Dissertation

Elucidating the possible Relation between Apolipoprotein A-IV and Prostaglandin D_2 in Cells involved in the Pathogenesis of Allergic Asthma and Rheumatoid Arthritis

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice”.

…… 17.08.2020 ……
date

…… David Roula ……
signature

Disclosures

Part of the data presented in this thesis have been published Allergy 2019 (Open Access):

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Abbreviations

Abbreviation	Meaning
AA	Arachidonic Acid
AC	Adenylyl cyclase
ACPA	Anti–citrullinated protein antibody
ACR	American College of Rheumatology
AIA^1	Antigen-induced arthritis
AIA^2	Adjuvant-induced arthritis
APC	Antigen presenting cell
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
Bmal1	Brain and muscle, ARNT-like
cAMP	Cyclic adenosine monophosphate
CCR	Chemokine receptor
CD	Cluster of differentiation
CE	Cholesteryl ester
CIA	Collagen-induced arthritis
clock	Circadian locomotor output cycles kaput
CNS	Central nervous system
COX	Cyclooxygenase
cPGES	Cytosolic PGE synthase
CRP	C-reactive protein
CSFR1	Colony stimulating factor 1 receptor
CX3CR1	CX3C receptor
DAG	Diacylglycerol
DMARD	Disease-modifying anti-rheumatic drugs
DP	D-type prostanoid receptor
ECP	Eosinophil cationic protein

- EDN — Eosinophil-derived neurotoxin
- EPO — Eosinophil peroxidase
- EULAR — European League Against Rheumatism
- FATP — Fatty acid transport proteins
- Fc — Fragment crystallizable region
- FcgRIIIa — Low affinity immunoglobulin gamma Fc region receptor III-A
- FcεRI — High-affinity IgE receptor
- FEV1 — Forced Expiratory Pressure in 1 Second
- FFA — Free fatty acids
- FLS — Fibroblast-like Synoviocytes
- GM-CSF — Granulocyte-macrophage colony-stimulating factor
- G-SCF — Granulocyte colony-stimulating factor
- HDL — High density lipoproteins
- HDM — House dust mite
- HFD — High-fat diet
- HLA-DRB1 — HLA class II histocompatibility antigen, DRB1 beta chain
- hPGDS — Hematopoietic PGD synthase
- HTGL — Hepatic triglyceride lipase
- IDL — Intermediate density lipoprotein
- IgE — Immunoglobulin E
- IgG — Immunoglobulin G
- IL — Interleukin
- IP3 — Inositol trisphosphate
- JAK — Janus kinase or Just another kinase
- JIA — Juvenile idiopathic arthritis
- kDa — Kilodalton

• LCAT	Lecithin–cholesterol acyltransferase
• LDL	Low density lipoproteins
• L-PGDS	Lipocalin-Type PGD Synthase
• LPL	Lipoprotein lipase
• LPS	Lipopolysaccharide
• Ly6C	Lymphocyte antigen 6 complex locus C1
• MAG	Monoacylglycerol lipase
• MAPK	Mitogen-activated protein kinase
• MBP	Major basic protein
• MCh	Methacholine
• MCP-1	Monocyte chemoattractant protein 1
• MHC	Major histocompatibility complex
• MLS	Macrophage-like Synoviocytes
• MMP	Matrix metalloproteinases
• mPGES-1	Microsomal PGE synthase-1
• mPGES-2	Microsomal PGE synthase-2
• NET	Neutrophil extracellular traps
• NR1D1	Nuclear receptor subfamily 1 group D member 1
• NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1
• NSAID	Nonsteroidal anti-inflammatory drug
• NSAR	Nichtsteroidales Antirheumatikum
• sterase	Non-specific esterase
• oxLDL	Oxidized low-density lipoprotein
• PAD4	Protein arginine deiminase 4
• PDK	Phosphoinositide-dependent kinase
• PGD_2	Prostaglandin D_2
• PGE_2	Prostaglandin E_2

• PI3K	Phosphatidylinositol 3-kinase
• PIP2	Phosphatidylinositol 4,5-bisphosphate
• PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
• PKA, -B, -C	Proteinkinase A, -B or -C
• PLC	Phospholipase C
• PMN	Polymorphonuclear neutrophils
• PTPN22	Protein Tyrosine Phosphatase Non-Receptor Type 22
• RA	Rheumatoid Arthritis
• RANKL	Receptor Activator of NF-κB Ligand
• RASF	Rheumatoid Arthritis Synovial Fibroblasts
• RF	Rheumatoid Factor
• TAG	Triacylglyceride
• TC2	Type 2 cytotoxic T cell
• TCR	T cell receptor
• Th	T helper cell
• TLR	Toll-like receptor
• TNF	Tumor necrosis factor
• VEGF	Vascular endothelial growth factor
• VLDL	Very-low density lipoprotein

Abstract

In the western world, allergies and autoimmune diseases are on the rise, including allergic asthma and rheumatoid arthritis (RA), respectively. Understanding how these pathologies develop is vital in the search for a cure. In recent years, a body of evidence accumulated that linked dysregulations in the immune system to dysregulations of metabolism. In both allergic asthma and rheumatoid arthritis, altered levels of apolipoproteins have been observed. Apolipoproteins are proteins present on the surface of lipoproteins and have long been considered to exclusively regulate lipid metabolism. Apolipoprotein A-IV (ApoA-IV) is a 46 kDa amphipathic protein present on chylomicrons, very low-density lipoproteins and in plasma. Its physiologic function has not been illuminated yet, but reports started to emerge that point to anti-inflammatory properties. ApoA-I, an apolipoprotein that is structurally related to ApoA-IV, has been shown to induce production of prostaglandin D_2 (PGD_2) *in vitro*. In both diseases, elevated levels of PGD_2 are present at the site of inflammation. PGD_2, an arachidonic acid-derived lipid mediator, possesses both pro- and anti-inflammatory properties, mediated through two surface receptors called DP1 and DP2. Receptor expression is widely distributed among leukocytes but also tissue cells.

The aim of this study was to elucidate a possible relation between ApoA-IV and PGD_2 in the context of allergic asthma and RA. To achieve this, a flow cytometric approach was used to test the effect of ApoA-IV on eosinophil and monocyte migration, macrophage polarization and neutrophil activation. In a small clinical cohort, we assessed DP2 receptor expression in leukocytes of patients suffering from RA. Western blot, quantitative RT-PCR and ELISA assays were used to measure protein expression, mRNA expression and cytokine production of the nuclear receptor NR1D1, prostaglandin synthases mPGES-1 and hPGDS and cytokines TNF-α and IL-10 respectively. RIA and ELISA were performed to measure production of prostaglandin E_2 and D_2. Murine models of airway hypersensitivity and experimental arthritis were used to measure the impact of ApoA-IV and DP2 antagonist OC000459 *in vivo* on pathophysiology, respectively.

We show that ApoA-IV potently suppresses eosinophil chemotaxis *in vitro* via NR1D1 and improves lung inflammation and pulmonary function *in vivo*. We show further that ApoA-IV potently suppresses monocyte activation, chemotaxis and induces PGE_2 production. In macrophages, ApoA-IV potently suppresses the pro-inflammatory M1 phenotype by decreasing surface expression of CD80 and production of TNF-α. The observed reduction of CD80 is mediated through NR1D1. In patients suffering from rheumatoid arthritis, we show that plasma ApoA-IV is decreased and that DP2 expression is reduced *ex vivo* monocytes and increased in basophils compared to healthy donors. Monocyte-derived M2 macrophages differentiated *in vitro* from monocytes of patients suffering from acute flairs show an increase in DP2 expression compared to patients in remission. Similar to ApoA-IV, PGD_2 and the selective DP1 agonist BW245C influences polarization in monocyte-derived macrophages from healthy donors.

In conclusion, these data suggest a potent anti-inflammatory role for the apolipoprotein A-IV in cells of the innate immune system, including eosinophils, monocytes and macrophages. The underlying pathway in this process may involve the nuclear receptor and clock gene NR1D1. Our clinical data suggest that DP2 receptor expression may play a role in non-classical monocytes, basophils and macrophages in rheumatoid arthritis.

Zusammenfassung

Allergien und Autoimmunerkrankungen, einschließlich allergisches Asthma und rheumatoide Arthritis, steigen in der westlichen Welt an. Auf der Suche nach Heilung ist es essenziell, die Entstehung dieser Pathologien zu verstehen. In den letzten Jahren wurde in einigen Studien gezeigt, dass Fehlregulationen im Immunsystem oft im Zusammenhang mit Fehlregulationen im Stoffwechsel stehen. Sowohl in allergischem Asthma als auch rheumatoider Arthritis wurden Veränderungen in den Konzentrationen von Apolipoproteinen im Plasma verzeichnet. Dabei handelt es sich um Proteine, die auf der Oberfläche von Lipoproteinen zu finden sind und für lange Zeit vorwiegend mit dem Fettstoffwechsel assoziiert wurden. Apolipoprotein A-IV (ApoA-IV) ist ein 46 kDa amphipathisches Protein, welches auf Chylomikronen, Lipoproteinen und im Plasma zu finden ist. Während seine physiologische Funktion noch nicht bekannt ist, konnten einige Studien zeigen, dass es anti-inflammatorische Eigenschaften besitzt. ApoA-I, ein Apolipoprotein welches strukturell mit ApoA-IV verwandt ist, induziert die Produktion von Prostaglandin D_2 (PGD_2) *in vitro*. In beiden Erkrankungen wurden erhöhte Werte von PGD_2 am Ort der Entzündung gemessen. PGD_2 ist ein Lipidmediator, der sowohl pro- als auch anti-inflammatorische Eigenschaften besitzt, mediiert durch die beiden Oberflächenrezeptoren DP1 und DP2. Sowohl Immunzellen als auch Gewebszellen exprimieren diese Rezeptoren.

Ziel dieser Studie war es, die mögliche Beziehung zwischen ApoA-IV und PGD_2 im Zusammenhang mit allergischem Asthma und rheumatoider Arthritis zu beleuchten. Um dies zu erreichen, wurde ein flowzytometrischer Ansatz verwendet, um den Effekt von ApoA-IV auf die Chemotaxis von Eosinophilen und Monozyten, die Polarisierung von Makrophagen und die Aktivierung von Neutrophilen zu testen. In einer kleinen klinischen Kohorte wurde die DP2 Rezeptor-Expression in Leukozyten von RA-Patienten überprüft. Western Blots wurden angewandt, um die Expression des nukleären Rezeptors NR1D1 zu prüfen, quantitative RT-PCR für die Expression von Prostaglandin-Synthasen mPGES-1 und hPGDS und ELISAs für die Bestimmung der Zytokine TNF-α und IL-10. RIA und ELISA wurden durchgeführt, um die Produktion von PGE_2 und PGD_2 zu quantifizieren. In Maus-Modellen wurde der Effekt von ApoA-

IV auf Überempfindlichkeit der Atemwege bzw. der Effekt von DP2-Antagonist OC000459 auf experimentelle Arthritis beleuchtet.

Wir zeigen, dass ApoA-IV die Chemotaxis von Eosinophilen über NR1D1 wirksam *in vitro* hemmt und weiters, dass es *in vivo* pulmonale Inflammation und Lungenfunktion verbessert. Außerdem zeigen wir das ApoA-IV wirksam die Monozyten-Aktivierung und -Chemotaxis hemmt und die PGE_2-Produktion anregt. In Makrophagen hemmt ApoA-IV den pro-inflammatorischen M1-Phänotyp, in dem es die Expression des Oberflächenmarkers CD80 und die Produktion von TNF-α senkt. Diese beobachtete Reduktion in CD80 ist NR1D1 mediiert. In Patienten, die unter rheumatoider Arthritis leiden, zeigen wir das Plasma-ApoA-IV reduziert ist und die Expression von DP2 in *ex vivo* Monozyten reduziert und in Basophilen erhöht ist, im Vergleich zu gesunden Kontrollen. In Patienten mit aktiver Arthritis finden wir einen Anstieg der DP2-Expression in M2-Makrophagen, im Vergleich zu Patienten in Remission. Ähnlich wie bei ApoA-IV, konnte PGD_2 und der selektive DP1-Agonist BW245C die Polarisierung von aus Monozyten-differenzierten Makrophagen von gesunden Spendern beeinflussen.

Wir schlussfolgern, dass diese Daten auf eine wirksame anti-inflammatorische Wirkung von Apolipoprotein A-IV in Zellen des angeborenen Immunsystems hinweisen, darunter Eosinophile, Monozyten und Makrophagen. Der zugrundeliegende Pfad in diesem Prozess involviert den nukleären Rezeptor und clock-Gen NR1D1. Unseren klinischen Daten suggerieren das die DP2-Expression eine Rolle in nicht-klassischen Monozyten, Basophilen und Makrophagen in rheumatoider Arthritis spielt.

1. Introduction

The consequence of having an adaptive immune system, which targets an almost infinite number of foreign epitopes, is the risk that these efforts stray sometimes into the wrong direction and lead to allergy or autoimmunity. Recently, these affections have been linked to disrupted or imbalanced metabolic processes such as apolipoprotein profiles. Understanding the underlying parameters is essential in order to find improvements in therapy. The difficulty herein lies in the fact that often immune disorders converge on an inflammatory crest, for instance production of prostaglandins and are therefore similarly interpreted. Based on the finding that apolipoprotein levels are altered in autoimmune and allergic diseases, we wanted to illuminate a potential connection between the anti-inflammatory apolipoprotein ApoA-IV and the pro-inflammatory prostaglandin PGD_2 in allergic asthma and rheumatoid arthritis, two immune disorders in which both apolipoprotein content and prostaglandin production seem to be out of balance.

1.1. Prostaglandins

Prostaglandins are a group of arachidonic acid (AA) derived lipid mediators that participate in tissue homeostasis in the steady state, but also play a role in the generation, propagation and resolution of inflammation [reviewed in (2)]. Arachidonic acid is an integral polyunsaturated omega-6 fatty acid found within the phospholipid fraction of cellular membranes, where it provides fluidity and flexibility. AA can be obtained from dietary sources (e.g. fish, meat, eggs) or be produced endogenously by desaturation and elongation of linoleic acid. Besides its function as part of the cell membrane, it participates in cell signalling as a second messenger and provides a substrate for the production of classical and non-classical eicosanoids [reviewed in (3)]. Release of AA from the cellular membrane is catalyzed by phospholipase A2 (PLA2), a hydrolase that cleaves between the fatty acid and the glycerol molecule of phospholipids. Arachidonic acid then serves as a substrate for eicosanoid production (see Figure 1). Essential for prostaglandin production is the enzyme Prostaglandin Endoperoxide H_2 synthase (PGHS), commonly known as cyclooxygenase (COX), consisting of two isoforms: COX-1 and COX-2.

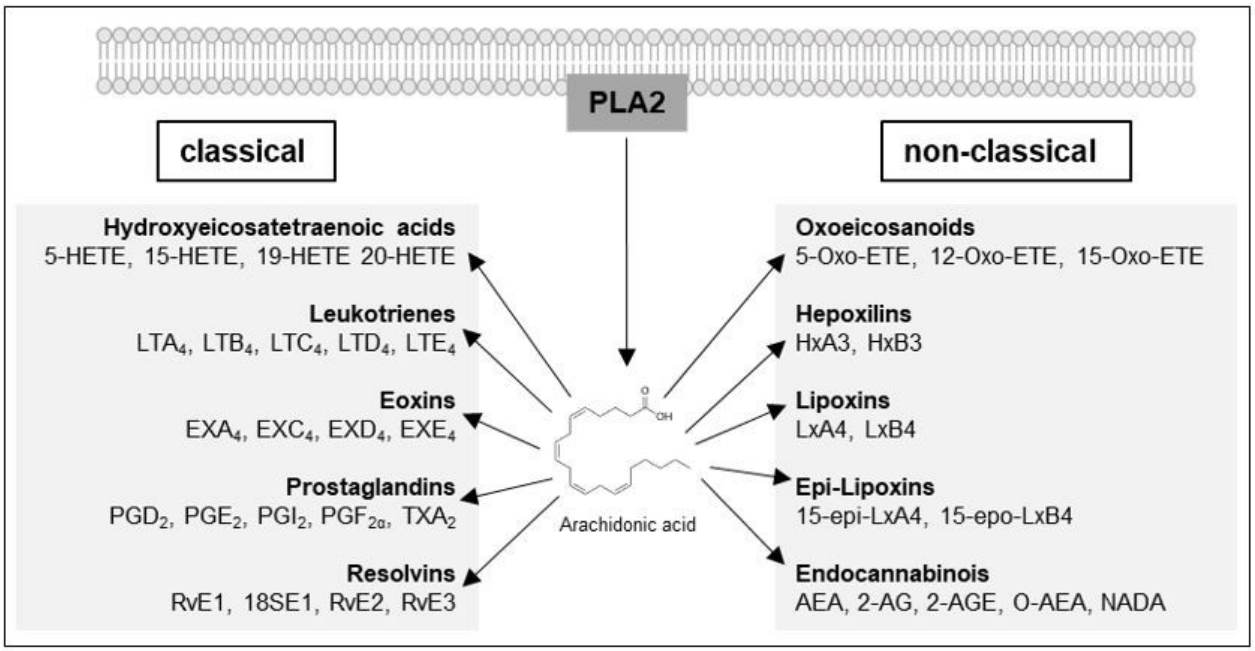

Figure 1: Overview of Classical and Non-Classical Eicosanoids

COX enzymes catalyze arachidonic acid twice, one time through their cyclooxygenase activity, resulting in the oxygenation of arachidonate (producing prostaglandin G_2) and a second time through their peroxidase activity (producing prostaglandin H_2), resulting in a substrate available for prostaglandin synthases [reviewed in (4)]. An overview of these PG synthases, their products and the receptors, which those products bind to, is provided in Table 1.

Table 1: Overview of Prostaglandin Synthases and Receptors

Prostanoid	Prostaglandin synthase	Targeted receptors
PGD_2	hPGDS L-PGDS	DP1, DP2
PGE_2	cPGES mPGES-1 mPGES-2	EP1, EP2, EP3, EP4
PGI_2	PGIS	IP
$PGF_{2\alpha}$	PGFS	FP_A, FP_B
$TXA_{2\alpha}$	TXS	TPα, TPβ

While COX-1 is ubiquitously expressed in most tissues, COX-2 expression depends highly on the presence of pro-inflammatory stimuli such as lipopolysaccharides (LPS), tumor necrosis Factor α (TNF-α) or interleukin-1 (IL-1) and can be suppressed by glucocorticoids and anti-inflammatory cytokines. Induction often follows the NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) or MAPK (mitogen activated protein kinases) pathway. As prostaglandins participate strongly in inflammation, treatments with non-steroidal anti-inflammatory drugs (NSAIDs) are often prescribed to alleviate fever and pain [reviewed in (5)]. However, outside of inflammation, prostaglandins participate in a variety of physiological processes such as gastrointestinal motility, blood pressure regulation or pregnancy, which is why PG synthase expression is highly regulated. In this part of the dissertation, all prostaglandins will be briefly introduced, however, PGD_2 will be the focal point of this chapter.

1.1.1. Prostaglandin D_2

Prostaglandin D_2 (PGD_2) is the product of PGH_2 conversion by two prostaglandin isomerases called hPGDS (hematopoietic Prostaglandin D synthase) and L-PGDS (Lipocalin-type Prostaglandin D synthase). hPGDS is, as its name suggests, expressed predominantly in cells of the hematopoietic system, including monocytes, macrophages, dendritic cells (DCs), eosinophils, basophils, Type-2 innate lymphoid cells (ILC2s), T-helper 2 cells (Th2) and mast cells [reviewed in (6)]. Outside of the immune system, hPGDS can be detected in epithelial cells, smooth muscle cells and chondrocytes (7–9). L-PGDS is expressed in the central nervous system (CNS), osteoblasts, chondrocytes, endothelial cells, fibroblasts, adipocytes and macrophages (10–16). PGD_2 function is multi-facetted and highly tissue and disease specific. While it exerts strong pro-inflammatory properties in allergic asthma, it has been reported to participate in the resolution of inflammation in peritonitis (17). As allergic asthma will be introduced in detail in Chapter 1.3, I will not focus on the role of PGD_2 to this disease in this chapter, but rather focus on its role in homeostasis, other diseases and its signalling. The prostaglandin binds two G-protein coupled receptors (GPCRs) named D-type prostanoid receptor 1 (DP1) and -2 (DP) with similar affinity, which will be discussed below.

1.1.1.1. D-type prostanoid receptor 1

DP1, coded by the PTGDR1 gene, is a surface GPCR that is widely expressed including eosinophils, basophils, DCs, monocytes, macrophages, platelets, vascular smooth muscle cells, in the intestine and the CNS, osteoblasts, osteoclasts, fibroblasts and chondrocytes (9,11,26,18–25). DP1 activation is best mapped out in eosinophils [reviewed in (27)]. Receptor binding results in a conformational change within the receptors structure, allowing adenylyl cyclase (AC) activation through the $G\alpha_s$ subunit. AC in turn increases intracellular cAMP (cyclic adenosine monophosphate), which is required as a substrate to activate the tetrameric protein kinase A (PKA), a cAMP-dependent serine/threonine kinase (28). PKA then enters the nucleus, where it phosphorylates cAMP response element binding proteins (CREB). CREB proteins can now bind to response elements on the DNA and induce gene regulations. There have also been reports of DP1 mediated calcium increase and the presence of a $G\alpha_{q/11}$ subunit, however, PKC signalling has not been observed so far (28,29). Contrary to DP2, which is considered a "functional" receptor that responds quickly (see 1.1.1.2), DP1 activation has long-term responses, including upregulation of anti-apoptotic proteins (30). An overview of DP1 signalling is displayed in Figure 2.

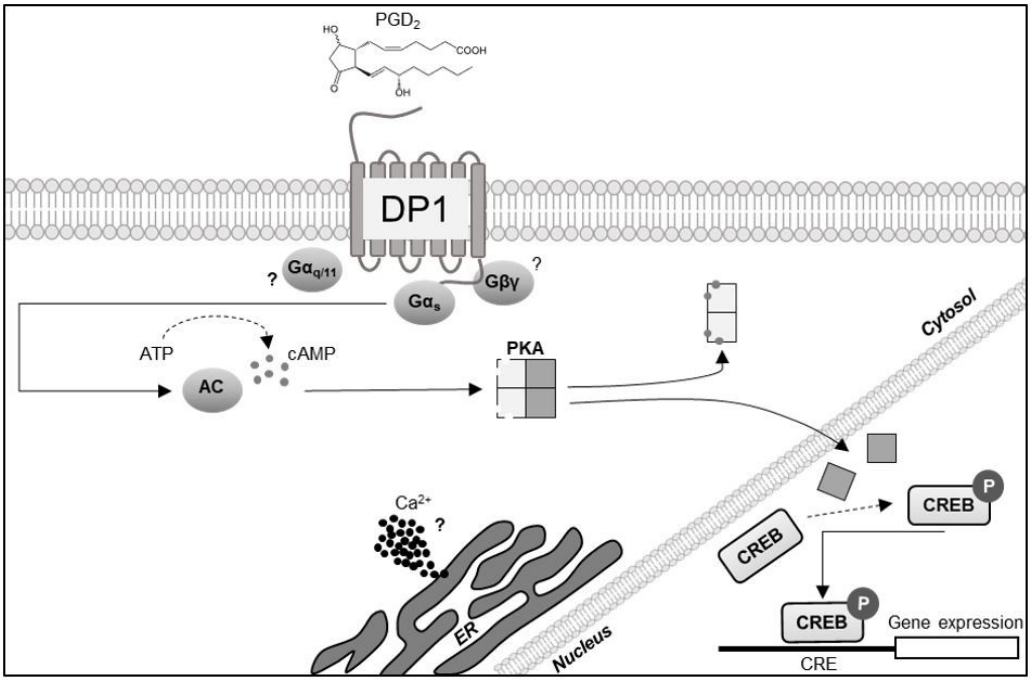

Figure 2: Signalling of D-type prostanoid receptor 1

DP1 is generally considered to be an anti-inflammatory receptor, as its blocking or deficiency often aggravate models of experimental inflammation [reviewed in (27)]. For instance, in experimental colitis, administering the DP1 selective agonist BW245C significantly reduced neutrophil inflammation (31). In a model of atopic dermatitis, DP1 activation reduced T cell activation and leukocyte recruitment into the dermis (32). DP1 deficient macrophages are prone to M1 differentiation (33). Contrary to this hypothesis, there are also reports of DP1 possessing inflammatory properties. Subjects carrying single nucleotide polymorphisms (SNPs) in DP1 seem to have a lower risk of developing allergic asthma (34). Receptor blockade can be achieved through binding of DP1 selective antagonist MK0524 (35).

1.1.1.2. D-type prostanoid receptor 2

DP2, also known as CRTH2 (Chemoattractant Receptor-homologous molecule expressed on T-Helper type 2 cells), PTGDR2, GPR44 or CD294 (cluster of differentiation), is the second cognate PGD_2 receptor. DP2 can be detected on the surface of many leukocytes, including eosinophils, basophils, Th2 cells, Type-2 cytotoxic T cells (TC2), monocytes, macrophages, ILC2, and mast cells (18,20,36–39). DP2 is also expressed in tissue cells, for instance in osteoblasts, osteoclasts, chondrocytes, keratinocytes or fibroblasts (11,25,40,41). As for DP1, most DP2 signalling research was performed in eosinophils.

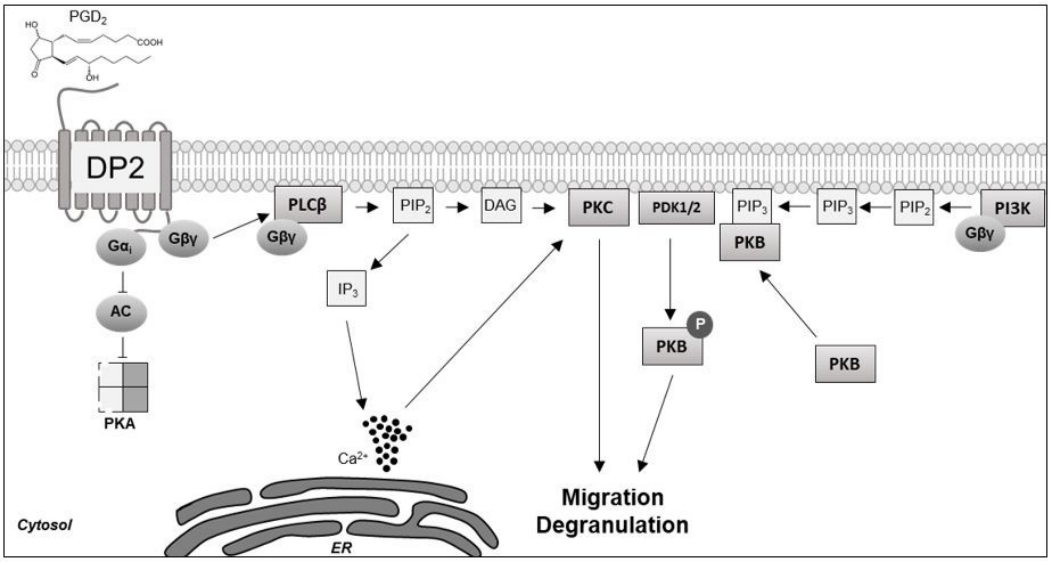

Figure 3: Signalling of D-type prostanoid receptor 2

DP2 activation results in conformational changes, causing the release of the Gα and Gβγ subunits of the G protein [reviewed in (27)]. Contrary to DP1, DP2 couples to $G\alpha_i$ subunit which suppresses AC activity (42). Gβγ in turn activates phospholipase C, resulting in calcium dependent PKC activation. Another mode of action of Gβγ is the phosphatidylinositol 3-kinase (PI3K) dependent PKB activation that has been observed in T cells (43). An overview of the known signalling is provided in Figure 3. Unlike DP1, DP2 is generally considered to be a pro-inflammatory receptor [reviewed in (44)]. In mice, it promotes macrophage dependent activation of neutrophils in a lung injury model and DP2 blocking improves symptoms of colitis, arthritis and skin inflammation (9,20,23,45,46). Receptor blockade can be achieved through binding of DP2 selective antagonists (e.g. OC-459 (47)), of which some have underwent clinical trials (48).

1.1.1.3. PGD_2 metabolism

PGD_2 has an *ex vivo* short half-life in plasma of 30 minutes and is then degraded enzymatically and non-enzymatically to biologically active metabolites (49). An overview of these metabolites and their targeted receptors is listed in Table 2.

Table 2: PGD_2 metabolites and their receptors

Type of degradation	Degrading enzyme	Product	Receptors	Literature
Enzymatically	Prostaglandin F synthase	11-epi-PGF_{2a}	DP1, DP2	(18)
	15-hydroxy Prostaglandin dehydrogenase	DK-PGD_2	DP2	(18)
Non-enzymatically	-	PGJ_2	DP1, DP2	(18)
		Δ^{12}-PGJ_2	DP1, DP2	(18)
		Δ^{12}-PGD_2	DP2	(50)
		15d-PGJ_2	DP2	(18)
		15d-PGD_2	DP2	(51)

These metabolites are often even further degraded to more stable isoforms. The most stable metabolite in urine is tetranor-PGDM which serves as a biological marker for PGD_2 synthesis (52).

1.1.1.4. DP1/DP2 interplay and the role of PGD_2 in inflammation

An interplay between both receptors, often termed the "DP1/DP2/PGD_2 axis", has been suggested, as both receptors bind PGD_2 with similar affinity and are known to co-localize on the cell membrane (18,29). However, as DP1 is often expressed to a smaller degree compared to DP2, it can be hypothesized that DP2 signalling can overshadow DP1 [reviewed in (27)]. Another factor that may contribute to this intricate signalling network is the availability of biologically active PGD_2 metabolites that prefer DP2 binding (see 1.1.1.3). Further, the receptors seem to depend on each other, as it was shown in a HEK293 cell line that calcium flux was DP2 mediated, but increased in the presence of DP1 expression and decreased when the latter was desensitized (29). How this axis contributes to inflammation, either through initiation, propagation or resolution, may depend on the tissue and the etiology of the disease. In 2007, a publication emerged in which Rajakariar and colleagues proposed a pro-resolving role for hPGDS derived PGD_2 in a murine peritonitis model (17). Although PGD_2 heavily accumulated in the initiation phase of inflammation, it induced the production of IL-10 and suppressed TNF-α and MCP-1 (Monocyte chemoattractant protein-1) and this regulatory role was lost in hPGDS KO mice – a process that was DP1 mediated. In the resolution phase of the inflammation, hPGDS KO mice failed to clear macrophages from the peritoneal cavity but this effect could be restored by BW245C treatment. While the pathological potential of PGD_2 in allergic asthma is evident (as discussed in Chapter 1.3.3), the prostaglandin may exert anti-inflammatory properties in other diseases.

1.1.2. Other prostaglandins and lipid mediators

PGE_2 is the most abundant prostaglandin and an ubiquitously expressed homeostatic regulator of blood pressure and cardiovascular homeostasis, kidney function, gastrointestinal integrity and fertility [reviewed in (2)]. PGE_2 is synthesized by three isoforms of PGE synthase, i.e. the cytosolic and ubiquitous cPGES, along with two membrane-associated isoforms (mPGES-1, associated with the nucleus and mPGES-2, associated with Golgi). cPGES is essential for development and predominantly couples with COX-1, suggesting that it plays a crucial role in maintaining homeostatic conditions, contrary to COX-2, which is upregulated during inflammation (53). mPGES-1 expression can be induced or suppressed by cytokines, indicating its important role in

inflammation (54). mPGES-1 deficient mice show a decreased severity in arthritis (55). mPGES-1 derived PGE_2 is most likely the culprit in the observed signs of inflammation, including redness and swelling, induced by vasodilation and increased vascular permeability, hyperalgesia, induced through expression of PGE receptors in the CNS, and fever, through its functioning as a pyrogen [reviewed in (56)]. The least well understood PGE synthase is mPGES-2, which is ubiquitously expressed and has no preference towards either COX-1 or -2. Deficient mice show no apparent phenotype, which is owed to the compensatory PGE_2 production of cPGES and mPGES-1 (57). PGE_2 exerts its effects through four GPCRs, named EP1, EP2, EP3 and EP4. Whereas the first two are scarcely expressed and show low affinity to the prostaglandin, EP3 and EP4 are widely distributed [reviewed in (58)]. PGE_2 possesses both pro- and anti-inflammatory functions and the impact of PGE_2 on inflammation may depend on expression of its cognate receptors in the tissue. For instance, autocrine PGE_2 activation as a result of IL-23 exposure drives pro-inflammatory Th17 differentiation in murine T cells (59). However, in monocytes, autocrine PGE_2 activation as a result of treatment with Imatinib, a protein kinase inhibitor, resulted in EP4 mediated suppression of TNF-α, IL-6 and IL-8 production (60).

PGI_2, better known as prostacyclin, is an important regulator of the cardiovascular system. It is produced by endothelial cells and vascular smooth muscle cells through PGI synthase [reviewed in (2)]. Prostacyclin is a strong vasodilator and inhibitor of platelet aggregation and adhesion of circulating immune cells (61). Its cognate receptor, IP, is expressed in the vasculature of many different organs, including kidney, liver and lung [reviewed in (62)]. Prostacyclin appears to play a beneficial role in inflammation, as it accumulates in the lung of asthmatics and IP deficient mice display more inflammation in an OVA model of experimental asthma as compared to WT mice (63,64). Similar reports have emerged in models of fibrosis, liver damage, experimental arthritis and others [reviewed in (65)].

$PGF_{2\alpha}$ is another arachidonic acid derived lipid mediator and is mostly associated with pregnancy [reviewed in (2)]. The prostaglandin is synthesized by PGF synthase and binds two distinct GPCRs called FP_A and FP_B. PGFS is mostly expressed in organs of the reproductive system, where it participates in ovulation, luteolysis and induction of labour. There are few reports available that link this prostaglandin to inflammation. In a model of bleomycin-induced pulmonary fibrosis, FP deficient mice showed reduced severity and inflammation compared to WT animals

(66). The group showed further that elevated levels of the prostaglandin were detected in the BAL (bronchoalveolar lavage) fluid of patients suffering from idiopathic pulmonary fibrosis. In a more recent report, it was shown that PGF_{2a} can induce production of pro-inflammatory cytokines and chemoattractants in human uterine smooth muscle cells (HUSMCs), isolated from pregnant women (67). Similar to PGE_2 and PGI_2, also PGF_{2a} is elevated in joint diseases like rheumatoid arthritis (68).

