Metabolism and lipid mediators as regulators of innate immune cell function: implications for inflammation and immune responses

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Implications for Inflammation and Immune Responses

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ISBN: 978-90-90423-86-9

Cover design: Luís Almeida

Layout: Luís Almeida

Printing: ProefschriftMaken

The work described in this thesis was performed at the Leiden University Centre for Infectious Diseases, at the Leiden University Medical Centre, Leiden, The Netherlands. This work was supported by funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant agreement No. 812890.

Metabolism and Lipid Mediators as Regulators of Innate Immune Cell Function

Implications for Inflammation and Immune Responses

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr. S. de Rijcke,
volgens besluit van het college voor promoties
te verdedigen op dinsdag 23 juni 2026
klokke 13:00 uur

door

Luís Pedro Ferreira de Almeida
geboren te Lissabon, Portugal
in 1996

Promotors:	Dr. B. Everts
	Prof. dr. M. Yazdanbakhsh
Leden promotiecommissie:	Prof. dr. M.A. Giera
	Prof. dr. C. Goodyear (University of Glasgow)
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I

General Introduction

Macrophages and Dendritic Cells as Key Regulators of Immune Responses

The immune system is the main line of defence against the millions of potential pathogens (1–3) and other challenges, such as possible cancerous cells and allergens, humans are exposed to on a daily basis (4–7). This system is a complex and intricate network of many different molecules, cells, tissues, and organs, all working in tandem, in a tightly regulated manner, albeit with different functions and mechanisms of action. The immune system can be divided into two main parts – the innate immune system and the adaptive immune system. The innate immune system acts in a fast and broad manner, with general specificity, being activated by generic conserved molecular motifs, such as PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular patterns). Conversely, the adaptive immune system, although slower to act, is highly precise and efficient, recognizing a specific antigen, thus being able to zone-in on an individual type of pathogen or molecule (8).

However, without the initial input of the innate immune system, the adaptive immune system cannot be activated (9,10). This makes the innate immune system a key player in the overall immune response. Therefore, unravelling the mechanisms of action which govern innate immune cell function poses as an imperative endeavour to understand both how we could manipulate the immune system to our advantage, such as by improving the immune response against infections, increasing vaccine efficacy, boosting the anti-tumoral response, or knowing how to introduce a break in certain hyperinflammatory responses (e.g. severe COVID-19) (11), or autoimmune diseases (e.g. rheumatoid arthritis), characterized by chronic inflammatory responses against self-antigens, due to the loss of self-tolerance (12).

Two key cell types of the innate immune system are macrophages and dendritic cells (DCs) (13). Macrophages are tissue resident immune cells responsible for the maintenance of homeostasis of tissues under steady state. In response to inflammatory signals (Fig. 1) they can adopt pro-inflammatory functions characterized by the synthesis of pro-inflammatory cytokines (e.g. TNF and IL-6) (14,15). A few examples of classical inflammatory signals in macrophages are:

- Stimulation of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) by PAMPs and DAMPs, which activate different inflammatory pathways, depending on the TLR being stimulated (16–18);
- Binding of the Fc portion of antibodies to Fc receptors (FcR) present in the macrophages (19,20);

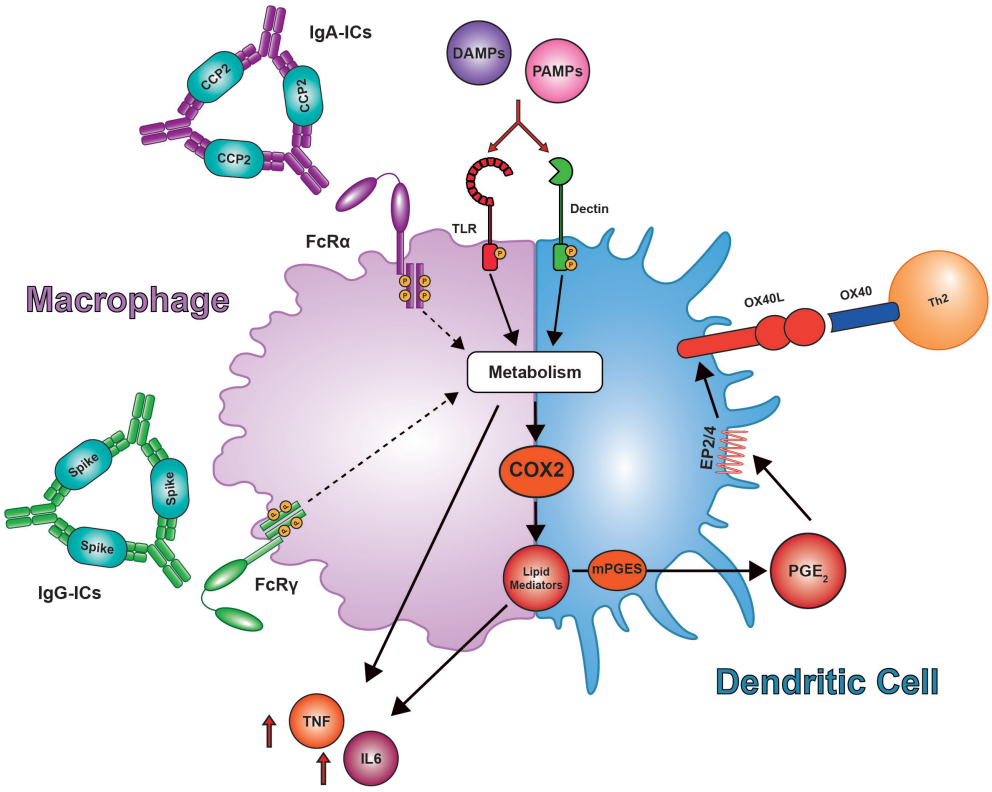

Figure 1: Metabolism and lipid mediators play a central role in innate immune cell function. Extrinsic stimuli induce metabolic changes in both macrophages and dendritic cells via PRRs (such as TLRs and Dectin), and/or via FcRs. These metabolic changes regulate the cellular immune response, via synthesis of COX2-dependent lipid mediators, and proinflammatory cytokines.

Predictably, these two stimuli can synergise and amplify the inflammatory phenotype of macrophages, thus mounting a stronger and more robust response against a pathogen, in cases where the Fc portion of the antibody engages an activating FcR (21–23). Alternatively, antibodies can also engage with inhibitory FcRs, thus dampening the overall inflammatory state of macrophages, and promoting tissue homeostasis (24).

While today it is known that macrophage differentiation and activation is a highly plastic process that exists on a spectrum, the two extremes of that spectrum are highly pro-inflammatory macrophages, commonly referred to as “M1-like”, and anti-inflammatory macrophages/macrophages that promote the resolution of the inflammatory process (i.e. pro-resolving macrophages), commonly referred to as “M2-like” (25). In general, macrophages with the M1-like phenotype are tasked with patrolling and killing

possible pathogens, through a process called phagocytosis and by the production of pro-inflammatory cytokines, while the M2-like promote wound healing, by producing anti-inflammatory cytokines, tissue regeneration and by engulfing dead cells, or cells undergoing cell death (i.e. apoptosis), through a process called efferocytosis (25).

On the other hand, the main function of DCs (Fig. 1) is to induce and direct the differentiation and activation of the adaptive immune system, especially through the presentation of antigens to T cells. Once DCs encounter an antigen, be it from an outside invader, such as a pathogen, or from an internal source (e.g. self-antigen or cancerous cell), they will undergo specific changes in activation, and either become immunogenic or tolerogenic. Immunogenic DCs will induce the differentiation and activation of cytotoxic CD8+ T cells, and/or CD4+ T helper cells, such as Th1, Th2 or Th17 cells, all with specific functions tailored to specific pathogens and immune challenges (26). Whereas tolerogenic DCs will instead induce the differentiation and activation of Treg cells, which work as built in immunologic brakes, that inhibit immune responses against a certain antigen, be it a self-antigen, to prevent autoimmunity, or against a foreign, but harmless, antigen such as foods (27,28).

In an impressive display of cellular coordination, the newly activated T cells, particularly Th1, Th2 or Th17, will migrate to the specific site where the presented antigen is located, and will oversee the immune response, by producing cytokines which will not only act on the innate immune system, such as the macrophages already present in the site, but also work as beacons to promote further migration of more innate immune cells to help fight against the specific threat (29–32).

Additionally, DCs can also activate Tfh cells (T follicular helper cells), which are intimately involved in initiating humoral immunity and shaping B cell responses (33,34). After this “activation trinity”, between B cells, DCs, and Tfh, B cells will migrate to the specific site the antigen presented by the DCs originated from, where they will then produce and secrete antibodies. These antibodies, usually in the form of IgG or IgA, are able to recognize the antigen and directly bind to it, forming immune complexes, which are then able to be recognized by macrophages, via Fc receptors (Fig. 1), thus helping these macrophages undergo further pro-inflammatory activation (35,36). These mechanisms put DCs as the first target in a strategy of immune manipulation, since they dictate the activation states of T cells and B cells, while it puts macrophages as the last target, since they are the final actors in this innate-adaptative-innate immunological chain.

Control of Macrophage and DC Function by Lipid Metabolism

One of the more recent fields of study in immunology is immunometabolism. This area arose from the discovery that, depending on the function being performed, immune cells will undergo specific metabolic reprogramming, favouring certain metabolic pathways over others, not only for their energetic needs, but also for the synthesis of crucial metabolites to be used in their activation and differentiation stages. For instance, it was described that, *generally*, pro-inflammatory macrophages and immunogenic DCs rely on glycolysis and fatty acid synthesis, while pro-resolving macrophages and tolerogenic DCs instead rely on fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) (37–39). With this knowledge also came the hypothesis that, by manipulating macrophage and DC metabolism, one could control their activation state. For example, by inhibiting metabolic pathways favoured by pro-inflammatory/immunogenic phenotypes, and promoting metabolic pathways favoured by pro-resolving/tolerogenic phenotypes, the cell would switch from a pro-inflammatory state to an anti-inflammatory state, and vice-versa.

One crucial pathway at the centre of immunometabolism is lipid metabolism. While lipids have long been seen as molecules for energy storage and building blocks for membranes, we now know that is not the case. Lipid metabolism plays a central role in the synthesis of metabolites used by immune cells for their respective functions. For example, fatty acid synthesis can lead to the formation of lipid droplets in macrophages and DCs, which have been shown to play a role in immune functions, such as cytokine production, phagocytosis and antigen presentation (40–42). Alternatively, FAO was shown to be important Acetyl-CoA from fatty acid oxidation can be used for histone acetylation and subsequent control of gene transcription (43,44).

Additionally, lipid metabolism also includes the synthesis of lipid mediators. Lipid mediators are a group of metabolites that play a highly important role in cell messaging. As the name suggests, these metabolites originate from the oxidation of fatty acids, usually derived from membrane phospholipids, and act as chemical transductors of messages (Fig. 1). These mediators can act on the cell itself (autocrine signalling), on cells close by to the original cell (paracrine) or on cells far away from the cell of origin (endocrine). These mediators are one of the main communicators between immune cells, being able to induce signalling pathways in macrophages and DCs that either promote the synthesis of proinflammatory cytokines and T cell priming, or instead dampen inflammation, by promoting tissue regeneration and tolerogenicity (45). Thus, unravelling the mechanisms which lead immune cells to synthesize these lipid mediators and how these mediators act on the immune cells themselves

is crucial to shed a light on possible therapeutic targets for intervention from an immunometabolism point of view.

Nevertheless, much still remains unknown. As mentioned above, *in general*, pro-inflammatory macrophages and immunogenic DCs tend to favour glycolysis and fatty acid synthesis, while anti-inflammatory macrophages and tolerogenic DCs tend to prefer oxidative phosphorylation and fatty acid oxidation. However, subsequent studies have suggested that this immunometabolic dichotomy is not always so, showcasing that the metabolic reprogramming that macrophages and DCs undergo upon activation is dependent on a panoply of factors, such as cell type, tissue location, activation stimulus, and even timing. Therefore, it is crucial to look at the cellular immunometabolic profile in each specific context. Indeed, recent literature has shown that FAO is important for NLRP3 inflammasome activation and anti-tumor functions (46,47), while fatty acid synthesis appears to impair anti-tumour responses and is important for M2-like activation in helminth infections (48,49).

This seemingly contradictory information also holds true for the role of lipid mediators. For example, while in some contexts one lipid mediator (e.g. Prostaglandin E_2) can induce a pro-inflammatory response in macrophages, or drive a Th1-priming response by DCs, in other contexts it can instead induce an anti-inflammatory response and/or drive a Th2 response (50–62).

Therefore, considering there is a crucial need to look into the metabolic requirements of macrophages and DCs in specific contexts, this thesis aimed to address a few unanswered questions, such as:

1. What does the current literature show regarding the role of lipid metabolism in regulating the functions of DCs and macrophages in pro- and anti-inflammatory contexts?
2. What are the metabolic requirements of macrophages during antibody-mediated inflammation and what roles do lipid mediators play in driving this inflammatory response? Is there a difference between antibody isotypes and classes, or do all antibodies use the same metabolic pathways?
3. What is the role of lipid mediators during antigen recognition by DCs in the context of Th2-priming driven by eggs during helminth infections? Can this be chemically targeted to shift the balance towards a Th1-priming phenotype in DCs?

Thesis Outline:

Chapter 2 constitutes a more in-depth theoretical introduction to this thesis. There you can find a background into the field of immunometabolism in the context of innate immunity, with specific focus on fatty acid metabolism in DCs and macrophages, and how both metabolic profile and immune function are intricately connected.

In **chapter 3** we studied the role of SARS-CoV-2 anti-spike IgG in promoting an hyperinflammatory state in macrophages. IgG achieves this by inducing specific metabolic changes that prime these macrophages for excessive pro-inflammatory cytokine expression. By inhibiting these metabolic pathways we should they could be chemically targeted to prevent IgG-induced hyperinflammation. On a similar note, in **chapter 4** we looked at the role of IgA against citrullinated peptides in driving chronic inflammation in the context of rheumatoid arthritis. IgA achieves this by inducing a state of hyperinflammation in macrophages that is both dependent on certain metabolic changes and the synthesis of lipid mediators downstream of cyclooxygenase-2, thus identifying chemical targets with possible therapeutic applications in treating auto-antibody-driven chronic inflammatory diseases.

In **chapter 5** we describe how DCs recognize soluble egg antigens from the parasitic helminth *Schistosoma mansoni* through Dectin-2, how they become licensed to induce the differentiation of Th2 cells, and how can DC lipid mediator synthesis be chemically targeted to mould their immune function within this context.

In **chapter 6** I provide an extensive discussion outlining the novel findings of this thesis, and delve into future research suggestions to further decode the nuances of immunometabolism in the context of inflammatory responses, and the possible practical applications that this scientific information may have on society as a whole.

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II

Fa(c)t Checking: How Fatty Acids Shape Metabolism and Function of Macrophages and Dendritic Cells

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First published: 31 March 2021

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European Journal of Immunology
DOI: 10.1002/eji.202048944

Abstract

In recent years there have been major advances in our understanding of the role of free fatty acids (FAs) and their metabolism in shaping the functional properties of macrophages and dendritic cells (DCs). This review presents the most recent insights into how cell intrinsic FA metabolism controls DC and macrophage function, as well as the current evidence of the importance of various exogenous FAs (such as polyunsaturated FAs and their oxidation products -prostaglandins, leukotrienes and pro-resolving lipid mediators) in affecting DC and macrophage biology, by modulating their metabolic properties. Finally, we explore whether targeted modulation of FA metabolism of myeloid cells to steer their function could hold promise in therapeutic settings.

Introduction

Macrophages are innate immune cells of myeloid origin with a diverse range of functions. They not only play a key role in maintaining tissue homeostasis under non-inflammatory conditions, but also in phagocytosis and killing of microbes during an infection and as well as in driving wound healing and tissue regeneration during the resolution phase of an inflammatory response (1,2). To be able to acquire these distinct functional traits, macrophages can adopt various polarisation states, which are imprinted by the local cues they are exposed to in the tissues where they reside (3–5). The most well-studied differentiation states are classically activated macrophages (also called M1 macrophages) and alternatively activated macrophages (also referred to as M2 macrophages), and both can be modelled in *in vitro* culture systems. Although these two polarisation states fail to capture the full diversity of functional states macrophages can adopt *in vivo*, they are commonly used as models for studying the pro-inflammatory and anti-inflammatory properties of these cells, respectively (6).

Like macrophages, dendritic cells (DCs) also belong to the group of myeloid innate immune cells. DCs sit at the crossroads between the innate and the adaptative immune system, working as specialised antigen-presenting cells (APCs) that are capable of initiating T cell responses (7). During steady state, DCs reside in peripheral tissues in a quiescent state. Upon sensing of pathogens or tissue-derived danger signals, DCs undergo a phenotypic and functional change involving enhanced internalisation and processing of antigens (Ags) (8–10) and migration towards tissue draining lymph nodes, where they can induce an adaptive immune response by priming and activating ag-specific T cells (9,11). Furthermore, DCs also play a role in the induction of tolerance during steady state due to exposure to tolerizing signals. These tolerogenic DCs can mediate tolerance by promoting peripheral T cell anergy and apoptosis, decreasing effector and memory T cell responses, and inducing the differentiation and activation of regulatory T cells (Tregs) (12–15).

Due to the central role played by DCs and macrophages in the immune response, it is important to understand how their function is regulated and what kind of stimuli are needed to initiate/sustain their activation and polarisation in specific scenarios. For instance, there has been a longstanding interest in defining the signalling pathways regulating macrophage and DC function in the context of “classical” immunological cues, such as cytokines, chemokines, and pathogen-associated molecular pattern (PAMPs) (16,17). More recently, there has been a growing appreciation that metabolic signals and alterations in cellular metabolism can also dictate immune cell function (see (18) for a detailed introduction to immune cell metabolism). Recent research about immunometabolism has contributed to the realisation that stimuli and changes

in the environment macrophages and DCs are exposed to, eventually converge into alterations in their metabolic properties. It has become clear that reprogramming of metabolic pathways such as glycolysis, oxidative phosphorylation (OXPHOS), fatty acid (FA) synthesis and oxidation (FAO) are not only associated with, but are also crucial for shaping functional responses of DCs and polarisation of macrophages to environmental cues (18).

The notion that FAs (both intracellular as well as extracellular) and their metabolism play a central role in shaping DC and macrophage biology has gained significant traction in recent years (19). Extracellular FAs can be synthesized *de novo* from carbons derived from other core metabolic pathways such as the TCA cycle, glycolysis and glutaminolysis, hydrolysed from intracellular lipid stores, or directly obtained from extracellular space (Fig. 1). These FAs play a pivotal structural role when used for incorporation into cellular membranes. Moreover, through their oxidation in mitochondria they serve an important role in generating energy via OXPHOS as well as in generating various TCA cycle intermediates that can act as signalling metabolites or that can be used for the synthesis of other macromolecules. As a result, metabolism of intracellular FAs is a central regulator of DC and macrophage function. In addition, various FAs present in the extracellular environment, released by other cells including adipocytes, tumour cells and other immune cells, or obtained through diet (20), have also been shown to have the potential to alter the functional properties of DCs and macrophages (20–22).

The most well-studied FAs in the context of myeloid cell biology are short-chain fatty acids (SCFAs), saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs), which could be mono-unsaturated (MUFAs) and poly-unsaturated (PUFAs). In addition, specialized pro-resolving mediators (SPMs) which are products from PUFA metabolism (mainly ω-3 and ω-6 PUFAs; see Fig. 1) (23), can also have strong modulatory effects on myeloid cells, including macrophages and DCs (23,24). Various mechanisms have been proposed through which these exogenous FAs can affect DC and macrophage function. These include acting as signalling molecules engaging receptors, serving as structural components, and - interestingly - altering the metabolism of these cells.

In this review we will discuss the most recent insights into how intrinsic FA metabolism controls DC and macrophage function, as well as the current evidence showing how various exogenous FAs (such as PUFAs and their oxidation products – prostaglandins, leukotrienes, and SPMs) affect DC and macrophage function, by modulating their metabolic properties.

Role of Intrinsic Fatty Acid Metabolism in Myeloid Cell Function

Fatty Acid Oxidation

Macrophages

Free FAs used for FAO can be acquired either by uptake of dietary fats and subsequent hydrolysis, by lipolysis of stored acylglycerols, or by *de novo* FA synthesis (20). These FAs can be oxidised either in peroxisomes, and/or be transported into the mitochondria by Carnitine Palmitoyltransferase (CPT) where they will undergo FAO. FAO results in the formation of multiple units of acetyl-CoA, which can serve as substrate in the TCA cycle to fuel OXPHOS. It is a tightly regulated pathway, with the rate limiting step being the transport of acyl-CoA into the mitochondrial matrix by CPT1. The control of CPT1 activity is, therefore, a key checkpoint for regulating mitochondrial FAO (Fig. 1) (25).

Murine macrophages stimulated with IL-4 i*n vitro* are characterized by increased FAO activity (26). Likewise, tumour-associated macrophages (TAMs) which share phenotypic and anti-inflammatory characteristics with *in vitro*-generated M2 macrophages (27) were shown to also have high levels of FAO (28). Moreover, increased FAO is also correlated with efferocytosis, a process used by M2-like macrophages to remove apoptotic cells to maintain tissue homeostasis (29). Initial studies have suggested that FAO itself is crucial for M2 polarisation, as pharmacological inhibition of CPT1 using etomoxir prevented M2 differentiation (26,30–32). However, more recent work has questioned these findings (33). Using genetic approaches, it was shown that conditional deletion of CPT1a had no effect on acquisition of an M2 phenotype. Moreover, etomoxir was found to have substantial off-target effects at concentrations used in these earlier studies (33). However, it should be noted that long-term genetic deletion of crucial FAO enzymes, such as CPT1a, may result in metabolic adaptation by usage of compensatory pathways to support cellular processes normally dependent on long-chain FAO, that cells may not be able to resort to upon acute pharmacological inhibition (34). These issues will need to be resolved to fully understand the importance of FAO in alternative activation of macrophages.

In vitro-generated murine M1 macrophages are characterized by high expression of inducible Nitric Oxide Synthase (iNOS) leading to the synthesis of Nitric Oxide (NO) (35), which is known to impair OXPHOS by inhibiting the electron transport chain (ETC) in an auto- or paracrine manner (Fig. 2A) (36), and promote the synthesis

of reactive oxygen species (ROS) that help in the microbicidal activity of M1 macrophages. As a consequence, FAO is severely compromised in these cells (30). However, LPS-stimulated peritoneal macrophages (pMacrophages) are characterized by increased OXPHOS (37), which may stem from a lower potential to produce NO by pMacrophages compared to their *in vitro* counterparts (30). Under certain conditions, FAO also be important for specific pro-inflammatory properties of macrophages. NLRP3 inflammasome activation, and synthesis of IL-1β was impaired following pharmacological inhibition of CPT1a with etomoxir suggesting dependency on FAO (38–40). However, the exact mechanism by which FAO boosts NLRP3 activations is still not clear, although increased FAO-fuelled mitochondrial ROS production has been implicated (40,41).

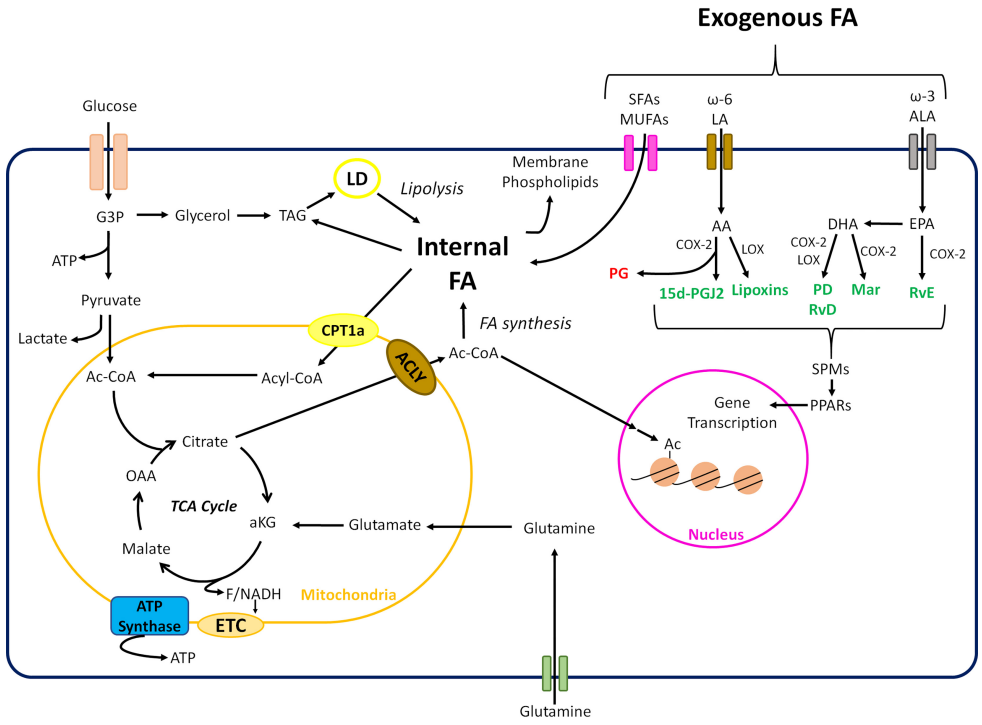

Fig. 1: **Cellular metabolism of intra- and extracellular fatty acids.**

A schematic overview of key processes involved in cellular FA uptake and metabolism. Core metabolic pathways connected to FA metabolism are indicated as well as the main processes involved in SPM synthesis from PUFAs such as w-3 and w-6 FAs. Specifically, PUFAs, many of which are essential fatty acids obtained from food, can be metabolised by cyclooxygenases (COX) and lipoxygenases (LOX) to give rise to SPMs. Main PUFAs that serve as substrate for these enzymes are linoleic acid (LA) and arachidonic acid (AA), which are ω-6 PUFAs, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both ω-3 PUFAs. Several enzymatic reactions lead to synthesis of SPMs (highlighted in green). The metabolism of EPA by COX-2 eventually gives rise to E-series Resolvins (RvE), while with DHA the products are more

diverse. The actions of LOX on DHA lead to products from the Maresin (MaR) family, while both LOX and COX-2 can give products from the Protectin family (PD) or D-series Resolvins (RvD). Protectin family (PD) or D-series Resolvins (RvD). In the case of ω-6 PUFAs, such as AA, COX and LOX give products such as Prostaglandins and Lipoxins, the former being mainly proinflammatory (red).

One of the mechanisms by which FA metabolism regulates macrophage polarisation involves changes in histone acetylation of IL-4-inducible genes fuelled by an increase in cellular concentrations of Acetyl-CoA (Ac-CoA) (31). It was found that IL-4 receptor (IL-4R) signalling increases ATP citrate lyase (ACLY) expression and activity (See Fig. 2B), resulting in an accumulation of nuclear and cytosolic Ac-CoA to enable efficient histone acetylation. Tracing experiments revealed that of glutamine, glucose and palmitate, the latter was the largest source of carbon for Ac-CoA (31). This may indicate that FAO in M2 macrophages fuels the TCA cycle to increase ACLY-driven Ac-CoA output to support histone acetylation required for expression of M2 macrophage-associated genes.

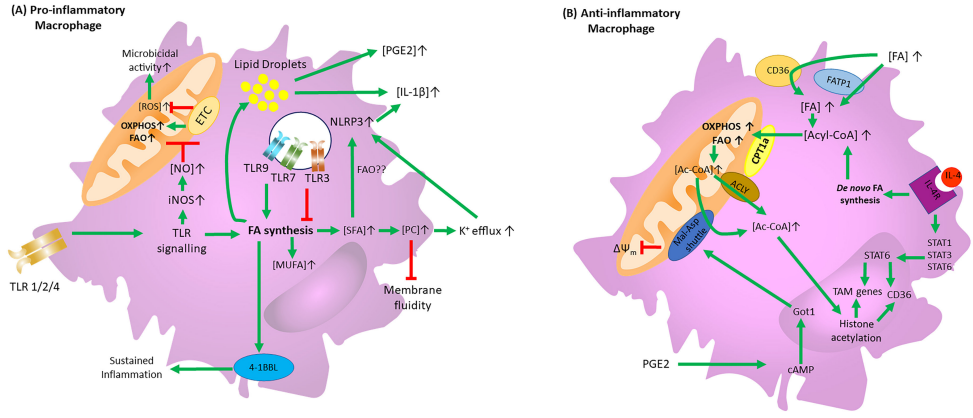

Fig. 2: **FA metabolism of pro- and anti-inflammatory macrophages**

Schematic depiction of how FA metabolism and uptake control the function of (A) pro- and (B) anti-inflammatory macrophages. Green lines indicate positive signalling, while red lines indicate an inhibitory effect. (A) pro-inflammatory macrophages increase FA synthesis upon TLR signalling. This increase in FA synthesis drives 4-1BBL activity which helps sustain inflammation. TLR-driven FA synthesis also leads to an increase in lipid droplets which is associated with increased PGE2 and IL-1β synthesis. Moreover, TLR signalling induces iNOS expression and subsequent NO synthesis which inhibits the ETC, thereby reducing FAO, and promoting ROS formation which helps with microbicidal functions. Increased FA synthesis upon TLR stimulation also leads to SFA synthesis, which increases PC levels in the cell membrane, leading to less fluidity and K^+ efflux, thereby activating NLPR3 and IL-1β synthesis. TLR1/2, TLR7 and TLR9 activation increased FA synthesis and *de novo* SFA and MUFA synthesis. TLR3 activation leads to the opposite effect, inhibiting FA synthesis, along with MUFA and SFA synthesis. (B) IL-4R signalling activates STAT1, STAT3, and STAT6 which promote the transcription of TAM genes and CD36. IL-4R signalling also promotes *de novo* FA synthesis which increases Acyl-CoA levels in the cell. Extrinsic FAs can also increase Acyl-CoA levels by being transported intracellularly by CD36 or FATP-1. Increased Acyl-CoA promotes OXPHOS and

FAO in a CPT1a-dependent manner. The increased flux in OXPHOS and FAO results in elevated levels of mitochondrial Acetyl-CoA which can be transported to the cytosol in an ACLY-dependent manner or through the Malate-Aspartate shuttle. Cytosolic Acetyl-CoA can participate in histone acetylation of M2-like and TAM genes. PGE2 impairs mitochondrial membrane potential in M2-like macrophages by dysregulation of the Malate-Aspartate shuttle by increasing cAMP-induced Got1 expression.

Thus, the relationship between macrophage phenotype and FAO does not appear to be as black and white, as initially thought. A picture is emerging that FAO can - depending on the context - support both pro- or anti-inflammatory properties by appropriating specific functions. For instance, in pro-inflammatory macrophages, FAO may be used to produce ROS to support activation of the NLRP3 inflammasome, as well as potentially fuel the TCA cycle to compensate for cataplerosis of intermediates that are being extracted from the TCA cycle for synthesis of amino acids and other macromolecules needed for pro-inflammatory activation (38–41). On the other hand, in M2 macrophages there is evidence that FAO, in addition to being involved in epigenetic remodelling by serving as a source of Ac-CoA required for acquisition of an M2 phenotype, also contributes to maintenance of anti-inflammatory activities, such as efferocytosis (29), by burning through FAs that otherwise accumulate in these cells as a consequence of this process. Moreover, β-oxidation of fatty acids from apoptotic cells enhanced IL-10 transcription and synthesis, thereby reinforcing their anti-inflammatory phenotype (42).

Dendritic Cells

Several studies have highlighted the importance of FAO in regulating the functional properties of DCs in a stimulus- and subset-specific manner (43–45). Different TLR stimuli engage FAO to a different extent in human moDCs (43). While TLR4 stimulation was found to induce glycolysis, TLR7/8 stimulation with pRNA increased FAO and OXPHOS in human moDCs (Fig 3A). This increase in FAO and OXPHOS was due to branched-chain alpha-keto acid dehydrogenase complex E1-alpha subunit (BCKDE1α) phosphorylation in a PTEN-induced putative kinase 1 (PINK1)-dependent manner. Interestingly, inducing PINK1 activity in tolerogenic DCs stimulated FAO and rendered these DCs immunostimulatory (43), suggesting FAO can promote pro-inflammatory functions in DCs. Other studies have also implicated FAO in supporting DC pro-inflammatory activation by showing that its pharmacological inhibition by etomoxir suppressed both murine cDC and pDC activation stimulated with a TLR9 agonist, as evidenced by decreased expression of costimulatory molecules and decreased synthesis of pro-inflammatory cytokines (44). Similarly, murine pDCs increase FAO upon stimulation of TLR9, which was found to be required for efficient type 1 interferon (I-IFN) production (46). Tumour cells may appropriate this mechanism by secreting α-Fetoprotein, which inhibits FAO and OXPHOS in DCs, leading to

impaired stimulation of Ag-specific effector functions (47). This points towards a link between FAO and triggering of endosomal TLRs, which may be explained by fact that engagement of endosomal TLRs generally lead to strong type 1-IFN production, which can promote FAO in an autocrine manner (41). Possibly, in this context, FAO may take over the role from glycolysis as main carbon source to fuel the synthesis of TCA cycle intermediates, which in DCs stimulated with cell membrane associated TLRs is required to support the anabolic demands of immunogenic DC activation (48).

However, increased FAO has also been implicated in supporting tolerogenic properties of DCs (Fig 2B). DCs rendered tolerogenic *in vitro* using Vitamin-D3 or *in vivo* through Wnt5a signalling in the tumour micro-environment, display increased FAO (49–51) on which they in part rely for induction of Tregs (51,52). In the latter study FAO was mechanistically shown to enhance indolamine 2,3-dioxygenase-1 (IDO) activity and suppress IL-6 and IL-12 cytokine expression by DCs, culminating in Treg generation. Additionally, failure of TLR-stimulated murine CD11c+ DCs or BM-DCs to switch from OXPHOS to glycolysis, due to deficiency in miRNA-142, which normally supresses CPT1a activity, locked these cells in a tolerogenic state with reduced synthesis of pro-inflammatory cytokines and reduced ability to activate T cells both *in vitro* and *in vivo* (53).

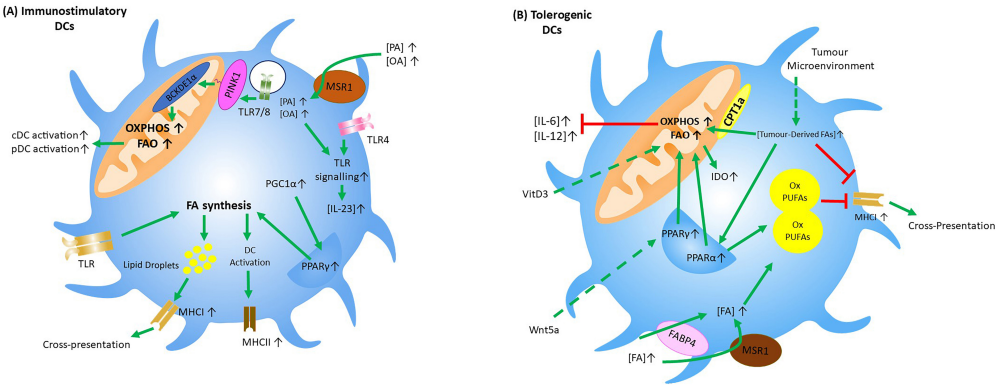

Fig. 3: **FA metabolism of immunostimulatory and tolerogenic DCs:**

Schematic depiction of how FA metabolism and uptake control the function of (A) immunostimulatory and (B) tolerogenic dendritic cells. Green lines indicate positive signalling, while red lines indicate an inhibitory effect. Dashed lines represent effects from exogenous stimuli. (A) In immunostimulatory DCs, TLR signalling increases FA synthesis to promotes ER expansion and LD formation, which contribute to upregulation of MHCII and MHCI. PGC1α and PPARγ activation is also associated with increased FA synthesis in immunostimulatory DCs. TLR7/8 stimulation can lead to the phosphorylation of BCKDE1α in a PINK-mediated manner. This results in increased OXPHOS and FAO which in turn is associated with increased activation of cDCs and pDCs. Additionally, extrinsic FAs, such as OA and PA, which are transported intracellularly by MSR1, can boost TLR4 signalling and increase IL-23 synthesis. (B) In tolerogenic DCs, increased OXPHOS and FAO are associated with reduced IL-6 and IL-12 synthesis and increased IDO expression. Tumour-derived FAs can increase OXPHOS and FAO by either being transported by CPT1a,

or by activating PPARα. This PPARα activation can also increase LD formation, which have a high content of oxidised PUFAs. Extrinsic FAs transported by MSR1 and FABP4 can feed these LDs and increase their oxidised PUFA content. These LDs, and tumour-derived FAs themselves, impair cross-presentation by suppressing MHCI surface expression. External stimuli such as VitD3 and Wnt5a can also increase FAO and OXPHOS, with Wnt5a doing so in a PPARγ-dependent manner.

Taken together, FAO may support, depending on the studied DC subset and/or nature of activating signal, either pro-inflammatory or anti-inflammatory properties of these cells. How exactly the same metabolic pathway can underpin these divergent immunological properties in DCs remains to be determined.

Fatty Acid Synthesis

De novo FA synthesis is a process in the cytoplasm whereby acyl chains are generated from Ac-CoA through the action of fatty acid synthases. Most of the Ac-CoA which is converted into FAs is derived from carbohydrates originating from the glycolytic pathway and TCA cycle [15], [47].

Macrophages

There are several studies that have linked *de novo* FA synthesis to supporting pro-inflammatory function of macrophages (Fig 2A). For instance, genetic models to block FA synthesis have shown that NO production and pro-inflammatory signalling are reduced in both human and murine macrophages upon TLR stimulation (54–57). Additionally, FA synthesis also appears to support TLR-driven 4-1BBL activity, a member of the TNF superfamily which regulates the sustained production of pro-inflammatory cytokines in TLR-activated macrophages (58). Consistent with this latter finding, inhibition of FA synthesis in a murine model of psoriasis, in which prolonged pro-inflammatory activity of M1 macrophages is a driving factor behind the disease, alleviated the symptoms (58). However, conditional deletion of ACC1, the enzyme that converts Acetyl-CoA into Malonyl-CoA used for FA synthesis, did not compromise pro-inflammatory macrophage responses (59). These discrepancies in outcome might, as alluded to above, arise from the difference in effects of instant pharmacological targeting with potential off target effects vs long-term deletion. In addition, different TLR ligands appear to have different effects on FA synthesis and the lipidome of macrophages (60). Stimulation of MyD88-dependent TLRs (i.e. TLR1/2, TLR7, or TLR9) increased *de novo* SFA and MUFA synthesis, while TLR3 stimulation reduced both SFA and MUFA synthesis. Moreover, inhibition of MUFA synthesis, without affecting SFA synthesis, disturbed the TLR-driven reprogramming of the lipidome, resulting in an increased inflammatory response (60).

Together, this links SFA synthesis to pro-inflammatory properties of macrophages. Several mechanisms have been proposed through which FA synthesis can support pro-inflammatory properties of macrophages. Firstly, it may help in changing membrane lipid composition to alter fluidity to facilitate membrane-associated effector functions such as phagocytosis (61–63), as has been shown following TLR4 stimulation (64). Here, sterol regulatory element binding protein-1a (SREBP-1a), a key transcriptional regulatory protein of fatty acid metabolism, was activated downstream of TLR4 and increased FA synthesis. Inhibiting this pathway led to defective phagocytosis, resulting from a reduction in the interaction between lipid rafts and the cytoskeleton, presumably due to reduced accumulation of newly synthesized fatty acyl chains within membrane phospholipids (64). Secondly, synthesized FAs can be used to form lipid droplets (LDs) that have a role in the first line of defence against pathogens, by serving as anchors for immune proteins and as docking sites for phagocytic membranes. This facilitates the encounter between immune proteins and phagocytosed pathogens, while also protecting the cell from possible unwanted damage due to the cytotoxic properties of these proteins (65). Additionally, LDs can change cellular metabolism by uncoupling themselves from mitochondria upon infection, to lower mitochondrial FAO. Finally, it was recently shown that LD development, as a consequence of commitment to triacylglycerol synthesis following TLR stimulation, was needed for increased synthesis of M1-associated inflammatory mediators, such as IL-1β and PGE2 (66).

Dendritic Cells

Studies with murine BMDCs have shown that LPS stimulation promotes *de novo* FA synthesis to support expression of cytokines and costimulatory markers required for potent T cell activation (48). Moreover, DC differentiation and subsequent upregulation of MHCII requires FA synthesis (67–69) (Fig. 3A). However, in contrast to pharmacological targeting of FA synthesis as used in the aforementioned study to interrogate the role of *de novo* FA synthesis, genetic targeting of ACC1 did not appear to affect DC cytokine expression (59).

Mechanistically, FA synthesis-driven DC activation was linked to increased ER and Golgi expansion to allow for efficient translation of these proteins (48). It is interesting to note that in this study increased LD formation dependent on FA synthesis was also observed. This increase in LD formation has been reported by others as well following LPS or IL-4 stimulation, both in *in vitro* cultured BM-DCs as well as primary CD11c+ murine splenic, and lymph node DCs (70), and was associated with enhanced T cell activation (48,70). This suggests that these LDs serve not just as passive lipid storage organelles but may also be linked to key processes fundamental to DC biology, such as Ag processing and presentation. Indeed inactivation of genes which regulate

the assembly of lipid bodies abrogated cross-presentation by DCs (71). The exact mechanism by which LDs participate in Ag cross-presentation is not yet known, but previous work showed that lipid composition of LDs affected MHC-I expression in DCs (72).

