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Abbreviations

µl – microliter

µM – micro-molar

µm – micrometer

A.dest. – distilled water

APS – ammonium persulfate

BAL – broncho-aleveolar-lavage

bmEos – bone marrow-derived eosinophils

C5a – complement 5a

Ca(2+) – calcium

CCR3 – C-C chemokine receptor type 3

CD – cluster of differentiation

COX – cyclooxygenase

CRTH2 – chemoattractant receptor-homologous molecule expressed on Th2 cells

ddH_2O – distilled water

DMSO – dimethyl sulphoxide

DP – D-type prostanoid receptor

ECP – eosinophilic cationic protein

EDN – eosinophil derived neurotoxin

EDTA – ethylendiaminetetraacetic acid

EGID – eosinophilic gastrointestinal disorder

Eos – eosinophils

EPO – eosinophil peroxidase

FBS – fetal bovine serum

FCS – fetal calf serum

FLT3-L – Fms-related tyrosine kinase 3 ligand

FSC – forward angle light scatter

GM-SCF – granulocyte-macrophage-colony-stimulating factor

H3 – histone 3

HCL – hydrogen chloride

HDAC – histone-deacytelases

HIL-3 sup – supernatant of a human adult T-cell leukemia cell line

HPC – hematopoetic progenitor cells

Ig – immunoglobulin

IFN-γ – interferon γ

IL – interleukin

LTB_4 – leukotrien B_4

MAP kinases – mitogen activated protein kinases

MBP – major basic protein

MCP – monocyte chemoattractant protein

Mg2+ – magnesium

MIP-1α – macrophage inflammatory protein - 1α

ml – milliliter

mM – millimolar

mSCF – mouse stem cell factor

NEAA – non essential amino acids

ng – nanogram

NSAID – non steroidal anti-inflammatory drug

PAF – platelet activating factor

PBS – phosphate buffered saline

PGD_2 – prostaglandin D_2

PGDS – prostaglandin synthase enzymes

PGE_2 – prostaglandin E_2

$PGF_{2\alpha}$ – prostaglandin $F_{2\alpha}$

PGG_2 – prostaglandin G_2

PGH2 – prostaglandin H_2

PGI_2 – prostaglandin I_2/prostacyclin

PI-3 kinase – phosphatidylinositol-3 kinase

PS – penicillin- streptomycin

pM – picomolar

RANTES – regulated on activation, normal T – cell expressed and secreted

rm – recombinant mouse

rpm – rounds per minute

SB – sodium butyrate

SCF – stem cell factor

SDS-PAGE – sodium-dodecyl sulphate-polyacrylamide gels

SEM – standard error of the mean

SSC – side angle light scatter

TEMED – tetramethylethylenediamine

TNFα – tumor necrosis factor α

TSA – trichostatin A

TXA_2 – thromboxane A_2

WB – whole blood

Figure Legend

Zusammenfassung

Da eosinophile Granulozyten nur einen geringen Anteil der peripheren Leukozyten ausmachen, ist ihre Isolierung und Anreicherung nur zu einem gewissen Grad möglich. Um die Erforschung der komplexen Funktionen eosinophiler Granulozyten zu erleichtern, ist die Optimierung neuer Kultivierungsmethoden, die die Generierung einer großen Anzahl von differenzierten Eosinophilen gewährleisten, notwendig.

In dieser Studie testeten wir zwei verschiedene Zellkultursysteme: einerseits arbeiteten wir mit Maus-Knochenmarks-Vorläuferzellen, die zu Eosinophilen differenziert wurden (BmEos), und andererseits mit einer humanen eosinophilen Leukämie Zelllinie, den EoL-1 Zellen. Um die Funktionalität der BmEos zu bestätigen, wurden die Effekte zweier Chemoattractants, Eotaxin und PGD_2, auf die ausdifferenzierten BmEos und auf frisch isolierte humane Eosinophile überprüft. Bei den EoL-1 Zellen untersuchten wir die Expression der beiden PGD_2 Rezeptoren, CRTH2 und DP, und überprüften ihre Aktivierbarkeit in verschiedenen funktionalen Assays.

Zur Differenzierung der BmEos wurde Knochenmark von BALB/c Mäusen isoliert und Vorläuferzellen wurden durch Kultivierung in Zytokin-Medium (FLT3-L und SCF) angereichert. Anschließend wurden diese in IL-5 Medium zu Eosinophilen ausdifferenziert. Für die Differenzierung der BmEos testeten wir Sera (FCS) unterschiedlicher Qualitätsgrade und variierten deren Konzentration, sowie die Menge von IL-5 im Medium.

Die Differenzierung der humanen EoL-1 Zelllinie in eosinophile Granulozyten wurde mit Natriumbutyrat induziert. Das verwendete Medium enthielt dabei unterschiedliche Konzentrationen an FCS und IL-5. Die Aktivierbarkeit der nicht-differenzierten und differenzierten Zellen wurde in Chemotaxis- und Calcium-Assays getestet. Mittels Durchflusszytometrie bestimmten wir die Expressionsmuster der CRTH2 und DP Rezeptoren auf den EoL-1 Zellen.

Bei unseren Tests fanden wir nur ein Serum, das die Proliferation und Differenzierung großer Mengen von Eosinophilen aus Maus-Knochenmarks-Vorläuferzellen in einem zwölf-tägigen Kultursystem unterstützte. Die ausdifferenzierten Zellen waren vom eosinophilen Phänotyp und exprimierten den Eosinophilen-Marker Siglec-F. Die Stimulierung mit Eotaxin führte zu einem klaren Anstieg der CD11b Oberflächenexpression, sowohl in den BmEos, als auch in humanen Vollblut-Eosinophilen. Stimulierung mit PGD_2 führte jedoch nur in humanen Eosinophilen zu einem Anstieg von CD11b. Ähnliche Ergebnisse ergaben auch die Chemotaxis-Versuche; Eotaxin induzierte sowohl in BmEos als auch in humanen

Eosinophilen eine chemotaktische Antwort, PGD_2 im Gegensatz führte nur bei humanen Eosinophilen zur Migration. Da wir jedoch mittels Western Blot das Vorhandensein des PGD_2 Rezeptors CRTH2 nachweisen konnten, gehen wir davon aus, dass durch die Kultivierung der BmEos in IL-5 der CRTH2 Rezeptor internalisiert bzw. inaktiviert wird und eine Stimulierung mit PGD_2 daher nicht mehr möglich ist.

Auf der Oberfläche der EoL-1 Zellen konnten wir CRTH2 nachweisen. Die Expression des DP Rezeptors zeigte sich jedoch als inkonsistent. Des Weiteren konnte mit PGD_2 eine chemotaktische Migration der EoL-1 Zellen induziert werden. Ein PGD_2-vermittelter Anstieg der intrazellulären Kalzium Konzentration wurde hingegen nicht beobachtet.

Abstract

Eosinophil granulocytes only comprise a small fraction of circulating peripheral blood leukocytes. Accordingly, the study of eosinophils is limited by difficulties in achieving sufficient cell number and purity. Thus, other models providing large quantities of fully differentiated eosinophils and permitting longer and stable cultivation for the in-depth study of eosinophil function are needed. Hence, we tested two different eosinophil cell culture systems: the murine bone marrow derived eosinophils (bmEos) and the human EoL-1 cell line. To explore the functional competence of these two cell lines, their responsiveness to eotaxin and PGD_2, two potent eosinophil chemoattractants, was assessed.

Unselected bone marrow was isolated from BALB/c mice. Progenitor cells were enriched in FLT3-L and SCF-containing medium for the first four days and thereafter differentiated into fully competent eosinophils in IL-5-medium. Different brands and quality grades of fetal calf serum (FCS) as well as different serum and IL-5 concentrations were tested. Using eotaxin and PGD_2 as stimulating agonists, we furthermore compared the responsiveness of human whole blood eosinophils and bmEos regarding their expression of the surface molecule CD11b, as well as their chemotactic responsiveness.

EoL-1 cells were cultured in a medium containing varying concentrations of FCS and IL-5 and differentiation was initiated using sodium butyrate. The expression patterns of the PGD_2 receptors CRTH2 and DP were investigated by flow cytometry and functional assays such as Ca(2+)-influx and chemotaxis were performed.

We observed that the development of bmEos strongly depends on the composition and the quality grade of the serum. We only found one serum that yielded large quantities of fully differentiated eosinophil granulocytes within 12 days of culture. Stimulation with eotaxin led to a significant upregulation of CD11b surface expression on bmEos, as well as on human whole blood eosinophils. In contrast, stimulation with PGD_2 only affected human whole blood eosinophils, but was without effect in bmEos. Similar results were obtained in the chemotaxis assay. Mouse bmEos as well as human eosinophils migrated towards eotaxin, whereas PGD_2 only induced chemotaxis in human eosinophils. A possible explanation for this finding might be the fact that IL-5 leads to internalization of CRTH2. Thus, we suggested that CRTH2 is internalized or inactivated due to the cultivation in IL-5 medium, since CRTH2 protein expression in bmEos was confirmed by western blotting.

Furthermore, we found CRTH2 being expressed on the cell surface of EoL-1 cells while levels of DP expression were inconsistent. The cells responded with chemotaxis to stimulation with PGD_2. In contrast, we found no PGD_2-induced Ca(2+) flux in EoL-1 cells.

1 Introduction

1.1 Eosinophil Granulocytes

The eosinophil granulocyte, first described in 1846 by Wharton Jones and later characterized by Paul Ehrlich (1879) plays multiple roles in the immune system, participating in various inflammatory responses as well as acting as a regulator in adaptive and innate defense (1,2). Under physiological conditions, the eosinophil granulocyte represents 1 – 5% of circulating blood leukocytes and has been identified to form an important fraction of the cellular population in the gastrointestinal tract (3-5).

Eosinophil granulocytes originate from hematopoietic progenitor cells (HPC) in the bone marrow. These cells are known as undifferentiated pluripotent stem cells, being capable of evolving into all cell types of the blood and having the ability of regeneration (6,7).
The viability and differentiation into certain cell types, depends on the influence of growth factors and cytokines. Stem cell factor (SCF) and IL-6 act directly on HPCs, as they are promoting their survival and regeneration. In contrast, granulocyte-macrophage-colony-stimulating factor (GM-CSF), IL-3 and IL-5, all known as pro-inflammatory cytokines, especially induce the differentiation of eosinophil granulocytes. Remarkably, these cytokines can be detected in allergic tissues, leading to a local increase of eosinophil production, as HPCs have the ability to differentiate as well in situ (6,7).
IL-33 was found to play another important role in the differentiation from progenitor cells into eosinophils, specifically in the course of eosinophil-mediated airway-inflammation (6,8). Conditions like parasitic infections, allergic disorders, neoplastic disorders and drug hypersensitivity lead to an augmentation of eosinophil production in the bone marrow (9).

Among all the factors promoting eosinophil differentiation, IL-5 takes a special position, as it triggers specifically eosinophil survival and development, both in human - as well as in mouse eosinophils (10). IL-5 itself is a glycoprotein, produced by various hematopoietic and non-hematopoietic cells, comprising natural helper cells, T-cells and granulocytes (9,11).
Besides its role in the final maturation of eosinophils, IL-5 plays many roles regarding eosinophil function, as it delays apoptosis, increases adhesion to endothelial cells, raises the effector function and increments degranulation (11,12).

1.1.1 The role of Eosinophil Granulocytes in the Immune System

Though the role of eosinophil granulocytes is being investigated intensively, their full function still is not completely clear. One of the main effects of eosinophil granulocytes derives from their granular proteins, including major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), eosinophilic cationic protein (ECP) and Charcot Leyden crystal protein. These proteins play a key role in the defence against pathogens, such as bacteria, parasites and viruses, and are able to stimulate an increased inflammatory mediator release from mast cells and basophils (13).
EPO produces halide acids and toxic hydrogen peroxide, which are responsible for cell damage (14). EDN and ECP are ribonucleases acting against viruses, as they are capable of degrading single-stranded RNA-containing viruses, such as respiratory syncytial virus (13,15). ECP together with MBP in contrast were found to work antibacterially (13). In general, MBP seems to play an important role as a pathogen, as it is cytotoxic itself and as it stimulates the degranulation of basophils and mast cells (16). Charcot Leyden crystal protein was found to be a remnant of eosinophil degranulation, and can therefore be identified in many tissues, secretions and body fluids associated with allergic inflammation (14,17). Concerning eosinophil function in helminthic infections, Butterworth et al. first demonstrated in 1975 that they are capable of killing parasites in their larval stage (18). In the course of the defence of parasites, increased peripheral eosinophilia and degranulation can be detected. The released granular proteins activate complement and antibody-dependent cell death, as well as they trigger pore formation of the cell membrane (13). Some studies also suggest a role for eosinophils in cancer, as in some types of cancer, for instance brain-, throat- and GI- cancer, peripheral eosinophilia was found to be associated with better prognoses. In the oral cell squamous carcinoma in contrast, eosinophilia was related with a worse prognosis (13,19).

Upon activation, eosinophils are capable of secreting lipid mediators such as leucotriene C4 and platelet activating factor, chemokines like RANTES and eotaxin-1 and a variety of pro-inflammatory cytokines, such as TGF-α, TGF-β, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16 and IL-18 (2). These molecules are involved in a variety of different inflammatory mechanisms, such as the regulation of cellular trafficking, the modulation of mucus secretion, smooth muscle constriction and vascular permeability (2,5).

Recent studies showed that eosinophils also play a role in the course of antigen presentation. They are able to process and present super-antigens, as well as different antigens from viruses, parasites and bacteria. In the course of antigen-presentation they were furthermore identified to mediate immune responses via enhancing Th2-cell responses (20).

1.1.2 Eosinophil specific chemoattractants

As a response to infections or tissue injury, inflammatory cytokines are set free, inducing the production of chemotactic factors and adhesion molecules of the surrounding cells, triggering the recruitment of leukocytes such as eosinophils out of the circulation to sites of inflammatory reactions (10).

Eosinophil chemoattractants, which were discovered first include PAF, LTB_4 and complement C5a, which are all acting on both eosinophil as well as neutrophil granulocytes. C5a and PAF were furthermore found to be involved in the transmigration of eosinophils across epithelial cells. Subsequently, IL-8, eotaxin, eotaxin-2 and eotaxin-3, MCP-3, MCP-4, TNFα, RANTES and PGD_2 were found to induce eosinophil migration. In addition, TNFα and C5a were described to play a special role in eosinophil adhesion, as they are acting together with $\alpha_5\beta_1$-integrin in the activation of the CD11b/CD18 adhesion molecule after eosinophil interaction with epithelial cells (21,22).

As described IL-5 is an important factor in eosinophil development and survival and was found to play an important role in eosinophil migration. After exposure to allergens, IL-5 triggers the mobilization of eosinophils into lung tissue from the bone marrow. However, as indicated by several trials using IL-5 lacking mice and applying anti IL-5 (Mepolizumab) in patients suffering from asthma, eosinophil recruitment can take place as well without the action of IL-5 (23-28).

Other cytokines, which were recognized to act in the activation of eosinophil migration are GM-CSF, IL-3 and IL-13 (21,22).

1.1.2.1 Prostaglandin (PG) D_2

Prostaglandins are autacoids, which derive from arachidonic acid, through subsequent metabolism by prostaglandin synthase and cyclooxygenase enzymes (29).