TXA_2 is a very short-lived thromboxane that is involved in cardiovascular homeostasis. It is produced by activated platelets and acts on a GPCR called TP, expressed in two isoforms TPα and TPβ [reviewed in (2)]. TXA_2 activates platelets and induces their aggregation, making it a key player in homeostasis. The thromboxane is synthesized by Thromboxane A synthase (TBXAS), which is mostly expressed in platelets, but also in monocytes and macrophages (69–71). It is therefore conceivable that TXA_2 participates in inflammation through the physiological changes it introduces, namely clot formation. After 30 seconds, TXA_2 is converted non-enzymatically to the biologically inactive TXB_2. Its role in inflammation has not been thoroughly elucidated. As a strong vasoconstrictor, it contributes to acute renal failure in an LPS induced sepsis model (72). TXB_2 is further metabolized enzymatically to a series of compounds, of which 11-dehydro-TXB_2 is the major product found both in plasma and urine. Of note, it has been shown previously that 11-dehydro-TXB_2 is a full agonist of the DP2 receptor and hence might cause DP2 activation in cellular contexts where PGD-synthase is not present (73).

5-OxoEXE is another arachidonic acid derivate and a strong chemoattractant for granulocytes. Contrary to prostaglandins, it belongs to the group of non-classical eicosanoids. It is synthesized via the 5-lipoxygenase pathway [reviewed in (74)]. It attracts eosinophils, neutrophils, basophils and monocytes and has been associated with asthma and rheumatoid arthritis (75,76). 5-OxoETE binds to the OXE receptor, which is present on platelets, leukocytes and cancer cells [reviewed in (74,77)]. Besides its effect as a chemoattractant, 5-OxoETE induces actin polymerization, calcium release, degranulation and CD11b upregulation in eosinophils and may act in concert with PGD_2 [reviewed in (78)]. Similar effects have been reported in neutrophils. However, in primed neutrophils, the lipid mediator induces a stronger production of superoxide and enhanced degranulation, suggesting that it may contribute to inflammation.

1.2. Apolipoprotein A-IV

Dietary lipids, besides proteins and carbohydrates, are essential sources for maintaining bodily functions like protein biosynthesis, cell proliferation, clearance of apoptotic cells, immune responses and communication between tissues and cell types. Due to their lipophilic character, lipids cannot be freely distributed through the circulation to reach their designated tissues but require an intricate transport mechanism. This transport is facilitated either by albumin, which binds directly free fatty acids (FFAs) or by a group of amphiphilic proteins called apolipoproteins (from Greek ἀπό, translating to "away" or "off"), that exert a range of metabolic functions, including acting as structural proteins, ligands for receptors or activators or inhibitor of enzyme reactions. One of those proteins is Apolipoprotein A-IV (ApoA-IV) and its physiological impact on the immune system, which exceeds its role as a metabolic regulator will be discussed in this chapter.

1.2.1. Dietary lipids, digestion and lipoproteins

Digestion of lipids depends on enzymes called lipases, which are present in saliva in the mouth and in the digestive juice secreted by the pancreas and endothelium derived lipases [reviewed in (79,80)]. While lipid digestion starts in the mouth and continues in the stomach, the latter being the major site for fat-soluble vitamin emulsification, most of lipid digestion occurs in the duodenum when the contents of the stomach are mixed with bile and pancreatic juice. As triacylglycerides (TAGs) cannot be directly absorbed by the intestine, the molecules are broken down to monoacylglycerides (MAGs) and FFAs. Activity of the pancreas lipase requires emulsification of those TAGs, which is enabled through bile salts. An impaired efficiency of bile-mediated emulsification results in reduced lipid absorption (81). These newly formed micelles, which consist of an amphiphilic exterior (phospholipids, FFAs, MGs) and a lipophilic core (free sterols, cholesteryl esters, fat-soluble vitamins, TAGs), are then absorbed by enterocytes in the jejunum, while the bile salts are reabsorbed in the ileum. In the endoplasmic reticulum of these cells, TAGs are resynthesized, and micelles are loaded with apolipoproteins. These newly formed particles are known as chylomicrons and vary in size between 100 and 1000 nm. Due to their size, they cannot

be directly taken up by liver cells and therefore enter circulation directly through the lymphatic system. In the capillaries, the TAGs within the chylomicrons are then hydrolysed by endothelial cell derived lipoprotein lipases (LPLs) and the resulting FFAs are taken up by adipocytes and muscle cells, a process that depends on fatty acid transport proteins (FATPs) and CD36. Following the TAG release, the smaller chylomicron remnants can be taken up by the liver.

The chylomicron remnants are rich in cholesteryl esters (CE), which are used for the production of bile acid. The remaining cholesterol, along with newly synthesized TAGs (derived from a surplus of glucose after a meal) are then redistributed in the blood in a similar way as chylomicrons: newly formed very low-density lipoproteins (VLDL) are secreted and supply fat- and muscle cells with TAGs, resulting in intermediate lipoproteins IDL. IDLs can scavenge cholesterol from the blood, converting them into low-density lipoproteins (LDL) or return to the liver in a similar fashion as chylomicron remnants. As most cells express the LDL receptor, these particles are highly mobile and accessible stores of cholesterol and CEs, which can be used for cellular membranes, steroid hormones or bile salts.

Table 3: Overview of Lipoproteins

Lipoprotein	Size	Source	Half-Life	Contents
Chylomicrons	100 – 1000 nm	Intestine	15 – 20 min	Apolipoproteins, TAGs, cholesterol
Chylomicron remnants	30 – 50 nm	Circulation	15 – 20 min	Apolipoproteins, cholesterol
VLDL	50 nm	Liver	2 – 4 h	Apolipoproteins, TAGs, cholesterol
IDL	20 nm	Circulation	2 – 4 h	Apolipoproteins, cholesterol
LDL	20 nm	Liver	2 days	Apolipoproteins, cholesterol
HDL	10 nm	Liver	4 days	Apolipoproteins, cholesterol

Contrary to TAG hydrolysis, which can be performed by almost any cell type, cholesterol is difficult to break down. The body excretes cholesterol through desquamation of mucosa cells that contain the lipid and through production of bile acids in the liver. To prevent accumulation, the body is tasked with constant surveillance of cholesterol quantities through production of High-density lipoproteins (HDL). HDL synthesis starts in the liver through synthesis of ApoA-I, which is then loaded with cholesterol and phospholipids. These nascent HDL molecules are secreted into

the blood stream, where they mature through uptake of apolipoproteins and cholesterol. Therefore, HDL is tasked with the removal of peripheral cholesterol to resupply the liver for bile acid production. An overview of lipoproteins is provided in Table 3. Apolipoproteins are essential components in this intricate process of TAG and cholesterol distribution in tissues and will be introduced below.

1.2.2. Apolipoproteins and their functions

$ApoB_{48}$ in one of the first apolipoproteins that is loaded in the intestine [reviewed in (80)]. Upon entering the circulation, these newly formed chylomicrons receive HDL-derived ApoC-I that prevents initial resorption in the liver, ApoC-II, which is an essential co-factor for LPL in the periphery and ApoE, which promotes endocytosis and will be important after TAG depletion. After TAGs are removed, the chylomicron remnant can now be resorbed by the liver, facilitated by binding of ApoE to the LDL receptor on liver cells. The remaining cholesterol that cannot be used up by the liver is now repacked into VLDL and loaded with $ApoB_{100}$, ApoC-II and ApoE and reintroduced into the circulation. VLDL are a second source of TAGs and after depletion, their remnants (IDL) either are resorbed by the liver or remain in the blood as much denser LDL.

Table 4: Overview of Apolipoproteins [reviewed in (80)**]**

Protein	Size	Source	Lipoprotein	Function
ApoA-I	28 kDa	Liver, intestine	HDL, chylomicrons	LCAT activator
ApoA-II	17 kDa	Liver	HDL, chylomicrons	HTGL activator
ApoA-IV	46 kDa	Liver, intestine	HDL, VLDL, chylomicrons	?
ApoA-V	39 kDa	Liver	HDL, chylomicrons	Co-factor for LPL
$ApoB_{48}$	241 kDa	Liver	Chylomicrons	unknown
$ApoB_{100}$	512 kDa	Liver	VLDL, IDL, LDL	Promotes endocytosis
ApoC-I	6.6 kDa	Liver	HDL, IDL, VLDL, chylomicrons/remnants	Inhibits liver resorption
ApoC-II	8.8 kDa	Liver	HDL, VLDL, chylomicrons	Co-factor for LPL
ApoE	34 kDa	Liver	HDL, IDL, chylomicrons/remnants	Promotes endocytosis

Now, the $ApoB_{100}$ is freely available as a ligand for the ubiquitous expressed LDL receptor, while ApoC and E are transferred onto HDL. LDLs remain in circulation for days, where they either supply cells in the periphery with cholesterol or are resorbed by the liver. HDL on the other hand serves various tasks. It can redistribute cholesteryl esters between chylomicrons/VLDL and LDL or TAGs between LDL and VLDL, it is essential for distribution of apolipoproteins C and E on lipid particles and lastly, the most important role is the reverse transport of cholesterol to the liver, facilitated by lecithin–cholesterol acyltransferase (LCAT). On its way to the liver, HDL supplies scavenger receptor-carrying cells locally with cholesterol. An overview of apolipoprotein functions is listed in Table 4. A major source of this chapter was the excellent review on lipid metabolism by Feingold (80).

1.2.3. ApoA-IV metabolism

ApoA-IV is a 46 kDa amphipathic protein that plays an important role in lipid metabolism [reviewed in (82)]. It has first been described in humans in chylomicrons and plasma and shortly after also in VLDL (83,84). While only a small fraction of plasma ApoA-IV is found on HDL and chylomicrons in fasted conditions, this can increase postprandially. The majority of plasma ApoA-IV is found in the lipid-free fraction (84). When chylomicrons are formed, they are loaded with ApoA-IV and enter the circulation. During TAG hydrolysis, most ApoA-IV molecules dissociate into plasma and whereas only 25 % are taken up by HDL, to reach higher stability (85). The ApoA-IV in the lipid-free fraction may promote HDL maturation (86). ApoA-IV is encoded by the APOA4 gene and several isoforms have been detected in humans (87). The intestine is the major site of ApoA-IV synthesis, but it may also occur in the liver (88). Outside of these tissues, ApoA-IV can be detected in the brain of rats where it is involved in energy homeostasis (e.g. glucose metabolism) and surprisingly, mRNA can also be detected in dendritic cells (89,90). Expression may be regulated by the cAMP response element pathway (90,91). The signalling of ApoA-IV has not been thoroughly elucidated as of yet and many questions, for instance, how it enters the cell, have not been answered. A possible target for ApoA-IV has been identified in liver cells, where the apolipoprotein reduced gluconeogenesis through the nuclear receptor NR1D1 (Nuclear receptor subfamily 1, group D, member 1), also known as Rev-ErbA alpha (92,93). This receptor

participates in circadian rhythm, including the sleep/wake cycle, blood pressure, body temperature and metabolism [reviewed in (94)]. Additionally, it was shown to modulate ApoA-CIII expression in rat hepatocytes and decrease toll-like receptor 4 (TLR4) expression in macrophages (95,96). ApoA-IV catabolism appears to occur in the liver and in the kidneys. In rats, serum HDL-ApoA-IV has a short half-life of roughly 8.5 hours. Four hours post-injection it starts to accumulate in the kidneys and the liver, the latter being the major catabolic site (97). In humans, ApoA-IV is at least partially degraded in the kidneys and excreted via urine (84,98).

1.2.4. Regulation of metabolism

ApoA-IV is strongly involved in a broad range of metabolic functions that exceed its role as a structural component of chylomicrons and HDL. First studies with ApoA-IV transgenic mice discovered that the animals were protected from developing atherosclerosis (99). Since this finding, many reports have emerged that reinforced ApoA-IV as a potent anti-atherogenic player in lipid metabolism. For instance: the protein enhanced cholesterol efflux in macrophages generated *in vitro* and this finding was later confirmed *in vivo* in mice (100,101). In ApoA-IV transgenic mice, LDL oxidation was significantly reduced, suggesting an anti-oxidative role (102). Further, ApoA-IV seems to be involved in glucose metabolism, as stimulated pancreatic islets responded to high glucose with a stronger insulin production, indicating that it may be crucial for blood glucose control as displayed in KO models (103). The group showed that ApoA-IV$^{-/-}$ mice show defective insulin production when fed with a high-fat diet (HFD). Other functions, like changes in intestinal lipid absorption or suppression of gluconeogenesis have been reported [reviewed in (82)]. ApoA-IV production depends highly on dietary lipids, as its mRNA levels are reduced during fasting conditions, but can be re-induced with a HFD (104). The discovery that ApoA-IV is expressed in the hypothalamus led to a series of animal experiments, where it was shown that a HFD does not affect intestinal or plasma ApoA-IV expression, but decreases hypothalamic ApoA-IV mRNA (105). Interestingly, this effect was due to the diet-induced obesity and not the high lipid content. Serum ApoA-IV levels increase during the dark phase in rats and hence partake in circadian rhythm, along with its assumed receptor NR1D1 (94,106). The observation that the protein is expressed in the hypothalamus and increases during the dark phase led to the discovery of ApoA-

IV being a satiety factor (107,108). These findings indicate that the impact of ApoA-IV exceeds much further than just lipid metabolism.

1.2.5. Regulation of the immune system

In atherosclerosis, macrophages ingest cholesterol, but due to a lack of feedback regulation, this uptake continues until they burst, releasing danger signals and causing inflammation. Through reverse cholesterol transport, HDL prevents this and is hence considered an anti-inflammatory mediator. However, HDL exerts also more direct anti-inflammatory features: it acts as an antioxidant to LDL and inhibits adhesion molecule expression on endothelial cells, thus reducing monocyte recruitment [reviewed in (109)]. This led to the discovery that apolipoproteins themselves exhibit anti-inflammatory properties, ApoA-I being the most studied member of this group (110,111). In 2004, Vowinkel and colleagues showed that ApoA-IV delayed the onset and decreased the severity of experimental colitis in mice (112). Since then, a growing number of reports about its protective role in allergy started to emerge (discussed in Chapter 1.3.4).

1.3. Allergic Asthma

Allergic asthma is a lung disease in which the immune system responds excessively to an exogenous environmental trigger with inflammation. The prevalence of asthma is very high in first world countries compared to more rural regions and is also increased in urban areas (113,114). In children, incidence is higher among boys but in adolescence, it increases in women (115,116). Similar to autoimmune disorders, a genetic predisposition which increases susceptibility and environmental factors to trigger the onset of the disease have been suggested. The word "asthma" derives from Greek and means short of breath and was first used by Henry Hyde Salter in the 19th Century [reviewed in (117)]. He published a book that depicted accurate illustrations of the affected lung. Its clinical symptoms were first described by Sir William Osler in 1892 and described bronchial muscle spasms, swelling of the bronchial mucous membrane and inflammation.

1.3.1. Development of Asthma

Although several forms of asthma have been identified, the focus of this Chapter will be on “Th2-high” asthma which involves eosinophils and Th2 associated cytokines. The dated dichotomy of T-cell immune responses distinguished Th1 cell mediated immunity, aiming to defend against intracellular pathogens and Th2 humoral mediated immunity, which aims to deal with exogenous parasites. While dysregulation of Th1 and the later identified Th17 are associated with autoimmune diseases, a perturbed Th2 response has been linked to the development of allergic asthma. Several stages of asthma development have been identified, namely the initiation or sensitization phase, in which the body recognizes a foreign antigen, the allergic reactions in which the body responds to these antigens and a chronic stage, in which tissue remodelling occurs, severely impairing lung function [reviewed in (118)].

1.3.1.1. Sensitization

Sensitization occurs when an allergen is presented by antigen presenting cells (APCs) to T cells, resulting in antibody production. These allergens often share similarities with enzymes expressed in parasites which enable them to enter tissues [reviewed in (119)]. While this indicates that the

risk to develop asthma depends on the type of allergen, it also depends on genetic factors, such as genes that regulate epithelial permeability [reviewed in (120)]. Given the presence of allergens and a permissive epithelium, the molecule can be taken up by DCs, which will then mature and migrate to adjacent lymph nodes to present the antigen to naïve T cells. Antigen presentation is facilitated when the T-cell receptor recognizes an epitope on the presented antigen, which is embedded in the MHC II molecule on the surface of the DC. Under the influence of IL-4 and several activation signals, the T cells will differentiate into the Th2 subset. These signals depend on upregulation of DC specific molecules, including CD80/CD86 (B7) which binds to the corresponding CD28 molecule on the T cell, as well as CD40 on the DC, which binds to CD40L on the T cell. The T cell will then in turn activate B cells, which works in a similar fashion via MHC II/TCR interaction and CD40/CD40L and B7/CD28 complex formation. Th2 cell derived IL-4 and IL-13 induce a class switch in B cells, resulting in the production of IgE antibodies. These newly secreted antibodies will re-enter circulation and bind on the surface of mast cells, basophils and eosinophils via the IgE specific antibody receptor FcεRI, thus rendering the organism sensitized for the allergen [reviewed in (121,122)].

1.3.1.2. Early-phase reactions

Upon re-exposure to the sensitized antigen, an early-phase reaction occurs within seconds to minutes [reviewed in (118)]. The IgE molecules present on saturated mast cells recognize their binding epitope on allergens that re-entered the system, which results in FcεRI cross-linking. This process in turn, activates the mast cell to release great amounts of pre-formed lipid mediators (e.g. prostaglandins and leukotrienes), biogenic amines (e.g. histamine) and cytokines (e.g. TNF-α) via their mast cell granules [reviewed in (123)]. The effect of these mediators is immediate, resulting in bronchoconstriction and vasodilation, which will temporarily impair lung function. Concomitantly, cross-linking also induces the production of cytokines, chemokines and growth factors, which will induce the late-phase reaction.

1.3.1.3. Late-phase reactions

As mentioned above, IgE activated mast cells are also a great source of lipid mediators, histamine, cytokines, chemokines and growth factors. However, only a fraction of these are pre-formed and

stored in mast cell granules, while many other signal molecules will be synthesized and secreted in a delayed manner, resulting in the biphasic response of mast cells, which occurs between 2 to 6 hours after allergen exposure [reviewed in (118,124)]. These mediators include cytokines like IL-1β, IL-4, IL-5, IL-6 and IL-13, reinforcing the Th2 milieu in the inflamed lung by attracting and maintaining high levels of recruited leukocytes. This process is fostered by an increase in vascular permeability and upregulation of adhesion molecules in the endothelium.

1.3.1.4. Chronic phase of allergic asthma

The continuous production of cytokines and chemokines will attract leukocytes to the lung, including granulocytes (eosinophils, neutrophils, basophils) and monocytes. Local activation of these cells will result in degranulation – a process in which a range of proteolytically active enzymes are released. These proteins, which aim to kill off large pathogens during parasite infections, will then cause tissue damage to the surrounding epithelium [reviewed in (118)]. A continuous cycle of re-exposure to sensitized allergens will result in tissue remodeling: Goblet cells, which are responsible for mucus production in the lung, will increase in numbers, subsequently resulting in elevated mucus secretion. Granulocyte derived enzymes will cause cell death in epithelial cells, thus decreasing barrier function. Lung function will be impaired: Fibroblast and myofibroblast metaplasia will result in the expansion of the *lamina reticularis*, a connective tissue consisting of reticular fibers and collagen which serves the role of a stabilizing structure in the lung. The continuous bronchoconstriction results in the proliferation of airway smooth muscle (ASM) cells, further impairing lung compliance [reviewed in (125)].

1.3.1.5. Eosinophils in allergic asthma

Eosinophils are competent drivers of lung inflammation and remodeling. Their pathogenic contribution was already known in the early stages of asthma research, as correlation studies between blood counts and disease severity were published (126). Blood eosinophils are recruited by a range of cytokines and inflammatory mediators, including PGD_2 and eotaxins (127). Like neutrophils and basophils, eosinophil cytoplasm contains basic granules which contain toxic proteins. These proteins include major basic protein (MBP), eosinophil cationic protein (ECP),

eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN) and degranulation of these proteins promotes severe damage to the surrounding tissue [reviewed in (128)]. Additionally, eosinophils can produce reactive oxygen species, leukotrienes, prostaglandins and cytokines (126,129–131). As eosinophils are short-lived, they constantly depend on the presence of pro-survival signals such as IL-5, IL-3 or GM-CSF (granulocyte-macrophage colony-stimulating factor) (132–134). Targeting these survival signals has been proven a successful treatment strategy that led to the approval of IL-5 antibodies mepolizumab and reslizumab, and IL-5 receptor antibody, benralizumab [reviewed in (135)].

1.3.2. Treatment of Asthma

Generally, four stages of asthma have been characterised, corresponding to disease severity. A major contributor to shortness of breath during an asthma attack is smooth muscle constriction, a process that depends on intracellular Ca^{2+} increase and inhibition of cAMP production [reviewed in (136)]. Bronchoconstriction can be induced through activation of several receptors that are present on the surface of ASM cells, including acetylcholine receptors (M_2, M_3), adenosine receptors (A_1) and leukotriene receptors (CysLT1) [reviewed by (137–139)]. Opposing this response, is the beta-adrenergic receptor, which acts via the cAMP pathway, causing relaxation of ASM (136). Bronchodilation can therefore be achieved by either blocking the former receptors or activating the latter. Agonists for the beta-adrenergic receptor are so-called β2 mimetics and include short-acting- (SABA), long-acting- (LABA) and ultra-long-acting- (uLABA) β2 agonists [reviewed in (140)]. Use of these mimetics depends on the asthma stage that has been diagnosed. In more severe cases, the use of glucocorticoids, which suppress cytokine production or biologics (e.g. targeting IgE or IL-5) can be prescribed [reviewed in (141,142)].

1.3.3. Prostaglandin D_2 in Asthma

IgE-mediated cross-linking of FcεRI on the surface of mast cells leads to degranulation and PGD_2 is the major prostaglandin released in this process (143). Expression of hPGDS has been observed in a range of leukocytes that participate in the development of asthma, including mast cells, DCs, eosinophils, basophils, neutrophils, Th2 cells, ILC2s, monocytes and macrophages [reviewed in (6)]. In DCs, it affects maturation through upregulation of B7 and enhancing production of Th2 cytokines, while at the same time, it inhibits DC migration via DP1 (19,144,145). In mast cells, DP2 is expressed intracellularly, however PGD_2 induced calcium release seemed to be DP2 independent (39). Eosinophils are recruited via PGD_2, a process that is DP2 mediated and results in shape change, calcium release and chemokine production (18). In contrast, eosinophil survival is enhanced through DP1 (18,30). Basophils are also recruited via DP2 and degranulate in response to receptor activation (146). In monocytes, both receptors are expressed on their surface and one group showed that these cells migrate towards PGD_2, although the chosen concentrations were very high (19,20). This expression is not lost when these cells differentiate into macrophages and PGD_2 serves as a chemoattractant and influences macrophage polarization (20,33,147). In the lymphocyte fraction, the DP2 receptor is present on Th2 cells, TC2 and ILC2, where it promotes chemotaxis and production of Th2 associated cytokines (18,38,148). Physiologically, PGD_2 is associated with the observed processes that favor tissue remodeling, including vasodilatation, bronchoconstriction, smooth muscle contraction and proliferation and indirectly goblet cell hyperplasia (21,149–152). For the interested reader, additional reviews are provided (6,153–155).

1.3.4. ApoA-IV in Asthma

Emerging evidence indicates a strong relation between apolipoproteins and the development of allergy. In 2006, hypercholesterolemia has been identified as a potential asthma risk factor, as asthmatics show a significant increase compared to healthy controls (156). This risk appears to be inversely related to levels of serum TAGs and non-HDL cholesterol (meaning all lipoprotein fractions except HDL), as both parameters are reduced in asthmatic patients (157). A closer look at apolipoprotein levels revealed a correlation between high plasma ApoA-I concentrations and

wheezing (158). Although this suggests that ApoA-I contributes to the symptoms of allergic asthma, Yao and colleagues showed that ApoA-I mimetics attenuate house dust mite (HDM)-induced airway hyperresponsiveness in mice (159). The group also identified the beneficial role of ApoE in experimental asthma, as ApoE deficient mice displayed increased airway inflammation, airway hyperreactivity and goblet cell hyperplasia, which could also be alleviated by an ApoE mimetic peptide. The positive effect of ApoA-I was later confirmed in humans, as serum levels of the protein correlated positively with a higher FEV_1 (forced expiratory pressure in 1 second) in atopic asthma (160). More studies followed, that indicated a lung-protective role for ApoA-I and ApoE [reviewed in (161)]. The possible mechanism underlying these findings is the function of apolipoproteins as inhibitors of inflammation. HDL and particularly ApoA-I possess strong anti-inflammatory properties (109–112). As ApoA-IV is structurally and functionally similar to ApoA-I, reports started to emerge that observed a similar property in this apolipoprotein. In ApoE deficient mice, ApoA-IV treatments protected the animals from LDL oxidation (102). This anti-atherogenic effect was confirmed in another $ApoE^{-/-}$ mouse experiment, in which human ApoA-IV significantly reduced pro-inflammatory cytokine levels after LPS treatment and increased oxidized LDL (oxLDL) autoantibodies (162). The group showed further *in vitro* that ApoA-IV reduced TNF-α production in LPS-stimulated monocytes, isolated from human PBMC. In 2010 it was shown that ApoA-IV serum levels rise in patients suffering from allergic rhinitis in response to sublingual immunotherapy (SLIT) and that ApoA-IV reduced histamine release from basophils *in vitro* (163). This finding led to our own publication in 2019, in which we show that human recombinant ApoA-IV inhibits eosinophil responsiveness and chemotaxis *in vitro* and suppresses airway hyperresponsiveness and airway eosinophilia *in vivo*. Moreover, ApoA-IV protein levels decrease in serum of allergic patients(1).

1.4. Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is an autoimmune disorder that affects the joints and leads to chronic inflammation, causing pain and decreased motility in people affected. Both words date back to ancient Greece: arthritis describes inflammation within the joints ("*arthra*") and rheuma translates to "to suffer from a flux", i.e. the release of bodily fluids. Paleopathological investigations discovered that bone erosion date back until 4000 BCE, however, it is difficult to distinguish between naturally occurring damage, RA and other joint diseases [reviewed in (164)]. For a long time, RA was not recognized as its own entity but attributed to the "rich man's disease" gout, which was already described in ancient Egypt. In the year 1800, French surgeon Jacob Landré-Beauvais discovered a distinct form of gout that affected the poor, with a higher incidence in women. This led to his dissertation in which he termed the disease "asthenic gout" (165). Roughly sixty years later, English physician Alfred Baring Garrod re-named Landré-Beauvais' discovery rheumatoid arthritis (166).

RA affects approximately 0.5 to 1 % of the world population and as autoimmune diseases are on the rise, this number may be growing in the near future (167). The three times higher incidence in women that was already described by Landré-Beauvais can be attributed to decreasing estrogen levels after menopause, as this sex hormone seems to possess anti-rheumatic properties [reviewed in (168)]. Disease onset occurs mostly within or after the sixth decade in life, however, RA markers can be measured in the pre-clinical phase (169). As proposed in a review, these markers may pre-date disease onset by up to 15 years (170). Clinical symptoms are mostly visible in the joint: bone erosion and cartilage degradation lead to a severe impairment of motility. Outside of the joint, RA affects the brain, the cardiovascular system, kidneys, lung, liver and other organs, however, these manifestations are less pronounced [reviewed in (171)].

1.4.1. Epidemiology and diagnosis of RA

As mentioned above, the overall world prevalence lies within 0.5 to 1 %. However, as paleopathological observations are difficult to interpret, it is not clear whether the disease is as old

as humankind or just developed recently. There are three schools of thoughts about its origin: the "recent origin" view argues that as RA has been only thoroughly described 200 years ago, it may be a new disease that developed due to exposure to environmental stimuli that were not present in ancient times. The "ancient origin" suggests that already our ancestors were affected by RA and this is based on the already mentioned paleopathological investigations in exhumed skeletons, but also corroborated by depictions of RA-like deformations in Renaissance artwork and mentions in ancient texts. A third school of thought is the "New World to Old World" view that suggests that RA developed in indigenous populations and spread to Europe (164). Diagnosis is based on physical examinations, leading to a RA score. An overview of the criteria is listed in Table 5.

Table 5: Overview of the 2010 Classification Criteria for Rheumatoid Arthritis(172)

Criteria	Description	Score
Morning stiffness	Clinical synovitis (joint swelling)	NA
Joint involvement	1 large joint	0
	2-10 large joints	1
	1-3 small joints	2
	4-10 small joints	3
	>10 joints	5
Serology	Negative RF and negative ACPA	0
	Low-positive RF or ACPA	2
	High-positive RF or ACPA	3
Acute phase reactants	Normal CRP and ESR	0
	Abnormal CRP and ESR	1
Duration of symptoms	<6 weeks	0
	≥6 weeks	1

The criteria in Table 5 are an updated version of the original RA classification score of 1987 (173). Although the previous classification criteria were sufficient to distinguish RA patients from patients that were affected by other joint diseases, it failed to diagnose patients in early phases, in which therapies would be most effective. The 2010 update enabled physicians to diagnose patients that pre-date clinical symptoms. One such criterion was the inclusion of anti-citrullinated protein antibodies (ACPAs): autoantibodies that bind epitopes carrying the amino acid citrulline. The

serology before 2010 only included rheumatoid factor (RF); autoantibodies that bind the Fc region of IgG antibodies. Both groups of antibodies can be present or absent in RA and their presence can predate disease onset by many years (169,170). While the RA classification criteria score is used for diagnosis, the disease activity score (DAS28) enables a validated and comparable observation of disease progression, for instance in response to therapies (174).

A specific cause for RA has not been identified. It is our understanding that a combination of genetic predispositions and environmental triggers may cause disease onset. Concordance studies in twins revealed genetic contributions to RA development. Among dizygotic twins, concordance rates of 5 % were identified. These rates increased to 15 to 30 % in monozygotic twins (175). However, the genetic predispositions are not linked to one single mutation but rather a group of alterations that can lead to different severities of the disease. People carrying the “shared epitope”, a common amino acid motif in the HLA-DRB1 region, display a higher susceptibility to develop RA, as it may increase the chance to develop APCAs (176). While the requirement of a genetic predisposition is evident, it has been hypothesized that if an adequate environmental trigger is absent, RA may not develop. For instance, while the use of oral contraceptives and pregnancy reduce the risk of RA, the postpartum period seems to increase it (177). Infection may also be a trigger, as the production of immune complexes may be favourable for RF development (177,178). Further, reports of the influence of the microbiome on RA development began to emerge [reviewed in (179,180). On a more biochemical level, pulmonary stress (caused by e.g. smoking) may promote RA development as well. The stress leads to tissue damage, resulting in leakage of cellular contents. Peptidyl arginine deiminase Type IV (PAD4), a protein associated with translational modification, is responsible for citrullination of proteins within the cell. In the extracellular space, it citrullinates mucosal proteins like vimentin, fibrin or fibrinogen, thereby producing a repertoire of possible autoantigens for ACPAs (171). If a number of these environmental cues occur, they may induce RA development in susceptible individuals.

1.4.2. Treatment of RA

There is a range of treatments available for patients suffering from RA. These treatments consist of three groups, namely glucocorticoids, NSAIDs (also known as NSARs) and Disease-Modifying Anti-Rheumatic Drugs (DMARDs). An overview of the available treatments is listed in Table 6.

Table 6: Overview of current treatments for Rheumatoid Arthritis

Group	Subgroup	Drug	Target
Glucocorticoids	-	Prednisone Prednisolone Hydrocortisone Dexamethasone Betamethasone	NR3C1
NSAIDs	Selective COX-2 inhibitors	Parecoxib Etoricoxib	COX-2
	Non-Selective NSAIDs	Salicylates (e.g. Aspirin) Propionic acid derivates (e.g. Ibuprofen) Acetic acid derivates (e.g. Diclofenac) Enolic acid derivates (e.g. Piroxicam)	COX-1, COX-2
DMARDs	Conventional	Methotrexate Leflunomide Sulfasalazine	Cytokine production Pyrimidine synthesis Inflammation
	JAK inhibitors	Tofacitinib Baricitinib	JAK1/2/3 JAK1/2
	Biologics	Infliximab, Etanercept Abatacept Tocilizumab Anakinra Rituximab Denosumab Mavrilimumab	TNF-α TNF-α Co-stimulation IL-6 IL-1 B-cell depletion RANKL GM-CSF

While glucocorticoids and NSAIDs are mostly prescribed to alleviate pain and inflammation, DMARDs are used to achieve remission and if remission is not possible, achieve low disease progression. DMARDs can be further divided in conventional DMARDs, targeted DMARDs and biologics. Treatment with the conventional drug methotrexate results in adenosine production, which is one mechanism of action, through which this drug achieves a decrease in inflammation (181). Targeted DMARDs and biologics on the other hand interfere directly with inflammatory signalling, resulting in its suppression.

1.4.3. Key Players in RA pathology

The articular joint consists of bones that are connected via the synovial capsule. The capsule comprises two layers: the outer layer consists of fibrous ligaments and supports the joint. The inner layer consists of the synovial membrane, in which an intima (lining layer) and a subintima (sublining layer) can be distinguished. The intima harbours two cell types, namely Macrophage-like Synoviocytes (MLS or "Type A" cells) and Fibroblast-like Synoviocytes (FLS or "Type B" cells). Under healthy conditions, MLS are tasked with clearance of debris and FLS with production of hyaluronan, a nutrient for the avascular cartilage and lubricin, a lubricant to enable low-friction environment. Both substances are secreted in the adjacent synovial fluid. The subintima consists of fibrous, areolar and adipose tissue, infused with blood and lymphatic vessels (182). In RA, the synovial membrane and fluid display severe changes in their cell repertoire. The synovial fluid is infiltrated by high numbers of neutrophils, the intima undergoes FLS hyperplasia and the subintima is infiltrated with B- and T cells, mast cells and monocytes. During the course of my experiments, I worked mostly with cells of the mononuclear phagocyte system and neutrophils, which is why these cells will be introduced more thoroughly.