Role of Exogenous Fatty Acids on Myeloid Cell Function

Apart from intracellular FA metabolism, free FAs present in the extracellular space, which can come from the diet or released by other cells, can also affect myeloid cell function (20,73,74). This can be either by serving as structural components, by being used as nutrients to fuel cellular metabolic pathways following uptake, or by acting as signalling molecules through engagement of surface or intracellular receptors. Here we will specifically focus on their effect on macrophage and DC metabolism and thereby function.

Fatty Acid Uptake

Macrophages

For many of their biological effects, extracellular FAs first need to be imported into the cell. Macrophages express various receptors and transporters that mediate this process (Fig. 2B). Studies have shown that fatty acid transport protein 1 (FATP1), an important transporter for FA uptake, plays a role in the metabolic reprogramming of macrophages during inflammation (75). FATP1 overexpression in murine BM-macrophages induced FAO and, in turn reduced an LPS-induced inflammatory response. Additionally, by inhibiting FATP1, instead a pro-inflammatory macrophage phenotype could be promoted. CD36, a scavenger receptor, is also involved in the transport of exogenous FAs into the cytosol (32), where they can fuel FAO as shown in IL-4-driven M2 differentiation (32). This was functionally important as CD36-deficient macrophages were impaired in their M2 polarisation. Likewise, human macrophages displayed a reduction in LPS-induced IL-12 and TNFα synthesis following exposure to FAs (76). Taken together, this suggests that uptake of exogenous FAs is generally linked to promotion of anti-inflammatory macrophage function.

Dendritic Cells

The role for FA uptake in regulating DC function seems to be more complex than in macrophages. On the one hand, activation of both *in vitro*-cultured BMDCs and primary CD11c+ murine DCs (isolated from spleen and lymph nodes) resulted in increased FA accumulation and LD formation (Fig. 3A), which was correlated with increased expression of scavenger receptors, such as Macrophage scavenger receptor 1 (MSR1) (70), suggesting FA uptake may support DC immunogenicity. On the other hand, there is also evidence for a tolerising effect of FA uptake by DCs, especially in the tumour microenvironment (21) (Fig. 3B). Tumour-associated DCs (TADCs) upregulate scavenger receptors including MSR1, fatty acid binding protein 4 (FABP4) and lipoprotein lipase (LPL), which promote exogenous FA uptake, (77–79), and correspondingly display high lipid content and LD accumulation. Functionally, these TADCs showed impaired Ag presentation ability and subsequent T cell stimulation. Interestingly, inhibiting FA synthesis or MSR1 activity restored their lipid content to normal levels and as well as their T cell-priming abilities (78). Recently, it was shown that FAs taken up by TADCs can serve as ligands for peroxisome proliferator-activated receptor alpha (PPARα) which is a member of ligand-activated nuclear transcription factors regulating lipid metabolism. PPARα binding promoted LD synthesis, as well as increased FAO, which resulted in reduced DC immunogenicity (80). Interestingly, correspondingly, inhibition of PPARα activation in this context, restored DCs function and enhanced anti-tumour immune responses in a therapeutic setting. Even though the anti-inflammatory properties of PPARα and PPARδ are well documented (as reviewed in (81)), in some contexts their activity is associated with inflammatory responses. For instance, a recent publication showed that deletion of PPARδ in CD11c+ cells in mice dampened palmitic acid-induced IL-12 and TNF synthesis, and upregulation of costimulatory molecules, resulting in attenuated development of atherosclerosis (82).

An explanation for why FA uptake and LD formation can, depending on the context, either support or interfere with DC immunogenicity, may come from the nature of the FAs these cells accumulate. LDs in TADCs were shown to contain high levels of oxidised PUFAs (83,84) compared to non-TADCs, which has been linked to tumour-derived molecules that prompt lipid peroxidation in TADCs (85). This was found to drive accumulation of MHC-I-peptide complexes in lysosomes and late endosomes, limiting cross-presentation and, subsequently, cytotoxic T-cell priming. This would be consistent with recent work by Ugolini *et al* (86) showing that uptake of oxidised truncated FAs impaired DC Ag cross-presentation in cancer, without affecting direct presentation.

Fatty Acids as Signalling Molecules

Saturated Fatty Acids

It is well described that in general signaling by exogenous SFAs exert pro-inflammatory effects on DCs and macrophages (87). Interestingly, several recent studies have now also revealed an important role for metabolic rewiring in this process. Activation of exogenous SFAs into Acyl-CoA, was shown to activate the NLPR3 inflammasome, driving an M1 type while UFAs prevented this (38) (Fig 2A). The authors showed that these SFAs promoted the synthesis of phosphatidylcholine, leading to loss of membrane fluidity and K+ efflux, enabling subsequent NLRP3 activation. UFAs were able to inhibit this effect by instead redirecting SFAs to triacylglycerol synthesis. Furthermore, exposure of macrophages to palmitate (PA), a SFA, was associated with impaired wound healing, a state of low-grade chronic inflammation and increased IL-1β and IL-23 synthesis (88–90). In an environment rich in FAs, DCs are also stimulated towards a pro-inflammatory phenotype. Specifically, accumulation of PA and oleic acid (OA) amplified TLR signalling and led to an increase in IL-23 expression, which in a model of psoriasis worsened disease progression (89) (Fig. 3A). This was linked to PA inhibiting hexokinase activity and perturbing TCA metabolism in TLR-activated cells, leading to an increase in mtROS and pro-inflammatory cytokines. Nevertheless, the exact mechanisms or receptors by which SFAs can modulate macrophage or DC function are still not fully elucidated. It was previously thought that SFAs could bind TLRs, thus activating a pro-inflammatory phenotype in macrophages. However, recent data shows that, while TLR4 signalling is needed for SFA-induced inflammation, SFAs do not bind directly to TLR4 (91).

Polyunsaturated Fatty Acids

One of the most well-studied bioactive FAs known to modulate myeloid cell function is Prostaglandin E_2 (PGE2), an oxidation product of AA that can bind specific receptors (Fig. 1). While PGE2 was already reported to inhibit murine BM-macrophage activation and polarisation both *in vitro* and *in vivo* (92), more recent work elucidated metabolic effects of PGE2 on M2 macrophages. The authors observed that PGE2, alongside a drop in expression of subset of M2 markers, caused a dissipation of the mitochondrial membrane potential in IL-4-stimulated M2 macrophages (93) (Fig 2B). This was due to PGE2 affecting the transcription of several genes related to maintenance of mitochondrial membrane potential in a cAMP-mediated manner. PGE2 initiated the transcription of genes which regulate the malate-aspartate shuttle, including Got1. Another PUFA, Leukotriene-B4 (LTB4) has also recently been linked to regulating

macrophage metabolism (94). Type 1 diabetic (T1D) mice have increased levels of circulating LTB4. Macrophages from these mice displayed increased FAO and CPT2 expression when compared to macrophages from control mice. This was associated with an increased pro-inflammatory signature. These effects were reduced upon blocking LTB4 signalling using a receptor antagonist.

Specialized Pro-Resolving Mediators

Recently, SPMs have received considerable attention given the growing evidence for their key role in active resolution of inflammation. There are already some strong correlations between deregulation of SPM metabolism, and certain chronic inflammatory diseases, such as Alzheimer's disease, atherosclerosis, arthritis, and type-2 diabetes (23) (Table 1). The pro-resolving properties of SPMs stem in a large part from their ability to suppress inflammatory properties of macrophages and DCs. SPMs promote the shift from M1-like to M2-like macrophages, increase phagocytic and efferocytotic activities of macrophages, and reduce IL-12 synthesis by DCs (95,96). However, up until now, little is known about whether SPMs may affect metabolism of these cells or how cellular metabolism of those cells affects SPM synthesis.

Given the known dampening effects of SPMs on pro-inflammatory DC and macrophage activation and the clear functional link between engagement of certain metabolic pathways and anti-inflammatory properties of these cells, it is tempting to speculate that a mechanism by which SPMs mediate these effects is by modulating DC and macrophages metabolism. One way SPMs might achieve this is by binding to PPARs. PPARs can be activated by many different ligands, including long-chain SFAs and UFAs, eicosanoids or other products of PUFA oxidation such as SPM 15-deoxy-δ-12,14-prostaglandin J2 and Marensin-1 (97–101). Interestingly, PPAR signalling is known to play a role in attenuating the inflammatory function of macrophages as well as DCs by regulating their metabolism (80,102). Additionally, SPMs can bind surface receptors within the family of G-protein-coupled receptors (GPRs) (103). Although it remains to be established whether signalling through GPRs that bind SPMs (e.g. GPR32, ALX/FPR2, ChemR23 (104,105)) could drive metabolic reprogramming, the fact that signalling through other GPRs, such as GPR120 and GPR40, has already been described to affect FA synthesis in adipocytes and hepatocytes, makes it tempting to speculate that SPMs may also alter macrophage and DC metabolism and thereby their function via GPR signalling (106,107).

Table 1: **SPMs and their association with protection against inflammatory diseases**

Disease	SPM	References
Alzheimer	RvD1, RvE1	(123,124)
Arthritis	17-HDHA, RvD1, RvE3	(120,125)
Atherosclerosis	Resolvins and Lipoxins	(126)
Colitis	RvE1	(104)
Diabetes	RvD1, RvE1, Lipoxins	(127)
Parkinson's	RvD1	(128)

Perspectives and Outlook

There is a growing body of evidence for a key role of FAs in the regulation of myeloid cell function and the inflammatory response by serving as nutrients, structural components of cells, signalling molecules and/or epigenetic regulators. Many studies point to the increasing importance of FA metabolism in the function of DCs, such as T-cell priming and Ag-presentation, and that of macrophages, such as microbicidal activity, phagocytosis, and efferocytosis. While pro- and anti-inflammatory macrophage and DC phenotypes are often characterised by, and dependent on, engagement of FA synthesis and FAO, respectively, it is becoming increasingly clear that , depending on the context, particularly FAO can support both pro- and anti-inflammatory properties of these cells. How exactly a single metabolic pathway can appropriate these different functions that allow it to support such diverse immunological responses is still poorly understood and it is one of the key outstanding questions that awaits to be addressed. However, one could hypothesise that the activity of metabolic pathways directly connected to FA metabolism, controlled by specific environmental cues and/or the nature of cell subset intrinsic metabolic imprinting, can play a decisive role in how products derived from FA metabolism are being redirected and used, and thereby what the final functional output is of such a pathway.

Many important insights in the effects of FAs on myeloid cell metabolism and function have been gained from *in vitro* studies. However, more in-depth *in vivo* studies will be crucial to fully capture the complexity of this interaction and will be needed to further the field. First, this pertains to the complexity of the FA composition and metabolic changes in the local micro-environment that are not easily reproduced *in vitro*. Metabolic conditions in general, and FA composition in particular - such as those found at sites of inflammation or the tumour microenvironment - are highly complex

and are likely fluctuating over time. Secondly, the diversity in macrophage phenotypes and DC subsets and associated metabolic properties found in tissues cannot be fully modelled *in vitro*. For instance, this is well illustrated by tissue-resident macrophages which have a very different metabolic profile than BM-macrophages generated *in vitro* (108). Likewise, evidence is accumulating that specific DC subsets found *in vivo*, have distinct metabolic properties and requirements for FA metabolism for their function (109). To what extent most aforementioned studies, often using *in vitro*-generated BMDCs, faithfully recapitulate the metabolic programs that are engaged and needed for primary DCs *in situ* is questionable. Moreover, what role FA metabolism plays in the biology of the recently discovered new DC subsets is yet to be addressed (110,111).

An important hurdle in studying FA metabolism of macrophages and DCs *in situ* is that they are generally present at very low frequencies. However, recent advances in single-cell technologies, such single cell RNA sequencing and high dimensional flow cytometry (112), are now making it possible to characterize in unprecedented depth phenotypes and metabolic characteristics of rare cell populations at single cell level in clinical and tissue samples (113). Moreover, imaging mass cytometry combined with imaging mass spectrometry could provide crucial additional spatial information about local metabolite and FA abundance and myeloid cell phenotype in tissues. These are some of the promising technological advances that will no doubt spur new discoveries in this exciting field of immunometabolism.

As exemplified in this review, there is a growing number of studies that show that extracellular FAs and SPMs can modulate DC and macrophage function by altering their metabolic properties. An aspect that has thus far received less attention is if and how cellular metabolism shapes the production and release of these lipid mediators. There are first indications that indeed synthesis of certain PUFAs is dependent on LD formation fuelled by *de novo* FA synthesis (66). Whether production of anti-inflammatory FAs such as SPMs is supported by different metabolic programs than pro-inflammatory FAs warrants further investigation.

Finally, there is increasing evidence that suggests that deregulated FA metabolism in macrophages and DCs can contribute to development of several inflammatory diseases. This link has been particularly well established in atherosclerosis and type 2 diabetes where dysfunctional lipid handling and metabolism by macrophages has been shown to be an important driver of pathology (i.e. plaque formation and tissue specific insulin resistance, respectively) (75,114,115). Moreover, altered lipid handling by macrophages and DCs due to hyperlipidaemia has in human studies been associated with, and in murine models causally linked to increased chances of developing autoimmune diseases, such as psoriasis, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (58,82,116). Therefore, therapies aimed at

targeting FA metabolism have proven their value in treatment of such disorders. An example are PPARγ agonists which are successfully used in the clinic to counteract hyperlipidaemia and hyperglycaemia. However, the beneficial effects of these drugs have been primarily attributed to alterations in FA metabolism of metabolic tissues rather than myeloid cells (117). Nonetheless, given the important role of FA metabolism in the regulation of macrophage and DC biology, now also efforts are undertaken to evaluate whether direct manipulation of FA metabolism of those cells could be used to shape their functional properties for therapeutic purposes. In this respect, there are several preclinical mouse studies that have given promising results. For instance, pharmacological inhibition of FAO with etomoxir in myeloid-derived suppressor cells enhanced the effectiveness of cancer therapies in mice (118). Additionally, etomoxir improved anti-tumour response following checkpoint blockade treatment *in vivo* (51), which was associated with a switch from a tolerogenic to an immunogenic TADC phenotype. Apart from targeting core FA metabolism in DCs or macrophages to shape their function for therapeutic purposes, there are also interesting developments aimed at promoting the synthesis of SPMs for treatment of inflammatory disorders. In this respect it is interesting to note that it is already known that aspirin, a common anti-inflammatory drug, triggers the synthesis of several SPMs by modifying COX-2 activity and inhibiting COX-1 (119). These data suggest the anti-inflammatory properties which have been attributed to aspirin lie, in part, in its ability to promote SPM synthesis. The therapeutic potential of SPMs has also been evaluated more directly. It was shown that RvD1 leads to cartilage protection and better disease outcome in a murine arthritis model (120). Additionally, through lipidomic analysis of human samples, 17-HDHA was shown to be associated with lower pain in arthritis patients, pointing towards a possible therapeutic application of these compounds in treating inflammatory diseases (121). Indeed, in a human skin blister model, it was shown that administration of SPMs into the inflamed site promoted resolution (122). These findings have paved the way for a phase I clinical trial (NCT04308889) that is currently ongoing, in which the effects of dietary supplementation with ω-3 FAs in a human inflammation blister model are assessed, to determine whether SPMs may promote resolution of inflammation. Additional work will be needed to establish to what extent the potential beneficial effects of these treatments are dependent on functional modulation of myeloid cells.

In conclusion, the intricate connection between FA metabolism and myeloid cell function, make it a highly interesting target for therapeutic intervention to modulate immune responses and to potentially treat diseases marked by a compromised inflammatory response, such as cancer, or by a failure to resolve inflammation, as occurs in various chronic inflammatory disorders.

Acknowledgements

This work was supported by funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 812890.

Conflict of Interest

The authors declare no financial or commercial conflict of interest

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III

Hyperinflammation by Human Macrophages Induced by SARS-CoV-2 Anti-Spike IgG Is Dependent on Glucose and Fatty Acid Metabolism

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First published: 19 December 2025, European Journal of Immunology

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European Journal of Immunology
DOI: 10.1002/eji.70087

Abstract

Severe COVID-19 is an immunological disorder characterized by excessive immune activation following infection with SARS-CoV-2, which typically occurs around the time of seroconversion. Anti-spike IgG of critically ill COVID-19 patients induces excessive inflammation by activation of Fc gamma receptors (FcγRs) on human alveolar macrophages, leading to tissue damage, pulmonary edema, and coagulopathy. While metabolic reprogramming of immune cells is critical for the induction of inflammatory responses, still little is known about the metabolic pathways that are involved in COVID-19-specific hyper-inflammation. In this study, we identified that anti-spike IgG immune complexes (ICs) induce rapid metabolic reprogramming of alveolar macrophages, which is essential for induction of inflammation. Through functional inhibition, we identified that glycolysis, fatty acid synthesis and pentose phosphate pathway (PPP) activation are critical for anti-spike IgG-induced hyper-inflammation. Remarkably, while excessive pro-inflammatory cytokine production by macrophages is critically dependent on simultaneous stimulation with viral stimuli and anti-spike IgG complexes, we show that the required metabolic reprogramming is specifically driven by anti-spike IgG complexes. These findings provide new insights into the metabolic pathways driving hyper-inflammation by macrophages in the context of severe COVID-19. Targeting of these pathways may reveal new possibilities to counteract pathological inflammatory responses in severe COVID-19 and related diseases.

Introduction

The recent pandemic has prominently shown how airborne viruses such as SARS-CoV-2 can cause a significant risk to public health [1]. Even though the fast development of highly efficient vaccines has successfully dissolved the acute situation in most countries, breakthrough infections and the occurrence of new variants of concern still especially endanger risk groups such as immunocompromised, obese, elderly or diabetic individuals [1-5]. It is thus crucial to further investigate the mechanisms underlying the development of severe COVID-19 as well as to find specific drugs to treat patients experiencing severe disease progression.

Anti-spike IgG immune complexes (ICs) play a key role in the development of severe COVID-19 progression by activation of Fc receptors (FcRs) [6-8]. Patients experiencing severe COVID-19 develop a pathological antibody response characterized by high titers of anti-spike IgG with a distinct glycosylation pattern of their Fc tail at position 297, with low amounts of fucose and high amounts of galactose [8]. Decreased fucosylation of IgG antibodies is generally observed in response to surface -exposed membrane-embedded antigens [9], which has been described to occur in a variety of anti-viral immune responses such as in dengue virus [10] or HIV infected individuals [11]. However, characteristic for severe COVID-19 is the induction of a hyper-inflammatory response by human alveolar macrophages through antibody-dependent inflammation (ADI), leading to tissue damage, pulmonary edema, and coagulopathy [6-8, 12].

In recent years, it has become increasingly clear that changes in cellular metabolism are an essential mechanism actively shaping the immune response [13]. Pro-inflammatory macrophages are characterized by an increased dependency on glycolysis and pentose phosphate pathway (PPP), combined with a decreased utilization of the TCA cycle, β-oxidation and oxidative phosphorylation (OXPHOS)[14-16]. In contrast, macrophages with a wound-healing phenotype mainly depend on an intact TCA cycle and OXPHOS as main energy source [15-17].

Crosstalk of IC-induced FcR signaling and Toll-like receptor (TLR) activation plays a crucial role in boosting effector function of myeloid cells such as pro-inflammatory cytokine induction in various tissues [18, 19]. For human dendritic cells, we previously identified that FcR-mediated cytokine secretion strongly relies on IRF5 regulated metabolic reprogramming towards a highly glycolytic phenotype [20]. Furthermore, rapid upregulation of glycolytic flux is an essential factor for fueling fatty acid synthesis and driving myeloid cell activation in a pro-inflammatory milieu [21].

Several studies have already revealed the impact of metabolic changes in SARS-CoV-2 infection and replication [22-24]. However, the role of metabolic reprogramming in pathogenic antibody-mediated inflammation in the context of severe COVID-19 is not yet well defined. In this study, we investigated metabolic reprograming of human alveolar-like macrophages upon exposure to pathological antibodies, and the role of these metabolic changes in the development of hyper-inflammation. We found that specifically anti-spike IgG, but not viral stimuli, induced pronounced changes in cellular metabolism in human alveolar-like macrophages. Moreover, we identified that inhibition of these metabolic pathways efficiently blocks anti-spike IgG induced pro-inflammatory cytokine induction, and thus may be a promising therapeutic target to counteract the effects of pathogenic antibodies in patients experiencing severe COVID-19.

Methods

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
COVA1-18 WT	P.J.M Brouwer et al. [25]	doi:10.1126/science.abc5902
Severe COVID-19 patient serum	Amsterdam UMC COVID-19 Biobank	N/A
Glut1 – Dylight 405	Heieis et al. [26]	doi:10.1038/s41467-023-41353-z
PKM – PE	Heieis et al. [26]	doi:10.1038/s41467-023-41353-z
SDHA – AF647	Heieis et al. [26]	doi:10.1038/s41467-023-41353-z
CPT1A – PE-Cy5	Heieis et al. [26]	doi:10.1038/s41467-023-41353-z
ACC1 – Pe-Cy7	Heieis et al. [26]	doi:10.1038/s41467-023-41353-z
G6PD – APC-Cy7	Heieis et al. [26]	doi:10.1038/s41467-023-41353-z
ATP5a – Dylight 488	Heieis et al. [26]	doi:10.1038/s41467-023-41353-z
CD36 – BV605	BD Biosciences	Cat# 563518
CD98 – BUV395	BD Biosciences	Cat# 744508
Human TruStain FcX™	Biolegend	Cat# 422301
HIF-1α	Invitrogen	Cat# 12-7528-80
FcIIb block	Biolegend	Cat# 398302

Chemicals, peptides, and recombinant proteins		
Human M-CSF	Miltenyi Biotec	Cat#130-096-491
Recombinant Human IL-10 Protein	R&D Systems	Cat# 217-IL-025/CF
Recombinant SARS-CoV2-Spike Wuhan Hu-1 Protein	T.Caniels et al. [27]	GenBank accession MN908947.3
Zombie NIR™ Fixable Viability Kit	Biolegend	Cat# 423106
2-DG	MedChemExpress.com	Cat# HY-13966
BPTES	MedChemExpress.com	Cat# HY-12683
C75	MedChemExpress.com	Cat# HY-12364
Etomoxir	MedChemExpress.com	Cat# HY-50202
6-AN	MedChemExpress.com	Cat# HY-W010342
Oligomycin	Sigma-Aldrich	Cat# 75351-5MG
UK-5099	MedChemExpress.com	Cat# HY-15475
polyinosinic:polycytidylic acid	Sigma-Aldrich	Cat#P1530
MitoTracker™ Deep Red FM	ThermoFisher	Cat#M224626
Critical commercial assays		
CD14 MicroBeads, human	Miltenyi Biotec	Cat#130-050-201
MitoProbe™ TMRM Assay Kit for Flow Cytometry	ThermoFisher	Cat#M20036
2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose) (2-NBDG)	ThermoFisher	Cat# N13195
4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene (BIODIPY)	ThermoFisher	Cat# D3821
ELISA MAX™ Standard Set Human IL-6	BioLegend	Cat#430501
Software and algorithms		
GraphPad Prism version 9.4.0	GraphPad Software	www.graphpad.com
SpetroFlo v.5	Cytek	www.cytekbio.com
FlowJo v. 10	BD Biosciences	www.flowjo.com

Monocyte-derived alveolar macrophage-like macrophages

Buffy coats from healthy anonymous donors were purchased from Sanquin blood supply (Amsterdam, NL). All donors provided written consent prior to blood donation. Briefly, monocytes were isolated via Lymphoprep isolation (Stemcell) followed by magnetic bead separation using the MACS cell separation system (Miltenyi). The purified monocytes were differentiated for 6 days Iscove's modified Dulbecco's medium (IMDM, Gibco) containing 5 % fetal calf serum (CAPRICORN) and Pencillin/ Steptomycin (Thermo Fisher) supplemented with 50 ng/ml human M-CSF (Miltenyi). The culture medium was refreshed after three culture days. To generate an alveolar macrophage like phenotype the medium was replaced by culture medium containing 50 ng/ml human IL-10 (R&D) 24 h prior stimulation.

Coating

Stabilized recombinant SARS-CoV-2 spike protein and monoclonal anti-spike IgG1 (COVA1-18) were generated as previously described [25, 27]. Spike protein specific ICs were generated by coating 2 µg/ml spike protein over-night on 96-well high affinity plates (Nunc). Subsequently, the plates were blocked with 10 % FCS in PBS for 1 hour at 37 °C to prevent unspecific binding. Monoclonal anti-spike IgG1 (2ug/ml) or severe COVID-19 patient serum (1% in PBS) was added to the stimulation plates and incubated for 1 h at 37 °C followed by washing the plates with PBS to remove unbound protein.

Cell stimulation and inhibitor treatment

All inhibitors were purchased in powdered form and dissolved according to the manufacturer's instructions. Human macrophages were diluted to a concentration of 277777 cells/ml and pre-treated with the indicated inhibitors (or DMSO as control) for 30 minutes at 37 °C. After pre-incubation, macrophages were added to the stimulation plate supplemented with either culture medium or with 20 µg/ml polyinosinic:polycytidylic acid (poly(I:C), Sigma-Aldrich). For FcγRIIb block, cells were pre-incubated with 20 µg/ml anti-FcγRIIb (Biolegend Cat# 398302) for 30 minutes at 4 °C. Prior to stimulation this was adjusted to a final concentration of 5 µg/ml.

Enzyme-linked immunosorbent assay (ELISA)

To determine cytokine production, supernatants of the simulated human macrophages were harvested after 6 h or 24 h. IL-6 concentration was measured using antibody pairs from Biolegend (ELISA MAX™ Standard Set Human IL-6, 430501).

Seahorse metabolic analysis

Spike protein specific ICs were generated in an XFe96 well Seahorse plate (Agilent) as described above. Prior to the experiment the Seahorse XFe96 cartridge was hydrated with Seahorse XF calibrant (Agilent). 50000 alveolar-like macrophages per

well were added to the Seahorse plate in XF assay medium on RPMI basis (Sigma) supplemented with 2 mM glutamine (ThermoFisher), 5 % FCS and with or without 20 µg/ml poly(I:C). Injection compounds were diluted in Seahorse XF medium and added to the previously prepared cartridge and loaded to the machine for calibration. Mito Stress Test Injections (final concentrations): 10 mM Glucose (Sigma Aldrich), 1.5 µM Oligomycin A (Cayman), 3 µM FCCP (Sigma Aldich), 1 µM Rotenone (Sigma Aldich) and Antimycin A (Sigma Aldich). Glycolysis stress test Injections: 10 mM Glucose (Sigma Aldrich), 1.5 µM Oligomycin A (Cayman), 10 mM 2-DG (Medchemxpress.com).

MitoTracker DR and TMRM staining

Cells were stimulated as described above. 15 min prior stimulation time end CCCP was added to the CCCP control conditions in a final concentration of 50 µM. Cells were harvested and washed with pre-warmed IMDM with-out supplements. Samples were stained with 2,5 nM MitroTracker DR and 3 nM TMRM for 15 min at 37 °C protected from light. After staining cells were once washed with IMDM + 1 % FCS and twice with cold PBS. Life cells were stained using Zombie Violet Dye (BioLengend). Cells were measured immediately after staining procedure at BD LSR Fortessa (BD Biosciences). Analysis was performed with FlowJo v.10 (BD Biosciences)

Glucose and fatty acid uptake

Alveolar-like macrophages were stimulated as mentioned above and cells were harvested after 1h of stimulation time. Cells were stained with 2-NBDG (ThermoFisher) diluted in pre-warmed 37 °C PBS in a concentration of 100 µM and BODIPY C16 (ThermoFisher) in a concentration of 20 nM. Live cells were stained using Zombie-NIR viability dye (Biolegend). Samples were acquired on a Cytek Aurora 5 L spectral flow cytometer. Spectral unmixing was performed with SpectroFlow v.5 and further analysis was executed with FlowJo v.10 (BD Biosciences).

Spectral flowcytometry

Antibodies for metabolic targets were generated as previously described [26]. 600000 cells per condition were pre-stained with viability dye (Biolegend) and Fc-blocking solution (Biolegend) for 20 min on ice. Following fixation with Fixation buffer (Biolegend) for 15 min on ice, surface marker staining was performed in FACS buffer for 1h on ice. Cells were permeabilized with 1 x Perm buffer (ThermoFisher). Followed by intracellular metabolic targets staining and HIF-1α (Invitrogen Cat# 12-7528-80) staining for 2 h at RT. Samples were acquired on a Cytek Auora 5L spectral flow cytometer. Spectral unmixing was performed using SpetroFlow v.5. Samples were further analyzed with FlowJo v.10 (BD Biosciences).

Propidium idodite (PI) assay

Human macrophages were cultured in presence of the selected metabolic inhibitors for 24 h. Following the inhibitor treatment the supernatant was replaced with serum free IMDM supplemented with 3 µM PI reagent (Sigma-Aldrich, P4170). After 30 minutes incubation under culture conditions, extracellular DNA content was measured by analyzing fluorescence intensities at $\lambda_{ex}/\lambda_{em}$ = 530/620 nm.

Lactate assay

Supernatant of stimulated human macrophages was deproteinized by addition of a final concentration of 3 % w/v metaphosphoric acid (MPA). Followed by incubation with 27 mM NAD (Roche) solution in 0.5M glycine-0.4M hydrazine buffer (pH 9.0). Lactate production was measured by lactate dehydrogenase (LDH, Roche) treatment. After 30 min incubation time with LDH at RT lactate content was measured by NADH fluorescence at $\lambda_{ex}/\lambda_{em}$ 340/450.

Quantification and Statistical Analysis

Statistical significance of the data was performed using GraphPad Prism 9.4.0 (GraphPad). The statistical analysis applied for each figure is stated in the corresponding Figure legend.

Results

Individual Stimulation With Anti-Spike IgG Rapidly Boosts Glycolysis and Oxidative Phosphorylation

To assess if stimulation with anti-spike IgG ICs changes core metabolic pathways in the context of severe COVID-19, we examined changes in glycolysis and oxidative phosphorylation (OXPHOS) of monocyte-derived alveolar-like macrophages after short time exposure. Previous data has shown that *in vitro* stimulation of monocyte-derived alveolar-like human macrophages with anti-spike IgG ICs serves as a promising experimental model to study antibody-induced inflammation of alveolar macrophages in severe COVID-19 [8]. We tested the effect of the recombinant anti-spike IgG COVA1-18, which we generated previously from B cells isolated from a patient with COVID-19 [28]. In addition, we generated anti-spike-IgG ICs by incubating SARS-CoV-2 spike–coated wells with diluted serum from patients with severe COVID-19 treated in the first pandemic wave at the intensive care unit (ICU) at the Amsterdam

University Medical Centers (UMC)[8]. In line with previous studies[8, 29], stimulation of alveolar-like macrophages with only viral stimulus poly(I:C) (reflecting the first phase of infection), did not elevate pro-inflammatory cytokine production. In contrast, upon co-stimulation of cells with a viral stimulus and anti-spike IgG ICs, mimicking the post-seroconversion phase of severe COVID-19 infection, high amounts of pro-inflammatory cytokine IL-6 were secreted (Fig. 1A,B).

Extracellular metabolic flux analysis of human macrophages treated with anti-spike IgG ICs showed an increased but not significant change in extracellular acidification rate (ECAR), indicative of a slightly enhanced glycolysis (Fig. 1C, Fig. S1 A).

In pro-inflammatory macrophages elevated glycolysis is associated with a decreased OXPHOS associated metabolism [15, 30]. Interestingly, the extracellular metabolic flux data revealed a rapid increase in oxygen consumption rate (OCR) representative of elevated OXPHOS utilization after anti-spike IgG IC stimulation (Fig. 1D,E, Fig. S1 B). Thus, our data indicates, that anti-spike IgG IC stimulation induces upregulation of both core metabolic pathways that boost ATP synthesis. Remarkably, in contrast to pro-inflammatory cytokine induction which requires a strong interplay of FcR signaling and TLR activating signal, anti-spike IgG ICs alone were sufficient to induce changes in glycolysis and basal respiration (Fig. 1C-E, Fig. S1 A,B) and poly(I:C) had no synergistic effect.

Macrophages resembling a pro-inflammatory phenotype are classically characterized by an increased dependence on glycolysis, fatty acid synthesis and the PPP [15, 17]. In this pro-inflammatory condition, pyruvate is reduced into lactate instead of being used to fuel the TCA cycle [15, 31]. Thus, lactate secretion can serve as a simple measure to determine alterations of the glycolytic pathway and reduced pyruvate metabolism in activated macrophages [32]. To further investigate the kinetics of anti-spike IgG ICs induced metabolic reprogramming we measured lactate secretion in the medium of anti-spike IgG stimulated macrophages after 1 h, 6 h and 24 h activation time. After 1 h of stimulation time, no differences between the stimulation conditions could be observed (Fig. 1F). In the next time frame, after 6 h of stimulation time, a small increase in lactate production in the samples stimulated with recombinant anti-spike IgG ICs or ICs derived from ICU patient serum could be detected (Fig. 1G). 24 h after activation the secretion of lactate in the anti-spike IgG ICs-treated cells was further elevated significantly (Fig. 1H). In line with the extracellular metabolic flux data these changes were mainly induced by anti-spike IgG stimulation, as the addition of a co-stimulatory TLR-activating agent did not further affect lactate secretion.

Since antibody stimulation significantly increased glycolytic activity and OXPHOS of alveolar-like macrophages, we assessed whether glucose and fatty acid uptake would

be increased upon stimulation as well. We visualized glucose and fatty acid uptake by tracking uptake of fluorescent glucose 2-NBDG and fluorescent BODIPY, respectively. There was a trend towards increased 2-NBDG uptake after 1 h of stimulation in the conditions stimulated with anti-spike IgG immune complexes (Fig. S1C), however, these changes did not reach statistical significance. Furthermore, no changes in fatty acid uptake were observed between the different stimulation conditions (Fig. S1D).

An important driver of glycolytic metabolism and release of pro-inflammatory cytokines is hypoxia induced factor (HIF)-1α [33, 34]. Therefore, we investigated whether anti-spike IgG IC stimulation changed HIF-1α expression in human alveolar-like macrophages. After 1h of stimulation time, no differences in HIF-1α protein expression were observed between the different conditions (Fig. S1E). In contrast, after 24 h of stimulation time there was a trend towards elevated HIF-1α expression in anti-spike IgG IC-treated conditions (Fig. S1F). Interestingly, as previously observed in the metabolic flux data, individual anti-spike IgG IC treatment was sufficient to induce this effect, and no further increase was observed in cells additionally treated with co-stimulatory signal poly(I:C).

Taken together these data indicate that anti-spike IgG IC stimulation induces upregulation of both metabolic core pathways boosting ATP synthesis, i.e. glycolysis and OXPHOS.

Anti-Spike IgG Induces Rapid Upregulation of Specific Metabolic Enzymes

To further characterize changes induced in relevant metabolic pathways upon anti-spike IgG IC stimulation, we analyzed the expression of a selection of core metabolic enzymes via Metflow analysis [26]. Briefly, human alveolar-like macrophages were stimulated with anti-spike IgG ICs as shown above (Fig. 1A,B). Following 6 h stimulation, metabolic targets were stained and measured using spectral flow cytometry (Fig. 2A). In line with the extracellular metabolic flux (Fig. 1) and 2-NBDG data (Fig S1D), stimulation of human macrophages with anti-spike IgG ICs significantly increased the expression of glucose transporter GLUT1 (Fig. 2B). Interestingly, even though stimulation with anti-spike IgG increased GLUT1 expression and elevated the glycolytic flux, PKM expression was not significantly elevated (Fig. 2C), potentially indicating an increased flux of glucose into upstream offshoots of glycolysis such as the PPP. Indeed, stimulation with anti-spike IgG ICs induced elevated expression of PPP associated enzyme G6PD (Fig. 2D). Furthermore, consistent with increased OXPHOS, anti-spike IgG ICs promoted expression of SDHA (Fig. 2E), an enzyme in

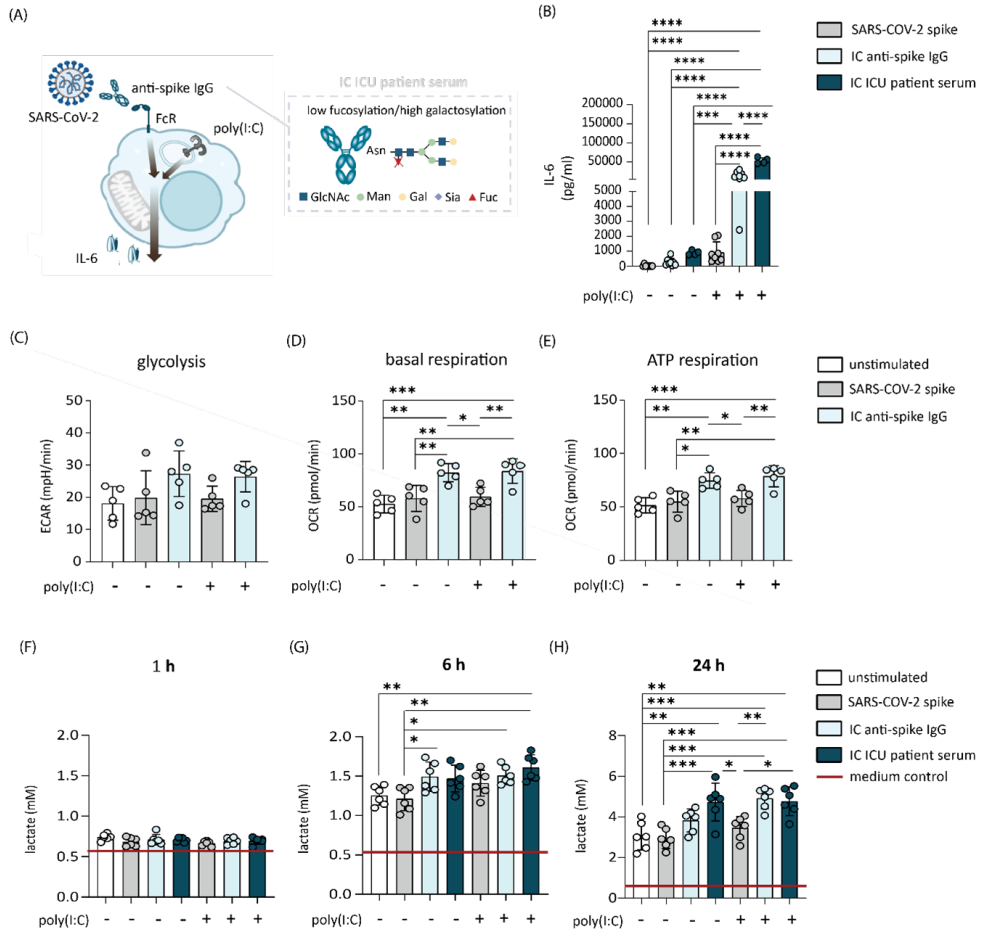

Fig. 1. Stimulation with anti-SARS-CoV-2 spike IgG rapidly boosts glycolysis and OXPHOS in human alveolar-like macrophages. (A) anti-spike IgG ICs induce pro-inflammatory cytokine induction of alveolar macrophages in combination with TLR activating signal (poly(I:C). Antibodies derived from COVID-19 ICU patients are characterized by an aberrant glycosylation pattern (B) IL-6 production by human alveolar-like macrophages after 24 h stimulation with ICs from recombinant anti-spike IgG or serum from unvaccinated patients diagnosed with severe COVID-19 (mean + SD). (C) glycolysis, (D) basal respiration and (E) ATP production of human alveolar-like macrophages. Stimulation for 1 h followed by seahorse assay (n = 5, mean + SD). (F-H) lactate production of human alveolar-like macrophages stimulated as indicated for 1 h, 6 h and 24 h (n = 6, mean + SD). IMDM medium with-out cells was added as control (red line). Significant differences were calculated with One-way ANOVA. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001

the TCA cycle and complex II enzyme of the respiratory chain. Finally, a trend towards increased expression of amino acid transporter CD98 (Fig. 2F) was visible after anti-spike IgG IC stimulation, which became more prominent after 24 h stimulation time (Fig. S2). No significant changes in the expression of metabolic enzymes CPT1A, ACC1, CD36 and ATP5a could be detected (Fig. 2G-J). In line with the extracellular

metabolic flux data and lactate assay the changes in expression of G6PD, CD98 and SDHA were mainly induced by anti-spike IgG IC stimulation and remained elevated for at least 24 h after stimulation (Fig. S2).

