The fate of cells

Interference and study of macrophage differentiation and activation

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Table of contents

The fate of cells

Interference and study of macrophage differentiation and activation

Table of contents

Chapter 1

Introduction

General introduction

Inflammation refers to the response of the immune system to invading pathogens or internal signals that result in the attack and clearance of these pathogens, necrosis of cells and finally tissue repair. This definition is simplistic; we know that the inflammatory process is more complex and includes synergy and interaction between molecular, immunological and physiological processes. The response is also heterogeneous in terms of the cells and molecular mediators involved [1]. Regardless of its complexity, the inflammatory response has four common components: inducers, sensors, mediators and target tissues.

Inducers are the signals, either endogenous or exogenous, that indicate malfunctioning, infection or stress [2]. Sensors are the cells, for instance, immune cells like macrophages that detect the inducers with specific receptors. The cells will respond by producing particular inflammatory mediators, like cytokines, in a repertoire depending on the inducers. These mediators act on the target tissues changing their functional state. This change in the functional state and the inability to restore homeostatic conditions is the core of pathologies related to inflammation [3].

1.Macrophages

In an inflamed state "the sensors" are the cells that detect and respond to inducers to initiate an inflammatory response. In this thesis, I will specifically focus on macrophages, as they play a crucial role in virtually any inflammatory disease.

Macrophages are phagocytic cells with a heterogeneous phenotype, that play a role in the activation and regulation of the immune response. These cells belong to the innate immune system, but they can activate and interact with the adaptive immune response, for example, by activating T cells. Macrophages play a central role in the development and resolution of inflammation. This suggests that modulation of the macrophage inflammatory response can be a treatment strategy for inflammatory diseases.

1.1 Macrophage's ontogeny

The macrophages present in the tissues can have different origins, which will impact the phenotype and function of these cells. The distinct macrophage's origins can be: yolk sac, foetal liver monocyte, which will become tissue-resident macrophages, or monocytes from the bone marrow, that will give rise to monocyte-derived macrophages (MDMs) [4]. Tissue-resident macrophages perform mainly functions related to homeostasis like, for example sensing changes in oxygen levels, osmolarity, and iron metabolism [5], while MDMs act especially in pathological states [6-8]. The proportion of the different macrophages varies during development. In the early stages, most of the macrophages in the body have yolk sac origin. During development, these cells are (partly) replaced by macrophages from foetal liver and, in an adult individual, MDMs can be found

in tissues [9]. Despite these changes, during development the proportion of macrophages from different origins in a tissue will depend on the tissue. For instance, the brain contains mainly tissue-resident macrophages (called microglia) from the yolk sac [10], the epidermis has a higher proportion of tissue-resident macrophages from monocytes originating from the foetal liver [11]. In the gut, the majority of macrophages are MDMs [12]. Other tissues present a balanced mix of macrophages from different origins.

MDMs are macrophages differentiated from circulating monocytes, originating in the bone marrow. The differentiation of monocytes into macrophages is induced by different growth factors, like macrophage colony-stimulating factor (M-CSF) or granulocyte colony-stimulating factor (GM-CSF), among other mediators [13, 14]. In the process of differentiation, monocytes change their functional repertoire and morphology, for instance to a more elongated one in the case of M-CSF-induced macrophages. With both M-CSF and GM-CSF there is an increase in adherence, change in protein markers (e.g. increased expression of CD68) among other phenotypic and functional changes that lead to the development of monocytes into mature macrophages [15]. When comparing M-CSF to GM-CSF derived macrophages, GM-CSF derived macrophages typically present a more pro-inflammatory phenotype compared to M-CSF derived macrophages [16]. After differentiation, macrophages can be activated towards different phenotypes and will produce different molecules like cytokines or chemokines.

1.2 Macrophage signalling pathways and cytokines in inflammation

The production of inflammatory mediators by immune or non-immune cells is initiated by the activation of pattern-recognition receptors (PRRs), either by exogenous or endogenous signals. There are different types of PRRs in macrophages: Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) [17, 18]. Receptor binding results in intracellular pathway activation to initiate an inflammatory response. Examples of intracellular pathways activated by PPRs in macrophages, and other cells, include the Mitogen-activated protein kinases (MAPK) pathway, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway.

The MAPK pathway consists of a series of serine/threonine protein kinases activated by various stimuli, including osmotic stress, heat shock, interleukin 6 (IL-6), interleukin 1 (IL-1) and tumour necrosis factor (TNF). This pathway regulates cell proliferation, apoptosis and differentiation [19]. Activation of this pathway leads to activation of the transcription factors Erk1/2, JNK and p38, which promote the expression of pro-inflammatory mediators [20].

