin	200 ng/mL	120	37	platelet activating factor (PAF)	10^{-7} M
EPO-release[1], MBP-release[1]	Cyclosporin H	10^{-6} M	10	4	aeroallergen	100 µg/mL
migration[1]	Cyclosporin H	10^{-6} M	5	37	aeroallergen	100 µg/mL
migration[1]	Cyclosporin H	10^{-6} M	5	37	fMLF, C5a, PAF	10^{-8} M
migration[1]	boc-MLF	10^{-5} M	5	37	aeroallergen	100 µg/mL
migration[1]	boc-MLF	10^{-5} M	5	37	fMLF, C5a, PAF	10^{-8} M
migration[3]	Cyclosporin H	10^{-6} M	10	4	eotaxin-1, -2, -3, fMLF, RANTES	10^{-8} M
migration[3]	Cyclosporin H	10^{-6} M	10	4	PAF, C5a	10^{-7} M
migration[3]	boc-MLF	10^{-5} M	10	4	eotaxin-1, fMLF	10^{-8} M
migration[1]	α-leukotriene B_4 receptor (α-LTB_4R)	5 µg/mL	30	4	leukotriene B_4	5×10^{-9} M
migration[1]	α-LTB_4R	5 µg/mL	30	4	fMLF	10^{-8} M
migration[1]	α-LTB_4R	5 µg/mL	30	4	aeroallergen	100 µg/mL
migration[1]	α-CCR3	5 µg/mL	30	4	eotaxin-1	10^{-8} M
migration[1]	α-CCR3	5 µg/mL	30	4	aeroallergen	100 µg/mL
migration[3]	α-CCR3	5 µg/mL	10	4	Eotaxin-1, -2, -3, fMLF, RANTES	10^{-8} M
migration[1]	staurosporine, PD098059, SB203580	10^{-7} M/10^{-5} M	30	4	fMLF, eotaxin-1	10^{-8} M
migration[1]	staurosporine, PD098059, SB203580	10^{-7} M/10^{-5} M	30	4	aeroallergen	100 µg/mL
migration[3]	staurosporine, PD098059, SB203580	10^{-5} M	10	4	eotaxin-1	10^{-8} M
migration[3]	staurosporine, PD098059, SB203580	10^{-5} M	10	4	fMLF	10^{-7} M
migration[1]	fMLF	10^{-7} M	10	4	fMLF, eotaxin-1	10^{-8} M
migration[1]	fMLF	10^{-7} M	10	4	aeroallergen	100 µg/mL
migration[1]	eotaxin-1	10^{-7} M	10	4	eotaxin-1	10^{-8} M
migration[3]	eotaxin-1	10^{-8} M	10	4	eotaxin-1, fMLF	10^{-8} M
migration[1]	eotaxin-1	10^{-7} M	10	4	aeroallergen	100 µg/mL
migration[1]	HDM	500 µg/mL	10	4	fMLF	10^{-8} M
migration[1]	HDM	500 µg/mL	10	4	HDM	100 µg/mL

Activation parameter	Pre-treatment	Conc.	time (min)	temp.	Stimulus	Conc.
migration[4]	Prostaglandin E_2	10^{-8} M	10	4	cod	3000 µg/mL
migration[4]	Prostaglandin E_2	10^{-8} M	10	4	Prostaglandin D_2	10^{-8} M
migration[4]	Prostaglandin D_2	10^{-7} M	10	4	Prostaglandin D_2	10^{-8} M
migration[4]	α-DP1	17 x 10^{-9} M	10	4	cod	3000 µg/mL
migration[4]	α-DP1	17 x 10^{-9} M	10	4	Prostaglandin D_2	10^{-8} M
migration[4]	α-DP2	6 x 10^{-9} M	10	4	cod	3000 µg/mL
migration[4]	α-DP2	6 x 10^{-9} M	10	4	Prostaglandin D_2	10^{-8} M
migration[4]	α-EP4	10^{-9} M	10	4	cod	3000 µg/mL
migration[4]	α-EP4	10^{-9} M	10	4	Prostaglandin D_2	10^{-8} M
calcium-flux[1, 5, 7]	Cyclosporin H	10^{-6} M	5	37	fMLF	10^{-8} M
calcium-flux[1, 5]	Cyclosporin H	10^{-6} M	5	37	HDM	100 µg/mL
calcium-flux[1, 6, 7]	WRWWWW	5 x 10^{-6} M	5	37	WKYMVM	10^{-8} M
calcium-flux[1, 6]	WRWWWW	5 x 10^{-6} M	5	37	HDM	100 µg/mL
calcium-flux[1, 7]	Cyclosporin H	10^{-6} M	5	37	aeroallergen	400 µg/mL
calcium-flux[1, 7]	WRWWWW	5 x 10^{-6} M	5	37	aeroallergen	400 µg/mL
calcium-flux[1, 7]	Cyclosporin H + WRWWWW	10^{-6} M + 5 x 10^{-6} M	5	37	aeroallergen	400 µg/mL
calcium-flux[3, 8]	Cyclosporin H	10^{-6} M	5	37	eotaxin-1, fMLF	10^{-7} M
calcium-flux[3]	α-CCR3	5 µg/mL	5	37	eotaxin-1, fMLF	10^{-7} M
calcium-flux[9]	Cyclosporin H + α-CCR3	10^{-6} M + 5 µg/mL	5	37	eotaxin-1, fMLF	10^{-7} M
respiratory burst[3]	Cyclosporin H	10^{-6} M	5	37	fMLF	10^{-7} M
respiratory burst[3]	Cyclosporin H	10^{-6} M	5	37	eotaxin-1, RANTES, C5a, PAF	10^{-8} M
respiratory burst[3]	fMLF	10^{-8} M	10	37	fMLF, eotaxin-1	10^{-8} M
respiratory burst[3]	eotaxin-1	10^{-8} M	10	37	fMLF, eotaxin-1	10^{-8} M
respiratory burst[3]	staurosporine, PD098059, SB203580[8]	10^{-5} M[2]	10	37	eotaxin-1	10^{-8} M
respiratory burst[3]	staurosporine, PD098059, SB203580[8]	10^{-5} M[2]	10	37	fMLF	10^{-7} M