Since Fc-mediated antibody effector functions correlate with expression and type of FcR, we measured expression levels of the different FcγR on human alveolar-like macrophages. As shown in Figure S4A FcγRI (CD64) and FcγIII (CD64) were expressed at similar levels, while FcγRIIb/c (CD32 b/c) showed a slightly lower and FcγRIIa/b (CD32a/b) increased expression on human alveolar-like macrophages (Fig. S4A). In contrast to other receptors of the FcR family expressed on human alveolar-like macrophages, FcγRIIb (CD32b) cross-linking inhibits activating signals by phosphorylation of its immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain [35]. We therefore determined whether block of FcγRIIb would further amplify the metabolic changes induced by anti-spike IgG IC. Notably, FcγRIIb inhibition indeed further increased GLUT-1 expression of alveolar-like macrophages, especially when additionally treated with costimulatory signal poly(I:C) (Fig. S4B).

Anti-Spike IgG Triggers Rapid Changes in Macrophage Mitochondria

To further understand effect of pathogenic anti-spike IgG on mitochondrial function of human alveolar-like macrophages in severe COVID-19, we analyzed mitochondrial mass and membrane potential in response to anti-spike IgG IC stimulation, using Mito Tracker Deep Red [36, 37] and Tetramethylrhodamine methyl ester (TMRM) probes [38], respectively. After 1 h of stimulation, human alveolar-like macrophages stimulated with recombinant anti-spike IgG and ICs derived from severe COVID-19 ICU patient serum showed a significantly increased Mito Tracker Deep Red (DR) signal (Fig. 3A), as well as an elevated mitochondrial membrane potential (Fig. 3B). After 6 h of stimulation time the changes in Mito Tracker DR accumulation remained stable while the TMRM signal was decreased (Fig. 3A,B).

Taken together, these data suggest that anti-spike IgG stimulation induces rapid changes in mitochondrial membrane potential and mass, suggesting first signs of mitochondrial damage, such as mitochondrial membrane depolarization.

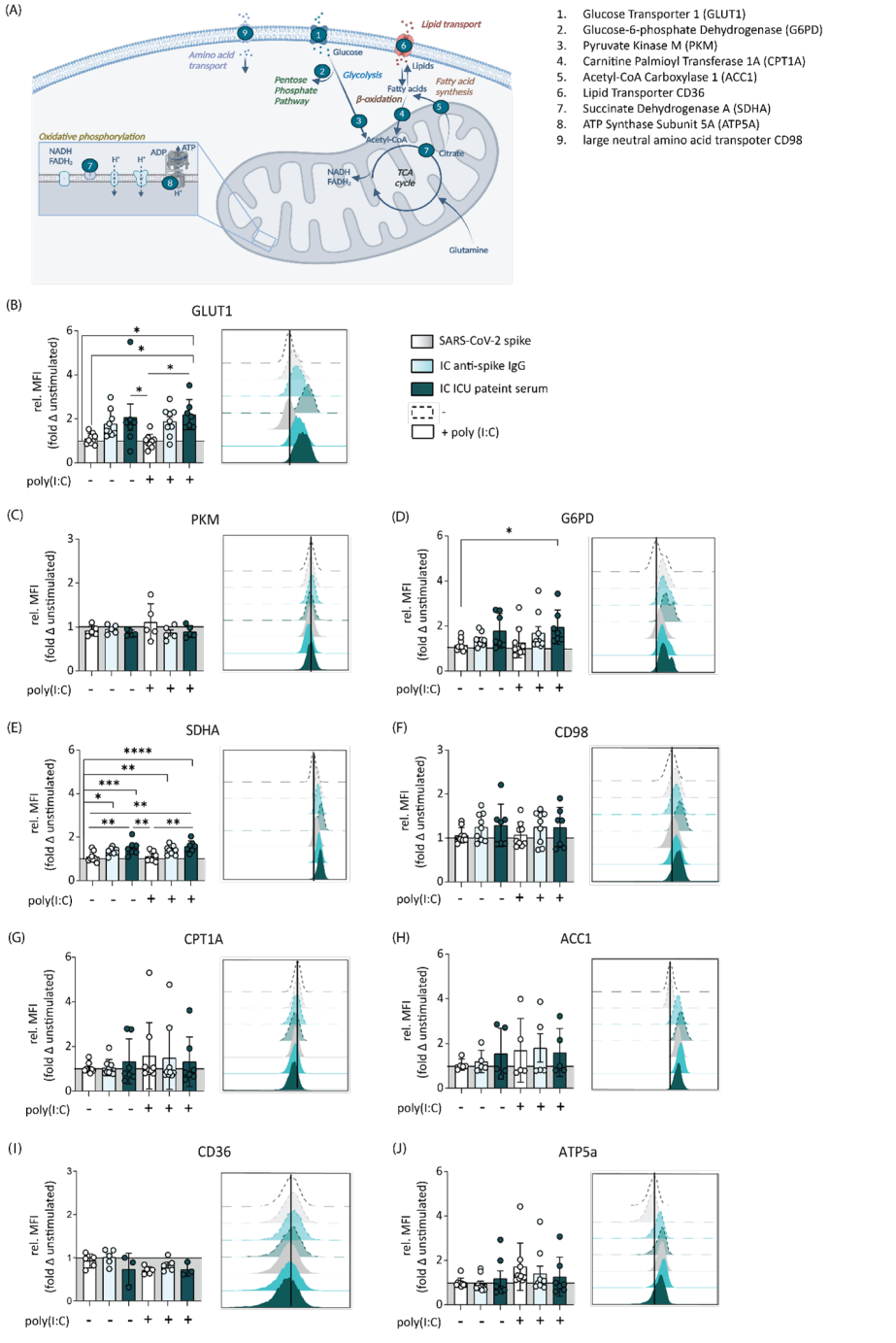

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Fig. 2. Anti-spike IgG induces upregulation of specific metabolic enzymes. (A) Schematic representation of Metflow analysis targets. Created in BioRender. Geyer, C. (2024) BioRender.com/h10r049 (B-J) Changes in relative MFI of metabolic enzymes of human macrophages after 6 h stimulation with ICs of recombinant IgG or ICU patient serum with or without viral stimulus poly(I:C). Representative histograms (unstimulated indicated as black line) and data points from individual donors (n = 5-8, mean + SD). Gating strategy is show in Figure S3. Significant differences were calculated with One-way ANOVA. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001

Anti-Spike IgG-Induced Hyper-Inflammation Is Functionally Dependent on Glycolysis, PPP, and Fatty Acid Synthesis

To assess if the identified changes in metabolic pathway activity are functionally required for anti-spike IgG IC-induced hyper-inflammation, we tested the effect of metabolic pathway inhibitors on the pro-inflammatory cytokine induction of human alveolar-like macrophages stimulated with anti-spike IgG ICs with or without poly(I:C). In line with the extracellular metabolic flux and metabolic enzyme expression data (Fig. 1,2), IL-6 production induced by co-stimulation of human alveolar-like macrophages was blocked by inhibition of glycolysis (by 2-DG) and PPP (by 6-AN) (Fig.4 A,B).

Interestingly, even though most donors did not show changes in ACC1 expression (Fig. 2H) blocking fatty acid synthesis with C75 efficiently reduced IL-6 production to the level of unstimulated macrophages, suggesting an important functional role from fatty acid synthesis in anti-spike IgG IC-induced inflammation. On other hand, neither inhibition of the mitochondrial pyruvate carrier by UK-5099 nor β-oxidation using Etomoxir affected the antibody induced pro-inflammatory cytokine production (Fig. 4 A,B). Despite the strong upregulation of basal respiration seen in the extracellular flux assay (Fig. 1D,E) blocking OXPHOS with oligomycin, only induced a small but still significant decrease in IL-6 induction (Fig. 4). This may be explained by the kinetics of the anti-spike IgG IC-induced metabolic reprogramming, which was dependent on OXPHOS mainly early after stimulation (Fig. 1 D,E). Of note, none of the inhibitors induced cell death at the indicated concentrations (Fig. S5).

Finally, we set out to validate these findings with anti-spike IgG from severely ill patients, by stimulating cells with serum obtained from first-wave COVID-19 ICU patients. Importantly, ICU patient serum displayed a similar dependency on glycolysis, fatty acid synthesis and PPP to promote elevated IL-6 induction (Fig. 4B). Moreover, when we compared the percentage of inhibition, the metabolic inhibitors suppressed cytokine induction by recombinant and patient-derived anti-spike IgG in a strikingly similar manner (Fig. 4C). This suggests that the higher cytokine induction by patient-derived IgG (Fig. 1B) results from quantitative, but not qualitative differences in metabolic reprogramming.

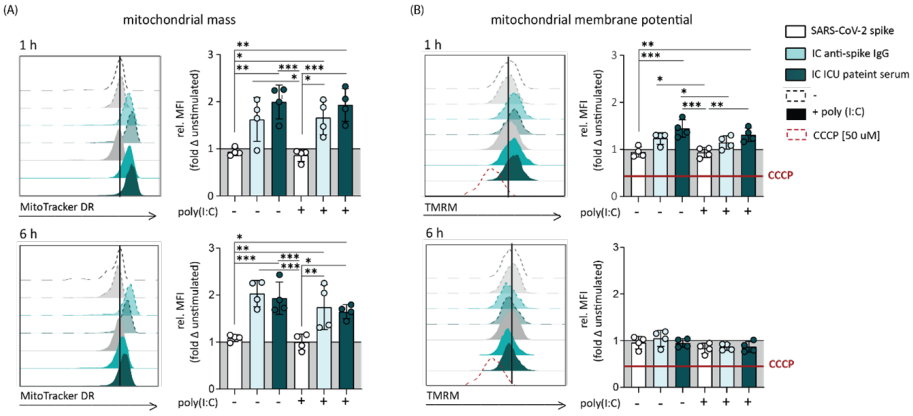

Fig. 3: Anti-spike IgG IC rapidly induces rapid changes in human macrophage mitochondria. Representative data of independent experiments (left, unstimulated indicated as black line) MitoTracker DR (A), TMRM (B). Data points from individual donors (right, n = 4, mean + SD). Human alveolar-like macrophages were stimulated as indicated and immediately stained with MitroTracker DR (3 nM) and TMRM (3 nM). 15 min prior stimulation end 50 uM CCCP was added to induce mitochondrial depolarization in TMRM samples as negative control. Gating strategy is show in Figure S3. Significant differences were calculated with One-way ANOVA. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001

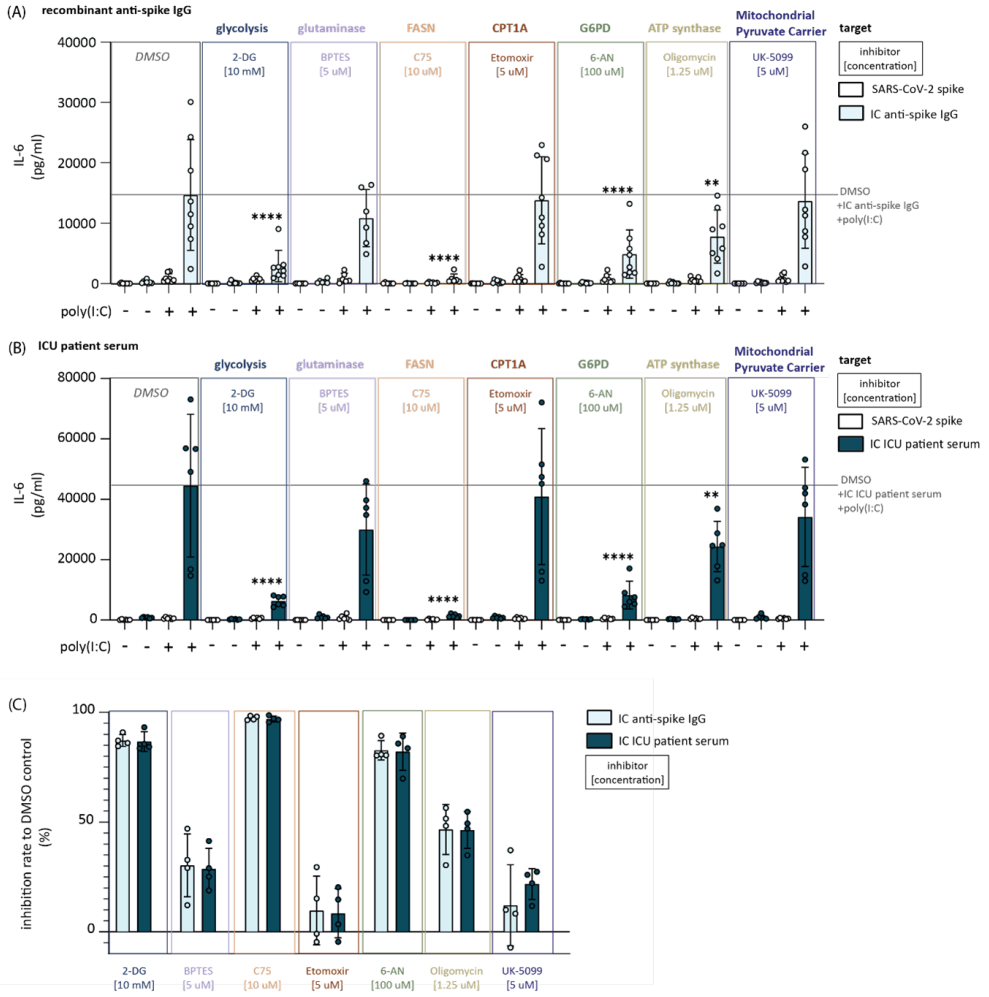

Fig. 4: Anti-spike IgG-induced pro-inflammatory cytokine induction is functionally depended on glycolysis, fatty acid synthesis and PPP (A) IL-6 secretion of human alveolar-like macrophages stimulated with anti-spike IgG IC and poly(i:C) after treatment with the indicated metabolic inhibitors. Each data point repesents one individual donor (n = 4-8, mean + SD). stimulation with immune complexes derived from recombinant anti-spike IgG (A) or ICU patient serum (B) inhibtion rate compared to DMSO control (C). Significant differences were calculated with one-way ANOVA. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Statistics indicate changes between inibitor treated stimulation condition compared to idenical stimulation condition in DMSO control sample.

Discussion

Severe COVID-19 is a immunological disorder, characterized by hyper-inflammation of macrophages and monocytes that cause tissue destruction [8, 39] edema, and coagulopathy [12, 40]. While metabolic reprogramming of immune cells is critical for immune activation [13], the metabolic pathways that enable hyper-inflammation in the context of severe COVID-19 are still largely unknown. In this study, we set out to investigate the role of antibody-induced metabolic reprogramming in severe disease progression to identify potential therapeutic targets for treatment of severe COVID-19.

In general, metabolic reprogramming towards high glycolysis provides important building blocks for transcription and promotes generation of reactive oxygen species which are beneficial for the host immune system leading to a rapid and effective pathogen clearance [15, 41]. In contrast, in severe COVID-19 patients alteration of glucose metabolism, such as increased glycolysis in BALF macrophages [42, 43] are associated with pathogenic over-activation of the immune response and correlated with a poor disease prognosis [43-46].

Here we show that anti-spike IgG ICs, as present upon seroconversion, may contribute to severe disease outcome by rapidly triggering metabolic reprograming of alveolar macrophages towards a highly glycolytic phenotype. Blocking this glycolytic reprograming by 2-DG treatment, counteracted the hyper-inflammatory immune reaction. Thus, 2-DG may be an efficient target to control the pathogenic over-activation in severe COVID-19. In phase II and III clinical trials preformed in India in patients with moderate-to-severe COVID-19 progression, treatment with 2-DG showed alleviation of symptoms [47, 48]. These studies thus provide first proof of principle that reducing glycolytic activity in severe COVID-19 patients could have therapeutic potential. However, previous studies done in cancer patients have shown that 2-DG can induce potential side effects including hypoglycemia, nausea and fatigue. However, these side effects were non-life-threatening if the doses were limited to a maximum of 63 mg/kg [49, 50]. Nevertheless, it is yet unclear whether 2-DG treatment would interfere with the standard treatment protocol of severe COVID-19 including antiviral drugs and systematic corticosteroids [51]. Therefore, while 2-DG is a potentially promising treatment for severe COVID-19, further evaluation of the optimal treatment doses, administration time and target patient group needs to be performed in additional randomized clinical trials.

Our experiments also show that anti-spike IgG IC strongly upregulates PPP-associated enzyme G6PD in human macrophages. In addition, our data demonstrate that antibody-induced cytokine secretion crucially depends on PPP activity, as inhibition

of this pathway via 6-AN treatment efficiently blocks anti-spike IgG IC-induced IL-6. Polidatin, a molecule naturally occurring in *Polygonum cuspidatum*, directly inhibits G6PD [52]. Phase II clinical studies testing supportive treatment with Polydatin in the context of inflammatory bowel disease and endometriosis-related pain indicated no toxic effects in humans for a treatment dose of 20-40 mg [53, 54]. Given the importance of the PPP for the generation of biosynthesis precursor molecules and NADPH, this pathway may be an additional promising therapeutic target to counterbalance the excessive cytokine induction in severe COVID-19 patients [23]. Even though the PPP inhibitor Polidatin is a promising drug candidate due to its low toxicity in humans, further *in vitro* studies followed by randomized clinical trials would be required to evaluate efficiency and specificity in severe COVID-19 disease treatment.

It has been established that inflammatory macrophages tend to rely on *de novo* fatty acid synthesis to maintain cellular functions, instead of increasing fatty acid uptake [55-60]. Indeed, several studies have identified fatty acid synthesis as an essential link of metabolic reprogramming and cytokine secretion in myeloid cells [21, 57, 61, 62]. By fostering ER and Golgi expansion, fatty acid synthesis supports cytokine induction of myeloid cells in a pro-inflammatory milieu [21, 63]. Inhibition via C75 furthermore blocks IL-1β secretion in murine macrophages in the context of sepsis [62]. This is further supported by recent studies where both ACC and fatty acid synthase (FASN) have been implicated in the metabolic rewiring of macrophages and in the subsequent synthesis of pro-inflammatory cytokines, such as IL-6 and IL-1β [64, 65]. In line with this, our data reveal no role for the process of lipid uptake in the inflammatory response (as evidenced by the lack of differences in BODIPY C16 staining), and instead show that inhibition of FASN via C75 effectively blocks anti-spike IgG IC-induced IL-6 cytokine secretion, suggesting an important role for *de novo* fatty acid synthesis in antibody-induced pro-inflammatory cytokine induction. Combined with the increased glycolysis and PPP enhancement, these data suggest a similar molecular mechanism underlying cytokine secretion as described for dendritic cells [21], thereby linking increased glycolytic flux and fatty acid synthesis as essential key pathways for pro-inflammatory cytokine production. Enhanced fatty acid synthesis was shown to induce NLRP3 inflammasome activation and pro-inflammatory cytokine synthesis [65]. Additionally, it has been suggested that fatty acid synthesis may also trigger increased membrane synthesis, supporting the ER and Golgi expansion required for the FcR-triggered increase of pro-inflammatory cytokine transcription [8, 20, 21]. The increased *de novo* fatty acid synthesis is fueled by NADPH produced by the elevated glycolytic flux enhancing PPP activation [21]. Interestingly, in our dataset, ACC1 expression showed a mixed pattern of increased expression in some donors upon anti-spike IgG IC stimulation, while in other donors the expression levels remained stable. ACC1 expression is reported to mainly reflect the cell's ability for fatty acid synthesis [66]. Since obese severely ill COVID-19 patients show aberrant levels of

blood lipids [67] and altered ACC1 levels have been associated with the development of obesity [68], the role of ACC1 expression in the development of severe COVID-19 disease progression may be of interest for further studies.

High levels of the pro-inflammatory cytokine IL-6 is a key factor in the progression of severe COVID-19, and is associated with increased mortality [69, 70]. Even though antibody-induced pro-inflammatory cytokine induction requires a strong cross-talk between FcR and TLR signaling [8], our data indicate that anti-spike IgG IC-mediated metabolic reprogramming is mainly controlled by FcR signaling. This is in line with a recent study that demonstrated that FcR-mediated increase in glycolysis boosts inflammation in lupus nephritis [71]. This FcR-mediated glycolytic switch, independent of a costimulatory signal, may therefore be relevant for inflammatory responses beyond the context of COVID-19, including rheumatoid arthritis (RA) [72], Sjögren's Syndrome [73] or systemic lupus erythematosus (SLE) [71].

IgG affinity towards FcRs and affiliated induction of effector functions are strongly dependent on changes in the antibody glycosylation pattern [74, 75]. Afucosylated antibodies from patients amplify inflammation by increased binding to FcγRIII [76], while recombinant anti-spike IgG (with conventional Fc glycosylation) predominantly activates FcγRIIa signaling [8]. Interestingly, in contrast to cytokine secretion, our data indicate that metabolic reprogramming induced by anti-spike IgG is not influenced by alterations in antibody glycosylation pattern (Fig. 4). This may relate to overlapping signaling pathways downstream of FcRs. Both FcγRIIa and FcγRIII activate Syk and PI3K signaling upon antibody crosslinking [77, 78], which has been identified as key molecules that mediate anti-spike IgG IC-induced hyper-inflammatory responses by alveolar macrophages [8, 29]. Data by Jing et al. [71] indicate that FcR-mediated glycolytic activation is functionally dependent on Syk and PI3K activation, suggesting a potential molecular link between FcγR activation and metabolic reprogramming via Syk and PI3K activation. Interestingly, as opposed to the activating FcγRs that promote metabolic rewiring, we find the inhibitory receptor FcγRIIb does the opposite, suggesting that the net degree and possible nature of FγcR-driven metabolic alterations are shaped by balance between engagement of activating and inhibitory FcγRs.

Mitochondrial dysfunction plays a role in increased stress and inflammation contributing to the worsening of symptom severity in COVID-19 patients [79-82]. In addition, risk groups such as elderly patients are characterized by generally reduced mitochondrial health [83]. Our data show that rapidly in the kinetics of the anti-spike IgG-induced inflammatory response, alveolar-like macrophage mitochondria show first signs of alternated membrane potential and function. After 1 h of stimulation, the TMRM signal and respiration in anti-spike IgG IC stimulated cells was increased, indicating that

the initial response is characterized by increased mitochondrial activity. However, this appeared not to be functionally relevant for heightened cytokine production as oligomycin treatment has minimal effects on this. Interestingly, after 6 h of stimulation the first signs of a loss of TMRM staining were visible, indicating the presence of depolarized mitochondria, and an indication of a loss of mitochondrial activity and potentially damage [84]. Of note, the Mito Tracker staining was increased at time points 1 h and 6 h upon stimulation with anti-spike IgG ICs. Given that mitochondrial biosynthesis in macrophages is reported to be occurring in a time frame of several hours [85], these changes are probably not induced by increased mitochondrial biosynthesis but may rather reflect changes in mitochondrial morphology. Since mitochondrial damage has been associated with symptoms persisting even after the virus has been cleared [86-88], further investigation of the role of anti-spike IgG ICs in the development of mitochondrial dysfunction may be of interest for further studies even beyond macrophages. For instance, platelets of severe COVID-19 patients have also been reported to display signs of damaged mitochondria and bioenergetics failure [89]. Given that platelets express FcγRIIa and that pathogenic antibodies of severe COVID-19 patients can directly activate platelets [90] to contribute to thrombosis in severe COVID-19 [12], it is conceivable that metabolic reprogramming by IgG IC during severe COVID-19 applies to a wider range of cells.

A limitation of our study is that the *in vitro* conditions of our model are not able to completely mimic the metabolic niche of alveolar macrophages in severe COVID-19 patients. Furthermore, since it was not possible to analyze bronchoalveolar lavage (BAL) macrophages derived from severe COVID-19 patients, we used a model closely mimicking alveolar macrophages based on transcriptomic signature [91] as well as on alveolar macrophage surface marker expression (data not shown). However, the use of healthy donors for the generation of alveolar-like macrophages is not taking into account potential pre-existing metabolic deficiencies in alveolar macrophages of severe COVID-19 risk groups individuals.

Taken together, we here show that the hyper-inflammatory response induced in the context of severe COVID-19 critically depends on anti-spike IgG IC-induced metabolic reprogramming of macrophages towards a highly glycolytic phenotype. This mechanism is mainly dependent on FcγR signaling and characterized by rapid kinetics. Moreover, we show that IgG IC-mediated inflammation can be counteracted by inhibiting glycolysis, fatty acid synthesis, and the pentose phosphate pathway. Therefore, these metabolic pathways may be promising targets to counteract the effects of pathological antibodies in patients suffering from severe COVID-19.

Acknowledgements

We acknowledge the Microscopy and Cytometry Core Facility at the Amsterdam UMC – Location VUmc for providing assistance in our cytometry work. We are grateful for the generous support from the Amsterdam UMC COVID-19 Biobank.

Statement of Ethics

This study protocol was reviewed and approved by Commissie Toetsing Biobanken (CTB, Amsterdam UMC, The Netherlands), approval number 2023.0978. All participants provided written informed consent to Amsterdam UMC COVID-19 Biobank. Buffy coats from healthy donors were purchased from Sanquin blood supply (Amsterdam, The Netherlands). Donors provided written informed consent prior blood donation to Sanquin.

Conflict of Interest

The authors have no conflicts of interest to declare.

Funding Sources

JdD was supported by ZonMW (10430 01 201 0008), Amsterdam Infection and Immunity COVID-19 grant (24184), AMC Fellowship (2015), European Union's Horizon 2020 research and innovation programme (847551), AGEM matching grant (2020) and Innovative Medicines Initiative 2 Joint Undertaking grant (831434). BE was supported by funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie Grant agreement no. 812890. The funders had no role in the design, data collection, data analysis, and reporting of this study.

Author Contributions

Conceptualization RHH, BE, and JdD; Methodology CEG, LA, GH, RHH, BE and JdD; Validation CEG; Formal analysis CEG; Investigation CEG, LA, LM and HJC; Resources LA, GH, JV, SWdT, TGC, TPLB, MJvG, MdW, Amsterdam UMC COVID-19 Biobank, JVdB Writing – Original Draft CEG; Writing – Review and Editing LA, LM, GAH, SWdT, HJC, TGC, TPLB, MJvG, MdW, JVdB, RHH, BE and JdD. Visualization CEG; Supervision RHH, BE and JdD; Funding Acquisition BE and JdD.

Data Availability Statement

All data associated with this study are presented in the paper or supplementary materials. The recombinant anti-Spike IgG1 antibody COVA1-18 is available upon request to the corresponding authors through a materials transfer agreement. Further information and requests for resources, and reagents should be directed to and will be fulfilled by the corresponding author, Jeroen den Dunnen (j.dendunnen@amsterdamumc.nl)

Supplementary Materials

Table S1. Members and affiliation of the Amsterdam UMC COVID-19 Biobank

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M. Beudel	Department of Neurology
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M.R. Smit	Department of Intensive Care
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P. Thoral	Department of Intensive Care
A.M. Tsonas	Department of Intensive Care
P.R. Tuinman	Department of Intensive Care
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W.J. Wiersinga	Department of Infectious Diseases
D. Wouters	Department of Clinical Chemistry
A.H. Zwinderman	Department of Clinical Epidemiology, Biostatistics and Bioinformatics

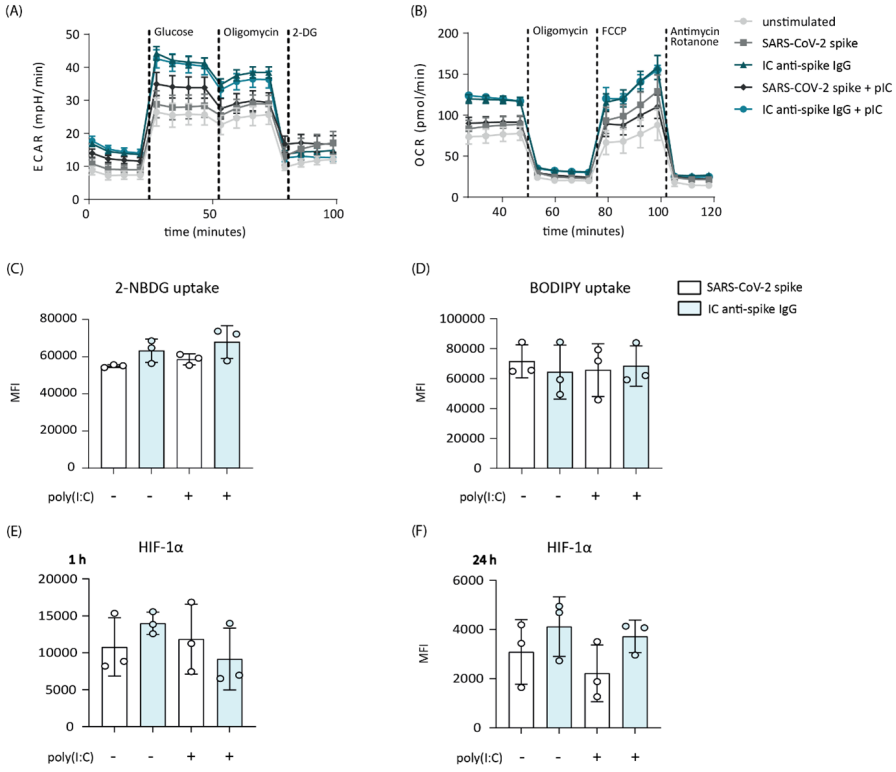

Figure S1: Metabolic changes of alveolar-like macrophages induced by anti-spike IgG stimulation (A) Real-time changes in ECAR (A) and OCR (B) of human alveolar-like macrophages stimulated with ICs from recombinant anti-spike IgG as indicated for a stimulation time of 1 h (Representative donor, mean + SEM). (C) glucose uptake quantified by glucose analogue 2-NBDG uptake (C), and fatty acid uptake, quantified by BIODIPY uptake (D) (n=3, mean + SD) of human alveolar-like macrophages after 1 h of stimulation time. (E) changes of HIF-1α expression in alveolar-like macrophages upon stimulation after 1 h and 24 h (n=3, mean + SD).

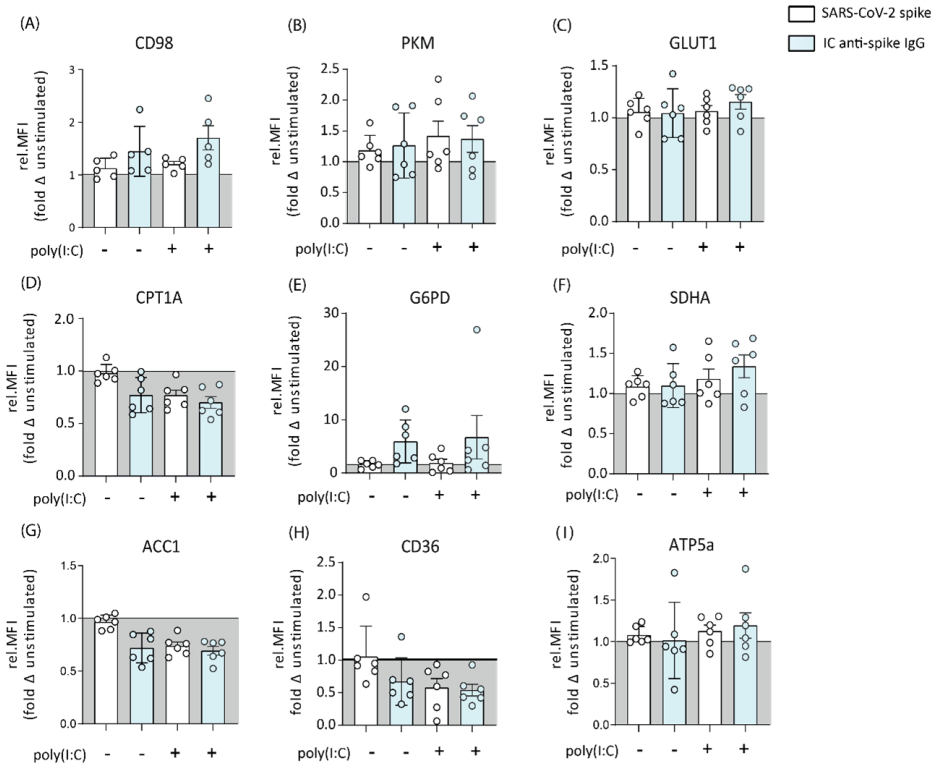

Figure S2. Anti-spike IgG induced change in metabolic enzymes after 24 h stimulation. (A-I) Changes in relative MFI of metabolic enzymes of human macrophages after 24 h stimulation with ICs of recombinant with or without viral stimulus poly(I:C). Data points from individual donors (n = 5, mean + SD).

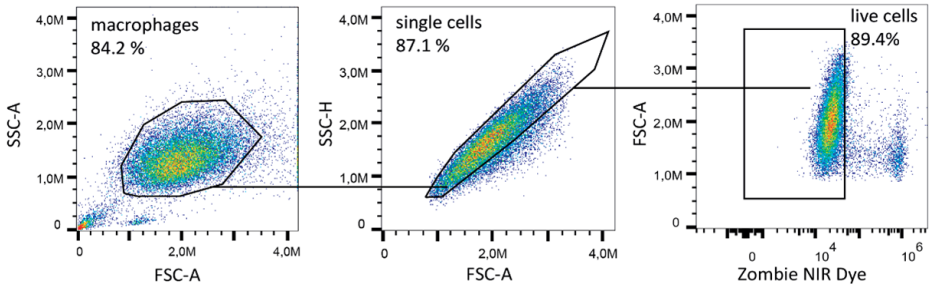

Figure S3: Gating strategy FACS. We selected human-like alveolar macrophages by applying a forward- and side-scatter gate. From this gate singles cells were selected followed by exclusion of dead cells by a live cell gate (Zombie NIR negative population).

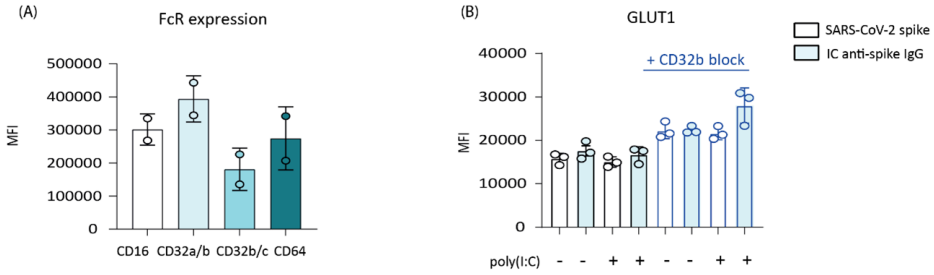

Figure S4. FcγR expression levels on human alveolar-like macrophages and effect of FcγRIIb blockage on anti-spike IgG IC induced changes in GLUT1 expression. (A) Human alveolar-like macrophages were harvested and stained with CD16-PE, CD32a/b-PerCP, CD32b/c-APC-A700, CD64-ECD to determine FcR expression levels. Representative data from n = 2 individual experiments (mean + SD). (B) Glut1 expression of alveolar-like macrophages treated with FcRIIb blocking antibody (blue) compared to untreated cells (black) . Samples were stimulated for 24 h stimulation with ICs of recombinant anti-spike IgG with or without viral stimulus poly(I:C) (n = 3, mean + SD).

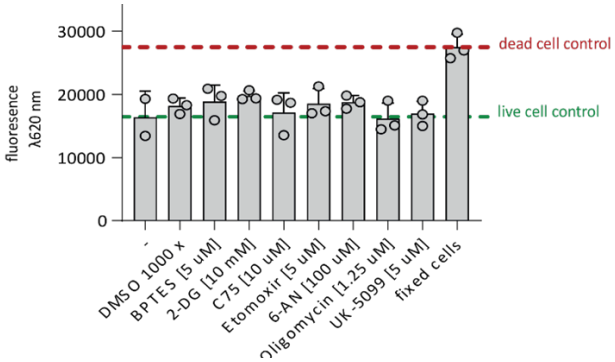

Figure S5. Effect of metabolic inhibitors on cell viability of human alveolar-like macrophages. Human macrophages were incubated with the indicated inhibitor concentration under culture conditions for 24 h. Afterwards, extracellular DNA content was determined via PI staining. Increased extracellular DNA content leads to elevated fluorescence at 620 nm. Representative data from n = 4 individual experiments (mean + SD).

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IV

IgA2 ACPA Drives a Hyper-Inflammatory Phenotype in Macrophages via ATP Synthase and COX2

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First published: 01 April 2025, European Journal of Immunology

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European Journal of Immunology
DOI: 10.1002/eji.202451586

Abstract

IgA can form immune complexes (ICs) and activate myeloid cells via Fc alpha receptor-mediated signalling to secrete pro-inflammatory cytokines. It was previously described that of the two IgA subclasses (IgA1 and IgA2), IgA2 is more inflammatory than IgA1. However, the mechanisms underlying this differential proinflammatory potential remain poorly defined. Using anti-citrullinated protein IgA1 and IgA2 antibodies (ACPA) that are commonly found in rheumatoid arthritis (RA) patients and linked to chronic inflammation, we show here that, in macrophages, IgA2-ICs boost TLR-induced TNF and IL6 secretion, COX2 expression, and production of COX2-dependent lipid mediators to a higher level than IgA1-ICs. Metabolically, we found the amplification of TLR-induced cytokine production and COX2 induction by IgA2-ICs to be dependent on mitochondrial ATP synthesis, but not glycolysis. Finally, we found the potentiation of TLR-induced cytokine production by IgA-ICs to be COX2-dependent. Together this work points towards a key role for mitochondrial ATP synthesis in driving COX2 expression and subsequent IgA2-IC-dependent potentiation of TLR-induced cytokine production by macrophages. As such, our work provides new insights into the mechanisms underlying IgA2-induced inflammation in the context of RA. Thus, this may hold novel clues to be explored as therapeutic possibilities to target antibody-driven inflammation in chronic inflammatory diseases.

Introduction

IgA is the most abundant antibody in the human body (1), primarily located in mucosal tissue as a dimer, where it plays an important role in both protection and maintenance of homeostasis (2). However, IgA is also the second most common circulating antibody, where, unlike in the mucosa, it exists as both a monomer or dimer, lacking the secretory component (3). This inherent characteristic of circulatory IgA allows it to bind to Fcα-receptor I (FcαRI) expressed by myeloid cells, such as macrophages, which has been linked to induce pro-inflammatory cytokines, such as TNF and IL-6 (4).

Two subclasses of IgA exist: IgA1 and IgA2. Recent studies have described that IgA2 potentiates TLR-induced inflammatory responses in macrophages, dendritic cells (DCs), monocytes, and neutrophils more strongly than IgA1 (5–9). However, the mechanisms by which IgA2 achieves this remain to be determined. IgA1 and IgA2 differ in their glycosylation profile and hinge regions. IgA1 has two conserved N-glycosylation sites and a hinge with an O-linked glycosylation rich structure, while IgA2 has four conserved N-glycosylation sites and no O-linked sites (10,11). Although it has been suggested that differences in glycosylation affect IgA-receptor interactions (11,12), it is still unknown how this translates into altered cellular responses in myeloid cells, leading to different inflammatory outputs.

It has been established that changes in the metabolism of myeloid cells are intimately linked to their function and activation states (13,14). In the context of FcR-driven inflammatory responses, it was shown that IgA immune complexes (ICs) enhance the synthesis of proinflammatory cytokines in dendritic cells (DCs), and that this is achieved through glycolytic reprogramming (6,15). In a similar fashion, it was demonstrated that IgG boosts TLR-induced cytokine synthesis in macrophages by inducing a switch towards glycolytic metabolism (16).
Moreover, IgG-ICs have been shown to induce prostaglandin E_2 (PGE_2) synthesis in macrophages (16). It is well known that lipid mediators, such as PGE_2, play an important role in regulating inflammatory responses, including that of macrophages, through autocrine and paracrine signalling (16–20). However, it is still unknown whether differential metabolic reprogramming or COX2/PGE_2 expression play a role in the ability of IgA1 and IgA2 to induce distinct inflammatory responses.

Therefore, in this study, we aimed to investigate the difference in inflammatory potential and underlying mechanisms of IgA1 and IgA2 in human monocyte-derived macrophages in the context of TLR co-stimulation. We found that IgA2-ICs synergize more strongly with TLR stimulation than IgA1-ICs, as evidenced by higher levels of

TNF and IL-6 induction. Additionally, we show a metabolic link between IgA2 signalling and COX2 induction, whereby ATP synthase is crucial for the aforementioned effects of IgA2, but not IgA1, on macrophage activation. We also show that this IgA2-induced phenotype is associated with increased PGE_2 synthesis and, correspondingly, with an augmented expression of COX2. Finally, we describe that, by targeting COX2 activity with indomethacin, we are able to abrogate the boosting effects of IgA2 in macrophage inflammatory potential, albeit this effect seems to be independent of PGE_2.

In summary, our work links IgA2-induced inflammation, with COX-dependent mediators, and ATP Synthase activity in macrophages.