Prostaglandin D_2 (PGD_2) in particular, as shown in Figure 1, is produced by specific terminal synthases as well as cyclooxygenase 1 and 2 (COX-1 & COX-2), as a result of various stimuli, like oxidative stress or exposure to allergens. Cells involved in its synthesis are mast cells, as well as eosinophils, macrophages, Th2-cells, dendritic cells and endothelial cells (30).

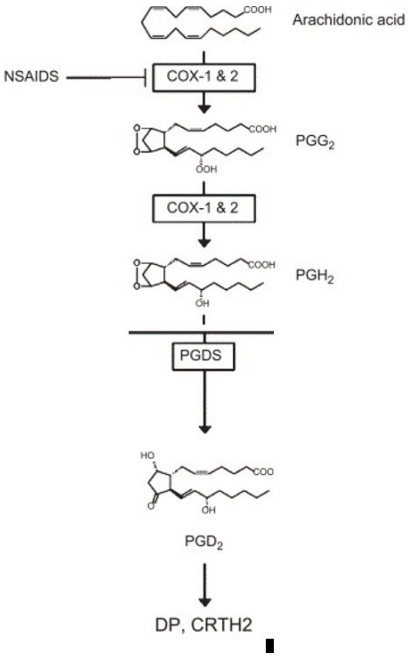

Figure 1. PGD_2 Synthesis – adapted from Hata 2004 (29). Arachidonic acid serves as a precursor for the biosynthesis of prostaglandin D_2. It is metabolized to PGH_2 via cyclooxygenase-1 or -2. Prostaglandin synthase enzymes (PGDS) then lead to the production of PGD_2.

In the initial step, arachidonic acid is set free by phospholipase A_2, which is then oxidized to PGG_2, followed by a reduction step, leading to the production of PGH_2. PGH_2 then acts as a substrate for the biosynthesis of the five main prostaglandins PGD_2, $PGF_{2\alpha}$, PGI_2, PGE_2 and TXA_2, via the prostaglandin synthase enzymes (PGDS) (29). As non-steroidal anti-inflammatory drugs (NSAIDs) are capable of blocking the synthesis of prostaglandins, they were firstly defined to act pro-inflammatory, but further investigation and findings have fuelled the awareness, that they also have anti-inflammatory properties. They were, for instance, found to suppress T-cell proliferation and inhibit macrophage cytokine synthesis (29).

Two seven-transmembrane G protein-coupled receptors are involved in the function of PGD_2: CRTH2 and D-type prostanoid receptor (DP), also known as DP1, which were both shown to bind PGD_2 with almost equal affinity (29-31).

The DP receptor was the first to be discovered, being expressed in several tissues, including intestine, nasal mucosa, lungs, central nervous system, retina and vasculature. Basophils, eosinophils, Th1 and Th2 cells as well as dendritic cells and platelets were shown to express DP (28,32-37).

Depending on the tissue, DP fulfils different functions. In intestinal mucosa, for instance, the DP receptor was identified to be involved in the regulation of mucous secretion. In platelets, DP was found to act as an inhibitor on aggregation (36,38).

Activation of the DP receptor was shown to be linked with elevated levels of intracellular Ca(2+) as well as elevated levels of cyclic AMP, which is related to the blocking of effector cell function in immune cells (32,39-41).

As the nomenclature of the CRTH2-*chemoattractant receptor-homologous molecule expressed on Th2 cells*- receptor suggests, this receptor was firstly found on Th2 cells, but subsequently has been identified on basophils and eosinophils. One of its functions is the regulation of chemotaxis towards PGD_2 (28,30,42).
Interestingly, stimulation of CRTH2 receptor leads to an activation of PLC, PI-3 kinase and MAP kinases in eosinophils, which are pathways responsible for eosinophil shape-change, CD11b up-regulation and actin polymerisation (29,43).

PGD_2 was identified to be increased especially in atopic and inflammatory conditions and was shown to be involved in the physiology of airways, by causing bronchoconstriction, vasodilation, mucous production and increase in capillary permeability (31). Patients suffering from asthma were found to have elevated levels of PGD_2 in the bronchoalveolar lavage (BAL) fluid. Accordingly 9α,11β-PGF_2 – PGD_2's main metabolite – was found to be increased in plasma and urine (31,44).
Hirai et al. underlined the role of CRTH2 and DP in the course of allergic reactions. They proposed that the production of PGD_2 through mast cells is responsible for the vasodilatation mediated by DP, which leads to leukocyte extravasation. Concerning the role of CRTH2, they indicated its involvement in the recruitment of basophils, Th2 cells and eosinophils through PGD_2, together with other chemotactic mediators such as eotaxin (28). Apart from its role in inflammation, PGD_2 also exerts its function on sleep induction, smooth muscle relaxation and contraction and inhibition of platelet aggregation (29,45,46).
Furthermore, it was discovered recently that DP is influencing CRTH2 mediated Ca(2+) mobilization. Therefore, it is suggested that CRTH2 and DP form heteromers, since they are affecting each other's signalling properties. Hence, blocking of CRTH2/DP heteromers could be an option for future therapeutic measurements against allergic diseases (47).

1.1.2.2 Eotaxins

Firstly, eotaxin-1 was discovered investigating the allergen-induced accumulation of eosinophils in lungs of guinea pigs (48,49).
Thereupon, two other chemokines with eosinophil-specific chemoattractant function were identified – being referred to as eotaxin-2 and eotaxin-3, which are only scarcely related to eotaxin-1, as they are located in another chromosomal location and their DNA sequence differs in 70% (24,25,50,51).
They are acting via selective expression of seven-transmembrane G protein-coupled receptor CCR3, which is mainly found on eosinophils (24,52,53).
Interestingly, the eotaxin chemokines are acting together with IL-5 in the initiation of eosinophilia in tissues, as well as IL-5 enhances the amount of eotaxin-responsive cells and triggers eosinophil responding to CCR3 ligands (50).

1.1.3 Eosinophil related diseases

1.1.3.1 Allergy

As eosinophilia is one of the main features of atopic conditions and the eosinophil granulocyte acts as an important effector cell in these syndromes, allergic diseases have been an important example for the study of eosinophil function.

About 20 – 30% of the population have a predisposition to atopy, which is linked to immoderate production of IgE antibodies as a response to antigens from the environment (16).

When exposed to allergens, many atopic patients evolve a clinical response, starting three to four hours after contact, reaching a climax approximately eight hours after contact, and declining after some days. This mechanism – called late-phase response – is linked to an accumulation of inflammatory cells, with eosinophils being the major effector cell (16).

Their role in allergy evolves from their diversity of inflammatory cytokines, responsible for the modulation of various pathways of the immune response (16).

In addition, eosinophils are capable of producing cysteinyl-leukotriene C_4, which together with its metabolites – leukotriene E_4 and leukotriene D_4 –enhances vascular permeability, provokes constriction of smooth muscles and is involved in the induction of mucus secretion. Therefore, these substances were identified to be slow reacting mediators involved in anaphylaxis (5,54,55).

1.1.3.2 Asthma Bronchiale

There are two different types of asthma, the allergic or extrinsic asthma and the non-allergic or intrinsic asthma. Extrinsic asthma, an IgE-mediated type 1 – allergic reaction develops after exposure to allergens such as pollen, chemicals and proteins of animals and plants. A consequence of this is a mast cell and eosinophil dominant inflammatory response, as well as bronchoconstriction. The intrinsic asthma in contrast is supposed to derive from respiratory viral infections, from chemical and physical irritations or exhaustion within adolescents. This mechanism is triggered on basis of congenital or acquired defects of the innervation of the bronchial system, leading to hyperreactivity (56).

Studies, which were performed to investigate the role of eosinophil granulocytes in the course of asthma, showed their dominance in the epithelium of the respiratory system and

they were also found to be the main cell in bronchoalveolar lavage (BAL) fluid of asthmatic patients. Especially increased concentrations of eosinophil granular proteins, like MBP, were discovered in the BAL-fluid, inducing toxic effects to lung epithelia cells and increasing the permeability of the airway epithelium in in vitro studies (13,16).
Apart from its cytotoxic activity, MBP enhances smooth muscle reactivity through inhibition of vagal muscarinic M2 receptors, leading to airway hyperreactivity, one of the main symptoms of asthma (57). Furthermore eotaxins 1-3 have been recognized as central regulators in the asthmatic response. They were found to play an important role in the development of allergic reactions, as they are produced by infiltrative allergy-triggered cells, such as macrophages and eosinophils, and by tissue based cells, like skin fibroblasts and respiratory epithelial cells. Challenging allergens in the human lung, eotaxin-1 was found to correlate with the early recruitment (6 hours) of eosinophils, eotaxin-2 in contrast correlates with eosinophilia after about 24 hours (5,50).
As described, eosinophil granular proteins, mediators and cytokines are all together influencing eosinophil cell function in asthma.

1.1.3.3 Inflammatory bowel disease

Eosinophils are the predominant cell - based in the lamina propria - of all segments of the gastrointestinal tract, except for the esophagus (58).
Remarkably, accumulation of eosinophil granulocytes in the gastrointestinal tract is a characteristic of several diseases, such as inflammatory bowel disease, eosinophilic gastroenteritis, allergic colitis, gastroesophageal reflux disease, helminthic infections and drug reactions (14).

In the 1950s eosinophils were first described to play a role in inflammatory bowel disease (IBD). In the 1960s Bercovitz and Sommers suggested their function being pathogenic, as they analysed biopsy specimens of patients with active ulcerative colitis, and found that elevated levels of eosinophils are related to necrosis (57).
In-depth knowledge has been gained, through immunohistochemical and microscopical analyses of biopsy specimens from patients suffering from ulcerative colitis, as well as Crohn's disease, demonstrating activation of eosinophils and increased levels of eosinophil granular proteins (57).

In addition, the presence of eosinophil granular proteins EPO, ECP, MBP and EDN in patients with ulcerative colitis was shown to correlate with disease severity, gastrointestinal dysfunction and changes of morphology (57).

Apart from IBD, gastrointestinal disorders named EGID (Eosinophilic Gastrointestinal disorders) were recognized, being defined as disorders with an increase of eosinophil count, but without any known reasons for the eosinophilia (e.g. helminthic infection, malignancies, drug abuse). EGIDs comprise diseases such as eosinophilic colitis, eosinophilic esophagitis and eosinophilic gastroenteritis. Symptoms of patients with EGIDs include abdominal pain, gastric dysmotility, irritability, diarrhoea, dysphagia and vomiting (14,59).
Even though the exact etiological cause is still unknown, it is suggested that an interplay of genetic and environmental factors play a role in the pathogenesis, as 75% of patients with EGIDs have been identified as atopics (14,60).
The pathogenic role of eosinophils in EGIDs is supposed to derive from their pro-inflammatory properties, as explained above.

1.2 Eosinophilic cell culture

1.2.1 Bone marrow derived Eosinophils (bmEos)

In 2008, Dyer et al. established a method for the isolation and ex-vivo differentiation of functional competent eosinophils from wild-type BALB/c mouse bone marrow. The bone marrow progenitor cells were extracted from tibiae and femurs and maintained in culture for 4 days in medium containing recombinant mouse stem cell factor (rmSCF) and recombinant mouse – FLT3-L (rmFLT3-L). Thereupon the cells were kept in culture with recombinant mouse IL-5, acting as the only cytokine (61). FLT3-L was identified to support the growth of progenitor cells. SCF likewise was described to play a role in forming eosinophils. (61-63).

The bmEos were demonstrated to be functionally competent, as they are undergoing chemotaxis, when stimulated with mouse-eotaxin. Additionally they yield cytokines in a way their natural equivalent does, as they produce MIP-1α, IL-4, IL-6, IL-9, IP-10 and IFN-γ. Furthermore, they express Siglec-F, CD11b, IL-5R α-chain and immunoreactive major basic protein. CCR3, the transcription factor GATA-1, mouse eosinophil peroxidase and the IL-3/IL-5/GM-CSF β-receptor chain were detected within mature bmEos (61).

From a morphological point of view, bmEos appeared to be similar to eosinophils from the peripheral blood of mice, with cytoplasmic granules and polymorphic nuclei, as they can be found in mature eosinophils (61). As described by Dyer et al., this procedure yields in bmEos, which were found to be fully differentiated with a purity of more than 90% by day 10, judged by their morphology, function and molecular expression. Concerning the cell count, a 10-fold increase was detected by day 17, compared to the start of the culture (61).

In 2009 Dyer et al. showed that this culturing method is not only limited to BALB/c mice but also applicable to wild-type C57BL/6 mice as well as to TLR-deleted mice (64).

Apparently, the generated bmEos are also capable of undergoing chemotaxis in vitro and in vivo, as showed by Sturm et al. in 2013. In this model, ex vivo generated bmEos were injected intravenously into eosinophil-deficient ΔdblGATA or Wild-type mice to study the migration into the lung, after chemotactic stimulation (65).

In summary, this method for the generation of bmEos offers the possibility of studying eosinophil function, hence eosinophil diseases, such as asthma, in many different ways.

1.2.2 Human eosinophilic leukemia cell line

The human eosinophilic leukemia cell line was first described by Saito et al in 1985, and derives from a patient who suffered from Philadelphia-chromosome negative eosinophil leukemia. The cells isolated from this patient, show myeloblast-like characteristics, containing already mature and immature eosinophils (66).
As several different culture conditions lead to their differentiation into mature eosinophils, they were recognized to be committed to the eosinophil lineage (66).
The cells were kept in culture with RPMI 1640 containing 10% FCS. After one year of cultivation, six different clones of cells were observed. Three of them have eosinophilic characteristics, named EoL-1, EoL-2 and EoL-3. The other three were called Eo-B, as they had B-cell features. The EoL lines differ in their growth patterns: EoL-1 grows in single cell suspension as well as in small clusters and needs 48 hours for duplication, EoL-2 and EoL-3, in contrast, have a doubling time of 72 hours and grow in clusters (66).
The EoL-1 cell line was noted to have a similar morphological aspect as the original leukemic cells, with 2% of the cells having already eosinophil-phenotype as they are containing cytoplasmic granules (66).

1.2.3 HL – 60 cell line

The HL-60 human promyelocytic cell line, first described by Collins et al in 1987, originates from a patient who suffered from acute myeloid leukemia. The leukocytes, isolated from the peripheral blood of this patient, show explicit myeloid features and growth factor independent proliferation with a doubling time between 20 and 45 hours. Morphological characteristics of HL-60 cell lines include blast-like cells, containing rounded large nuclei, azurophilic granules and a basophilic cytoplasm (67,68).
Fischkoff et al. demonstrated that a modification of the pH-value of the growth medium induces diverse differentiation paths: Neutrophil cells result at a pH of 7.2, eosinophil cells in contrast, at a pH of 7.6, when treated with 0.5mM butyric acid (67). As a consequence of this knowledge, Fischkoff et al. established the eosinophil subline HL-60 clone 15, through the permanent cultivation of Hl-60 cells at a pH of 7.6 (69). Several trials were performed, varying the concentration of butyric acid, but 0.5mM appeared to be the optimal concentration, as higher levels led to a rapid decline in cell count (70).

Apart from butyric acid, other substances can be used for the induction of differentiation, leading to different cell types. Vitamin D and phorbol esters trigger the differentiation of macrophages. Retinoic acid, polar-planar compounds like dimethyl sulphoxide (DMSO) and purin/pyrimidine analogues, in contrast, induce neutrophil differentiation (67,70).