The NF-κB pathway is formed by five proteins of the NF-κB family (P50, p52, p65, RelB and c-Rel [21]). Upon activation of this pathway, the phosphorylation and consequent degradation of

IκB releases the NF-κB factors. These transcription factors then translocate into the nucleus thereby inducing expression of inflammatory cytokines [22, 23].

Finally, the JAK-STAT pathway is a conserved pathway used to control gene expression by extracellular signals. When signals like interferons activate this pathway, the receptor associated JAK proteins get phosphorylated. STAT proteins bind to phosphorylated JAKs and get phosphorylated, forming dimers that will bind the DNA and initiate gene transcription to generate a specific response [24, 25]. The activation of these pathways leads, among other things, to the production of cytokines and chemokines, which are essential mediators in the inflammatory response.

Cytokines and chemokines are proteins that are mainly produced by immune cells, including macrophages. These proteins are classified in interleukins (ILs), interferons (IFNs), tumour necrosis factor (TNFs), colony stimulation factors (CSFs) and chemokines, and they can either be pro or anti-inflammatory. Chemokines mainly act as recruitment signals for other immune cells [26]. Cytokines produced by macrophages have functions in many inflammatory diseases, with interleukin 1 beta (IL-1β), TNF and IL-6 being the most common ones associated with diseases. IL-6 is particularly interesting due to its dual role as a mediator of both anti- and pro-inflammatory responses [27, 28].

Another central pro-inflammatory cytokine is interferon gamma (IFNγ). This cytokine is produced by T cells when activated towards a Th1 phenotype [29]. Macrophages are not primary producers of IFNγ, but their function is strongly affected by shifting the cells towards a pro-inflammatory phenotype [30]. This is applicable in many inflammatory diseases where a positive feedback loop of continuous signalling between cells causes a chronic inflamed state.

Besides pro-inflammatory functions, cytokines produced by macrophages, like interleukin 10 (IL-10) are also involved in immunoregulatory activities. IL-10 blocks the differentiation and pro-inflammatory activation of T cells and modulates the differentiation of macrophages to cells with alternatively activated properties [31-33].

2. Epigenetic regulation of the macrophages

Modulation of the response of macrophages might be a useful approach in the treatment of various inflammation-related diseases. One of the main mechanisms that control macrophage function is by epigenetic processes.

2.1 Epigenetic regulation

Epigenetics involves all changes associated to the DNA or DNA-associated proteins that do not affect the sequence of the DNA itself. Epigenetic modifications lead to changes in transcription

factor binding and therefore affect gene expression. Regulation of gene expression is achieved among other things by chemical modification of DNA and histones. Histones are proteins that form octamer protein-complexes termed nucleosomes around which DNA is wrapped. This histone-DNA structure is known as chromatin. Chromatin can be found in two different states, heterochromatin and euchromatin. Heterochromatin is the closed conformation of chromatin and is linked to gene repression while euchromatin is the open state and is linked to gene expression due to the accessibility of genes. Histone modifications tightly regulate DNA accessibility and they can be either activating or repressive depending on the type and position of the modification. Histone acetylation is in general associated with gene expression as the chromatin acquires a less condensed structure, whereas histone methylation can be activating (H3K4me/H4K20me) or repressive (H3K27me/H3K9me) [34-36]. Epigenetic modifications are carried out by epigenetic enzymes that can be divided in writers, that add modification; erasers, that remove them; and readers, which act as detectors of these modifications [37]. The epigenetic changes and the associated effects on gene expression impact on the inflammatory phenotype of cells [38]. Macrophages are also regulated by epigenetic modifications.

Histone deacetylases (HDACs) are epigenetic enzymes that remove acetyl groups from histones. There are 18 HDACs identified, and the protein family is sorted into four different classes; Class I (HDAC1, 2, 3 and 8), class II, that is further divided into class IIa (HDAC4, 5, 7 and 9) and class IIb (HDAC6 and 10), class III, also known as sirtuins (SIRT1, 2, 3, 4, 5, 6 and 7), and class IV (HDAC11) [39]. In macrophages, this protein family has been associated with specific inflammatory phenotypes. The absence of HDAC3 in macrophages has been linked with reduced expression of LPS-induced genes. Conversely, these cells are more reactive to IL-4 and present an enhanced alternatively activated phenotype [40, 41]. Other HDACs have also been linked to inflammation; overexpression of HDAC5 leads to increased expression of monocyte chemoattractant protein-1 (MCP-1) and TNF [42]. Similarly, overexpression of HDAC11 leads to inhibition of IL-10 production and induces macrophage phenotype that induces the activation of naïve T cells [43]. In general, HDACs have been shown to be implicated in a lot of different inflammatory diseases including atherosclerosis, cancer, etc [44, 45]. Other examples of epigenetic enzymes are the histone acetyl transferases (HATs). In chronic obstructive pulmonary disease, the alveolar macrophages show increased acetylation in the promotors of pro-inflammatory genes (*IL1B*, *IL2*, *IL8* and *IL12B*) induced by the CBP-p300 HAT, which it is linked to higher expression of these genes [46].