Results

IgA2 Synergizes With TLR2/1 Signalling to Induce a More Inflammatory Phenotype in Macrophages via Syk

We first aimed to assess the inflammatory potential of IgA1 and IgA2 on human macrophages. We decided to focus on IgA ACPAs, which are known to play an important role in the inflammatory response in RA. This disease is characterized by chronic inflammation of the joints, specifically the synovium. One of the hallmarks of this inflammatory environment in the synovium is the crosstalk between macrophages and ACPAs. Importantly, the levels of IgA ACPAs and rheumatoid factor specific IgA in the synovium are significantly increased in patients with RA and are associated with RA flares (21,22). Moreover, higher IgA2:IgA1 ratios have been associated with a worse disease score in RA patients (5), making IgA1 and IgA2 ACPAs highly clinically relevant to study this interaction. To do so, we employed an *in vitro* model whereby we cultured human monocyte-derived macrophages in plates pre-coated with citrullinated antigen (CCP2), and with equal concentrations of either monoclonal IgA1 or IgA2 ACPA (Fig. S1 A-C) – both having the same patient derived variable domain sequence – to simulate ACPA-ICs, in the presence or absence of PAM3CSK4 (PAM), a TLR2/1 ligand, since many danger associated molecular patterns associated with RA have been shown to signal via this TLR (23). Furthermore, we opted by differentiating macrophages with GM-CSF due to its role in RA in driving macrophage differentiation, M1-polarization, Fcα receptor expression, and subsequent inflammation and cartilage degradation (24–30). Additionally, GM-CSF has been implicated in regulating macrophage proliferation, glycolysis, lipid metabolism, and mitochondrial function (31,32). Therefore, macrophages from a GM-CSF background present themselves as a relevant model to study macrophages in the context of RA and IgA binding, along with their metabolism and inflammatory responses.

Upon dual stimulation with PAM and IgA, we found that IgA2, but not IgA1, synergized

with the TLR signalling to induce TNF and IL-6 (Fig1. A, B). In addition, COX-dependent lipid species, including PGE_2, were also more strongly upregulated following IgA2 stimulation (Fig1. C and Fig. S2 A, B). However, this was not the case for LOX-dependent species (Fig1. D). The increased production in PGE_2 was paralleled by potentiated COX2 expression (Fig1. E). Finally, we found the effect of IgA2 to be dependent on syk, a crucial mediator of FcR-signalling, as syk inhibition abrogated IgA2-driven potentiation of TLR-induced cytokine synthesis and COX2 expression, with no decrease in cell viability (Fig. S3 A-D).

Together, these data show that IgA2 has a stronger proinflammatory effect than IgA1 on macrophages and further suggest that this is syk-dependent.

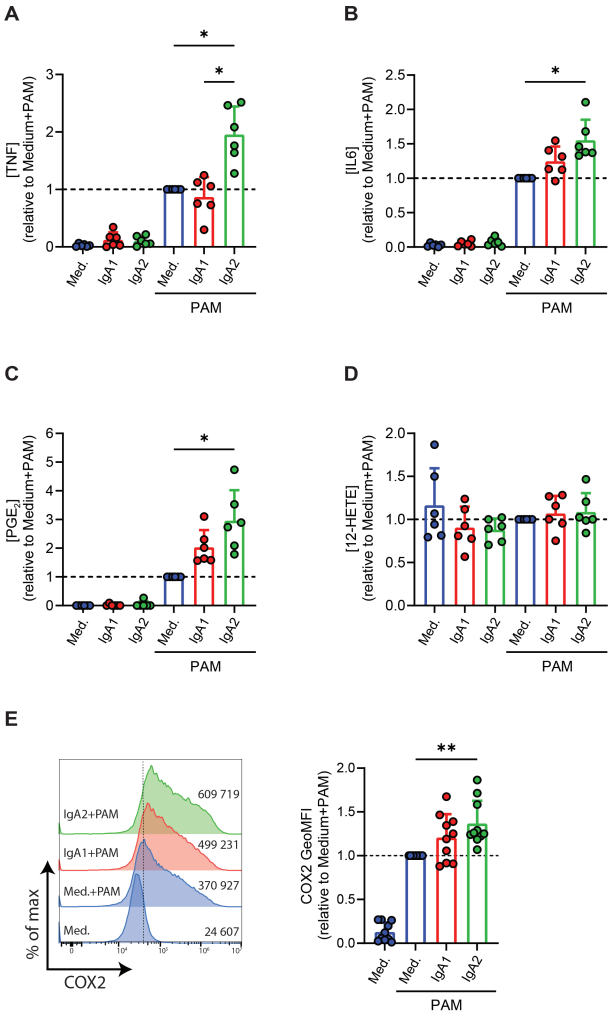

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Fig1: **IgA synergizes with PAM to potentiate TLR-induced cytokine production and COX2 expression in macrophages.** Levels of (A) TNF, (B) IL-6, (C) PGE_2, and (D) 12-HETE measured in supernatants following stimulation of cells with indicated compounds. Levels of (A) TNF and (B) IL-6 were measured with ELISA as described in materials and methods. (E) Representative histogram of COX2 expression for each indicated stimulus (left); values represent the pooled average raw GeoMFI of all experiments. (Right) normalized COX2 GeoMFI of cells treated with indicated stimuli. (A)-(D) are representative plots of 6 donors from 2 independent experiments (mean ± SD). (E) is a representative plot of 10 donors from 5 independent experiments (mean ± SD). All data were analysed using a paired one-way ANOVA. * $p < 0.05$, ** $p < 0.01$.

IgA2 and PAM Co-Stimulation Induces Metabolic Changes in the Mitochondrial Compartment

Since IgA has been previously shown to reprogram DCs into a pro-inflammatory phenotype by potentiating glycolysis (15), and since PGE_2 was shown to have an effect on mitochondrial metabolism in macrophages (33), we aimed to explore whether IgA2-driven inflammatory responses were accompanied by, and dependent on, metabolic rewiring. Using a metabolic flux analyser to measure OCR (oxygen consumption rate) (Fig2. A) and ECAR (extracellular acidification rate) (Fig. S4 A), we found that IgA1 and IgA2 did not promote significant changes in glycolysis (Fig. S4 B-D), basal respiration (Fig2. B), or ATP synthesis (Fig2. C) 24h after stimulation, although IgA2-stimulated cells appeared to have a lower respiratory spare capacity (Fig2. D). This was also associated with trends towards lower expression of mitochondrial enzymes 24h following stimulation with IgA2 (Fig2. E-H), albeit no change was observed in mitochondrial membrane potential (Fig. S4 E). SCENITH assay to assess the dependence on mitochondrial metabolism and glycolytic metabolism for ATP-dependent translation, revealed no clear differences (Fig2. I-L). Together this indicates that, in human macrophages, IgA1 and IgA2, except for altered mitochondrial respiratory spare capacity, induce minor changes in core metabolic pathways.

Inhibition of Mitochondrial Respiration Abrogates the Pro-Inflammatory Effects of IgA2

Next, we evaluated whether the potentiated inflammatory profile induced by IgA2 was dependent on any core metabolic pathway activity, using various inhibitors. While blocking glycolysis with 2-Deoxy-D-Glucose (2-DG), slightly affected TNF production, blocking glycolysis with 2-DG, or fatty acid oxidation with etomoxir, did not affect the production of IL-6, nor COX2 expression (Fig. S5 A-C). Interestingly, blocking ATP Synthase with oligomycin abrogated the potentiating effects of IgA2 on both TNF (Fig3. A) and IL-6 (Fig3. B) production. Additionally, oligomycin also prevented the boosting effect of IgA2 on COX2 expression (Fig3. C). Correspondingly, oligomycin, and the general COX inhibitor indomethacin (Indo), but not 2-DG or etomoxir, were able to block IgA2-induced PGE_2 synthesis, and other COX2-dependent mediators (Fig4. A-C). Of note, there were no effects on cellular viability by the inhibitors used,

apart from minimal effects by 2-DG (Fig. S6). Interestingly, oligomycin showed no effect on IgA1-induced potentiation of COX2-derived products by TLR-stimulated macrophages (Fig4. D-F). Additionally, LOX-dependent mediators were unaffected by oligomycin treatment upon IgA2 or IgA1 stimulation (Fig4. G, H). This together suggests that the effects of IgA2 on TLR-induced cytokine production and COX2 activity are dependent on ATP Synthase activity.

The Pro-Inflammatory Effects of IgA2 Are Supported by COX2 Activity

As PGE_2 is a well-known signalling lipid in macrophages (16–20), we wondered if this IgA2-potentiated inflammatory profile was dependent on COX2 induction and subsequent PGE_2 synthesis. To test this, we stimulated cells with IgA in the presence of PAM and indomethacin. Upon inhibiting COX activity, the enhancement of IgA2 on TNF (Fig5. A) and particularly IL-6 (Fig5. B) production was reduced. Furthermore, in a similar fashion as observed with oligomycin (Fig3. B), Indomethacin not only prevented IgA2 potentiation on IL-6 production, but it also decreased the production of IL-6 by PAM in the absence of IgA, thus showing that PAM as well as the potentiated response by IgA2 requires COX2 activity for IL-6 production by these cells.

Since IgA2 was boosting COX2 expression and PGE_2 synthesis, and indomethacin reduced the pro-inflammatory effects of IgA co-stimulation with TLR2/1 and PGE_2 synthesis, we tested whether we could rescue these effects by adding PGE_2 to the medium of these cells. Interestingly, the addition of PGE_2 had no effect on the synthesis of IL-6 upon inhibition of COX with indomethacin or oligomycin (Fig. S7), indicating the synergistic effect of IgA2 and TLR2/1 on the pro-inflammatory phenotype of macrophages is dependent on COX, but possibly independent of PGE_2 synthesis.

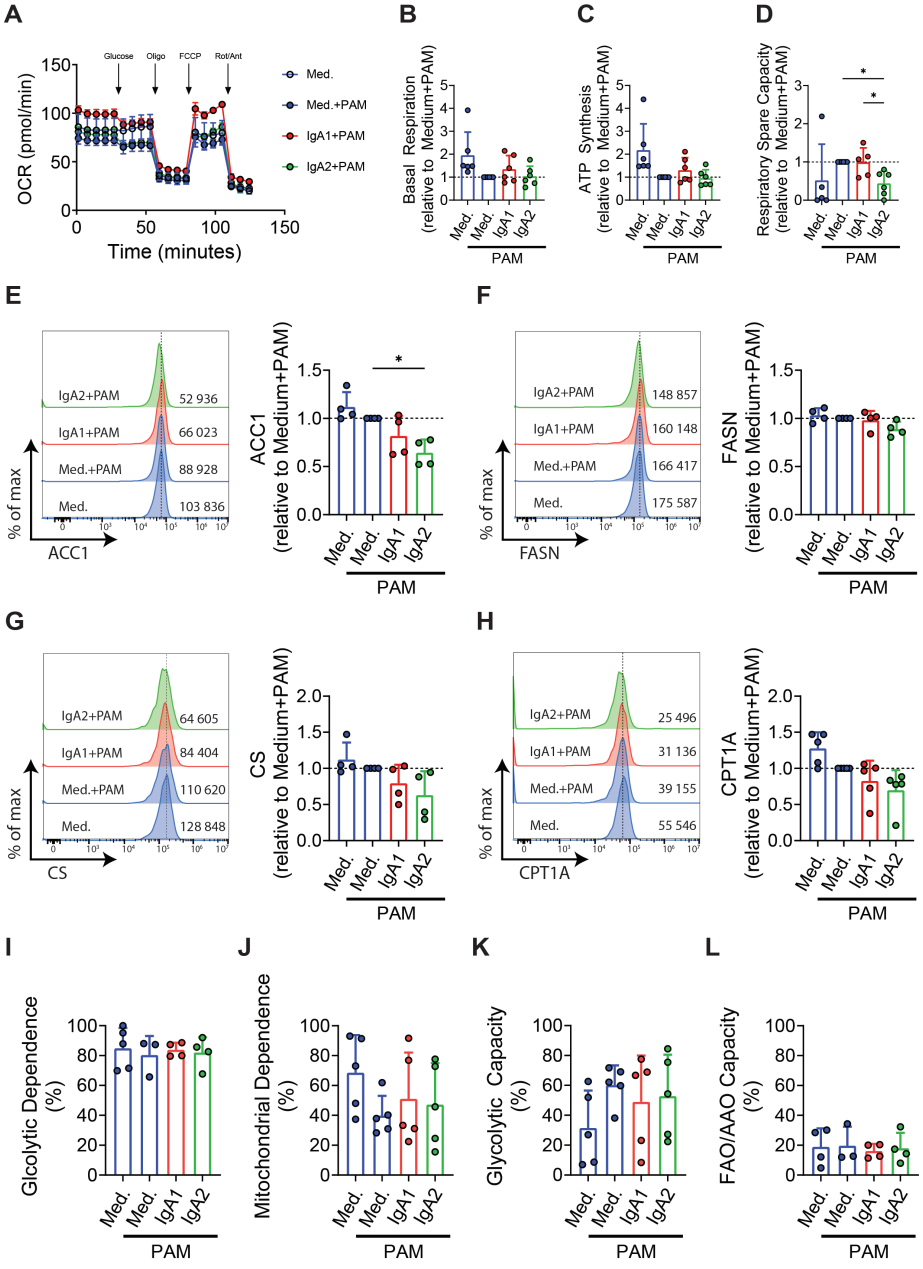

Fig2: **IgA2-PAM co-stimulation induces metabolic changes in the mitochondrial compartment.** Representative histograms (left) and normalized GeoMFI (right) of (E) ACC1, (F) FASN, (G) CS, and (H) CPT1A measured via FACS following 24h stimulation of cells with indicated stimuli. Values in histograms represent the pooled average raw GeoMFI of all experiments. (I) Glycolytic Dependence, (J) Mitochondrial Dependence, (K) Glycolytic Capacity, and (L) Fatty Acid and Amino Acid Oxidation (FAO/AAO) capacity measured using SCENITH and calculated as described in materials and methods. (A)-

(D) are representative plots of 6 donors from 3 independent experiments with outliers removed (mean ± SD). (E)-(G) are representative plots of 4 donors from 2 independent experiments (mean ± SD). (H) is a representative plot of 5 donors from 3 independent experiments (mean ± SD). (I)-(L) are representative plots of 5 donors from 2 independent experiments (mean ± SD). All data were analysed using a paired one-way ANOVA. * $p < 0.05$.

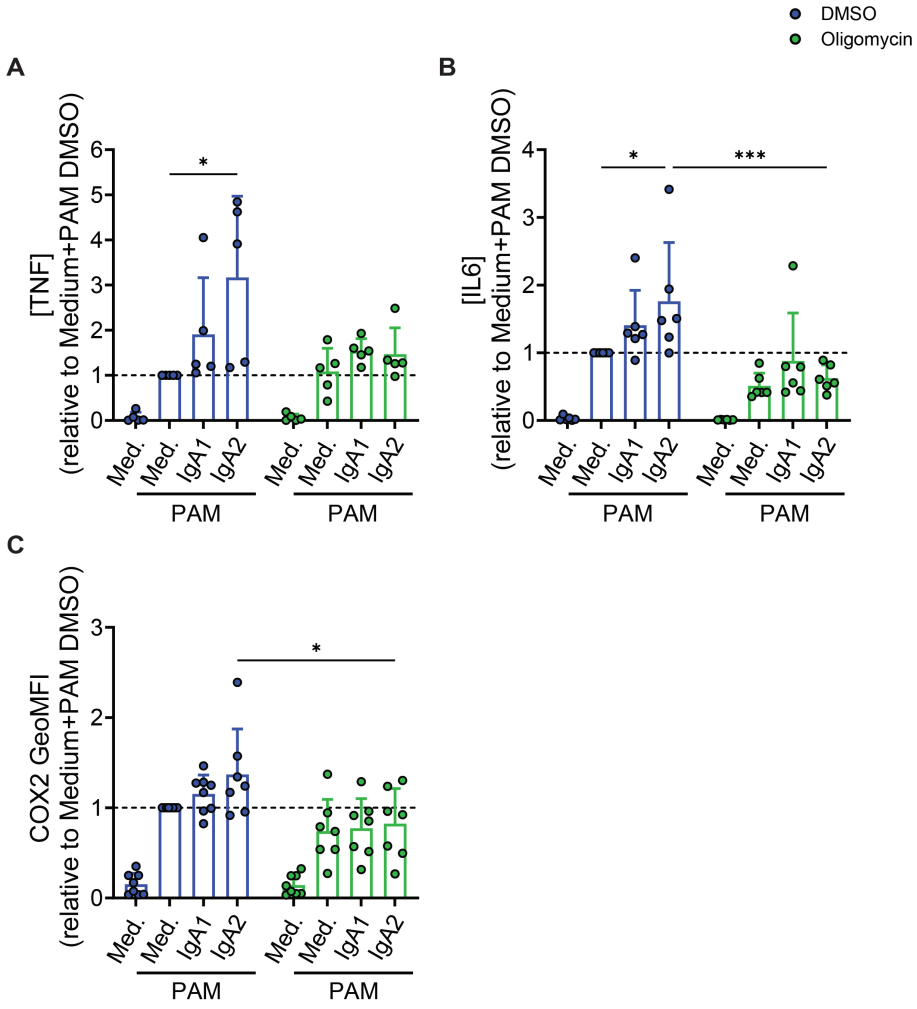

Fig3: **Inhibition of ATP Synthase with oligomycin abrogates the pro-inflammatory potentiation effects of IgA2.** Levels of (A) TNF and (B) IL-6 measured in supernatants with ELISA following stimulation of cells with indicated stimuli in the presence of DMSO (blue) or Oligomycin (green). (C) Normalized COX2 GeoMFI of cells treated with indicated stimuli in the presence of DMSO (blue) or Oligomycin (green). (A) is a representative plot of 5 donors from 3 independent experiments (mean ± SD). (B) is a representative plot of 6 donors from 3 independent experiments (mean ± SD). (C) is a representative plot of 8 donors from 4 independent experiments with outliers removed (mean ± SD). All data were analysed using a paired two-way ANOVA, with matched values both stacked and spread across a row, using a Tukey's multiple comparisons test, with a single pooled variance. * $p < 0.05$, *** $p < 0.001$.

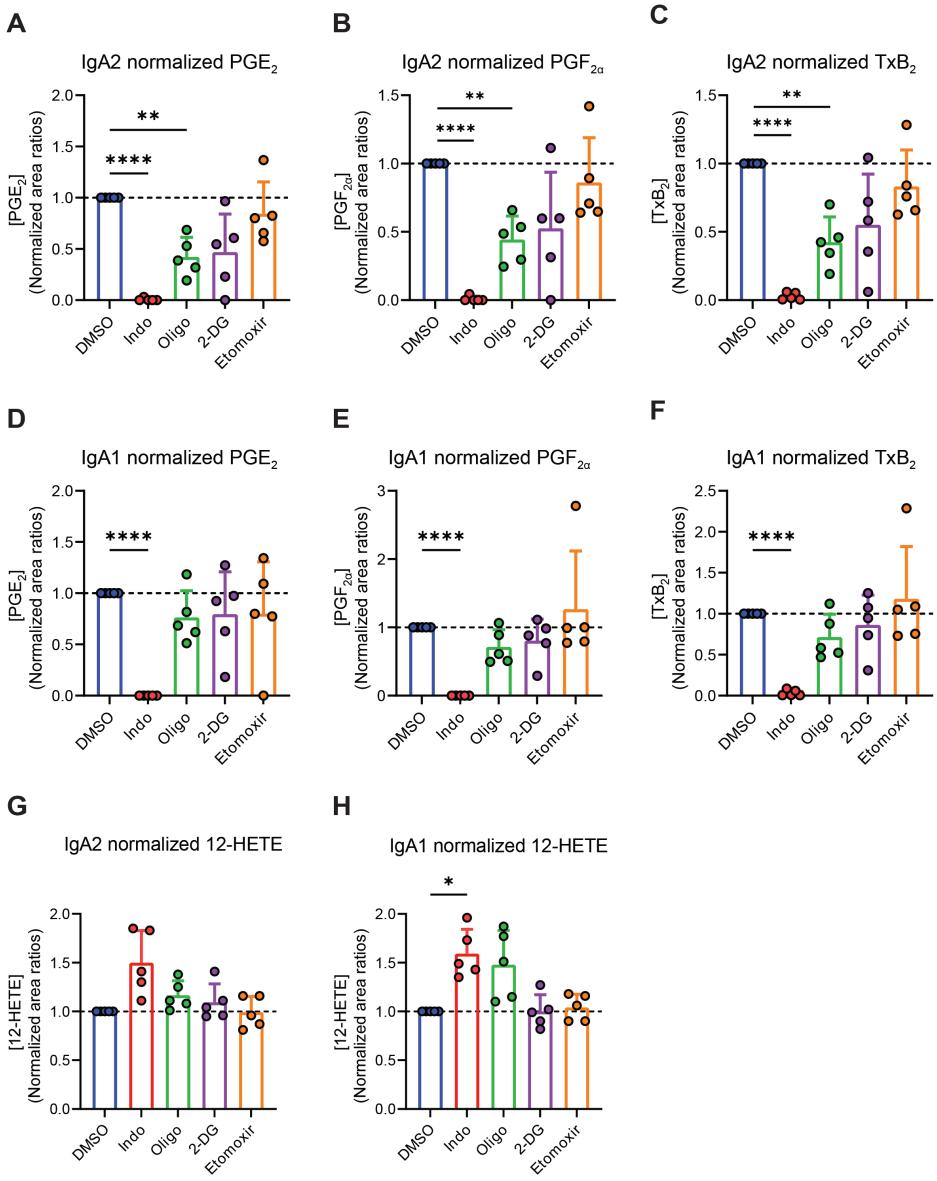

Fig4: **IgA2 requires ATP Synthase for potentiation of PAM-driven synthesis of COX2-dependent lipid mediators.** Levels of (A) PGE_2, (B) $PGF_{2\alpha}$, and (C) TxB_2 measured in supernatants of cells co-stimulated with IgA2 and PAM and indicated inhibitors. Levels of (D) PGE_2, (E) $PGF_{2\alpha}$, and (F) TxB_2 measured in supernatants of cells co-stimulated with IgA1 and PAM and indicated inhibitors. Levels of COX2-independent 12-HETE measured in supernatants of cells co-stimulated with PAM and (G) IgA2, or (H) IgA1, and indicated inhibitors. (A)-(H) are representative plots of 5 donors from 2 independent experiments (mean ± SD). All data were analysed using a paired one-way ANOVA with Dunnett's post-hoc test. The mean of each column was compared with the mean of the control column (DMSO). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

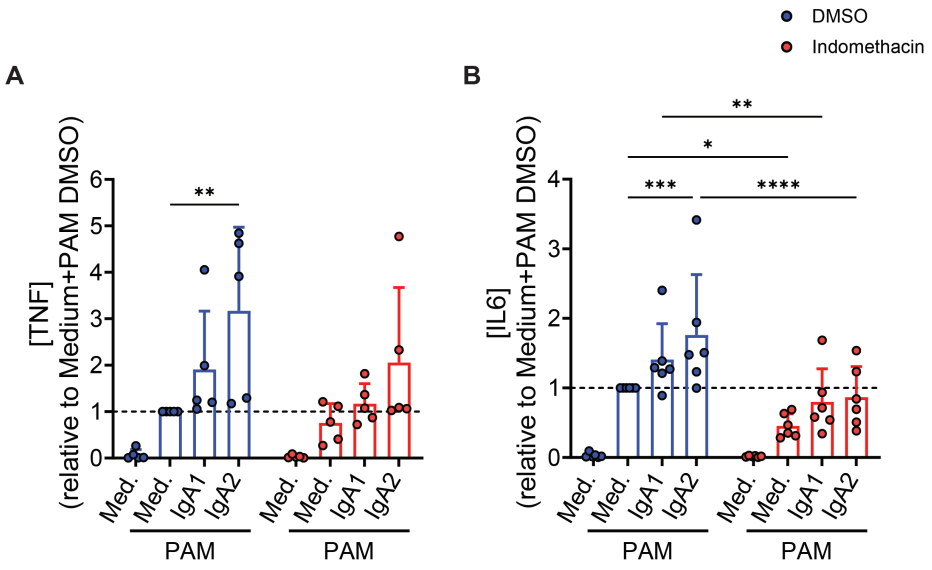

Fig5: **IgA2 requires COX2 activity for potentiation of TLR-induced cytokine production.** Levels of (A) TNF and (B) IL-6 measured in supernatants with ELISA following stimulation of cells with indicated stimuli in the presence of DMSO (blue) or Indomethacin (red). (A) is a representative plot of 5 donors from 3 independent experiments (mean ± SD). (B) is a representative plot of 6 donors from 3 independent experiments (mean ± SD). Respective DMSO controls are the same as the ones shown in Fig3. All data were analysed using a paired two-way ANOVA, with matched values both stacked and spread across a row, using a Tukey's multiple comparisons test, with a single pooled variance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Discussion

Previous studies have shown that IgA2 drives stronger pro-inflammatory signalling in both macrophages and neutrophils, compared to IgA1 (5). Correspondingly, higher ratios of IgA2:IgA1 ACPA have been correlated with worse RA scores (5), pointing to a more detrimental role of IgA2 than IgA1 in RA pathology. In our study we have further extended these findings, by showing that, in macrophages, IgA2 ACPA has the ability to induce higher levels of TNF and IL-6, both when compared to TLR-2 ligation alone or TLR-2 and IgA1 ACPA co-stimulation. Furthermore, we also found that this increased inflammatory signalling was associated with both higher expression of COX2 and higher levels of COX2-dependent lipid mediators, specifically PGE_2. Interestingly, while no major changes were seen in OCR or Glycolysis, IgA2 did show a decrease in respiratory spare capacity. These data showing reduced spare capacity align with previous work done by van den Bossche *et al* (34) showing that M1-polarized macrophages both present some mitochondrial disfunction and display a lower metabolic elasticity, unlike M2 which can more easily adapt their metabolism if one pathway is blunted. However, despite a lack of clear reprogramming of core

metabolic pathways by these macrophages, we observed a dependence of IgA2 on ATP Synthase for TLR-induced cytokine production, COX2 expression and subsequent PGE_2 levels. We also report that indomethacin, a general COX2 inhibitor, was able to reduce the inflammatory potential of IgA2.

The distinct inflammatory effects of IgA2 and IgA1 have been postulated to be driven by differences in their glycosylation profiles. These differences may alter interaction strength with the FcαR, and thus also downstream signalling pathways, and ultimately TLR-induced cytokine production. IgA has been shown to also signal via MAPK and MyD88 (35,36), which are known mediators of TLR-driven cytokine responses and COX2 expression (37–42). This overlap of signalling pathways, together with the recent findings that IgA2 induces stronger binding and activating signalling than IgA1 (5), could explain why IgA2 induces higher expression of COX2 and drives a stronger inflammatory response than IgA1.

We have previously shown that IgA-ICs induce, and depend on, glycolytic reprogramming to render tolerogenic DCs proinflammatory (15), with no effect or dependency on mitochondrial metabolism. In contrast, our current observations in macrophages show no role for glycolysis, but instead point to a crucial role for mitochondrial ATP synthase in supporting the pro-inflammatory effects of IgA2. This is in line with other studies that have shown that in a L929 fibrosarcoma cell line, a functional electron transport chain (ETC) is required for proper NFκB activation, and TNF and IL-6 synthesis. Furthermore, tampering with either ETC complexes or ATP Synthase resulted in a reduction of these cytokines in whole blood (43,44). An additional explanation as to why ATP synthase is required for IgA2-driven inflammatory responses in macrophages, may be connected to mTOR and its role in cytokine and COX2 expression, and promotion of inflammation (42,45). Since mTOR is inhibited in conditions of low ATP:AMP ratio, it is conceivable that oligomycin, by blocking ATP synthase, decreases the ATP:AMP ratio, leading to mTOR inhibition and thus lower cytokine and COX2 expression. This, however, remains to be explored in future studies.

Additionally, work by Sanin *et al* (33) showed an effect of PGE_2 on mitochondrial membrane potential, which in turn affected gene expression in macrophages. However, in our work, we did not find alterations in membrane potential despite high levels of PGE_2 production. This can probably be explained by differences in macrophage models used, as the authors used murine M2-like macrophages from MCSF (macrophage colony-stimulating factor) cultures, while in our study we used human GM-CSF stimulated M1-like macrophages, which have been shown to respond differently to inflammatory signals (46).

Interestingly, it was shown that, in RA synoviocytes, mitochondrial dysfunction increases COX2 expression (47). In these studies, it was indicated that oligomycin increased ROS (reactive oxygen species) production, which in turn stimulated the expression of COX2, again indicating that different cell types can respond in opposite ways to the same metabolic manipulation (14).

As shown previously for IgG, IgA, and IgE (Immunoglobulin E) stimulation of monocytes (48,49), we here report that IgA2 synergizes with TLR ligands to promote COX2 and PGE_2 synthesis, and that the former is required for boosting the proinflammatory profile of macrophages induced by IgA2. However, how COX2 achieves this, in an apparently PGE_2-independent manner, remains to be determined. Possibly, there is another COX2-dependent mediator responsible for the IgA2-induced inflammation, or the effects of PGE_2 are time- and/or dose-dependent in a way that cannot be mimicked by a single supplementation in culture. This phenomenon has previously been shown in different contexts, where PGE_2 plays distinct roles depending on the concentration and the stimulation timepoint (50–53). Our data also showed $PGF_{2\alpha}$ and TxB_2 to be decreased following oligomycin treatment, as such one could speculate that one of these plays a role in supporting the proinflammatory effect of IgA2. Indeed, it was shown that both $PGF_{2\alpha}$ and Thromboxane A_2 (the active precursor of TxB_2) are present in higher levels in patients with RA compared to either healthy controls or RA patients undergoing treatment (54–56). Furthermore, previous studies suggest that prostaglandin I_2, another lipid mediator downstream of COX2, plays a role in driving inflammation in the context of RA (57,58), identifying prostaglandin I_2 as another interesting candidate to further study in the context of IgA2-driven inflammation.

In conclusion, we find a crucial role for mitochondrial ATP synthase-dependent COX2 induction in promoting a proinflammatory cytokine response following co-stimulation with PAM and IgA2, revealing a novel interplay between metabolism, lipid mediator production and cytokine expression that shapes the inflammatory profile of macrophages. Thus, our work warrants exploration of the potential of targeted manipulation of macrophage mitochondrial metabolism, as a novel strategy, to dampen IgA-driven inflammation in inflammatory disorders, such as RA.

Materials and Methods

Generation of IgA1 ACPA and IgA2 ACPA

All monoclonal antibodies had identical variable domains corresponding to the anti-citrullinated protein antibody (ACPA) clone 1C11. This clone was derived from cyclic citrullinated protein 2 (CCP2) reactive B-cells, isolated by single-cell sorting from an

ACPA-positive patient. The heavy and light chain variable domains of the B-cell receptor were sequenced as previously described (59). The ACPA 1C11 IgA variable gene sequences, linked to the lambda light chain (LC), IgA1 heavy chain (HC), or IgA2m1 HC constant domains, along with the leader peptides MELGLSWVFLVVILEGVQC (for HC) or MAWIPLFLGVLAYCTDIWA (for LC), the Kozak sequence, and BamHI and XhoI restriction sites, were codon-optimized by GeneArt (Life Technologies) and ordered from IDT. The 1C11 IgA HC and LC constructs were cloned into the pcDNA3.1(+) expression vector and co-transfected into FreestyleTM 293-F cells (Gibco) as previously reported (60). Supernatants from transfected and non-transfected cells (as negative controls) were collected and 0.45 µM filtered 5–6 days later. The supernatants were then diluted to achieve an IgA concentration of 2 µg/mL, as measured by ELISA using light chain detection antibody to check for equal coating.

Coating of plates with IgA1 ACPA or IgA2 ACPA

For regular culture and stimulation, 96-well streptavidin microplates (Microcoat, Bavaria) were coated with 50 µL of 1 µg/mL biotinylated CCP2 antigen in sterile PBS/0.1% BSA for 1h at room temperature. After 3 washes with 150 µL sterile PBS, 50 µL of IgA1, IgA2 or negative control HEK cell supernatant (Med.) were added and incubated for 1h at 37°C. The plate was washed 3 times with sterile PBS prior adding the cells.

For seahorse and SCENITH experiment, untreated 6 well culture plates (Corning) were coated with 1.5 mL of 1 µg/mL streptavidin (Invitrogen) in sterile coating buffer (0.1 M Na2CO3 0.1 M NaHCO3 in H_2O Mili-Q) and incubated overnight at 4°C. After 3 washes with 2 mL sterile PBS, 1 mL of 1 µg/mL biotinylated CCP2 antigen in sterile PBS/0.1% BSA were added per well. The wells were washed 3 times with 2 mL sterile PBS and 800 µL of HEK cell supernatant were added for 1h at 37°C. the wells were washed 3 more times with sterile PBS prior cells stimulation.

Lambda Light Chain ELISA

96-well streptavidin microplates (Microcoat, Baviera) were coated as described above. 50 µL of Sheep anti-lambda-light chain-HRP (polyclonal, abcam) in PBS/0.05% Tween/1% BSA were added for 1h at 37°C. The plate was washed 3 times with PBS/0.05% Tween and absorbance was measured at 415 nm using ABTS and H_2O_2 (Fig. S1 A-C).

Human Macrophage culture and stimulation

Peripheral blood mononuclear cells were isolated from the venous blood of healthy volunteers by density centrifugation in Ficoll as described before (61). Monocytes were isolated by positive magnetic cell sorting using CD14-microbeads (Miltenyi Biotech, 130-097-052) and cultured in complete RPMI medium (RPMI containing 10% FCS, 100 U/mL penicillin/streptomycin, and 2 mM L-glutamine) supplemented with

20 ng/mL rGM-CSF (ThermoFisher, PHC2011). On day 2/3, the medium, including supplements, was replaced.

Macrophages were stimulated on day 5 in the presence or absence (if indicated) of 2 µg/mL PAM3CSK4 (InvivoGen, tlrl-pms) in wells previously coated with HEK cell medium, IgA1 or IgA2, along with the indicated reagents: 50 µM Indomethacin (Sigma-Aldrich, I7378), 2 µM Oligomycin (Cayman, 11342), 1 µM 2-DG (Sigma-Aldrich, D8375), 3 µM Etomoxir (Sigma-Aldrich, E1905), 1 µM Entosplenetinib (SelleneckChem, S7523), 10 µM Prostaglandin E_2 (Cayman, 14010).
After 24h of stimulation, supernatant was collected and stored at -20°C, if for ELISA, or at -80°C, if for lipidomics. Cells were harvested and stained with the viability dye Zombie NIR (Biolegend, 423106) for 15 minutes at 4°C in the dark, before being fixed with 2% PFA for 10 minutes, in the dark, at room temperature.

Staining protocol and analysis

Fixed cells were washed once in 1x permeabilization buffer (ThermoFisher, 00-5523-00) before staining intracellular targets in 1x permeabilization buffer, containing Fc-block, for 2 h in the dark at room temperature. The expression of intercellular enzymes was determined by flow cytometry (Aurora; Cytek, Amsterdam, The Netherlands) using the following antibodies: COX2 (clone AS67, BD 565125), ACC1, (Abcam, ab272704), FASN (Abcam, ab128870), CPT1A (Abcam, ab235841) CS (Abcam, ab129088). Only single live cells that were negative for Zombie NIR were included in the analysis. Gating strategy and FMOs can be seen in Fig. S8. The acquired samples were unmixed using SpectroFlo version 3 and analysed with FlowJo version 10.10.0.

TNF and IL6 cytokine ELISA

Supernatants from cell cultures stored at -20°C were slowly thawed at 37°C and used to measure concentration of TNF, with BD OptEIA™ Human TNF ELISA Set (BD Biosciences, 555212), and IL-6, with BD OptEIA™ Human IL-6 ELISA Set (BD Biosciences, 555220) according to manufacturer instructions.

Measurement of lipid mediators

Lipid mediators (LMs) and polyunsaturated fatty acids (PUFA) were measured using reverse-phase liquid chromatography coupled to tandem mass spectrometry (RPLC-MS/MS) as previously described (62), with some modifications. Briefly, 2 µL internal standard (IS) mix of deuterated lipid standards consisting of PGE_2-d_4, 15-HETE-d_8, Leukotriene B_4-d_4, DHA-d_5, 8-iso-$PGF_{2\alpha}$-d_4, and 14(15)-EET-d_{11} (50 ng/mL in methanol (MeOH)) was added to 400 µL culture supernatants. Lipids were extracted and purified by solid-phase extraction (SPE) after protein precipitation with 1.2 mL MeOH. The dried extracts were reconstituted in 100 µL 40% MeOH and transferred into a micro-vial glass insert. Furthermore, a 40-µL sample was injected and analysed using a Shimadzu Nexera LC40 system with an autosampler coupled to a QTrap 6500 mass

spectrometer (Sciex). Kinetex C18 50 × 2.1 mm, 1.7 µm column, and C8 precolumn (Phenomenex) were used for LC separation. LC–MS/MS chromatograms were integrated manually using Sciex OS (Sciex). The results were reported as relative peak area of lipids to the internal standards. PGE_2-d_4 IS was used for reporting the area ratios of PGE_2, TxB_2, and $PGF_{2\alpha}$.

Extracellular Flux Assay (Seahorse)

5×10^5 macrophages were plated in an XFe96 well Seahorse plate (Agilent) after being stimulated overnight. Medium was replaced, after washing 2x with PBS, with 180 µL XF assay made from base RPMI without HEPES and $NaHCO_3$ (Sigma, R6504) supplemented with 5% FCS and 2 mM L-Glutamine, and incubated in a non-CO_2 37 °C incubator for 1 h. As cells were incubating, injected compounds were diluted in XF media (without FCS) and added to the hydrated cartridge, after which the cartridge was immediately loaded into the Seahorse for calibration. 10 mM Glucose (Sigma, G8644), 1,5 µM Oligomycin (Cayman, 11342), 3 µM FCCP (Sigma, C2920), 1 µM Rotenone (Sigma, 557368) and 1 µM Antimycin A (Sigma, A8674).

Homopropargylglycine uptake (SCENITH)

$5x10^5$ macrophages were plated in a 96-well untreated V-bottom plate, washed with PBS, and plated in 90 µL of methionine-free medium (Sigma, R7513) supplemented with 65 mg/L L-cystine dihydrochloride (Sigma, C6727), 2 mM L-Glutamine (Sigma, G3126), and 10% dialyzed FCS (ThermoFisher, A3382001). Cells were starved of methionine for 45 min at 37°C, before addition of 10 µl of indicated inhibitor(s) (medium, 2 µM Oligomycin, 100 mM 2DG, or 2 µM Oligomycin and 100 mM 2DG) and subsequently incubated another 15 min at 37°C. Homopropargylglycine (Click Chemistry Tools, 1067) was added at a final concentration of 100 µM and incubated for 30 min at 37°C before being washed 2x with cold PBS, live/dead stain with Zombie NIR at 4°C for 15 minutes, washed 2x with cold PBS, and fixed with 2% PFA for 10 min.

Click Chemistry Reaction

Cells fixed after Homopropargylglycine uptake were permeabilized with PBS containing 1% BSA/0.1% Saponin for 15 min and washed 2x in Click buffer (100 mM Tris-HCl, pH 7.4) before the addition of Click reaction mix. The reaction mix was made by sequential addition of 10 mM Sodium Ascorbate (Sigma, A7631), 2 mM THPTA (Click Chemistry Tools, 1010), 0.5 µM AFdye488 azide plus (Click Chemistry Tools, 1475) and 1x click buffer to $CuSO_4$ (0.5 mM final conc., [Sigma, 209198]). Samples were incubated for 30 min in the dark at room temperature. Cells were washed with FACS buffer and measured on the Aurora. The acquired samples were unmixed using SpectroFlo version 3 and analysed with FlowJo version 10.10.0. Calculations of metabolic capacities and dependences were done as previously described (63).

Statistical Analysis

The statistical tests used are indicated in the figure legends. Generally, data were compared using one-way ANOVA for more than two groups or two-way ANOVA for comparing multiple parameters across two or more groups, with Tukey's *post-hoc* test for multiple comparison. *p*-values <0.05 were considered significant ($*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$). All statistical analyses were performed using GraphPad Prism v.10.2.3.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Statement

The studies involving humans were approved by Sanquin National Blood donation Bank. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from voluntary blood donations to Sanquin Blood bank. Written informed consent for participation was not required from the participants or the participants' legal guardians/ next of kin in accordance with the national legislation and institutional requirements.

Author contributions

LA: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. AB: Formal Analysis, Investigation, Methodology, Writing – review & editing. MGh: Data curation, Formal Analysis, Investigation, Methodology, Writing – review & editing. AH: Investigation, Methodology, Writing – review & editing. RT: Conceptualization, Resources, Supervision, Writing – review & editing. MGi: Conceptualization, Resources, Supervision, Writing – review & editing. BE: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft.

Acknowledgements

Many thanks to Dr. Jeroen den Dunnen, Chiara Geyer, and Lynn Mes for their support and collaboration. We would also like to thank our colleagues from the Leiden

University Centre for Infectious Diseases, and Dr. Luís Almeida from Mainz University for their continual scientific discussions. We would also like to acknowledge the LUMC Flow Core Facility operators for the continual maintenance and troubleshooting of the Cytek Auroras.

This work was supported by funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant agreement No. 812890.