[1]Paper I [2]Aeroallergen refers to either aqueous extracts of house dust mite (HDM) or birch pollen [3]Paper II [4]Paper IV [5]HL- 60-FPR1 [6]HL-60-FPR2 [7]Neutrophils [8]Incubation with CsH followed by stimulation with fMLF was also performed with HL-60-FPR1 cells. [9]SB203580 was also tested at 10^{-7} M.

Release of reactive oxygen species

An enhanced chemiluminescence system was used to determine eosinophilic production of reactive oxygen species. Isoluminol is a substrate which is unable to enter the cell, thus it can only react with extracellular reactive oxygen species [161]. The assay is based on the fact that oxygen radicals excite isoluminol. When the molecule returns to its ground state, light is emitted. Horseradish peroxidase was also added as a peroxidase source, needed for the energy transfer from the superoxide anion to the isoluminol. In short, 5×10^4 eosinophils resuspended in KRG were preincubated with isoluminol [2×10^{-5} M] and horseradish peroxidase [4 U] for 5 minutes at 37ºC before the addition of the various chemoattractants. The chemiluminescence activity was continuously measured in a Biolumat LB 9505 (Berthold Co., Wildbad, Germany) and expressed as megacounts/ min (MCPM).

Isolation of cellular mRNA and PCR analysis (Paper I)

Total RNA was isolated from highly purified eosinophils (>98%), neutrophils and monocytes, using the RNeasy Mini Kit from Qiagen. Contaminating DNA was removed by the use of DNA-*free*™ (Ambion), and the RNA yield and purity were determined by spectrophotometry. Using the First Strand cDNA Synthesis Kit, with an oligo$(dT)_{18}$ primer cDNA was synthesized using the RNA as a template. This cDNA was used for PCR amplification of mRNA transcripts for FPR1, FPR2, FPR3, and the housekeeping gene GAPDH. FPR1 transcripts were amplified with the forward primer 5'-TACCCAGAGCAAGACCACA-3' and the reverse primer 5'- AAAAGGCTGCTGCGACAA-3', FPR2 with 5'-TGCTGGTGCTGCTGGCAA-3' (forward) and 5'-AATATCCCTGACCCCATCCTCA-3' (reverse), FPR3 with 5'-AGTTGCTCCACAGGAATCCA-3' (forward) and 5'-GCCAATAATGAAGTGGAGGATCAGA-3' (reverse), and GAPDH with the primers 5'-

GGCTGCTTTTAACTCTGG-3' (forward) and 5'-GGAGGGATCTCGCTCC-3' (reverse). The amplification was performed on a Mastercycler gradient (Eppendorf Nordic). The number of cycles was optimized for the different cell types and primers, respectively. The PCR products as well as a molecular size standard (ΦX174 RF DNA/*Hae* III Fragments; Invitrogen), were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining.

Measurement of intracellular calcium transients (Paper I, II and IV)

By using the two cell-permeable dyes Fura red™ and fluo-3 it is possible to measure intracellular calcium transients in live cells, simultaneous as this occurs, and thus monitor activation of receptors which signal through the calcium-release pathway. When calcium is bound by Fura red™ there is a change in the wavelength of the light emitted by the compound. This causes the signal recorded by the flow cytometer to diminish in strength. Conversely, as fluo-3 binds calcium the intensity of the signal is increased. By dividing the increasing signal (fluo-3) by the decreasing signal (Fura red™) those changes are further magnified.