3.Inflammatory diseases

Inflammatory responses can be acute or chronic [47]. Inflammation and its deregulation have been linked to a wide range of diseases and macrophages play an important role in many of them.

3.1 Atherosclerosis

Inflammation is key in all phases of atherosclerosis development. Atherosclerosis is characterized by a narrowing of the lumen in the arteries due to the build-up of an atherosclerotic plaque [48]. The rupture of the plaques can cause thrombus formation and occlusion of downstream vessels, leading to myocardial infarction or stroke. The formation of atherosclerotic plaques starts with the accumulation of lipoproteins (LDL) in the arterial wall. Within the arterial wall, LDL can be modified (e.g. oxidized) resulting in endothelial and immune cell activation. Macrophages recruited from the circulation can phagocytose oxLDL in an unrestricted way and thereby become so called foam cells [49]. Macrophages and foam cells release cytokines like IL-1β, IL-6, and chemokines like CCL2, that will attract more monocytes to the atherosclerotic lesion. This is the beginning of a positive feedback loop where new monocytes will be recruited, engulf the oxLDL and become foam cells that will again release mediators of atherosclerosis development. Excessive uptake of cholesterol can also lead to apoptotic macrophages, which if not efficiently cleared, results in secondary necrosis. The presence of necrotic cells will further enhance the pro-inflammatory response leading to a general inflamed state in the blood vessel [6]. Other immune cells, like T cells, are later also recruited to the lesions where they are activated towards pro-inflammatory phenotypes producing IFNγ and participating in the inflammatory response [50].

3.2 Cancer

Pre-existing chronic inflammation promotes the development, progression and metastasis of different types of cancer [51]. Chronic inflammation is thus considered a risk factor and it has been linked to mutations in cells that lead to transformation into cancerous cells [52]. The tumour micro-environment (TME) is formed by the cells and the mediators associated to the tumour. Tumour-associated macrophages are part of the TME and these cells produce cytokines and prostaglandins that cause immunosuppression, allowing the malignance to escape from host defence mechanisms [53]. Pro-inflammatory mediators like IL-6, TNF and IL-1β have been linked to the progression of cancer by promoting stromal development, tissue remodelling and growth stimulation of cancer cells [54]. Therapies inhibiting IL-1β like by Canakinumab have been shown to lead to a better outcome of disease [55].

3.3 Psoriasis

Psoriasis is an inflammatory skin disease characterized by lesions in the skin, increased number of keratinocytes and infiltration of immune cells, especially T cells and DCs. Plasmacytoid DCs can produce IFNα which leads to the recruitment and maturation of myeloid DCs. These cells release inflammatory mediators like IL-6, IL-12, IL-23 and IFNγ, which cause the activation of T cells and affect the function of macrophages activating them towards a pro-inflammatory phenotype. These cells will release more pro-inflammatory mediators, generating an inflamed state in the dermis [56]. Inflammatory mediators (e.g. TNF, IFNγ, IL-2, IL-17, IL-23, IL-12 and IL-1β) have

been shown to be present in the dermis and serum of the patients [57]. The inflammatory state leads to the proliferation of keratinocytes and the emergence of the typical skin lesions [58]. This disease is considered a risk factor for cardiovascular diseases and presents comorbidity with atherosclerosis, due to the presence of common mediators and a general inflammatory state [59].

3.4 Rheumatoid arthritis

In rheumatoid arthritis (RA) the inflammation process mainly appears in the synovial fluid of the joints, leading to their destruction [60]. The synovial fluid inflammation is maintained by the continuous recruitment of immune cells by macrophages. T cells attracted by the presence of chemokines which are produced by macrophages [61], collaborate with the macrophages in maintaining the inflamed state. Similar to psoriasis, RA has been linked to an increased risk for atherosclerosis. This is due to the general inflammatory state and the common mediators between the two diseases, like TNF and IL-6.