Supplementary Materials

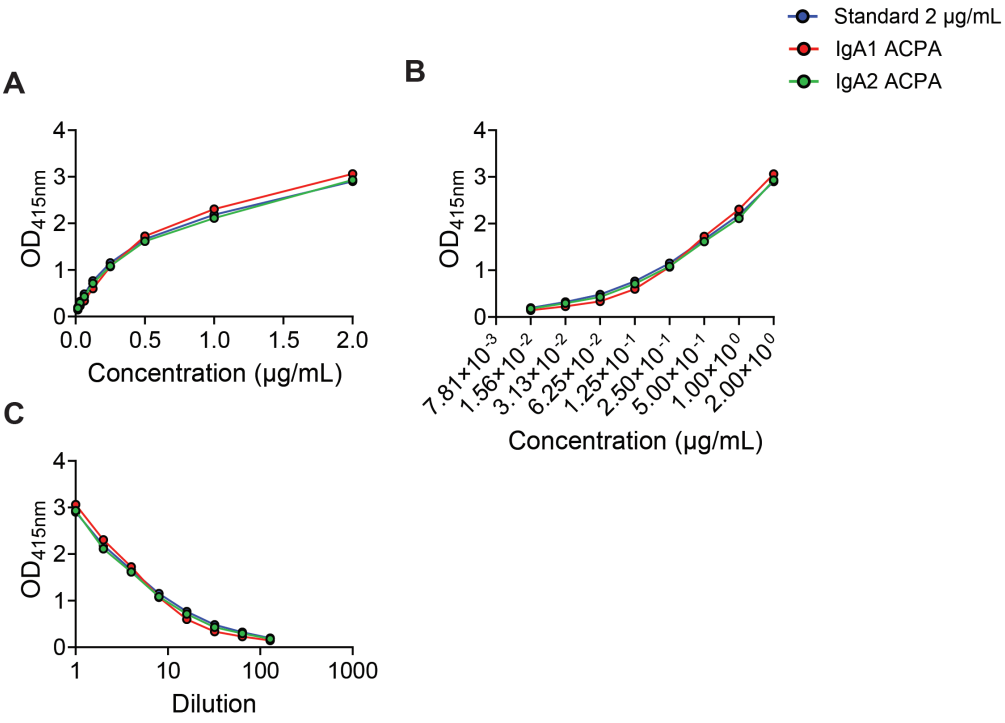

Fig. S1: **Coating of the monoclonal IgA1 ACPA and IgA2 ACPA.** Antibody binding to the CCP2 antigen was measured by ELISA using anti-lambda light chain detection antibody as described in materials and methods. Optical Density at 415nm (OD_{415nm}) and corresponding linear (A) and log_2 (B) concentration of antibody used. (C) Optical Density at 415nm and corresponding log_{10} dilution. (A)-(C) are representative plots of 2 replicates from 1 experiment (mean ± SD).

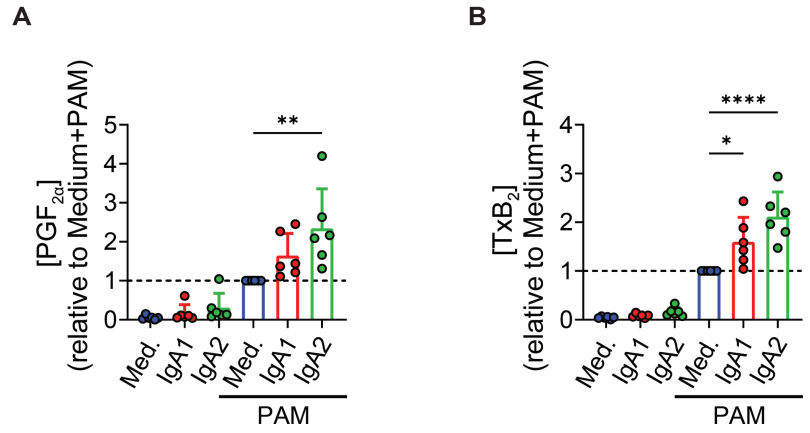

Fig. S2: **IgA synergizes with PAM to potentiate synthesis of COX2-dependent lipid mediators.** Levels of (A) $PGF_{2\alpha}$, and (B) TxB_2 measured in supernatants following stimulation of cells with indicated compounds. (A)-(B) are representative plots of 6 donors from 2 independent experiments (mean ± SD). All data were compared using an ordinary one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

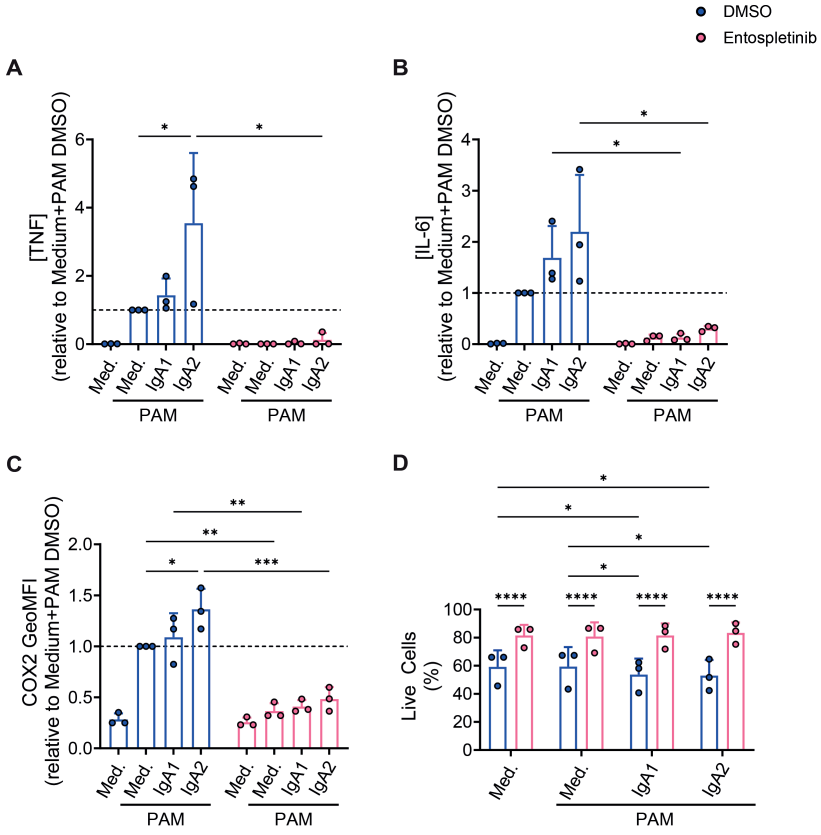

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Fig. S3: **IgA1 and IgA2 signal through Syk.** Levels of (A) TNF and (B) IL-6 measured in supernatants with ELISA following stimulation of cells with indicated stimuli in the presence of DMSO (blue) or the Syk inhibitor Entospletinib (lilac). (C) Normalized COX2 GeoMFI of cells treated with indicated stimuli in the presence of DMSO (blue) or Entospletinib (lilac). (D) Viability of cells treated with indicated stimuli in the presence of DMSO (blue) or Entospletinib (lilac). (A)-(D) are representative plots of 3 donors from 2 independent experiments (mean ± SD). All data were analysed using a paired two-way ANOVA, with matched values both stacked and spread across a row, using a Tukey's multiple comparisons test, with a single pooled variance. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

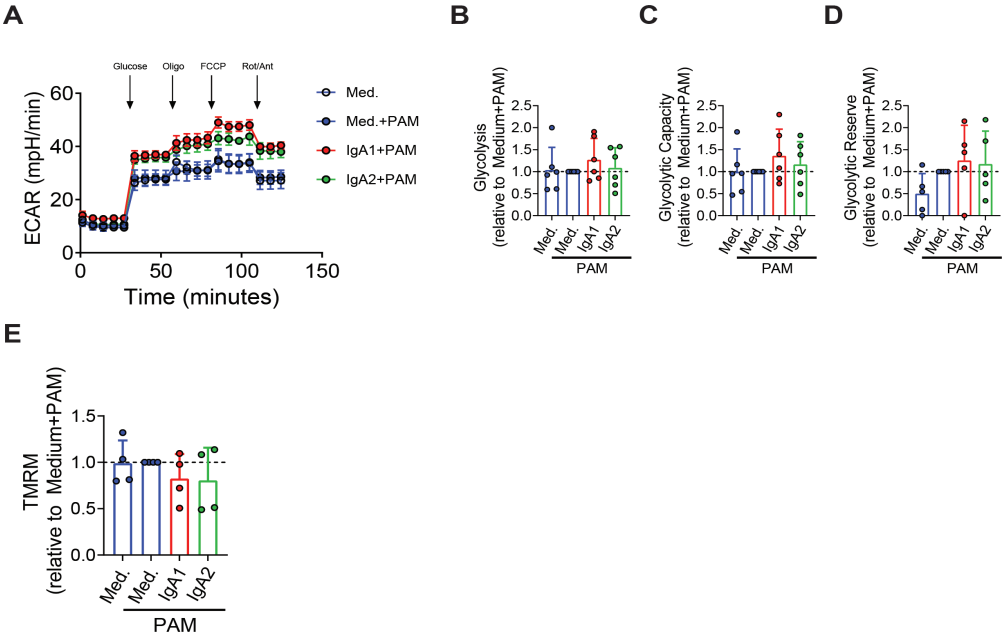

Fig. S4: **IgA2-PAM co-stimulation does not affect glycolysis or mitochondrial membrane potential.** (A) Representative plot of extracellular acidification rate (ECAR) of cells stimulated for 24h with indicated stimuli, measured using an extracellular flux analyser (Seahorse). (B) Basal glycolysis, (C) Glycolytic capacity, and (D) Glycolytic reserve. (E) Mitochondrial membrane potential measured with TMRM (Tetramethylrhodamine). (A)-(D) are representative plots of 6 donors from 3 independent experiments with outliers removed (mean ± SD). (E) is a representative plot of 4 donors from 2 independent experiments (mean ± SD). All data were analysed using a paired one-way ANOVA.

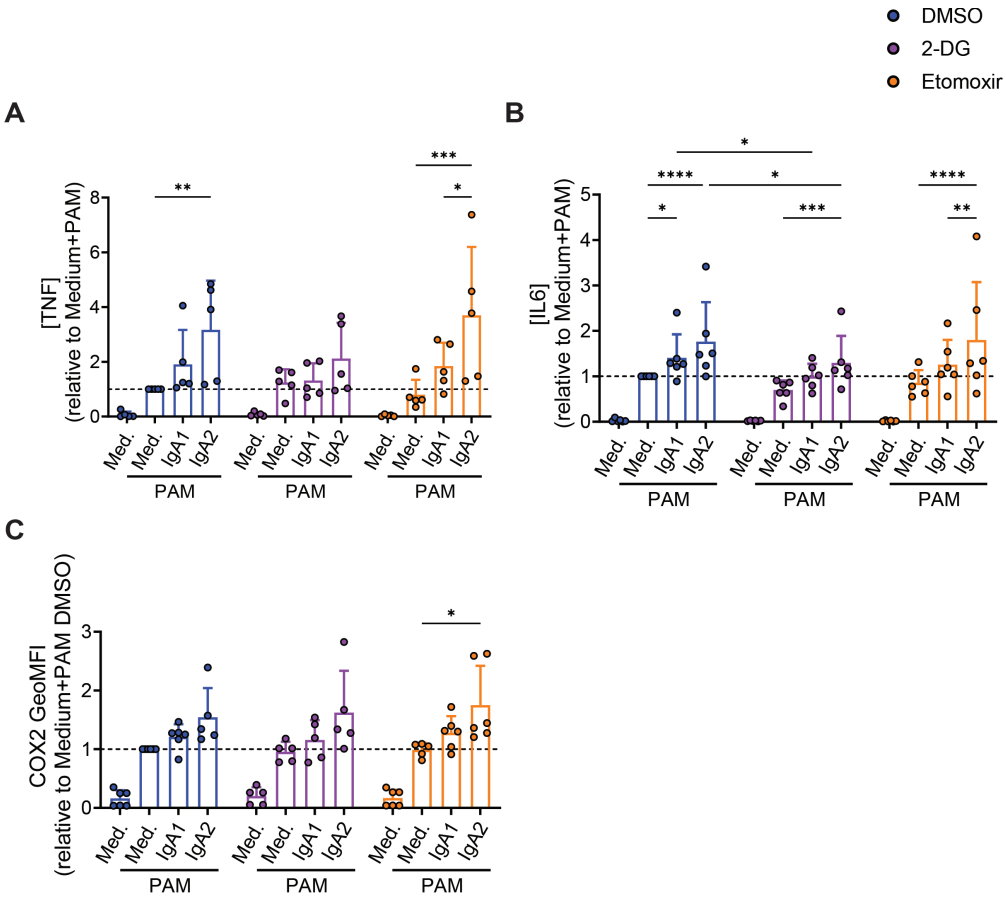

Fig. S5: **Inhibition of glycolysis with 2-DG or inhibition of fatty acid oxidation with Etomoxir does not affect IgA1- and IgA2-dependent inflammation.** Levels of (A) TNF and (B) IL-6 measured in supernatants with ELISA following stimulation of cells with indicated stimuli in the presence of DMSO (blue), 2-DG (purple) or Etomoxir (orange). (C) Normalized COX2 GeoMFI of cells treated with indicated stimuli in the presence of DMSO (blue), 2-DG (purple) or Etomoxir (orange). (A) is a representative plot of 5 donors from 3 independent experiments (mean ± SD). (B)-(C) are representative plots of 6 donors from 3 independent experiments with outliers removed (mean ± SD). Respective DMSO controls are the same as the ones shown in Fig3. All data were analysed using a paired two-way ANOVA, with matched values both stacked and spread across a row, using a Tukey's multiple comparisons test, with a single pooled variance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

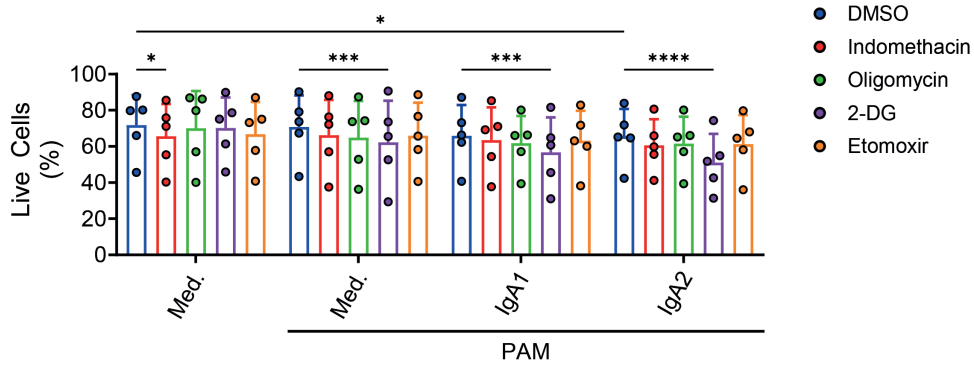

Fig. S6: **Viability of inhibitor-treated cells.** Viability of cells treated with indicated stimuli in the presence of DMSO (blue), Indomethacin (red), Oligomycin (green), 2-DG (purple) or Etomoxir (orange), measured with flow cytometry following Live/Dead staining with Zombie NIR as described in materials and methods. Representative plots of 5 donors from 3 independent experiments (mean ± SD). Data were analysed using a paired two-way ANOVA, with matched values both stacked and spread across a row, using a Tukey's multiple comparisons test, with a single pooled variance. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

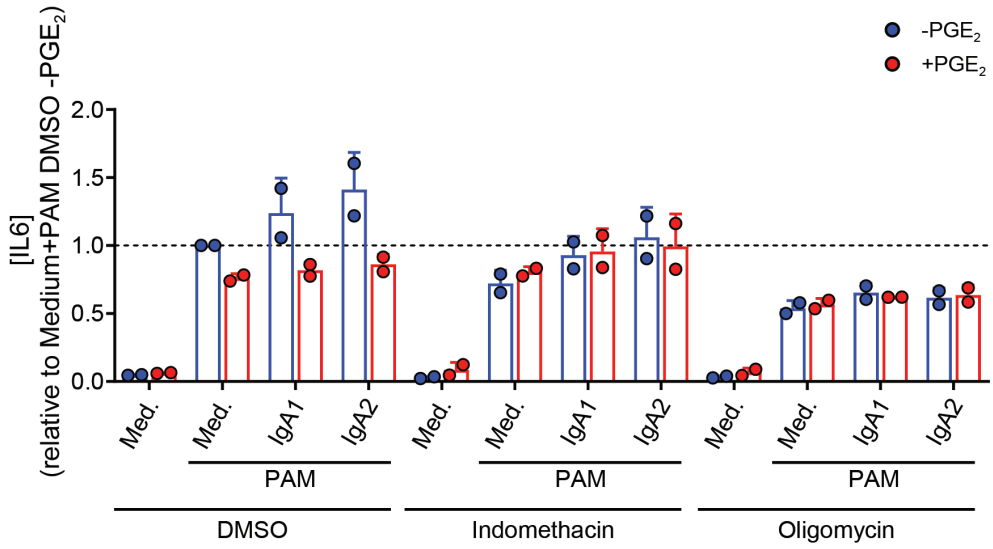

Fig. S7: **Pro-inflammatory potentiation driven by IgA2 is independent of PGE2.** Levels of IL-6 measured in supernatants following stimulation of cells with indicated stimuli in the absence (blue) or presence (red) of exogenous PGE2. Representative plot of 2 donors from 1 experiment (mean ± SD).

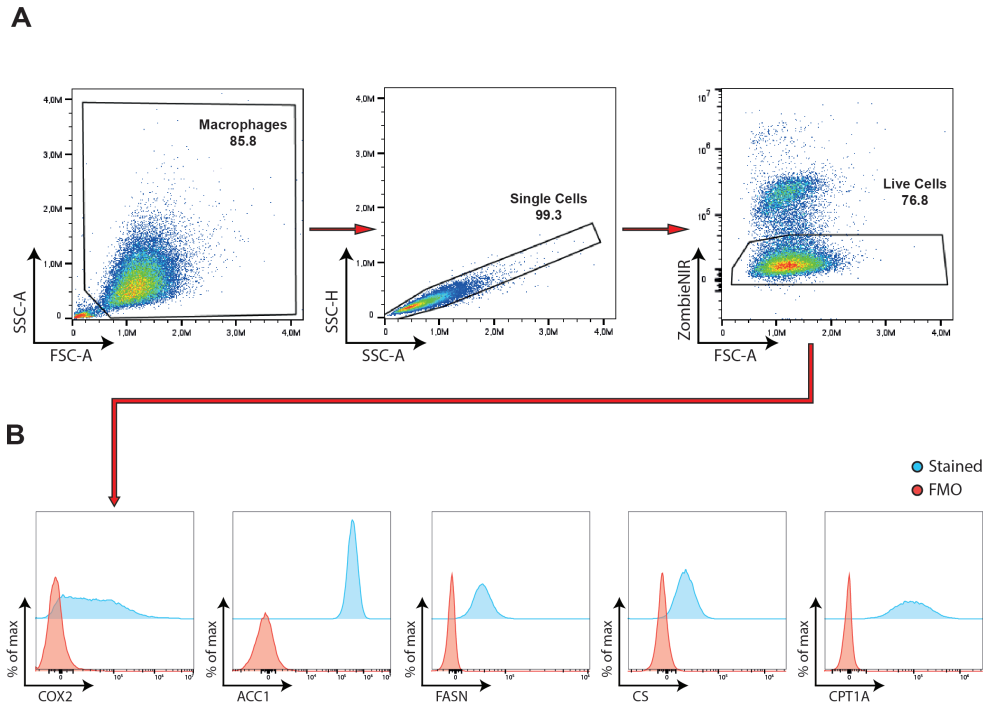

Fig. S8. **Gating strategy and FMOs.** Cells were gated according to FSC-A and SSC-A parameters (A), followed by exclusion of doublets, and selection of live cells, according to Zombie NIR staining. (B) Expression of COX2, ACC1, FASN, CS, and CPT1A, measured via flow cytometry, in live PAM-treated GM-CSF macrophages, compared to respective FMO (Fluorescence Minus One) controls.

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High-Mannose Glycans From *Schistosoma mansoni* Eggs Are Important for Priming of Th2 Responses via Dectin-2 and Prostaglandin E_2

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First published: 29 April 2024, Frontiers in Immunology

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Frontiers in Immunology
DOI: 10.3389/fimmu.2024.1372927

Abstract

The parasitic helminth *Schistosoma mansoni* is a potent inducer of type 2 immune responses by stimulating dendritic cells (DCs) to prime T helper 2 (Th2) responses. We previously found that *S. mansoni* soluble egg antigens (SEA) promote the synthesis of Prostaglandin E_2 (PGE2) by DCs through ERK-dependent signalling via Dectin-1 and -2, that subsequently induces OX40L expression, licensing them for Th2 priming. Yet, the ligands present in SEA involved in driving this response, and whether specific targeting of PGE2 synthesis by DCs could affect Th2 polarization are unknown. We here show that the ability of SEA to bind Dectin-2, drive ERK phosphorylation, PGE2 synthesis, OX40L expression, and Th2 polarization is impaired upon cleavage of high mannose glycans by Endoglycosidase H treatment. This identifies high mannose glycans present on glycoproteins in SEA as important drivers of this signalling axis. Moreover, we find that OX40L expression and Th2 induction are abrogated when microsomal prostaglandin E synthase-1 (mPGES) is selectively inhibited, but not when a general COX-1/2 inhibitor is used. This shows that *de novo* synthesis of PGE2 is vital for the Th2-priming function of SEA-stimulated DCs, as well as pointing to the potential existence of other COX-dependent lipid mediators that antagonize PGE2-driven Th2 polarization. Lastly, specific PGE2 inhibition following immunization with *S. mansoni* eggs dampened the egg-specific Th cell response. In summary, our findings provide new insights in the molecular mechanisms underpinning Th2 induction by *S. mansoni* and identifies druggable targets for potential control of helminth driven-Th2 responses.

Introduction

Helminth parasites are known to provoke strong T helper 2 (Th2) cell–polarized immune responses, which can contribute to protective immunity, but may also lead to immunopathology. Yet, the underlying molecular mechanisms through which helminths activate this type of immune response are still incompletely understood. A better understanding of how Th2 responses are initiated by helminths may help to identify pathways that could be targeted to shape Th2 responses in therapeutic settings, not only in the context of helminth infections, but also inflammatory disorders such as allergies that are characterized by aberrant Th2 responses.

Dendritic cells (DCs) play a highly important role in the immune system by functioning as a bridge between the innate and adaptive immune system. Their specialized role as antigen presenting cells (APCs) allows them to prime responses depending on the pathogen or stimulus encountered (1,2). Upon helminth infection, DCs are key players in inducing the differentiation and activation of Th2 cells (3,4). As such, elucidating the mechanisms by which parasite antigens license DCs to induce Th2 responses will be key to unravel how helminths activate this type of immune response.

Schistosoma mansoni soluble egg antigens (SEA) are one of the most commonly used antigen preparations to study the immune response against helminths (5–7). SEA is complex mixture of highly immunogenic antigens that are capable of activating DCs, driving robust Th2 polarized immune responses (7,8). Within SEA, omega-1 (ω1), a glycoprotein with T2 Ribonuclease activity, has been identified as a key driver of Th2 responses (9), both *in vitro* and *in vivo*, by conditioning DCs for Th2 priming in a mannose receptor-dependent fashion

Importantly however, SEA depleted of ω1 (Δω1-SEA), can still promote a Th2 response, highlighting the existence of additional omega-1-independent mechanisms through which *S. mansoni* eggs can condition DCs for Th2 priming (5). More recently, we identified that components in SEA, independently from omega-1, can trigger a Dectin-1 and -2-dependent signalling pathway, involving Syk-dependent ERK phosphorylation, to increase COX1/2 activity, resulting in elevated oxidation of arachidonic acid and *de novo* PGE2 synthesis. This PGE2 in turn acts in an autocrine manner on DCs to induce OX40L expression, thereby endowing DCs with the ability to prime a Th2 response (10). However, the molecular determinants in SEA that interact with Dectin-1 and -2 to induce this signalling cascade remained unidentified.

Many proteins in SEA are heavily glycosylated and their reported immunomodulatory effects are in many cases glycan dependent (11,12). Correspondingly, on DCs, SEA

has been shown to interact with and signal through several glycan-binding C-type lectin receptors, such as the macrophage galactose-type lectin (MGL), CD209 (DC-SIGN) and CD206 (Mannose Receptor) to modulate TLR-induced cytokine production and T cell-priming capacity (13–17). The main glycan moieties present in SEA that are thought to mediate interaction with these receptors are Galβ1,4(Fucα1,3)GlcNAc (LeX), GalNAcβ1,4(Fucα1,3)GlcNAc (LDNF), and GalNAcβ1,4GlcNAc (LDN) (13,18). However, it is currently unknown which component(s) in SEA act as ligands for Dectin-1 and Dectin-2. Both transmembrane C-type lectins are well known to bind β-Glucan and high mannose glycans, respectively (19,20). As SEA has been shown to contain high mannose glycans (21), and since Δω1-SEA requires Dectin signalling (10), we here explored whether SEA components were capable of binding to, and subsequently activating signalling downstream of Dectin-1- and Dectin-2.

Additionally, it remains to be determined whether pharmacological targeting of the PGE2/OX40L signalling pathway in DCs, in particular PGE2 synthesis, could be used to manipulate the egg-induced Th2 response. We previously found that while antibody-mediated PGE2 neutralization was able to fully block omega-1 independent Th2 priming by DCs, COX1/2 inhibition only had a partial effect (10). This prompted us to explore the possibility that a more targeted pharmacological intervention is needed, i.e. by selectively inhibiting PGE2 synthesis to effectively block Th2 priming.

Materials and Methods

Preparation and purification of S. mansoni egg–derived antigens

SEA and Δω1-SEA from *S. mansoni* eggs were prepared and isolated as described previously (22)

EndoH treatment of SEA and Δω1-SEA

SEA and Δω1-SEA were treated with Endo-H (NEB #P0702S) according to their non-denaturing protocol conditions. Succinctly, for both SEA and Δω1-SEA, 500 µg was treated with Endo-H and 500 µg was mock-treated. Samples were not denatured. Buffer was directly added along with 12.500 units of Endo-H. Samples were then incubated at 37 °C for 24h. Removal of oligomannose N-glycans was confirmed by mass spectrometry (FigS1).

Purification of Mannose-9 from human serum

High mannose glycans were cleaved from human serum proteins, using Endo-H (NEB #P0702S) and incubating at 37°C for 48h. Released glycans were purified by

application of C18 SPE-(J.T.Baker, #7020-03) and Carbon SPE columns (Supelco, #57088) and labelled with anthranilic acid (AA) by reductive amination. To remove labelling reagent excess, Acetonitrile (ACN) was added to a final concentration of 75%, and the sample loaded onto Bio-Gel P10 Gel resin (catalog no.: 1504144; Bio-Rad) previously conditioned with 80% ACN. Glycans were eluted with MQ and dried using a Speedvac. Glycans were then further fractionated using reverse phase HPLC (RP-HPLC) and analysed by MALDI-TOF MS, yielding pure Man9 glycan. Above methodology is described in more detail by Petralia *et al* (23)

AA to AEAB label conversion

2-amino-N-(2-aminoethyl)-benzamide (AEAB) labelled Man9 glycans generated from AA labelled Man9 as previously described (24). In short, 20 µg of both Maltopentose-AA (generated from maltopentose (Sigma #SMB01321) as described for Man9 above) and Man9-AA were mixed with 50 µL EDC (10 mg/mL in DMSO) and 50 µL HOBt (10mg/mL in DMSO). Subsequently, we added 10 µL of 5% (v/v) EDA and 0.5 M MES buffer (pH = 6.5). Samples were then vortexed for 1 min and incubated at room temperature for 3h and then quenched with 1.1 mL of cold ACN. Samples were then vortexed and stored at -20 °C for 30 minutes. The cloudy reaction mixture was centrifuged for 10 min at 10 000 x g. Supernatant was discarded and the precipitate was dried under vacuum. Once dry, it was dissolved in 100 µL of Mili-Q and applied to a RP-HPLC C18 column for purification.

Generation of Man9-labelled NHS gold nanoparticles

Both Man9-AEAB and Maltopentose-AEAB were conjugated to 100nm NHS-Activated Gold Nanoparticles using a kit (Cytodiagnostics #CGN10K-100-1) and following the manufacturers protocol. As a deviation to the protocol, glycans were diluted to 0.1 µg/µL using protein re-suspension buffer and 1x PBS was used instead of conjugate storage buffer. Reaction efficiency was estimated to be ≈58% via RP-HPLC with fluorescence detection to quantify the percentage of recovered uncoupled material.

Human DC culture, stimulation, and analysis

Peripheral blood mononuclear cells were isolated from venous blood of healthy volunteers by density centrifugation in Ficoll as described before (25). Monocytes were isolated by positive magnetic cell sorting using CD14-microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) and cultured in 10% FCS RPMI medium supplemented with 20 ng/mL rGM-CSF (BioSource/Invitrogen, Carlsbad, CA) and 0.86 ng/mL of rIL-4 (R&D Systems, Minneapolis, MN). On days 2-3, medium, including supplements was replaced.

Immature moDCs were stimulated on day 5-6 in the presence or absence (if indicated) of 25 ng/mL ultrapure LPS (Escherichia coli 0111 B4 strain; InvivoGen, San Diego, CA), along with indicated reagents: SEA (20 µg/mL), Δω1-SEA (20 µg/mL), 50 µg/mL Zymosan (Z4250; Sigma-Aldrich, St. Louis, MO), CIII (10 µM), Indomethacin (Sigma-Aldrich #I7378, 50 µM), 5 µg/mL, 2 µg/mL or 0.2 µg/mL of either Man9- or Maltopentose-bound nanoparticles, or inactivated nanoparticles.

After 48h of stimulation, surface expression of co-stimulatory molecules was determined by flow cytometry (FACS-Canto; BD Biosciences, Breda, the Netherlands or Aurora; Cytek, Amsterdam, the Netherlands) using the following antibodies: CD1a (clone HI149), CD14 (clone MΦP9), CD86 (clone 2331 FUN-1), CD40 (clone 5C3), and CD80 (clone L307.4) (all BD Biosciences); HLA-DR (clone LN3) CD83 (clone HB15e) (both eBioscience, San Diego, CA); and CD252/OX40L (clone ANC10G1; Ancell, Bayport, MN). Only live cells that were negative for Zombie NIR (BioLegend Europe BV, Amsterdam, the Netherlands) were included in the analysis. Acquired samples were unmixed using SpectroFlo version 3 (if measured with Aurora), and analyzed with FlowJo.

Human DC and T cell coculture and determination of T-cell polarization

For analysis of T-cell polarization, 5×10^3 moDCs pulsed for 48 h were cultured with 2×10^4 allogenic naïve CD4+ T cells for 7 days in the presence of Staphylococcal enterotoxin B (10 pg/mL). On day 7, T cells were replated and rhuIL-2 (10 U/mL; R&D Systems) was added to expand the T cells. On day 9-10 T cells were split with medium containing the same concentration of rhuIL-2. Intracellular cytokine production was analyzed on day 12 after restimulation with 100 ng/mL phorbol myristate acetate, 2 µg/mL ionomycin and 10 µg/mL brefeldin A for 4 h. Subsequently, the cells were fixed with 2% paraformaldehyde (all Sigma-Aldrich). The cells were permeabilized with permeabilization buffer (eBioscience #00-5523-00) and stained with antibodies against IL-4 and IFN-γ, respectively (BD Biosciences). Acquired samples were unmixed using SpectroFlo version 3 (if measured with Aurora), and analyzed with FlowJo.

Measurements of PGE2 levels in culture supernatants

Lipid mediators (LM) and polyunsaturated fatty acids (PUFA) were measured using reverse-phase liquid chromatography coupled to tandem mass spectrometry (RPLC-MS/MS) as previously described (26), with some modifications. Briefly, 2 µL internal standard (IS) mix of deuterated lipid standards consisting of PGE2-d_4, 15-HETE-d_8, Leukotriene B_4-d_4, DHA-d_5, 8-iso-PGF2a-d_4 and 14(15)-EET-d_{11} (50 ng/mL in MeOH) were added to 400 µL culture supernatants. Lipids were extracted and purified by solid phase extraction (SPE) after protein precipitation with 1.2 mL MeOH. The dried

extracts were reconstituted in 100 µL 40% MeOH and transferred into a micro vial glass insert. 40 µL sample was injected and analyzed using a Shimadzu Nexera LC40-system with an autosampler coupled to a QTrap 6500 mass spectrometer (Sciex). Kinetex C18 50 × 2.1 mm, 1.7 µm column and C8 precolumn (Phenomenex) were used for LC separation. LC-MS/MS chromatograms were integrated manually using Sciex OS (Sciex). The results were reported as relative peak area of lipids to the internal standards. PGE2-d_4 IS was used for reporting area ratios of PGE2, TxB2 and PGF2a.

ERK phosphorylation

For detection of ERK phosphorylation (pERK), 2.5×10^4 immature moDCs were seeded overnight in a 96-well flat-bottom plate. moDCs were stimulated with SEA (25 µg/mL), Δω1-SEA (25 µg/mL), for indicated periods, and the moDCs were fixed for 15 min with 4% ultrapure formaldehyde (Polysciences, Warrington, PA) directly in the plate. The cells were harvested and washed first with PBS and then with 0.5% of saponin for permeabilization. Cells were intracellularly stained with anti–phospo-p44/42 MAPK (Erk1/2) (clone E10) (both Cell Signalling Technology). Following 2 h incubation at room temperature, cells were washed with 0.5% of saponin, and ERK phosphorylation was determined by flow cytometry.

Dectin ELISA

96-well high-binding half-area microplates (Corning #10052511) were used for the Dectin-1/2-hFc binding ELISAs. The appropriate antigens were coated in 50 µL TSM (20mM Tris-HCl, 150mM NaCl, 2mM $CaCl_2$ and 2mM $MgCl_2$ at pH 7.4) overnight at 4°C; SEA of *Schistosoma mansoni* (50 µg/mL), Mock or Endo-H treated SEA of *Schistosoma mansoni* (50 µg/mL), Zymosan (20 µg/mL), NHS gold nanoparticles (5 µg/mL), or 1% BSA in TSM. After overnight coating, the plate was washed 3 times with an excessive amount of TSM/0.005% Tween. After washing, the plate was blocked for 1 h with 100 µL TSM/1% BSA at room temperature. After blocking, the plate was washed 3 times, again with an excessive amount of TSM/0.005% Tween. Following the washing, 50 µL Dectin-1-hFc (Sino Biological #10215-H01H) or Dectin-2-hFc (Sino Biological #10250-H01H) at a concentration of 10 µg/mL in TSM/0.005% Tween was incubated for 2h at room temperature. After incubation, the plate was washed 5 times again with an excessive amount of TSM/0.005% Tween. Next, 50 µL of Monoclonal Biotin Mouse anti-Human IgG1-Fc (Invitrogen #05-3340) (1:500 in TSM/0.005% Tween) was added to the plates, along with HRP-Strep (BD 51-9002813) and incubated for 1 h. The plate was then washed again for 6 times. We used 50 µL of TMB ELISA substrate solution (ThermoFisher #34021) for 30 min, followed by 25 µL of H_2SO_4 1.8 M to stop the coloring reaction. ELISA readout was performed at 450 nm, with absorbance correction at 570 nm, by MultiskanTM FC Microplate Photometer

(ThermoFisher, type 357).

Mice

Wild type (WT) mice, both male and female and all on a C57BL/6J background, were bred under SPF conditions at the Leiden University Medical Center (LUMC), Leiden, The Netherlands. Mice were culled through cervical dislocation. Animal experiments were performed when the mice were between 8 and 16 weeks old. Animal experiments were performed in accordance with local government regulations, EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, as well as approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD). Animal license number AVD116002015253.

SEA immunization

Mice were injected subcutaneously with 5000 *S. mansoni* eggs, in the hind footpad, and either injected i.p. with vehicle, or 1 mg CIII/20 g of body weight. Injections with either CIII or vehicle were then repeated on day 2 and day 4 post-immunization. Seven days later, mice were sacrificed and cells from both draining and nondraining lymph nodes were isolated and analysed as described below.

Analysis of murine T-cell responses

Antigen-specific responses were determined by culturing 5×10^5 LN cells per well in high binding 96-well flat-bottom plates (Corning #3590) in 200 µL complete medium (RPMI containing 10% FCS, 100 U/mL penicillin/streptomycin, and 2 mM L-glutamine) in the presence of 20 µg/mL SEA along with 2.5 µg/mL IL-4R blocking antibody to retain IL-4 in culture supernatants. After 48 h, culture supernatants were stored for cytokine determination. Cell culture supernatants were analyzed for cytokines using the Cytokine Bead Array (BD) according to the manufacturer's recommendation. Samples were analyzed on a BD Canto II Flow Cytometer. Alternatively, cytokine production was assessed by intracellular staining of T cells from LNs after polyclonal restimulation in 96-well flat-bottom plates for 4 h with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL), ionomycin (1 µg/mL), and brefeldin A (10 µg/mL; all from Sigma-Aldrich). Afterwards, cells were fixed with 2% PFA and subsequently stained in eBioscience permeabilization buffer: IL-4 (11B11), IFN-γ (XMG1.2), IL-13 (eBio13A), IL-17A (TC11-18H10.1), and CD4 (RM4-5), IL-10 (JES5-16E3) (all BD Bioscience or BioLegend). Samples were analyzed on a BD Canto II Flow Cytometer or Cytek Aurora. Acquired samples were unmixed using SpectroFlo version 3 (if measured with Aurora), and analyzed with FlowJo.

Statistical analysis

Data were tested for normality using the Shapiro–Wilk test. Statistical tests used are indicated in the figure legends. Generally, data were compared using one-way ANOVA for more than two groups, or two-way ANOVA for comparing multiple parameters across two or more groups, with Tukey's post-hoc test for multiple comparison. If comparing parameters within the same sample, a paired or repeated-measures test with Geisser-Greenhouse correction was used. p values < 0.05 were considered significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). All statistical analyses were performed using GraphPad Prism v.9.0.

Results

SEA Binds to Dectin-2 in a High-Mannose-Dependent Manner

We previously reported that blocking antibodies against Dectin-1 and -2 in DCs were able to impair omega-1-independent Th2 priming by SEA (10). However, it was not assessed whether components in SEA were directly interacting to Dectin-1 and/or -2 to promote this response. To determine this directly we performed a binding ELISA with a construct consisting of the carbohydrate binding domain of Dectin-1 or Dectin-2 coupled to a human IgG1 Fc domain. Zymosan, a known ligand for Dectin-1 and -2 was taken along as positive control. We observed that both Dectin-1 and Dectin-2 were directly able to bind to components present in SEA (Fig1. A, B).

These receptors are C-type lectins that preferentially bind to sugar residues. It was shown in previous studies (8) that high mannose glycans (e.g. Man9), known to be Dectin-2 ligands (20,27), are present in high frequency in the mixture. To assess if the presence of these high mannose moieties was required for SEA to interact with Dectin-1 and -2, we treated SEA with endoglycosidase-H (EndoH), to specifically remove N-linked oligomannose glycans, including high mannose, without affecting complex N-linked glycans or O-linked glycans (FigS1). EndoH hydrolyses the glycosidic linkage of high mannose glycans between GlcNAc1 and GlcNAc2, resulting in free glycans with only one GlcNAc residue present in the core, while PNGase-A cleaves off glycans between GlcNAc 1 and asparagine, resulting in free glycans with two GlcNAc residues present in the core. The success of EndoH treatment can thus be confirmed in the top spectrum due to the absence of high mannose glycans with two GlcNAc residues., indicating that all high mannose glycans were released by Endo-H before the PNGase-A treatment.

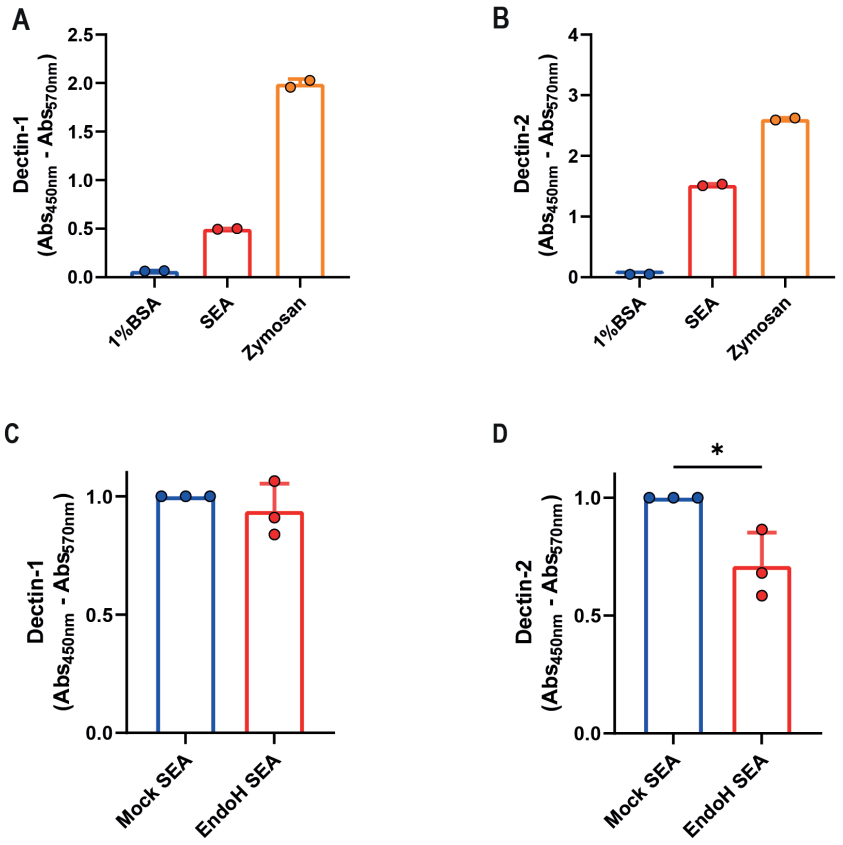


Figure 1. Dectin-1 and Dectin-2 directly bind to SEA
Binding ELISA of indicated molecules was performed as described in Materials and Methods for (A) Dectin-1, and (B) Dectin-2. Binding ELISA of indicated molecules for (C) Dectin-1 and (D) Dectin-2. (A), (B), are representative plots of 3 experiments (n = 2 per experiment, mean ± SD). (C) and (D) represent pooled data of 3 independent experiments shown with data normalized by the Mock SEA condition and compared using a paired one-tailed t-test (n = 2 -3 per experiment, mean ± SD). *p < 0.05.