HL-60 cells were resuspended in cell-loading medium (KRG with Ca^{2+} + 1% FCS) and loaded with the fluorescent dyes fluo-3, AM and Fura Red™, AM (Molecular Probes, Eugene, OR) for 30 min at 37°C. Following this, excess dye was removed and the cells were resuspended in cell loading medium and kept on ice until five min. prior to analysis, when the cells were warmed to 37°C in a water bath. The fluorescence emission of fluo-3 was monitored in the FL-1 channel and that of Fura Red™ in the FL-3 channel. First, baseline fluorescence was established for 20 s before the stimulant was added, and fluorescence emission was recorded for a total of 300 seconds. Data was collected using the software CellQuest, BD and re-

analyzed using the, at the time, latest version of the analysis software Flow Jo (Tree Star Inc., OR). The relative Ca^{2+} concentration was expressed as the ratio between Fluo-3 and Fura Red (FL-1/FL-3) MFI over time.

p38 MAPK immunoblots (Paper II)

We wanted to determine if eosinophils used the mitogen-activated protein kinase (MAPK) p38 pathway to signal the presence of fMLF and eotaxin-1. This was done by stimulating the cells for various periods of time and assaying for phosphorylated p38 as a marker of activation. Eosinophils were incubated with fMLF or eotaxin-1 for 0, 1, 3 and 10 min at 37° C. The cells were lysed and intracellular reactions were stopped by the addition of ice-cold RIPA lysis buffer and the cells were then incubated in the buffer for additional 20 minutes. The lysates were spun down at 12000 x *g* for 5 min at 4°C, and the supernatants were collected. Sample buffer was added and the mixture was heated to 70 °C for 10 min. The cytosolic extracts were run on a NuPAGE Bis-Tris 4-12% gel together with a protein standard (MagicMark XP, Invitrogen), transferred onto a nitrocellulose membrane and blocked in TBS (20 mM Trizma base, 137 mM NaCl with 1% BSA) for 1 h at room temperature. Primary antibody, p38 MAPK (pT180/pY182) mouse IgG1 isotype, was diluted in TBS-0.05% Tween-0.1% BSA (TTB buffer) and incubated at 4 °C overnight. The membrane was then incubated with HRP-conjugated goat-anti mouse IgG, diluted in TBS-0.05% Tween buffer for 1 h at room temperature before addition of 1-4-α-chloronaphtol and H_2O_2. Lysed anisomycin-treated HeLa cells were used as a positive control for phosphorylated p38 protein expression.

Receptor density assay (Paper II)

We wondered whether internalization of CCR3 was responsible for the lack of activation following stimulation with fMLF or eotaxin-1. Purified eosinophils were incubated with medium, fMLF, eotaxin-1 or CsH for 10 min at 37° C. This was followed by labeling with either α-FPR-PE or α-CCR3-PE, clone 5E8 for 20 min at 4° C and analyzed by flow cytometry (FACSCanto II, BD Biosciences). The median fluorescence intensity (medianFI) of mock treated cells was compared to the medianFI of cells treated with stimuli or inhibitor. A decrease in medianFI of treated cells would indicate a decrease in the amount of surface receptors. This method measures relative amounts of the protein labeled, but due to differing base line quantities of CCR3 and FPR1 and differing affinities of the antibodies it is not possible to compare the values recorded for the two receptors.

Eotaxin binding assay (Paper II)

By assessing the capacity of eotaxin-1 to bind its cognate receptor CCR3 following treatment with fMLF or CsH, we hoped to determine whether our previous results could be due to a diminished affinity between receptor and ligand.

Purified eosinophils were incubated with medium, fMLF [10^{-7} M], CsH [10^{-6} M] or anti-eotaxin (Human CCL11/Eotaxin Biotinylated Fluorokine Kit (R&D Systems Inc.) for 10 min at 4° C, followed by stimulation with biotin-conjugated eotaxin (R&D Systems Inc.) for 5 min, and labeling with avidin-FITC (R&D Systems Inc.) for 15 min at 4 ° C. The samples were analyzed by flow cytometry (FACSCanto II, BD Biosciences). Two negative controls were also used, biotin conjugated soy bean trypsin inhibitor (STI-biotin) was used as a negative background control and anti-eotaxin, a negative specificity control provided by the

manufacturer (R&D Systems Inc) which was pre-incubated for 10 min with the eotaxin-biotin conjugate prior to incubation with eosinophils. The median fluorescence intensity (medianFI) of the cells was compared; a decrease in medianFI relative to medianFI would indicate decreased binding capacity.

Acceptor depletion fluorescence resonance energy transfer (adFRET) (Paper II)

Fluorescence resonance energy transfer (FRET) is a method to measure whether two molecules are in close proximity to each other. Energy is transferred from an excited donor fluorophore to another fluorophore (the acceptor) if the two receptors are within 100 Ångström from one another [162]. If FRET occurs, the donor signal will be quenched and the acceptor signal will be increased [163]. In acceptor depletion (ad)FRET, the donor fluorescence is measured before and after photobleaching of the acceptor. Photobleaching leads to the loss of fluorescence and only fluorescence emitted from the unquenched donor will be registered [164].