1.2.3.1 Mechanisms of differentiation

There are different methods which were reported to trigger the differentiation of EoL-1 cells into mature eosinophils. Saito et al. first described the use of N-butyrate for the induction of differentiation in 1985 (71).

In 2006, Ishihara et al. tried to elucidate the mechanisms of eosinophil differentiation by studying EoL-1 cells and Hl-60 clone 15 cells, an eosinophil subline of Hl-60 cells (69,71). They suggested that the continuous inhibition of histone-deacytelases (HDAC) triggers the differentiation of EoL-1 cells. Therefore, they compared the effects of N-butyrate, Apicidin and Trichostatin A (TSA), three inhibitors of histone acetylases, on the induction of differentiation (71). The assays were performed with N-butyrate at a concentration of 0.5mM, 100nM of Apicidin and TSA at 30nM. The inhibition of the HDAC was measured via the expression of acetylated Histone 4 (H4) and Histone 3 (H3) by western blotting. The differentiation into mature eosinophils was determined via the expression of the eosinophil markers CCR1, CCR3 and integrin β7. As a result, Apicidin and N-butyrate led to a continuous acetylation of H3 and H4 and to differentiation into mature eosinophils. TSA in contrast, did not induce any differentiation and evoked only a transient acetylation of H3 and H4. Consequently, TSA was identified to have a half-life of 14.7 hours in cell culture. Because of that, Ishihara et al. increased the treatment of Hl-60 clone 15 cells with TSA, up to three times in 12 hours, ultimately leading to differentiation into eosinophil cells (71-73). In summary, Ishihara et al. demonstrated that Apicidin and N-butyrate induce eosinophil differentiation, presumably through their effects of continuous acetylation on H3 and H4. TSA does not trigger any differentiation, as it only leads to a transient acetylation (71).

Another factor involved in the proliferation and differentiation of EoL-1 cells was found to be the gene product of the FIP1L1-PDGFRA fusion-kinase, referred to as FIP1L1-PDGFRAα. Therefore Kaneko et al. tried to investigate the effects of N-butyrate, TSA and Apicidin on FIP1L1-PDGFRAα (72,74).

As a result of various experiments, including western-blotting, the treatment of EoL-1 cells with Apicidin and N-butyrate, led to a decrease of FIP1L1-PDGFRAα, suggesting that the

effect of the HDAC inhibitors on the FIP1L1-PDGFRA fusion –kinase is associated with EoL-1 differentiation (72).

1.2.4 Human bone marrow Eosinophils

Another novel method to obtain mature eosinophils, in this case from human bone marrow, was described by Wong et al. in 2013. This model does not focus on the differentiation of eosinophils from progenitor cells, but rather on the purification of eosinophils from human bone marrow in an eight-day culture system (75).

Wong et al. first tried to purify the bone marrow cells, using the anti-CD16 negative selection method, a reliable technique to define the eosinophil fraction in peripheral blood. This procedure normally results in an eosinophil $CD16^-$ fraction with a purity of 95% (75).

As CD16 is expressed in the final steps of the differentiation of neutrophils, this protocol was not suitable for the use of bone marrow cells and led to an incomplete separation of neutrophils and eosinophils (75). Then the group established a protocol for the cultivation and purification of bone marrow cells, containing different factors supporting the differentiation and growth of eosinophils, but lacking factors necessary for the survival of neutrophils (75). The medium used for the cultivation consisted of RPMI 1640, FCS at a concentration of 20%, l-glutamine and antibiotic supplements. Apart from this, 1ng/ml human recombinant IL-5 was added. The first four days the cells were incubated at 37°C without any disturbance. The separation of dead cells and cellular debris from viable cells was then performed between day 4 and 8 via the exchange of culture flasks, applying conventional centrifugation and Percoll-density gradient centrifugation. The cell-count at the beginning of the cultivation was between 46 to 95 million granulocytes, with an eosinophil fraction of 3-13%. At the end of cultivation, at day 8, the purity of the eosinophil fraction reached almost 100% with a cell count of $2.26x\ 10^6$ viable cells (75).

Although this procedure results in fully differentiated eosinophil granulocytes, the application is somehow limited, as it depends on donors of human bone marrow. Overall, however, it could provide a useful tool in laboratories with easy access to human bone marrow (75).

2 Aim of the study

As eosinophil granulocytes comprise just a small fraction of circulating blood leukocytes, isolation of human peripheral eosinophils is only possible to a certain extent. Though methods do exist and are frequently applied, the amount of eosinophils yielded in these procedures is very rare and also donor-dependent. Furthermore, the possibilities of long-term culturing of human eosinophils are limited through their short viability and the difficulties of acquiring large quantities, which would be necessary for the in-depth study of eosinophil function. Therefore, other models, providing large numbers of eosinophils, as well as offering the possibility of longer and steady cultivation, are needed.

In this work, we studied two distinct types of eosinophilic cells. On the one hand, primary mouse bone marrow-derived eosinophils (bmEos) and on the other hand EoL-1 cells, an eosinophilic cell line, originating from a patient with Philadelphia-chromosome negative eosinophil leukemia.
The main aim of this work was to establish and optimize theses cell culture systems, yielding in large amounts of fully differentiated and functional competent cells. In bmEos, we also investigated their responsiveness to the chemoattractants eotaxin and PGD_2 by means of CD11b upregulation and chemotaxis. Regarding EoL-1 cells, our objective was to investigate the surface expression of the PGD_2 receptors CRTH2 and DP, as well as their functional responses to PGD_2 by means of chemotaxis and calcium flux.

3 Material

3.1 *Chemicals*

- Ethanol (Lactan)
- Pluronic F127 (Sigma Aldrich)
- Fluo 3 AM (Invitrogen)
- Ammoniumchloride (Sigma Aldrich)
- Isoflurane (Abbott)

3.2 *Reagents*

- Recombinant mSCF (Peprotech)
- Recombinant mIL-5 (R&D Systems)
- Recombinant mFLT3-L (Peprotech)
- PGD_2 (Cayman Chemicals)
- Eotaxin (Peprotech or R&D Systems)
- Ultra V Block (Thermo Scientific)
- Probenecid (Sigma Aldrich)
- Calcium Ionophore A23187 (Sigma Aldrich)
- Hemacolor® – Staining kit (Merck)

3.3 *Cell culture material*

- Penn-Strep (PAA)
- Sodium Pyruvate (PAA)
- NEAA (PAA)

Serums for differentiation of bmEos:

- FBS – Gold (PAA)
- Sigma (Sigma Aldrich)
- Thermo Scientific HyClone Standard FBS (Fisher Scientific)
- HyClone Research grade (Fisher Scientific)

Base Medium – bmEos:

- 20% FCS
- 25mM Hepes (PAA)
- 1% Penicillin/Streptomycin (PAA)
- 1% Glutamine (PAA)
- 1% NEAA (PAA)
- 1mM Sodium pyruvate (PAA)
- 50µM β-Mercaptoethanol (Sigma)
- RPMI 1640 (PAA)

3.4 *Antibodies*

- Mouse anti-human CD16 – PE CY5 (Becton Dickinson)
- Rat anti-mouse CD11b (Becton Dickinson)
- Anti-human CD11b – PE (Biolegend)
- Anti CD11b – monoclonal – produced in mouse – FITC (Sigma)
- Rat anti-mouse CD16/CD32 (Becton Dickinson)
- Rat anti-mouse Siglec-F – PE (Becton Dickinson)
- Rat IgG2a,K Isotype Control – PE (Becton Dickinson)
- Rat anti-human CD294 – Alexa Fluor 647 (Becton Dickinson)
- Rat anti-mouse CD11b – Alexa Fluor 647 (Becton Dickinson)
- Rat IgG2 - Isotype control (R&D)
- Goat anti-rabbit IgG – Alexa Fluor 647 (Invitrogen)
- Antibody to CD294 – polyclonal (Acris)
- HRP Goat anti-rabbit (Jackson ImmunoResearch)
- Rabbit anti-goat IgG – Alexa Fluor 647 (Invitrogen)
- Goat anti-DP1 – polyclonal (Santa Cruz Biotechnology)

3.5 *Buffer and media solutions*

- PBS with Ca2+/Mg2+ (PAA or Gibco)
- PBS without Ca2+/Mg2+ (PAA or Gibco)
- RPMI 1640 (PAA)
- HEPES (PAA)

+ Buffer – assay buffer (pH 7.4)

- PBS with Ca2+/Mg2+ (PAA or Gibco)
- 10mM Glucose- Monohydrate (Lactan)
- 0.1% Bovine serum albumin (Sigma Aldrich)
- 10mM HEPES (PAA)

- Buffer – washing buffer (pH 7.4)

- PBS without Ca2+/Mg2+ (PAA or Gibco)
- 10mM Glucose- Monohydrate (Lactan)
- 0.1% Bovine serum albumin (Sigma Aldrich)
- 10mM HEPES (PAA or Gibco)

Fixative Solution

- 75% FacsFlow (Becton Dickinson)
- 22.5% A.dest. (Fresenius)
- 2.5% Cellfix (Becton Dickinson)

Western Blot:

Extractionbuffer

- 0.61g/100ml 50mM TRIS (Roth)
- 0.37g/100ml 10mM EDTA (Sigma)
- 1ml/100ml Triton X (Fisher Scientific)
- Proteaseinhibitor 1:20 (Roche)
- PhosSTOP – Phospataseinhibitor (Roche)

Sample buffer

- 17.5% 0,5M TRIS/HCL (Roth)
- 50% SDS 10% (Roth)
- 15% Glycerol (Sigma)
- 12.5% Bromphenolblue (Sigma)
- Before use add Mercapthoethanol (5%)

Separating buffer

- 1.5M TRIS/HCL pH=8,8
- Aqua dest.

Stacking buffer

- 0.5M TRIS/HCL pH=6,8
- Aqua dest.

Separating gel

- 2.95ml ddH_2O
- 2.17ml Separating buffer
- 3.48ml Acrylamide (Sigma)
- 86µl 10% SDS (Roth)
- 4.36µl TEMED (Sigma)
- 76µl 10%APS (Sigma)

Stacking gel

- 1.45ml Acrylamide/Bisacrylamide (Sigma)
- 2.17ml Stacking buffer (Roth)
- 4.95ml ddH_2O
- 86µl 10%SDS (Roth)
- 8.72µl TEMED (Sigma)
- 76µl 10%APS (Sigma)

10% SDS

- 10g SDS / 100ml ddH_2O

10% APS

- 1g APS / 10ml ddH_2O

3.6 *Equipment*

FACSCalibur	BD Biosciences
NucleoCounter®NC-100	ChemoMetec
Micro Boyden Chemotaxis chamber	NeuroProbe
Cytospin Centrifuge	Shandon
Nunclon-cell culture flasks	Sigma Aldrich
Holten Horizontal Cabinet	Thermo Fisher Scientific

3.7 *Cells*

Female BALB/c bone marrow derived eosinophils

EoL-1 cell line – human acute myeloid (eosinophilic) leukemia

4 Methods

4.1 Cell culture methods

4.1.1 Cell isolation from mouse bone marrow

Six- to eight-week-old female BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Experimental procedures were approved by the Austrian Federal Ministry of Science and Research (protocol number: BMWF-66.010/0143-II/3b/2012) and performed in accordance with international guidelines.

The following protocol, elaborated by Dyer et al. 2008 (61), was used for the differentiation of eosinophils from unselected adult mouse bone marrow. Prior to collecting bone marrow from the mice, base medium was prepared, containing 20% FCS, 25mM Hepes, 1% Pen-Strep, 1% Glutamine, 1% NEAA, 1mM Sodium pyruvate, 50µM β-mercaptoethanol and RPMI 1640. To achieve optimal growth and differentiation conditions for the cells, sera from different companies as well as different concentrations of the serum and of IL-5 were tested. Female BALB/c mice were euthanized by isoflurane inhalation and cervical dislocation. They were bathed in ethanol, their hind legs were removed and put in RPMI-1640. The flesh was removed from the bones and the tips of femur and tibia were cut off with a scalpel. The bone marrow was flushed with 3mls of RPMI 1640/bone, using 25 gauge needles and then collected in 15ml tubes. The bone marrow of each mouse was put in a particular tube.

After all bone marrow was collected, the tubes were centrifuged. To lyse red blood cells, samples were resuspended in 9mls of dH_20 and 1ml of 10xPBS was added for neutralization. After the cells were centrifuged again, the cells were counted with the cell counter NucleoCounter®NC-100. Depending on the isolation quality and the age of the mouse, between 12 and 18 million cells could be collected from one animal. Cells were transferred into cell culture flasks and cultured at 10^6 cells/ml media. For cultivation, Nunclon-cell culture flasks Thermo Scientific were used. Recombinant mSCF and mFLT3-L, both at a concentration of 100ng/ml, were added to the base medium for the first four days to support granulocyte progenitor survival.

4.1.2 BmEos cell differentiation

On day 2, half of the mSCF/FLT3-L medium was changed and after determining the cell count, the amount of medium was adjusted. On day 4, the mSCF/FLT3-L medium was removed and replaced with base medium containing recombinant mIL-5 at 10ng/ml. Half of the medium was replaced every second day with fresh IL-5 medium, and the amount of medium was adjusted depending on the cell count. On day 8, the cells were transferred into new culture flasks. Eosinophil cell's morphology, granularity and differentiation state were assessed by visual inspection of stained cells. Therefore, bmEos were cytospun onto slides for 5 minutes at 300rpm using a Shandon Cytospin centrifuge. The slides then were fixated and stained using the Hemacolor® stain kit, a modified staining method according to May-Gruenwald-Giemsa, originally developed by Romanovsky (76). The percentage of fully differentiated eosinophils was determined by counting a total of 500 cells/slide.

4.1.3 EoL-1 culture

The EoL-1 cell line, consisting of human acute myeloid (eosinophilic) leukemia cells, was purchased by DSMZ. RPMI 1640 with glutamine, containing 1%PS and 10%FBS was used as cultivating medium. The cells were maintained at about 0.5 million to 1 million cells per millilitre. Cell numbers were determined using the cell counter NucleoCounter®NC-100 and depending on the density of the EoL-1 culture, the cells were split every third day, refilling the discarded amount of medium with fresh medium. To induce differentiation 5 x 10^5 cells were cultured in 0.5 mM Sodium Butyrate containing basal medium for up to 12 days. Varying FBS concentrations were tested in different experiments, but stayed within 10 and 20%. Cell numbers were adjusted every three days. Cell morphology, granularity and differentiation state were assessed by visual inspection of stained cells as explained above. The following flow cytometric analyses were conducted between day 6 and day 9 of differentiation.

4.2 Flow cytometry

Flow cytometry is an instrument, based on laser technology, used to measure the constitution of biological particles, comprising physical, biochemical and morphological characteristics. As it permits the analysis of thousands of cells in a few seconds, it is an important tool in research and clinic (77).

While the cells pass the flow cytometer one by one, the laser beams measure the scattered lights and fluorescence emissions at different angles (78).

A flow cytometer is composed of three interrelated components, which are common to all cytometers. Initially there is the fluidic system, allowing the transport of the cells from the sample through the cytometer by hydrodynamic focusing (78). As illustrated in Figure 2, this process arranges the particles like a string of pearls, as the pressure of the sheath fluid acts on the suspended particles. Sheath fluid is a liquid, most likely a diluent, which is directed into the flow chamber via air pressure to keep the particles moving (77).