As assessed in a Dectin-1 and -2 binding ELISA, the hydrolysis of high mannose glycans in SEA did not affect binding by Dectin-1. However, this treatment did significantly decrease binding of SEA by Dectin-2, suggesting that SEA directly interacts with the latter in a partly high mannose-dependent manner (Fig1. C, D).

High-Mannose Glycans in SEA Are Required, but Not Sufficient, to Induce PGE2 and OX40L Expression and Th2 Priming by moDCs

Considering SEA requires the presence of oligomannose glycans, including Man9, to bind to Dectin-2, we wondered if the presence of these glycans was important for induction of the signalling cascade leading to OX40L expression and Th2 polarization

by moDCs. EndoH-treated SEA lost its ability to induce ERK phosphorylation (Fig2. A), OX40L expression (Fig2. B), and subsequent Th2 polarization by moDCs (Fig2. C). The latter two readouts were performed in the presence of LPS as neutral DC maturation factor (5). Correspondingly, the synthesis of PGE2 was also reduced in moDCs stimulated with EndoH-treated SEA (Fig2. D). These effects were not confounded by the presence of omega-1 (ω1) as moDCs stimulated with SEA depleted of ω1 (Δω1-SEA), which had undergone EndoH treatment, were also compromised in their ability to induce a Th2 response compared to DCs stimulated with mock treated control Δω1-SEA (Fig2. E). These data suggest that high mannose residues are important for ω1-independent Th2 polarization by SEA.

To investigate if high mannose glycans themselves are sufficient to recapitulate the effects of SEA, we coupled AEAB-labelled mannose-9 (Man9) oligosaccharides isolated from human serum to N-Hydroxysuccinimide (NHS)-activated gold nanoparticles. Interestingly, while these loaded nanoparticles were efficiently covered with high mannose glycans (FigS2. A-D) and were able to directly bind to Dectin-1 and -2 as determined by ELISA (FigS3. A, B), they were not able to replicate the effects of SEA on moDCs in terms of inducing OX40L on moDCs or condition them for Th2 polarization in a concentration of glycans labelled to NPs ranging from 0.2-5 μg/mL (Fig2. F, G). This indicates that, while high mannose glycans are required for the activation of the dectin-OX40L axis by SEA, they are not sufficient to activate this pathway.

Selective Inhibition of PGE2 Synthesis Impairs OX40L Expression, and Th2-Priming by moDCs

We previously found that SEA-driven Th2 polarization via Dectin-1/2 is critically dependent on PGE2, one of the downstream products of COX. However, in contrast to PGE2 neutralization experiments, COX inhibition was only able to modestly decrease Th2 priming (10). We wondered whether this could be explained by the fact that COX inhibition does not only inhibit PGE2 synthesis, but also affects the synthesis of other COX products that may affect the Th2 priming capacity of DCs. To test this and avoid this potentially confounding issue, we used CIII, an inhibitor that specifically targets microsomal Prostaglandin E synthase-1 (mPGES) without affecting other COX-derived products (28) (FigS4).
Corresponding with inhibition of PGE2 synthesis by both drugs, their incubation reduced the Δω1-SEA-driven expression of OX40L by moDCs (Fig3. A). While no statistical difference was found when comparing CIII to Indomethacin ($p = 0.442$) (Fig3. B), CIII treatment significantly lowered the Th2-priming ability of Δω1-SEA-treated moDCs. In contrast, this was not the case for treatment with Indomethacin, suggesting that targeting PGE2 synthesis itself is superior in modulating Th2 priming in this setting to targeting COX further upstream.

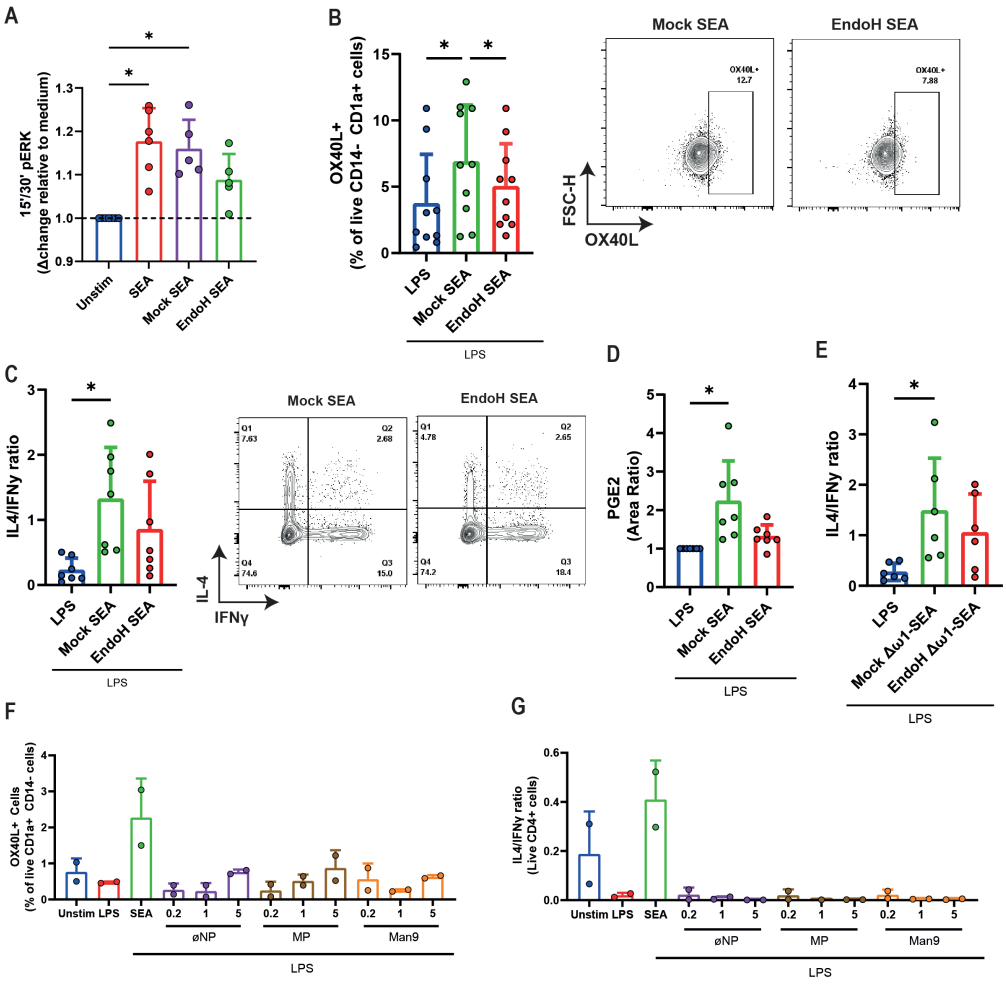


Figure 2. Man9 is required, but not sufficient, to induce OX40L in moDCs and subsequent Th2-priming

(A) ERK phosphorylation was measured with FACS after stimulating moDCs for 15 and 30 minutes with the indicated stimuli. (B) OX40L expression by moDCs was measured via FACS in m after 48h of stimulation with mock-treated SEA or with EndoH-treated SEA, all in the presence of LPS. (C) Th1- and Th2-priming abilities of moDCs treated with indicated stimuli were analysed as described in Materials and Methods. The ratios of IL4+IFNy- percentage over IL4-IFNy+ percentages are based on intracellular staining following PMA/Iono/BrefA stimulation. (D) PGE2 levels (measured in area ratio) in supernatants from moDC cultures after stimulation with indicated stimuli. (E) IL4/IFNy ratio of CD4+ T cells primed by moDCs treated for 48h with indicated stimuli. (F) moDCs were stimulated with inactivated nanoparticles (øNP) Maltopentose-coated nanoparticles (MP) or Man9-coated nanoparticles and OX40L expression was measured via FACS. (G) Th1- and Th2-priming abilities of moDCs treated with indicated nanoparticles were also measured, as mentioned in (C) and (E). (A)-(D) data points represent individual donors pooled from 3-5 experiments with data compared using a paired one-way ANOVA (n = 5-10, mean ± SD). (F) and (G) data points represent data from 2 individual donors. *p-value <0.05.

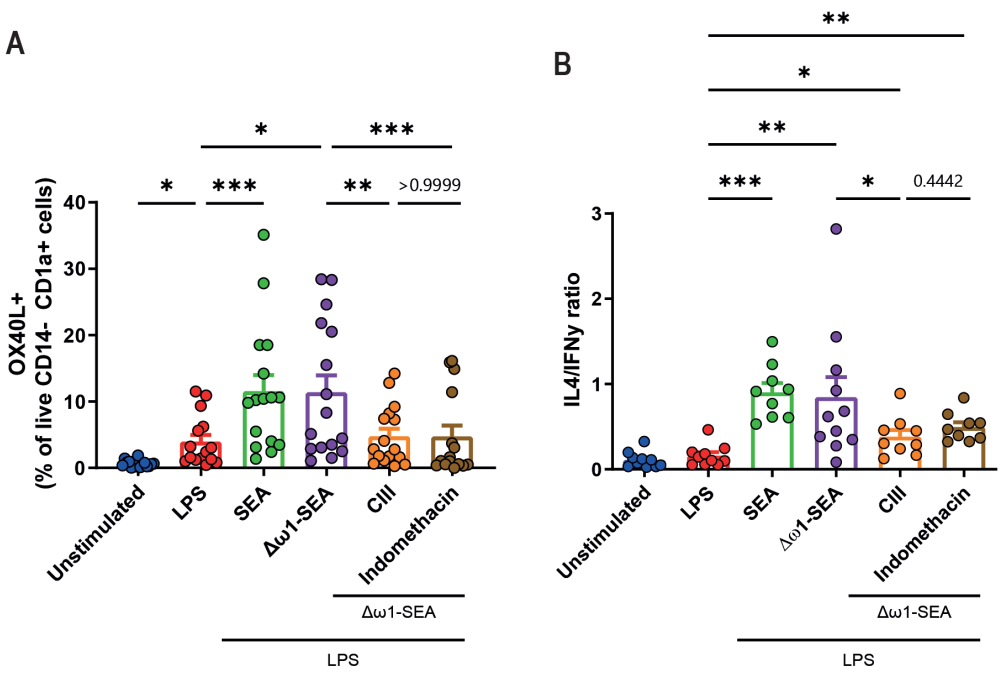


Figure 3. Selective inhibition of mPGES, but not general COX inhibition, in moDCs reduces Th2 priming following SEA stimulation
(A) OX40L expression in moDCs following 48h stimulation with indicated stimuli and (B) Th1- and Th2-priming abilities as described in Fig2. Data points in (A)-(B) represent data 7 to 16 individual donors. Data were compared using a paired one-way ANOVA (median ± SD). *p <0.05, **p < 0.01 ***p < 0.001.

Inhibition of PGE2 Synthesis Impairs the T Cell Response to S. mansoni Eggs In Vivo

In view of these results we obtained *in vitro*, we wondered if PGE2 synthesis inhibition would also reduce Th2 polarization *in vivo*. To test this, we injected *S. mansoni* eggs in the footpad of wild type mice, followed by i.p. injections of CIII day 0, 2 and day 4 post-challenge. On day 7 mice were sacrificed and the $CD4^+$ T cell response in the draining and non-draining lymph nodes was characterized.

Mice challenged with *S. mansoni* eggs and treated with CIII displayed an overall lower number of $CD4^+$ T cells in draining LNs (Fig4. A), resulting in a lower number of IL4-, IL13-producing Th2-polarized T cells upon polyclonal restimulation, when compared to egg immunized mice injected with the vehicle control. However, this was also true for the number of IFNγ- and IL-17-producing Th cells. As a consequence, CIII treatment did not alter the ratio between IL4- and IFNγ-producing $CD4^+$ T cells (Fig4. B-F) No difference was seen in IL10-producing $CD4^+$ T cells (Fig4. G). To assess antigen-specific cytokine responses, cells that had been isolated from the LNs of

immunized mice were restimulated with SEA. While no difference was seen in the IL-4 response (Fig4. H), cell cultures from egg immunized mice receiving CIII displayed a near significant lower IL-13 (Fig4. I) and a decreased IFNγ (Fig4. J) response to the antigens, while the IL-17 (Fig4. K) and the IL-10 (Fig4. L) levels remained unaffected. This indicates that optimal priming of Th2 cell responses upon *S. mansoni* egg challenge *in vivo*, but also that of other concomitant egg-induced Th cell responses, rely on *de novo* PGE2 synthesis.

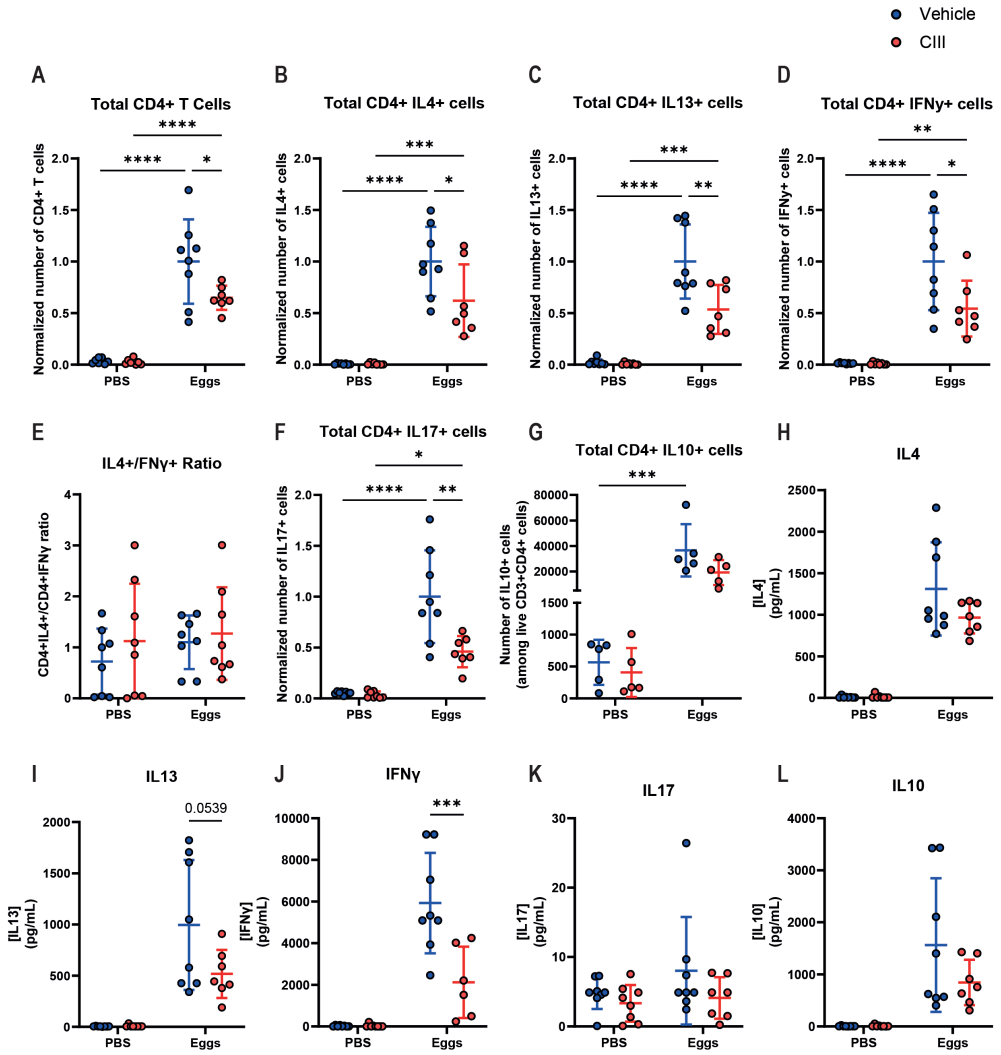


Figure 4. *De novo* PGE2 synthesis is required for optimal induction of CD4⁺ T cell responses by *S. mansoni* eggs *in vivo*

(A) Total number of live CD4⁺ T cells in non-draining and draining lymph nodes in mice injected with CIII or Vehicle following injection with *S. mansoni* eggs, and total number of live CD4⁺ T cells producing IL4 (B),

IL13 (C), and IFNy (D), along with the ratio of IL4+/IFNy+ (E) are shown. CD4+ T cells producing IL17 (F), and IL10 (G) were also measured. (H)-(L) Antigen-specific cytokine production of indicated ex vivo T cells was measured using a CBA assay in supernatant collected after 24h stimulation with SEA. Data points represent individual mice from 2 experiments. Data were compared using a 2-way ANOVA (n = 7-8, mean ± SD). Number of cells was normalized by using the average of vehicle + eggs condition from each respective experiment. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Discussion

It has previously been shown that SEA drives type 2 immune responses, even if depleted of ω-1, one of its main Th2-inducing molecules (5,10). While it was demonstrated that this ω-1-independent Th2-priming was reliant on dectin signalling and PGE2 synthesis in DCs, the ligands in SEA that trigger this signalling cascade had not been identified, nor had it been determined if selective chemical inhibition of PGE2 synthesis in DCs would impair their Th2-priming abilities following SEA stimulation.
Here we have shown that Dectin-1 and -2 are able to directly bind components in SEA, and that for Dectin-2 this is in part dependent on the presence of high mannose N-glycans. Dectins are traditionally associated with anti-fungal responses (29–31), however we show that both Dectin-1 and Dectin-2 can also bind to helminth-derived glycoproteins. This aligns with previous work showing that both Dectin-1 and Dectin-2 play a role in driving the immune response against other helminths (32,33).

While Dectin-2 is known to bind to mannose residues (20,27), we have demonstrated that Dectin-1, which typically binds to β-Glucans (34), can also bind to SEA, albeit in a high mannose-independent manner. The motifs in SEA behind Dectin-1 binding remain elusive, as SEA does not contain β-glucans typically found in fungi (21). However, there is data showing that Dectin-1 can bind to N-glycans present on tumour cells (35), and to an unknown ligand present in T cells that was resistant to tunicamycin/N-glycosidase treatment, but susceptible to trypsin treatment (36), suggesting Dectin-1 may bind to other carbohydrates than classically thought, and perhaps even non-glycan components.

The importance of high mannose glycans present in SEA in mediating binding to Dectin-2, was further extended in functional studies in which treatment of SEA with the enzyme EndoH, to hydrolyse high mannose glycans from its glycoproteins, not only reduced Dectin-2 binding but also translated into lower pERK levels, PGE2 synthesis, OX40L expression, and, subsequently, impaired Th2-priming ability by moDCs. However, while we here provide evidence for a requirement of high mannose glycans in promoting this Dectin-2/PGE2/OX40L signalling axis by SEA, these glycan moieties alone do not appear to be sufficient to drive this response, as loading gold nanoparticles with Man9 residues did not mimic the effects seen with SEA. This might

be due to possible differences in coating density of Man9 glycans between the native proteins and the nanoparticles (37), as glycan density may influence the extent to which multimers of Dectin-2 (or other glycan-binding receptors) can be formed, which can affect the signalling strength downstream of those receptors (38). Alternatively, there might be a contribution of an unknown co-receptor engaged by other glycans or proteins in SEA to the signalling cascade. For instance, we previously reported that CD206 on moDCs is needed for optimal expression of PGE2 following SEA stimulation (10), which may suggest that Dectin-2 may act in concert with other glycan-binding receptors to drive this response. Finally, a not mutually exclusive possibility is that Man9 facilitates Dectin-2-dependent endocytosis of a carrier protein that subsequently modulates DC function for Th2 priming, analogous to what previously had been described for ω-1, that requires its glycans to be internalized after which its ribonuclease activity modulates DC function (9).

In addition, we demonstrate that selective chemical inhibition of PGE2 synthesis is superior in inhibiting Th2 polarization by DCs stimulated with helminth antigens, compared to targeting COX. Some conflicting results have been published regarding the effects of COX2 inhibition on Th1 and Th2 responses. While some studies indicate COX2 activity/PGE2 synthesis induce a Th2 response (39–42), some point to the opposite, showing instead an inhibition of the Th2 response and an induction of Th1 activity (43–46). We postulate that these diverse outcomes in part stem from effects on synthesis of lipid mediators downstream of COX2 other than PGE2, such as PGD_2 and PGI_2, which have been shown to exert diverse immunomodulatory effects , that includes modulation of Th1 and Th2 differentiation (47–54).

These *in vitro* findings were largely recapitulated *in vivo*, as targeted PGE2 inhibition reduced the Th2 response following immunization with *S. mansoni* eggs. We found there was lower Th2 cell expansion in immunized mice treated with the inhibitor, as well as reduced Th2 cell cytokine production following antigen-specific restimulation. Of note, also egg-induced Th1- and Th17-associated cytokine production was reduced, suggesting a more general dampening effect of mPGES inhibition on *S. mansoni* egg-driven Th cell priming, that is not limited to the Th2 response specifically. It is known that PGE2 signalling can contribute to DC maturation, antigen uptake, and migration to lymph nodes (55–57), and, as such, additional studies would be required to evaluate to what extent the effects of PGE2 inhibition on T cell priming, in the context of *S. mansoni* egg challenge, are secondary to changes in DC biology as a whole.

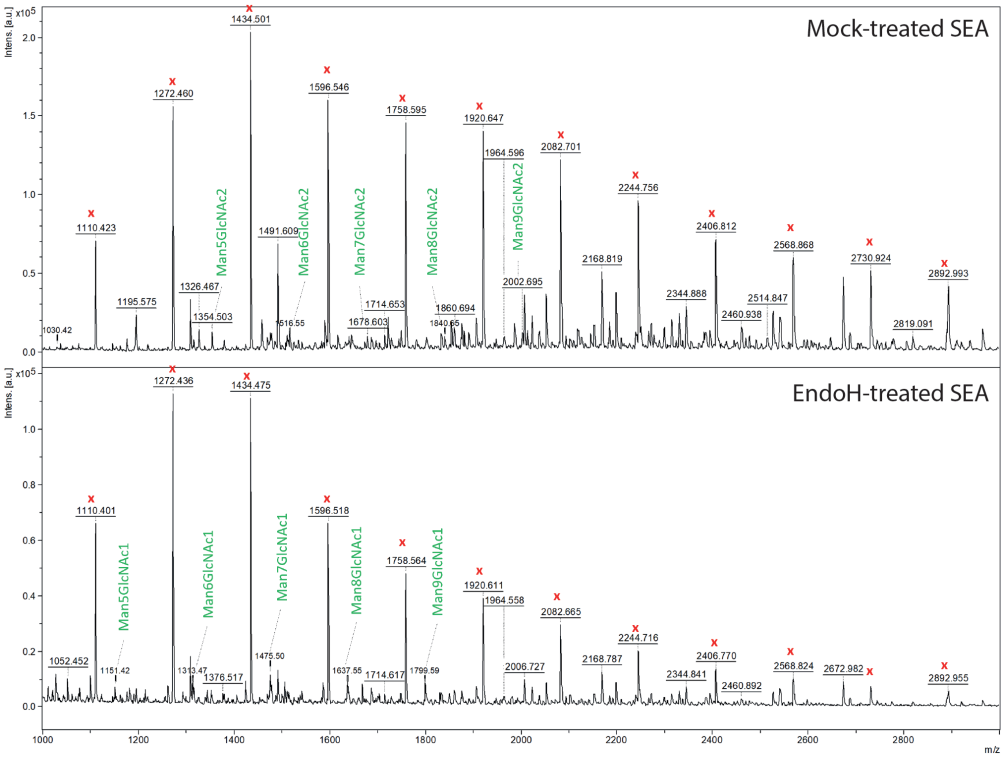
In conclusion, we show here that SEA is able to directly bind to Dectin-1 and -2 on moDCs, the latter in a partially high mannose-dependent manner. We also reported on the importance of high mannose residues present in SEA in inducing the

previously identified PGE2/OX40L signalling axis (10), that licenses DCs to promote a Th2 immune response. Additionally, we found that the specific targeting of PGE2 synthesis, by chemical inhibition of mPGES, is able to impair Th2 priming by DCs both *in vitro* and *in vivo*. As the expression of high mannose glycans is shared with several other parasitic helminths, such as *Fasciola hepatica* (58) and *Brugia malayi* (23), it will be interesting in future studies to explore whether high mannose glycan-driven Dectin-2/PGE2/OX40L signalling axis is a more common pathway through which helminths elicit Th2 responses. Finally, our data provide first proof of principle that targeting PGE2 synthesis with specific chemical inhibitors could be a strategy to dampen pathological type 2 immunity in the context of schistosomiasis, but possibly also in other type-2 immunity driven diseases, such as allergies.

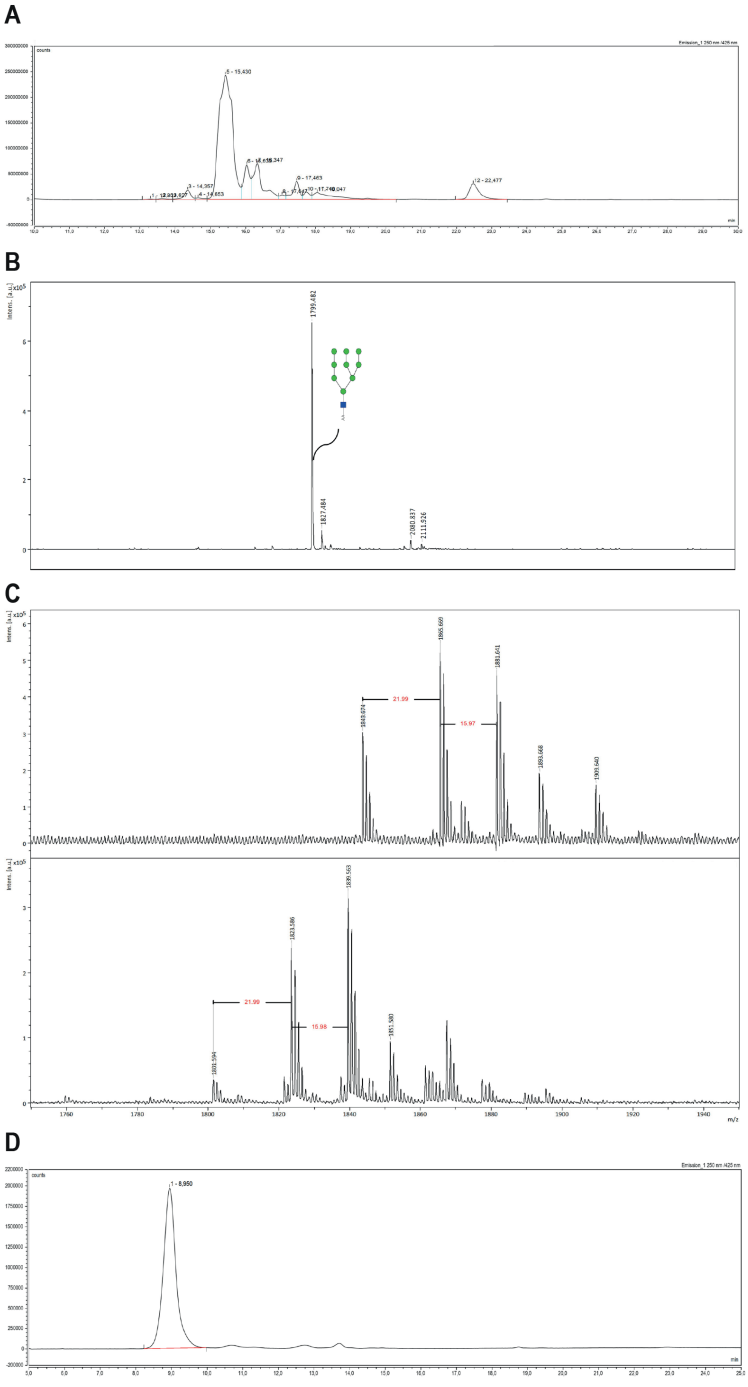
Acknowledgements

This work was supported by funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant agreement No. 812890. Many thanks to Jochem Grossouw for his contributions, and to our colleagues at the department of Parasitology from the LUMC and Dr. Luís Almeida from Mainz University for their continual scientific discussions. We would also like to acknowledge the LUMC Flow Core Facility operators, for the continual maintenance and troubleshooting of the Cytek Auroras.

Supplementary Material



Supplemental Figure 1. MALDI-TOF MS of Mock- and EndoH-treated SEA N-glycans
MALDI-TOF mass spectra of PNGase-A released AA-labelled N-glycans of Mock (top) or EndoH (bottom) treated SEA.



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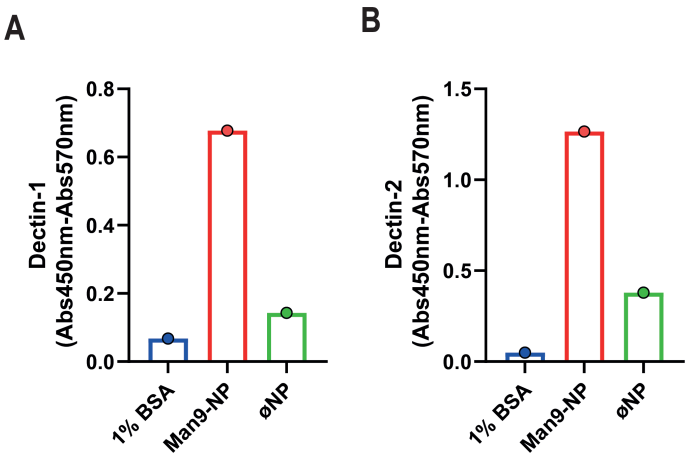
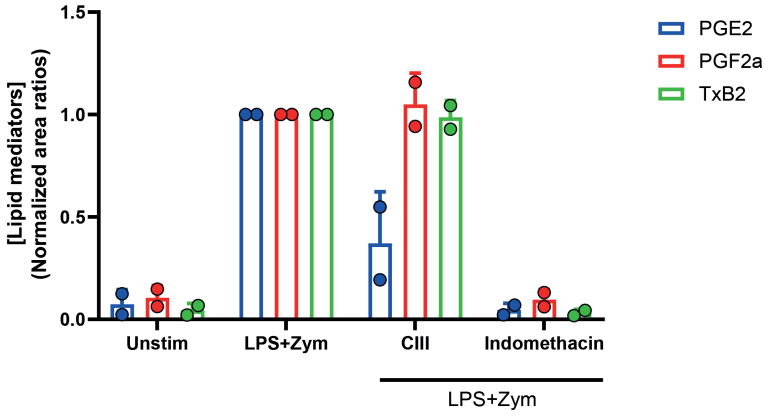
Supplemental Figure 2. Purification of Man9 from human serum and AEAB labelling
(A) RP-HPLC profile of high mannose glycans cleaved from human serum with EndoH, and (B) MALDI-TOF mass spectrum of Man9 after purification, as described in materials and methods. (C) MALDI-TOF mass spectra of AEAB-labelled Man9 (top) and AA-labelled Man9 (bottom). Successful AEAB labelling, as described in materials and methods, can be identified by an increase of 42 m/z ratio of the peaks, with Man9-AEAB having a m/z ratio of 1843.674 (top) and Man9-AA a m/z ratio of 1801.594 (bottom). (D) RP-HPLC profile of Man9-AEAB generated and purified as described in materials and methods.



Supplemental Figure 3. Nanoparticle Dectin binding ELISA
Dectin-1 (A) and Dectin-2 (B) binding ELISA to Man9-coated nanoparticles (Man9-NP) or inactivated nanoparticles (øNP). Data are from one experiment.



Supplemental Figure 4. Effects of COX and mPGES inhibitor on lipid species synthesis
(A) PGF2a, PGE2 and TxB2 concentrations in supernatants from moDC cultures after stimulation with indicated reagents. Cells were stimulated with Zymosan and LPS to induce high levels of COX-dependent lipid species, along with either CIII to inhibit mPGES or Indomethacin to inhibit COX. To confirm the specificity of CIII for inhibition of PGE2 synthesis, we measured PGE2 levels, but also mPGES-independent, COX-dependent lipids, such as PGF2a and TxB2 and compared the effect of CIII to Indomethacin. Data points represent data from 2 individual donors.

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VI

General Discussion

Introduction

In this thesis we have touched upon the topics of both cell intrinsic and paracrine effects of lipid metabolism (Fig. 1). In **chapters 3** and **4** we described how IgG and IgA induce distinct metabolic reprogramming via Fc receptors, with IgG needing FA synthesis, and IgA requiring mitochondrial respiration and COX2 activity to boost macrophage inflammatory responses. Finally, in **chapter 5** we reported how pathogen-derived glycans, following binding to Dectin-1 and -2, instruct DCs to oxidize lipids via COX 2, resulting in the synthesis of PGE_2 followed by autocrine signalling, licensing DCs for Th2 priming.

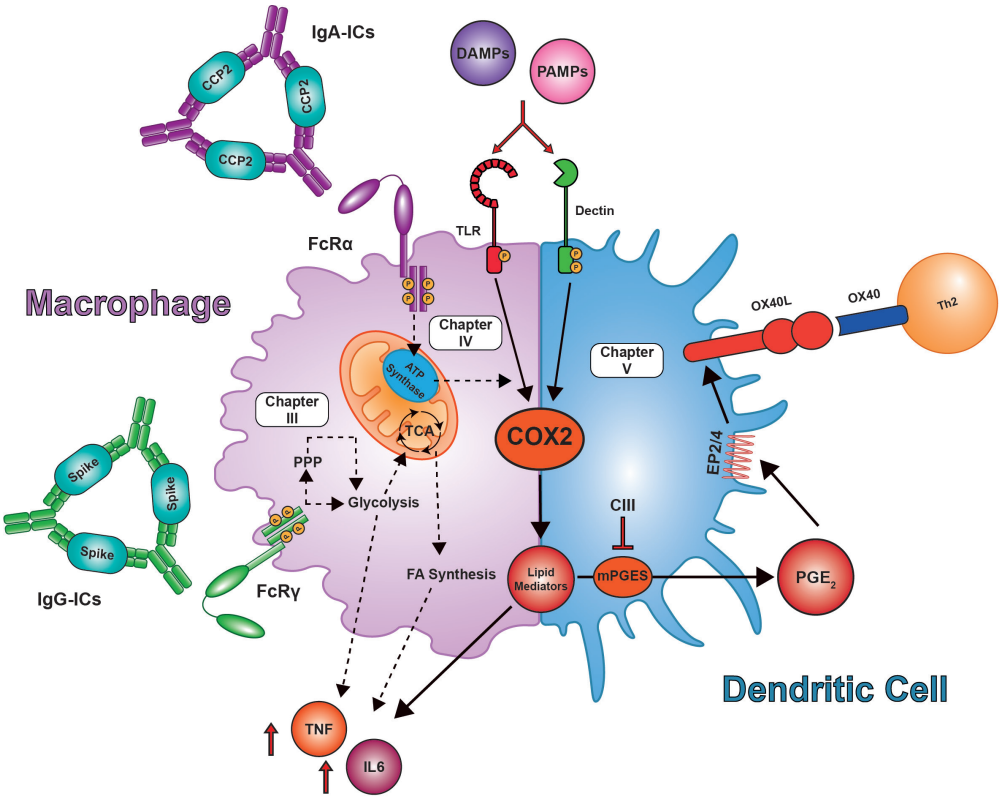

Figure 1: Macrophage and Dendritic Cell functions are regulated by metabolism and lipid mediators. Anti-Spike IgG-ICs reprogramme macrophage metabolism via Glycolyis, PPP and FA synthesis to induce a hyper-inflammatory state. Similarly, anti-CCP2 IgA-ICs boost the inflammatory potential of macrophages via ATP synthase and COX2, resulting in increased synthesis of lipid mediators, TNF and IL6. In DCs, antigens from *S. mansoni* eggs bind to Dectin and induce OX40L expression and subsequent Th2 priming in a PGE_2-dependent manner, which can be chemically targeted with CIII, a specific inhibitor of mPGES.

Lipid Metabolism Within Myeloid Cells

The work presented in this thesis point to a central and vital role of intrinsic lipid metabolism in driving myeloid cell immune responses, and showcases its plasticity, resulting in drastically different immune cell phenotypes, depending on the stage of cellular differentiation and which stimulus the cell encounters.

We have seen that antibodies induce a different metabolic reprograming in macrophages via FcR engagement, depending on both their isotype and subclass. IgG seems to rely on glycolysis, PPP and FA synthesis to induce an inflammatory phenotype in macrophages. However, we found that IgA, contrary to what we observed for IgG in macrophages, and contrary to what was seen for IgA in DCs (1), does not require glycolysis to increase the inflammatory potential of macrophages. Interestingly, not only that, but we observed that within the two IgA subclasses, i.e. IgA1 and IgA2, different metabolic requirements exist for cell activation. While IgA1 does not seem to rely on any specific metabolic pathway, IgA2 seems to be dependent on mitochondrial metabolism, more specifically, ATP Synthase activity, to induce a hyper-inflammatory phenotype in macrophages. Together, these studies point towards a complex relationship between pathologies, cellular metabolism and immune function.

Key factors that are likely to underpin these differences in metabolic reprogramming and dependencies are tissue microenvironment, cell type and nature of Fc receptor signalling. For example, while generally macrophages rely on glycolytic metabolism to mount a pro-inflammatory response in response to pathogens (2) this is not always the case and can be dictated by the tissue microenvironment they reside in (3). Alveolar macrophages, as opposed macrophages in other tissues, are well known for relying on OXPHOS instead of glycolysis for their inflammatory response (4), due to being adapted to an oxygen-rich environment. In the case of RA, during inflammation, the synovium undergoes repeated cycles of hypoxia and re-oxygenation (5–8). These hypoxia conditions have been shown to induce COX2 expression (9), and the repeated oscillations of oxygen availability was reported to induce the production of ROS with a HIF1a-stabilizing effect (10–12). The expression of COX2, and hypoxia-driven ROS and subsequent HIF1α activation were shown to drive inflammation in both macrophages and neutrophils (9,13,14). Therefore, one could hypothesise that this IgA2-ATP Synthase-COX2 axis in macrophages is part of a broader RA-specific physiological mechanism of inflammatory potentiation to induce mitochondrial ROS, HIF1α stabilization and subsequent proinflammatory cytokine synthesis (15,16).

As for the case of Fc receptor signalling, it is known that, in humans, only certain cell types express FcαRI (e.g. monocytes, macrophages or DCs) (17). Even within

those populations, the expression of FcαRI is variable, e.g. while it is expressed by most macrophage populations, it is not expressed by intestinal macrophages, while for DCs its expression is mainly restricted to interstitial DCs, monocyte-derived DCs and CD34+-derived DCs (17–19). This showcases that, depending on the tissue localization of the cell, they may reduce, or even lose, the expression of IgA-binding receptors. This can lead us to hypothesize that, if an immune cell population expresses IgA-binding receptors to different degrees depending on their tissue niche, they might also have a different metabolic response to IgA, depending on the function of the cell, and the tissue where it resides. Additionally, it has been described that different Fc receptors have different signalling pathways (20), so it is conceivable that FcαRI stimulation might induce a different metabolic reprogramming than FcγR stimulation. Secondly, it was already shown, for IgG, that different isotypes induce different signalling pathways, different metabolic reprogramming, and subsequently differences in cytokine production (21). Our work suggests a similar phenomenon applies to IgA1 and IgA2. This, however, raises more questions – Is this due to differences in binding strength/affinity between IgA subclasses and/or different glycosylation profiles and the FcαR, analogous to what has already been described for IgG (22,23)? Hypothetically, could there also be an IgA2-specific receptor/signal transducer that induces certain complementary activation states or, on the other hand, is there an IgA1-specific receptor/signal transducer that inhibits certain key signals that induce these changes? Alternatively, could this be due to time differences in phosphorylation signals, such as what was already seen for mTOR (24)?

It would be interesting to explore these questions in future studies, for example by mapping subclass-specific phosphorylation signalling in the cell, and also study the effects of IgA1 and IgA2 in FcαRI-KO and in Fcα/μR-KO macrophages, as these (CD89 and CD351 respectively) are the only currently known receptors able to bind IgA. Furthermore, it is fundamental to take into account that mice have a different expression pattern of Fc receptors than humans. Especially when it comes to IgA, unlike humans, mice do not express a homologue of the FcαRI (25–27). Therefore, not only is it important to explore these questions within a specific cell type (e.g. FcR signalling in macrophages vs DCs), but it is also crucial to do it within an appropriate physiological context, for example by using human cells or humanized mouse models.