Eosinophils were incubated with eotaxin-1, fMLF or buffer on poly L–lysine-coated cover slips for 10 min at 37°C, followed by fixation in 3% paraformaldehyde in Pipes buffer (pH 6.8) for 5 min at RT, followed by 3% paraformaldehyde in Borax buffer for 10 min at RT. Cover slips were incubated with 10 μg/ml of either α-FPR-1 mab, clone 350418 mouse IgG 2a or α-CCR3, clone 5E8 mouse IgG 2bκ, for 30 min at RT in PBS containing 0.1% BSA then incubated with Alexa 488-goat anti-mouse IgG (1:200; Invitrogen) at RT. Alternately, cover slips were blocked with 2% BSA in PBS at 4°C overnight, incubated with either FPR1 mab or CCR3 as described above, followed by Cy3-labeled donkey anti-mouse IgG (1:400; Jackson Immuno Research) Finally, cover slips were mounted in Mowiol antifadent and

images collected on an inverted Zeiss Axioplan microscope with a LSM510 laser scanning system with wideopen pinholes (3µm) to ensure co-localization. adFRET was carried out as described previously [164].

Quantification of Th1/Th2 cytokines by cytokine bead array (Paper III)

The amounts of IL-2, IL-4, IL-5, IL-10, tumor necrosis factor (TNF) and interferon γ (IFN-γ) in supernatants from eosinophils stimulated with either medium or allergen (100 µg/mL) were measured simultaneously using the Cytokine Bead Array (CBA) Human Th1/Th2 cytokine kit (BD Biosciences, San Diego, CA, USA) and analyzed by flow cytometry (FACScan, Becton Dickinson) using CBA Software (BD Biosciences).

The CBA-method is based on capture-antibody labeled beads of discrete fluorescence intensities. PE-conjugated antibodies with the same specificity as the antibodies on the beads are also supplied. When beads, free PE-labeled antibodies and cytokine are mixed in solution, a sandwich construct is formed of bead-cytokine-PE-labeled antibody. The signal read-out from each bead increases with the amount of cytokine and matching antibodies which are bound. This quantification is calibrated using a cytokine standard and set-up beads specific for each batch produced. CBA makes it possible to analyze multiple proteins simultaneously, using very small (50 µL) volumes of supernatant.

The limits of detection for the individual cytokines were 2.4 pg/mL for IL-5, 2.6 pg/mL for IL-2 and IL-4, 2.8 pg/mL for IL-10 and TNF, and 7.1 pg/mL for IFN-γ, according to the manufacturer.

Leukotriene C_4 and Prostaglandin E_2 release (Paper IV)

Supernatants from eosinophils stimulated for 15 min with food extracts and other agents, were analyzed using the Cysteinyl-Leukotriene C_4 EIA kit or the Prostaglandin E_2 EIA Kit (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturers' recommendations.

Leukotriene C_4, Prostaglandin D_2 and Prostaglandin E_2 in allergen extracts (Paper IV)

The endogenous amounts of leukotriene C_4, and prostaglandin E_2 in food allergen extracts were measured using the same EIA-kits as described above. Likewise, the amount of prostaglandin D_2 was measured by a PGD_2- enzyme immunoassay from Cayman Chemical Company, following the manufacturer's instructions.

To further confirm the results obtained by the enzyme inhibition assays, samples were subjected to liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). Positive samples were quantitated by LC- single reaction monitoring (SRM). Both procedures were performed in the TSQ Quantum Ultra™.

Statistics

Statistical analyzes were performed using GraphPad Prism 3.0-5.0 software. The non-parametric Mann- Whitney *t*-test or one-way ANOVA with Newman- Keuls post test was employed. $p < 0.05$ was used to indicate statistical significance.

Results and discussion

Previous work by our group has shown that eosinophils are capable of sensing danger associated molecular patterns (DAMPs) from various species of bacteria [100], damaged epithelial cells [103] and airborne allergens [101] in a serum-free system. The activation pattern of the eosinophils was dependent on the stimuli used, e.g. house dust mite elicited release of both eosinophil peroxidase (EPO) and major basic protein (MBP) while birch pollen gave rise to EPO release, but not MBP [101]. Also, this activation was not dependent on any traditional helper cells such as CD4+ T cells, as we only used purified eosinophils in the assays.

FPR1 and FPR2 are the receptors used to signal the presence of house dust mite and birch allergens (Paper I)

After discovering that eosinophils could react independently to airborne allergens [101], the next step was to identify the receptor/s responsible for transducing this information. We decided to focus on the response to two aeroallergens, house dust mite (HDM) and birch.

The major allergen of the European house dust mite, *Dermatophagoides pteronyssinus* is the protein Der p1. This protein has been shown to have proteolytic properties disrupting tight junctions between cells [165], facilitating the sensitization process. There are additional proteins in HDM extract which, though lacking proteolytic activity have been shown to stimulate airway-epithelia into secreting pro-inflammatory cytokines [166]. Our group has investigated the effect of one such protein, Der p 2, but not been able to evoke an eosinophilic response [101].

Birch pollen has been shown to attract and activate eosinophils by both us and others [101, 167] and they appear to do so by pollen-associated lipid mediators (PALMs) which resemble eicosanoids. PALMs also appear to stimulate dendritic cells to secrete Th2 cytokines [168] making this likely to be the first step of allergic inflammation and recruitment of eosinophils and other mediators of late events of inflammation [169].