4.Therapies targeting cytokines and epigenetics enzymes in inflammatory diseases

Considering the importance of epigenetics in the regulation of inflammatory responses and the role of inflammatory mediators in disease, therapies to modulate these two elements are explored and applied as treatments in inflammation-related diseases.

4.1 Anti-inflammatory mediator therapy

Blockade of inflammatory mediators could help to avoid the establishment of the inflamed state. Canakinumab is a monoclonal antibody that blocks IL-1β; this molecule is being used in the clinic for the treatment of rheumatoid arthritis [62]. In cardiovascular disease (CVD) patients, Canakinumab leads to a decrease in cardiovascular events, IL-6 and protein C-reactive (CRP) levels without affecting circulating levels of LDL-C (CANTOS study) [63]. Other therapies targeting IL-1β like Anakinra (IL-1β receptor inhibitor) also showed beneficial effects on the treatment of acute and chronic inflammatory diseases [64]. Another molecule that regulates inflammation, colchicine, has been tested in CVD patients after myocardial infarction (MI) in the COLCOT trial. Patients that were treated with colchicine after MI showed a 23% reduction of the primary composite endpoint (cardiovascular death, resuscitated cardiac arrest, MI, stroke, or urgent hospitalization for angina necessitating coronary revascularization) and a reduction of 34% in the total burden of ischemic cardiovascular events, compare to the placebo group [65].

Anti-TNF therapy has been widely studied and several molecules are available for the treatment of RA: Infliximab, Adalimumab, Etanercept, Golimumab, Certolizumab. The majority of these molecules are monoclonal antibodies that bind and neutralize TNF or its receptor (TNFR2) [66]. Besides RA, anti-TNF therapy is also being considered for other diseases like gastrointestinal, dermatologic and inflammatory lung diseases [67-69]. Tocilizumab, is an IL-6 receptor antagonist

used in the treatment of RA alone or in combination with other anti-inflammatory mediators therapy, like anti-TNF [70, 71].The importance of IFNγ in inflammation has been previously described, therefore blockage of this cytokine is being studied with the aim of investigating its therapeutic potential. Anti-IFNγ therapy is being tested in inflammatory T cell response-related diseases, for example in hemophagocytic lymph histiocytosis, RA, psoriasis and type I diabetes among others. Anti-IFNγ therapy has been shown to improve the clinical outcome of patients with less side effects than anti-TNF treatment [72-75]. Anti-cytokine therapy has also been investigated for its therapeutic potential in cancer. These therapies target different cytokines, including IL-6, IL-8 and receptors CXCR1/2 and IL17A [76].Even though these therapies have shown good results, there may be a concern that the systemic blockage of cytokines could create an imbalance as these cytokines are important in maintaining homeostasis. This imbalance could lead to side effects like encephalitis and susceptibility to infection or sepsis among others [77].

4.2 Targeting epigenetics

Different epigenetic enzymes are related to the initiation, regulation and termination of the immune response [78, 79]. Considering this importance of epigenetic enzymes, targeting them in inflammatory diseases is a field in expansion with a promising future for therapies.

One promising inhibitor for inflammatory diseases are the HDAC inhibitors (HDACi). Currently six HDAC inhibitors (vorinostat, belinostat, panobinostat, romidepsin, valproic acid and sodium butyrate) have been approved by the FDA for the treatment of cancer (cutaneous T cell lymphoma, peripheral T cell lymphoma, multiple myeloma) and neurological disorders [80-83]. All HDAC inhibitors bind to the zinc-containing catalytic domain of the histone deacetylase and block the deacetylase activity [84]. There are different types of HDAC inhibitors: hydroxamic acid-based, cyclic tetra/depsipeptides, amino-benzamide-based, short-chain fatty acid-derived inhibitors and more recently hydrazide-based HDACs inhibitors [81]. The different types of inhibitors have more affinity towards specific classes of HDACs, e.g. amino-benzamide-based HDAC inhibitors show more affinity for class I HDACs [85].

Besides their applications in cancer, HDAC inhibitors have also been studied as possible treatment for inflammatory diseases. Givinostat has been tested in patients with juvenile idiopathic arthritis showing improvement in mobility and joint inflammation [86]. Two other HDAC inhibitors applied in patients are TSA and SAHA. TSA is an hydroxamic acid that targets class 1, 2 and 4 HDACs with nanomolar potency [87]. SAHA is also and an hydroxamic acid but targets class II HDACs [88]. Both compounds show promising results in type I diabetes, multiple sclerosis, IBD, SLE, RA and sepsis [89-91]. Inhibition of NF-κB transcriptional activity is implicated in their mechanism to block the expression of cytokines such as IL-6, IL-8, IL-10 and IL-12 [44] in macrophages. SAHA has also been tested in atherosclerosis models, showing a reduction of atherosclerotic lesions and inflammatory markers in atherosclerotic mice [92].