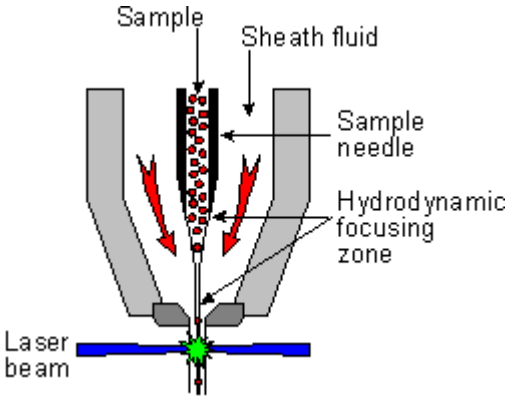

Figure 2. Fluidic System (101).
As the sheath fluid acts onto the injected sample cells, they are aligned like a string of pearls. Laminar flow and surface tension cause a slow moving sample stream within the centre of a faster moving sheath stream. Through modification of the velocity of the two streams, the cells are aligned within the centre stream, before passing the laser beam. (Hydrodynamic focusing zone)

The cell suspension is being pressurized after entry into the flow cytometer and inserted into a sheath core prior to passing a nozzle. The nozzle aperture should be narrow and perfectly calibrated for obtaining laminar fluxes and to centralize the particles into the middle of the stream (78).

Most of the flow cytometers use this hydrodynamic focusing to transport the cells, but other machineries are available as well, among which micro capillary technology or acoustic focusing, permitting the alignment of the cells via sound waves (78).

After the fluidic system, the cells pass the optical system with the flow chamber. The flow chamber is known as the heart of the flow cytometer, as it is the place where the interaction between the cells and the laser beam lead to scattered lights (78).

After the particle is focused hydrodynamically, it is struck by the laser beam, which emits scattered light in all directions. There are different types of parameters, which can be detected from the scattered light. The two main properties are light scattering properties, called forward angle light scatter (FSC) and side angle light scatter (SSC). The FSC originates in diffracted light, as it strikes the cell directly or at small angles, and gives information about basic morphological features of the cell, like the cell size. The SSC in comparison strikes the cell at 90°, which gives more information about the surface and the insight of the cell, such as irregularities of the membrane and granularity. Figure 3 shows a schematic representation of this interaction. Via the correlation of FSC and SSC a discrimination between living and dead cells can be made, as well as cellular debris can be identified (77).

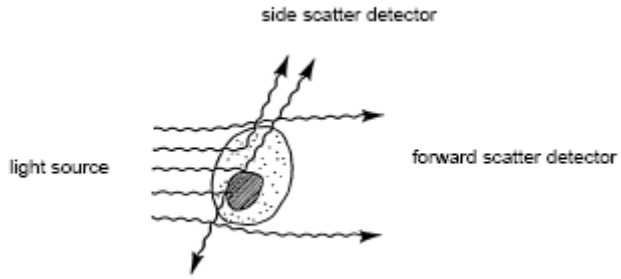

Figure 3. Forward Scatter - Side Scatter (101). As the cell is struck by the laser beam, light is scattered in all directions. The forward scatter detector acquires data from the light, scattered at smaller angles or directly –FSC. The side scatter detector acquires data from light, scattered at larger angles –SSC.

In addition to the scattered lights, fluorescent lights can be detected, which are emitted, as the laser beam strikes fluorochrome-labelled cells. Adding different fluorochromes to the cells, offers the possibility to study more characteristics of the analysed probe, than using FSC and SSC. Apart from surface properties, internal molecules can be studied and analysis of DNA, RNA, chromosomes, hormones and cytokines becomes possible. Measurements of DNA-cell-cycle, DNA synthesis, membrane potentials and calcium flux are just some examples for the wide range of the investigative potential, offered by the use of fluorochromes in flow cytometry (77,78).

The third part of the flow cytometer is the electronic system. As the laser beam strikes the cells, photons are emitted. This light signal is digitalized for data acquisition and analysis with the computer (78).

4.2.1 CD11b receptor staining

4.2.1.1 Whole blood

The staining of CD11b was performed as described by Konya et al. 2010 (79) and Luschnig-Schratl et al. 2011 (80). Blood was taken from healthy donors and citrated to avoid coagulation. To identify neutrophil granulocytes, Anti CD16-PECY5 was added at a concentration of 1:50. Anti-CD11b-PE was added at a concentration of 1:25. Afterwards the whole blood was incubated for 10 minutes at room temperature in the dark. As stimulating agonists, serial dilutions of eotaxin and PGD_2, 100µl each, were prepared and mixed with 100µl whole blood. The highest concentration of eotaxin was 60nM and the dilution series of PGD_2 started with 100nM. As a negative control, one sample of whole blood was mixed with assay buffer. After 20 minutes of incubation in the water bath at 37°C, the samples were transferred on ice to terminate the reaction. 250µl fixative solution was pipetted into each sample and kept on ice for 10 minutes. To lyse the erythrocytes, 3ml of 1x ammoniumchloride-lysing solution was added. After centrifugation, the cells were washed once with wash buffer and fixed again with 300µl fixative solution.
After the sample preparation, the cell samples were analyzed immediately by the FACSCalibur flow cytometer.

4.2.1.2 Bone marrow Eosinophils (bmEos)

To perform the CD11b staining on the bmEos, IL-5 media was removed and cells were resuspended in base medium for two hours to adapt to IL-5-free conditions. Unspecific binding sites were blocked with anti-mouse CD16/CD32 (1:100) and anti-mouse CD11b-PE was added at a concentration of 1:25. Apart from this step, the procedure was identical to the one explained above.

4.2.2 Siglec–F staining with bmEos

The Siglec-F staining was performed as described by Sturm et al. 2013 (65) and Dyer et al. 2008 (61). IL-5 media was removed and cells were resuspended in assay buffer. To obtain the Siglec-F receptor expression on bmEos, a rat anti-mouse Siglec-F-PE antibody and the respective isotype control were used at a concentration of 1:200. Simultaneously, unspecific binding sites were blocked with anti-mouse CD16/CD32 (1:100). After 30 minutes of incubation, the samples were analyzed on a FACSCalibur flow cytometer. Siglec-F expression was assessed as an increase in the FL-2 channel.

4.2.3 CRTH2 and DP1 receptor staining on EoL-1 cells

4.2.3.1 CRTH2 staining

The EoL-1 cell suspension was centrifuged and washed with PBS-. To block unspecific binding sites all samples were resuspended in 100µl of ultra-V block and incubated at 4°C for 30 minutes. Then the cells were washed again and the antibodies were added. The Alexa Fluor 647 rat anti-human CRTH2 antibody and the respective isotype control (rat IgG2) were diluted in antibody diluent (Dako) at a concentration of 10 µg/ml. After incubating for 30 minutes at 4°C the cells were washed twice with PBS, and 150µl fixative solution was added.

4.2.3.2 DP1 staining

After the EoL-1 cell suspension was centrifuged and washed with PBS-, the cells were blocked with ultra V-block at 4°C for 30 minutes. After washing cells were loaded with the goat polyclonal anti-human DP antibody or the respective isotype control at a concentration of 20 µg/ml and incubated for 30 minutes at 4°C. After the cells were washed, the secondary antibody – rabbit anti-goat IgG Alexa Fluor 647 was added 1:2000 and the cells were labeled for 45 minutes at 4°C. After two washing steps with PBS-, the cells were fixed with 150µl fixative solution, and the measurements were conducted on a FACSCalibur flow cytometer in the FL-4 channel.

4.2.4 Calcium ion flux with EoL-1 cells

The intracellular free calcium levels of EoL-1 cells were investigated, as described by Heinemann et al. 2003 (81) and Schuligoi et al. 2008 (82). The EoL-1 cell suspension was centrifuged, washed and resuspended with PBS without Ca^{2+}/Mg^{2+}. The calcium indicator Fluo3 AM was added at a concentration of 4 µM in the presence of 0.04% pluronic F127. To avoid the effusion of the fluorophor, Probenecid was added at a concentration of 2.5 mM. After an incubation period of one hour in the dark at room temperature, the cells were washed again and resuspended in PBS with Ca^{2+}/Mg^{2+}. Changes in intracellular Ca(2+) levels were determined upon stimulating the cells with PGD_2 or vehicle. Maximal Ca(2+) responses were detected by stimulation with the ionophor A23187 at a concentration of 300 nM. Samples were analysed on a FACSCalibur flow cytometer, tracing the fluorescent baseline for one minute before adding the agonists and continuing with the measurement for another two minutes. The calcium levels were detected as changes of fluorescence in the Fl-1 channel.

4.2.5 Chemotactic migration of EoL-1 cells and bmEos

BmEos. Mature bmEos were washed and resuspended at 1x 10^6 cells/ml in assay media (RPMI 1640/1% FCS/10 mM Hepes). Hundred µl of cell suspensions were placed into the upper wells, and 100 µl agonist or vehicle controls were placed into the bottom wells of an equilibrated 96-well chemotaxis plate (Corning Life Sciences, Radnor, PA).

EoL-1. The chemotactic migration assay was performed as described by Schratl et al. 2007 (83) and Schuligoi et al. 2008 (82). Cell suspensions were centrifuged and resuspended in respective media. 2x 10^5 EoL-1 cells were applied to the top wells of a MicroBoyden chemotaxis chamber. Agonist or vehicle containing medium was added to the bottom wells with a 5µm -pore size polycarbonate filter in between.

Chemotaxis of bmEos and EoL-1 cells was stimulated for 3h at 37°C in a humidified CO_2 incubator. After the incubation period, the cells of the bottom wells were collected, centrifuged and fixated with 150µl fixative solution. Migrated bmEos and EoL-1 cells were enumerated for 30 s at high flow rate by flow cytometric counting on a FACSCalibur.

Chemotaxis of differentiated versus undifferentiated EoL-1 cells was induced by 100 nM of PGD_2. Chemotaxis of bmEos was investigated in response to eotaxin and PGD_2 at concentrations of 10 – 1000 nM.

4.3 Western blot

Western Blot, also referred to as Immunoblot, is a method for the detection of proteins. It derived from Southern Blot, used for the detection of DNA, and Northern Blot, used for the detection of RNA. The procedure was introduced by Towbin et.al. in 1979, enabling the electrophoretical separation of proteins in polyacrylamide urea-gels. Burnette then adapted this method in 1981 starting to use sodium-dodecyl sulfate-polyacrylamide gels (SDS-PAGE), which are used up till today (84,85).

SDS is a strong anionic detergent. Thus, it charges the proteins negatively and denatures them. This procedure allows the separation of the proteins by molecular weight. Further denaturing and unfolding of the native constitution of the protein, results from treatment with reducing agents and boiling. The gel is loaded with the samples and a molecular weight marker, to determine the molecular weight of the protein of interest (85).

Depending of the concentration of acrylamide in the gel, the velocity of the proteins running in electrophoresis changes. After the electrophoretic separation of the proteins, they are transferred from the gel onto a membrane, most commonly a nitrocellulose membrane. Membranes produced of activated paper, activated nylon or polyvinylidene difluorid can be used (84,85).

The transfer can be conducted in different ways with electroblotting being the most common. It uses electric currents to drag the proteins onto the membrane. As binding to non-specific binding sides can lead to false positive results, the membrane is put into a dilute solution of dry milk and bovine serum albumin, which blocks these binding sites and leads to a clear result. Subsequently the membrane is incubated with the primary antibody, which binds to the target protein. Afterwards, the membrane is incubated with the secondary antibody, which is normally attached to horseradish peroxidase. It is utilised to cleave chemiluminescent agents, allowing the visualisation of proteins. Depending of the quantity of protein, this reaction produces illuminiscence, which can be detected with a photographic film in the dark chamber (85).

4.3.1 Western blot of CRTH2 receptor with bmEos

The method used to prove the presence of CRTH2 on bmEos was the SDS-PAGE method, based on the model of Towbin et al. 1979 (86), as described above. The procedure was conducted with 50 million bmEos, which were lysed prior to the SDS-PAGE. Polyacrylamide was used at a concentration of 12% in the gel. Rabbit anti-mouse CRTH2 primary antibodies (1:1000, Acris) and goat anti-rabbit secondary antibodies (1:7500, Jackson Immuno Research) were applied for protein detection.

5 Results

5.1 Bone marrow derived Eosinophils

5.1.1 Mouse bone marrow

A

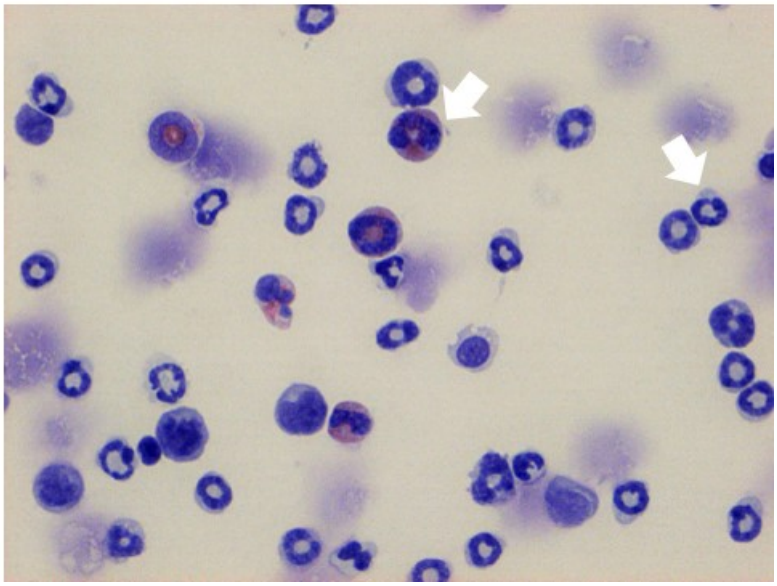

Figure 4. Mouse bone marrow. Freshly isolated bone marrow cells were stained with Hemacolor®**.** The left arrow in the figure shows one of the few eosinophil granulocytes already present within bone marrow progenitor cells. The right arrow shows a neutrophil granulocyte. Cells were evaluated using light microscopy.

Figure 4 shows a smear of freshly isolated mouse bone marrow, already containing some eosinophil granulocytes, identifiable through their red died granules in the cytoplasm, as well as some neutrophil granulocytes. The cells have been cytospun onto the slides immediately after isolation and then stained with Hemacolor®.