Successful signal blockade with pertussis toxin (PTX) confirmed that the danger signaling evoked by birch and HDM was relayed through a G-protein coupled receptor (GPCR). One particular GPCR of interest was the formyl peptide receptor 1 (FPR1) which we had studied previously. By using the FPR1-specific antagonist cyclosporine H (CsH) we could successfully inhibit EPO release and migration in response to both birch and HDM in human blood-derived eosinophils from healthy subjects.

Even though our experimental results clearly indicated FPR1 as being involved in signaling the presence of HDM and birch, we performed a series of additional experiments to ascertain this. The first step was to use another antagonist of FPR1, namely boc-MLF. Boc-MLF did also inhibit migration towards fMLF, birch and HDM, in a similar manner as CsH. Chemotaxis towards two other GPCR-ligands, C5a and PAF, was also performed in order to exclude the possibility that CsH and boc-MLF non-specifically inhibited all GPCRs. Neither antagonist had any effect on the migratory capacity of eosinophils towards either C5a or PAF. Finally, using antibodies to block the eotaxin-1 receptor, CCR3, or the leukotriene B_4 receptor (LTB_4R), two other GPCRs of importance in eosinophil biology, again had no influence on the migration of eosinophils towards HDM or birch. LTB_4R had previously been shown to mediate eosinophil activation in response to pollen extracts [167].

So far our results pointed to FPR1 being the receptor responsible for recognizing birch and HDM. However, FPR2 shares approximately 70% sequence homology with FPR1, and is also

a receptor for fMLF, although a low affinity one. We therefore had to consider signaling through FPR2 as well.

In order to study signaling through one receptor at a time, we used the human leukocyte cell line HL-60 transfected with either FPR1 or FPR2. Both HL-60-FPR1 tranfectants and HL-60-FPR2 transfectants showed a clear migration to their ligands fMLF and WKYMVM, respectively, but not towards the allergens. This could be viewed as confirmation of the specificity of the transfected cells, but unfortunately those results did not further our understanding of the mechanisms behind eosinophilic responses to aeroallergens.

Another marker of activation of GPCRs is the release of intracellular calcium stores. By measuring the concentration of cytosolic calcium response to stimulation we could determine that while birch did not cause calcium release, HDM could activate both FPR1 and FPR2 HL-60 transfectants. This activation by HDM could be inhibited by the respective antagonists CsH and WRWWWW (WRW^4). .

Eosinophils are, though their exact function is unknown, key players in allergic conditions. We and others have shown them capable of responding to allergen extracts rather than purified allergenic proteins [101, 170], as opposed to T and B cells which preferentially become activated by protein epitopes.

The recognition of HDM and birch pollen extracts by FPR1 and FPR2, receptors which are believed to signal the presence of bacteria and cell damage, is curious. The two allergens do not share any common features with each other nor with the cognate ligands of the formyl peptide receptors. Clearly, more studies into the activating properties of allergens are needed.

Eosinophils express FPR1 and FPR2, but not FPR3 (Paper I)

As we could infer from our experimental data that responses to birch and HDM were elicited by engagement of FPR1 and FPR2, we wanted to confirm the expression of those receptors in eosinophils, and also determine whether FPR3, the third member of the human formyl peptide receptor family, was expressed. To our knowledge this had previously been investigated in neutrophils and monocytes [171], but not in eosinophils. We therefore set up a PCR-system to detect FPR1, FPR2 and FPR3 and used monocytes and neutrophils as positive controls. As expected, we could detect all three FPRs in the monocyte preparation. The eosinophil preparations showed the same expression pattern of FPR1 and FPR2 as neutrophils, and this did not vary between the several eosinophil donors tested. We thus concluded that eosinophils express functional FPR1 and FPR2, but not FPR3.

Since our study was published, Devosse *et al.* have contradicted our findings and shown that eosinophils express functional FPR3 [172]. They used a combination of PCR, FACS of α-FPR3 labeled eosinophils and a functional assay where the chemoattractivity of the FPR3-ligand F2L was evaluated. An explanation for the differing results between our study and theirs might be a combination of several differences in methodology. Firstly, there are some differences in the purification protocols, one being the choice of magnetically labeled antibodies used for the separation step and the choice of ammonium chloride for lysis of erythrocytes. This is known to affect the responsiveness of eosinophils e.g. decreasing the antigen presenting ability of eosinophils [16]. Another difference between the two studies is the choices of primers for FPR3. The primers used by our group were matched to sequences close to the 5' end, the group of Parmentier chose a primer pair situated closer to the middle. While we did confirm the presence of FPR3 in monocytes using our system, it is possible there are differences in the protein expressed by eosinophils as compared to monocytes.