1.4.3.1. Monocyte and Macrophages

Three types of myeloid derived phagocytes can be observed in the inflamed synovium of RA patients, namely Macrophage-like Synoviocytes, Monocytes and actual Macrophages. As these cell populations have redundant markers, it is difficult to distinguish easily between them.

Macrophage-like Synoviocytes. MLS or "Type A" cells display, as their name suggests, macrophage-like properties e.g. expression of MHC II and hence contribute to the pool of antigen presentation within the synovium (183). Similar to FLS, they were termed "macrophage-like", although recent reports showed that these cells are derived from embryonic and bone marrow macrophages (184). However, as they are seeded during development, MLS will be recognized as a distinct population and monocytes and macrophages will be introduced below. Until recently, only few reports described this phagocyte population within the joints. Due to their low numbers and low rate of proliferation, *in vitro* studies have been proven difficult. MLS cells are found in the upper part of the intima and can be identified by non-specific esterase (NSE), cathepsins B, D and L and FcgRIIIa (part of CD16) [reviewed in (182,185)]. In RA, these cells may contribute to the pool of inflammatory cytokines that activate FLS, recruit monocytes and neutrophils and promote Th17 differentiation, however, as they are overshadowed by *in situ* differentiated macrophages, it is difficult to pinpoint which population is the key producer [reviewed in (186)]. In a murine model of arthritis it was shown that MLS suppress $Ly6C^{lo}$ monocytes, which correspond to non-classical monocytes in humans and skew M1 macrophages towards a M2 phenotype, indicating their regulatory character (187). MLS may interact with FLS through activation marker CD97 (188). Fate mapping in mice showed that intimal MLS are positive for CX_3CR1, negative for CSF1R and have a half-life of 5 weeks with no proliferative potential. These cells are replaced by interstitial $CX3CR1^-CSF1R^+$ macrophages in the steady state. Both populations are maintained independently of blood monocytes (189). Upon induction of arthritis, CX_3CR1^+ lining MLS maintain their position but change their morphology, while CX_3CR1^- interstitial MLS start to proliferate. Further, CX_3CR1^+ lining MLS display an epithelial-like phenotype through expression of tight-junction and gap-junction proteins and corresponded to $TREM2^+MHCII^-$ macrophages in human synovial tissue of OA patients. These cells were outnumbered by $TREM2^-$ macrophages in RA which may explain that the RA synovium is no longer sterile (190). To this date, at least three monocyte-independent macrophage populations have been identified within the synovial membrane [reviewed in (191)]. However, as macrophage numbers vastly increase in RA due to monocyte recruitment, it is difficult to determine how these cells contribute to disease progression.

Monocytes. Cells of the monophagocytic system (MPS), consisting of monocytes and macrophages, have always been considered key players in RA, as great numbers infiltrate the synovial intima during the onset of the disease (192). Recruitment of monocytes is facilitated through production of chemoattractants like MCP-1 or CX_3CL_1, also known as fractalkine (193,194). To this date, three major monocyte populations have been identified, namely classical $CD14^{++}CD16^{-}$ monocytes, intermediate $CD14^{++}CD16^{+}$ monocytes and non-classical $CD14^{lo}CD16^{+}$ monocytes [reviewed in (195,196)]. While the classical monocyte is also termed "inflammatory monocyte", indicating these cells are ready to enter tissues, the non-classical monocyte was dubbed "patrolling monocyte", as these cells were assumed to stay in circulation. There are conflicting reports on monocyte distribution within the blood and synovium in RA patients compared to healthy subjects: while one study claims that classical monocytes are expanded in RA patients, another report shows that non-classical monocyte levels are elevated (197,198). The distinction between these populations is often solely based on flow cytometric detection of CD14, a co-receptor of TLR4 to detect bacterial LPS, and CD16 (FcγRIII), one of three Fc receptors that recognize antibodies of the IgG isotype. As these gating strategies were never officially pre-determined, overlaps within the populations often occur, although recently, new strategies were proposed (199,200). Understanding the contribution of monocytes to RA pathology is crucial, as the subsets clearly perform distinguishable tasks: for instance, classical monocytes in RA display higher expression of CD11b and produce greater levels of IL-1β and IL-6, while non-classical monocytes are the major subset identified at the bone pannus in RA patients and appear to drive inflammatory arthritis in mice (187,201,202). Besides their distinguishable tasks, monocytes can differentiate into a range of cells: macrophages (see below) osteoclasts, dendritic cells [reviewed in (203,204)] and fibrocytes [reviewed in (205)], which all contribute in varying degrees to RA pathophysiology. Single cell RNA sequencing and mass cytometry approaches revealed many more monocyte subsets [reviewed in (206)]. These findings merit a closer look in how these cells differentiate differently into the aforementioned effector cells and whether some monocytes remain undifferentiated in RA tissue.

Macrophages. While FLS are considered to be the main source of IL-6, monocytes and macrophages are assumed to produce the highest levels of TNF-α and IL-1β in the inflamed

synovium of RA patients [reviewed in (207)]. The pool of macrophages in the synovial intima and subintima consists of tissue resident macrophages and macrophages derived from monocytes, the latter will be introduced in this sub chapter. To this day, three cytokines have been identified that induce macrophage differentiation: GM-CSF, M-CSF and IL-34, all of which are present in the synovial fluid of RA patients (208,209). Resembling the Th1/Th2 dichotomy in T-lymphocytes, M1 and M2 phenotypes for macrophages were characterized (210). M1 or "classically activated macrophages" exert a pro-inflammatory phenotype, displayed by marked expression of CD80/CD86 and production of TNF-α and IL-1β. M1 polarization is achieved by exposing monocyte-derived macrophages (M0) to the Th1 cytokine IFN-γ and LPS. On the other side of the dichotomy are "alternatively activated macrophages" (M2), polarized with Th2 associated cytokines IL-4 and/or IL-13. While M1 macrophages are assumed to drive the inflammatory phase, M2 macrophages are assumed to be tasked with resolving inflammation; through production of anti-inflammatory cytokines like IL-10 and uptake of apoptotic cells, proteins and debris through upregulation of CD163, CD200R, CD204 and CD206. The M1/M2 model was lately refurbished and extended (211). To this day, four M2 macrophage subsets have been classified. M2a, which are induced by IL-4 or IL-13 are associated with Th2 responses to kill parasites, M2b, induced by immune complexes, act as immunoregulators, M2c, polarized through IL-10, participate in tissue remodelling and the most recent addition M2d, induced through adenosine, display an angiogenic phenotype through production of VEGF and IL-10 (211,212). On the M1 side of the spectrum, the scientific community is hesitant to classify them to a similar extent and rather speak of activation rather than polarization (213,214). A publication in 2014 reported that by using different stimuli, up to 10 transcriptionally distinctive populations could be observed and the authors push for the implementation of a spectrum model instead of a polarization model (215). Macrophages are strongly affected by the cytokines and mediators they are exposed to and it is likely that the ability to polarize is not restricted into one direction, but rather can be interfered with at any time to shift into a different direction. While these markers are very useful targets to observe macrophage polarization *in vitro*, the impact of their heterogeneity in RA is difficult to illuminate. In the inflamed synovium, a broad range of macrophages accumulates, including macrophage-like synoviocytes (derived from bone marrow and embryonic macrophages) and monocyte-derived macrophages (polarized into short-lived M1 or M2). Further, there are reports of monocytes that

retain their phenotype upon tissue entry and monocyte-derived macrophages that assume tissue residency and longevity [reviewed in (216)]. As many of these cells arise from similar precursors, several markers to distinguish them from another may be redundantly expressed, although to varying degrees. It is therefore not surprising, that synovial macrophages were never defined to be solely M1- or M2-like. While CD68, an intracellular glycoprotein that is expressed in endosomes was often used to stain macrophages, a report that showed its expression in FLS rendered it obsolete (217). The high levels of TNF-α and IL-1β suggest that M1 macrophages are predominant in RA, but both anti-inflammatory cytokines like IL-10 and M2 markers like CD163 have been identified in this tissue (217,218). Contrary to T cell depletion, targeting macrophages directly or indirectly alleviates disease progression. CC chemokine receptor 2 (CCR2) deficient mice, which lack $Ly6C^{hi}$ classical monocytes, are protected from arthritis in the K/BxN serum transfer model (187). However, the same knock-out mice were not protected from developing antigen-induced arthritis (AIA^{1}) (219). Although both models show contradicting data regarding the question how relevant classical monocytes may be in arthritis, the overall conclusion of monocyte participation is evident. Suppression of macrophage differentiation using either a blocking antibody to prevent CSF1R activation or directly targeting GM-CSF using biologic DMARDs ameliorated murine and human arthritis (220,221). Lastly, direct targeting of tissue resident macrophages (MLS) through CD64 neutralization and inflammatory macrophages (MDM) through folate receptor β relieved synovial tissue *in vitro* and inhibited murine arthritis (222–224).

1.4.3.2. Neutrophilic Granulocytes

Neutrophilic granulocytes, also known as polymorphonuclear neutrophils (PMN) are cells of the innate immune system that are primarily charged with immune surveillance. PMN represent about 70-80 % of the total leukocyte numbers in circulation and about 10^{11} cells are produced daily in the bone marrow [reviewed in (225)]. They mature in the bone marrow, are released into the circulation, enter tissues and are cleared off by macrophages, in the absence of stimuli often within the same day. Upon exposure to pathogen-associated molecular patterns and danger-associated molecular patterns, these cells will respond through three modes of action: production of reactive oxygen and nitrogen species, phagocytosis and the most recent addition, formation of neutrophil

extracellular traps (NET) (226). Neutrophil granulocytes are the predominant leukocyte in the synovial fluid of RA patients (227). Further, these cells have recently been proclaimed to be key players in RA as well [reviewed in (228)]. Circulating PMN upregulate a range of chemokine receptors and adhesion molecules to enhance their migratory properties in RA (229). Their survival in the synovial fluid is enhanced through the presence of anti-apoptotic signals like G-CSF (granulocyte colony-stimulating factor) or GM-CSF (208,230). In the RA synovium, PMN contribute to inflammation through cytokine production and tissue remodelling through induction of matrix metalloprotease (MMP) expression (228,231). NET formation is relevant in RA as these traps contain citrullinated motifs (232). As RA neutrophils are more prone to spontaneous NET production, these citrullinated sites provide a source of autoantigens for ACPA production (233,234). Further, synovial fluid PMN express MHC II molecules and may be able to contribute to antigen presentation at sites of inflammation (235,236).

1.4.3.3. Other Key players

Fibroblast-like Synoviocytes. FLS or "Type B" cells are mesenchymal cells that comprise most of the synovial intima. They are distinguished from fibroblasts, as they behave differently in *in vitro* settings compared to classical fibroblasts, but share similar markers (237). In RA, these cells undergo a series of changes in their phenotype that remind of malignancy, including anchorage-independency, invasiveness, loss of contact inhibition, promotion of neoangiogenesis, seeding and enhanced survival [thoroughly reviewed in (238–240)]. These so-called Rheumatoid Arthritis Synovial Fibroblasts (RASFs) are highly susceptible to cytokines and are also a great source for them, suggesting a vicious cycle of autocrine pro-inflammatory signalling that perpetuates the disease (241–243). Two phenotypically distinct subsets of RASFs, one that is embedded in the intima and one that is floating in the synovial fluid, have already been described in 2009 (244,245). Recently, Croft et. al identified two distinct RASF populations within the synovial linings using mass cytometry (246).

T-lymphocytes. As a source of cytokines, RASF contribute strongly to leukocyte recruitment from the circulation. The predominant cell type in the subintima of RA patients is the T cell (247)

and its involvement in the disease has long been suggested [reviewed in (248)]. Early treatment approaches aimed therefore at T cell depletion and when these therapies failed to achieve favourable results, T cells were considered to be irrelevant bystanders [reviewed in (249)]. Genetic investigations discovered that many subjects suffering from the disease displayed genetic alterations. Most predominant were changes in the human leukocyte antigen-DR (HLA-DR), namely the "shared epitope" (see Chapter 1.4.1) that rendered subjects more susceptible to RA, especially smokers (250,251). While the risk contribution of the shared epitope is evident, the process underlying the pathophysiology is elusive. Is has been hypothesized that alterations in the amino acid motif leads to altered antigen recognition by T cells which enables production of autoantibodies (252).

B-lymphocytes. B cells are essential for autoantibody production. In rheumatoid arthritis, B cells can be found in the subintima, inside of lymphoid follicles containing germinal centers (253). These consist of CD4 T cells, CD8 T cells, DCs and B cells (253,254). As B cells are more predominant than DCs, they have been considered the primary source of antigen presentations in later phases of the disease (255). The inflamed synovium harbours plasmablasts and plasma cells that provide B cell chemoattractants and survival factors [reviewed in (256)]. Besides their function as APCs, B cells are able to produce pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-8, IL-12, TNF-α), anti-inflammatory cytokines (e.g. IL-10, TGF-β) and tissue remodelling promoting factors (receptor activator of NF-κB ligand or RANKL, VEGF, G-CSF, GM-CSF) and are therefore potent contributors for the propagation of inflammation [reviewed in (257)]. Their most prominent role, however, is their differentiation to autoantibody producing plasma cells. RF and ACPAs can predate disease onset by up to 15 years (170). It is therefore tempting to assume that if this process of autoantibody production is interrupted, disease progression will not reach the clinical phase.

Eosinophils. Eosinophil granulocytes are mostly associated with Th2 associated diseases. Their contribution to RA progression is not well illuminated to this day. They appear to be absent on disease onset, but mild eosinophilia may predict a severe clinical outcomes of RA (258). Eosinophil arthritis may be a separate entity of unknown aetiology but there are also cases of established RA with observed eosinophilia and in those, it serves as a disease marker (259,260).

Basophils. Basophil granulocytes are mostly associated with anaphylaxis, asthma and helminth infections and absent in RA synovial fluid. Their contribution to RA progression is assumed to be of systemic nature. Basophils are strong immunoregulators that are able to skew Th0 responses towards Th2 responses and it has been hypothesized that they play a crucial role in juvenile idiopathic arthritis (JIA), as most patients suffering from this disease display elevated basophil counts and a Th2 cell-mediated immune response, whereas decreased levels of circulating basophils and a dominant Th1 response have been reported in adult patients with RA (261).

Osteoclasts. Osteoclasts are bone-resorbing cells differentiated from monocytes. They are the sole contributors to bone loss in arthritic diseases, as osteoclasts are observed in all murine arthritis models and RANKL knockout mice are protected from bone destruction, despite ongoing inflammation (262). Osteoclastogenesis, the process of osteoclast differentiation, depends on M-CSF and RANKL, which both are produced in the synovial environment by FLS, B- and T cells (257,263–265). Further, other cytokines and mediators (e.g. TNF-α, IL-1β, ACPAs, IL-17) promote or enhance osteoclastogenesis, either directly or indirectly and thus support the cytokine network hypothesis [reviewed in (266)]. Recently it was shown that non-classical monocytes are the predominant monocyte subset in the vicinity of bone erosions and that these cells are most effective in differentiating into osteoclasts (202).

1.4.4. Prostaglandin D_2 in RA

COX-1 and COX-2 inhibition using NSAID therapies has long been established in RA and helps to reduce pain and suppress inflammation (see 1.4.2). COX-2 is strongly upregulated in synovial tissue of RA patients [reviewed in (267)]. The predominant prostaglandin is PGE_2, which exerts both pro- and anti-inflammatory properties, depending on its environment, the targeted receptor, the targeted cells and co-stimulatory molecules [reviewed in (268)]. In a model of collagen antibody-induced arthritis (CAIA), EP1-EP3 deficient mice were unaffected, while EP4 knockouts displayed a decreased incidence of the disease (269). In RA patients, mPGES-1 is strongly expressed in the synovial intima (270). These and other findings shifted the focus of prostaglandin research in RA strongly towards PGE_2, although other prostanoids were also detected in the

synovial fluid, including PGD_2 (268). Roughly 30 years later, a paper emerged that correlated PGD_2 production with the disease progression in collagen-induced arthritis (CIA) (9). In this report, it was shown that PGD_2 levels rise in hind paws and serum of CIA mice, along with expression of hPGDS, L-PGDS, DP1 and DP2 in cartilage. Blocking of DP1 aggravated the disease, while DP2 blocking had no impact. Lastly, the group showed that PGD_2 injections or DP1 activation alleviated the symptoms of CIA. This clearly indicated a pathological role for DP2 in experimental arthritis but was later challenged in a model of adjuvant-induced arthritis (AIA^2), in which DP2 deficiency resulted in a more severe progression of arthritis (271): CFA injection into the ankle joints induced PGD_2 production and paw swelling. While the inflammatory arthritis was less severe in WT mice, DP2 deficient mice displayed a more severe course of the disease, which was mediated by bone marrow-derived macrophages and could be blocked by targeting these cells directly. Both reports indicate a pro-resolving role of PGD_2 in experimental arthritis – an effect that has been reported in other murine models of inflammation (17). This hypothesis is supported by a recent publication by Tsubosaka et. al, in which the authors show that hPGDS deficiency aggravates arthritic parameters in AIA^2 (272). DP1 deficiency also contributed to disease progression, while DP1 activation resulted in alleviation. Interestingly, in osteoarthritis, DP1 activation also alleviated the symptoms and a recent report showed that L-PGDS deficient mice had a more severe incidence compared to WT animals (273,274). Outside of animal experiments, there are few reports showing PGD_2 involvement in RA in humans. In 2017, one group showed that PGD_2 and not PGE_2 was the predominant prostaglandin in RA synovial fluid and that synovial fluid monocytes expressed less DP2 mRNA than their counterparts in circulation (275). To elucidate the role of the PGD_2-DP1-DP2 axis in rheumatoid arthritis, it is crucial to identify senders and receivers in the synovium. A range of cells present in the RA synovium express hPGDS or L-PGDS, including dendritic cells, monocytes, macrophages, chondrocytes, osteoblasts and FLS (11,12,272,275,276). The predominant producer of PGD_2 in asthma have long been supposed to be mast cells (see Chapter 1.3.3), which are also present in the synovial fluid of RA patients and may be activated through ACPAs (277). After PGD_2 is metabolized, it can be measured as urinary tetranor-PGDM. A study of 60 RA patients showed that one third of those patients had elevated levels of tetranor-PGDM compared to healthy subjects, further indicating that PGD_2 plays a role in this disease (278). However, as it is important to illuminate which cells are a source for PGD_2, it is of similar

importance to identify cells that are affected by it. There are no reports available that directly address DP1/DP2 expression on mRNA or protein levels in MLS or FLS. However, as both cells share many similarities to macrophages and fibroblasts, respectively, it is tempting to assume that they express PGD_2 receptors. Monocytes express DP1 and DP2, however, in which subset, to what extent and what their function is, has not been elucidated (20,279). Macrophages express both receptors and in mice it was shown that DP2 is involved in migration (147). A later report showed that activation of both receptors leads to calcium flux and chemotaxis (20). Further, DP2 activation prior to LPS exposure resulted in higher concentrations of TNF-α. In fibroblasts, DP1 and DP2 expression have been reported in the lung, eye and nasal polyps where they exert anti-migratory properties or release substances like hyaluronan, VEGF or collagen (26,280–282). In human T cells, DP2 expression is restricted to CD4 positive Th2 and CD8 positive Type 2 cytotoxic T cells (TC2) where it also participates in recruitment (36,37). Interestingly, this Th2 restricted expression is not apparent in the mouse, where DP2 mRNA was also detected in Th1 cells (283). Lastly, both receptors are expressed in osteoclasts, where they inhibit osteoclastogenesis and induce apoptosis (25,284). Other cells within the synovium that have not been discussed in this introduction also express DP1 and/or DP2, including dendritic cells, osteoblasts and chondrocytes (9,11,275).

1.4.5. ApoA-IV in RA

A direct link between ApoA-IV and RA has not been established. One of the comorbidities in RA is atherosclerosis and cardiovascular events [reviewed in (285)]. This may be explained through alterations in lipid levels of RA patients. In a 2009 review, Choy and Sattar propose that the observed reduction in HDL cholesterol, LDL cholesterol and total cholesterol may contribute to the promotion of atherosclerosis (286). Before the onset of arthritis, LDL- and total cholesterol decrease (287). A closer look at apolipoprotein levels revealed that RA patients express less serum ApoA-I compared to healthy controls (288). Interestingly, ApoA-I accumulates in the inflamed RA synovium where it may be involved in an attempt to mitigate the chronic inflammation (289). One of these modes of action may be the production of PGE_2 and PGD_2 by monocyte-derived macrophages, suggesting that apolipoproteins are directly involved in PGD_2 signalling in RA (290). In experimental arthritis, ApoA-I along with statin treatment prevented the onset of CIA

(291). It is therefore tempting to assume that ApoA-IV also plays a part in RA or other immune disorders like allergy. Elevated levels of ApoA-IV were detected in patients suffering from chronic rhinosinusitis (1). In the same disease, PGD_2 synthase and receptor expression has been observed (7,292). It is tempting to assume that ApoA-IV incites *de novo* PGD_2 synthesis in a similar way as ApoA-I (290). As mentioned in Chapter 0, ApoA-IV binds to NR1D1. A very recent study in Cell Death & Disease links NR1D1 signalling to RA (293). Like ApoA-I, NR1D1 accumulates in synovial tissue where it might exert resolution of inflammation. In RASFs, receptor activation decreased cytokine and MMP expression, in macrophages, it inhibited M1 polarization and in monocytes, it suppressed osteoclastogenesis. The group also showed that CIA severity was ameliorated through NR1D1 activation. Taken together, these data suggest that apolipoproteins play an important immune regulatory role in RA.

1.5. Aims and Hypothesis

Immune disorders are major health issues in which the immune system is imbalanced and responds strongly to non-pathogenic antigens of foreign nature (allergy) or against self-antigens (autoimmunity). Due to the complexity of the biological processes that facilitate these conditions, it is difficult to find a permanent cure and achieving drug-mediated remission is often easier to accomplish. It is therefore crucial to understand which processes are affected and how this imbalance occurs. Both allergic asthma and rheumatoid arthritis are diseases in which the immune system seems out of balance, partly due to elevated levels of prostaglandins that contribute to inflammation. As recent reports show that apolipoprotein levels are also altered in both conditions, we set out to investigate whether there is a relation between the pro-inflammatory lipid mediator PGD_2 and the anti-inflammatory apolipoprotein ApoA-IV in the context of allergic asthma and rheumatoid arthritis. To accomplish this task, we tested the impact of both substances on cell activation, recruitment, differentiation, production of cytokines both *in vitro* and *in vivo*. We anticipated an immunosuppressive effect of ApoA-IV and a concomitant production of prostaglandins.

2. Material and Methods

2.1. Materials

2.1.1. Antibodies

Table 7: Antibodies used in this dissertation

ID	Name	Company	Cat. #	Concentration
AB01	Brilliant Violet 421™ anti-human CD14 Antibody	BioLegend	301830	70 µg/mL
AB02	PE anti-human CD11b	BioLegend	301306	150 µg/mL
AB03	PE Mouse IgG1, κ isotype Ctrl	BioLegend	400112	200 µg/mL
AB04	PerCP anti-human CD14 Antibody	BioLegend	325632	300 µg/mL
AB05	Human TruStain FcX™ (Fc Receptor Blocking Solution)	BioLegend	422302	-
AB06	Alexa Fluor® 488 anti-human CD16 Antibody	BioLegend	302019	200 µg/mL
AB07	APC/Fire™ 750 anti-human CD3 Antibody	BioLegend	344840	200 µg/mL
AB08	Alexa Fluor® 488 anti-human CD4 Antibody	BioLegend	317420	50 µg/mL
AB09	Brilliant Violet 510™ anti-human CD8 Antibody	BioLegend	344732	100 µg/mL
AB10	Brilliant Violet 421™ anti-human CD56 (NCAM) Antibody	BioLegend	318328	60 µg/mL
AB11	APC anti-human CD19 Antibody	BioLegend	302212	50 µg/mL
AB12	PE/Cy7 anti-human CD123 Antibody	BioLegend	306010	200 µg/mL
AB13	PerCP/Cy5.5 anti-human HLA-DR Antibody	BioLegend	307630	200 µg/mL
AB14	PE anti-human CD294 (CRTH2) Antibody	BioLegend	350106	200 µg/mL
AB15	PE Rat IgG2a, κ Isotype Ctrl Antibody	BioLegend	400508	200 µg/mL
AB16	Zombie Green™ Fixable Viability Kit	BioLegend	423111	-
AB17	PerCP/Cyanine5.5 anti-human CD16 Antibody	BioLegend	302027	200 µg/mL
AB18	Brilliant Violet 421™ anti-human CD80 Antibody	BioLegend	305221	160 µg/mL
AB19	APC/Cyanine7 anti-human CD206 (MMR) Antibody	BioLegend	321119	400 µg/mL
AB20	Brilliant Violet 421™ Mouse IgG1, κ Isotype Ctrl Antibody	BioLegend	400157	100 µg/mL
AB21	APC/Cy7 Mouse IgG1, κ Isotype Ctrl Antibody	BioLegend	400127	200 µg/mL
AB22	BD Pharmingen™ PE Mouse Anti-Human CD80	BD Pharmingen	557227	-
AB23	PE Mouse Anti-Human CD206	BD Pharmingen	555954	-
AB24	BD Pharmingen™ PE Mouse IgG1, κ Isotype Control	BD Pharmingen	559320	-
AB25	DP (C-15) Antibody	Santa Cruz	sc-55812	200 µg/mL
AB26	normal goat IgG : sc-2028	Santa Cruz	sc-2028	400 µg/mL
AB27	Alexa Fluor® 647 Rat Anti-Human CD294	BD Pharmingen	558042	200 µg/mL
AB28	Alexa Fluor® 647 Rat IgG2a, κ Isotype Control	BD Pharmingen	557906	200 µg/mL

AB29	Alexa Fluor® 488 Rabbit anti-Goat IgG (H+L)	Invitrogen	A11078	200 µg/mL
AB30	PE/Cy5 anti-human CD16 Antibody	BioLegend	302010	60 µg/mL
AB31	PE/Cy5 anti-human CD4	BioLegend	317411	25 µg/mL
AB32	Anti-COX2 / Cyclooxygenase 2 antibody	abcam	ab15191	-
AB33	beta-Actin Antibody (AC-15)	Novus Biologicals	MB600-501	-
AB34	Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP	ThermoFisher Scientific	32430	-
AB35	Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	111-035-045	-
AB36	Arthrogen-CIA® 5-Clone Cocktail Kit, 10 mg	Chondrex	53010	10 mg/mL
AB37	PE/Cyanine5 anti-mouse CD45 Antibody	BioLegend	103109	200 µg/mL
AB38	Brilliant Violet 421™ anti-mouse CD3	BioLegend	100227	50 µg/mL
AB39	FITC anti-mouse Ly-6G	BioLegend	127605	500 µg/mL
AB40	PE/Cy7 anti-mouse Ly-6C	BioLegend	128017	200 µg/mL
AB41	APC/Fire™ 750 anti-mouse F4/80	BioLegend	123151	200 µg/mL
AB42	PE anti-mouse CD117 (c-kit)	BioLegend	105807	200 µg/mL
AB43	TruStain FcX™ (anti-mouse CD16/32) Antibody	BioLegend	101319	500 µg/mL
AB44	PE Rat Anti-Mouse Siglec-F	Becton Dickinson	552126	200 µg/mL
AB45	FITC anti-mouse Ly-6C Antibody	Biozym Biotech	128005	500 µg/mL
AB46	APC anti-mouse Ly-6G Antibody	Biolegend	127613	200 µg/mL
AB47	PE-Cy™5 Hamster Anti-Mouse CD3e	Becton Dickinson	553065	200 µg/mL
AB48	Brilliant Violet 510TM anti-mouse I-A/I-E Antibody	Biolegend	107635	100 µg/mL
AB49	PE-Cy™7 Rat Anti-CD11b	Becton Dickinson	552850	200 µg/mL
AB50	BV421 Hamster Anit-Mouse CD11c	Becton Dickinson	562782	200 µg/mL
AB51	rev-erba-3 1 y6d-rabbit mab	Cell Signaling	13418S	293 µg/mL

2.1.2. Kits

Table 8: Kits used in this dissertation

Name	Company	Cat. #
Eosinophil Isolation Kit, human	Miltenyi Biotec	130-092-010
High-Capacity cDNA Rev. Transcription Kit	Thermo Fisher Scientific	4368814
Human Apolipoprotein A-IV ELISA Kit	abcam	ab214567
Human IL-10 Quantikine ELISA Kit	R&D Systems	D1000B
Human TNF-α Standard ABTS ELISA Development Kit	PeproTech	900-K25
Pierce™ BCA Protein Assay Kit	Life Technologies	23228
Prostaglandin D2-MOX ELISA Kit	Cayman Chemical	512011
SsoAdvanced™ Universal SYBR® Green Supermix	Bio-Rad	1725270

2.1.3. Chemicals & Substrates

Table 9: Chemicals and substances used in this dissertation

Name	Company	Cat. #
13,14-dihydro-15-keto Prostaglandin D_2	Cayman Chemical	12610
2-Mercaptoethanol	Sigma-Aldrich	M3148
5-OxoETE | CAS 106154-18-1	Sanova	Cay34250
Apolipoprotein A-IV Recombinant Protein	ProSci	91-720
Apolipoprotein AIV, Human Plasma	AthensResearch	16-16-120104
Aqua Bidest "Fresenius" for Parenteralia	Fresenius	-
Bovine Serum Albumin - heat shock fraction	Sigma-Aldrich	A7906
BW 245C (72814-32-5)	Cayman Chemical	12050
Clarity Western ECL	Bio-Rad	170-5061
Collagenase from Clostridium histolyticum	Sigma-Aldrich	C2139
Dextran from Leuconostoc spp.	Sigma-Aldrich	31392
DPBS, no calcium, no magnesium	Thermo Fisher Scientific	14190169
FACS Lysing Solution	BD	349202
Fetal calve serum	Thermo Fisher Scientific	10270106
Fluo-3, AM, FluoroPure™ grade	Life Technologies	F23915
GSK 4112	Tocris	3663
Halt™ Protease and Phosphatase Inhibitor Cocktail	Thermo Fisher scientific	78440
HEPES Buffer Solution	Thermo Fisher Scientific	15630080
Histopaque®-1077	Sigma-Aldrich	10771
Human AB serum	Sigma-Aldrich	H4522
Hyaluronidase from bovine testes	Sigma-Aldrich	H3506
LPS	Sigma-Aldrich	L8880
MEM Non-essential Amino Acid Solution	Sigma-Aldrich	M7145
Novex™ 4-20% Tris-Glycine Mini Gels	Life Technologies	XP04205Box
OC-000459	Cayman Chemical	12027
Penicillin-Streptomycin	Pan-Biotech	p06-07100
Pluronic® F-127	Sigma-Aldrich	P2443
Prostaglandin D_2 | CAS 41598-07-6	Cayman Chemical	12010
Prostaglandin E_2 (363-24-6)	Cayman Chemical	14010
Prostaglandin E_2, [5,6,8,11,12,14,15-3H(N)]	PerkinElmer	NET428025UC
Recombinant C5a	PeproTech	300-70
Recombinant Human IL-1β	PeproTech	200-01B-10UG

Recombinant Human IL-6	PeproTech	200-06-20UG
Recombinant Human MCP-1 (CCL2)	PeproTech	300-04
Recombinant Human M-CSF	PeproTech	300-25-10UG
Recombinant Human TNF-α	PeproTech	300-01A-50UG
rh CXCL8	Immunotools	11349084
rh Eotaxin / CCL11	Immunotools	11343212
rh GM-CSF	Immunotools	11343123
rh IFN-gamma	Immunotools	11343534
rh IL-4	Immunotools	11340043
RIPA Lysis and Extraction Buffer	Thermo Fisher Scientific	89900
RPMI 1640 Media	Life Technologies	11875093
Sodium pyruvate solution	Sigma-Aldrich	S8636
SR8278	Sigma-Aldrich	S9576
tri-Natriumcitrat-Dihydrat min. 99 %	Lactan	35804

2.1.4. Equipment

Table 10: Equipment used in this dissertation

Name	Company
0.5 mL Eppendorf Tubes	Greiner Holding AG
1 mL syringes	Braun
1.5 mL Eppendorf Tubes	Greiner Holding AG
15 mL Cellstar Tubes	Greiner Holding
48-Well Micro Chemotaxis Chamber	Neuro Probe
50 mL Cellstar Tubes	Greiner Holding
5mL Polystyrene Round Bottom Tube	ThermoFisher
Allegra X-12 Centrifuge	Beckman Coulter, Inc.
ChemiDoc™ Imagers	Bio-Rad
Corning® CellBIND® Multiple Well Plate	Sigma
FACS Canto II	BD Biosciences
Falcon® 70 µm Cell Strainer, White, Sterile	Corning
FlexiVent system	Scireq
iBlot® Dry Blotting System	Invitrogen
Neubauer haemocytometer	Marienfield Superior
Pipetman pipettes	Gilson, Inc.
Polycarbonate Filters 5 µm Pores	Neuro Probe

Transwell® with 5.0µm Pore Polycarbonate Membrane Insert	Szabo Scandic
Transwell® with 8.0µm Pore Polycarbonate Membrane Insert	Szabo Scandic

2.1.5. Primers

Table 11: Primers used in this dissertation

Gene	Direction	Sequence	Designer
Beta actin	FWD	CCTCACCCTGAAGTACCCCA	Endo et. al (294)
	REV	TGCCAGATTTTCTCCATGTCG	
COX-2	FWD	GAATCATTCACCAGGCAAATTG	Endo et. al (294)
	REV	TTTCTGTACTGCGGGTGGAAC	
mPGES-1	FWD	CTGCTGGTCATCAAGATGTACG	Endo et. al (294)
	REV	GGTTAGGACCCAGAAAGGAGT	
hPGDS	FWD	ACCAGAGCCTAGCAATAGCAA	Sonja Rittchen (unpublished data)
	REV	AGAGTGTCCACAATAGCATCAAC	

2.1.6. Buffers

FIX

- 60 mL FACS Flow
- 20 mL Aqua dest.
- 2 mL BD CellFIX

CTX buffer

- 25 mg BSA
- 45 mg Glucose
- 19 mg EDTA
- 250 µL HEPES
- 25 mL $PBS^{-/-}$

-buffer

- 25 mg BSA
- 45 mg Glucose
- 250 µL HEPES
- 25 mL $PBS^{-/-}$

+buffer

- 25 mg BSA
- 45 mg Glucose
- 250 µL HEPES
- 25 mL $PBS^{+/+}$

10x NH_4Cl lysis

- 9 g ammonium chloride
- 1 g potassium bicarbonate
- 37 mg EDTA
- 100 mL Aqua dest.