5.1.2 Morphological differentiation of bmEos

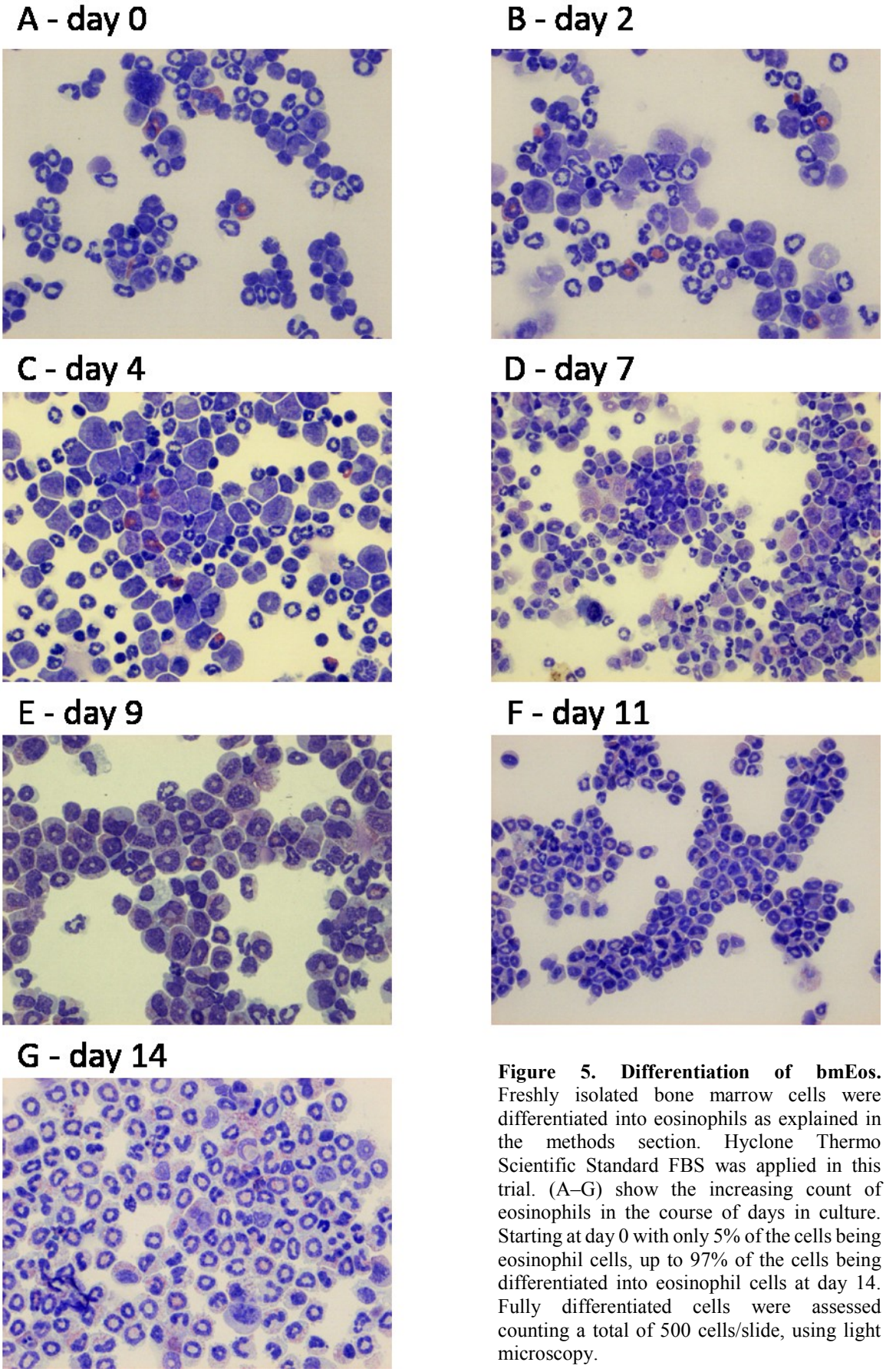

Figure 5. Differentiation of bmEos. Freshly isolated bone marrow cells were differentiated into eosinophils as explained in the methods section. Hyclone Thermo Scientific Standard FBS was applied in this trial. (A–G) show the increasing count of eosinophils in the course of days in culture. Starting at day 0 with only 5% of the cells being eosinophil cells, up to 97% of the cells being differentiated into eosinophil cells at day 14. Fully differentiated cells were assessed counting a total of 500 cells/slide, using light microscopy.

Figure 5 above shows various stages of differentiation of bmEos. The serum, used in this trial was HyClone Thermo Scientific Standard FBS.

The eosinophil cells were identified by counting the cells containing granules in their cytoplasm, which appear red, using Hemacolor® staining. As seen above, the cells containing red granules increase day by day (A –G), with almost all cells having differentiated into eosinophil cells at day 14 (G)

Judging the cells morphologically, hardly any apoptotic cells can be identified and the cells do not show any sign of deterioration.

5.1.3 Comparison of serums from different companies

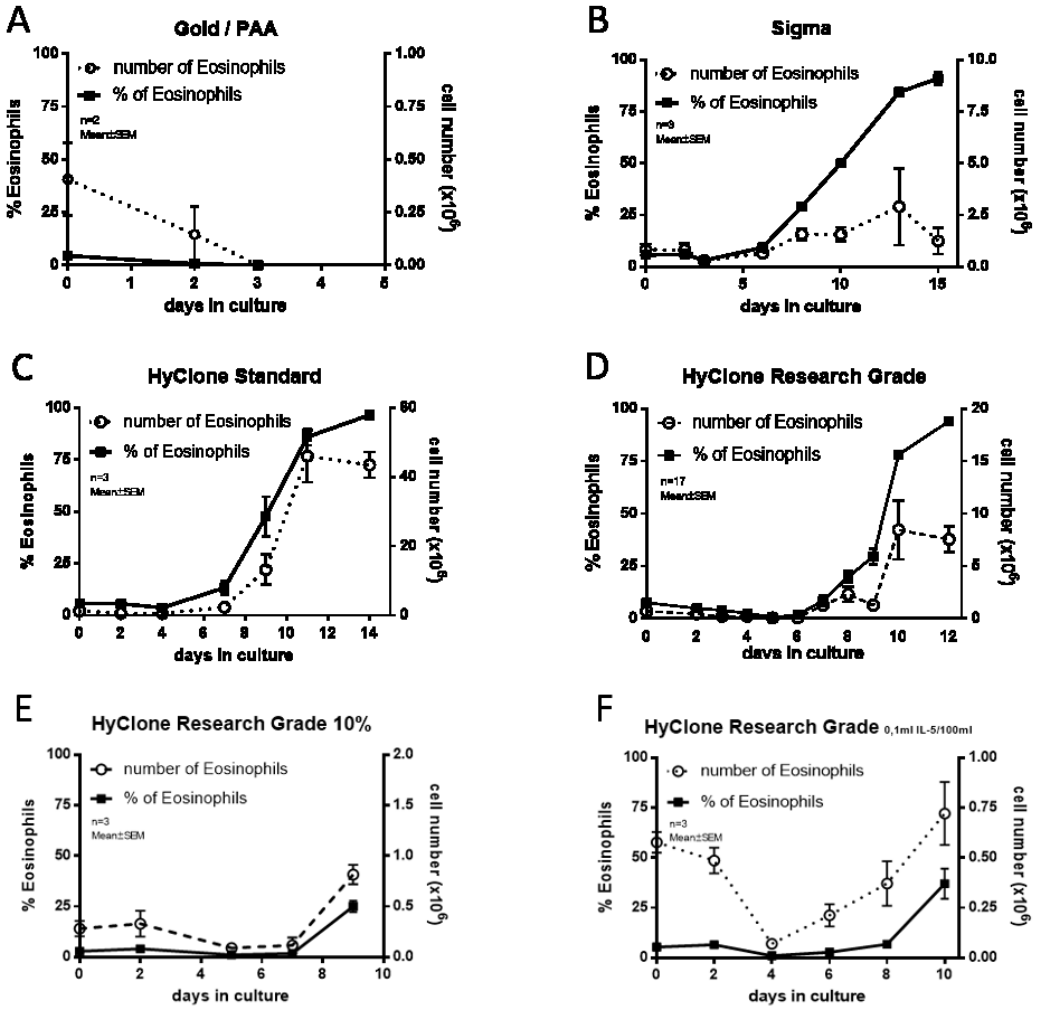

Figure 6. Comparison of different serums (FCS). Freshly isolated bone marrow eosinophils were maintained in culture, using a medium containing 20% FCS, except for one trial, where only 10% of FCS was applied. (A – F) show six different culture conditions and their effects on the viability and differentiation of eosinophils. Different sera and different concentrations of sera were applied, as well as one trial was performed varying the quantity of IL-5 (F). Number of eosinophils and fraction of eosinophils in percent are displayed in the course of the days in culture. Data are shown as means ± SEM.

In order to define optimal culture conditions that yield large amounts of fully differentiated bmEos, serums from different companies were tested in our cell culture system (Figure 6) The cultures were initiated with a cell count between 12 and 15 million cells at day 0:

(A) Using heat inactivated (56°C for 60 minutes) Gold/PAA serum, the experiment had to be cancelled, as 95% of the cells died until day 6. The percentage of eosinophils also constantly decreased from day 0 (5%), until day 3, where no eosinophil cells were detectable.

(B) Using the Sigma serum, the cells differentiated until day 15, with 94% of the cells being differentiated, but the total cell count decreased day by day; starting with 14.8 million cells on day 0, and ending with 2 million cells on day 15.

(C) Using the HyClone Standard Serum, the cells differentiated continually. Additionally the total cell count increased gradually, with 98% of the cells being differentiated into eosinophilic cells and the total cell count reaching 45 million cells at day 14.

(D) Using the HyClone Research grade Serum, the cells started to differentiate on day 6 with 2.5% being eosinophil cells, up to 98% eosinophil cells on day 12. But the total cell count started to decrease between day 4 and 6, reaching 7 million on day 12.

(E) In this experiment, 10% instead of 20% serum was used in the culture media, leading to a decrease of the total cell count starting at day 4, with almost all cells being defunct at day 10. The percentage of eosinophils however, increased, as shown in figure (E), with 25% eosinophils on day 10.

(F) Besides testing different serums, we also tried to reduce the amount of IL-5 in the culture system. One experiment was performed using half of IL-5 (0.1ml/IL-5 per 100 ml serum). The total cell count in this trial started to decrease at day 4. On day 10 the experiment had to be cancelled, due to the death of virtually all cells. In contrast to the decreasing total cell count, the percentage of eosinophils increased, with 30% being differentiated into eosinophils at day 10.

5.1.5 Siglec-F

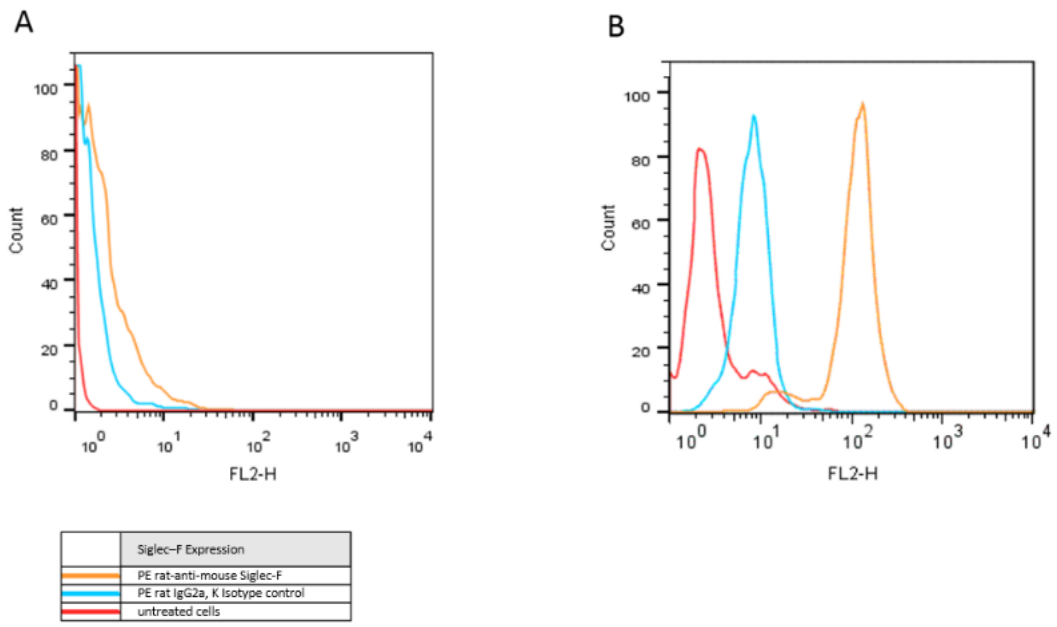

Figure 7. Siglec-F Expression. Mouse bone marrow eosinophils were stained with rat-anti-mouse Siglec-F (PE-conjugated) and isotype-matched control antibodies. Siglec- F expression was assessed as an increase in the FL-2 channel, analyzed in a FACSCalibur Flow Cytometer. (A) Freshly isolated bone marrow cells having only very basal expression of Siglec-F. (B) Fully differentiated bmEos having high Siglec-F expression.

The sialic acid-binding immunoglobulin-like lectin Siglec-F is a mouse functional paralog of human Siglec-8 and is now routinely used as a reliable marker for detecting eosinophils (87).

To compare the undifferentiated bone marrow cells with fully differentiated bmEos, Siglec–F staining was performed with freshly isolated bone marrow cells at day 0 (A) and with differentiated eosinophil cells at day 12 (B). Figure 7 shows the results of this assay. The measurements were conducted on the FACSCalibur flow cytometer. Therefore, cells were stained with PE-conjugated anti-Siglec-F antibodies. Siglec-F surface expression was detected in the Fl-2 channel, showing a clear shift to increased expression of Siglec-F within the differentiated cell population, with a fold change of 31, compared to the isotype control (B). The undifferentiated progenitor bone marrow cells in contrast, showed an increased expression of Siglec-F being twice as high, than the isotype control. (A).

5.1.6 CD11b upregulation WB + bmEos

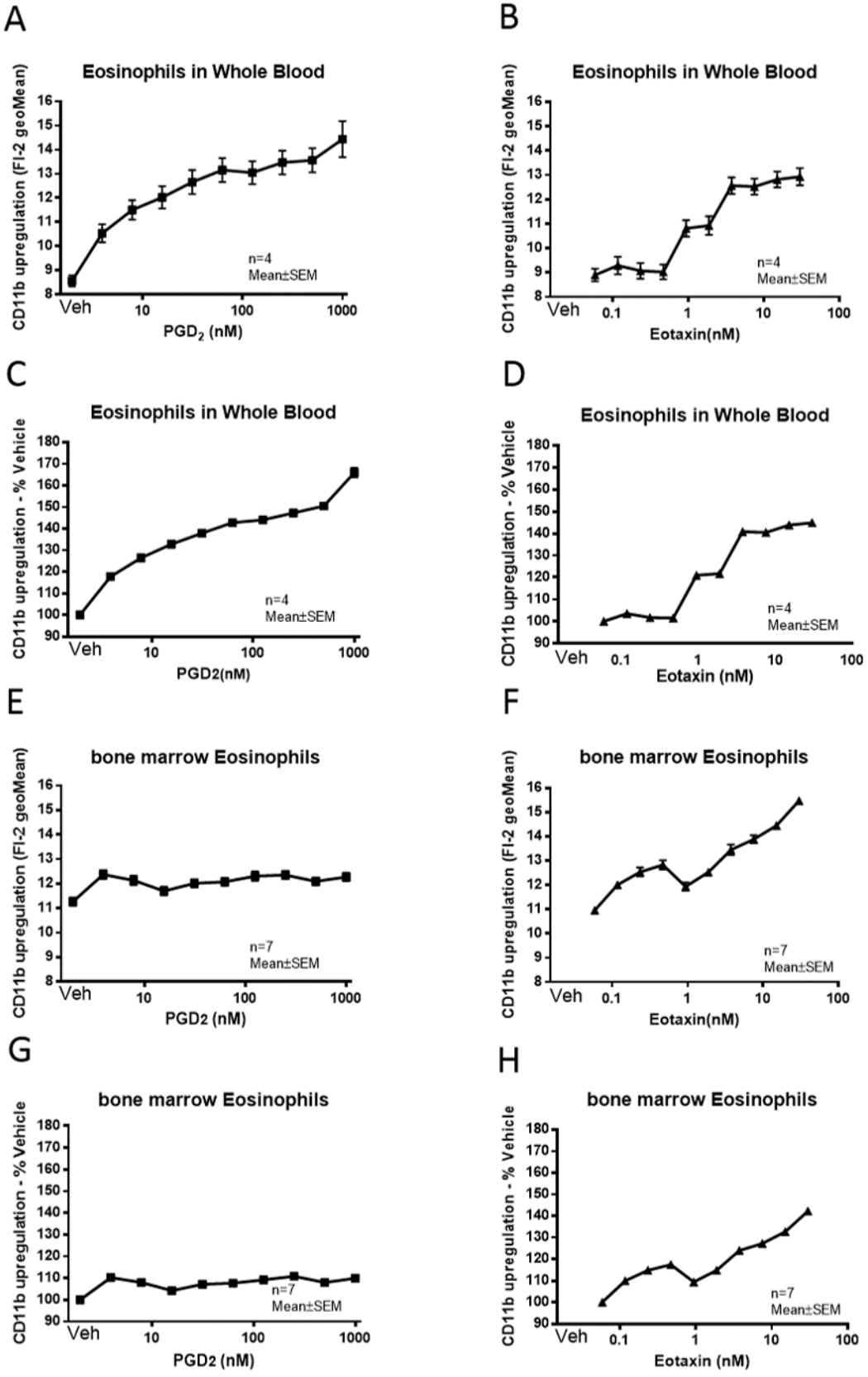

Figure 8. CD11b upregulation. Whole blood eosinophils and mouse bone marrow eosinophils were stained with anti-CD11b (PE-conjugated). As stimulating agonists, increasing concentrations of the chemoattractants PGD_2 or eotaxin were added. The samples were analyzed in the FL-2 channel of a FACSCalibur flow cytometer. Stimulation with eotaxin led to an upregulation of the adhesion molecule CD11b within bmEos and whole blood eosinophils (B+D+F+H). Stimulation with PGD_2 in contrast only affected whole blood eosinophils (A+C). Data are shown as means ± SEM.