There is interplay between the eotaxin receptor CCR3 and the formyl peptide receptor FPR1 (Paper II)

As eosinophils are a major part of the immune cells involved in allergy it is important to elucidate which receptors and intracellular signaling pathways are used by those cells in the response to allergens. We knew the presence of HDM to be signaled through FPR1 and FPR2, and had discovered that pre-incubation of eosinophils with the FPR agonist fMLF inhibited the migration towards HDM and birch pollen **(Paper I)**. Our next step was to see if engagement of FPR1 could affect another GPCR, CCR3. CCR3, also called the eotaxin receptor, can bind eotaxin-1, -2, and -3 (CCL11, CCL24 and CCL26) and "regulated on activation, normal T cell expressed" (RANTES/CCL5). This receptor is vital for the migration of eosinophils as mice with a deletion of CCR3 lack gastrointestinal eosinophils [173]. Also, disruption of CCR3 expression by antisense oligonucleotide therapy reduces the early asthmatic response in human subjects [174]. Increased knowledge about those two receptors could give rise to new treatment strategies for allergies and other diseases characterized by eosinophilic infiltration.

Treatment of eosinophils with the FPR1 antagonists cyclosporinH (CsH) and boc-MLF did abrogate migration towards fMLF, as expected. However, migration towards eotaxin-1 was also completely inhibited! CsH did in fact decrease migration towards all three eotaxins and RANTES, clearly affecting the signaling capacity of CCR3 somehow. There was no discernable effect on the response towards either PAF or C5a, the latter ligands engage two other GPCRs. Blocking of CCR3 using α-CCR monoclonal antibodies showed the expected decrease in migration towards the eotaxins and RANTES, but the response to fMLF was unaffected. It thus appeared as if inhibition of FPR1 also blocked CCR3 with regard to migration, but not the other way around. Further, CsH inhibited calcium flux in response to

eotaxin-1, whereas α-CCR3 did not affect calcium release in cells stimulated with fMLF. Interestingly, the inhibitory effect of CsH on CCR3-signaling was not seen with regard to oxidative burst.

The inherent trait of GPCRs to become desensitized by activation, and thus unable to respond again for a period of time, can be used to study which receptor/s is/are engaged by a specific ligand. Incubating eosinophils with fMLF decreased migration towards both fMLF (homologous desensitization) and eotaxin-1 (heterologous desensitization), indicating that both FPR and CCR3 are somehow affected by fMLF. As incubation with eotaxin-1 only affected migration towards eotaxin-1, there was apparently no heterologous desensitization affecting FPR1 in this case. When we repeated the same type of desensitization experiment regarding respiratory burst we could only achieve homologous desensitization for either substance. There appears to be at least two different signaling mechanisms in response to fMLF and eotaxin-1. One pathway signaling calcium-flux and migration, where engagement of FPR1 somehow appears to block CCR3. The other pathway does not exhibit this type of uni-directional heterologous desensitization and causes the activation of oxidative burst.

In order to study the signaling pathway used in response to eotaxin-1 and fMLF, we used specific pharmacological inhibitors and studied their effects on migration and release of oxygen radicals. We achieved similar degrees of inhibition in all experiments except one. While SB203580, the inhibitor of mitogen activated protein kinase 14 (MAPK14)/p38, completely abolished respiratory burst in response to eotaxin-1, the response to fMLF was only inhibited by approximately 60%. Western blot of eosinophils stimulated with eotaxin-1 and fMLF showed activation of p38 by both substances, but eotaxin-1 appeared to elicit a stronger response.

GPCRs is a large group of receptors, but the magnitude of differential responses to different stimuli cannot be accounted for by the numbers of G-proteins available. There must be additional factors guiding the cellular responses and it appears as if our current knowledge of those events is only the tip of the iceberg.

Eosinophil reactivity does not appear to be affected by the atopic status of the donor (Paper III)

Our discovery of aeroallergens activating eosinophils [101] also raised a question. Since the cells used in those experiments were purified from anonymously donated blood, the atopic status of the donor was unknown. As many as 25% of blood donors in Scandinavia express IgE specific to common aeroallergens [109] and this sensitization might act as a priming environment for the eosinophils prior to isolation. We decided to collect eosinophils from donors with a history of allergic reactions and confirmed sensitization and compare the reactivity of their cells to cells from non-atopic donors, focusing on individuals with birch pollen allergy.

Comparing the eosinophilic reactivity of those two groups to birch pollen and cat dander we saw no differences regarding release of either eosinophil peroxidase (EPO) or major basic protein (MBP).

The presence of eosinophils at sites of allergic inflammation and the many cytokines eosinophils are able to release into their surroundings raises the question as to which cytokines they *do* release in response to aeroallergens. We found the Th2 cytokine IL-4 was secreted both spontaneously and in response to stimulation by aeroallergens by almost half of our donors, irrespective of which group they belonged to. This correlates with the finding of Moqbel *et al.* [175] where IL-4 was detected in eosinophils from roughly 30% of the tested

donors, both atopic asthmatics and non-atopic individuals. IL-4 is considered a typical Th2 cytokine which induces B cells to switch to IgE-production, skews naïve T cells into Th2 cells and inhibits macrophage activation [64]. One could easily assume this cytokine to be expressed by cells involved in an ongoing allergic inflammation, but why it is expressed in non-atopic individuals is enigmatic. However, the levels detected were not above 15 pg/mL, probably less than a tenth of what a corresponding number of T cells are capable of secreting [176].