Lipid Metabolism and Its Autocrine and Paracrine Effects

While part of this thesis was focused on intrinsic lipid metabolism, it is important to also keep in mind that lipids and lipid-metabolism-derived molecules also play very important roles in cell messaging, both in a paracrine as well as an autocrine fashion. A key enzyme involved in this process is COX2.

In **chapters 3-5**, the data showed that cells undergo different metabolic reprograming and acquire distinct metabolic phenotypes depending on the signal and cellular context of the pro-inflammatory stimuli. However, despite this, there seems to be a convergence towards COX2 upregulation and the synthesis of lipid mediators. As seen in **chapter 4**, IgA2 induced a hyper-inflammatory state in macrophages that was dependent on ATP Synthase activity, but also on COX2 expression. This was correlated with increased production of lipid mediators downstream of COX2, especially PGE_2. While PGE_2 is a well-known factor linked to inflammatory responses, it does not seem to be required for IgA2-driven potentiation of inflammation, since supplementing cells after COX2 inhibition with PGE_2 did not rescue the effects of IgA2. However, since the net effects of PGE_2 signalling are dependent on a number of factors, such as timing, concentration and duration of the stimulus, a potential contribution of PGE_2 to the proinflammatory effects of IgA2 stimulation cannot be totally ruled out yet. Since COX2 is crucial for the synthesis of many lipid mediators, including PGE_2, one could speculate that perhaps other immunomodulatory COX2 products, such as $PGF_{2\alpha}$, TxA_2, or PGI_2, (28–32) are contributing to, or even responsible for, this effect. This warrants further study.

Additional work highlighting the importance of COX2-dependent mediators in cellular function was shown in in **chapter 5**. While DCs require PGE_2 production and subsequent autocrine signalling to induce a Th2 inflammatory response following stimulation with *S. mansoni* egg antigens, they also require other COX2-dependent lipid mediators to ensure a proper Th1 response to LPS. Indeed, this was evidenced by the fact that general COX inhibition in DCs with indomethacin affected both Th1 and Th2 priming, while targeted mPGES inhibition mostly affected Th2 priming.
It is, therefore, interesting to note that while there is a central role for COX2 in supporting both type 1 and type 2 immune responses in macrophages and DCs, these cells are not dependent on the same COX2-dependent mediators, i.e. IgA-driven type 1 inflammation in macrophages being independent of PGE_2, while type 2 inflammatory responses by DCs, in the context of helminth infections, are specifically PGE_2-dependent.

Bridging Findings From Fundamental Studies to Clinical Implications

These studies highlight the importance of the contributions of metabolic programs and lipid mediators to immune cell function are highly in cell- and context-specific and advocate, for design of tailored targeting approaches of these processes to influence immune responses for future therapies. While IgG-dependent pathologies might benefit from targeted modulation of glycolysis, PPP and/or FA synthesis in macrophages, IgA-dependent pathologies, specifically IgA2, might instead benefit from a two-pronged strategy, by targeting glycolysis in DCs on one hand (1), and mitochondrial metabolism in macrophages on the other, thus ensuring the most optimal outcome to reign in uncontrolled inflammation.

These data also open interesting additional exploratory paths for the design of immunometabolic therapies. If inhibiting the aforementioned metabolic pathways in IgG- and IgA-dependent pathologies could be beneficial in curtailing unchecked inflammatory responses, it is tempting to speculate the opposite approach may also be possible: could inflammatory responses be improved by boosting these pathways in the context of immunocompromised patients, immune-evasive pathogens, and antibiotic-resistant infections, as host directed therapy? Could glycolysis and/or mitochondrial respiration be hyperactivated in situations where there are issues with mounting a robust protective inflammatory response to pathogens?

While it is an interesting idea to explore, its conceptualization presents as a hurdle. While genetic approaches to overexpress certain key enzymes to increase metabolic flux for specific pathways work well *in vitro*, their applicability in an *in vivo* setting would be highly complex, if not impossible. One alternative could be the use of allosteric regulators that are known to increase metabolic flux, such as Fructose-2,6-bisphoshphate (F-2,6-P_2) for glycolysis and AMP, NAD+, CoA and/or lactate in the case of oxidative phosphorylation/ATP synthase activity. Another strategy could be to use drugs that have been shown to activate pathways, be it directly or indirectly, such as the HIF-1α stabilizer BAY 85-3934, to boost glycolysis, or the fatty acid synthesis activator GGTI-298. Additionally, while targeted delivery of these compounds poses an issue, the recent use of liposomes to deliver drugs and metabolites to specific cells (33) could be exploited in the context of metabolic manipulation, to specifically deliver pathway activators and/or inhibitors to the target cells (e.g. macrophages, DCs, Th cells), to ensure an optimal immune response for each distinct context. For instance, therapies that involve the use of monoclonal IgG could be used in tandem with F-2,6-P_2/ BAY 85-3934 and GGTI-298, to respectively boost glycolysis and FA synthesis in macrophages, while AMP, NAD+, CoA and/or lactate could be used to increase ATP

Synthase activity and help in boosting the IgA-driven response by macrophages. Indeed, some metabolic activators have already been used in a pre-clinical context. For example, dichloroacetate, an activator of the Pyruvate Dehydrogenase Complex, which promotes oxidation of glucose derived pyruvate in the mitochondria, has been used in macrophages to improve anti-tumour immunoreactivity (34).

Finally, in the case of macrophages we found that certain IgG- and IgA-driven inflammatory responses were dependent on certain metabolic pathways, while with DCs we found that type-2 immune responses rely on PGE_2. As such it would be interesting to see if this could be applied in future therapies, such as in supplementing anti-parasitic vaccines with PGE_2 to ensure a more robust induction of type 2 immunity, thus potentially avoiding the immune-evasive mechanisms of parasites. Alternatively, PGE_2 could be inhibited as a way to downregulate uncontrolled type 2 responses, such as in the cases of asthma and allergy, provided this principle applies to other type 2 diseases. As type 1 responses are inhibited by type 2 responses, this means that specifically inhibiting PGE_2, while leaving other COX2-dependent mediators intact, could potentially induce a more robust type 1 inflammatory response, which could be beneficial in cases such as cancer or vaccines against bacteria and viruses. However, it is important to take into account any off-target effects of PGE_2 that could potentially affect cells other than DCs. For instance, induction of PGE_2 synthesis in macrophages was shown to impair Th2 responses during helminth infections (35). Therefore, it is crucial to develop therapies that are not only disease-specific but also cell-specific, to ensure the most efficient outcome.

Future Perspectives

Metabolism of the lipidome and its wide range of functional roles in shaping innate immune system is only starting to be uncovered. One conclusion that can be taken from the work done in **chapters 3**, **4**, and **5**, is that changes in cellular metabolism are highly context- and cell-dependent. As such, to bolster our arsenal of therapies against infection and/or inflammation, it is important to have a closer look at cellular immunometabolism and, particularly, lipid metabolism. Recent advances in the field of lipidomics (36–38) have opened the door to perform single-cell lipidomics, thus being able to better unravel the heterogeneity in immunometabolic phenotype of diverse and rare immune cell populations. It would be interesting to pair this new approach with spatial lipidomics (39–41) to get a better picture of the lipidome of specific cells/cellular populations but also their distribution within the tissue/milieu, as the immediate environment of a cell will be an important a factor in shaping its metabolism and subsequent activation state.

Furthermore, while this thesis is focused on lipid metabolism and lipid mediators, it also begs a question that has been gaining more and more relevance in our day and age – What are the immunological implications of whole body lipid imbalance, such as what is seen in obesity (42–44) and type 2 diabetes (45,46)? What about localized lipid abnormalities, such as those seen in the tumour microenvironment (TME) (47–49)? It is known that obesity is a risk factor for the development of cancer and pathologies associated with chronic inflammation, such as Alzheimer's or rheumatoid arthritis (50–52). It is also associated with impaired immune protection against infectious diseases, as clearly evidenced with the recent COVID19 pandemic (53). Obesity is a result of long-term intake of excessive calories, which results in increased accumulation of lipids in the liver and adipose tissue (54,55). Due to drastic diet and lifestyle changes in modern society, and the widespread availability of high-fat/calorically dense foods, obesity is on the rise, having been declared a global epidemic by the World Health Organization (56,57). It has been suggested that the expansion of adipose tissue due to prolonged intake of excess calories could cause hypoxia and mechanical stress to adipose tissue macrophages (ATMs), subsequently inducing inflammation and apoptosis (58). Not only is this conducive of inflammation within the adipose tissue itself, but it also leads to foam cell formation, due to efferocytosis of dead ATMs, thus contributing to further tissue dysfunction (59). Additionally, dietary fatty acids, particularly saturated fatty acids (SFAs), have been shown to skew ATMs towards a proinflammatory phenotype (60,61). During homeostasis, ATMs are somewhat metabolically inert, presenting low rates of both glycolysis and OXPHOS (62). However, ATMs from obese mice fed with a high-fat diet and exposed to SFAs were shown to have increased rates of glycolysis, and OXPHOS, and an accompanying proinflammatory phenotype (62–64).

Notably, there have been studies connecting obesity and adipose tissue dysfunction with arthritis. A recent study (65) showed that ACPAs lead to defective differentiation of adipocytes, promote M1 differentiation of macrophages, and increase inflammatory cytokine production. Moreover, cohort studies have suggested that obesity increases the chance of developing RA (66–70). Furthermore, it has already been extensively reviewed (71,72) that the AT can secrete molecules (i.e. adipokines) that increase inflammation and impair anti-RA therapies. Curiously, it has been hypothesized that this impairment, particularly when it comes to anti-TNF therapies, is due to increased Fc-receptor expression in omental adipocytes (71,73,74). As such, building on the work done in this thesis and further investigating the interplay between inflammation, antibody signalling, and lipid dysfunction, might prove invaluable in helping to find new strategies to tackle antibody-driven chronic inflammation.

In addition to obesity, lipid metabolism in myeloid cells also plays an important role in cancer. It is well known that tumour-associated macrophages (TAMs) are

characterized by having high expression of lipid transporters, such as CD36, and fatty acid binding proteins (FABPs), which results in increased fatty acid (FA) uptake and lipid accumulation in the cytosol (75,76). It has been described that tumour-derived lipid mediators, namely PGE_2, are capable of inducing an M2-like/tolerogenic phenotype in macrophages (77–79). Moreover, recent studies have shown that tumour-derived FAs, particularly monounsaturated long-chain FAs, induce an immunosuppressive phenotype in TAMs (76). Furthermore, it has been shown that lipid-laden TAMs display increased expression of immuno-inhibitory proteins, such as PD-L1, and decreased phagocytosis, therefore presenting a defective anti-tumour function (80). Additionally, it is already known that lipid-laden macrophages express high levels of PPARy, which can increase the expression of CD36 and lead to further lipid uptake (81,82), thus possibly creating a positive feedback loop that boosts the immunosuppressive phenotype in TAMs. Interestingly, there have been quite some anti-tumour strategies dependent on FcR signalling in TAMs, which work by overcoming their immunosuppressive phenotype and "kick starting" their inflammatory response. Indeed, these studies showed that using therapeutic antibodies against "don't eat me" signals, in conjugation with M1-derived exosomes, resulted in FcRs in TAMs binding to the Fc portion of the aforementioned therapeutic antibodies, leading to repolarization of TAMs, along with antibody-dependent cytotoxicity and antibody-dependent phagocytosis of tumour cells (83–85). However, to what extent this boosting of TAM inflammatory potential is dependent on metabolic reprogramming remains an open question.

Likewise, similar findings have been made in regard to tumour-associated DCs (TADCs). A recent study showed that tumour-derived PGE_2 induces cDC1 dysfunction and impairs anti-cancer T cell responses (86). It has also been suggested that TADCs develop a tolerising phenotype by uptaking FAs in the TME (87). TADCs upregulate several FA scavenger receptors, such as FABP4 and CD204, and display high lipid content and lipid-droplet accumulation (88–90). These TADCs were shown to have impaired Ag presentation and T cell priming abilities. Moreover, lipid droplet content in TADCs was shown to differ from non-TADCs, whereby TADC lipid droplets exhibit higher levels of oxidized polyunsaturated fatty acids (91,92), which has been suggested to impair cross-presentation by TADCs and subsequent priming of anti-tumour T cells (93). Furthermore, anti-tumour vaccination strategies relying on DC FcR signalling, although limited, have been proposed and tested in a clinical trial setting (94,95).

In conclusion, this thesis contributes to the growing recognition of the importance of lipid metabolism and Fc receptors in macrophage and DC biology, in the context of autoimmunity, chronic inflammation, cancer, obesity, and infection. Therefore, studying the effects of lipid mediators, FcR signalling, and the baseline immunometabolic

phenotype of tissue-associated myeloid cells in the context of each disease, in tandem with the aforementioned lipidomics tools, could give us an insight into the mechanism underlying the cause of these pathologies, help predict disease development and progression, and potentially lead to new therapies to prevent, treat or cure these diseases.

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Appendix

English Summary

Background

The immune system is the main line of defence against the millions of potential pathogens and other dangers, such as possible cancerous cells and allergens, humans are exposed to on a daily basis. This system is a complex and intricate network of many different molecules, cells, tissues, and organs, all working in tandem, in a tightly regulated manner, albeit with different functions and mechanisms of action. The immune system can be divided into two main parts – the innate immune system and the adaptive immune system. The innate immune system acts in a fast and broad manner, with general specificity, being activated by generically conserved molecular patterns, such as PAMPs (pathogen-associated molecular patterns), which are present in pathogens/microbes and DAMPs (damage-associated molecular patterns), which are released by our own cells where they are damaged and/or attacked. Conversely, the adaptive immune system, although slower to act, is highly precise and efficient, recognizing a specific antigen, thus being able to zone-in on an individual type of pathogen or molecule.

However, without the initial input of the innate immune system, the adaptive immune system cannot be activated. This makes the innate immune system a key player in the overall immune response. Therefore, studying the mechanisms of action which govern innate immune cell function poses as an imperative endeavour to understand how we could enhance the activity of our immune system for our advantage, such as by improving the immune response against infections, increasing vaccine efficacy or boosting the anti-tumoral response. Conversely, knowing how to introduce a brake on immune responses could be important as well, such as in certain hyperinflammatory (e.g. severe COVID-19), or autoimmune diseases (e.g. rheumatoid arthritis), characterized by chronic inflammatory responses against our own antigens (also called self-antigens), which happens because our own immune cells start attacking our own body, due to loss of immune tolerance – in simple terms, our immune cells start seeing our own body as a threat which needs to be eliminated.

Two key cell types of the innate immune system are macrophages and dendritic cells (DCs), two main types of myeloid cells (Fig. 1). Macrophages are tissue resident immune cells responsible for the maintenance of homeostasis of tissues. In response to inflammatory signals, they can adopt pro-inflammatory functions characterized by the synthesis of pro-inflammatory cytokines (e.g. TNF and IL-6). A few examples of classical inflammatory signals in macrophages are:

- Stimulation of toll-like receptors (TLRs) by PAMPs and DAMPs, which activate different inflammatory pathways, depending on the TLR being stimulated;
- Binding of the Fc portion of antibodies (commonly called the "tail" of the *Y-shaped* antibody (Fig. 1)) to Fc receptors (FcR) present on the macrophages;

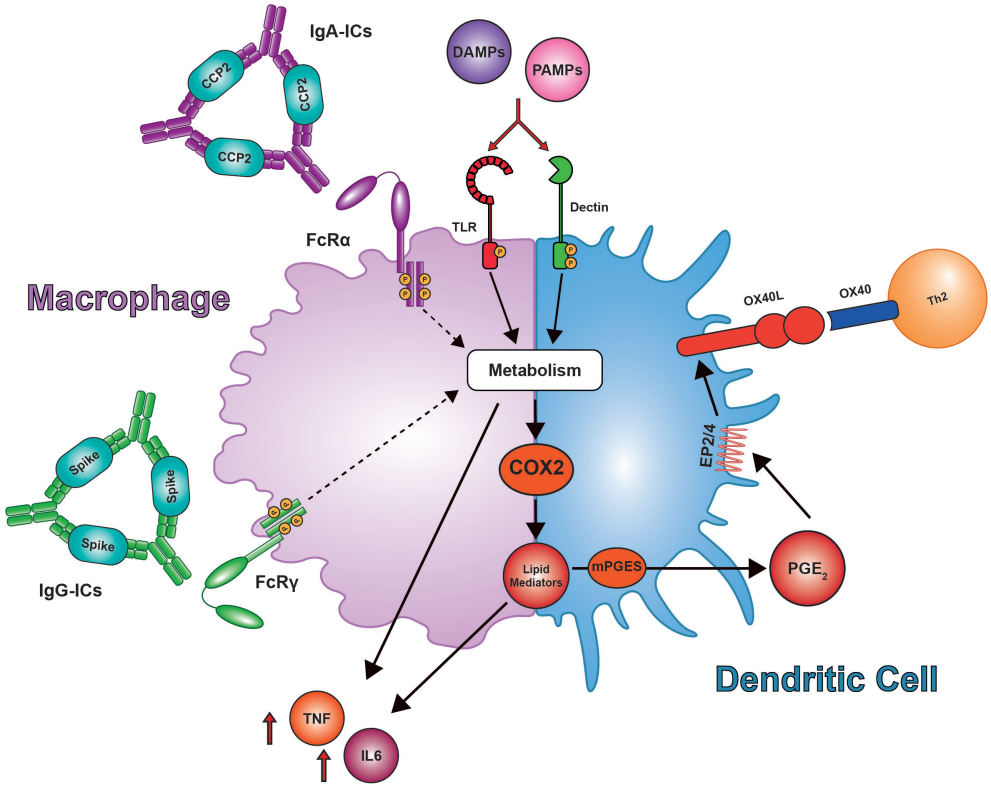


Figure 1: **Metabolism and lipid mediators play a central role in innate immune cell function.** Extrinsic stimuli induce metabolic changes in both macrophages and dendritic cells via PRRs (such as TLRs and Dectin), and/or via FcRs. These metabolic changes regulate the cellular immune response, via synthesis of COX2-dependent lipid mediators, and proinflammatory cytokines.

Predictably, these two stimuli can synergise and amplify the inflammatory phenotype of macrophages, thus mounting a stronger and more robust response against a pathogen, in cases where the Fc portion of the antibody engages an activating FcR.

While today it is known that macrophage differentiation and activation is a highly plastic process that exists on a spectrum, the two extremes of that spectrum are highly pro-inflammatory macrophages, commonly referred to as "M1-like", and anti-inflammatory macrophages/macrophages that promote the resolution of the inflammatory process (i.e. pro-resolving macrophages), commonly referred to as "M2-

like". In general, macrophages with the M1-like phenotype are tasked with patrolling and killing pathogens, through a process called phagocytosis and by the production of pro-inflammatory cytokines, while the M2-like promote wound healing, by producing anti-inflammatory cytokines, tissue regeneration and by engulfing dead cells, or cells undergoing cell death (i.e. apoptosis), through a process called efferocytosis.

On the other hand, the main function of DCs is to induce and direct the differentiation and activation of the adaptive immune system, especially through the presentation of antigens to T cells. Once DCs encounter an antigen, be it from an outside invader, such as a pathogen, or from an internal source (e.g. self-antigen or cancerous cell), they will undergo specific changes in activation, and either become immunogenic or tolerogenic. Immunogenic DCs will induce the differentiation and activation of cytotoxic CD8+ T cells, and/or CD4+ T helper cells, such as Th1, Th2 or Th17 cells, all with specific functions tailored to counter specific pathogens and immune challenges. Whereas tolerogenic DCs will instead induce the differentiation and activation of Treg cells, which work as built in immunologic brakes, that inhibit immune responses against a certain antigen, be it a self-antigen, to prevent autoimmune diseases, or against a foreign, but harmless, antigen such as those in foods.

In an impressive display of cellular coordination, the newly activated T cells, will migrate to the specific site where the presented antigen is located, and will oversee the immune response, by producing cytokines which will not only act on the innate immune system, such as the macrophages already present in the site, but also work as beacons to promote further migration of more innate immune cells to help fight against the specific threat, in the case of Th1, Th2 and Th17, or to instead stop the inflammatory immune response and promote tolerance, in the case of Tregs.

Additionally, DCs can also activate Tfh cells (T follicular helper cells), which are intimately involved in initiating and shaping responses by the *other branch* of the adaptive immune system – B cells. After this "activation trinity", between B cells, DCs, and Tfh, B cells will migrate to the specific site the antigen presented by the DCs originated from, where they will then produce and secrete antibodies. These antibodies, usually in the form of Immunoglobulin G (IgG) or Immunoglobulin A (IgA), are able to recognize the antigen and directly bind to it, forming immune complexes, where the *two little arms* of the Y-shaped antibody bind the antigen, while its "*tail*" i.e. its Fc part, sticks out (Fig. 1). These immune complexes are then able to be recognized by macrophages, via Fc receptors, which bind the Fc part, thus helping these macrophages undergo further pro-inflammatory activation. These mechanisms put DCs as the first target in a strategy of immune modulation, since they dictate the activation states of T cells and B cells, while it puts macrophages as the last target, since they are the final actors in this innate-adaptative-innate immunological chain.

One of the more recent fields of study in immunology which tries to understand how to modulate immune responses is immunometabolism. This area arose from the discovery that, depending on the function being performed, immune cells will undergo specific metabolic reprogramming, favouring certain metabolic pathways over others, not only for their energetic needs, but also for the synthesis of crucial metabolites to be used in their activation and differentiation stages. For instance, it was described that, *generally*, pro-inflammatory macrophages and immunogenic DCs rely on glycolysis and fatty acid synthesis, while pro-resolving macrophages and tolerogenic DCs instead rely on fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS). With this knowledge also came the hypothesis that, by modulating macrophage and DC metabolism, one could control their activation state. For example, by inhibiting metabolic pathways favoured by pro-inflammatory/immunogenic phenotypes, and promoting metabolic pathways favoured by pro-resolving/tolerogenic phenotypes, the cell would switch from a pro-inflammatory state to an anti-inflammatory state, and vice-versa.

One crucial pathway at the centre of immunometabolism is lipid metabolism. While lipids have long been seen as molecules for energy storage and building blocks for cell membranes, we now know that is not the case. Lipid metabolism plays a central role in the synthesis of metabolites used by immune cells for their respective functions. For example, fatty acid synthesis can lead to the formation of lipid droplets (small round pools of lipid molecules inside the cell, much like the oil droplets we see on the surface of soups and stews) in macrophages and DCs, which have been shown to play a role in immune functions, such as cytokine production, phagocytosis and antigen presentation. Alternatively, FAO was shown to be important as Acetyl-CoA from fatty acid oxidation can be used for histone acetylation, which is a way for the cell to control which genes are being expressed and/or repressed.

Additionally, lipid metabolism also includes the synthesis of lipid mediators. Lipid mediators are a group of molecules that play a highly important role in cell messaging. As the name suggests, these molecules originate from the oxidation of fatty acids, and act as chemical messengers. These mediators can act on the cell itself (autocrine signalling), on cells close by to the original cell (paracrine) or on cells far away from the cell of origin (endocrine signalling). These mediators are one of the main communicators between immune cells, being able to induce signalling pathways in macrophages and DCs that either promote the synthesis of proinflammatory cytokines and T cell priming, or instead dampen inflammation, by promoting tissue regeneration and immune tolerance. Thus, unravelling the mechanisms which lead immune cells to synthesize these lipid mediators and how these mediators act on the immune cells themselves is crucial to shed a light on possible therapeutic targets for intervention from an immunometabolism point of view.

Nevertheless, much still remains unknown. As mentioned above, *in general*, pro-inflammatory macrophages and immunogenic DCs tend to favour glycolysis and fatty acid synthesis, while anti-inflammatory macrophages and tolerogenic DCs tend to prefer oxidative phosphorylation and fatty acid oxidation. However, some studies have suggested that this immunometabolic dichotomy does not apply to all settings, showcasing that the metabolic reprogramming that macrophages and DCs undergo upon activation is dependent on a panoply of factors, such as cell differentiation state, tissue location, activation stimulus, and timing. Therefore, it is crucial to look at the cellular immunometabolic profile in specific contexts.

This context specificity also holds true for the role of lipid mediators. For example, while in some contexts one lipid mediator (e.g. Prostaglandin E_2) can induce a pro-inflammatory response in macrophages, or drive a Th1-priming response by DCs, in other contexts it can instead induce an anti-inflammatory response and/or drive a Th2 response.

Therefore, considering there is a crucial need to look into the metabolic requirements of macrophages and DCs in specific situations, this thesis aimed to map metabolic profiles and dependencies in different inflammatory and cellular contexts.

Outline of Thesis Chapters:

Chapter 2 constitutes a more in-depth theoretical introduction to this thesis. There you can find a background into the field of immunometabolism in the context of innate immunity, with specific focus on fatty acid metabolism in DCs and macrophages, and how both metabolic profile and immune function are intricately connected. We addressed the current evidence showcasing the importance of fatty acids (e.g. polyunsaturated fatty acids) and lipid mediators (e.g. prostaglandins) in affecting macrophage and DC function and metabolism. We also explored the multifaceted role of fatty acid metabolism in both promoting and inhibiting inflammation, having reached the conclusion that this showcases the need to study each specific context if we want to get a complete overview of the metabolic needs of immune cells and to find a way of efficiently modulating immune responses by targeting metabolism as a therapeutic approach.

In **chapter 3** we studied the role of SARS-CoV-2 anti-spike IgG (Fig. 1) in promoting an hyperinflammatory state in macrophages. IgG achieves this by inducing specific metabolic changes that prime these macrophages for excessive pro-inflammatory cytokine expression. By chemical inhibition of these metabolic pathways we could prevent IgG-induced hyperinflammation. On a similar note, in **chapter 4** we looked at

the role of IgA against CCP2 (citrullinated peptides, which are common self-antigens in the joints (Fig. 1)) in driving chronic inflammation in the context of rheumatoid arthritis. IgA achieves this by inducing a state of hyperinflammation in macrophages that is both dependent on certain metabolic changes and the synthesis of lipid mediators downstream of cyclooxygenase-2, thus identifying druggable targets with possible therapeutic applications in treating auto-antibody-driven chronic inflammatory diseases.

In **chapter 5** we describe how DCs recognize soluble egg antigens (SEA) from the parasitic helminth *Schistosoma mansoni* through Dectin-2 (a receptor on the cell surface of DCs), how they become licensed to induce the differentiation of Th2 cells, and how can DC lipid mediator synthesis be chemically targeted to mould their immune function within this context. It was previously shown that SEA signalled via Dectin-2 in DCs to induce the synthesis of PGE_2 (a type of lipid mediator called prostaglandin, downstream of an enzyme called cyclooxygenase-2). This PGE_2 acted autocrinally on the DCs and promoted the expression of OX40L, which is a molecule that licenses the DCs to prime a Th2 response.

We were able to build on that work and show that Dectin-2 directly binds to SEA in a manner that is dependent on the presence of high mannose glycans (molecules composed of several mannose sugar residues bound to each other). In the absence of these glycans, the binding of Dectin-2 was decreased, along with subsequent synthesis of PGE_2, the expression of OX40L and also the ability to prime Th2. Furthermore, we were also able to show that, when stimulating DCs with SEA, chemically inhibiting the synthesis of PGE_2 also decreased the expression of OX40L and their ability to prime Th2 cells. So, in short, this work provides new insights on how *Schistosoma mansoni* licences DCs to prime a Th2 response, and we were also able to identify possible molecular targets to control helminth-driven Th2 immune responses.

In **chapter 6** I provide an extensive discussion outlining the novel findings of this thesis and delve into future research suggestions to further decode the nuances of immunometabolism in the context of inflammatory responses, and the possible practical applications that this scientific information may have on society as a whole. I expose how lipid metabolism is intimately connected with macrophage and DC function, not only due to the metabolic reprogramming these cells undergo, but also via the synthesis of lipid mediators, which can act on the cells themselves (autocrine effect) and on their neighbours (paracrine effect). Specifically, I show how mediators downstream of cyclooxygenase-2 play a central role in driving both type 1 and type 2 immune responses, with DCs requiring PGE_2 to drive Th2 priming (type 2 immune response), but macrophages *seemingly* requiring mediators other than PGE_2 to acquire an *M1-like* phenotype (type 1 immune response). I also touch upon the

subject of how metabolic reprogramming is not a black and white picture and that we need to study metabolic requirements in specific contexts.

This was evidenced by that fact that the same stimulus (IgA) requires different metabolic pathways in different cells to induce inflammation (glycolysis in DCs vs mitochondrial metabolism in macrophages), or the fact that the same cell (macrophages) will use different metabolic pathways when stimulated with different antibodies to drive inflammation (mitochondrial metabolism for IgA vs glycolysis, pentose phosphate pathway and fatty acid synthesis for IgG). Finally, I also hypothesise about future applications for these discoveries, and how we could bridge these findings into clinical applications, potentially through the use of chemical inhibitors and/or activators to inhibit specific unwanted pathways and/or promote desired pathways to obtain a specific immune response – for example promote glycolysis and fatty acid synthesis in tandem with IgG-based therapies, or promote mitochondrial activity in tandem with IgA-based therapies to have a more robust immune response by macrophages, or inhibit PGE_2 in DCs, to downregulate unwanted type-2 immune responses and/or promote type-1 immune responses. However, it is important to always keep in mind that these ideas might not universally apply to all pathological contexts. Thus, after suggesting future perspectives on how to move forward, such as by incorporating spatial lipidomics with functional immune assays, and exploring what the results of this thesis might mean in the context of current urgent pathologies, such as cancer, obesity and age-associated neurological diseases, I conclude by reinforcing the message that to find efficient therapeutic targets we need to study this in each specific pathological context and have a cell-targeted approach.

Conclusion

In conclusion, this thesis contributes to the growing importance of lipid metabolism and Fc receptors in macrophage and DC biology, in the context of autoimmunity, chronic inflammation, cancer, obesity, and infection. Therefore, studying the effects of lipid mediators, FcR signalling, and the baseline immunometabolic phenotype of tissue-associated myeloid cells in the context of each disease, in tandem with lipidomics tools, could give us an insight into the mechanism underlying the cause of these pathologies, help predict disease development and progression, and potentially lead to new therapies to prevent, treat or cure these diseases.

Nederlandse Samenvatting

Achtergrond

Het immuunsysteem vormt de belangrijkste verdedigingslinie tegen de miljoenen potentiële pathogenen en andere gevaren, zoals kankercellen en allergenen, waaraan mensen dagelijks worden blootgesteld. Dit systeem is een complex en verfijnd netwerk van vele verschillende moleculen, cellen, weefsels en organen, die allemaal gelijktijdig functioneren binnen een strikt gereguleerd geheel, zij het met verschillende functies en werkingsmechanismen.

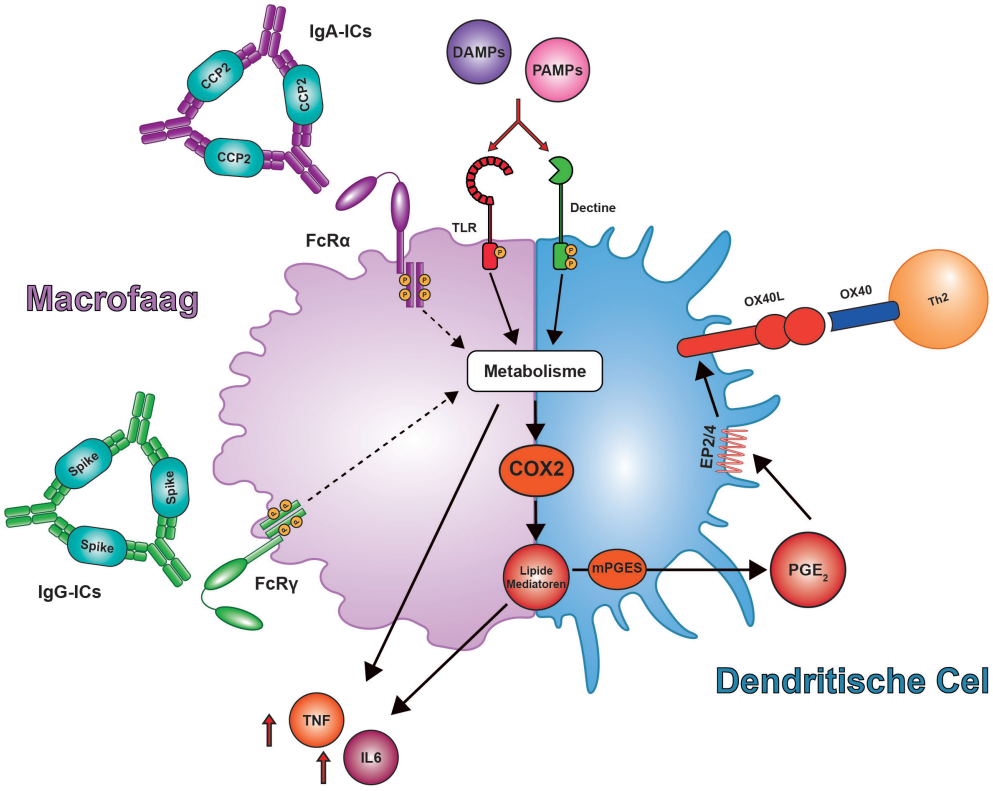
Het immuunsysteem kan worden onderverdeeld in twee hoofdonderdelen – het aangeboren immuunsysteem en het adaptieve immuunsysteem. Het aangeboren immuunsysteem werkt op een snelle en brede manier, met een algemene specificiteit, en wordt geactiveerd door generiek geconserveerde moleculaire patronen, zoals PAMP's (pathogeen-geassocieerde moleculaire patronen), die aanwezig zijn in pathogenen/micro-organismen, en DAMP's (schade-geassocieerde moleculaire patronen), die vrijkomen uit onze eigen cellen wanneer deze beschadigd en/of aangevallen zijn. Het adaptieve immuunsysteem daarentegen, hoewel trager in werking, is zeer precies en efficiënt, doordat het een specifiek antigeen herkent en zich daardoor kan richten op een individueel type pathogeen of molecuul.

Zonder de initiële input van het aangeboren immuunsysteem kan het adaptieve immuunsysteem echter niet worden geactiveerd. Dit maakt het aangeboren immuunsysteem een sleutelcomponent in de algehele immuunrespons. Daarom is het bestuderen van de werkingsmechanismen die de functie van aangeboren immuuncellen reguleren een essentiieelom te begrijpen hoe het immuunsysteem in ons voordeel kan worden gemanipuleerd. Het versterken zou de immuunrespons tegen infecties kunnen verbeteren, de effectiviteit van vaccins te verhogen, de antitumorale respons te bevorderen. Vice versa, het remmen zou kunnen helpen bij het voorkomen van bepaalde inflammatoire reacties (bijv. ernstige COVID-19), of op auto-immuunziekten (bijv. reumatoïde artritis), die worden gekenmerkt door chronische ontstekingsreacties tegen lichaamseigen antigenen (ook wel zelfantigenen genoemd). Dit ontstaat doordat immuuncellen het eigen lichaam beginnen aan te vallen als gevolg van verlies van immuuntolerantie – in eenvoudige termen: immuuncellen gaan het eigen lichaam zien als een bedreiging die geëlimineerd moet worden.

Belangrijke cellen van het aangeboren immuunsysteem zijn twee type myeloïde cellen: macrofagen en dendritische cellen (DC's), (Fig. 1). Macrofagen zijn immuuncellen aanwezig in weefsels en verantwoordelijk voor het handhaven van de homeostase van deze weefsels. In reactie op inflammatoire signalen kunnen zij pro-inflammatoire

functies aannemen, gekenmerkt door de synthese van pro-inflammatoire cytokinen (bijv. TNF en IL-6). Enkele voorbeelden van klassieke inflammatoire signalen in macrofagen zijn:

- stimulatie van toll-like receptoren (TLR's) door PAMP's en DAMP's, die verschillende inflammatoire signaalroutes activeren, afhankelijk van welke TLR wordt gestimuleerd;
- binding van het Fc-gedeelte van antilichamen (vaak aangeduid als de "staart" van het *Y-vormige* antilichaam (Fig. 1)) aan Fc-receptoren (FcR) die aanwezig zijn op macrofagen;



Figuur 1: **Metabolisme en lipidemediatoren spelen een centrale rol in de functie van aangeboren immuuncellen.** Extrinsieke stimuli induceren metabole veranderingen in zowel macrofagen als dendritische cellen via patroonherkennende receptoren (PRR's; zoals TLR's en Dectine) en/of via Fc-receptoren (FcR's). Deze metabole veranderingen reguleren de cellulaire immuunrespons, onder andere via de synthese van COX-2-afhankelijke lipidemediatoren en pro-inflammatoire cytokinen.

Deze twee stimuli kunnen synergetisch werken en het inflammatoire fenotype van macrofagen versterken, waardoor een sterkere en robuustere respons tegen een pathogeen wordt gegenereerd, in gevallen waarin het Fc-gedeelte van het antilichaam

een activerende Fc-receptor bindt.

Tegenwoordig is bekend dat macrofaagdifferentiatie en -activatie een zeer plastisch proces is dat zich op een spectrum bevindt. twee uitersten op dat spectrum zijn sterk pro-inflammatoire macrofagen, doorgaans aangeduid als “M1-achtig”, aan de een kant, en anti-inflammatoire macrofagen/macrofagen die de resolutie van het inflammatoire proces bevorderen (d.w.z. pro-resolverende macrofagen), doorgaans aangeduid als “M2-achtig”, aan de ander kant Over het algemeen zijn M1-achtige macrofagen belast met het patrouilleren en elimineren van mogelijke pathogenen via een proces dat fagocytose wordt genoemd en door de productie van pro-inflammatoire cytokinen, terwijl M2-achtige macrofagen wondgenezing bevorderen door de productie van anti-inflammatoire cytokinen, weefselregeneratie en door het opnemen van dode cellen of cellen die celdood ondergaan (d.w.z. apoptose) via een proces dat efferocytose wordt genoemd.

De belangrijkste functie van DC’s het induceren en sturen van de differentiatie en activatie van het adaptieve immuunsysteem, met name via de presentatie van antigenen aan T-cellen. Zodra DC’s een antigeen tegenkomen, hetzij afkomstig van een externe indringer zoals een pathogeen, hetzij van een interne bron (bijv. een zelfantigeen of een kankercel), ondergaan zij specifieke veranderingen in activatie en worden zij ofwel immunogeen ofwel tolerogeen. Immunogene DC’s zullen de differentiatie en activatie van cytotoxische CD8+ T-cellen en/of CD4+ T-helpercellen induceren, zoals Th1-, Th2- of Th17-cellen, elk met specifieke functies die zijn afgestemd op het bestrijden van specifieke pathogenen en immuunuitdagingen. Tolerogene DC’s daarentegen zullen de differentiatie en activatie van Treg-cellen induceren, die functioneren als ingebouwde immunologische rem die immuunresponsen tegen een bepaald antigeen beperken, hetzij een zelfantigeen om auto-immuunziekten te voorkomen, hetzij een vreemd maar onschadelijk antigeen zoals die in voedsel voorkomt.

In een indrukwekkend staaltje van cellulaire coördinatie migreren de nieuw geactiveerde T-cellen naar de specifieke plaats waar het gepresenteerde antigeen zich bevindt, en sturen zij de immuunrespons door cytokinen te produceren die niet alleen inwerken op het aangeboren immuunsysteem, zoals de macrofagen die reeds op de betreffende locatie aanwezig zijn, maar ook -in het geval van Th1-, Th2- en Th17-cellen - signalen afgeven om verdere migratie van aanvullende aangeboren immuuncellen te bevorderen om de specifieke dreiging te bestrijden, , of juist om de inflammatoire immuunrespons te beëindigen en tolerantie te bevorderen, in het geval van Treg-cellen.

Daarnaast kunnen DC’s ook Tfh-cellen (T folliculaire helpercellen) activeren, die nauw betrokken zijn bij het initiëren en vormgeven van responsen van de *andere*

tak van het adaptieve immuunsysteem – de B-cellen. Na deze "activatietrinititeit" tussen B-cellen, DC's en Tfh-cellen migreren B-cellen naar de specifieke plaats waar het door DC's gepresenteerde antigeen vandaan kwam, waar zij vervolgens antilichamen produceren en uitscheiden. Deze antilichamen, meestal in de vorm van immunoglobuline G (IgG) of immunoglobuline A (IgA), zijn in staat het antigeen te herkennen en er direct aan te binden, waarbij immuuncomplexen worden gevormd. Hierbij binden de *twee kleine armpjes* van het Y-vormige antilichaam het antigeen, terwijl de "*staart*", d.w.z. het Fc-gedeelte, uitsteekt (Fig. 1). Deze immuuncomplexen kunnen vervolgens worden herkend door macrofagen via Fc-receptoren, die het Fc-gedeelte binden, en zo bijdragen aan verdere pro-inflammatoire activatie van deze macrofagen. Deze mechanismen positioneren DC's als het eerste doelwit binnen een strategie van immuunmodulatie, aangezien zij de activatiestatus van T-cellen en B-cellen bepalen, terwijl macrofagen als het laatste doelwit worden beschouwd, aangezien zij de uiteindelijke uitvoerders zijn in deze aangeboren-adaptieve-aangeboren immunologische keten.