It is well known that specifical genetic, biochemical, molecular, and morphological differences exist between human and mouse eosinophils. Thus we explored the functional differences between human peripheral blood eosinophils and mouse bmEos by means of CD11b upregulation (10).

Figure 8 above shows the upregulation of the adhesion molecule CD11b on human whole blood eosinophils and bmEos in response to the chemoattractants PGD_2 and eotaxin.

The first part of the figure (A-D) shows the effects of PGD_2 and eotaxin on human whole blood cells, leading to a clear climax of the CD11b molecule (A+B). (C+D) demonstrate the percentage ratio of the stimulated whole blood cells. The cells stimulated with PGD_2 (C) showed an increase up to 66% in comparison to the non-treated cells. The cells stimulated with eotaxin (D) showed an increase of 45%.

The second part of the figure (E-H) shows the effects of PGD_2 and eotaxin on bmEos. As seen in (E) and (G), the stimulation with PGD_2 did not show any effect. In contrast, the increasing concentrations of eotaxin showed a clear upregulation (F) in CD11b surface expression up to 42% in comparison with the non-treated cells (H).

5.1.7 Western Blot

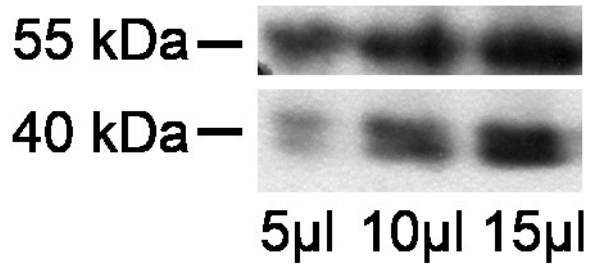

Figure 9. Western Blot with bmEos. CRTH2 protein was detected, using a rabbit anti-mouse CRTH2 primary antibody and a goat anti-rabbit secondary antibody. BmEos were lysed during the process, using the wet-blot method. CRTH2 expression is visualized as an increase of band density at 41kDa and 55kDa, correlating with increasing quantities of protein suspension – 5µl to 15µl.

It is a common fact, that CRTH2 is present on eosinophils, therefore we wanted to investigate if this also applies to bmEos (42).

Western Blot analysis of CRTH2 protein expression was performed with 50 million bmEos, which were lysed in 300 µl lysis buffer. The protein suspension was further processed using the wet-blot method, as explained in the methods section, to determine the presence of the CRTH2 receptor in bmEos.

As seen in Figure 9, an increase of the quantity of protein suspension leads to an increase of the band density at approximately 41kDA and 55kDA, demonstrating expression of CRTH2 (88).

5.1.8 Chemotactic migration with bmEos

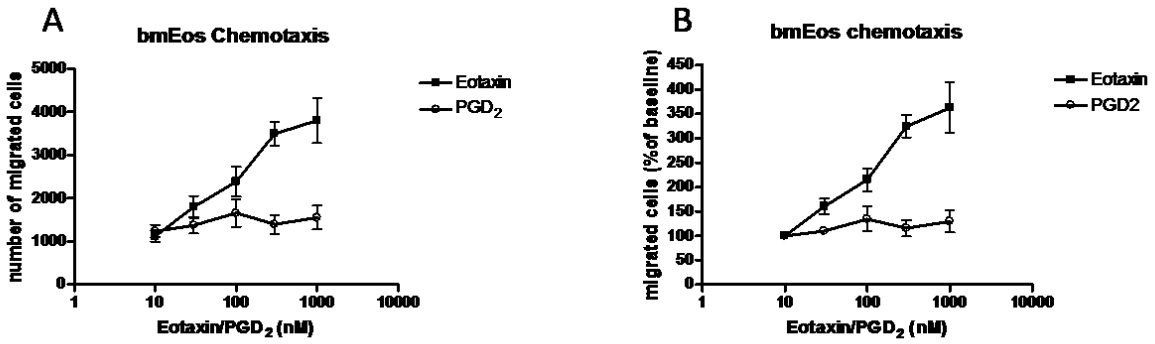

Figure 10. Chemotactic migration with bmEos. Chemotactic migration assay was performed using 96-well transwell plates, loaded with 100.000 bmEos/well. Migration was induced via stimulation with increasing quantities of either eotaxin or PGD_2. Migrated cells of the bottom wells were counted using a FACSCalibur Flow Cytometer. Results are shown by absolute numbers (A) and in percent of baseline (B). Data shown as MEAN ± SEM.

Eotaxin, as well as PGD_2 are known to act as potent eosinophil chemoattractants. We compared those substances regarding their function on bmEos. Figure 10 shows the results of a chemotactic migration assay, which was performed with bmEos, stimulated with increasing concentrations of PGD_2 and eotaxin. As seen in (A) PGD_2 does not affect the chemotactic ability of bmEos. Eotaxin, in contrast, clearly increases the amount of migrated cells, correlating with increasing concentrations. (B) Migrated cells are expressed as percent of baseline, demonstrating an increase up to 362% (28,48).

5.2 EoL-1

5.2.1 Morphology of EoL-1 cells

To visualize morphological features of EoL-1, cell cytospins were performed on glass slides and cells were stained with Hemacolor® after fixation as described in 4.1.2. (Fig.11).
EoL-1 cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 1% PS. Under basal conditions two distinct populations of EoL-1 cells were found. The predominant population showed a homogeneous myeloblast-like morphology, as already described by Kaneko et al. (72), with few cells containing cytoplasmic vacuoles. The minor population consisted of much smaller lymphocyte-like cells with a condensed nucleus.

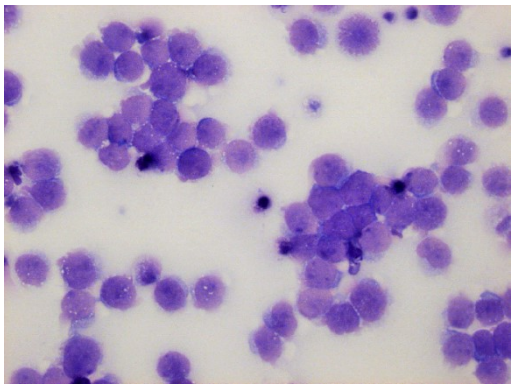

Figure 11. Morphology of EoL-1 cells under basal culture conditions. EoL-1 cells were maintained in culture with RPMI - 1640 medium containing 10% FBS and 1% PS. After centrifugation on glass slides, cells were fixed and stained with Hemacolor®.

5.2.2 Differentiation of EoL-1 cells

For induction of differentiation we used Sodium Butyrate (SB), which is a well-established method for the induction of differentiation within eosinophil cell lines. $5x10^5$ cells were cultured in basal medium supplemented with 0.5 mM SB for up to 9 days (Fig. 12). The formation of granules, a sign of differentiation, was detected at day 6 (Fig. 12, A), as well as at day 9 (Fig.12, B). At day 6, cells already showed morphologically marks of apoptosis, i.e. shrinking of cells and protrusion of the plasma membrane, a sign called ‘bleb’. At day 9, hardly any viable cells could be identified. The apoptotic cells showed increased staining, as they became more and more tightly packed with nuclear fragments and cell organelles, in the course of apoptosis (89).

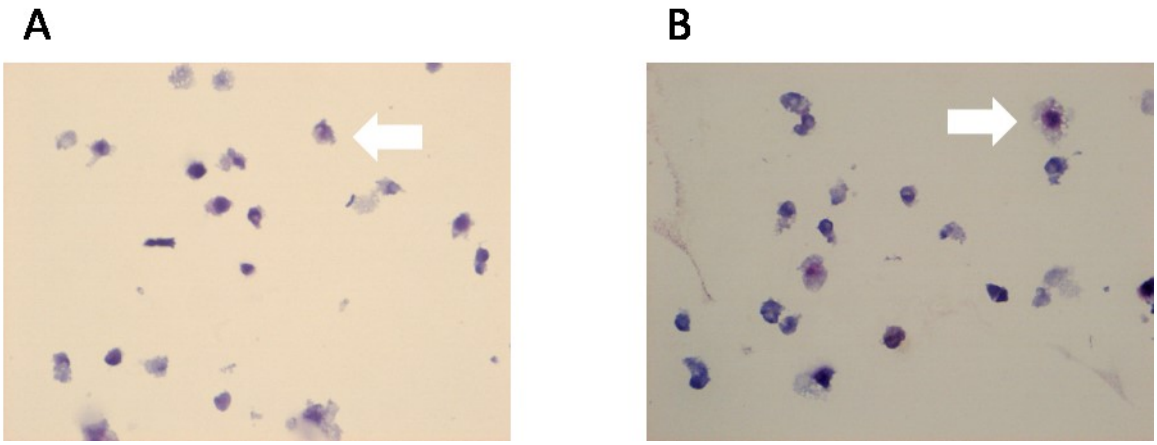

Figure 12. Sodium butyrate-induced differentiation of EoL-1 cells. For induction of differentiation EoL-cells were cultured with RPMI-1640 medium containing 10% FCS supplemented with 0.5 mM SB. Cytospins and Hemacolor® stainings were performed on day 6 (A) and day 9 (B) of culture. The arrows show two EoL-1 cells containing red died granules in their cytoplasm, the cell pointed at in (B), also contains vacuoles.

As a next step, culture conditions were altered in order to enhance cell survival under the differentiation pressure. Thus, the FCS concentration was increased up to 15% (Fig. 13) and IL-5 was added for differentiation (Fig. 14), this has been shown to support eosinophil survival and development (10,70).

The increased FCS concentration during differentiation obtained the myeloblast-like morphology of cells, seen under basal conditions. At day 6 (Fig 13.A) the cells showed vacuoles and isolated granules. The same morphology was observed at day 9 (Fig. 13B). However, enhanced differentiation signs were not detected. In the course of the differentiation, particularly from day 6 on, more and more cells became apoptotic, as seen in Fig. 13 B, where several dead cells as well as cellular debris are visible.

The effect of IL-5 on survival of EoL-1 cells during differentiation was tested in further experiments (Fig. 14) and revealed increasing size of the cytoplasmic vacuoles in the course of differentiation (Fig. 14 A-C). In contrast, no significant increase in eosinophilic granules was detected.

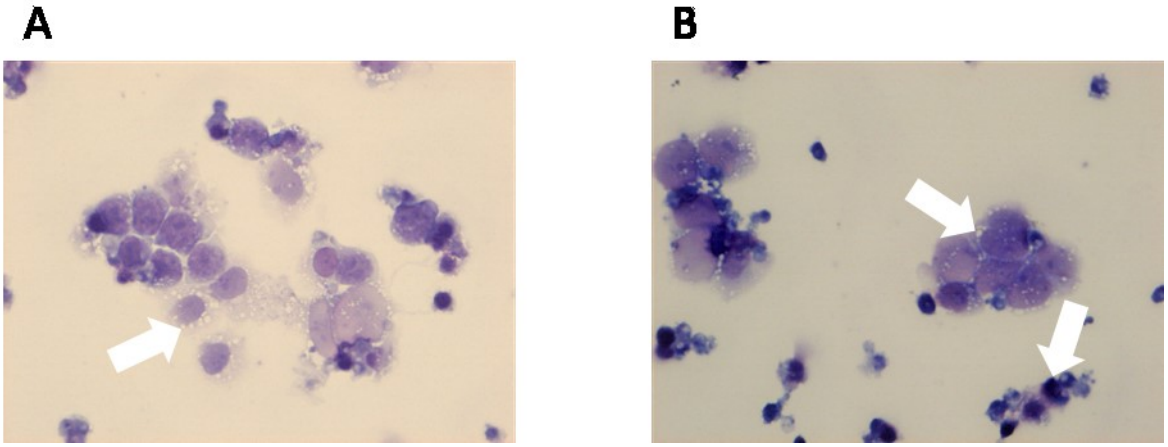

Figure 13. Optimization of cell survival during sodium butyrate-induced differentiation of EoL-1 cells. For induction of differentiation, EoL-1 cells were cultured with RPMI-1640 medium containing 15% FCS supplemented with 0.5 mM SB. Cytospins and Hemacolor® stainings were performed on day 6 (A) and day 9 (B) of culture. The arrows are pointing at vacuoles within EoL-1 cells. The bottom arrow in (B) shows a cell containing red died granules in its cytoplasm.