As opposed to the finding of IL-4, we also found the pro-inflammatory cytokine TNF in the supernatants of eosinophils stimulated with the aeroallergens birch and HDM, birch eliciting the strongest response. Again, we could not see a difference in response between the two groups of subjects, but as with IL-4 the amounts of TNF were close to the detection level. The main source of TNF is LPS-stimulated macrophages, which release it to recruit monocytes and neutrophils. A recent study has shown this also up-regulates the adhesion molecule CD54/ inter-cellular adhesion molecule (ICAM-1) on eosinophils [177].

Allergen induced activation patterns are influenced by natural exposure to birch pollen (Paper III)

During our study of potential differences between atopic and non-atopic subjects, we also had the opportunity to compare the reactivity of those two groups not only to each other, but also between two different points of time. By collecting blood samples both during the winter and later in the spring during the ongoing birch pollen season, we could study the effect of natural exposure to an aeroallergen on eosinophilic reactivity to allergens in vitro.

While eosinophils from atopic and non-atopic individuals had similar activation patterns, we did see a difference in response between cells collected during the winter and during the birch

pollen season. A low-grade release of TNF could be seen during the winter, most strongly after stimulation with birch, but this was also noted for some of the donors after mock treatment of the cells and after stimulation with cat dander, HDM or grass pollen. This was almost completely abrogated by the natural exposure to birch pollen during the spring. We also investigated the migratory capacity towards the eosinophil-specific chemoattractant eotaxin-1 and towards house dust mite (HDM). Although it has been suggested that eosinophils from allergic individuals express a primed phenotype [178], thus being more sensitive to chemoattractants such as eotaxin-1, we could not discern such a pattern. Neither could we detect any differences in the migratory response towards HDM between the two groups. Eosinophils from atopics did not migrate to a greater degree than those from the non-atopic group, nor did the former respond to a lower concentration of HDM than the latter.

Many studies have investigated the differences between allergic and non-allergic individuals, usually focusing on the eosinophil, or eosinophilic protein, as markers of allergic inflammation. While there is a positive correlation between eosinophil infiltration into tissues and allergic inflammation, a recent study showed increased eosinophil infiltration into the lungs of both non-atopic and atopic asthmatics, while ECP levels were elevated in atopic individuals without asthma compared to the control group [179] Trials with anti-IL-5 monoclonal antibodies report decreased blood eosinophil counts and prevention of eosinophilia in allergic subjects following allergen exposure. However, no effects were seen regarding the late asthmatic response, or histamine-triggered AHR [180]. A later trial, where anti IL-5 therapy was given to asthmatic patients, reported reduced levels of eosinophils in blood and in sputum, but no change in clinical markers of asthma such as morning peak expiratory flow, daily β_2-agonist use or the number of exacerbations, or the perceived quality of life of the patients [181].

Eosinophils are activated by whey and cod extracts (Paper IV)

As we had shown that aeroallergens were capable of directly activating eosinophils, and as the eosinophil mostly resides in the gastrointestinal tract, it was logical to investigate the reactivity of eosinophils to food allergens. Eosinophils are traditionally believed to mediate late phase allergic reactions, but eosinophil activation can occur within 1 hr after exposure to foods [86]. Also, milk proteins appear rapidly in the serum after oral ingestion in both healthy and allergic individuals [182], and fish allergens can be indirectly detected in serum already 10 min after ingestion [183]. We focused on whole milk, milk whey proteins, egg white, soy bean, peanut and cod fish because they are common food allergens in Sweden.

Eosinophils responded to milk and whey by secretion of eosinophil peroxidase (EPO) and stimulation with cod fish extract caused migration and calcium release. Those allergens were the ones which caused the strongest responses in eosinophils, thus we focused on them in our continued studies. Neither of the tested allergens gave rise to oxidative burst, or release of either ECP or EDN. We also measured release of leukotriene C_4 and prostaglandin E_2 from stimulated eosinophils. Both eicosanoids were secreted in low amounts in response to cod, and dose responses could be seen after stimulation with whey. Milk elicited a dose response release of PGE_2, but only measurable levels of LTC_4 at the highest concentration tested. Analysis of milk, whey and cod extracts revealed considerable amounts of LTC_4 in all extracts, and of PGE_2 in the cod extract. When analyzed by liquid chromatography followed by tandem mass-spectrometry we could only verify the presence of PGE_2 in the cod extract.

The EPO release was stronger in response to whey proteins than to milk, even though we attempted to use whey concentrations corresponding to those found in milk. Twenty percent of total milk proteins can be found in the whey fraction, and most of those are globular proteins. We investigated the two major whey proteins α-lactalbumin and β-lactoglobulin, but

neither protein could elicit EPO-release, either separately or in combination. While neutrophils become primed by α-lactalbumin and β-lactoglobulin, exhibiting increased responses to fMLF and more vigorous phagocytosis [184], the eosinophil-activating component requires further investigation.