BAL buffer

- 29 mg EDTA
- 100 mL $PBS^{-/-}$

2.2. Methods

Some of the texts in the material and methods section have been published (1), and are thus similar in wording and content as may be expected. Other methods that have been used and adapted, which were already described elsewhere were cited accordingly.

2.2.1. Ethical approvals

Blood samples of patients suffering from rheumatoid arthritis were obtained with informed consent at the Division of Rheumatology and Immunology at the Medical University of Graz (29-101 ex 16/17). Blood samples of healthy donors were obtained at the Otto Loewi Research Centre, Division of Pharmacology (EK 17-291 ex 05/06). Mouse experiments using BALB/c were performed with the approved ethical vote BMWFW-66.010/0020-WF/V/3b/2015 and experiments with DBA/1J mice were performed with approved ethical vote BMWF-66.010/0045/WF/V/3B/2017.

2.2.2. Isolation of peripheral blood mononuclear cells from blood

For isolation of human peripheral blood mononuclear cells, 70 mL blood was drawn from healthy donors after obtaining informed consent according to a local ethics committee–approved protocol. Blood coagulation was prevented by adding 3.8 % sodium citrate. The mixture was centrifuged at 400 xg (low brake) for 20 minutes. Platelet-rich plasma was removed and dextran sedimentation (6 % dextran, filled up with 0.9 % saline solution) followed. The samples were incubated for 30 minutes at room temperature, facilitating erythrocyte aggregation. The upper phase containing

leukocytes was transferred on top of 15 mL Histopaque, a polysucrose solution with a defined density of 1.077 g/ml used to separate cells by centrifugation (400 xg, 20 min, low break) according to their density. The saline phase was discarded and the interphase containing the PBMCs was transferred into a new Falcon. The cell pellet containing granulocytes was also used (see Isolation of Polymorphonuclear Cells). Wash buffer was added to reach a volume of 30 to 40 mL and the cells were centrifuged at 400 xg for 7 minutes. The supernatant was discarded, and cells were washed again in 20 mL wash buffer before being centrifuged a final time for 7 minutes. For counting, cells were stained with Kimura stain (1:10) and counted using a Neubauer haemocytometer (1).

2.2.3. Isolation of Polymorphonuclear Leukocytes from blood

The cell pellet obtained after density centrifugation was resuspended in 10 mL Wash buffer. Cells were again centrifuged at 400 xg (maximum brake) for 7 minutes. To remove remaining erythrocytes, hypotonic shock lysis was induced by resuspending the cell pellet with 0.2 % saline. Physiological conditions were re-established after adding 1.6 % saline (same volume as 0.2 % saline). Cells were pelleted at 400 xg (maximum brake) for 7 minutes and then resuspended. Lastly, cells were stained with Kimura stain (1:10) and counted using a Neubauer haemocytometer (1).

2.2.4. Isolation of Eosinophils Granulocytes from PMNL

Eosinophil granulocyte isolation was performed according to the manufacturer's protocol. In short, PMNL cell number was determined, and cells were centrifuged at 400 xg for 7 minutes. The cell pellet was resuspended in 40 µL -buffer per 10^7 total cells. 10 µL of a cocktail of biotin-conjugated monoclonal antibodies (containing CD2, CD14, CD16, CD19, CD56, CD123, and CD235a) was added and cells were incubated at 4°C. After ten minutes, 30 µL -buffer were added along with 20 µL of an anti-biotin antibody conjugated to MicroBeads. Cells were mixed and incubated for additional 15 minutes. Cells were washed and centrifuged at 400 xg for 7 minutes and resuspended in 500 µL per 10^8 cells. To isolate eosinophils, LS columns (developed for gentle isolation of Microbead-labelled cells) were washed with 3 mL -buffer, then cell suspension was added and the

eluent was collected by adding additional 3 mL for 3 times in total. After a final centrifugation step, cells were stained with Kimura stain (1:10) and counted using a Neubauer haemocytometer (1).

2.2.5. Monocyte adhesion

PBMC were seeded at densities ranging between 5 to 10 x 10^6 cells per mL in 12-well CellBIND plates in adhesion medium (RPMI-1640, 1 % Pen/Strep, 1 % sodium pyruvate, 1 % non-essential amino acids, 0.5 % HEPES, 5 % human AB serum) and incubated for 1 hour at 37°C (5 % CO_2), allowing monocytes to attach to the well surface (20).

2.2.6. Macrophage differentiation and polarization

Following monocyte adhesion, medium was aspirated, and cells were washed in $PBS^{-/-}$ 3 times in 1 mL each, before differentiation medium (RPMI-1640, 1 % PS, 10 % FCS, 10.8 nM M-CSF) was added. Every 2nd to 3rd day, an additional mL of medium was added. After 7 days, monocytes were fully differentiated into macrophages. Monocyte-derived macrophages required no activation and were incubated in RPMI medium containing 10 % FCS. M1 macrophage medium was supplemented with 20 ng/mL IFN-γ and 100 ng/mL LPS, while M2 macrophage medium was supplemented with 20 ng/mL interleukin-4. Macrophages were incubated for 2 days at 37°C. Additional treatments during polarization are mentioned below (20).

2.2.6.1. Treatments with PGD_2 or DP receptor agonists

During polarization to M1 or M2 or as a control, unpolarised MDM, cells were treated with 1 µM of PGD_2, BW245C or DK-PGD_2. On day 1, the treatments started and were given for 2 consecutive days at the same time in the morning. The polarization was induced only in the afternoon of day 1. On day 3 in the afternoon, supernatants and cells were harvested.

2.2.6.2. Treatments with ApoA-IV and NR1D1 agonists/antagonists

During polarization, medium was supplemented with either vehicle (aqua dest. or DMSO), 3 µg/mL plasma ApoA-IV, selective NR1D1 agonist GSK4112 (100 nM – 100 µM) to activate this nuclear receptor and mimic ApoA-IV signaling or selective NR1D1 antagonist SR8278 (10 nM – 100 µM) to test toxicity of this substance. When testing the antagonistic effect of SR8278, cells were pre-treated with 1 µM SR8278 or vehicle for 30 minutes, before being stimulated with 3 µg/mL plasma ApoA-IV. These treatments were started on day 0 and continued on day 1 and day 2. On day 0, five hours after the first treatment, macrophages were polarised using IFN-γ and LPS.

2.2.7. Surface stainings in Whole Blood

2.2.7.1. DP2 staining in whole blood of RA patients

Patient blood or blood of healthy donors containing 3.8 % sodium citrate was distributed in polystyrene round bottom tubes (from now on called FACS tubes and blocked with Fc block [AB05] (1:200) for 5 minutes at room temperature before being stained with an antibody cocktail containing CD4 [AB08], CD16 [AB06], CD3 [AB07], CD8 [AB], CD56 [AB10], CD19 [AB11], CD123 [AB12] and HLA-DR [AB13] (see Table 7). 2.5 µL of DP2 [AB14] antibody or the corresponding Isotype Control [AB15] were added in their designated tubes. Cells were incubated for 30 minutes at room temperature without light exposure. RBC were lysed using the FACS Lysing Solution for 10 minutes before centrifugation at 400 xg. Supernatant was decanted, cells were washed in 500 µL $PBS^{-/-}$ and centrifuged again before being fixed with 200 µL FIX.

2.2.7.2. CD11b staining in whole blood of healthy donors in response to PGD_2

Whole blood obtained from healthy donors was blocked with Fc-Block [AB05] (dilution 1:200) for 30 minutes at 37°C. Next, the blood was distributed in FACS tubes and samples were treated with either 1 µM of PGD_2 or vehicle (EtOH). At the same time, antibodies were added (dilution 1:50 for each): CD14 [AB01] and CD16 [AB06] to distinguish monocyte populations and, CD11b [AB02] or corresponding Isotype Control [AB03] (see Table 7). Samples were incubated for another 30 minutes at 37°C before RBC were lysed by addition of 2 mL BD Lysis Solution (diluted

1:10 in aqua dest.) for 10 minutes at room temperature. Lastly, a washing step was performed and samples were measured (295).

2.2.7.3. CD11b staining in whole blood of healthy donors in response to ApoA-IV

Whole blood was transferred to FACS tubes and cells were stained with CD11b [AB02] (dilution 1:50) (see Table 7). To measure ApoA-IV mediated CD11b upregulation, ApoA-IV (3 mg/mL) dissolved in +buffer or vehicle were added immediately and cells were incubated at 37°C for 30 minutes, 3 hours or 6 hours. Afterwards, whole blood was lysed by addition of 1 mL BD Lysis Solution (diluted 1:10 in Aqua Dest.) for 10 minutes at room temperature. Cells were pelleted at 400 xg (maximum brake) for 7 minutes and then washed in 1 mL PBS. Lastly, cells were fixed and measured using flow cytometry (295).

2.2.8. Shape change in PMNL

2.2.8.1. Shape change in RA PMNL in response to lipid mediators

Polymorphonuclear leukocytes were collected at a density of 1.5 x 10^7 and resuspended in 1.5 mL +buffer. Cells were treated with 5-OxoETE or PGD_2 as indicated for 4 minutes at 37°C before being transferred immediately on ice. 250 µL of pre-cooled FIX was added and granulocyte shape change was measured using Flow Cytometry. For this, eosinophil granulocytes were distinguished from neutrophils through their auto-fluorescent properties, observable in the FL2 (585/42 nm) channel and both cell populations were then measured for increase in FSC, indicating rearrangement of cytoskeleton (296).

2.2.8.2. Shape change in neutrophils in healthy donors in response to IL-8

PMNL of healthy donors were resuspended in -buffer and distributed in FACS tubes (350.000 cells per tube). Vehicle or 3 µg/mL ApoA-IV were administered and cells were incubated at 37°C. After 30 minutes, cells were washed, distributed in small FACS tubes and treated with IL-8 as indicated. After 20 minutes at 37°C, cells were fixed on ice and measured (296).

2.2.9. Reactive oxygen species production in PMNL and monocytes

2.2.9.1. ROS production in PMNL in response to lipid mediators

Cells were collected at a density of 5 x 10^6 and resuspended in 500 µL -buffer before being stained with CD16 antibody [AB30] (1:500) at room temperature without light exposure (see Table 7). After 10 minutes, cells were washed in -buffer and centrifuged at 400 xg for 7 minutes. Cells were resuspended in 500 µL -buffer containing 5 µM of DHR123 and incubated for at 37°C. After 5 minutes, cells were stimulated as indicated for 20 minutes at 37°C before being transferred on ice and fixed (295).

2.2.9.2. ROS production in PMNL in response to C5a

Cells were collected at a density of 7 x 10^6 and resuspended in 700 µL $PBS^{-/-}$ before being distributed in two FACS tubes (3 Mio per tube). Cells were then treated with either vehicle or 3 µg/mL ApoA-IV. After 20 minutes, cells were stained with CD16 antibody [AB30] (1:500) at room temperature without light exposure (see Table 7). After 10 minutes, cells were washed in -buffer and centrifuged at 400 xg for 7 minutes before being exposed to 5 µM DHR123 for 5 minutes at 37°C. After this incubation period, cells were treated as indicated for 20 minutes at 37°C prior to fixation and measurement (295).

2.2.9.3. ROS production in monocytes in response to C5a

PBMC at a density of 1.8 x 10^7 were collected and resuspended in 450 µL -buffer. CD14 antibody [AB01] was added (1:50) and cells were distributed directly into FACS tubes (8 Mio cells per tube) (see Table 7). Vehicle or 3 µg/mL ApoA-IV were added and cells were incubated at 37°C without light exposure. After 30 minutes, cells were washed and exposed to 5 µM DHR123 for 5 minutes at 37°C, before. After this incubation period, cells were transferred to small FACS tubes and treated with zymosan A as indicated for 60 minutes at 37°C, before being fixed and measured (295).

2.2.10. Monocyte chemotaxis

Monocyte chemotaxis was performed using transwell plates. The plates were pre-incubated in $PBS^{-/-}$ containing 1 % BSA for 30 minutes prior to the experiment (297).

2.2.10.1. Chemotaxis to increasing concentrations of PGD_2

4 x 10^7 PBMC were collected from RA patients or healthy donors. Cells from healthy donors were distributed in FACS tubes and pre-treated with either Vehicle (AD) or 20 nM of either TNF-α, IL-1β or IL-6 for 1 hour at 37°C, whereas samples from RA patients were used without pre-treatments. After a washing step in $PBS^{-/-}$, cells were transferred in duplicates into the Transwells (4 x 10^6 cells per well) of 24 well Transwell plate (8 µM filter) and allowed to migrate towards vehicle, PGD_2 or MCP-1 as indicated. After one hour at 37°C, cells were washed and stained with CD14 [AB04] (dilution 1:200) before measurement.

2.2.10.2. Chemotaxis to increasing concentrations of C5a

8 x 10^6 PBMC isolated from healthy donors were resuspended in 2 mL CTX buffer (containing EDTA to prevent adherence), distributed in FACS tubes, and treated with vehicle or ApoA-IV (3 µg/mL) for 30 minutes at 37°C. After this incubation period, cells were transferred in the top wells of the Transwell plate (4 x 10^5 cells per well) and allowed to migrate towards vehicle or increasing concentrations of C5a (1 nM, 3 nM or 10 nM) by passing through a 5 µM filter. After one hour at 37°C, the top wells were aspirated and the filters were rinsed with the content of each corresponding bottom well. Cells were centrifuged (400 xg, 7 minutes, room temperature) and stained in CTX buffer containing CD14 antibody [AB04] (dilution 1:200) for 10 minutes on ice. Lastly, samples were washed and measured in 150 µL CTX buffer.

2.2.11. Eosinophil chemotaxis

Prior to the experiment, Boyden chamber was incubated at 37°C for a couple of hours and filters were engulfed in +buffer for 30 minutes before the experiment. Isolated eosinophils were collected in the desired amount and resuspended in +buffer. Cells were transferred in FACS tubes and treated

according to the protocol (see below). After the incubation period, cells were transferred onto the assembled Boyden chamber (100.000 cells per well) and allowed to migrate for 60 minutes at 37°C. After that, upper wells were aspirated, and the content of the lower wells was fixed in 150 µL FIX and measured using flow cytometry (1).

2.2.11.1. Chemotaxis to CCL11 and PGD_2 after ApoA-IV treatment

Eosinophils were resuspended in +buffer and exposed to either vehicle or 3 µg/mL ApoA-IV at 37°C. After 30 minutes, cells were directly transferred into the upper wells of the Boyden chamber and allowed to migrate towards CCL11- (3 nM) or PGD_2 (30 nM). Each experiments were performed in triplicates.

2.2.11.2. Chemotaxis to CCL11 after ApoA-IV or GSK4112 treatment

Eosinophils were resuspended in +buffer and exposed to either vehicle, 3 µg/mL ApoA-IV or 10 µM of GSK4112 for 30 minutes at 37°C prior to being allowed to migrate towards 3 nM of CCL11. Each experiments were performed in triplicates.

2.2.11.3. Chemotaxis to CCL11 after SR8278 pre-treatment followed by ApoA-IV treatment

Eosinophils were pre-exposed to either vehicle or 1 µM of SR8278 for 30 minutes at 37°C prior to being stimulated with Vehicle or 3 µg/mL ApoA-IV. After 30 minutes at 37°C, cells were allowed to migrate towards 3 nM of CCL11. Each experiment was performed in triplicates.

2.2.12. Surface stainings in differentiated macrophages

MDM, M1 and M2 macrophages were harvested by incubating the cells with pre-warmed Accutase per well for 15 minutes at 37°C. To fully detach the macrophages, the tip of a 1 mL syringe plunger was used for scraping. The cells were then washed and resuspended in $PBS^{-/-}$ containing Fc-Block [AB05] (1:250). After 5 minutes at room temperature, Zombie Live Dye [AB16] was added and cells were incubated for another 15 minutes at room temperature in the dark (see Table 7). Afterwards, cells were fixed and distributed into FACS tubes to receive antibody stainings.

2.2.12.1. Macrophage phenotyping based on surface marker expression

Macrophages were stained with CD80 [AB18] and CD206 [AB19] (1:50) or their respective isotypes [AB20, AB21]. Cells were stained for 30 minutes at room temperature in the dark, then washed in $PBS^{-/-}$ and measured.

2.2.12.2. DP2 expression in monocyte-derived macrophages

For DP2 staining, cells were distributed in two FACS tubes after fixation. The DP2 antibody [AB27] and its isotype [AB28] were diluted 1:5 in $PBS^{-/-}$. Cells were incubated at room temperature without light exposure for 30 minutes and then washed in $PBS^{-/-}$ prior to measurement.

2.2.13. Surface stainings in isolated PBMC

2.2.13.1. CD4 stainings in monocyte populations in response to ApoA-IV

7 x 10^6 PBMC were resuspended in 700 µL -buffer, transferred in FACS tubes and stained with antibodies for CD4 [AB31], CD14 [AB01], CD16 [AB06] (each 1:50 diluted). Immediately, cells were treated with vehicle or 3 µg/mL ApoA-IV. After the indicated timepoints, cells were washed, fixed and measured.

2.2.13.2. DP2 staining in monocytes populations in response to ApoA-IV

1 x 10^7 PBMC were collected and resuspended in RPMI containing 1 % PenStrep and 10 % FCS. Cells were distributed in FACS tubes and treated with either vehicle or 3 µg/mL ApoA-IV for the timepoints indicated. Afterwards, cells were washed and resuspended in $PBS^{-/-}$ containing Fc-Block [AB05] (1:200). After five minutes, a Zombie Live Dye [AB16] (1:500) was added and cells were kept in the dark at room temperature. After 15 minutes, cells were stained with CD14 and CD16 [AB01, AB17] (1:50 diluted) to distinguish monocyte populations and DP2 [AB14] and its corresponding isotype control [AB15] (1:50 diluted). After 15 minutes at room temperature in the dark, cells were washed in $PBS^{-/-}$, fixed and measured.

2.2.14. Surface stainings in isolated PMNL

2.2.14.1. GM-CSF induced upregulation of HLA-DR

PMNL were resuspended in 650 µL RPMI containing 1 % PenStrep and 10 % FCS and distributed in FACS tubes. Cells were treated with either vehicle or 3 µg/mL ApoA-IV for 30 minutes at 37°C, before being transferred in fresh tubes containing increasing GM-CSF concentrations as indicated. Cells were incubated for 48 hours, before being stained with CD16 [AB30] (1:500) and HLA-DR [AB13] (1:300) for 30 minutes. Afterwards, cells were washed and fixed before sample measurement(298).

2.2.15. PGE_2 radioimmunoassay

2.2.15.1. In monocytes

Isolated PBMCs were seeded at a density of 7 x 10^6 cells per 500 µL in RPMI-1640 supplemented with 1 % PenStrep, 1 % non-essential amino acids, 1 % sodium pyruvate, 0.5 % HEPES, 5 % human male AB serum and incubated at 37°C in a humidified atmosphere containing 5 % CO_2. After one hour, non-adherent cells were removed by gentle washing. Remaining adherent monocytes were supplied with RPMI growth medium containing 1 % Pen/Strep, 1 % non-essential amino acids, 1 % sodium pyruvate and 0.5 % HEPES. Monocytes were treated with either vehicle or 3 µg/mL ApoA-IV for 24 hours. Immunoreactive prostaglandin E_2 was determined in the supernatant as described previously(299), using [5,6,8,11,12,14,15-^{3}H(N)] prostaglandin E_2 as a tracer and synthetic prostaglandin E_2 as standard. The detection limit, defined as 10 % inhibition of binding was at 10.52 ± 0.59 pg/mL. The EC_{50} values were 80.84 ± x 1.10 pg/mL(60).

2.2.15.2. In M1 macrophages

Isolated PBMCs were seeded at a density of 7 x 10^6 cells per 500 µL in RPMI-1640 supplemented with 1 % PenStrep, 1 % non-essential amino acids, 1 % sodium pyruvate, 0.5 % HEPES, 5 % human male AB serum and incubated at 37°C in a humidified atmosphere containing 5 % CO_2. After one hour, non-adherent cells were removed by gentle washing. Remaining adherent monocytes were supplied with RPMI growth medium containing 1 % Pen/Strep, 10 % FCS and

10.8 nM M-CSF. After 7 days, monocytes were fully differentiated into macrophages. For polarization, see 2.2.6. Medium was supplemented with either vehicle (aqua dest.) or 3 µg/mL plasma ApoA-IV. The treatment was repeated at the same time for two consecutive days. The first ApoA-IV treatment was administered 5 hours prior to cytokine activation(60).

2.2.16. Enzyme-linked immunosorbent assay

2.2.16.1. ApoA-IV ELISA in human plasma or serum

Plasma or serum was collected from RA patients or healthy controls. The ApoA-IV ELISA was performed according to the manufacturer's protocol.

2.2.16.2. Interleukin-10 ELISA in ApoA-IV treated monocytes

Isolated PBMCs were seeded at a density of 7 x 10^6 cells per 500 µL in RPMI-1640 supplemented with 1 % PenStrep, 1 % non-essential amino acids, 1 % sodium pyruvate, 0.5 % HEPES, 5 % human male AB serum and incubated at 37°C in a humidified atmosphere containing 5 % CO_2. After one hour, non-adherent cells were removed by gentle washing. Remaining adherent monocytes were supplied with RPMI growth medium containing 1 % PenStrep, 1 % non-essential amino acids, 1 % sodium pyruvate and 0.5 % HEPES. Medium was supplemented with either vehicle or 3 µg/mL ApoA-IV. After 24 hours, supernatants were collected. Enzyme-linked immunosorbent assay was performed according to the manufacturer's protocol.

2.2.16.3. Interleukin-10 ELISA in ApoA-IV treated macrophages

For macrophage differentiation, polarization and ApoA-IV treatments, see chapter 2.2.6 and 2.2.6.2, respectively. Supernatants were collected and enzyme-linked immunosorbent assay was performed according to the manufacturer's protocol.

2.2.16.4. Tumour necrosis factor-α ELISA in ApoA-IV treated monocytes

Isolated PBMCs were seeded at a density of 5 x 10^6 cells per 500 µL in RPMI-1640 supplemented with 1 % PenStrep, 1 % non-essential amino acids, 1 % sodium pyruvate, 0.5 % HEPES, 5 % human male AB serum and incubated at 37°C in a humidified atmosphere containing 5 % CO_2.

After one hour, non-adherent cells were removed by gentle washing. Remaining adherent monocytes were supplied with RPMI growth medium containing 1 % PenStrep, 1 % non-essential amino acids, 1 % sodium pyruvate and 0.5 % HEPES. Medium was supplemented with either vehicle or 3 µg/mL ApoA-IV. After 24 hours, supernatants were collected. Enzyme-linked immunosorbent assay was performed according to the manufacturer's protocol.

2.2.16.5. Tumour necrosis factor-α ELISA in ApoA-IV treated macrophages

For macrophage differentiation, polarization and ApoA-IV treatments, see chapter 2.2.6 and 2.2.6.2, respectively. Supernatants were collected and enzyme-linked immunosorbent assay was performed according to the manufacturer's protocol.

2.2.16.6. PGD_2 MOX ELISA in ApoA-IV treated monocytes

Isolated PBMCs were seeded at a density of 1 million cells/mL in RPMI-1640 supplemented with 1 % PenStrep, 1 % non-essential amino acids, 1 % sodium pyruvate, 0.5 % HEPES, 5 % human male AB serum and incubated at 37°C in a humidified atmosphere containing 5 % CO_2. After one hour, non-adherent cells were removed by gentle washing. Remaining adherent monocytes were supplied with RPMI growth medium containing 1 % PenStrep, 1 % non-essential amino acids, 1 % sodium pyruvate and 0.5 % HEPES. Medium was supplemented with either vehicle or 3 µg/mL ApoA-IV. After 24 hours, supernatants were collected. Enzyme-linked immunosorbent assay was performed according to the manufacturer's protocol.

2.2.16.7. PGD_2 MOX ELISA in ApoA-IV treated monocytes

For macrophage differentiation, polarization and ApoA-IV treatments, see chapter 2.2.6 and 2.2.6.2, respectively. Supernatants were collected and enzyme-linked immunosorbent assay was performed according to the manufacturer's protocol.

2.2.17. Western blot analysis

For Western blot analysis, cells were harvested using Accutase and centrifuged for 7 minutes at 400 xg. Supernatant was aspirated and the pellet was lysed in pre-cooled RIPA buffer containing

1x Phosphatase inhibitor. Lysates were kept on ice for 20 minutes and centrifuged at maximum spin for 10 minutes at 4°C. Afterwards, samples were transferred into fresh Eppendorf tubes and either stored at -70°C or directly used to measure protein content using BCA assay. For this, the manufacturer's protocol was strictly followed, and protein content was measured in a photometer. Next, between 5 and 15 µg of protein were mixed with 6x Laemmli buffer containing 5 % β-mercaptoethanol and samples were cooked at 95°C for 5 minutes. Afterwards, samples were briefly centrifuged and then loaded onto Novex 4-20 % Tris-Glycine gels. Gels were run 125 Volt for 1.5 hours before being washed and blotted onto a nitrocellulose membrane, using iBlot technology. After the transfer, membranes were blocked in TBST containing 5 % milk for 2 hours at room temperature and then incubated with the primary antibody over night at 4°C. On the next morning, membranes were washed 3 times in TBST, and membrane was incubated with a secondary antibody. After another 3 washing steps, membrane was engulfed in HRP solution for 5 minutes before HRP signal was measured.

- COX-2 [AB32]: 1:1000 in 1% milk (dissolved in 1x TBST)
- β-Actin [AB33]: 1:1000 in 1% milk (dissolved in 1x TBST)
- NR1D1 [AB51]: 1:1000 in 5% BSA (dissolved in 1x TBST)
- Anti-mouse HRP [AB34]: 1:5000 in 5% milk (dissolved in 1x TBST)
- Anti-rabbit HRP [AB35]: 1:5000 in 10% milk (dissolved in 1x TBST)

2.2.18. RNA isolation and RT-qPCR

Monocytes were collected at a density of 7 x 10^6 as described above (see Chapter 2.2.5) and incubated either in the presence or absence of 3 µg/mL ApoA-IV for 24 hours, before being harvested using Accutase. Macrophages were collected and treated at a density of 5 x 10^6 as described above (see Chapter 2.2.6 and 2.2.6.2, respectively) prior to being harvested using Accutase. Pellets were then resuspended in 500 µL Trizol and stored at -70°C until RNA was isolated. For isolation, 100 µL of chloroform was added and samples were vortexed for 15 seconds. After 2-3 minutes at room temperature, samples were centrifuged at 12000 xg for 15 minutes in a

pre-chilled centrifuge set to a temperature of 4°C using a RNA collection column. Flow-through was discarded, pellets were dissolved in isopropanol and centrifuged for another 10 minutes. Afterwards, pellets were washed two times (7500 xg for 5 minutes each). RNA concentration was measured using Nanodrop. For cDNA synthesis, RNA samples were processed according to manufacturer's protocol. The following primer were used in the subsequent RT-qPCR: β actin, COX-2, mPGES-1 and hPGDS. qRT-PCR was performed according to manufacturer's protocol. To measure Δct, the genes of interested were subtracted from β actin and relative mRNA expression was measured by using $2^{\Delta ct}$.

2.2.19. Neutrophil calcium flux

1.1 x 10^6 PMNL of healthy donors were collected and resuspended in 1 mL -buffer. Fluo-3AM (1:1000) and F-127 (1:1000) were added and cells were incubated in the dark at room temperature. After 60 minutes, cells were washed, transferred in FACS tubes and treated with either vehicle or 3 µg/mL ApoA-IV at 37°C. After 20 minutes, CD16 [AB30] (1:500 diluted) was added and cells were stained for 10 minutes at 37°C, washed two times in -buffer and transferred into small FACS tubes. To measure intracellular calcium release, baseline was recorded for 30 seconds, after which the cells were stimulated with IL-8 as indicated. Calcium release was measured for two minutes (297).

2.2.20. Animal experiments

2.2.20.1. House dust mite-induced allergic lung inflammation

Eight-week-old female Balb/C mice were ordered (Charles River) and housed in individually ventilated cages under constant temperature (21°C), air humidity (50 %) and a 12-hour light/dark cycle. Standard chow and water were provided *ad libitum*. The experimental procedure was approved by the Austrian Federal Ministry of Science, Research and Economy (BMWFW-66.010/0020-WF/V/3b/2015) conform to Directive 2010/63/EU and was performed in accordance with national and international guidelines. To assess leukocyte infiltration in the BAL fluid, eight-week-old female mice were sensitized with 1 µg HDM intranasally on day 1. Starting on day 7,

mice were challenged daily with 10 µg HDM intranasally until day 11 and received concomitantly either 100 µL aqua dest. (vehicle) or aqua dest. containing 10 µg recombinant ApoA-IV until day 14. On day 15, mice were anaesthetized, and BAL fluid was collected. For this, the fur in the chest region was removed and the tracheal cannula was inserted and fixed. Blood was collected through cardiac puncture and the lung was rinsed. BAL fluid was collected by injecting 3 mL of pre-chilled BAL buffer through the cannula. Next, the BAL fluid was transferred into a 50 mL Falcon and centrifuged along with the collected whole blood at 400 xg for 7 minutes at 4°C. After centrifugation, 5 mL of 1x NH_4Cl lysis was added to cell pellets and remaining erythrocytes were lysed for 5 minutes on ice. Plasma of the whole blood samples was stored at -70°C. Cells were blocked in PBS containing Fc-Block [AB43] (1:200) for 15 minutes on ice, before being washed in PBS. Afterwards, cells were stained with an antibody cocktail, containing Siglec-F [AB44] (1:100), Ly6C [AB45] (1:600), Ly6G [AB46] (1:500), CD3e [AB47] 1:100, I-A/I-E (MHC II) [AB48] (1:200), CD11b [AB49] (1:200) and CD11c [AB50] (1:200) for 30 minutes at 4°C without light exposure (see Table 7). Afterwards, cells were washed, fixed and measured on the following day.

To assess lung function, eight-week-old female mice were sensitized with 1 µg HDM intranasally on day 1. Starting on day 7, mice were challenged daily with 10 µg HDM intranasally until day 11 and received. Between day 10 and day 14, mice received either 100 µL aqua dest. (vehicle) or aqua dest. containing 10 µg recombinant ApoA-IV. On day 15, mice were anaesthetized, and airway resistance and lung compliance were measured in response to increasing concentrations of methacholine through a FlexiVent system (1).

2.2.20.2. Collagen antibody-induced arthritis

Six-week-old male and female DBA/1J mice were ordered (Jackson Laboratory) and housed in individually ventilated cages under constant temperature (21°C), air humidity (50 %) and a 12-hour light/dark cycle. Standard chow and water were provided *ad libitum*. The experimental procedure was approved by the Austrian Federal Ministry of Science, Research and Economy (BMWFW-66.010/0045-WF/V/3b/2017) conform to Directive 2010/63/EU and was performed in accordance with national and international guidelines. The ethical vote was later extended (GZ

66.010/0187-WF/V/3b/2017, GZ 66.010/0168-V/3b/2018 and GZ 2020-0.220.296). Eight male mice (between 25 to 26 weeks old) were randomly chosen for the CAIA experiment (300). On day 0, mice were treated with 1.5 mg CAIA cocktail [AB36] intraperitoneally (see Table 7).

Table 12: Arthritis Score

Score	Symptom
0	no swelling/redness
1	redness/swelling in one digit
2	redness/swelling in more than one digit
3	redness/swelling in all digits

On the same day, mice were treated with either Vehicle or 0.1 mg/kg DP2 antagonist OC000459 twice daily subcutaneously. Weight and arthritic score were measured daily (see Table 12). To assess thermal pain sensitivity, the plantar test was used as described elsewhere (301). On day 3 and day 10, inflammation was boosted with 50 µg LPS (intraperitoneally). On day 14, mice were sacrificed. Blood was collected by cardiac puncture of anesthetized mice, centrifuged and plasma was stored at -70°C. Joint infiltrate was harvested as described previously (302). In short, hind limbs were cut 0.7 cm above the ankle joint using scissors, muscle and fat tissue was removed and bone marrow was flushed and collected. Ankles were digested in RPMI containing hyaluronidase and collagenase VIII and 10 % FCS for one hour at 37°C. Afterwards, cells were collected using a 70 µM cell strainer and suspension was washed in RPMI containing 10 % FCS. Lastly, joint infiltrate, blood leukocytes and bone marrow cells were centrifuged at 400 xg for 7 minutes at 4°C prior to being using for Flow Cytometry. After centrifugation, 5 mL of 1x NH_4Cl lysis was added to joint infiltrate, bone marrow and blood leukocytes and remaining erythrocytes were lysed for ten minutes on ice. Cells were blocked in PBS containing Fc-Block [AB43] (1:200) for 15 minutes on ice, before being washed in PBS. An antibody cocktail, consisting of CD45 [AB37] (1:100), CD3 [AB38] (1:200), Ly6G (1:100) [AB39], Ly6C [AB40] (1:50), F480 [AB41] (1:50) and c-kit [AB42] (1:100), was added and cells were stained for 30 minutes at 4°C without light exposure (see Table 7). Afterwards, cells were washed, fixed and measured on the following day.