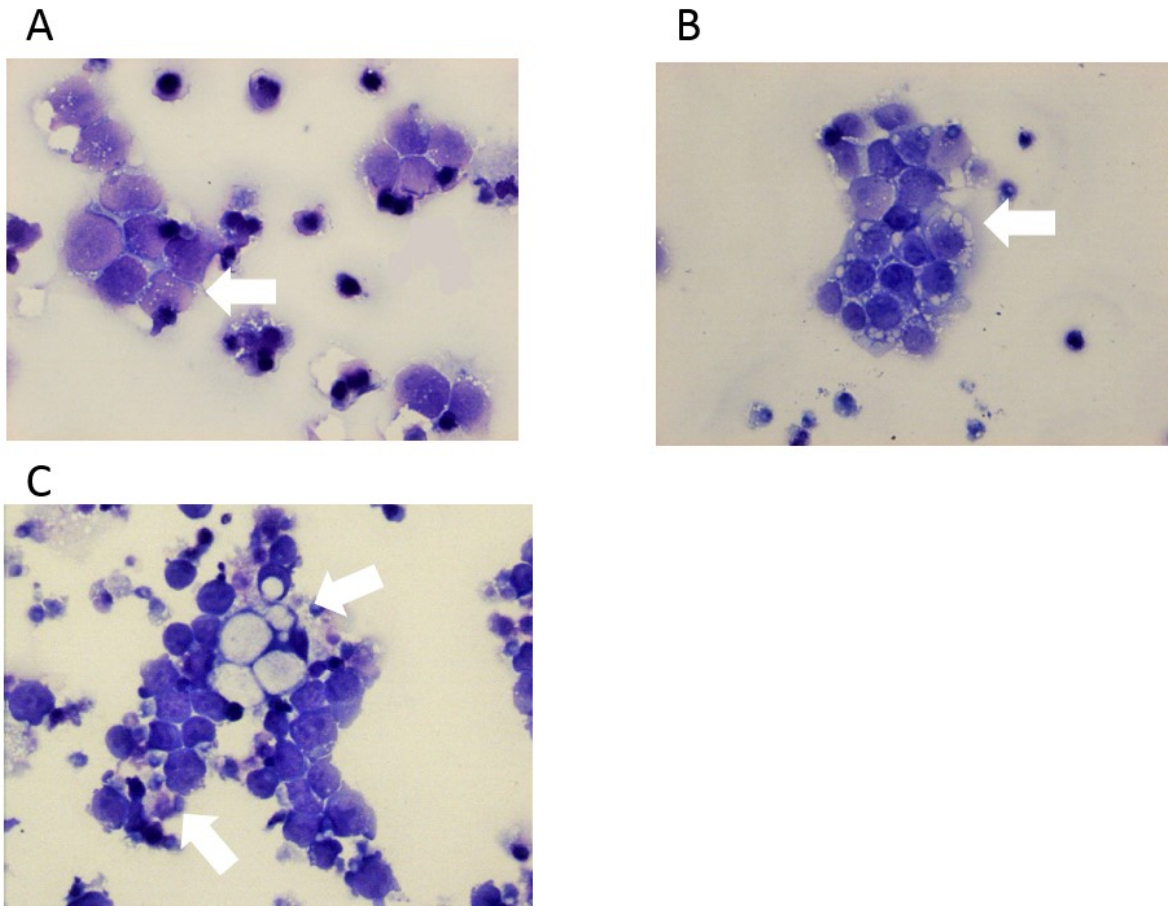

Figure 14. Effect of IL-5 on cell survival and sodium butyrate-induced differentiation of EoL-1 cells. For induction of differentiation EoL-1 cells were cultured with RPMI-1640 medium containing 15% FCS supplemented with 0.5 mM SB and 10 pM IL-5. Cytospins and Hemacolor® stainings were performed on day 3 (A), day 6 (B) and day 9 (C) of culture. The arrows in (A+B) and the upper arrow in (C) point at vacuoles within EoL-1cells. The bottom arrow in (C) shows a cell containing red died granules.

5.2.3 Flow cytometric analysis of EoL-1 cells

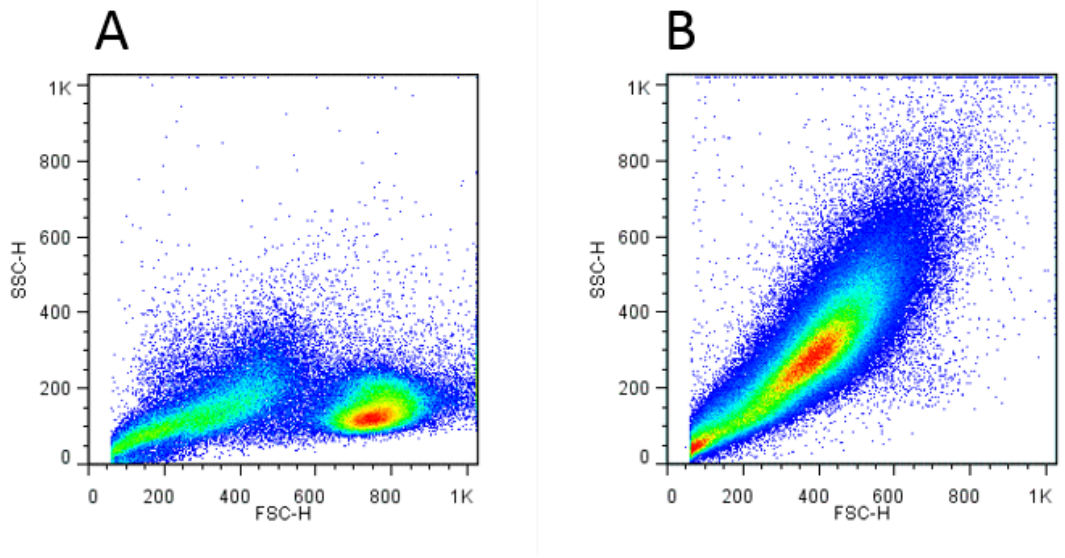

Figure 15. EoL-1 cells in the FACSCalibur Flow Cytometer. Morphology of EoL-1 cells under basal conditions (A) and differentiated cells (B) was analyzed using a FACSCalibur Flow Cytometer and correlating FSC and SSC. The colour red indicates high density, whereas the blue colour on the contrary indicates low cell density.

In addition to the Hemacolor® stainings, we wanted to have a look at the cells on a more molecular basis, using FSC and SSC correlations of the FACSCalibur Flow Cytometer, shown in Figure 15.

(A) shows EoL-1 cells under basal culture conditions, (B) shows the differentiated EoL-1 cells at day 6, treated with 15% FBS, 0.5mM SB and 10pm Il-5. In (A), two different populations of the undifferentiated cells can be clearly distinguished. In contrast, in (B) the two cell populations merge into one, showing dispersion and an increase in the side scatter.

5.2.4 Expression of the PGD_2 receptors CRTH2 and DP1 on EoL-1 cells

CRTH2 and DP1 are co-expressed in eosinophils (28,34). While CRTH2 has been described to be expressed in the EoL-1 cell line, the abundance of the DP1 receptor has not been investigated, yet. In this part of the work we aimed to determine (i) the expression level of CRTH2 and DP1 receptors in the EoL-1 cell line, as well as (ii) whether their expression pattern differs between cells under basal conditions versus differentiated cells, by means of flow cytometry as described in the Methods section (see 4.2.3). To this end, undifferentiated and differentiated cells were stained with anti-CRTH2 and anti-DP1 receptor antibodies at day 6 of sodium butyrate-induced differentiation (Fig 16). Figure 16 exemplarily shows a trial, where we found CRTH2 as well as DP being expressed within undifferentiated and differentiated cells. Unstained cells are displayed as gray. Both cell populations, the undifferentiated EoL-1 cells (A) as well as the differentiated cell population (B) show expression of the CRTH2 receptor. However, no valid conclusion regarding the abundance of CRTH2 comparing the two populations can be made, as the expression patterns varied within different trials. Concerning DP, we only found inconsistent receptor levels. In some trials, we were able to confirm expression of DP, in others, in contrast, we did not find any expression. The lower section of Figure 16 exemplarily shows a trial, where we were able to detect DP.

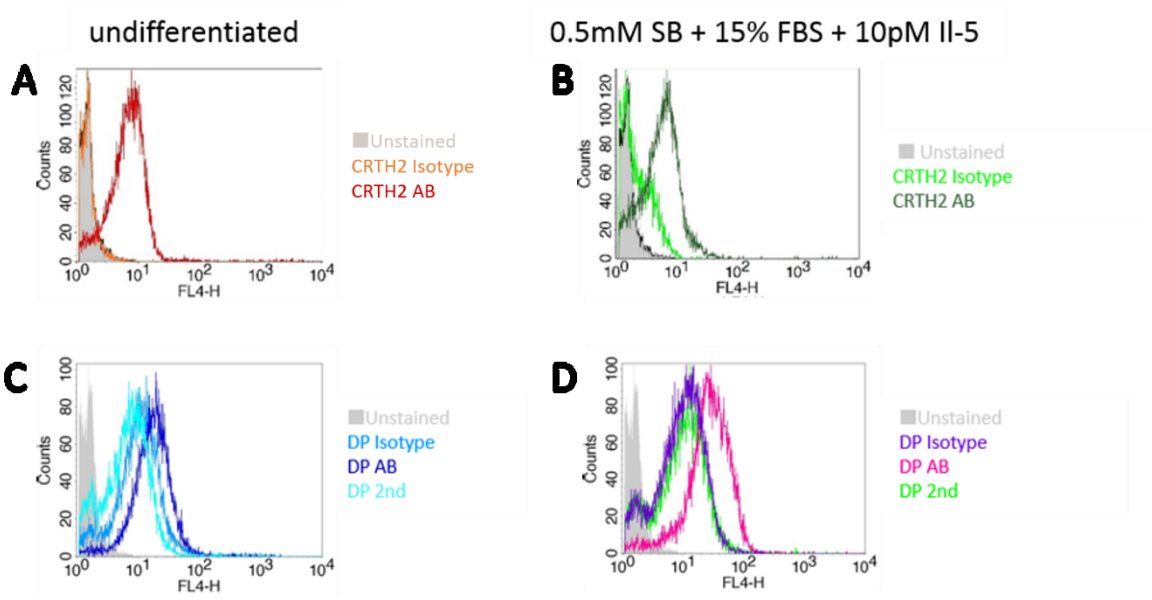

Figure 11. CRTH2 and DP1 Staining with EoL-1 cells. Undifferentiated and differentiated EoL-1 cells were stained with anti-CRTH2 antibody (A+B), anti DP antibodies (C+D) and their respective isotype controls. (A) shows the CRTH2 expression within undifferentiated and (B) within differentiated cells. Both populations show comparable expression of CRTH2. (C+D) exemplarily shows a trial where we found DP being expressed within undifferentiated (C) and differentiated (D) EoL-1 cells. Unstained cells are displayed as grey.

5.2.5 PGD_2-induced calcium flux in EoL-1 cells

Recently, two PGD_2 receptors - CRTH2 and DP - were described to cooperatively mediate PGD_2-induced Ca(2+) mobilization (47). As we intended to gain in depth-knowledge on the influence of CRTH2 and DP on cellular Ca(2+) release, we investigated the changes of intracellular calcium levels in EoL-1 cells in response to PGD_2. Using the calcium indicator Fluo-3 at a concentration of 1:500 we did not detect any change in intracellular calcium levels, therefore we altered the concentration up to 1:300, again without result. Untreated cells served as a negative control. Figure 17 shows one of the trials performed.

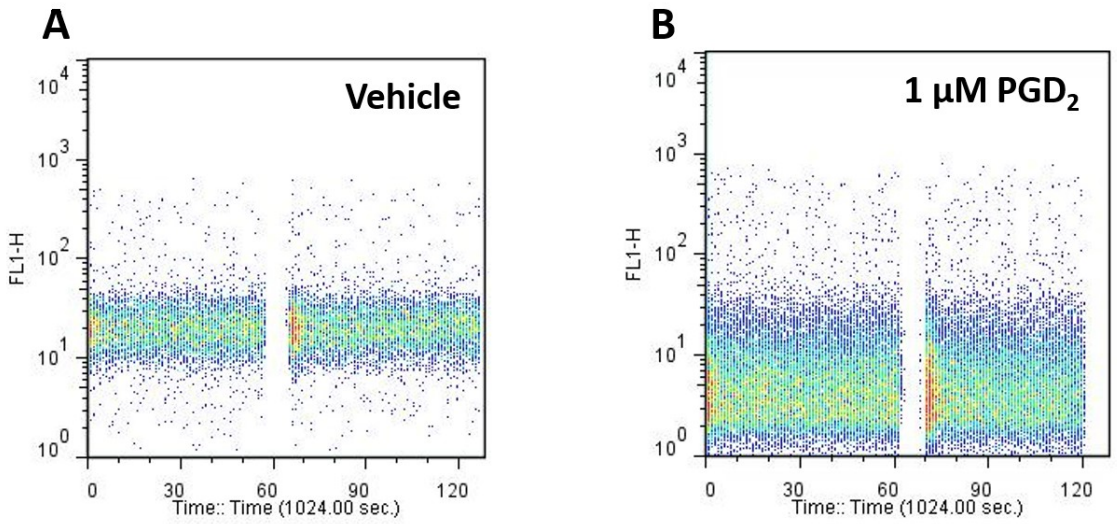

Figure 17. Calcium Flux in differentiated EoL-1 cells. Differentiated EoL-1 cells were treated with the calcium indicator Fluo-3. Baseline was recorded for one minute before adding vehicle (A) or agonist (B). Measurements were continued for another two minutes. Calcium levels were detected as changes of fluorescence in the Fl-1 channel. No changes in intracellular calcium levels were found.

5.2.6 PGD_2-induced chemotaxis of EoL-1 cells

PGD_2 is well known to induce chemotaxis of eosinophils via CRTH2 (28). Similarly, the involvement of DP within eosinophil recruitment was recently reported by Schratl et al.(83). Hence, we investigated chemotactic responses of EoL-1 cells to PGD_2 (Fig. 18).

Figure 18 shows the results of a chemotaxis assay, performed with undifferentiated and differentiated EoL-1 cells, using 100nM PGD_2 as a chemotactic stimulus. The results are displayed as percentage ratio, comparing the stimulated cells with the untreated cells (basal). As seen within the undifferentiated cell population, the cells incubated with PGD_2 clearly showed increased migration, being 130% higher than within the basal cell population. Within the differentiated cell population, in contrast, the migration of the basal cells only differs slightly to the cells incubated with PGD_2.

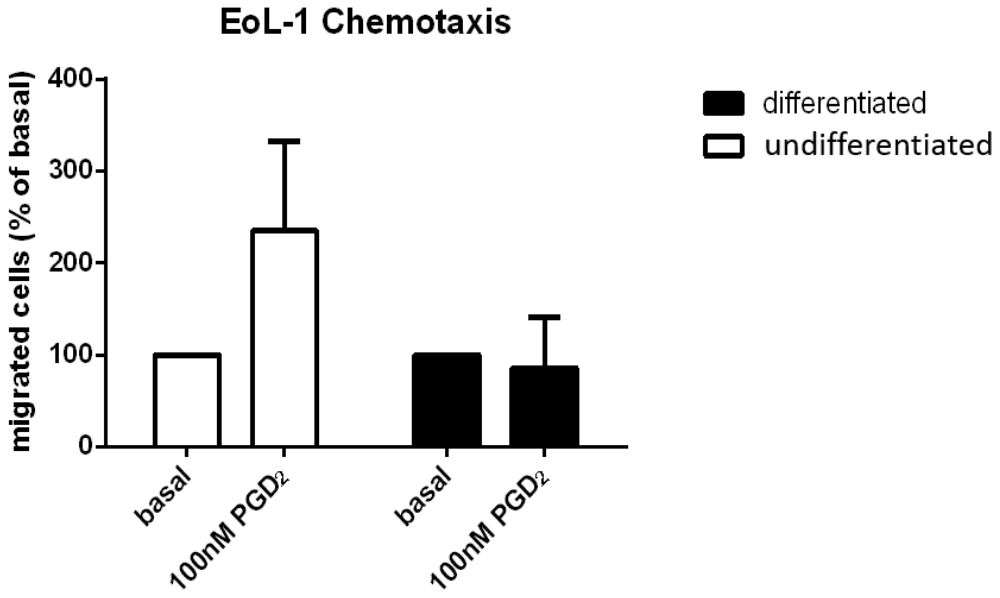

Figure 18. Chemotactic Migration with EoL-1 cells. Differentiated and undifferentiated EoL-1 cells were loaded into the top wells of a MicroBoyden chemotaxis chamber. Chemotactic migration was induced using 100nM PGD_2. After an incubation period of three hours, migrated cells of the bottom wells were counted using a FACSCalibur Flow Cytometer. Results are shown as an increase of migrated cells, comparing untreated (basal) to stimulated (100nM PGD_2) cells. Data shown as MEAN ± SEM.