Parvalbumin is the major fish allergen [185-189] and even though the structure can differ between different fish species, the parvalbumins are more or less recognized by IgE purified from fish allergic individuals [188]. However, this was not the case regarding eosinophils, as cod fish parvalbumin did not elicit eosinophil activation.

The activating substance in cod fish extract share many properties of PGD_2 (Paper IV)

The presence of PGE_2 in cod extract led us to assess the migratory capacity of purified PGE_2 using the structurally similar prostanoid PGD_2 as a second positive control. PGD_2 is a potent mediator of allergic inflammation [190-191], and an eosinophil chemoattractant [192]. While eosinophils did not migrate towards PGE_2, this prostanoid had previously been found to inhibit migration of eosinophils towards eotaxin, PGD_2 and C5a [38]. In our hands, PGE_2 inhibited the migration of eosinophils towards both cod extract and PGD_2 in a similar manner, indicating usage of the same receptor. We thus focused on PGD_2 or PGD_2-like substances in cod as a possible activating candidate.

Initial enzyme-linked immunosorbent assay (ELISA) analysis of the cod extract revealed presence of PGD_2, but mass spectrometry could not verify this leading us to the conclusion that cod contains a PGD_2-like substance responsible for the activation of eosinophils.

PGD_2 signals through the two receptors Prostglandin D_2 receptor 1 (DP1) and DP2/CRTH2 (chemoattractant receptor homologous molecule expressed on Th2 cells), and DP2 has been

shown to be active on eosinophils [193]. The similarities in the inhibitory pattern after blocking of DP2 further strengthens the theory that DP2 was the receptor responsible for mediating the activation of eosinophils by cod extract. However, the exact pathways mediating activation in response to cod remain to be determined in future studies.

Eosinophils are, as previously mentioned, capable of interacting with particular antigens [194] while B- and T cells are only able to respond to soluble ones. This would make eosinophils ideal in capturing and responding to food allergens which are detected by conventional antigen-presenting cells. Most research into food allergies are aimed at IgE-mediated allergies, though reactions caused by IgE-independent routes may also be of consequence to the quality of life for those suffering from them. Further research will increase our understanding of the role played by innate immunity in allergies. This will open up new treatment possibilities for allergies and other inflammatory conditions where eosinophils appear to be effector cells.

Personal reflections

The eosinophil is a beautiful little cell with its pink granules and oddly shaped nucleus. It is also quite the ambiguous little cell. Though it was discovered far earlier than many other players of the immune system, our understanding of its functions is still very incomplete. However, the field is changing and the classical view of eosinophils, based mostly on the effect of their specific granule proteins, is under revision. The role of eosinophils has been considered to be protection against parasites and the causing of tissue damage in allergic disease, but with increased research emerges the role of an immunomodulatory cell involved in remodeling and repair [195]. There is also evidence that eosinophil granule proteins can be synthesized in the granules of cells which have left the bone marrow [196], indicating more adaptability to external signals than previously assumed. New effector functions are also discovered, such as the ability to release mitochondrial DNA which forms extracellular structures with granule proteins, binding to and killing bacteria [197].

Our discoveries of eosinophils recognizing both airborne and food derived allergens are not surprising in the light of the multitude of receptors expressed by those cells. But what is the function of this recognition?

If eosinophils can influence their environment into a Th2 response and sustain this [198], it should also be possible for eosinophils to initiate this type of reaction. We did not see a difference in the reactivity of eosinophils to allergen based on the atopic phenotype of the donor. However, the volunteers in this study were tested for IgE, and while IgE-mediated allergies are the most common ones, there are apparently other mechanisms at play in adverse reactions to the environment. It may be that eosinophils in IgE-mediated allergies are attracted to sites of ongoing allergic inflammation and the Th2 milieu stimulates the cells to express the same type of cytokines. This would not be dependent on hyperreactive eosinophils, but a

response to the microenvironment. In another microenvironment, caused by bacterial infection, eosinophils have been noted to express IL-12 and IFN-γ [114, 199], contributing to a Th1 type of response. Eosinophil chemoattractants are secreted by epithelial cells and other immune cells as part of a response to many types of local inflammation, of both Th1 and Th2 types. Taken together, the eosinophil appears to be somewhat of a chameleon cell, lending support to the type of reaction it is attracted to.

The reactivity to allergens mediated by signaling through FPR1, FPR2 and other, as yet unidentified receptors, may be viewed as coincidence. A theory proposed by Polly Matzinger is that PRRs were not originally intended as such, but instead as receptors of injured self [200]. During evolution the role of those receptors has changed, and the recognition of allergens might be one such alteration. The response elicited from eosinophils is probably not sufficient to drive an inflammatory response by itself, but instead starts a chain reaction where damaged epithelial cells recruit additional cells of both innate and adaptive immunity, giving rise to a local inflammation. Again, it is not the response by the eosinophils themselves, but the subsequent overreaction of surrounding cells which cause the inflammation.

There is much more to discover concerning the actions and interactions of eosinophils, and the perception of the eosinophil will shift more than once regarding its capacities.

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