3. Results

Some of the data presented in the results section have been previously published (1).

3.1. ApoA-IV potently suppresses eosinophil chemotaxis via NR1D1

Eosinophils are drivers of tissue remodelling in allergic asthma and eosinophil infiltration occurs between 2 to 6 hours after allergen exposure [reviewed in (118,124)]. In the last two decades, reports started to emerge that linked altered lipoprotein levels to allergic asthma. ApoA-I, a protein that is essential in lipid metabolism, was identified as a potential anti-allergic agent in mice and humans (159,160). After that, papers were published that suggested a similar role for a structurally related protein called ApoA-IV and its anti-inflammatory properties (162,163). For this reason, we set out to investigate whether ApoA-IV was able to suppress eosinophil migration using an *in vitro* assay. We stimulated isolated eosinophils with either vehicle or 3 µg/mL ApoA-IV for 30 minutes. After that, we let them migrate towards 3 nM of CCL11, which is a strong chemoattractant. ApoA-IV significantly suppressed CCL11-induced chemotaxis in eosinophils by 32.5 % (67.5 ± 22.6 % of vehicle response) (Figure 4A). In a similar setup, we used PGD_2 as chemoattractant and in a similar fashion, ApoA-IV significantly suppressed chemotaxis by 20.2 % (79.8 ± 15.1 % of vehicle response) (Figure 4B). Next, we were interested how ApoA-IV exerts its anti-migratory properties. ApoA-IV has recently been found to bind a nuclear receptor called Rev-ErbA alpha (NR1D1) in hepatocytes (92,93). When we exposed eosinophils to the NR1D1 agonist GSK4112, we observed a significant reduction by 46.2 % (53.8 ± 30.9 % of vehicle response) (Figure 4C). To verify our finding, we pre-treated eosinophils with a NR1D1 selective antagonist called SR8278 for 30 minutes prior to ApoA-IV treatment. While ApoA-IV significantly suppressed CCL11-mediated chemotaxis (66.8 ± 22.9 % of vehicle response), this effect was lost when the cells were pre-exposed to SR8278 (Figure 4D). In summary, we showed that ApoA-IV potently suppresses CCL11- and PGD_2-induced chemotaxis in eosinophils and that this inhibitory effect is mediated via the nuclear receptor NR1D1.

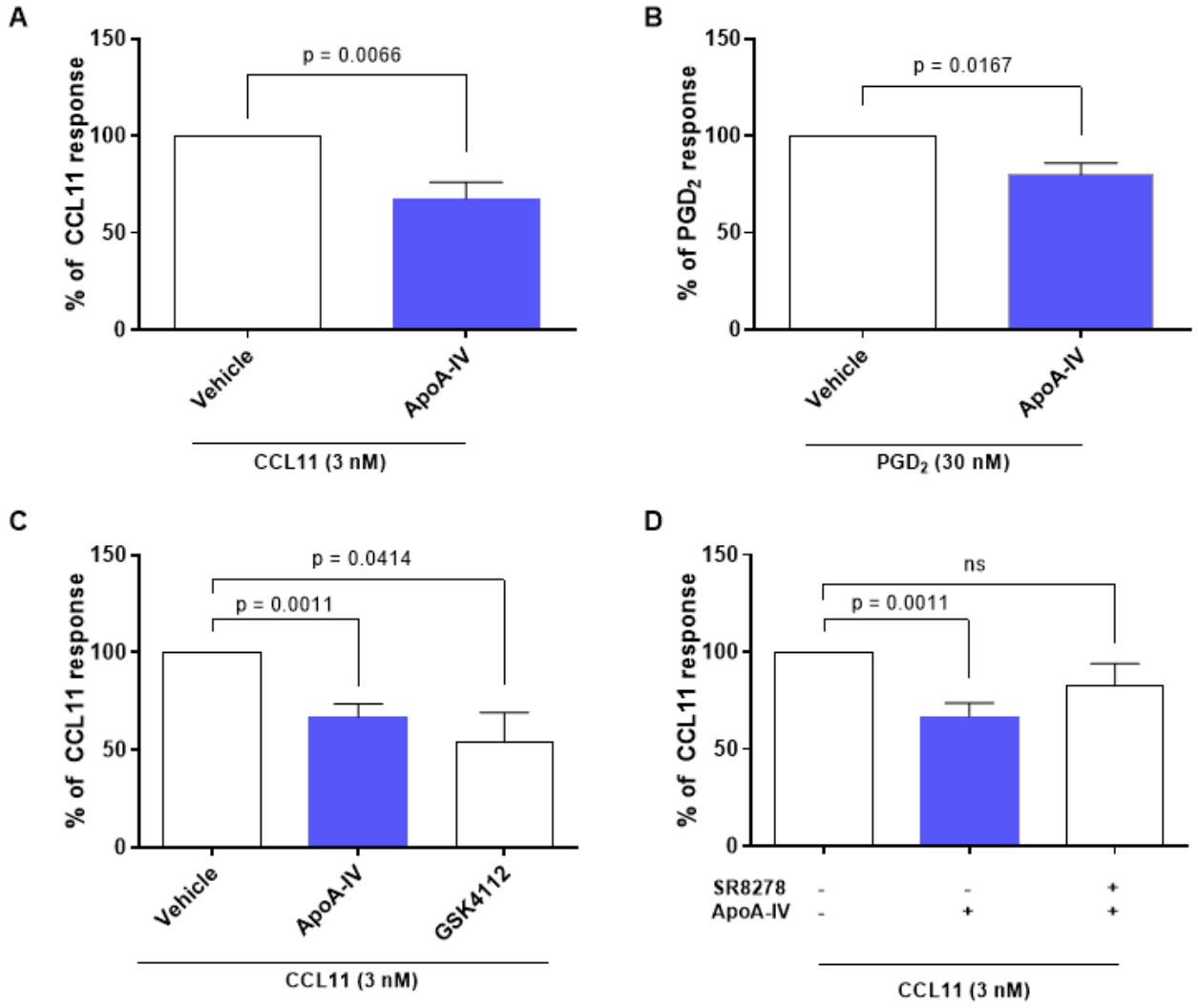

Figure 4: Effect of ApoA-IV on eosinophil chemotaxis. 100.000 Eosinophils were isolated from PMNL of non-allergic donors and allowed to migrate to CCL11 or PGD_2 for one hour at 37°C. Percentage of CCL11 or PGD_2 response is shown. (A) Eosinophils were treated with either vehicle or 3 µg/mL ApoA-IV for 30 minutes prior to chemotaxis towards CCL11 (n = 8). (B) Eosinophils were treated with either vehicle or 3 µg/mL ApoA-IV for 30 minutes prior to chemotaxis towards PGD_2 (n = 7). (C) Eosinophils were treated with either vehicle, ApoA-IV (3 µg/mL) or NR1D1 agonist GSK4112 (10 µM) for 30 minutes prior to chemotaxis towards CCL11 (n = 5-12). (D) Eosinophils were exposed to NR1D1 antagonist SR8278 (1 µM) for 30 minutes before being treated with either vehicle or 3 µg/mL ApoA-IV. After 30 minutes, cells were allowed to migrate towards CCL11 (n = 5-12). Statistical analyses were performed with paired two-tailed Student's t test (A-B) or one-way ANOVA followed by Dunnett's post-test. Iris Red or Kathrin Rohrer isolated eosinophils.

3.2. ApoA-IV suppresses lung inflammation in experimental airway hyperreactivity

To verify our *in vitro* findings *in vivo*, we used a HDM-induced model of experimental airway hyperreactivity to test whether ApoA-IV protects against eosinophilic inflammation in mice.

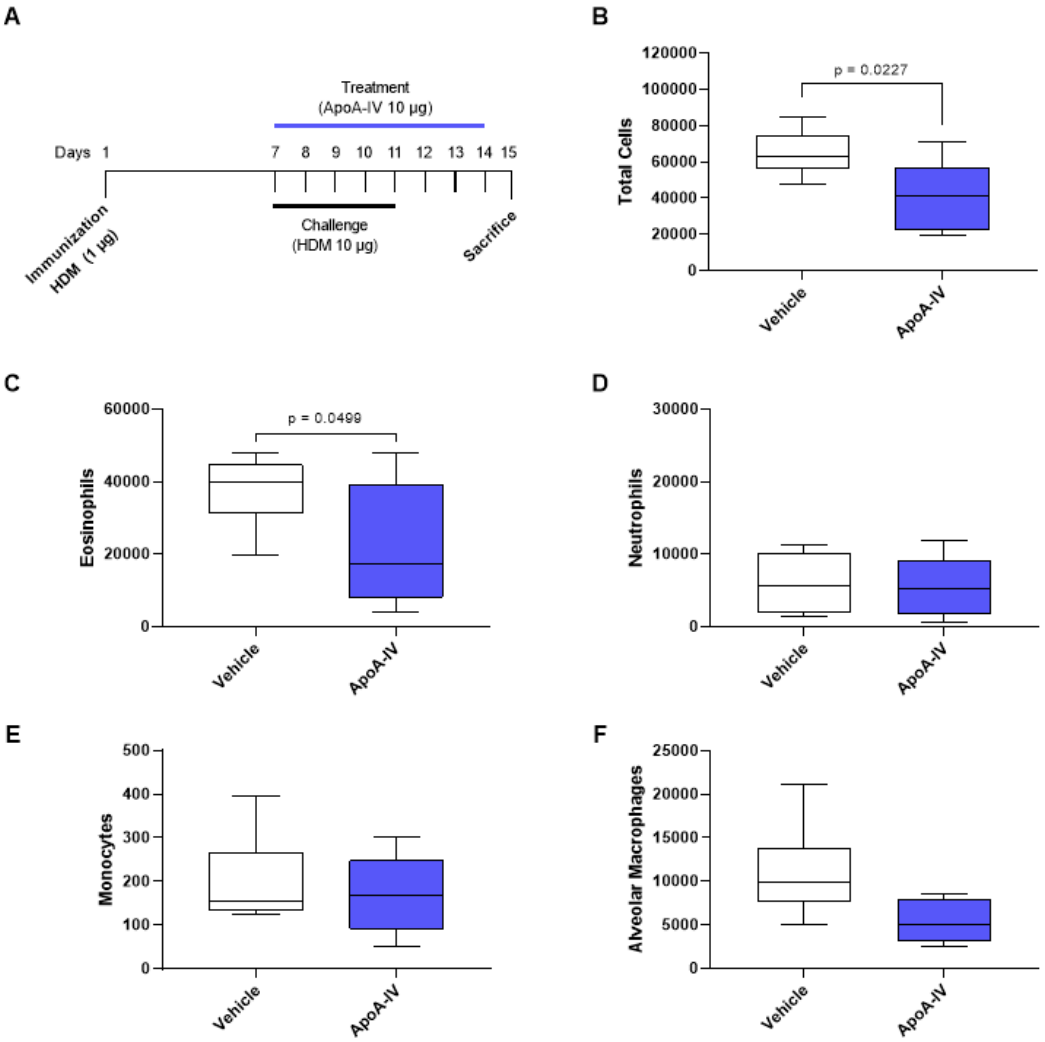

Figure 5: Effect of ApoA-IV on lung infiltration in experimental airway hyperreactivity. (A) Mice were immunized intranasally with 1 µg HDM on day 1. Seven days later, mice were challenged daily with 10 µg HDM intranasally until day 11. Between day 7 and day 14, mice received either vehicle or 10 µg ApoA-IV intraperitoneally. On day 15, mice were sacrificed and BAL fluid was collected (n = 6). Using Flow Cytometry, total cell (B), eosinophil (C), neutrophil (D), monocyte (E) and alveolar macrophage (F) counts were assessed. Statistical analyses were performed with paired two-tailed Student's t test. This figure was adapted from (1). Anna Theiler performed BAL fluid extraction and leukocyte staining.

We immunized eight-week-old female Balb/C mice with 1 µg HDM intranasally on day 1 (Figure 5A). After seven days, mice were challenged daily with 10 µg HDM from day 7 until day 11. Prior to each challenge, mice were injected with either vehicle or 10 µg ApoA-IV from day 7 until day 14. On day 15, mice were sacrificed and BAL fluid was collected. When we measured infiltrating immune cells in the BAL fluid, we observed in the vehicle-treated group a total number of 6.5 x 10^4 cells and in the ApoA-IV treated group a significant reduction to 4.12 x 10^4 cells (Figure 5B). Next, we wanted to assess which kind of immune cells are affected by ApoA-IV. For this, we stained the CD45 positive immune cells with markers to distinguish further between cell populations. In the vehicle-treated group, we counted 3.7 x 10^4 eosinophils. This number was significantly reduced to 2.2 x 10^4 cells in the ApoA-IV group (Figure 5C). We found no significant differences in neutrophils (Figure 5D) and monocytes (Figure 5E). In alveolar macrophages, we observed a visible trend, but it failed to reach statistical significance (Figure 5F) (1).

A hallmark of allergic asthma is lung remodeling in the chronic phase, resulting in the impairment of lung function. To investigate whether ApoA-IV displays beneficial effects also on a physiological level in experimental airway hyperreactivity, we assessed lung function. For this, eight-week-old female Balb/C mice were immunized with 1 µg HDM on day 1 and after one week, were challenged daily with 10 µg HDM for five consecutive days. On day 10 until day 14, mice were treated with either vehicle or 10 µg ApoA-IV intraperitoneally. Lung function was tested by challenging mouse lungs with increasing concentrations of methacholine, a potent bronchoconstrictor. Lung resistance was assessed using a FlexiVent system (Figure 6A). We observed at 30 mg/mL methacholine (MCh) a significant reduction in the ApoA-IV group (2.32 ± 0.34 cm H_2O.s/mL) compared to the vehicle group (7.81 ± 4.01 H_2O.s/mL). This significant difference remained even at the highest concentration of 100 mg/mL. When we looked at lung compliance, we observed that ApoA-IV treated mice showed a significant better lung function at 10 mg/mL MCh challenge (0.90 ± 0.06 mL/cm H_2O) than the vehicle group (0.62 ± 0.17 mL/cm H_2O) (Figure 6B) (1).

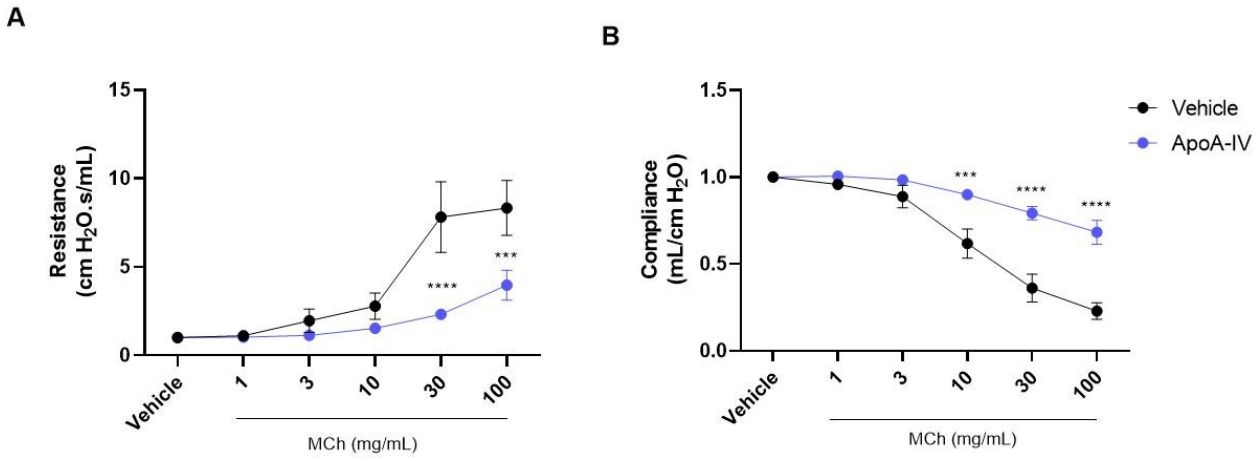

Figure 6: Effect of ApoA-IV on lung function in experimental airway hyperreactivity. Mice were immunized intranasally with 1 µg HDM on day 1. Seven days later, mice were challenged daily with 10 µg HDM intranasally until day 11. Between day 10 and day 14, mice received either vehicle or 10 µg ApoA-IV intraperitoneally. On day 15, lung function was observed (n = 6-8). For this, increasing concentrations of methacholine were administered while airway resistance and compliance were measured using a FlexiVent system. Statistical analyses were performed with two-way ANOVA followed by Sidak post-test. Statistical significance is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, **** < 0.0001. This figure was adapted from (1). Lung function assessment was performed by Petra Luschnig and Kathrin Rohrer.

This lung protective ability of ApoA-IV was also observed at 30 and 100 mg/mL MCh challenge. In summary, we show that ApoA-IV potently mitigates HDM-induced experimental lung inflammation and hyperreactivity in mice, as shown in a reduction in infiltrating total cells and eosinophils in the BAL fluid as well as in lung function assessment.

3.3. ApoA-IV suppresses monocyte chemotaxis and CD11b upregulation, but does not affect basal production of IL-10 or TNF-α

We next shifted our focus towards cells involved in the pathogenesis of the autoimmune disease rheumatoid arthritis. As monocyte infiltration correlates with disease progression, we wondered whether ApoA-IV would have a similar effect on these cells as on eosinophil migration (Figure 4). We treated isolated PBMC with 3 µg/mL ApoA-IV and transferred them into Transwells, where they were exposed to increasing concentrations of C5a, a strong chemoattractant that is present in RA synovial fluid (303). ApoA-IV treatment significantly reduced the chemotactic index from 9.3 ± 10.5 (vehicle) to 4.8 ± 5.0 (Figure 7A). To support our findings, we looked at CD11b expression,

which is highly involved in migration. We treated PBMC with 3 µg/mL ApoA-IV for either 30 minutes, 3 hours or 6 hours and measured CD11b (Figure 7B). CD11b was significantly upregulated after 30 minutes of treatment (94.76 ± 28.5-fold increase) compared to vehicle-treated cells (67.83 ± 19.1-fold increase). This upregulation was lost at the 3-hour time point. After 6 hours, ApoA-IV treated cells showed a significant reduction in CD11b expression (41.99 ± 17.26-fold increase) compared to vehicle-treated cells (61.46 ± 28.74-fold increase). Next, we wanted to test whether ApoA-IV exerted anti-inflammatory effects on monocytes. For this, we stimulated adherent monocytes with ApoA-IV and measured the basal production of interleukin (IL)-10 and TNF-α (Figure 7C-D). No significant changes in the production of both cytokines were observed, although there was a trend towards an increase in ApoA-IV-treated cells. Next, we wanted to assess whether the apolipoprotein could dampen pro-inflammatory responses. For this purpose, we pre-treated monocytes with 3 µg/mL of ApoA-IV before stimulating the cells with zymosan-A, a fungal derived TLR2 agonist that induces production of reactive oxygen species (ROS) in monocytes. However, no differences between the treatments could be detected (Figure 7E). Thereafter, we looked at expression of surface molecules. CD4 is a well-known immune receptor that is predominantly associated with T lymphocytes. While its signalling and function have been extensively researched in T cells, its presence on the surface of innate immune cells such as monocytes, macrophages and dendritic cells could not be explained since its discovery on these cells (304). It has been shown that CD4 ligation in monocytes leads to macrophage differentiation (305). As monocyte-to-macrophage transition is a common hallmark in the pathophysiology of RA, we hypothesized that ApoA-IV could influence CD4 expression on the surface of all three monocyte populations. However, we found no significant changes in CD4 expression after 30 minutes (Figure 7F), 3 hours or 6 hours (data not shown) of ApoA-IV treatment. In summary, ApoA-IV suppressed monocyte chemotaxis and activation, but had no impact on IL-10, TNF-α or ROS production in these cells.

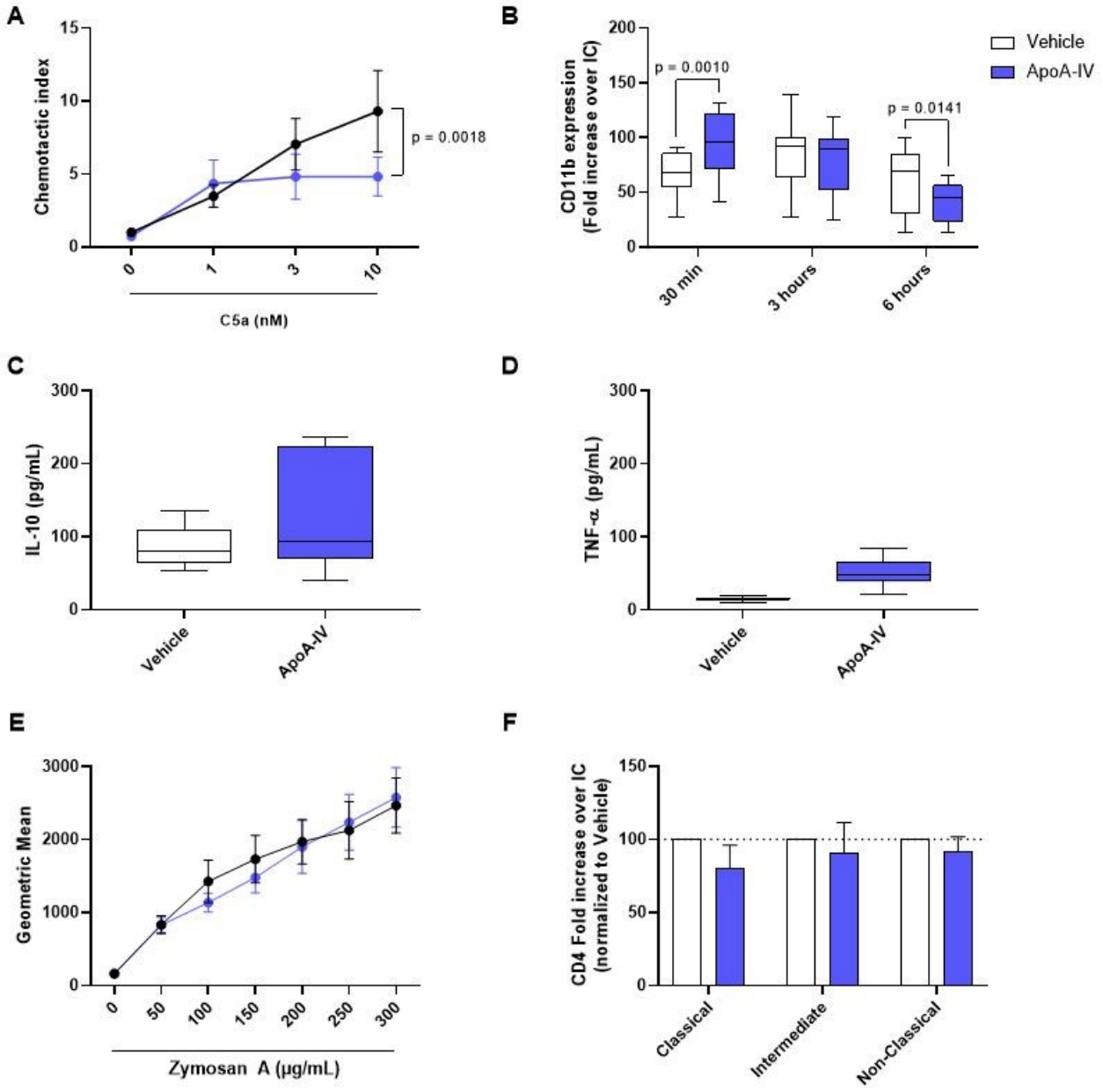

Figure 7: Effect of ApoA-IV on monocyte activation and cytokine production. (A) Isolated PBMC were pre-treated with 3 µg/mL ApoA-IV or vehicle for 30 minutes before being exposed to increasing concentrations of chemoattractant C5a (n = 15). (B) CD11b expression was measured in monocytes after being exposed to vehicle or 3 µg/mL ApoA-IV for 30 minutes, 3 hours or 6 hours using flow cytometry (n = 9). (C) IL-10 production was measured in monocyte supernatants using ELISA (n = 9-10). (D) TNF-α production was measured in monocyte supernatants using ELISA (n = 2-6). (E) Zymosan A-induced ROS production was measured using flow cytometry (n = 6). (F) CD4 expression was measured using flow cytometry (n = 3). Statistical analyses were performed with two-way ANOVA followed by Sidak post-test (A-B, E-F) or paired two-tailed Student's t test (C-D). Iris Red or Kathrin Rohrer isolated PBMC.

3.4. ApoA-IV acts as a phenotype switch in macrophages

While we observed no anti-inflammatory effects of ApoA-IV in monocytes, we wondered whether the apolipoprotein exerted a stronger response in macrophages. For this, we differentiated isolated PBMC with M-CSF to monocyte-derived macrophages ("MDM") and polarized them either with LPS and IFN-γ ("M1") or IL-4 ("M2"). As a control, unpolarized macrophages were retained in the MDM state ("M0"). For the experiment, the polarization medium was supplemented with either vehicle or 3 µg/mL ApoA-IV. When we looked at CD80, we found that ApoA-IV drastically altered the expression levels of this surface marker (Figure 8A). While no differences could be observed in MDM, the pro-inflammatory M1 macrophages displayed a significant reduction in the fold increase (11.20 ± 2.11) compared to vehicle-treated cells (17.05 ± 4.12). To our surprise, CD80 expression in ApoA-IV treated M2 macrophages increased (8.33 ± 2.37) compared to vehicle-treated cells (5.28 ± 1.65). Next, we looked at the expression of the anti-inflammatory M2 marker CD206 (Figure 8B). While MDM and M1 macrophages were unaffected by ApoA-IV treatment, we observed a significant reduction of CD206 in M2 (35.96 ± 18.68) compared to vehicle-treated cells (58.72 ± 33.70). This suggests that ApoA-IV may provoke a phenotype switch in macrophages. To verify our findings, we next sought to test whether this "de-polarization" also had an effect on cytokine production. For this, we first tested whether ApoA-IV would increase basal production of anti-inflammatory interleukin-10 (Figure 8C), but we failed to detect any significant changes. Next, we shifted our focus towards pro-inflammatory cytokines and measured TNF-α (Figure 8D). We found that TNF-α levels in the macrophage supernatants were significantly lower in ApoA-IV treated cells (344.06 ± 224.0 pg/mL) compared to our vehicle control (1168.08 ± 480.1 pg/mL). In summary, we showed that ApoA-IV suppresses CD80 expression and TNF-α production in M1 macrophages. In M2 macrophages, we showed a significant upregulation of CD80 and a concomitant downregulation of CD206.

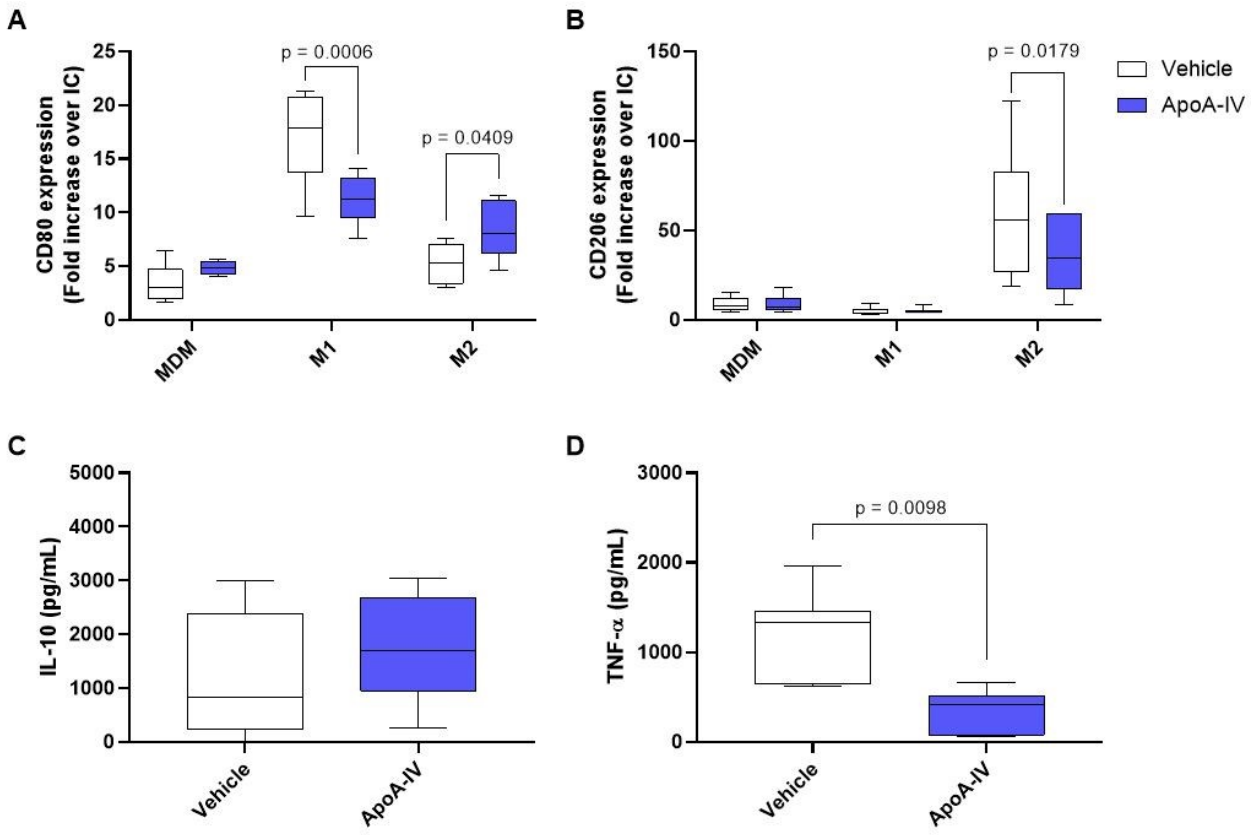

Figure 8: Effect of ApoA-IV on M1 macrophage polarization and cytokine production. Isolated PBMC were differentiated to macrophages in the presence of M-CSF. After one week, macrophages were polarized to M1 or M2 while being exposed to either vehicle or 3 µg/mL ApoA-IV. (A) CD80 expression was measured using flow cytometry (n = 6). (B) CD206 expression was measured using flow cytometry (n = 6). (C) Basal IL-10 production was measured in M1 macrophage supernatants using ELISA (n = 9-10). (D) Basal TNF-α production was measured in M1 macrophages using ELISA (n = 7). Statistical analyses were performed with two-way ANOVA followed by Sidak post-test (A-B) or paired two-tailed Student's t test (C-D). Iris Red or Kathrin Rohrer isolated PBMC.

3.5. ApoA-IV exerts its anti-inflammatory effects via NR1D1 in macrophages

Next, we wanted to elucidate the signaling underlying the observed effects of ApoA-IV in macrophages. As we showed in eosinophils that ApoA-IV acts via NR1D1 (Figure 4C-D), we tested whether this was also the case for macrophages. First, we stained for NR1D1 in untreated MDM, untreated M1 and ApoA-IV treated M1 macrophages via Western blotting and observed a strong band at 67 kDa, indicating that this nuclear receptor is present in these cells (**Figure 9**A). ApoA-IV treatments had no significant effect on protein expression (**Figure 9**B).

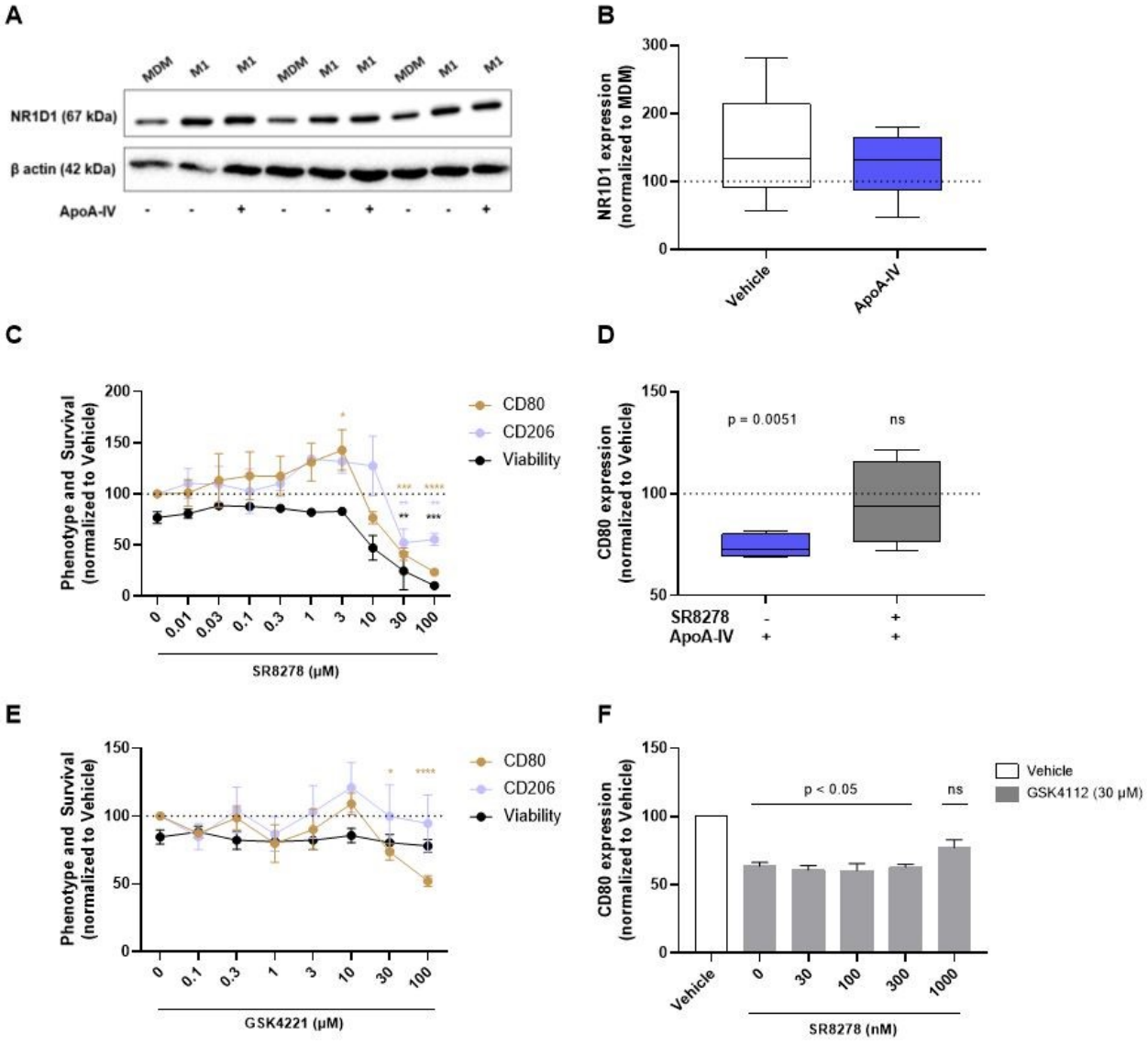

Figure 9: Effect of NR1D1 signalling on M1 polarization. Isolated PBMC were differentiated into macrophages and polarized to M1 with LPS and IFN-γ. Medium was supplemented with either vehicle, 3 µg/mL ApoA-IV or varying concentrations of SR8278 and GSK4112. (A-B) NR1D1 expression was measured in either vehicle-treated MDM or M1 or ApoA-IV treated M1 macrophages using Western blotting (n = 5). (C) M1 polarization medium was supplemented with either vehicle or increasing concentrations of NR1D1 antagonist SR8278 (n = 2). (D) M1 macrophages were pre-treated with either vehicle or 1 µM of SR8278 before being treated with 3 µg/mL ApoA-IV (n = 4). (E) M1 polarization medium was supplemented with either vehicle or increasing concentrations of NR1D1 agonist GSK4112 (n = 4). (F) During M1 polarization, macrophages were pre-treated with either vehicle or increasing concentrations of SR8278 for 30 minutes and then treated with vehicle or 30 µM of GSK4112 (n = 3). Statistical analyses were performed with paired two-tailed Student's t test (B), one-way ANOVA followed by Dunnett's post test (D, F) or two-way ANOVA followed by Dunnett's post-test (C, E). If p value is not stated, statistical significance is indicated as follows: *p < 0.05,**p < 0.01, ***p < 0.001, **** < 0.0001. Iris Red or Kathrin Rohrer isolated PBMC.