6 Discussion

This study was performed to identify optimal conditions for culturing and generating large quantities of fully differentiated eosinophils, using two different cell culture systems: (1) murine bone marrow-derived eosinophils (bmEos) and (2) EoL-1 cells, a leukemic cell line. Furthermore, insight into their functional roles was gained.

6.1 Bone marrow derived Eosinophils

Animal models, in particular murine models, are widely used to investigate the pathophysiological entities underlying human disease. Reasons therefor derive from their similarities concerning physiological, biochemical and cellular pathways, as well as their close genetic match (90-93).

Human and murine eosinophils have many features in common. Lee et al., recently compared human and mouse eosinophils. They described many similarities including eosinophilopoiesis, maturation, morphology, peripheral distribution and function, as well as differences between these two species, which were described as of unknown significance or 'cosmetic' in character. Furthermore, based on other reviews discovering significant similarities between human and mouse eosinophils, the usefulness of mouse-based studies for the understanding of human diseases associated with eosinophils, was highlighted (10).

Concerning their morphology, human eosinophils are slightly larger than mouse eosinophils. Their size varies from 12 – 15 μm, compared to 9 – 12 μm in mice. Moreover, granules in the cytoplasm of human eosinophils are packed more densely than granules of mouse eosinophils. The nuclei of both species show a polymorph character and undergo transformation during differentiation. Nuclei of progenitor cells change from spherical appearance over more elongated to cylindrical forms, including stab and band forms. In humans, the nuclei of the metamyelocytes are known to develop multiple lobes. In murine eosinophils, the nuclei evolve a ring-like segmented form (10), that is also observable in our ex-vivo generated bmEos.

In this work, we used a protocol, elaborated by Dyer et al. in 2008 for the isolation and cultivation of primary bone marrow cells of BALB/c mice (61).

In general, primary cell cultures are preferred over continuous immortalized cell lines, as primary cells are considered to be more physiologically similar to in-vivo cells. In addition, cell lines cultured for extended periods of time can undergo phenotypic and genotypic changes, leading to discrepancies when comparing results from different cell line passages. Furthermore, many cell types are not available as continuous cell lines. However, primary cells are more difficult to maintain than continuous cell lines as they are only capable of a limited number of cell divisions, after which they enter a non-proliferative state and eventually die.

Apart from other crucial supplements, such as the eosinophils growth factor IL-5 in our primary culture, the main component of the culture medium is the fetal calf serum (FCS), as it contains a large number of essential, but not well-defined cytokines and embryonic growth factors, which significantly contribute to the differentiation and survival of primary cells. Unfortunately, the content of these components varies not only from company to company, but also from one serum charge to another. Therefore, we tried to identify a serum that permits us to generate a large number of fully differentiated murine bone marrow-derived eosinophils. Thus, we tested four different sera from three different companies and also varied their concentrations, as well as the amount of IL-5 in the differentiation medium. We noticed that bmEos are very sensitive to FCS, as for some sera, all cells died within a few days and many apoptotic cells were visible in Hemacolor® stained samples (according to May-Gruenwald-Giemsa).

Of four tested sera, only three supported the proliferation of bmEos. In one serum, almost no viable cells could be detected by day 5. Using other sera, the cells stopped to proliferate and became apoptotic at some point, except for one serum – HyClone Standard – which we now use routinely for the differentiation, leading to continuous proliferation and differentiation of bmEos.
Interestingly, we found that independently of the total cell count, the number of differentiated eosinophils still augmented day by day, suggesting that a specific component of the serum is required to support and enrich progenitor cells, rather than eosinophil differentiation.

Compared to the original report of Dyer et al., where a differentiation rate of 90% was shown by day 10, the amount of differentiated cells in our optimized culture system was delayed by

approximately two days. As we used the identical protocol, this effect may again be due to the serum composition (61).

Thus, our cultures yielded high purity (~ 90%) bone-marrow derived eosinophils (bmEos) by day 12, as determined by visual inspection of Hemacolor® stained cells. Furthermore we confirmed high expression of the sialic acid-binding immunoglobulin-like lectin Siglec-F, the mouse paralog of the human Siglec-8, which is used regularly for the detection of eosinophils (87).

To verify the specificity of Siglec-F as a marker for mature eosinophils, flow cytometric analyses of Siglec-F expression were performed in fully differentiated bmEos as well as in freshly isolated bone marrow cells. As expected, only a weak Siglec-F expression was found on freshly isolated bone marrow cells, whereas a 30-fold increase over isotype control was detected in mature bmEos. These results well correlated with our morphological observations, where we could only identify very few eosinophils within fresh bone marrow, but almost only eosinophils by day 12 of culture.

Apart from the differences and similarities of human and mouse eosinophils explained above, we wanted to gain insight into the functional role of bmEos in comparison to human whole blood (WB) - eosinophils, concerning two chemoattractants, PGD_2 and eotaxin by means of CD11b upregulation and chemotaxis. As expected, eotaxin induced a significant upregulation of CD11b molecule in human WB – eosinophils, as well as in bmEos.

PGD_2, in contrast only stimulated human WB – eosinophils, leading to a clear increase of the CD11b adhesion molecule, but was without effect in bmEos. Similar results were found in the chemotaxis assay: eotaxin induced a distinct chemotactic response in bmEos, confirming previous observations by Dyer et al. (61). Although both agonist, eotaxin and PGD_2 are potent chemoattractants for human eosinophils, bmEos did not respond to PGD_2 in the chemotaxis assay.

Eotaxin is acting via the CCR3 receptor, which is expressed in human as well as mouse eosinophils, explaining why stimulation with eotaxin does also affect bmEos. Interestingly, thus far three human eotaxins – eotaxin-1,-2 and -3 have been described– but only two murine eotaxin-family members – eotaxin-1 and -2 exist, as in mice only a non-functional eotaxin-3 pseudogene is present (10,94).

PGD_2, in contrast, acts via DP and CRTH2 receptors which have been described for human and mouse eosinophils. In human eosinophils, CRTH2 is known to mediate CD11b

upregulation via the activation of PLC, PI-3 kinase and MAP kinase, as well as chemotaxis (10,28,43,95).

Accordingly, bmEos should respond to stimulation with PGD_2, but in respect to our data, they do not. As the expression of CRTH2 on the protein level was clearly confirmed by western blotting, we hypothesize that the non-response of the bmEos is due to the culture conditions we used: IL-5, which is an important component of our cell culture system, was shown to reduce the surface expression of CRTH2 in human eosinophils (96). As the bmEos are differentiating from day 4 onwards in a medium containing 10ng/ml IL-5, this could certainly lead to receptor internalization, reduction or pre-activation of the CRTH2 receptor. Thus, separated western Blotting of cytoplasmatic and membrane-associated proteins as well as intracellular fluorescence staining of CRTH2 would be helpful tools to define the exact localization of the receptor in bmEos. Moreover, cultivation of bmEos in cytokine-free base media prior to the assays may lead to an increase in CRTH2 surface expression and possibly restore the responsiveness to PGD_2. It must be noted, however, that IL-5 is not only crucial in eosinophil priming as a growth factor, but also essentially important for the survival of mature eosinophils. Consequently, mature bmEos start dying when cultured in IL-5-free medium for several hours.

In summary, in this first part of the study we found one serum supporting proliferation and differentiation of unselected murine bone marrow cells into mature functionally competent bmEos. The differentiated bmEos showed a high expression of the eosinophil-specific marker Siglec-F and expressed CD11b. Additionally, they underwent chemotaxis in response to stimulation with eotaxin, but not with PGD_2.

We discovered that CRTH2 is expressed in murine bmEos on the protein level, but is internalized or down-regulated due to cultivation in IL-5, explaining the missing responsiveness to PGD_2.

6.2 EoL-1

Concerning EoL-1 cells, we made different attempts to define an ideal differentiation method.

The undifferentiated cells were maintained in culture media supplemented with 10% FCS and antibiotics. To induce differentiation, we used sodium butyrate (SB) at a concentration of 0.5mM, a method, which was described by Fischkoff et al. in 1985. Apart from SB, other

reagents leading to eosinophil differentiation, such as Apicidin and dbcAMP do exist (71,97).

To monitor cell viability as well as morphological appearance, we performed Hemacolor® stainings on day 3, 6 and 9 of culture.
The results of the differentiation with 0.5mM alone were not satisfying at all, as the cell count decreased day by day, and more and more apoptotic bodies were detectable.
For this reason, we tried different concentrations of the FCS, varying between 10 and 20%, and added IL-5, which was identified to enhance the life-time of eosinophils by withholding apoptosis (98).
The best outcome of differentiation was reached with 15% FBS, 0.5mM SB and 10pM IL-5. Thus we decided to conduct the ongoing experiments with this method.

EoL-1 cells maintained at standard culture conditions were described to show myeoloblast-like features with a round nucleus but without any granules – characteristics we were able to observe in our EoL-1 cells (99). As previously described by Saito et al., our undifferentiated cells were growing in single-cell suspension in a time-dependent manner. Due to this increase in cell density, splitting cells was necessary every 2 to 3 days. Upon induction of differentiation, they started to form clusters (66).

Concerning the proliferation and cell viability within the course of differentiation, Morita et al. induced differentiation with culture supernatants from a human ATL cell line (HIL-3 sup.) and described the viability and the growth rate of the EoL-1 cells to decrease between day 6 and day 9 (99). Ishihara et al. used SB at a concentration of 0.5mM – as we did in our cell culture – and reported the viability of the EoL-1 cells being decreased approximately 15% by day 8, but the proliferation being inhibited significantly starting with addition of SB. Within our cell culture system, we did not notice any proliferation but observed a much more drastic decrease of cell viability in differentiating cells, starting by day 3, with a sharp decline of viable cells between day 6 and 9, with almost all cells being dead by day 9 (71). Thus, we decided to conduct the functional assays on day 5 and day 6 of culture.
Regarding the appearance of eosinophil granules, Saito et al. reported 2% of cells having an eosinophil phenotype with cytoplasmic granules. Later, they described differentiated EoL-1 cells possessing many granules, after incubation with SB (66,100).

Morita et al. claimed that undifferentiated EoL-1 cells would not have any granules, but granules-containing cells would increase up to 24% until day 9 (99).
Similarly, our undifferentiated cells did not contain any granules, but starting at day 3 after induction of differentiation, granularity increased until day 9, but in general of low quantity.

One thing we noticed within our EoL-1 cells was the appearance of vacuoles, which were already present within the untreated cells, but they increased in size and quantity in the course of differentiation. This was also described by Wong et al.,1999 within EoL-1 cells, differentiated via the use of dbcAMP. These findings suggest, that in the course of differentiation the alteration of granule matrix as well as dispersion of crystalloid material into the matrix leads to the presence of refractile vacuoles, visible in the light microscope. As vacuoles were also found to appear within human eosinophils, this was assumed to be one sign of differentiation (97).

In addition to the differentiation, we investigated expression patterns of CRTH2 and DP receptors, as well as responses of EoL-1 cells to the CRTH2/DP1 receptor ligand PGD2 in two functional assays, i.e. chemotaxis and Ca(2+) flux.

CRTH2 and DP are both known to be present on eosinophils and involved in the action of PGD_2 (29).
In this work, we tried to confirm the expression of CRTH2 and DP on protein level in EoL-1 cells. Concerning CRTH2, we found clear expression within the undifferentiated cell population as well as within the differentiated cell population. Comparing the two populations regarding their abundance of CRTH2, their expression levels did not show any significant difference.
Concerning the DP receptor, in contrast, we only found inconsistent levels - within some trials we found slightly expression of DP, within others we did not find any expression of DP.
In another step, we aimed to investigate the functionality of EoL-1 cells in terms of PGD_2-mediated chemotaxis and Ca(2+) influx.
Since we could not obtain any viable data in the first trials, which we performed to investigate the changes of intracellular Ca(2+) levels in EoL-1 cells, we altered the amount of the calcium indicator Fluo-3, similarly without any result.

A reason for their non-responsiveness to PGD_2 could be their lack of abundance of DP. Similarly, an optimization of the loading of the cells as well as a more sensitive imaging method could lead to different results. An option would be the single-cell calcium imaging. This method is more sensitive, as it offers the possibility to look at the change of intracellular calcium levels of every single cell via the use of a fluorescence microscope. As a requirement for the use of this technique, the cells have to be adherent onto a cover glass. This would be a problem using our EoL-1 cells, as they are growing in suspension. In general, the use of single-cell calcium imaging with cells in suspension is complicated and the application of special coating is only effective to a limited extent.

Within the chemotaxis assay we performed, only the undifferentiated cells responded to stimulation with PGD_2. They demonstrated an increase of migrated cells of 100%. The differentiated cells, in contrast, did not respond to the stimulation with PGD_2. A reason therefore might be the bad viability of the differentiated cells, as their cell count started to decrease drastically upon induction of differentiation. Within Hemacolor® stainings, we also saw increasing count of apoptotic bodies as well as cellular debris in the course of differentiation.
The correlation of FSC and SSC in the FACSCalibur displayed two clearly distinguishable cell populations within the undifferentiated cell population. Within the differentiated cell population in contrast, these two populations merged into one big spreading cell population, also indicating a heterogeneous and rougher cell surface, which could be a factor inhibiting stimulation via PGD_2.

In summary, EoL-1 cells do possess CRTH2, with comparable expression levels within undifferentiated cells and differentiated cells. Regarding DP, no expression could be confirmed.
Investigating their functionality, we could not detect any change of intracellular calcium levels as a response to PGD_2. As DP is known to intensify CRTH2 mediated Ca(2+) influx, we suggested that the lack of DP abundance within the EoL-1 cells is responsible for their inability of PGD_2 induced Ca(2+) mobilization (47).
Concerning the chemotaxis assay, only the undifferentiated cells showed increased migration after stimulation with PGD_2, the differentiated cells did not respond to PGD_2. As we found CRTH2 expression to a comparable level within differentiated as well as undifferentiated cells, both populations should actually migrate as a response to PGD_2.

Therefore, we hypothesize that the bad viability of the differentiated cells is the reason for their non-responsiveness to PGD_2.

As the life-span of human eosinophils is very limited and it is difficult to obtain high quantities of human eosinophils, some experiments like transfections are difficult to perform. The establishment of a cell culture system, such as with the EoL-1 cell line could give new perspectives to study eosinophils as well as the interactions between the PGD_2 receptors CRTH2 and DP in more detail.

Further aims in the studying of EoL-1 cells would be the attempt of detecting DP on mRNA-level and thereupon a receptor knock-out of DP. As recently DP and CRTH2 were identified to form heteromers, as well as the presence of DP was shown to intensify the CRTH2 mediated Ca(2+) influx, further data would be needed to gain in-depth knowledge about their functional interaction (47).

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