Een van de meer recente onderzoeksgebieden binnen de immunologie dat tracht te begrijpen hoe immuunresponsen gemoduleerd kunnen worden, is immuunmetabolisme. Dit veld is ontstaan uit de ontdekking dat immuuncellen, afhankelijk van de functie die zij vervullen, specifieke metabole herprogrammering ondergaan, waarbij bepaalde metabole routes worden bevoordeeld boven andere, niet alleen om in hun energiebehoefte te voorzien, maar ook voor de synthese van cruciale metabolieten die worden gebruikt tijdens hun activatie- en differentiatiestadia. Zo is beschreven dat pro-inflammatoire macrofagen en immunogene DC's *over het algemeen* afhankelijk zijn van glycolyse en vetzuursynthese, terwijl pro-resolverende macrofagen en tolerogene DC's juist afhankelijk zijn van vetzuuroxidatie (FAO) en oxidatieve fosforylering (OXPHOS). Met deze kennis ontstond ook de hypothese dat, door het metabolisme van macrofagen en DC's te moduleren, men hun activatiestatus zou kunnen sturen. Bijvoorbeeld door stofwisseling belangrijk voor het ondersteunen vanpro-inflammatoire/immunogene fenotypes te remmen of metabole routes die bijdragen aan pro-resolverende/tolerogene fenotypes te stimuleren, zou de cel kunnen omschakelen van een pro-inflammatoire naar een anti-inflammatoire toestand en vice versa.

Een veel bestudeerde stofwisselingsroute binnen het immuunmetabolisme veld is het lipidenmetabolisme. Hoewel lipiden lange tijd werden beschouwd als moleculen voor energieopslag en als bouwstenen voor celmembranen, is inmiddels duidelijk dat hun rol veel breder is. Lipidenmetabolisme speelt een centrale rol in de synthese van metabolieten die door immuuncellen worden gebruikt voor hun specifieke functies. Zo kan vetzuursynthese leiden tot de vorming van lipidedruppels (kleine, ronde aggregaten van lipidemoleculen binnen de cel, vergelijkbaar met de oliedruppels

die zichtbaar zijn op het oppervlak van soepen en stoofgerechten) in macrofagen en DC's, waarvan is aangetoond dat zij betrokken zijn bij immuunfuncties zoals cytokineproductie, fagocytose en antigeenpresentatie. Daarentegen is aangetoond dat FAO belangrijk is omdat acetyl-CoA, afkomstig van vetzuuroxidatie, kan worden gebruikt voor histonacetylering, een mechanisme waarmee de cel reguleert welke genen tot expressie komen en/of worden onderdrukt.

Daarnaast omvat het lipidenmetabolisme ook de synthese van lipidemediatoren. Lipidemediatoren vormen een groep moleculen die een zeer belangrijke rol spelen in cellulaire communicatie. Deze moleculen ontstaan uit de oxidatie van vetzuren en functioneren als chemische boodschappers. Deze mediatoren kunnen inwerken op de cel zelf (autocriene signalering), op nabijgelegen cellen (paracriene signalering) of op cellen op grotere afstand (endocriene signalering). Deze mediatoren behoren tot de belangrijkste communicatiemiddelen tussen immuuncellen en zijn in staat signaalroutes te induceren in macrofagen en DC's die ofwel de synthese van pro-inflammatoire cytokinen en T-celpriming bevorderen, dan wel ontsteking dempen door weefselregeneratie en immuuntolerantie te stimuleren. Het ontrafelen van de mechanismen die leiden tot de synthese van deze lipidemediatoren en de wijze waarop zij inwerken op immuuncellen zelf, is daarom cruciaal om inzicht te krijgen in potentiële therapeutische aangrijpingspunten vanuit een immuunmetabool perspectief.

Desondanks blijft er nog veel onbekend. Zoals hierboven vermeld, neigen pro-inflammatoire macrofagen en immunogene DC's *in het algemeen* naar glycolyse en vetzuursynthese, terwijl anti-inflammatoire macrofagen en tolerogene DC's eerder oxidatieve fosforylering en vetzuuroxidatie prefereren. Sommige studies suggereren echter dat deze immuunmetabole dichotomie niet altijd opgaat, wat aangeeft dat de metabole herprogrammering die macrofagen en DC's ondergaan bij activatie afhankelijk is van een breed scala aan factoren, zoals celtype, weefsellocatie, activatiestimulus en zelfs tijdsverloop. Daarom is het essentieel om het cellulaire immunometabole profiel in elke specifieke context te bestuderen.

Dit geldt eveneens voor de rol van lipidemediatoren. Zo kan één en dezelfde lipidemediator (bijv. prostaglandine E2) in bepaalde contexten een pro-inflammatoire respons in macrofagen induceren of een Th1-primingrespons in DC's stimuleren, terwijl deze in andere situaties juist een anti-inflammatoire respons kan induceren en/of een Th2-respons kan bevorderen.

Daarom had deze thesis, gezien de noodzaak om de metabole vereisten van macrofagen en DC's in specifieke situaties te onderzoeken, tot doel te bestuderen hoe metabolisme en lipidemediatoren de functie van immuuncellen vormgeven en reguleren in verschillende inflammatoire en cellulaire contexten

Opzet van de Hoofdstukken van de Thesis:

Hoofdstuk 2 vormt een meer diepgaande theoretische introductie tot deze thesis. Hierin wordt achtergrondinformatie gegeven over het veld van immuunmetabolisme in de context van aangeboren immuniteit, met specifieke aandacht voor vetzuurmetabolisme in DC's en macrofagen, en de nauwe verwevenheid tussen metabole profielen en immuunfuncties. We hebben het beschikbare bewijs besproken dat het belang aantoont van vetzuren (bijv. meervoudig onverzadigde vetzuren) en lipidemediatoren (bijv. prostaglandinen) in het beïnvloeden van de functie en het metabolisme van macrofagen en DC's. Tevens hebben we de veelzijdige rol van vetzuurmetabolisme in zowel het bevorderen als remmen van ontsteking onderzocht, en geconcludeerd dat dit de noodzaak onderstreept om elke specifieke context afzonderlijk te bestuderen om een volledig beeld te krijgen van de metabole behoeften van immuuncellen en om immuunresponsen effectief te kunnen moduleren via metabole interventies.

In **hoofdstuk 3** hebben wij de rol van SARS-CoV-2 anti-spike IgG (Fig. 1) onderzocht in het bevorderen van een hyperinflammatoire toestand in macrofagen. IgG bereikt dit door specifieke metabole veranderingen te induceren die deze macrofagen voorbereiden op overmatige expressie van pro-inflammatoire cytokinen. Door deze metabole routes te remmen, kunnen we de IgG-geïnduceerde hyperinflammatie te voorkomen. Op vergelijkbare wijze hebben wij in **hoofdstuk 4** de rol onderzocht van IgA tegen CCP2 (gecitrullineerde peptiden die veelvoorkomende zelfantigenen in de gewrichten zijn (Fig. 1)) in het bevorderen van chronische ontsteking bij reumatoïde artritis. IgA induceert een hyperinflammatoire toestand in macrofagen die afhankelijk is van zowel specifieke metabole veranderingen als de synthese van lipidemediatoren stroomafwaarts van cyclo-oxygenase-2, waarmee potentiële aangrijpingspunten worden geïdentificeerd voor de behandeling van deze door auto-antilichamen aangedreven chronische ontstekingsziekten.

In **hoofdstuk 5** beschrijf ik hoe DC's oplosbare ei-antigenen (SEA) van de parasitaire worm *Schistosoma mansoni* herkennen via Dectine-2 (een receptor op het celoppervlak van DC's), hoe zij worden geprogrammeerd om de differentiatie van Th2-cellen te induceren, en hoe de synthese van lipidemediatoren in DC's chemisch kan worden gemoduleerd om hun immuunfunctie in deze context te sturen. Eerder is aangetoond dat SEA via Dectine-2 op DC's signaleert om de synthese van PGE_2 (een type lipidemediator, behorend tot de prostaglandinen, stroomafwaarts van het enzym cyclo-oxygenase-2) te induceren. Dit PGE_2 werkt autocrien op DC's en bevordert de expressie van OX40L, een molecuul dat DC's in staat stelt een Th2-respons te initiëren.

We hebben dit werk verder uitgebreid door aan te tonen dat Dectine-2 direct bindt aan

SEA op een wijze die afhankelijk is van de aanwezigheid van hoog-mannoseglycanen (moleculen opgebouwd uit meerdere mannosesuikerresiduen die onderling verbonden zijn). In afwezigheid van deze glycanen was de binding van Dectine-2 verminderd, evenals de daaropvolgende synthese van PGE_2, de expressie van OX40L en het vermogen om Th2-cellen te primen. Bovendien hebben we aangetoond dat, bij stimulatie van DC's met SEA, chemische remming van de synthese van PGE_2 eveneens leidde tot een verminderde expressie van OX40L en een afgenomen capaciteit om Th2-cellen te primen. Samengevat hebben we nieuwe inzichten verkregen in de wijze waarop *Schistosoma mansoni* DC's conditioneert om een Th2-respons te induceren, en hebben we potentiële moleculaire doelwitten geïdentificeerd voor het reguleren van door wormen geïnduceerde Th2-immuunresponsen.

In **hoofdstuk 6** geef ik een uitgebreide discussie waarin de nieuwe bevindingen van deze thesis worden uiteengezet en ga ik in op toekomstige onderzoeksrichtingen om de complexiteit van immuunmetabolisme in inflammatoire contexten verder te ontrafelen, evenals de mogelijke maatschappelijke en klinische toepassingen van deze kennis. Ik laat zien hoe lipidenmetabolisme nauw verweven is met de functie van macrofagen en DC's, niet alleen via metabole herprogrammering, maar ook via de synthese van lipidemediatoren die zowel autocriene als paracriene effecten uitoefenen.

Specifiek toon ik aan dat mediatoren stroomafwaarts van cyclo-oxygenase-2 een centrale rol spelen in zowel type 1- als type 2-immuunresponsen, waarbij DC's PGE_2 nodig hebben voor Th2-priming (type 2-respons), terwijl macrofagen andere mediatoren dan PGE_2 nodig hebben om een *M1-achtig* fenotype (type 1-respons) te verkrijgen. Tevens bespreek ik dat metabole herprogrammering geen binair proces is en dat de metabole vereisten per context verschillen. Dit blijkt onder meer uit het feit dat dezelfde stimulus (IgA) verschillende metabole routes vereist in verschillende celtypen om ontsteking te induceren (glycolyse in DC's versus mitochondriaal metabolisme in macrofagen), evenals uit het feit dat één celtype (macrofagen) verschillende metabole routes gebruikt afhankelijk van het type antilichaam (mitochondriaal metabolisme voor IgA versus glycolyse, pentosefosfaatroute en vetzuursynthese voor IgG).

Ten slotte formuleer ik hypothesen over toekomstige toepassingen van deze bevindingen en de mogelijke vertaling naar klinische toepassingen, bijvoorbeeld door gebruik te maken van chemische remmers en/of activatoren om specifieke metabole routes te onderdrukken of te stimuleren en zo gerichte immuunresponsen te bewerkstelligen. Voorbeelden hiervan zijn het stimuleren van glycolyse en vetzuursynthese in combinatie met IgG-gebaseerde therapieën, het bevorderen van mitochondriale activiteit in combinatie met IgA-gebaseerde therapieën om een sterkere macrofaagrespons te verkrijgen, of het remmen van PGE_2 in DC's om

ongewenste type 2-responsen te onderdrukken en type 1-responsen te bevorderen. Tegelijkertijd benadruk ik dat dergelijke benaderingen contextafhankelijk zijn en niet universeel toepasbaar.

Na het bespreken van toekomstige perspectieven, waaronder de integratie van ruimtelijke lipidomics met functionele immuunassays en de relevantie van deze bevindingen voor actuele pathologieën zoals kanker, obesitas en leeftijdsgerelateerde neurologische aandoeningen, concludeer ik dat het identificeren van effectieve therapeutische doelwitten vereist dat elke pathologische context afzonderlijk wordt bestudeerd, met een cel-specifieke benadering.

Conclusie

Samenvattend draagt deze thesis bij aan het groeiende inzicht in het belang van lipidenmetabolisme en Fc-receptoren in de biologie van macrofagen en dendritische cellen, binnen de context van auto-immuniteit, chronische ontsteking, kanker, obesitas en infecties. Het bestuderen van de effecten van lipidemediatoren, FcR-signalering en het basale immunometabole fenotype van weefsel-geassocieerde myeloïde cellen, in combinatie met lipidomics-technieken, kan waardevolle inzichten opleveren in de onderliggende mechanismen van deze pathologieën, bijdragen aan het voorspellen van ziekteontwikkeling en progressie, en uiteindelijk leiden tot nieuwe therapeutische strategieën voor preventie, behandeling en genezing.

Resumo em Português

Introdução

O sistema imune é a primeira linha de defesa contra os milhões de potenciais patógenos e outros perigos, tais como possíveis células cancerosas e alérgenos, a que os humanos estão expostos no dia-a-dia. Este sistema é uma rede complexa de várias moléculas, células, tecidos e órgãos, todos a trabalhar em conjunto, de forma altamente regulada, embora com diferentes funções e mecanismos de acção.

O sistema imune pode ser dividido em duas principais partes – o sistema imune inato e o sistema imune adaptativo. O sistema imune inato actua de uma forma rápida e ampla, com uma especificidade geral, sendo activado por padrões moleculares genericamente conservados, tais como PAMPs (padrões moleculares associados a patógenos), que estão presentes em patógenos/micróbios, e DAMPs (padrões moleculares associados a dano), que são libertados pelas nossas próprias células quando são danificadas e/ou atacadas. Em contrapartida, o sistema imune adaptativo, apesar de ser mais lento a actuar, é altamente preciso e exacto, sendo capaz de reconhecer um antígeno específico, tendo assim a habilidade de se focar num tipo individual de patógeno ou molécula.

No entanto, sem o contributo inicial do sistema imune inato, o sistema imune adaptativo não consegue ser activado. Isto faz com que o sistema imune inato seja uma peça fundamental na resposta imune como um todo. Logo, estudar os mecanismos de acção que controlam as funções celulares do sistema imune inato é uma tarefa imperativa para conseguir compreender como podemos aperfeiçoar a actividade do nosso sistema imune em nosso proveito, tal como melhorar a resposta imune contra infecções, aumentar a eficácia de vacinas, ou estimular a resposta anti-tumoral. No entanto, pode ser também importante saber como introduzir um travão nas respostas imunes, tal como em certas respostas hiperinflamatórias (ex. COVID-19 severa), ou doenças auto-imunes (ex. artrite reumatóide), caracterizadas por respostas de inflamação crónica contra os nossos próprios antígenos (também chamados auto-antígenos), que acontece porque as nossas células imunes começam a atacar o nosso próprio corpo, devido à perda de tolerância imunológica – ou seja, as nossas células imunes começam a ver o nosso próprio corpo como uma ameaça que tem que ser eliminada.

Duas células fundamentais do sistema imune inato são os macrófagos e as células dendríticas (DCs), dois tipos de células mielóides (Fig. 1). Os macrófagos são células residentes de tecido responsáveis pela manutenção da homeostase dos tecidos. Em resposta a sinais inflamatórios, eles podem adquirir funções pró-inflamatórias,

caracterizadas pela síntese de citocinas pró-inflamatórias (ex. TNF e IL-6). Alguns exemplos de sinais clássicos de inflamação em macrófagos são:

- A estimulação de receptores tipo toll (TLRs), por PAMPs e DAMPs, que activam diferentes vias inflamatórias, dependendo do TLR que está a ser estimulado
- A ligação da porção Fc dos anticorpos (frequentemente chamada de "cauda" do anticorpo em forma de Y (Fig. 1)) aos receptores Fc (FcR) presentes nos macrófagos

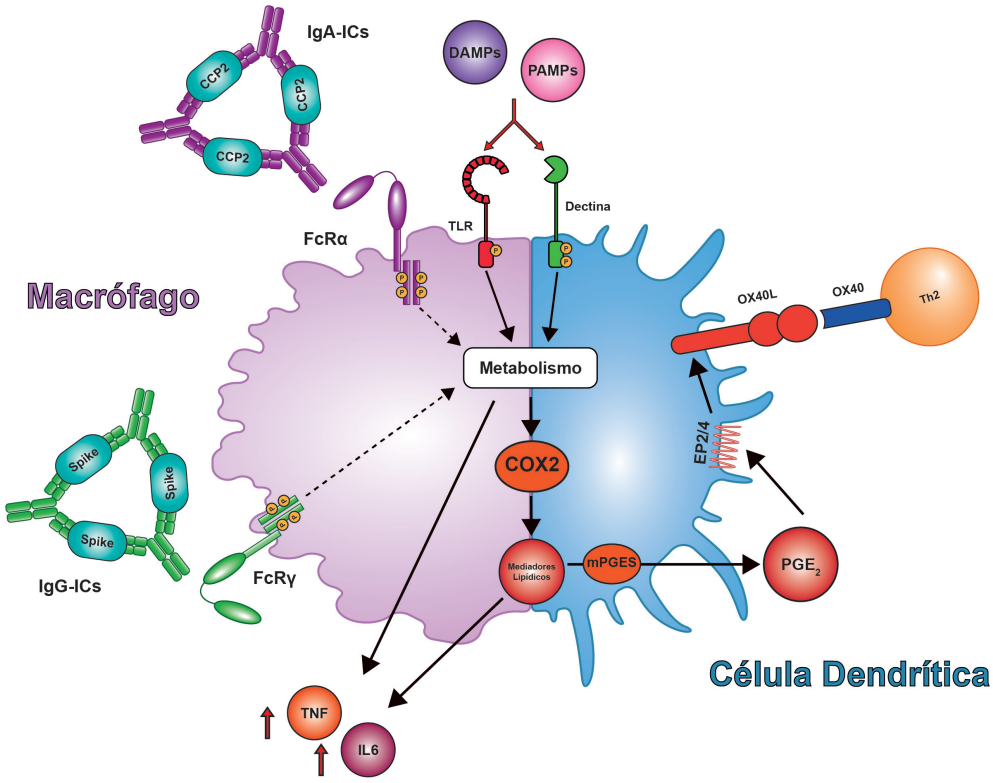


Figura 1: **Metabolismo e mediadores lipídicos desempenham um processo central na função de células imune inatas.** Estímulos extrínsecos induzem mudanças metabólicas tanto em macrófagos como em células dendríticas através de PRRs (tal como TLRs e Dectina) e/ou através de FcRs. Estas alterações metabólicas regulam a resposta imune das células através da síntese de mediadores lipídicos dependentes de COX-2 e citocinas pró-inflamatórias.

Como seria de esperar, estes dois estímulos podem criar sinergia e amplificar o fenótipo inflamatório dos macrófagos, resultando assim numa resposta imune mais forte e mais robusta contra um patógeno, em casos em que a porção Fc do anticorpo interage com um FcR activador.

Apesar de hoje em dia já se saber que a diferenciação e activação de macrófagos é um processo altamente plástico que se encontra num espectro, os dois extremos desse espectro são macrófagos fortemente inflamatórios, normalmente referidos como "tipo M1", e macrófagos anti-inflamatórios/macrófagos que promovem a resolução do processo inflamatório (ou seja, macrófagos pró-resolventes), normalmente referidos como "tipo M2". Em geral, macrófagos com o fenótipo tipo M1 têm a tarefa de patrulhar e matar patógenos, através de um processo chamado fagocitose e pela produção de citocinas pró-inflamatórias, enquanto que os tipo M2 favorecem a cicatrização de feridas, através da produção de citocinas anti-inflamatórias, regeneração do tecido e por engolirem células mortas ou células em vias de morte (ou seja, em apoptose), através de um processo chamado eferocitose.

Por outro lado, a principal função das DCs é induzir e orientar a diferenciação e activação do sistema imune adaptativo, especialmente através da apresentação de antígenos a células T. Quando as DCs encontram um antígeno, seja de um invasor externo, tal como um patógeno, ou de uma fonte interna (ex. auto-antígenos ou células cancerosas), passam por mudanças específicas durante a sua activação e, ou se tornam imunogénicas, ou tolerogénicas. DCs imunogénicas induzem a diferenciação e activação de células T citotóxicas CD8+ e/ou células T auxiliares CD4+, tais como células Th1, Th2 ou Th17; todas com funções específicas e especializadas em contra-atacar patógenos e desafios imunológicos individuais. Em contrapartida, as DCs tolerogénicas induzem a diferenciação e activação de células Treg, que funcionam como travões imunológicos que inibem respostas imunes contra um certo antígeno, seja ele um auto-antígeno, para prevenir doenças auto-imunes, ou contra antígenos externos, mas inofensivos, como é o caso dos dos alimentos.

Numa impressionante exibição de coordenação celular, as células T recém-activadas migram para o local específico onde se encontra o antígeno apresentado e supervisionam a resposta imune, produzindo citocinas que, não só actuam no sistema imune inato, tais como em macrófagos já presentes no local, mas também funcionam como faróis que promovem migração adicional de mais células imunes inatas para ajudar a lutar contra uma ameaça específica, no caso de células Th1, Th2 e Th17, ou para parar a resposta inflamatória e promover tolerância, no caso de Tregs.

Adicionalmente, as DCs também podem activar células Tfh (células T auxiliares foliculares), que estão intimamente envolvidas em dar início e em moldar respostas imunes pelo *outro ramo* do sistema imune adaptativo – células B. Após esta "trindade de activação" entre células B, DCs e Tfh, as células B migram para o local de origem específico do antígeno apresentado pelas DCs, onde irão produzir e secretar anticorpos. Estes anticorpos, normalmente sob a forma de imunoglobulina G (IgG) ou imunoglobulina A (IgA), são capazes de reconhecer o antígeno e ligarem-se

directamente a ele, formando complexos imunes em que os *dois pequenos braços* do anticorpo em forma de Y se ligam ao antígeno, enquanto a sua "cauda", ou seja, a sua porção Fc, fica virada para fora (Fig. 1). Estes complexos imunes são depois capazes de serem reconhecidos pelos macrófagos, através dos receptores Fc, que se ligam à porção Fc, ajudando assim estes macrófagos a passarem por ainda outra activação pró-inflamatória adicional. Estes mecanismos colocam as DCs como o alvo inicial de uma estratégia de modulação imune, visto que ditam os estados de activação das células T e células B, enquanto coloca os macrófagos como alvo final, visto que são os últimos actuadores nesta cadeia imunológica de inato-adaptativo-inato.

Uma das áreas mais recentes no estudo de imunologia que tenta desvendar como modular respostas imunes é a área de imunometabolismo. Esta área surgiu da descoberta de que, dependendo da função que está a ser desempenhada, as células imunes sofrem reprogramações metabólica específicas, favorecendo certas vias metabólicas em vez de outras, não apenas para as suas necessidades energéticas, mas também para a síntese de metabolitos cruciais para serem usados durante as suas etapas de activação e diferenciação. Por exemplo, foi descrito que, *em geral*, macrófagos pró-inflamatórios e DCs imunogénicas dependem da glicólise e da síntese de ácidos gordos, enquanto macrófagos pró-resolventes e DCs tolerogénicas dependem da oxidação de ácidos gordos (FAO) e da fosforilação oxidativa (OXPHOS). Com este conhecimento nasceu a hipótese de que, modulando o metabolismo de macrófagos e DCs, seria possível controlar o seu estado de activação. Por exemplo, ao inibir vias metabólicas preferidas por fenótipos pró-inflamatórios/imunogénicos e ao promover vias metabólicas preferidas por fenótipos pró-resolventes/tolerogénicos, a célula mudaria de um estado pró-inflamatório para um estado anti-inflamatório e vice-versa.

Uma via fulcral que se encontra no centro de imunometabolismo é o metabolismo de lípidos. Apesar de lípidos terem sido vistos tradicionalmente como moléculas para armazenamento de energia e como materiais para a síntese de membranas celulares, hoje sabemos que não é bem assim. O metabolismo de lípidos tem um papel central na síntese de metabolitos usados pelas células imunes para desempenharem as suas respectivas funções. Por exemplo, a síntese de ácidos gordos pode levar à formação de gotículas lipídicas (pequenas bolhas de moléculas lipídicas dentro da célula, semelhantes às gotas de gordura que podemos observar na superfície de sopas e de guisados) em macrófagos e DCs, tendo sido identificadas como tendo um papel em funções imunes, tais como a produção de citocinas, fagocitose e apresentação de antígenos. Em contrapartida, FAO foi demonstrada como sendo importante, visto que Acetil-CoA resultante da oxidação de ácidos gordos pode ser usado para acetilação de histonas, que é uma maneira que a célula tem para controlar que genes são expressos e/ou reprimidos.

Para além disso, o metabolismo de lípidos inclui também a síntese de mediadores lipídicos. Os mediadores lipídicos são um grupo de moléculas que desempenham um papel altamente importante na comunicação entre células. Como o nome sugere, estas moléculas têm origem na oxidação de ácidos gordos e actuam como mensageiros químicos. Estes mediadores podem actuar na própria célula (sinalização autócrina), em células próximas da célula de onde originaram (sinalização parácrina), ou em células longe da célula original (sinalização endócrina). Estes mediadores são um dos principais mensageiros entre células imunes, sendo capazes de induzir vias de sinalização em macrófagos e DCs que, ou promovem a síntese de citocinas pró-inflamatórias e activação de células T, ou, em vez disso, abafam a inflamação, promovendo a regeneração do tecido e tolerância imune. Como tal, elucidar os mecanismos que levam células imunes a sintetizar estes mediadores lipídicos e compreender como é que estes mediadores actuam nas células imunes é fundamental para desvendar possíveis alvos terapêuticos para uma intervenção de um ponto de vista imunometabólico.

No entanto, ainda há muito que não se sabe. Como foi acima mencionado, *em geral*, macrófagos pró-inflamatórios e DCs imunogénicas tendem a favorecer glicólise e síntese de ácidos gordos, enquanto macrófagos anti-inflamatórios e DCs tolerogénicas tendem a preferir fosforilação oxidativa e oxidação de ácidos gordos. Contudo, há alguns estudos que sugerem que esta dicotomia imunometabólica não se aplica a todas as situações, demonstrando que a reprogramação metabólica pela qual os macrófagos e as DCs passam após activação é dependente de uma panóplia de factores, tais como o estado de diferenciação da célula, a localização no tecido, o estímulo e o momento de activação. Como tal, é fundamental olhar para o perfil imunometabólico das células em contextos específicos.

Esta especificidade de contextos também se verifica no papel dos mediadores lipídicos. Por exemplo, enquanto nalguns contextos um mediador lipídico (ex. Prostaglandina E_2) pode induzir respostas pró-inflamatórias em macrófagos, ou promover uma resposta activadora de células Th1 por parte de DCs, noutros contextos pode induzir uma resposta anti-inflamatória e/ou promover uma resposta Th2.

Logo, considerando que existe uma necessidade crucial de estudar os requisitos de macrófagos e DCs em situações específicas, esta tese teve como objectivo mapear os perfis e as dependências em diferentes contextos inflamatórios e celulares.

Delineação dos Capítulos da Tese

O **capítulo 2** constitui uma introdução teórica mais aprofundada a esta tese. Nele, podemos encontrar um enquadramento da área de imunometabolismo no contexto da imunidade inata, com especial foco no metabolismo de ácidos gordos em DCs e macrófagos, e na forma como o perfil metabólico e a função imune estão intrinsecamente ligados. Abordámos os dados actuais que demonstram a importância dos ácidos gordos (ex. ácidos gordos poli-insaturados) e dos mediadores lipídicos (ex. prostaglandinas) na modulação da função e do metabolismo dos macrófagos e das DCs. Explorámos também o papel multifacetado do metabolismo de ácidos gordos, tanto na promoção, como na inibição da inflamação, tendo chegado à conclusão de que tal coloca em evidência a importância de estudar cada contexto específico, se queremos obter uma visão completa das necessidades metabólicas das células imunes e encontrar formas de modular eficientemente as respostas imunes através da utilização de metabolismo como abordagem terapêutica.

No **capítulo 3**, estudámos o papel da IgG anti-spike de SARS-CoV-2 (Fig 1.) na promoção de um estado hiper-inflamatório em macrófagos. A IgG causa isto através da indução de mudanças metabólicas específicas que preparam estes macrófagos para uma expressão excessiva de citocinas pró-inflamatórias. Através de inibição química destas vias metabólicas conseguimos prevenir a hiper-inflamação induzida pela IgG. Semelhantemente, no **capítulo 4**, observámos qual o papel da IgA contra CCP2 (péptidos citrulinados que são auto-antígenos comuns nas articulações (Fig. 1)) em promover inflamação crónica no contexto de artrite reumatóide. A IgA causa isso ao induzir um estado de hiper-inflamação em macrófagos que tanto depende de certas mudanças metabólicas, como da síntese de mediadores lipídicos após cicloxigenase-2, identificando assim alvos terapêuticos com possíveis aplicações no tratamento de doenças de inflamação crónica causadas por auto-anticorpos.

No **capítulo 5**, descrevemos como é que DCs reconhecem antígenos solúveis de ovos (SEA) do parasita *Schistosoma mansoni* através da Dectina-2 (um receptor na membrana celular das DCs), como é que elas ficam habilitadas a induzir a diferenciação de células Th2, e como é que a síntese de mediadores lipídicos pela parte de DCs pode ser quimicamente modulada para modificar a sua função imune dentro deste contexto. Foi demonstrado previamente que os SEA sinalizam através da Dectina-2 em DCs de forma a induzir a síntese de PGE_2, a expressão de OX40L e também a habilidade de activar células Th2. Para além disso, nós fomos capazes de demonstrar também que, após estimular DCs com SEA, inibir quimicamente a síntese de PGE_2 também diminuiu a expressão de OX40L e a sua habilidade de induzir células Th2. Em suma, este estudo oferece novos conhecimentos sobre como é que *Schistosoma mansoni* habilita DCs de forma a induzir uma resposta

Th2 e fomos também capazes de identificar possíveis alvos terapêuticos de forma a controlar respostas Th2 induzidas por helmintos.

No **capítulo 6**, apresento uma discussão extensa delineando as novas descobertas desta tese e exploro futuras questões científicas para continuar a descodificar as nuances de imunometabolismo no contexto de respostas inflamatórias e as possíveis aplicações que este conhecimento científico pode ter na sociedade como um todo. Exponho como é que o metabolismo de lípidos está intimamente conectado com a função de macrófagos e DCs, não apenas devido à reprogramação metabólica pela qual passam estas células, mas também através da síntese de mediadores lipídicos que podem actuar nas próprias células (efeito autócrino) ou em células vizinhas (efeito parácrino). Especificamente, demonstro como é que mediadores abaixo de cicloxigenase-2 desempenham um papel central na promoção de respostas imunes tipo 1 e tipo 2, com DCs a precisar de PGE_2 para induzir uma resposta Th2 (resposta imune tipo 2), mas com macrófagos a necessitar *aparentemente* de outros mediadores que não PGE_2 para adquirir um fenótipo tipo M1 (resposta imune tipo 1). Também abordo o tópico de como reprogramação metabólica não é algo preto e branco e que é preciso estudar os requisitos metabólicos em contextos específicos. Isto foi evidenciado pelo facto de que o mesmo estímulo (IgA) necessita de vias diferentes em células diferentes de forma a induzir inflamação (glicólise em DCs vs metabolismo mitocondrial em macrófagos), ou o facto de que a mesma célula (macrófagos) usa diferentes vias metabólicas quando estimulado com diferentes anticorpos para promover inflamação (metabolismo mitocondrial para IgA vs glicólise, via dos fosfatos de pentose e síntese de ácidos gordos para IgG). Por fim, hipotiso também sobre as futuras aplicações destas descobertas e como é que as podemos transformar em aplicações clínicas, potencialmente através do uso de inibidores e/ou activadores químicos de forma a inibir vias metabólicas indesejadas e/ou de forma a promover vias metabólicas desejadas para obter uma resposta imune específica – por exemplo, promover glicólise e síntese de ácidos gordos juntamente com com terapisas baseadas no uso de IgG, ou promover actividade mitocondrial em conjunto com terapias baseadas no uso de IgA, de forma a ter uma resposta imune mais robusta pela parte de macrófagos, ou inibir PGE_2 em DCs, para inibir respostas tipo 2 indesejadas e/ou promover respostas imune tipo 1. No entanto, é importante ter sempre em mente que estas ideias podem não ser extensíveis a todos os contextos patológicos. Como tal, após sugerir várias futuras perspectivas sobre como avançar a partir daqui, tal como incorporar lipidómica espacial com ensaios imunes funcionais e explorar o que é que podem significar os resultados desta tese no contexto de patologias urgentes, tais como cancro, obesidade e doenças neurológicas associadas à idade, concluo ao reforçar a mensagem de que, para encontrar alvos terapêuticos eficazes, precisamos de estudar cada contexto patológico específico e ter uma abordagem focada em cada tipo de célula.

Conclusão

Em suma, esta tese contribui para a importância crescente do metabolismo de lípidos e de receptores Fc na biologia de macrófagos e DCs, no contexto de auto-imunidade, inflamação crónica, cancro, obesidade e infecção. Como tal, estudar os efeitos de mediadores lipídicos, sinalização de FcR, e o padrão imunometabólico de células mielóides associadas a tecidos no contexto de cada doença, em conjunto com ferramentas de lipidómica, pode dar-nos a compreender o mecanismo subjacente às causas destas patologias, ajudar a prever o desenvolvimento e a progressão de doenças e potencialmente levar a novas terapias para prevenir, tratar ou curar estas doenças.

Curriculum Vitae

Luís Pedro Ferreira de Almeida was born on 7th June 1996 in Lisbon, Portugal. He obtained his highschool diploma at the Escola Secundária de Miraflores (now called Escola Secundária Professor Santana Castilho) in 2014. A few months later he started his bachelor's in Biochemistry at the Faculty of Sciences of the University of Lisbon. He was the only one in a class of >70 Biochemistry students to obtain the maximum possible grade (20 out of 20) in the Bioenergetics and Metabolic Regulation course. After successfully completing his bachelor's in 2017, and not being able to decide between immunology and metabolism, he decided to set a course towards infectious diseases and immunometabolism. That is why, still in 2017, he joined a binational master's programme in infection biology, where he studied for one year at the Catholic University of Córdoba, in Argentina, and for another year, at the Hannover Medical School, in Germany. It was also in Hannover where he did is master's thesis, at the TWINCORE institute, in the lab of Prof. dr. med. Tim Sparwasser, where he studied how proteins from tuberculosis modulated the differentiation and activation of T cells. In 2019 he successfully completed his master's, having defended his thesis with the title "The immunomodulatory properties of *Mycobacterium tuberculosis* secretory proteins". Still in 2019, he started his PhD, as part of a Marie Skłodowska-Curie consortium, in the group of Dr. Bart Everts, at the department of Parasitology (now called LUCID), at the Leiden University Medical Centre. There he studied how metabolism and lipid mediators could regulate the function of macrophages and dendritic cells in the context of infectious diseases and chronic inflammation. The results of his research are described here in this thesis. Luís plans to use the knowledge and skills he obtained during his PhD to continue advancing translational research and finding possible new therapeutic strategies to treat immune-related diseases.

Portfolio

Mandatory courses

- Leiden University Onboarding Programme Inform & Connect 2021
- Basic Methods and Reasoning in Biostatistics 2022
- Responsible Research
  - Research Regulations and Practical Implications in the LUMC 2023
  - Data Management Workshop 2024

Generic/disciplinary courses

- Advanced Course Infection, Immunity and Tolerance 2020
- ArthritisHeal Biostatistics and Bioinformatics Workshop 2021
- Business and Career Development Workshop 2022

Attended lectures, LUMC presentations, participation in meetings

- Rheumatology Workshop 2020
- ArthritisHeal Consortium Final Meeting 2022

Congress attendance and poster or oral presentations

- LIPID MAPS Spring School 2021 2021
- 34th European Macrophage and Dendritic Cell Society 2021
- VIB Translational Immunology Conference 2021 2021
- NVVI Annual Meeting 2021-2022 2022
- 8th European Workshop on Lipid Mediators 2022
- NVVI Annual Meeting 2022 2022
- Joint Belgian-Dutch Immunology Meeting 2023 2023

Traineeship abroad

- Beckman Coulter Secondment (Marseille) 2020

Lecturing, lab assistance, student supervision

- B.Sc. Jochem Grossouw 2022
- Half-Minor HIV Flow-Cytometry Demonstration 2023 2023
- Half-Minor HIV Flow-Cytometry Demonstration 2024 2024

List of Publications

Bacon A*, **Almeida L***, Ghorasaini M, Toes REM, Everts B, Giera M. Comprehensive lipidomic profiling reveals distinct metabolic remodeling during differentiation and polarization of human monocyte-derived macrophages. J Proteome Res. 2026. *Shared first authorship

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Acknowledgements

While many think a PhD is an individual achievement, those that go through its motions quickly realise that *no man is an island*, and that a PhD cannot be done without the help and support of many.

Bart, thank you for your constant support, both professional and personal, this PhD is not only proof that you helped me evolve as a scientist, but also as a person. As I said several times over the years, I couldn't have asked for a better supervisor, and if there's one thing I'll miss from the PhD, it will definitely be our one-on-one meetings, not just for the nice scientific discussions, but for all the talks, and funny stories.

Maria, thank you for the constant inspiration, for always having your door open, and for all the wonderful conversations we had, be it about science, culture, food, travel, music, and many other mutual interests.

Bruno, thank you for all the metabolic discussions, for all the amazing suggestions, ideas and, especially, the BBQs.

Joost and Nikolas, my two paranymphs. Joost, for all the coffee breaks, the jokes, and the talks that could go from Lord of the Rings and ancient history to food recipes and world politics. Nikolas, for all our gastronomic pilgrimages, our classical concert attendances, and our literary and philosophical discussions, especially the ones in the evenings after a hearty meal.

Thiago and Graham, the group's two resident postdocs. Thiago, for always being willing to lend a hand with anything, for being the only other Lusophone with whom I could talk in Portuguese (my accent permitting), and for all the talks and jokes about Brazil and Portugal. Graham, for being the only other group member on the macrophage camp, for all the talks going from science to literature and, above all, for having introduced me (and many others) to the wonders of Scotland.

Eline, thank you for the sailing trips, your amazing friendship and inspiration; you made my PhD memorable, and continue to inspire me with your positivity and confidence (except when you deny that macrophages > DCs).

Irene, thank you for the jokes, the conversations, the dinners and *especially* the Iberian delicacies, which helped kill the *saudades* of this expatriated Portuguese.

Natalia, my Gen Z friend. Thank you for all the moments, the interesting talks, the memes and the witty banter.

Anna, Miriam, and Rike, thank you for your support and friendship, and for all the moments we shared both in the office, in the lab, and on the outside. You made me

feel at home when I arrived to what was an entirely new country for me.

Chanel, Dennis, Emma, Eva, and Roos, thank you for all the coffee breaks, the nice talks, the parties and for the overall *gezelligheid.*

The ArthritisHeal group, Alice, Mohan, Benedict, Celia, Chiara, Daniela, Fei, Henneke, Jianyang, Konstantina, and Patrícia. For all the comradery and for all the wonderful and unique experiences we shared that came along with being ESRs in the same consortium.

Alwin, thank you for all the good talks, the jokes, and the help in solving problems of almost any nature. Frank, thank you also for all the nice talks and for always being willing to help in the lab. Marjolein, for the "m*oaning meetings"* we shared during our coffee breaks.

Jeroen, Chiara, and Lynn, thank you for the amazing scientific discussions and all the great work we did together.

The people of *the department formerly known as PARA*, thank you for helping make my PhD such a memorable time.

To the FAMIBA, Elia, Letícia, Luise, Matias, Pia, and Sinja. For the moments we shared all over the world and for proving that strong friendships can resist the longest of distances.

To my Portuguese friends, Afonso, Ana, Bruno, Cátia, Costa, Filipa, Hélvio, Ivo, Joana, Mafalda, Miguel, Ni, and Rita. As I'm writing this, I realise we've been friends for more than a decade; and even though we are all in different corners of the world, living our respective "adult" lives, I would like to thank you for your friendship, your support, the moments we shared (some of them against my will), and the ones that are yet to come.

Por fim, à minha família, cuja ajuda e sacrifícios tornaram isto possível. Aos meus avós, Guilhermina, Gracinda e Henrique, por toda a comida deliciosa, por tomarem conta de mim, tanto em criança como em adulto, e por toda a sabedoria e valores que me ensinaram. Aos meus pais, Luís e Teresa, que garantiram que eu tive a possibilidade de ir atrás de todas as oportunidades que me foram apresentadas.[1] To my siblings, Luís Miguel, Filipa, and Raquel, and to my sister-in-law Ayesha; family by chance but friends (and also annoyers and *annoyees*) by choice.

1 Finally, to my family, without whose help and sacrifices none of this would have been possible. To my grandparents, Guilhermina, Gracinda, and Henrique, for all the delicious food, for taking care of me, both as a child and as an adult, and for all the wisdom and values they taught me. To my parents, Luís and Teresa, who made sure I had the ability to pursue all the opportunities that were put in front of me.