Next, we wanted to test whether NR1D1 was involved in ApoA-IV signaling in M1 macrophages. To rule out unspecific effects of the NR1D1 antagonist SR8278, we tested increasing concentrations of this substance and measured CD80 expression, CD206 expression and cell viability (**Figure 9**C). We found that at a concentration of 3 µM, CD80 expression was significantly increased by roughly 43 % compared to vehicle-treated cells. At 10 µM, cell viability started to decline and was significantly reduced at 30 µM and 100 µM. Therefore, we determined that the highest concentration to use was 1 µM of this antagonist. To test whether SR8278 could inhibit ApoA-IV mediated CD80 downregulation, we compared cells that were exposed to either vehicle or 1 µM of SR8278 prior to ApoA-IV treatment (**Figure 9**D). We observed that ApoA-IV alone suppressed CD80 expression by 26 % (74.13 ± 5.0 % of response of untreated M1 macrophages), but this reduction was no longer significant, when these cells were pre-exposed to the antagonist (95.05 ± 4.9 % of response of untreated M1 macrophages). To verify that ApoA-IV signals through NR1D1, we compared its effect with the NR1D1 agonist GSK4221. First, we tested increasing concentrations to see whether it exerts similar effects on CD80 expression in M1 macrophages as observed with ApoA-IV (**Figure 9**E). We found that at a concentration of 30 µM, GSK4112 could significantly decrease CD80 expression by 27 % (73.5 ± 10.5 % of response of untreated M1 macrophages) compared to vehicle-treated cells. This reduction was even greater (48 %) at 100 µM. Cell viability was unaffected at all conditions. Concluding from these data, we decided to use 30 µM of GSK4112 for our next experiments. Lastly, we tested whether antagonist SR8278 could block the observed effect of GSK4112 (**Figure 9**F). We found that 30 µM GSK4112 significantly reduced CD80 expression and this effect could be prevented with 1 µM of the NR1D1 antagonist SR8278. In summary, we observed that NR1D1 is expressed in macrophages but is unaffected by ApoA-IV treatment in M1. In these cells we showed further that the observed suppressive effect of the apolipoprotein on CD80 expression is mediated via the NR1D1 pathway.

3.6. ApoA-IV does neither affect IL-8 induced shape change or calcium flux nor C5a induced ROS production nor GM-CSF mediated HLA-DR up-regulation in neutrophils

Neutrophil granulocytes are the predominant leukocytes in the synovial fluid of RA patients (227). We were therefore curious to see whether ApoA-IV displays similar effects on neutrophils as observed in eosinophils (Figure 4). For this purpose, we devised a range of experiments to test whether neutrophil activation and functionality could be suppressed in the presence of ApoA-IV. First, we tested whether interleukin-8 induced shape change could be affected by ApoA-IV (Figure 10A). Neutrophils responded strongly through cytoskeletal rearrangements to IL-8. At a concentration of 1 nM of IL-8 we observed a small but significant rise in the Geometric Mean in our ApoA-IV treated group (280.0 ± 31.18) compared to the vehicle-treated group (266.1 ± 24.1). However, this effect was lost at a higher concentration of 10 nM. When we looked at IL-8 induced calcium release in neutrophils, no changes between the treatments could be observed (Figure 10B). Next, we assessed C5a mediated production of reactive oxygen species, however, neutrophils continued to be unaffected by ApoA-IV in these experiments (Figure 10C). Lastly, we looked at GM-CSF-induced up-regulation of HLA-DR. It has been recently discovered that neutrophils acquire the capacity to present antigens to T cells by up-regulation of HLA-DR (236). As GM-CSF is a strong inducer of HLA-DR expression and elevated in the synovial fluid of RA patients(306), we were interested whether this up-regulation could be interfered with by ApoA-IV treatment.

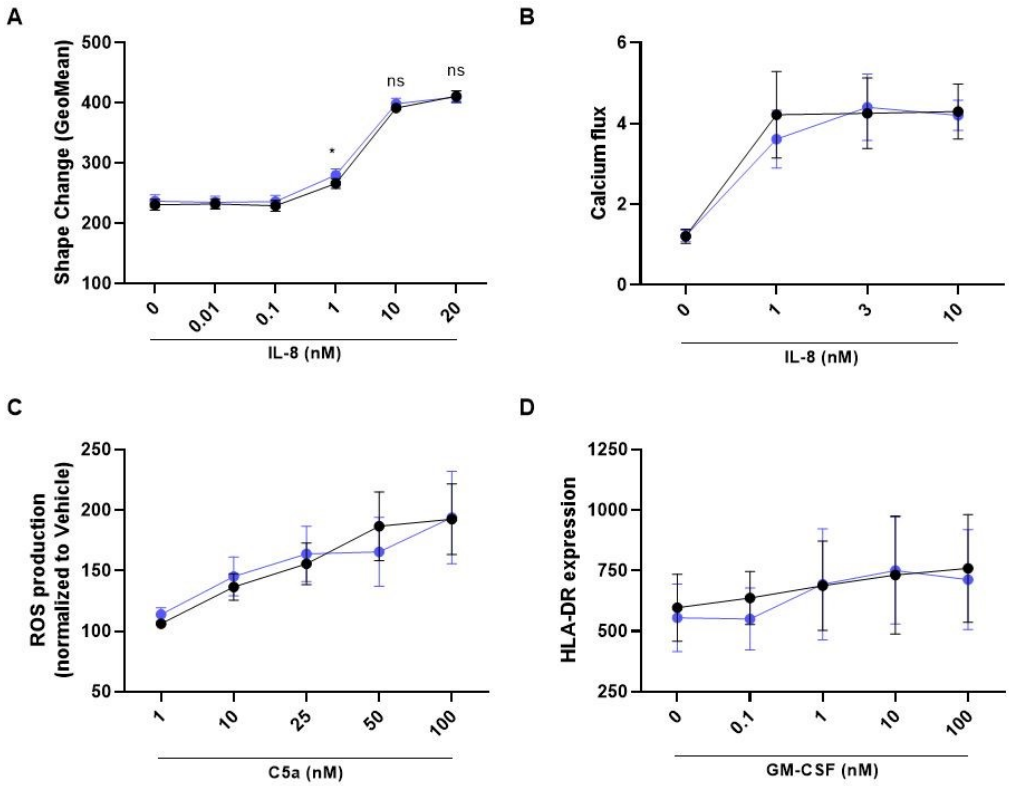

Figure 10: Effect of ApoA-IV on neutrophil activation. Isolated PMNL from healthy donors were pre-treated either with vehicle or 3 µg/mL of ApoA-IV before assays were performed. (A) Shape change of neutrophils in response to increasing concentrations of IL-8 (n = 4). (B) Neutrophil calcium release in response to increasing concentrations of IL-8 (n = 3). (C) Neutrophil ROS production in response to increasing concentrations of C5a (n = 9). (D) GM-CSF induced upregulation of HLA-DR (n = 3). Statistical analyses were performed two-way ANOVA followed by Sidak post-test. Statistical significance is indicated as follows: *p < 0.05. Iris Red or Kathrin Rohrer isolated PMNL.

For this, we treated the cells with ApoA-IV or vehicle and incubated them in the presence of increasing concentrations of GM-CSF for 24 hours, before we measured up-regulation using flow cytometry (Figure 10D). However, ApoA-IV failed to suppress GM-CSF-induced up-regulation of HLA-DR. In summary, ApoA-IV did not affect IL-8 induced shape change, chemotaxis or calcium release. Further, no changes in C5a mediated ROS production or GM-CSF-induced HLA-DR upregulation could be observed.

3.7. Plasma ApoA-IV levels are reduced and DP2 expression is altered in rheumatoid arthritis

ApoA-I, which is structurally and functionally similar to ApoA-IV, is reduced in the serum of RA patients but accumulates in the inflamed joint (288,289). As we observed potent suppressive effects of ApoA-IV on cells that drive this disease, we wanted to assess the impact of this apolipoprotein in a clinical setting. For this, we looked at plasma levels of ApoA-IV in RA patients. We observed that healthy donors displayed higher plasma concentrations of the apolipoprotein (1587.8 ± 154.8 ng/mL) compared to RA patients (1075.2 ± 377.6 ng/mL) (Figure 11A). ApoA-I has been shown to induce COX-2 mediated PGD_2 production in macrophages and since elevated PGD_2 levels have been detected in the synovial fluid of RA patients - the same site where ApoA-I accumulates - we wanted to investigate a possible interrelation between both ApoA-IV and PGD_2 (275,290). Therefore, we wanted to measure surface expression of PGD_2 receptor DP2, which is present on the surface of eosinophils, basophils, DCs, Th2 cells, mast cells and monocytes (27). We compared the expression levels of RA patients suffering from active disease ("flare"), patients that received treatments and show signs of remission ("remission") and healthy donors as controls ("healthy"). In the PMNL fraction, we found that eosinophils expressed DP2 but there were no differences between the three groups observed (Figure 11B). When we looked at the PBMC fraction that contained CD3 expressing cells, we found that samples of the "remission" group displayed a significant fold increase of 1.3 ± 0.75 in DP2 expression in $CD8^+$ T cells compared to the fold of 0.8 ± 0.40 in the "healthy group" (Figure 11C). In monocytes, DP2 was only expressed in the non-classical $CD14^-CD16^+$ population (Figure 11D). While the "healthy" group displayed a fold increase of 2.2 ± 0.52, this expression of DP2 was significantly reduced in the "flare" group (1.36 ± 0.41).

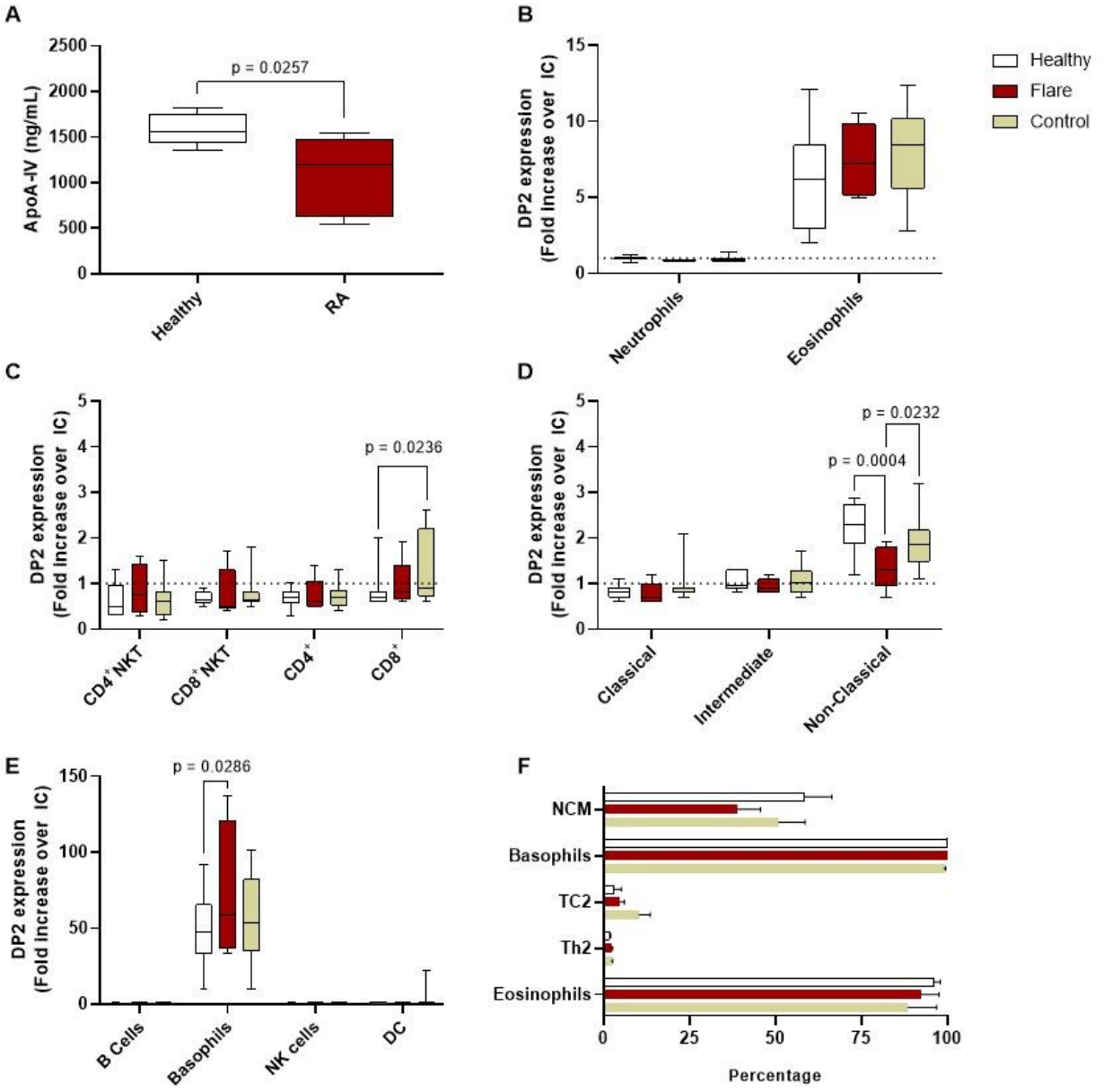

Figure 11: Plasma ApoA-IV levels and DP2 expression in circulating leukocytes of RA patients. (A) Plasma was collected from healthy donors and RA patients (flare and remission were pooled) and ApoA-IV levels were measured using an enzyme-linked immunosorbent assay (n = 5-7). (B-F) Blood was drawn from healthy donors or patients suffering from rheumatoid arthritis. RA samples were grouped in patients with active disease (flare) or patients in remission. DP2 expression was measured in (B) granulocytes, (C) NK-T and T cells, (D) monocytes and (E) CD3 negative cells of the PBMC fraction (n = 5-12). (F) Comparison of receptor distribution within DP2 expressing populations (n = 5-12). Statistical analyses were performed with paired two-tailed Student's t test (A) or two-way ANOVA followed by Tukey post-test (B-F). RA samples were provided by Martin Stradner and Angelika Lackner of the Division of Rheumatology and Immunology, Medical University of Graz.

The “remission” group displayed a similar expression as the “healthy” group (1.93 ± 0.59). In the CD3 negative fraction of PBMCs, we observed a strong expression of DP2 in basophils (Figure 11E). Contrary to the surface expression in monocytes, DP2 expression was significantly increased in basophils of the “flare” group (72.15 ± 39.8) compared to the “healthy” group (49.0 ± 22.9). As we found DP2 expression in non-classical monocytes, basophils, CD4 and CD8 T cells and eosinophils, we were curious whether the percentage of DP2 positive cells was differently distributed in RA. However, we found no differences between the “healthy”, “flare” or “remission” groups (Figure 11F). In summary, we observed that RA patients display a significant reduction in plasma ApoA-IV levels compared to healthy donors and that DP2 expression is altered in CD8 T cells, non-classical monocytes and basophils in patients suffering from RA.

3.8. Classical monocytes and $CD4^+$ T helper cells are expanded in the blood of RA patients and RA monocytes do not migrate towards PGD_2

Next, we looked further within the cell populations to see if their distribution is different between the three groups. We found no significant changes in eosinophils or neutrophils between the three groups (Figure 12A). When we looked at CD3 positive cells of the PBMC fraction, we observed a small but significant increase in T Helper cells of the “flare” group (69 % of $CD3^+$ cells) compared to the cells of the “healthy” group (61 % of $CD3^+$ cells) (Figure 12B). In the CD3 negative populations of PBMC we detected non-significant alterations (Figure 12C). Next, we assessed whether there was a similar change of distribution within the subpopulations of monocytes (Figure 12D). We found that classical $CD14^{++}CD16^-$ monocytes are expanded in the “flare” group (74 % of monocytes) compared to the “healthy” group (65 % of monocytes). Next, we wanted to investigate the effect of PGD_2 on monocyte migration. PGD_2 has been shown to attract macrophages *in vitro* and since this lipid mediator is present in the inflamed synovium, we wanted to investigate whether it also attracts monocytes (20,147,275). When we assessed chemotaxis of $CD14^+$ monocytes in response to increasing concentrations of PGD_2, we found no statistically significant changes between the “healthy”, “flare” or “remission” groups (Figure 12E). When PGD_2 failed to attract unstimulated monocytes, we exposed isolated PBMC from healthy donors in our next experiment to IL-1β, IL-6 or TNF-α prior to chemotaxis to emulate a pro-inflammatory

environment. We found no significant changes in the chemotactic index (Figure 12F). In summary, we found that in RA patients suffering from flares, CD4 T helper cells and classical monocytes are expanded compared to healthy controls and that PGD_2 does not attract monocytes.

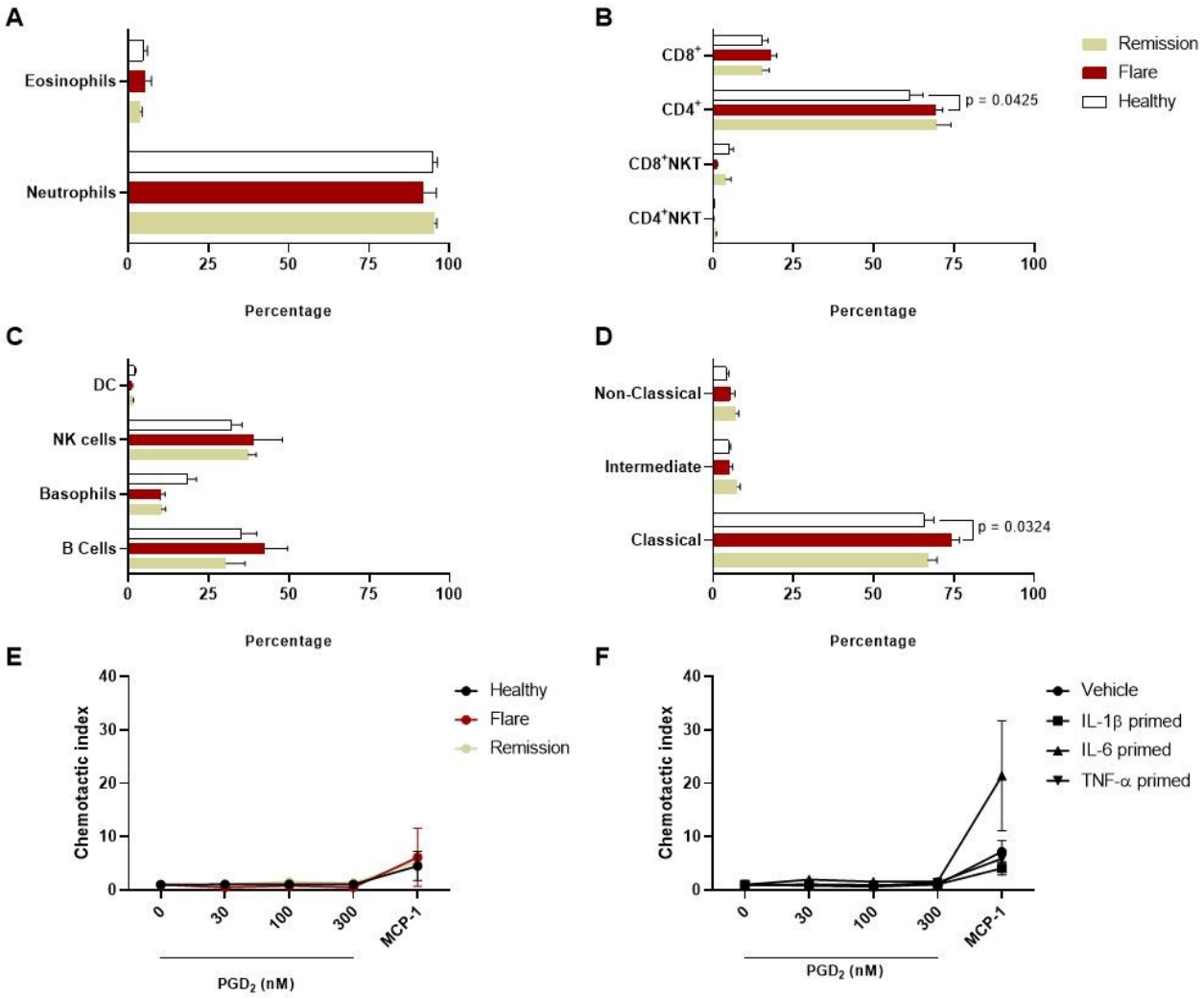

Figure 12: Distributions of circulating leukocytes in RA patients and effect of PGD_2 on monocyte migration. Blood was drawn from healthy donors or patients suffering from rheumatoid arthritis. RA samples were grouped in patients with active disease (flare) or patients in remission. The distribution of (A) granulocytes, (B) NK-T and T cells, (C) CD3 negative cells of the PBMC fraction and (D) monocytes was also observed (n = 5-12). (E) Isolated PBMC from RA patients and healthy donors were allowed to migrate towards increasing concentrations of PGD_2 (n = 3-9). (F) Isolated PBMC from healthy donors were pre-treated with either IL-1β, IL-6 or TNF-α before being allowed to migrate towards PGD_2 (n = 5-7). Statistical analyses were performed with two-way ANOVA followed by Tukey post. RA samples were provided by Martin Stradner and Angelika Lackner of the Division of Rheumatology and Immunology, Medical University of Graz.

3.9. Granulocyte shape change in response to 5-OxoETE and PGD_2 is unaffected in Rheumatoid Arthritis

While we observed no chemotactic response to PGD_2 in monocytes, we hypothesized that synovial fluid derived lipid mediators may act on other innate immune cells that are involved in RA pathogenesis. We were therefore interested whether eosinophil and neutrophil responses are altered when compared to cells from healthy donors. When granulocytes encounter chemoattractants, they are quickly activated and migrate towards increasing concentrations of these stimulants to fulfil their purpose as effector cells.

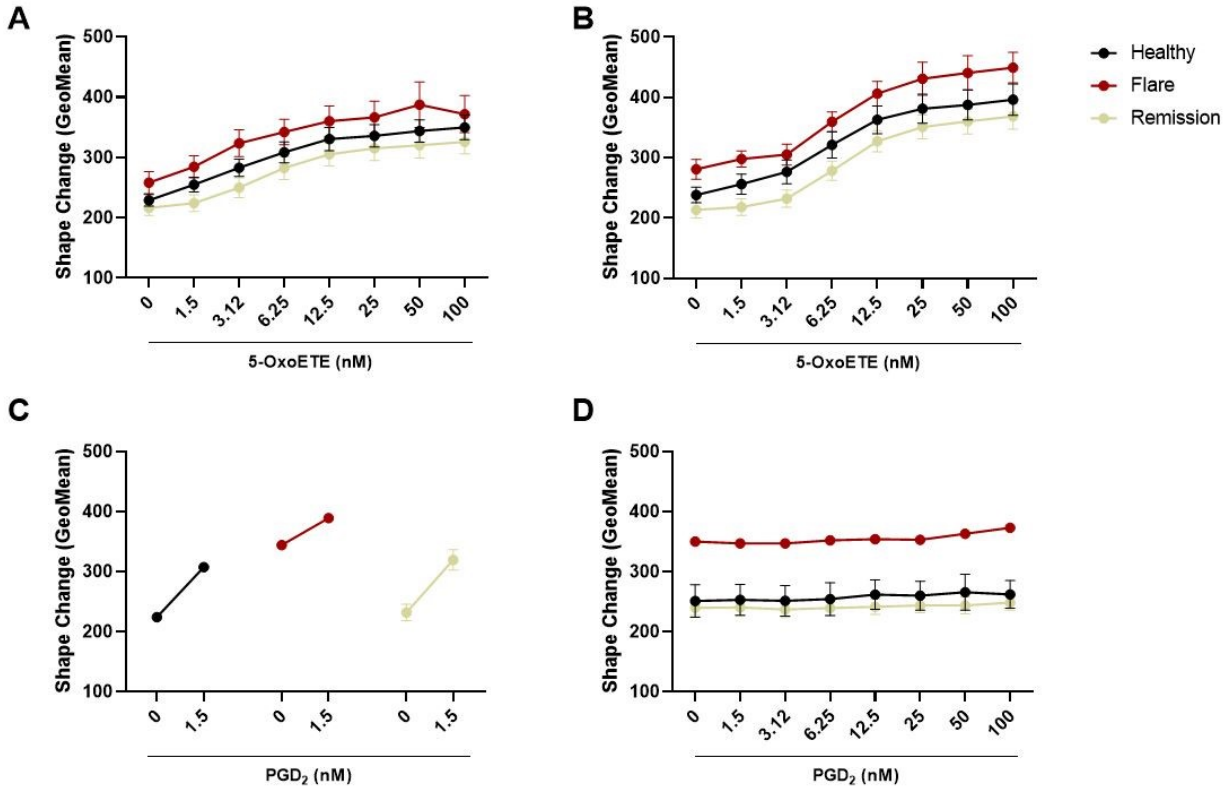

Figure 13: Eosinophil and neutrophil shape change in response to 5-OxoETE and PGD_2 in RA patients. PMNL were isolated from human blood of healthy donors, patients suffering from acute flares or patients in remission. Cells were then treated with lipid mediators and shape change was assessed by Flow Cytometry in (A) eosinophils in response to 5-OxoETE (n = 4-15), in (B) neutrophils in response to 5-OxoETE (n = 4-15), in (C) eosinophils in response to PGD_2 (n = 1-5) or in (D) neutrophils in response to PGD_2 (n = 1-5). Statistical analyses were performed with two-way ANOVA followed by Tukey post-test. RA samples were provided by Martin Stradner and Angelika Lackner of the Division of Rheumatology and Immunology, Medical University of Graz. PMNL isolation and shape change experiment were performed by Kathrin Rohrer.

5-OxoETE and PGD_2 are both lipid mediators that display strong chemotactic properties on eosinophils and act on cells of the mononuclear phagocyte system (20,307). For production of these lipid mediators, the enzymes 5-LOX and hPGDS are required, respectively. Both enzymes are present in the inflamed synovium of RA patients (275,308). We therefore set out to test whether the response to these substances is altered in granulocytes of RA patients. For this, we measured changes on their surface using flow cytometry. These "shape changes" were observed in response to increasing concentrations of 5-OxoETE in eosinophils (Figure 13A) and neutrophils (Figure 13B). We found no significant changes between the three groups in any of these cell types. When we looked at granulocyte shape change in response to PGD_2, we also could not detect any differences in eosinophils (Figure 13C) or neutrophils (Figure 13D). In summary, granulocytes of patients suffering from rheumatoid arthritis display no altered response to increasing concentrations of 5-OxoETE or PGD_2.

3.10. Granulocyte ROS production in response to 5-OxoETE and PGD_2 is unaffected in Rheumatoid Arthritis

Excessive production of reactive oxygen species has been observed in RA and contributes to the exacerbation of synovitis (309). Therefore, we set out to investigate whether PMNL isolated from RA patients produce more ROS compared to granulocytes from healthy donors. We treated eosinophils and neutrophils with increasing concentrations of 5-OxoETE but could not find any altered response between the "healthy", "flare" or "remission" group (Figure 14A-B). Similarly, we exposed eosinophils and neutrophils to increasing concentrations of PGD_2. While the ROS baseline was visibly but not significantly increased between RA samples and samples of healthy donors, no difference in response to the lipid mediators was detected (Figure 14C-D). In summary, 5-OxoETE and PGD_2 could not induce a stronger ROS production in RA granulocytes when comparted to samples of healthy donors.

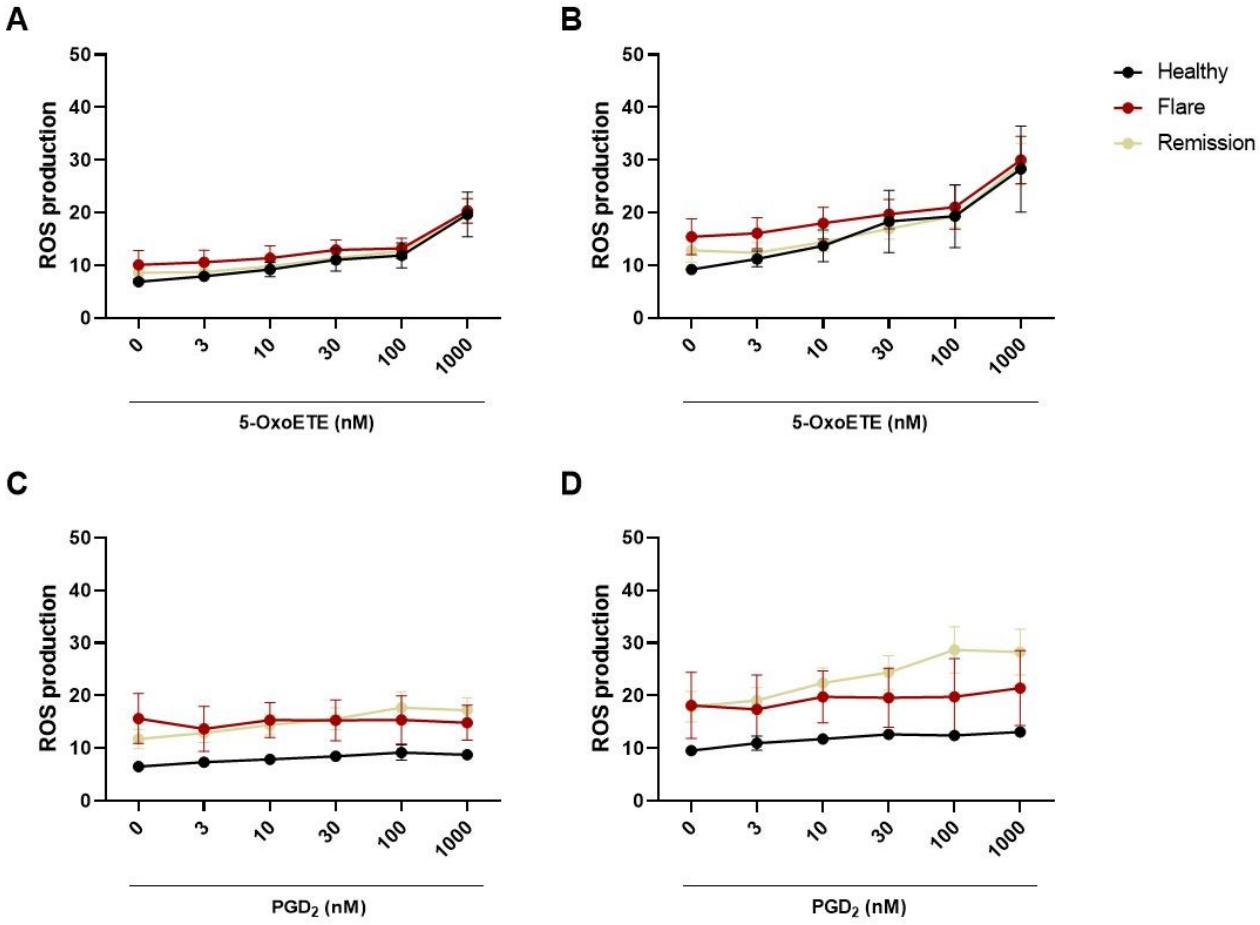

Figure 14: Eosinophil and neutrophil ROS production in response to 5-OxoETE and PGD_2 in RA patients. PMNL were isolated from human blood of healthy donors, patients suffering from acute flares or patients in remission. Cells were then treated with lipid mediators and shape change was assessed by flow cytometry in (A) eosinophils in response to 5-OxoETE (n = 5-15), in (B) neutrophils in response to 5-OxoETE (n = 5-15), in (C) eosinophils in response to PGD_2 (n = 2-6) or in (D) neutrophils in response to PGD_2 (n = 2-6). Statistical analyses were performed with two-way ANOVA followed by Tukey post-test. RA samples were provided by Martin Stradner and Angelika Lackner of the Division of Rheumatology and Immunology, Medical University of Graz. PMNL isolation and ROS production measurement were performed by Kathrin Rohrer.

3.11. DP2 expression is altered in M2 macrophages of RA patients

Next to synovial fibroblasts, macrophages are considered key players in the development and progression in rheumatoid arthritis. Therefore, we set out to investigate how PGD_2 receptors are expressed on their surface and whether PGD_2 could affect their polarization. First, we assessed whether DP1 and DP2 were expressed on MDM, M1 or M2 and compared the fold increase

between the three groups. We discovered that DP1 was weakly expressed on all macrophage populations, but we observed no changes between the patient groups and our control group (Figure 15A). When we looked at DP2, we found that this receptor was much stronger expressed on these cells (Figure 15B). Interestingly, the receptor expression was significantly increased in M2 macrophages of patients suffering from acute flares (8.45 ± 3.0-fold increase) compared to macrophages differentiated from patients in remission (4.93 ± 1.39-fold increase). This fold increase was also higher than in the control group but failed to reach significance (6.35 ± 2.24-fold increase). As DP2 is generally considered a pro-inflammatory receptor, a higher expression in M2 macrophages was quite surprising. This led to the question whether these M2 macrophages actually display an anti-inflammatory phenotype. To rule out that they polarized insufficiently towards M2, we compared the expression levels of both markers (CD80 and CD206) in M1 and M2 macrophages and normalized them to their basal expression in MDM (Figure 15C-D). To our surprise, we observed that CD80 expression was significantly reduced in the "remission" group (747.53 ± 431.15-fold increase) compared to our control group (1874.0 ± 456.17-fold increase) and the "flare" group (1430.6 ± 538.32-fold increase), indicating that these cells polarized with varying sufficiency (Figure 15C). When we looked at the anti-inflammatory marker CD206, we detected no differences between the three groups (Figure 15D). As DP2 receptor expression and M1 markers are differently expressed in RA patients, we wondered whether PGD_2 signalling could have an impact on macrophage polarization. Therefore, we treated our cells with this lipid mediator during polarization. To determine which PGD_2 receptor mediates a response in macrophages, we also treated the cells with the DP1 selective agonist BW245C or the DP2 selective agonist DK-PGD_2 (Figure 15E-F). We observed that PGD_2 significantly upregulated CD80 expression in M2 macrophages (135.8 ± 11.8 % of Vehicle-treated cells). This effect was even stronger in response to BW245C (176.0 ± 47.9 % of Vehicle-treated cells), suggesting that the observed PGD_2 effect was mediated by DP1 (Figure 15E).

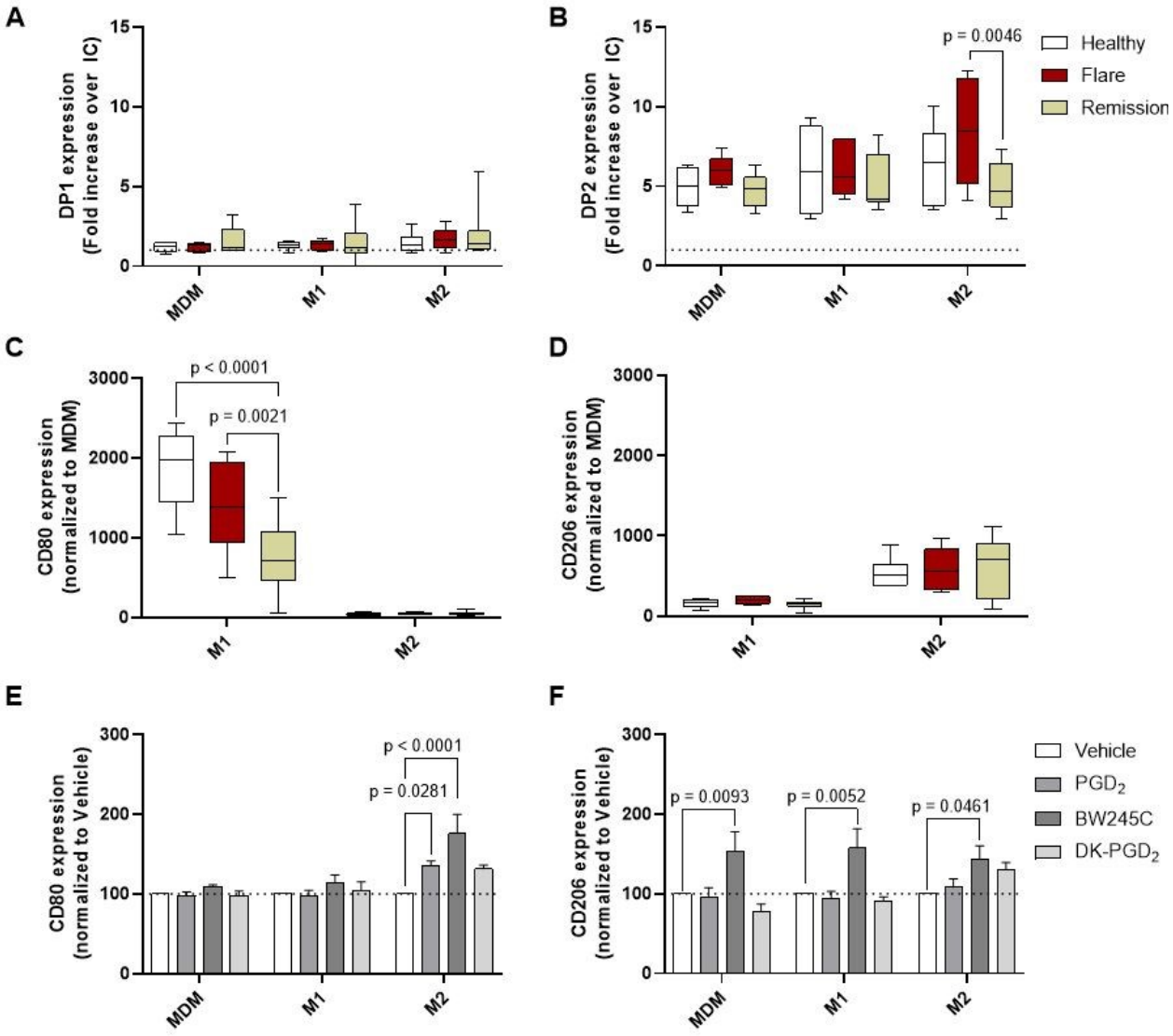

Figure 15: PGD_2 receptor expression in macrophages of RA patients and macrophage polarization. Isolated PBMC from either healthy donors, patients suffering from acute flares or patients in remission were differentiated to macrophages using M-CSF and polarized to M1 or M2. (A) DP1 expression in MDM, M1 or M2 macrophages of RA patients (n = 5-9). (B) DP2 expression in MDM, M1 or M2 macrophages of RA patients (n = 5-9). (C) CD80 expression in M1 and M2 macrophages from RA patients (n = 5-12). (D) CD206 expression in M1 and M2 macrophages from RA patients (n = 5-12). (E) CD80 expression in response to PGD_2, BW245C or DK-PGD_2, compared between MDM, M1 or M2 macrophages (n = 5). (F) CD206 expression in response to PGD_2, BW245C or DK-PGD_2, compared between MDM, M1 or M2 macrophages (n = 5). Statistical analyses were performed with two-way ANOVA followed by Tukey post-test. RA samples were provided by Martin Stradner and Angelika Lackner of the Division of Rheumatology and Immunology, Medical University of Graz. PBMC isolation was performed by Kathrin Rohrer or Iris Red.

While this proposes a pro-inflammatory role for PGD_2 in macrophage biology, we were surprised to find that BW245C induced a significant CD206 upregulation in MDM (153.8 ± 48.1 %), M1

(157.6 ± 48.4 %) and M2 (143.0 ± 34.9 %) macrophages (Figure 15F). In summary, we showed that M2 macrophages differentiated from RA patients suffering from acute flares display an increase in DP2 expression compared to patients in remission. Further, M1 macrophages from patients in remission showed a clear reduction in CD80 expression compared to cells differentiated from healthy controls and the "flare" group, indicating varying polarization sufficiency. Lastly, we show that the DP1 selective agonist BW245C significantly upregulated CD206 in MDM, M1 and M2 macrophages along with CD80 in M2 macrophages.

3.12. DP2 antagonist OC-459 displays a weak protection against collagen antibody-induced arthritis

Next, we were interested whether DP2 antagonist would have a protective effect against the development of CAIA. In collagen-induced arthritis, an earlier version of the CAIA model, PGD_2 levels were observed in paws and serum and DP2 knockout mice exert a more severe course of adjuvant-induced arthritis, indicating an involvement of this lipid mediator in these disease models (9,271). For this reason, we tested whether the DP2 antagonist OC-459 would have a beneficial effect on joint inflammation. We choose the CAIA model instead of the older CIA model, as it offers simpler handling and a higher disease incidence, while at the same time having a much shorter duration compared to CIA (300). 25 to 26-week-old male DBA/1J mice were immunized with a 1.5 mg of cocktail, containing antibodies against collagen. On day 3 and day 10, inflammation was boosted through intraperitoneal injection of LPS. To assess DP2 involvement in the disease progression, mice were injected daily with 0.1 mg/kg OC-459 subcutaneously. After 14 days, mice were sacrificed, and blood and joint tissue were processed. When we compared the weight of OC-459 treated mice with a vehicle-treated group, we observed on day 7, day 8 and day 9 that OC-459 treated mice retained significantly more weight compared to the vehicle group (Figure 16A). This effect was lost, as the experiment progressed. When we compared the RA score, we observed an earlier disease onset in the OC-459 group and this effect was significant on day 4 (2.75 ± 1.3 RA score) (Figure 16B). However, no significant reduction in RA scores were observed in the OC-459 group. After the mice were sacrificed, we measured how many cells infiltrated the joints and compared these data between both groups. In total cell counts, we observed a significant

reduction in neutrophils in the OC-459 group (1.44 x 10^4 cells) compared to the vehicle group (42.4 x 10^4 cells) (Figure 16C). As the procedure to measure joint infiltrates is prone to varying efficiency, we normalized the identified cell populations to the total cell counts to obtain percentages (Figure 16D). Here, we observed a significant increase in mast cells (12.3 ± 2.15 % vehicle vs. 24.3 ± 2.0 % OC-459) and decrease in neutrophils (63.9 ± 6.1 % vehicle vs. 35.0 ± 5.3 % OC-459). In summary, we observed that DP2 antagonist OC-459 protected for a short time against weight loss, but at the same time, induced an earlier disease onset compared to the vehicle-treated group. However, neutrophil infiltration was significantly reduced in the OC-459 group.

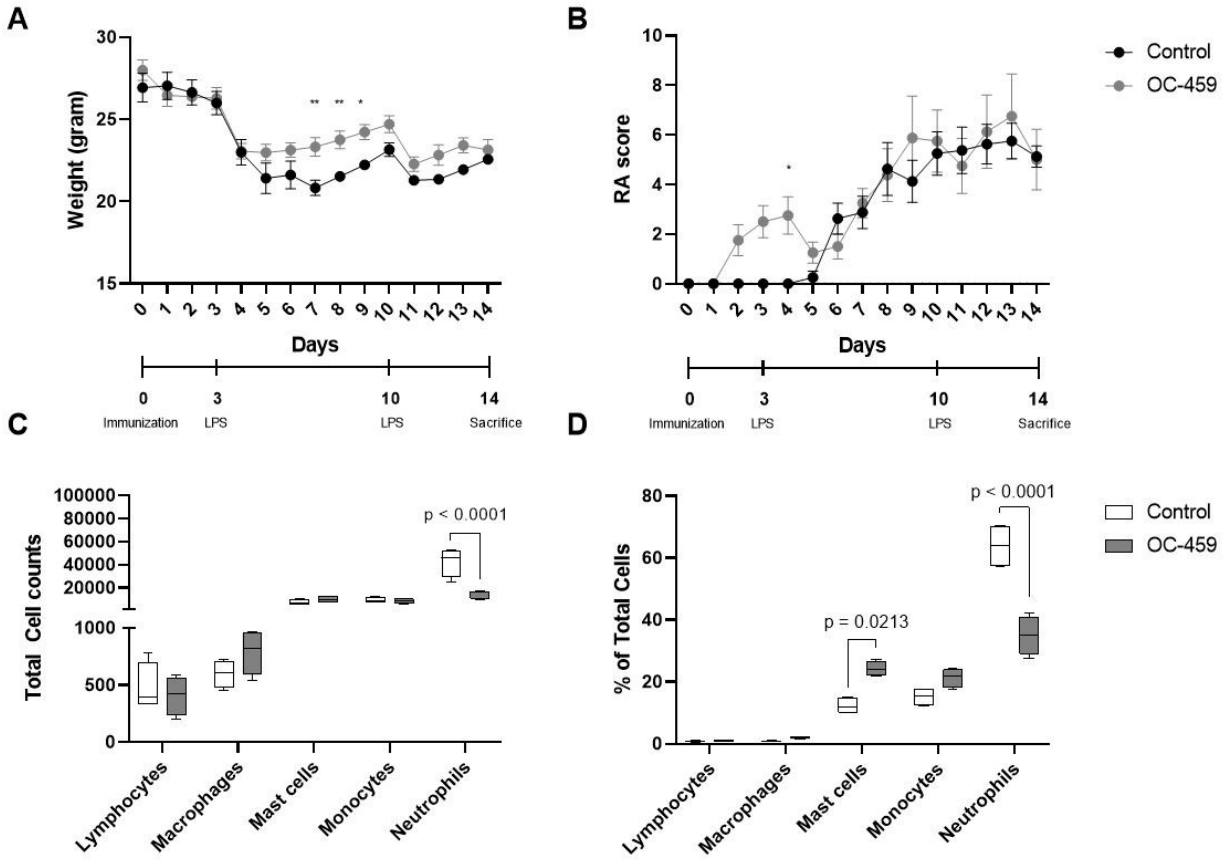

Figure 16: Effect of DP2 antagonist OC-459 on phenotype in experimental murine arthritis. DBA/1J mice were immunized with 1.5 mg CAIA cocktail and treated daily twice with either vehicle or 0.1 mg/kg OC000459. On day 3 and day 10, the immune response was boosted with 50 µg LPS (n = 4). (A) Weight was measured daily prior to the first injection. (B) Arthritis score was measured daily on the front and hind paws. (C) Total cell counts were measured using flow cytometry. (D) Percentages were calculated from total cell counts. Statistical analyses were performed with two-way ANOVA followed by Sidak post-test. If p value is not stated, statistical significance is indicated as follows: *p < 0.05,**p < 0.01, ***p < 0.001, **** < 0.0001.

3.13.ApoA-IV enhances basal PGE_2 production in monocytes

As we observed a decrease in plasma ApoA-IV and altered PGD2 receptor expression in samples of RA patients, we set out to investigate a possible connection between both. In our last experiments, we hypothesized that ApoA-IV directly impacts PGD_2 biology in cells of the mononuclear phagocyte system. This hypothesis is based on the reports that ApoA-I, which is structurally similar to ApoA-IV, accumulates in the synovial joints of RA patients and has been shown to elicit prostaglandin production in macrophages *in vitro* (289,290). First, we treated isolated PBMC with either vehicle or 3 µg/mL ApoA-IV and stained for DP2 in non-classical monocytes, but observed no changes between the two groups (Figure 17A). As stimulated monocytes are strong sources of PGE_2 and express hPGDS, we were curious whether the apolipoprotein affected prostaglandin production (310,311). We incubated adherent monocytes in either the presence or absence of ApoA-IV and harvested the cells and supernatants after 24 hours. Although not significant, we observed a visible upregulation of COX-2 in ApoA-IV treated monocytes (Figure 17B). Although COX-2 is associated with inflammation, PGE_2 is generally considered an anti-inflammatory mediator. Following up on our data that showed suppression of monocyte migration and activation by ApoA-IV, we hypothesized that it may also affect prostaglandin production. When we looked at basal PGE_2 production, we observed a significant increase in ApoA-IV treated monocytes (306.32 ± 119.99 pg/mL) compared to vehicle-treated cells (142.76 ± 68.52 pg/mL) (Figure 17C). Next, we were also curious whether ApoA-IV induces PGD_2 production. When we compared basal levels of PGD_2 in the supernatant of ApoA-IV treated monocytes and compared it to a vehicle-treated group we observed no statistically significant differences (Figure 17D). In summary, we show that ApoA-IV elicits production of PGE_2 in adherent monocytes.

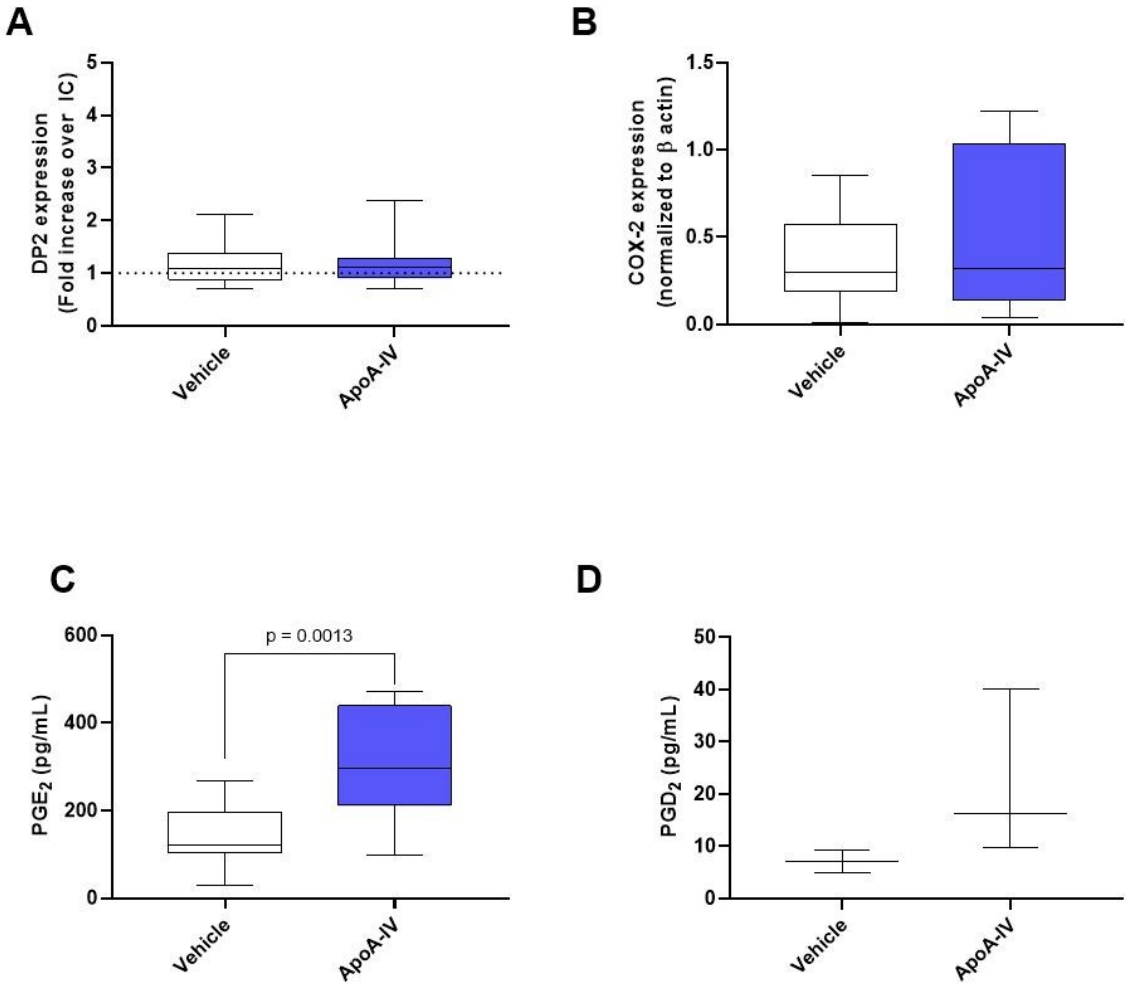

Figure 17: Effect of ApoA-IV on DP2 expression and prostaglandin production in monocytes. Isolated PBMC or adherent monocytes were treated with either vehicle or 3 µg/mL ApoA-IV for 30 minutes (A) or 24 hours (B-D). (A) DP2 expression was measured in non-classical monocytes using flow cytometry (n = 12). (B) COX-2 expression was measured using Western blotting (n = 9). (C) Basal PGE_2 production was measured using radioimmunoassay in the supernatant of adherent monocytes that were incubated for 24 hours in the presence of vehicle or 3 µg/mL ApoA-IV (n = 9). (D) Basal PGD_2 production was measured using MOX ELISA in the supernatant of adherent monocytes that were incubated for 24 hours in the presence of vehicle or 3 µg/mL ApoA-IV (n = 3). Statistical analyses were performed with paired two-tailed Student's t test. PBMC isolation was performed by Kathrin Rohrer or Iris Red (A), COX-2 expression was measured by Wolfgang Platzer (B), PGE_2 production was measured by Ilse Lanz (C)

3.14. ApoA-IV increases COX-2 expression and PGE_2 production in M1 macrophages

Lastly, we investigated whether ApoA-IV treatment could influence prostaglandin biology in macrophages. Macrophages were polarized either in the presence or absence of 3 µg/mL ApoA-IV cells and supernatants were harvested for further processing. First, we compared the levels of COX-2 expression. We observed a significant increase in COX-2 mRNA in ApoA-IV treated cells compared to the vehicle-treated group (Figure 18A). To verify our results, we measured COX-2 expression also on a protein level and observed a similar up-regulation of this enzyme in ApoA-IV treated cells (0.86 ± 0.45 over β actin) compared to vehicle-treated macrophages (0.50 ± 0.28 over β actin) (Figure 18B). COX-2 up-regulation is a clear indicator of prostaglandin synthesis, and since PGD_2 and PGE_2 levels are elevated in the synovial fluid of RA patients and the enzymes required for these prostaglandins are present in this tissue, we next tested for synthesis of both lipid mediators (275). As we hypothesized that ApoA-IV exhibits anti-inflammatory properties, we focused on its effect on M1 macrophages and measured mPGES-1 mRNA and basal PGE_2 levels in these cells (Figure 18C-D). Although we observed no significant changes in mPGES-1 mRNA levels (Figure 18C), we measured a basal PGE_2 production of 55.21 ± 30.2 pg/mL in vehicle-treated cells. When these macrophages were exposed to ApoA-IV, they significantly increased their production to 105.53 ± 62.8 pg/mL. Next, we looked at basal PGD_2 production in M1 macrophages. Although we observed an apparent trend for up-regulation, we neither detected significant changes in hPGDS mRNA or PGD_2 levels in ApoA-IV treated cells compared to the vehicle group (Figure 18E-F). In summary, we observed that ApoA-IV significantly reduced CD80 expression in M1 macrophages, while elevating it in M2 macrophages, indicating that it may act as a phenotype switch. This was confirmed by ApoA-IV mediated COX-2 upregulation and subsequent PGE_2 synthesis.

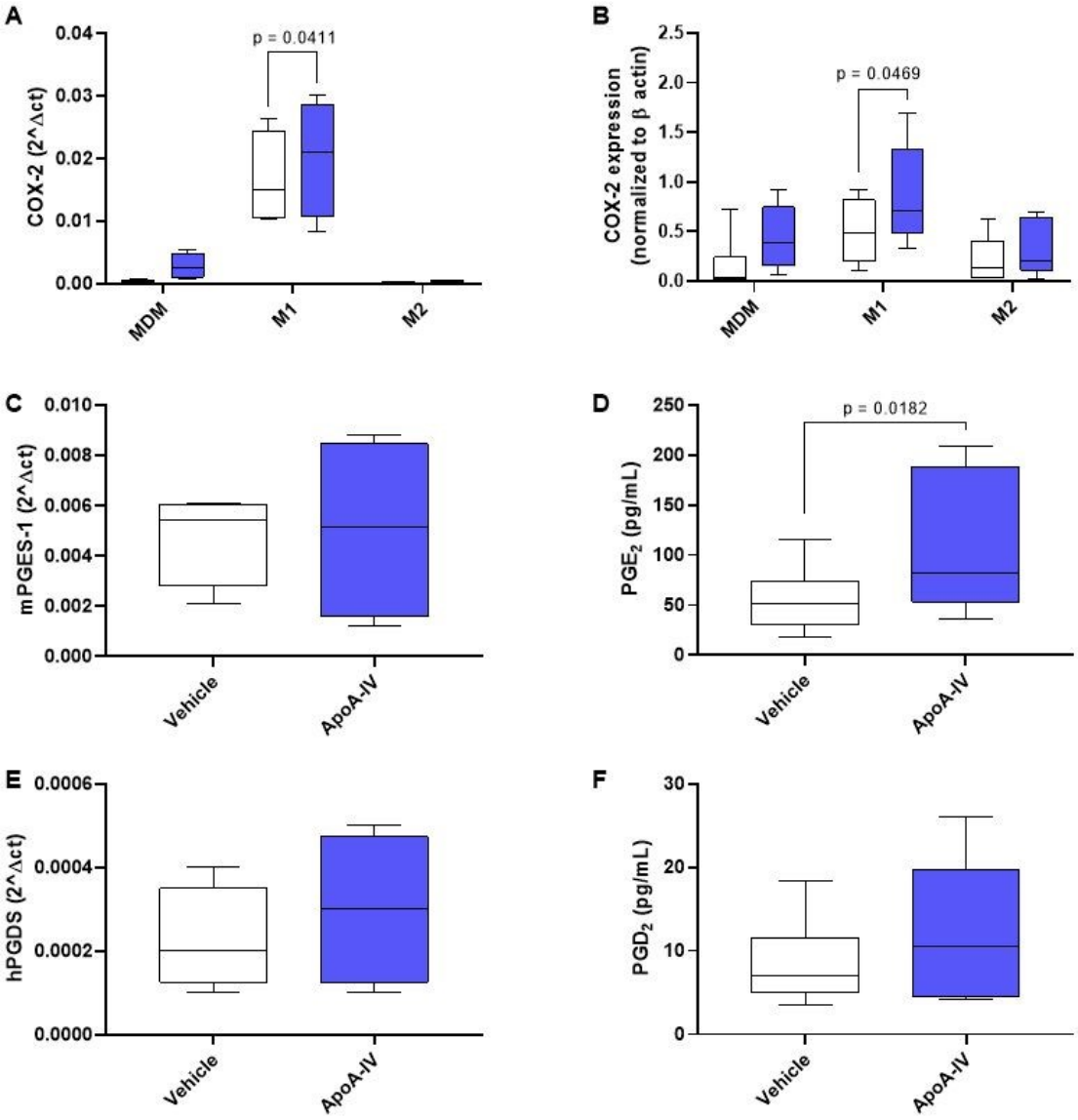

Figure 18: Effect of ApoA-IV on COX-2 expression and prostaglandin synthesis in M1 macrophages. (A) COX-2 mRNA expression was measured using RT-qPCR (n = 4). (B) COX-2 expression was measured using Western blotting (n = 6). (C) mPGES-1 mRNA expression was measured using RT-qPCR (n = 4). (D) Basal PGE_2 production was measured using radioimmunoassay in the supernatant of M1 macrophages that were incubated for 24 hours in the presence of vehicle or 3 µg/mL ApoA-IV (n = 7). (E) hPGDS mRNA expression was measured using RT-qPCR (n = 4). (F) Basal PGD_2 production was measured using MOX ELISA in the supernatant of M1 macrophages that were incubated for 24 hours in the presence of vehicle or 3 µg/mL ApoA-IV (n = 6). Statistical analyses were performed with two-way ANOVA followed by Sidak post-test (A-B) or paired two-tailed Student's t test (C-F). PBMC isolation was performed by Iris Red or Kathrin Rohrer. RT-qPCR and PGE_2 RIA were performed by Ilse Lanz. COX-2 expression was measured by Wolfgang Platzer.

4. Discussion

Parts of the results depicted in this dissertation have been published in an original article (1).

The aim of this dissertation was to illuminate a potential interrelation between the pro-inflammatory lipid mediator Prostaglandin D_2 and the anti-inflammatory apolipoprotein ApoA-IV. In order to elucidate this putative connection, we devised a range of experiments in the context of a Th1 driven disease (rheumatoid arthritis) and a Th2 driven disease (allergic asthma).

In our first experiment, we wanted to investigate whether ApoA-IV has any impact on eosinophil migration. In recent reports it was shown that ApoA-I and ApoE mimetics had beneficial effects in murine models of experimental asthma (159,160). Eosinophil granulocytes are cells of the innate immune system, tasked with the defence against exogenous parasites such as helminths and they strongly contribute to the lung remodelling observed in patients suffering from chronic asthma (118). Eosinophil recruitment into the lung is mediated through a range of mediators, including eotaxin-1 (CCL11) and prostaglandin D_2 (PGD_2) (312,313). In the first experiments we assessed eosinophil recruitment via chemotaxis and hence, exposed resting eosinophils with either vehicle or 3 µg/mL of ApoA-IV. The chosen concentration of 3 µg/mL was determined in preliminary experiments (data not shown). After 30 minutes of incubation, the eosinophils were transferred and allowed to migrate towards 3 nM of CCL11 (Figure 4A). We observed that ApoA-IV potently suppressed CCL11-induced chemotaxis compared to vehicle-treated cells. To confirm our findings, we tested PGD_2 in the same setting and observed that ApoA-IV treated eosinophils also displayed reduced migration towards PGD_2 (Figure 4B). Next, we wanted to assess through which pathway ApoA-IV exerts its anti-migratory effect. ApoA-IV has recently been found to bind a nuclear receptor called Rev-ErbA alpha, also known as NR1D1 (92,93). This receptor is involved in circadian rhythm, which controls the sleep/wake cycle and influences physiological processes such as blood pressure, body temperature and metabolism [reviewed in (94)]. This is achieved by cycling expression of clock genes (e.g. CLOCK, Bmal1) that impact these processes. To shut down this process, these genes regulate the expression of protein-mediated feedback loops which in turn inhibit clock gene transactivation. One of such repressors is fluctuating NR1D1 which inhibits

Bmal1 expression. NR1D1 suppresses ApoC-III expression in rat hepatocytes, which is important in regulating LDL metabolism. Therefore, it has been suggested that activation of this nuclear receptor may contribute to the risk of atherosclerosis (95). Further, NR1D1 has been identified in macrophages (96). As both eosinophils and macrophages belong to the innate immune system and NR1D1 is a conserved receptor, we investigated whether we could reproduce our ApoA-IV mediated suppression of chemotaxis in eosinophils by stimulated them with a NR1D1 selective agonist GSK4112. When we exposed eosinophils to 10 µM of GSK4112 in a similar fashion as with ApoA-IV and let them migrate towards CCL11, we observed a similar suppression in chemotaxis (Figure 4C). If ApoA-IV acted via the NR1D1 pathway, we should be able to disrupt the anti-migratory effect by blocking NR1D1 prior to ApoA-IV treatment. At a concentration of 1 µM of selective NR1D1 antagonist SR8278, we detected that the effect of ApoA-IV was no longer significant when compared to vehicle-treated cells (Figure 4D). Taken together we observed that ApoA-IV potently suppresses CCL11- and PGD_2-induced chemotaxis and this suppression is mediated via the NR1D1 pathway.

Although *in vitro* experiments enable the observer to test a biological effect in an isolated setting under highly controlled conditions (one cell type, one stimulant), we wanted to verify our finding in a murine model of HDM-induced experimental airway hyperreactivity. Lung infiltration is defined as the infiltration of immune cells or cancer cells into pulmonary tissue. This in turn can introduce changes into the pulmonary environment, such as tissue remodeling, resulting in a decline in gas exchange. A common method to indirectly estimate lung infiltration is collecting the BAL fluid. When we examined the cellular content of the BAL fluid of ApoA-IV-treated mice, we observed a significant reduction in total cells and eosinophils, but not in neutrophils, monocytes or alveolar macrophages (Figure 5B-F). This indicates that the observed anti-migratory effect of ApoA-IV on eosinophils was also observable in an *in vivo* approach. In a similar experiment we wanted to assess lung function by measuring airway hyperresponsiveness to the bronchoconstrictor methacholine (MCh). Airway hyperresponsiveness describes an increased response to an inhaled bronchoconstrictor or a non-specific stimulus and is used in asthmatics for diagnosis, but is also an important clinical feature of the disease. First, we looked at airway resistance, which is the resistance of the respiratory tract to airflow during inhalation or exhalation. During an asthma

attack, the airways constrict causing an increase in resistance. This in turn has a disadvantageous effect on spirometry values, such as the FEV_1 (forced expiratory pressure in 1 second). Potent asthma therapy results in improved spirometry values. When we challenged Balb/C mice with increasing concentrations of MCh we observed a significant reduction in airway resistance in the ApoA-IV treated group compared to the vehicle group (Figure 6A). Next, we assessed lung compliance, which is defined as the ability of the lung to stretch and expand. A low compliance indicates a stiff lung and can be observed in pulmonary fibrosis due to the scarring of the lung tissue. During an asthma attack, patients also have a decreased lung compliance, however this is caused by the bronchospasm-induced hyperinflation of the lung. When we tested this parameter, we observed that ApoA-IV treated mice displayed a much better pulmonary compliance in response to MCh challenge as the vehicle group (Figure 6B). In our *in vivo* experiments using the model of HDM induced experimental airway hyperreactivity in Balb/C, we confirmed our *in vitro* data in which we show that ApoA-IV potently suppresses eosinophil migration. Lung infiltration, airway resistance and pulmonary compliance were all improved in the ApoA-IV treated group compared to the controls. This indicates that ApoA-IV may have a beneficial role in allergic asthma. In 2010 a study emerged that showed that ApoA-IV levels increase in the serum of allergic rhinitis patients after sublingual immunotherapy (163). Following up on these data, our own lab showed that serum ApoA-IV levels are decreased in treatment-naïve patients suffering from chronic rhinosinusitis (1). It is therefore conceivable that ApoA-IV treatment may provide a potential treatment strategy for mitigating allergic responses (1).

Next, we wanted to assess the impact of ApoA-IV and its potential correlation to PGD_2 in cells involved in the pathogenesis of the autoimmune disease rheumatoid arthritis (RA). With the rise of autoimmune disorders and a relatively high incidence rate of 0.5 to 1 %, new RA treatments and therapies are drastically required (167). Standard therapies include a group of chemical substances called DMARDs (disease modifying anti-rheumatic drugs) that include methotrexate, leflunomide and sulfasalazine. These drugs predominantly suppress purine and pyrimidine synthesis and how they elicit their anti-rheumatic actions is still subject of research. NSAIDs (non-steroidal anti-inflammatory drugs) are also highly prescribed due to their COX-inhibiting and therefore pain-relieving effects. In recent years, biologics have become more feasible as RA therapies. These

antibodies target TNF-α, IL-1β or the IL-6 receptor and have proven to counter-act the pathophysiology observed in affected joints. Another approach is to target the pro-inflammatory signalling with the use of janus kinase (JAK) inhibitors [reviewed in (314)]. Although the overall life quality of RA patients has vastly improved due to the wider availability of treatments, these drugs target specific symptoms but fail to elicit a long-lasting resolution of inflammation. A better understanding how the disease develops is required to find a permanent cure.

Altered levels of apolipoproteins have been observed in RA patients (288). ApoA-I, a protein essential in lipid metabolism, is decreased in serum of patients but accumulates in the synovial lining of the inflamed joint (289). As ApoA-I shares functional and structural similarities with ApoA-IV and the latter has been shown to elicit anti-inflammatory effects in monocytes (162), we started to investigate how much impact this apolipoprotein has on cells involved in the pathogenesis of RA. Therefore, we aimed to look whether ApoA-IV influences monocyte migration, activation or affects cytokine production (Figure 7). First, we exposed monocytes to 3 µg/mL ApoA-IV prior to migration towards C5a and observed a significant reduction in the chemotactic index (Figure 7A). C5 is a protein of the complement system, which is cleaved into C5a and C5b by C5 convertase, which act as a chemoattractant and part of the complement membrane attack complex, respectively. C5a is elevated in the synovial fluid of RA patients and may be one of the effectors that lead to monocyte recruitment from the circulation (303). To confirm that ApoA-IV suppresses monocyte migration, we next looked at CD11b expression. CD11b upregulation is generally interpreted as a sign of activation. When we treated monocytes with ApoA-IV, we observed a significant rise in CD11b expression after 30 minutes (Figure 7B). This was surprising, as the same dose and incubation time was used in the chemotaxis experiment. Interestingly, this effect was not only lost after 3 hours of incubation, but even reversed after 6 hours. CD11b is part of the integrin $\alpha_M\beta_2$, also known as macrophage-1 antigen (Mac-1) and strongly expressed in cells of the innate immune system. Its major function is the regulation of leukocyte adhesion, migration and phagocytosis, but also the suppression of pro-inflammatory cytokine production has been observed (315,316). Further experiments are required to delineate the downstream signalling following these CD11b changes.

In order to identify whether ApoA-IV exerts anti-inflammatory properties in monocytes, we assessed cytokine production. First, we performed an interleukin-10 ELISA in the supernatant of ApoA-IV treated monocytes and compared the results to a vehicle group (Figure 7C). Although we observed a visible trend towards increased IL-10 production, the results failed to reach significance. Next, we assessed the impact of ApoA-IV on basal TNF-α production but could not detect any significant changes (Figure 7D). On a more functional side, we tested whether ApoA-IV could suppress zymosan-A induced ROS production, but failed to detect a significant change between the treatments (Figure 7E). Zymosan-A is a cell wall-derived glucan that is isolated from yeast. It promotes TNF-α production in monocytes, which is likely to be mediated through TLR2 (317,318). The inability of ApoA-IV to suppress zymosan-A induced ROS production could be explained through the high potency of the glucan. The concentrations chosen elicited already at the lowest concentration of 50 µg/mL a strong increase in the geometric mean. Working with lower doses to observe at which concentration the cells start to produce reactive oxygen species would help to investigate whether ApoA-IV is able to exert suppressive functions in this context.

Next, our focus shifted to surface marker expression in monocytes. Monocytes differentiate to macrophages when exposed to a range of mediators, including M-CSF, GM-CSF and interleukin-34 (319,320). Besides those cytokines, other modes of differentiation have been observed. For instance, monocytes express CD4 and ligation of the receptor leads to macrophage differentiation (304,305). We were curious whether ApoA-IV could affect the expression of this receptor. We found that CD4 is expressed most strongly in $CD14^{++}CD16^{-}$ classical monocytes and at lower levels in $CD14^{++}CD16^{+}$ intermediate and $CD14^{-}CD16^{+}$ non-classical monocytes (data not shown). Although a trend towards reduction in this expression could be observed in all groups, it failed to reach significance (Figure 7F). In general, very little is known about the role of CD4 in monocytes. Previous reports suggest, that there are no differences in CD4 expression between healthy donors and HIV-1 patients and that the receptor is internalized, once these cells start to differentiate into macrophages (321,322). As the view on monocytes being nothing more than precursors of macrophages shifted within the last decade, more research is warranted to delineate CD4 signalling in monocyte populations.

While ApoA-IV showed no effect on CD4 expression in monocytes, we wondered whether it could affect macrophage polarization. We differentiated monocytes to macrophages and polarized them to either M1 or M2 macrophages or retained them at the M0 state (Figure 8A-B). When the polarization medium was supplemented with ApoA-IV, we observed that CD80 was significantly down-regulated in M1 macrophages, while CD206 expression was unaffected. CD80 is a surface protein on antigen presenting cells and is required for T cell activation. Macrophages upregulate this protein in response to LPS, indicating a bacterial infection. IFN-γ, derived from the now activated T cell, acts on the macrophage in turn through upregulation of pro-inflammatory mediators, which is why these cells are considered pro-inflammatory. CD206 on the other hand recognizes mannose, a sugar molecule present on microorganisms. M2 macrophages are therefore responsible for resolving inflammation and pathogen clearance. A significant reduction in CD80 expression in M1 macrophages therefore suggests that ApoA-IV exerts anti-inflammatory signalling in these cells, driving M1 towards M0. In M2 macrophages, we observed the opposite: CD80 expression was markedly increased and to our surprise, CD206 was decreased, suggesting that it drives M2 towards the M1 phenotype. This suggests a phenotype switch in macrophage differentiation. While the M1/M2 model is useful to distinguish between two polar opposites, namely pro- and anti-inflammatory functioning, it is obsolete as it does not take into account the plasticity of these cells, which is much higher than anticipated. To date, four M2 macrophage subsets have been classified. M2a, which are induced by IL-4 or IL-13 and are associated with Th2 responses to kill parasites, M2b, induced by immune complexes and act as immunoregulators, M2c, polarized through IL-10 and participate in tissue remodelling and the most recent addition: M2d, induced through adenosine, which display an angiogenic phenotype through production of vascular endothelial growth factor (VEGF) and IL-10 (211,212). On the M1 side of the spectrum, the scientific community is hesitant to classify them to a similar extent and rather speak of activation rather than polarization (213,214). A publication in 2014 reported that by using different stimuli, up to 10 transcriptionally distinctive populations could be observed and the authors push for the implementation of a spectrum model instead of a polarization model (215). Macrophages are strongly affected by the cytokines and mediators they are exposed to and it is likely that the ability to polarize is not restricted into one direction, but rather can be interfered with at any time to shift into a different direction.

Next, we wanted to confirm that the observed "de-polarization" can also be assessed through cytokine production. Therefore, we exposed M1 macrophages to either vehicle or ApoA-IV and collected the supernatants for ELISA. Although we observed a trend towards elevated IL-10 production, these data failed to reach significance (Figure 8C). Next, we wanted to test whether ApoA-IV has any impact on basal TNF-α in M1 macrophages. When M1 polarization medium was supplemented with ApoA-IV, macrophages produced significantly less TNF-α compared to vehicle-treated cells (Figure 8D). This confirmed our finding that ApoA-IV exerted anti-inflammatory effects on M1 macrophages.

In a similar fashion as in eosinophils, we wanted to investigate whether ApoA-IV signals in macrophages through NR1D1. NR1D1 has been observed in macrophages and receptor activation resulted in a reduction in TLR4 expression (96). As TLR4 is tasked with detecting LPS, thus a reduction of TLR4 surface expression may result in a reduced ability to differentiate towards the LPS-dependent M1 phenotype. Therefore, we investigated whether ApoA-IV had an impact on NR1D1 expression in macrophages. We differentiated PBMC using M-CSF and polarized them towards M1 or retained them at M0. The M1 macrophages were polarized either in the presence or absence of ApoA-IV. We detected in MDM, vehicle-treated M1 and ApoA-IV-treated M1 macrophages a strong band at 67 kDa (**Figure 9**A). We observed no significant changes between vehicle- and ApoA-IV treatments (**Figure 9**B). Next, we wanted to test whether NR1D1 antagonist SR8278 had any unspecific effect on CD80 and CD206 expression or cell viability (**Figure 9**C). At a concentration of 3 µM, CD80 expression increased significantly. This effect was lost at 10 µM and although not significantly, cell viability began to decline. At a concentration of 30 µM, CD80, CD206 and cell viability were significantly decreased. We therefore settled on 1 µM as our maximal concentration for further experiments. Next, we tested whether this concentration was sufficient to abolish ApoA-IV induced reduction of CD80 expression in M1 macrophages (**Figure 9**D). While ApoA-IV alone significantly reduced CD80, this effect was lost when the cells were pre-treated with 1 µM of SR8278, indicating that the observed effect was NR1D1 mediated. Next, we wanted to verify our findings by directly activating NR1D1. For this, we tested increasing concentrations of GSK4112 during M1 polarization (**Figure 9**E). At a concentration of 30 µM, we saw a clear reduction in CD80 expression. This effect was even more prominent at 100 µM.

Survival was unaffected by all used concentrations of the agonist. We concluded that 30 μM is a good concentration to test this agonist in further experiments.

Lastly, we tested which concentration of the antagonist is required to block the observed CD80 downregulation by GSK4112. We found that at a concentration of 1 μM SR8278, the GSK4112 mediated reduction in CD80 was no longer significant (**Figure 9**F). These data suggest that ApoA-IV elicits its immunosuppressive effects through this nuclear receptor, however, further experiments are required to prove this hypothesis. Although 1 μM of SR8278 abolished the ApoA-IV observed effect, we do not show how ApoA-IV and NR1D1 directly interact with each other. The importance of NR1D1 in the inflammatory context of rheumatoid arthritis was proven recently, as silencing this receptor in fibroblast-like synoviocytes enhanced expression of pro-inflammatory cytokines, NF-κB and matrix metalloproteinases. Further, activation inhibited M1 polarization and osteoclastogenesis (293). However, off-target effects of GSK4112 are also possible, as the agonist concentration is rather high in comparison to concentrations used in published literature (323–325).

While monocyte-derived macrophages are the predominant cell type recruited from circulation in the inflamed synovium, neutrophils are the major leukocyte found in the synovial fluid (227). As we showed that ApoA-IV potently suppressed CCL11-induced chemotaxis in eosinophils (Figure 4), we wondered whether ApoA-IV exhibited suppressive effects on neutrophil function First, we tested whether IL-8 induced shape change could be suppressed with ApoA-IV (Figure 10A). However, we could not detect that apolipoprotein treatment affected neutrophil shape change in response to increasing IL-8 concentrations. Next, we tested more functional assays to determine whether this protein would affect neutrophil behaviour. We observed that ApoA-IV was incapable of inhibiting IL-8 induced calcium flux (Figure 10B) and C5a induced ROS production (Figure 10C) in these cells. Lastly, we looked at HLA-DR upregulation, as neutrophils express MHC II in the RA synovium and are able to present antigens to T cells (235,236). When we induced HLA-DR expression with GM-CSF, we found that ApoA-IV was unable to influence this process (Figure 10D). We observed that ApoA-IV could not affect neutrophil behaviour in any of our experiments and it is therefore reasonable to assume that ApoA-IV does not exert anti-inflammatory properties in these cells. However, further experiments would be required support this preliminary conclusion.

ApoA-I levels are reduced in serum of patients suffering from RA (289). Therefore, we were interested whether ApoA-IV was in a similar fashion reduced when compared to healthy controls of the same age group. Due to a low number of samples within the "acute" group, we pooled the data from both the "remission" and "flare" groups (Figure 11A). We observed a significant reduction in ApoA-IV protein in the plasma of RA patients. ApoA-IV exerts anti-inflammatory effects in human PBMC (162,163). It is therefore tempting to assume that ApoA-IV metabolism is somehow impaired in chronic inflammation. However, whether this is due to impaired ApoA-IV synthesis or increased protein catabolism needs to be identified.

Based on the finding that ApoA-I can elicit a COX-2 mediated prostaglandin response and shares functional and structural similarities with ApoA-IV, we started to investigate a possible connection between both PGD_2 and ApoA-IV. The role of PGD_2 in RA has not been elucidated yet. While it has been abundantly described in asthmatic disease (326), its function in rheumatoid arthritis is not elucidated yet. In serum and synovial fluid of RA patients, PGD_2 levels were elevated and in a murine model of experimental arthritis, PGD_2 levels correlated with progression of the disease (9,275). While studies suggest that DP1 plays a beneficial and DP2 an aggravating role in collagen-induced arthritis, deficiency in DP2 exacerbated adjuvant-induced arthritis (271). A very recent publication showed that hPGDS knock-out mice also showed an aggravated phenotype in this model (272).

First, we wanted to investigate the expression levels of Prostaglandin D_2 receptor DP2 (alternatively known as CRTH2 or GPR44) in circulating leukocytes in rheumatoid arthritis. DP2 is expressed predominantly in immune cells, namely eosinophils, basophils, T cells, monocytes and macrophages, but also in osteoblasts, osteoclasts and lung fibroblasts (44). For this, we collected whole blood from patients suffering from RA and healthy donors. The RA patient samples were further divided into a "flare" group, which displayed acute symptoms, either because RA was just diagnosed at this point or because patients suffered an inflammatory flare despite receiving treatments. The second group termed "remission" consisted of patients that received treatments (e.g. DMARDs or NSAIDs) and therefore displayed an attenuated state. We evaluated DP2 expression in neutrophils, eosinophils, T cells, B cells, basophils, natural killer cells, dendritic cells and monocytes (Figure 11). We found DP2 to be expressed in eosinophils, CD8 cells, non-classical monocytes and basophils. While we detected no differences in eosinophils between the

three groups (Figure 11B), we saw a significant upregulation in $CD8^+$ cells in the "remission" group, when compared to the "healthy" group (Figure 11C). As this up-regulation is only present in the remission group, it can be hypothesised that it is associated with the patients' treatments. As these experiments were of a pioneer nature, we did not stratify the patients further in regard to these treatments. DP2 is also expressed in $CD4^+$ T helper cells, although only in Th2 cells, where the receptor was originally discovered. As the majority of CD4 cells is DP2 negative, we surmise that these cells overshadow the signal of the $DP2^+$, thus showing no expression.

When we looked at the expression in monocytes, we showed for the first time that non-classical monocytes express DP2 and further, that this expression is altered in RA patients (Figure 11D). Compared to the "healthy" group, samples of the "flare" group displayed a significant reduction in DP2 expression. This decrease is diminished in patients of the "remission" group. Blood and synovial fluid non-classical monocytes are expanded in rheumatoid arthritis and their levels correlate with disease activity (197). Close studies at monocyte distribution within the inflamed tissue showed that although $Ly6C^+$ classical monocytes are present, only $CD115^+$ non-classical were detected near bone erosions (202). Further, these cells displayed a much higher osteoclastogenic potential as compared to classical monocytes. This suggests that non-classical monocytes are predominantly responsible for refuelling the osteoclast supply in the inflamed synovium. It is therefore conceivable, that DP2 down-regulation plays a role in this process. In 2008 it was shown that osteoclasts express both DP1 and DP2 and osteoclastogenesis from PBMC was suppressed when either of these receptors were activated (25). Further, DP2 activation leads to osteoclast apoptosis (284). It is tempting to assume that PGD_2 participates in bone protection by inducing osteoclast apoptosis and preventing osteoclast differentiation, but this process is impaired by down-regulation of DP2 in infiltrating non-classical monocytes in rheumatoid arthritis. Further experiments are necessary to prove this hypothesis.

When we looked at CD3 negative cells within the PBMC fraction, we observed a strong DP2 expression in basophils (Figure 11E). This expression was increased in "acute" patients when compared to "healthy" controls. Basophil granulocytes are mostly associated with anaphylaxis, asthma, and helminth infections. However, as they are not infiltrating the inflamed synovium, their

contribution to RA progression, if there is any, is assumed to be of systemic nature. Basophils are strong immunoregulators that are able to skew Th0 responses towards Th2 responses and it has been hypothesized, that they play a crucial role in juvenile idiopathic arthritis, as most patients suffering from JIA display elevated basophil counts and a Th2 cell-mediated immune response [reviewed in (261)]. Although their numbers are decreased in normal RA and they display an activated state, their role in this mostly Th1 driven disease is elusive. In another autoimmune disease, systemic lupus erythematosus, basophils are known to migrate towards secondary lymphoid organs where they most likely exert immunoregulatory functions (327). In a recent publication, it was shown that basophils of SLE patients express higher levels of DP2 compared to healthy controls and have increased levels of PGD_2 metabolites in their plasma (328). Activation of both DP1 and DP2 induced externalization of CXCR4, promoting accumulation in lymph nodes. As PGD_2 levels are also increased in plasma of RA patients and we show here that basophils display DP2 upregulation, it is tempting to assume that basophils will display a similar behaviour as in SLE. Further mouse experiments will help elucidating their impact on disease progression.

With the same data set, we looked at distributions of leukocytes in RA patients and compared those to our healthy controls. First, we looked at DP2 distribution within leukocytes that stained positive for this receptor, to determine whether this was also affected (Figure 11F). However, we found no expansion of DP2 receptor expressing cells. Next, we looked closer within the populations. While neither eosinophil nor neutrophil distribution was altered within the three groups (Figure 12A), we observed that CD4 T cells were markedly increased in samples of the "flare" group compared to healthy donors (Figure 12B). In RA, a subset of CD4 cells is expanded; namely $CD4^+CD28^-$ cells (329). These cells lack CD28 and are therefore independently activated from B7/CD28 and can induce cytolytic cell death. It is likely that the increase in total CD4 cells can be attributed to these cells, however, this is speculative, as we did not stain for CD28 or any marker to distinguish between T-helper populations.

When we looked at cells of the lymphocyte fraction that did not carry CD3, we did not observe significant differences in dendritic cells, natural killer cells, B-cells or basophils (Figure 12C). Basophils have been reported to be reduced in RA (261). Although not significant, there was a

trend towards reduction in samples of the "flare" and "remission" group, compared to healthy donors. We observed a significant increase in $CD16^{-}CD14^{++}$ classical monocytes in patients of the "flare" group when compared to healthy controls (Figure 12D). As mentioned above, non-classical monocytes are expanded in RA (197). However, in another study, classical monocytes were found to be elevated in RA patients (198). Those observed differences could be explained through different gating strategies, resulting in contaminating intermediate monocytes or neutrophils, subsequently altering the monocyte numbers. On the other hand, altered differentiation could also be a cause. Although the existence of non-classical monocyte precursors has been suggested, the general understanding is that non-classical monocytes arise from classical monocytes, switching first to intermediate state and eventually to the non-classical state (206). However, under inflammatory conditions like RA, reports show that immature monocytes are released from the bone marrow, causing a so called "left-shift" (330). This could in part explain different levels of monocyte populations.

It is well established that differentiated macrophages show migratory behaviour in response to PGD_2 (20,147). We wondered whether this was also the case for circulating monocytes as these cells actively migrate towards the inflamed synovium and contribute to the tissue-resident pool of macrophages and osteoclasts. We therefore performed chemotaxis experiments with PBMC from RA patients and compared them with PBMC from healthy donors (Figure 12E). To distinguish monocytes from other PBMC, we stained them with CD14 prior to measurement. We observed no differences between patients in remission, with acute flares or our healthy controls. Next, we tested whether priming with pro-inflammatory cytokines (IL-1β, IL-6 or TNF-α) could induce a stronger response to PGD_2, but the results were similar (Figure 12F). However, these data are difficult to interpret, as there was a broad variety between responses from different donors. One explanation could be that monocytes become quickly adherent when activated and are thus more difficult to measure in an experiment like this. A second explanation, why we observed so little monocyte migration, could be that at the time of these experiments we were unaware of the aforementioned DP2 restriction to non-classical monocytes (Figure 11D), and thus, staining solely for CD14 only provided us with classical monocytes.

Next, we looked at neutrophil and eosinophil responses to lipid mediators (Figure 13 and Figure 14). First, we assessed cytoskeletal rearrangements ("shape change") in response to 5-OxoETE and PGD_2 as both lipid mediators are strong chemoattractants to PMNL and can activate cells of the mononuclear phagocyte system (20,307). In allergic asthma, 5-OxoETE is hypothesized to be an influential contributor to eosinophil lung infiltration, where it acts in concert with PGD_2 [reviewed in (78)]. As PGD_2 levels are increased in the synovial fluid of RA patients and 5-lipoxygenase, the enzyme required for 5-OxoETE production, is expressed in the RA and OA synovium, we wanted to assess whether RA granulocytes act differently in response to both eicosanoids (76,275). Although eosinophils and neutrophils reacted strongly to our positive controls (data not shown), we found that their response to this lipid mediator was similar in all three groups. This was also the case for PGD_2 treatments. As assumed, neutrophils did not show chemotactic behaviour towards any PGD_2 concentration, as DP2 is not expressed in neutrophils (27). To compare with a more functional approach, we tested whether ROS production in response to both substances was altered in RA patients (Figure 14). In all experiments, the cells responded strongly to our positive controls (data not shown) and to our treatments (except for PGD_2 in neutrophils), but no significant changes between the "flare", "remission" or "healthy" group could be observed. This suggests that pre-activated neutrophils do not respond stronger towards either of these substances, although limitations of the method should be considered. Particularly in the shape change assay, both lipid mediators display a high efficacy and maximal responses were already reached at concentrations in the nanomolar range. Thus, to determine possible differences between groups at lower concentrations, titrating into the picomolar range would be necessary. Another limitation is the low sample number of three repetitions.

Although some monocytes retain their phenotype in RA, the fate of most of these cells is to differentiate into macrophages. Therefore, we designed a range of experiments to test the impact of PGD_2 on macrophages. First, we assessed the expression of both DP1 and DP2 on the surface of M-CSF differentiated monocyte-derived macrophages and compared it between all three groups (Figure 15A-B). We observed that both receptors were present on MDM, pro-inflammatory M1 and anti-inflammatory M2 macrophages. While DP1 expression remained unaltered between all three groups, we detected a significant upregulation of DP2 in M2 macrophages of the "flare"

group compared to the "remission" group (Figure 15B). As DP2 is mostly considered a pro-inflammatory receptor, this was surprising. However, there are reports in which DP2 downregulation in eosinophils correlated with disease severity. In ulcerative colitis, DP2 surface expression on blood eosinophils is reduced due to receptor internalization in response to the elevated serum PGD_2 levels (45). To rule out that DP2 upregulation is an indicator that these cells are driven towards a M1 state, we looked at the surface expression of CD80 and CD206 (Figure 15C-D). When we compared CD80 expression in LPS/IFN-γ polarised M1 macrophages, we observed that samples from healthy donors showed the strongest expression, while samples from patients in remission showed a significant reduction (Figure 15C). Between the "flare" group and the "remission" group we also observed a significant decrease. This suggests that the ability to polarize towards M1 is decreased in rheumatoid arthritis. As the anti-inflammatory marker CD206 did not rise in these cells, it is more likely that M1 are prone to retain a "M0" state, which refers to unpolarised monocyte-derived macrophages, rather than differentiated towards M2. When we looked at IL-4 polarised M2 macrophages, we neither found significant changes in CD80 nor in CD206 expression (Figure 15D). To further elucidate this peculiar up-regulation of DP2 in M2 macrophages, we approached this issue with PGD_2 receptor agonists. During polarization towards M1 or M2 (or retaining them at M0), the medium was supplemented with PGD_2, DP1 receptor agonist BW245C or DP2 receptor agonist DK-PGD_2 and afterwards, surface marker expression of CD80 and CD206 was measured (Figure 15E-F). PGD_2 elicited a significant upregulation of CD80 in M2 macrophages. This effect was even more pronounced in BW245C-treated M2 macrophages, suggesting it was DP1 mediated. When we looked at CD206 expression, we observed that BW245C upregulated this receptor in MDM, M1 and, surprisingly, in M2 as well. This phenotype switching reminds of the effects of ApoA-IV on macrophage polarization, where we saw that the apolipoprotein reduced CD80 expression in M1 macrophages, while in M2 this pro-inflammatory marker was increased (Figure 8A-B). Like ApoA-IV, BW245C induced CD80 upregulation in M2 macrophages (M2 → M1). Further, it upregulated CD206 in MDM (M0 → M2) and M1 (M1 → M2). Thus, it seems possible that there is a connection between PGD_2 and ApoA-IV signalling, however, this observation could be simply of correlative rather than causative nature.

Nonetheless, the concomitant upregulation of CD206 along with CD80 in M2 macrophages is more difficult to interpret. As mentioned above, CD80 upregulation indicates a commitment

towards a pro-inflammatory phenotype and CD206 upregulation the opposite. Thus, concurrent upregulation of both markers in the same cell population is puzzling but supports the hypothesis of the existence of a mixed M1/M2 phenotype, which has already been described in many pathological situations, such as cancer, inflammatory and autoimmune disorders, and chronic infections. Further experiments (e.g. cytokine production) are required to define their actual state on the M1-M2 spectrum.

Lastly, we wanted to investigate the effect of DP2 antagonist OC-459 on collagen antibody-induced arthritis. For this, we immunized 25-26 week-old DBA/1J mice with a 1.5 mg of cocktail, containing antibodies against collagen. On day 3 and day 10, inflammation was boosted through intraperitoneal injection of LPS. To assess DP2 involvement in the disease progression, mice were injected daily with 0.1 mg/kg OC-459 subcutaneously. After 14 days, mice were sacrificed, and blood and joint tissue were processed. We observed a weak protection against weight loss in the OC-459 group compared to the vehicle group on day 7 until day 9, but this effect was lost after that (Figure 16A). Interestingly, we observed a significantly earlier disease onset in the OC-459 group (Figure 16B). This was surprising, as DP2 blockade did not have an impact in the model of collagen-induced arthritis (9). It should be mentioned, however, that these arthritis models work differently and drawing accurate comparisons between them can often be a challenging task. In a model of adjuvants-induced arthritis for example, DP2 deficiency resulted in a more severe phenotype (271). When we compared the levels of total leukocytes in the joint, we observed a visible reduction in neutrophils in the OC-459 group (Figure 16C). Given the fact that neutrophils do not express DP2 (27), these data are difficult to interpret. A possible explanation could be that neutrophil infiltration in the CAIA model requires leukocytes that are DP2 dependent and due to the receptor blockade, neutrophil recruitment is impaired. When we looked at percentages, we observed a similar reduction in neutrophils and a rise in mast cells (Figure 16D). Contrary to neutrophils, mast cells express DP2, although intracellularly (39). It is therefore unlikely that this local increase in mast cells can be attributed to OC-459 directly. As PGD_2 possesses pro-resolving properties in collagen-induced arthritis and hPGDS deficiency aggravated adjuvant-induced arthritis, it is conceivable that the purpose of this lipid mediator in the inflamed joint is to resolve

inflammation (9,272). However, as blocking DP2 via OC-459 mimics the absence of PGD_2, an undiscovered regulatory loop could attract mast cells, which are potent PGD_2 producers.

While we saw that ApoA-IV levels are reduced in RA patients and that these patients also display altered expression of PGD_2 receptor DP2, we tested the direct effect the apolipoprotein has on these cells in the context of prostaglandin production. In our last set of experiments, we wanted to delineate a possible connection between both findings by exposing monocytes and macrophages to ApoA-IV and measuring subsequent changes in prostaglandin production.

As previously shown, DP2 expression in non-classical monocytes and ApoA-IV levels in plasma are reduced in RA patients (Figure 11A and Figure 11D). We therefore investigated whether there was a connection between these two findings. We stimulated monocyte populations with ApoA-IV for 30 minutes and observed DP2 expression in classical, intermediate and non-classical monocytes (Figure 17A). We found that ApoA-IV did not alter the expression levels of this receptor in these cells. As monocytes express hPGDS and are efficient producers of PGD_2 ([reviewed in (6)] and unpublished data), we next set out to investigate whether prostaglandin production was affected by the apolipoprotein. First, we compared vehicle-treated monocytes with cells that were exposed to 3 µg/mL ApoA-IV and looked at COX-2 expression (Figure 17B). Although not significant, we observed a visible upregulation of this protein in ApoA-IV stimulated monocytes. Next, we measured PGE_2 and PGD_2 production in the supernatant of ApoA-IV stimulated cells and compared it to a vehicle group (Figure 17C-D). Adherent monocytes treated with ApoA-IV for 24 hours produced significantly more PGE_2 than vehicle-treated cells (Figure 17C). PGE_2 can act in anti-inflammatory or pro-inflammatory manner, depending on the tissue and PGE_2 receptor expression (2). When we compared PGD_2 levels between stimulated and unstimulated monocytes, we observed no statistically significant differences, although a visible trend towards a rise in PGD_2 was detected in the ApoA-IV group (Figure 17D). It is conceivable that more repetitions would reveal a significant rise in PGD_2 production, as the sample number of three repetitions is rather low to compare prostaglandin production.

Following up on these data, we also investigated the impact of ApoA-IV on prostaglandin production in macrophages. First, we assessed whether ApoA-IV influenced COX-2 expression. In compliance with literature, we only observed COX-2 expression in M1 macrophages (331). There, we detected a significant upregulation of both COX-2 mRNA (Figure 18A) and protein

(Figure 18B), compared to vehicle-treated cells. As COX-2 expression is restricted to M1 macrophages, it is crucial to ascertain whether ApoA-IV induced COX-2 upregulation is an indicator of a M1→M2 transition or whether it enhances the pro-inflammatory repertoire of M1 macrophages. Of note, our lab could recently show that PGE_2 production in monocyte-derived macrophages attenuated LPS induced TNF-α production through an autocrine mechanism (60). To confirm our data, we next looked at prostaglandin synthesis. Although we could only detect a visible but not significant rise in mPGES-1 mRNA (Figure 18C), we observed a significant increase in basal PGE_2 levels when these cells were exposed to ApoA-IV (Figure 18D). Albeit it could be argued that the low sample number is responsible for lack of significance, the unchanged mPGES-1 expression may be explained functionally as well. PGE_2 has a relatively long half-life of roughly nine hours (332). Due to the fact that the supernatants were collected after 48 hours post-stimulation, PGE_2 levels could remain in the supernatant for a couple of hours after mPGES-1 was exhausted. Next, we wanted to assess PGD_2 production and first looked at hPGDS mRNA expression in response to ApoA-IV (Figure 18E). We could, however, observe no change in mRNA expression between both groups. When we looked at PGD_2 levels in the supernatant, we also failed to detect significant changes between the treatments (Figure 18F). As a trend towards elevation is apparent, it can be hypothesized that a comparison between a higher sample number would reach significance, but more repetitions are required to confirm this.

Conclusion

In summary, in the first part of our study we focused on the interplay between apolipoprotein ApoA-IV and allergic asthma, in which the prostaglandin D_2 plays a crucial role in the development and propagation of lung inflammation and remodelling. We showed that ApoA-IV potently suppresses CCL11- and PGD_2-induced eosinophil migration *in vitro* and later confirmed our data *in vivo* in a model of HDM-induced experimental airway hyperreactivity, where ApoA-IV protected against eosinophil lung infiltration and ameliorated impairment of lung function. The underlying pathway responsible for the observed anti-migratory effect of ApoA-IV was that of nuclear receptor NR1D1, as shown in experiments using selective NR1D1 agonists and antagonists. We conclude that ApoA-IV, among other apolipoproteins, displays anti-inflammatory properties and may be a possible therapeutic target in the search for drugs to ameliorate chronic asthma.

In the second part of our study we focused on cells involved in the pathogenesis of the autoimmune disorder rheumatoid arthritis, in which altered serum levels of apolipoproteins and prostaglandins have been observed. We show in monocytes that ApoA-IV potently suppresses chemotaxis and CD11b upregulation. We show further, that similar to ApoA-I, ApoA-IV induces COX-2 mediated PGE_2 release in monocytes and macrophages (290). This PGE_2 release may be due to a phenotype switch, as ApoA-IV decreased CD80 expression and TNF-α production in pro-inflammatory M1 macrophages. The process underneath these anti-inflammatory effects seems to involve NR1D1, which is present in M1 macrophages. Similar to eosinophils, we show that the NR1D1 selective agonist GSK4221 mimics the reduction of CD80 in pro-inflammatory macrophages and the use of the antagonist SR8278 reverses the effect of ApoA-IV.

In a clinical study, we show that the PGD_2 receptor DP2 is differentially expressed in immune cells of RA patients and healthy controls. While non-classical monocytes of patients suffering from acute disease showed a marked reduction, basophil granulocytes display an increase in DP2 in these patients. As only non-classical monocytes are assumed to differentiate into osteoclasts in RA and osteoclastogenesis in turn is inhibited by PGD_2, it may be possible that PGD_2 elicits an inhibitory effect on these cells to retain non-classical monocytes in circulation (202,284). However, further experiments would be necessary to prove this hypothesis. When we looked at differentiated

macrophages, we again observed a significant DP2 upregulation in M2 macrophages in the acute group. However, as this was a pilot study, we were unable to examine if this affects macrophage behaviour in terms of cytokine release for instance. Reports on how macrophages or monocytes respond to PGD_2 treatment are scarce and without understanding how healthy cells behave in general, we were unable to delineate whether it promotes or ameliorates pro-inflammatory signalling in these cells(20). It is however an interesting finding that M1 macrophages in RA display a reduced CD80 expression and further, that CD80 as well as CD206 expression can be increased by PGD_2 or BW245C. Lastly, we observed that RA patients have lower levels of ApoA-IV circulating in plasma as healthy controls.

We conclude that ApoA-IV possesses potent anti-inflammatory properties on immune cells like eosinophils and cells of the mononuclear phagocyte system through a possible NR1D1/COX-2/PG axis.

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