Improvements in HDAC inhibition treatment could be obtained by using inhibitors for specific HDACs rather than pan HDAC inhibitors [93] or targeting specific cell types like macrophages [94, 95]. Besides HDAC inhibition, other epigenetic enzymes are also being explored as possible treatments for inflammatory diseases.

Bromodomain and, within them, the extra terminal (BET) proteins are epigenetic readers. BET proteins are a family of four epigenetic readers (BRD2, BRD3, BRD4 and BRDT). These proteins contain two bromodomains (BD1 and 2) that bind acetylated lysines. There are two main types of bromodomain inhibitors depending on their ability to act as acetylated lysine mimetics or not [96]. Most of the studies with these inhibitors are focused on their application in cancer, but new studies show that these compounds could also be used in inflammatory diseases. For instance, in RA, BET inhibition (with JQ1 or I-BET151) showed reduction of pro-inflammatory mediator (e.g. TNF) production via blockage of the NF-κB pathway [97, 98]. Similar results were observed by various BET inhibition studies on periodontitis, retinal inflammatory diseases, type 2 diabetes and allergic lung inflammation [99-102]. The BET inhibitor JQ1 has shown promising results in different cardiovascular diseases by decreasing atherosclerosis, angiogenesis, intimal hyperplasia, pulmonary arterial hypertension, and cardiac hypertrophy [103]. Another BET inhibitor, apabetalone (RVX-208) is already in clinical trials. This compound has passed phase I and II clinical trials (ASSERT, ASSURE, SUSTAIN) where it showed that treated CVD patients showed decreased CRP levels and reduced major adverse cardiovascular events [104]. The BETonMACE phase III study assesses the effect of treatment with apabetalone in patients with type 2 diabetes with acute coronary syndrome (ACS) [105].

Aim and outline of the thesis

The aim of this thesis was to study small molecule drugs that affect monocyte to macrophage differentiation and activation and assess their effect in inflammatory conditions. The first part of the thesis is focused on understanding how different mediators are capable of inducing monocyte to macrophage differentiation and their phenotype. In the second part, various small molecule inhibitors are tested for their capacity to block macrophage differentiation or activation.

Chapter 2 is a review on macrophage origin and function. It discusses how mediators other than M-CSF and GM-CSF are able to induce macrophage differentiation and discusses *in vitro* models of generating human macrophage models. In **Chapter 3**, the capacity of IFNγ as a macrophage differentiation mediator is investigated. In this chapter, macrophages differentiated from monocytes using IFNγ as a differentiation factor are characterized and compared to macrophages differentiated with M-CSF or GM-CSF. We discuss how these IFNγ generated macrophages can be used as an *in vitro* model for psoriasis.

In order to find small molecules that affect the process of monocyte-to-macrophage differentiation in **Chapter 4** we performed a CRISPR-Cas9 screen in the THP-1 monocytic cell line. Based on

the findings from the screen, small molecules that target the identified proteins are used to modify macrophage differentiation in THP-1 cells and primary M-CSF differentiated macrophages. Finally, the effect of the inhibitors on pro-inflammatory responses was assessed.

Chapter 5 describes IBET-151 (BET inhibitor) that blocks GM-CSF but not M-CSF induced human monocyte differentiation. Under heterogeneic conditions, IBET-151 skewed monocyte differentiation towards homeostatic M-CSF derived macrophages that are less pro-inflammatory and do not activate autologous T lymphocytes. Chip-seq experiments demonstrated that IBET-151 displaced BET proteins actively recruited to promoters and enhancers of GM-CSF-induced genes. We tested this inhibitor in a rat CIA model of arthritis, as GM-CSF is known to have a pro-inflammatory function and therefore GM-CSF macrophages are thought to play an important role in joint destruction. We found that IBET-151 abrogated arthritis without impacting the number of macrophages in the joint but rather through dampening the pro-inflammatory phenotype. In **chapter 6,** we used an HDAC inhibitor that specifically targets myeloid cells (ESM-HDAC528). We found that ESM-HDAC528 impairs the differentiation of peritoneal macrophage and its pro-inflammatory response in a model of acute inflammation. Based on these results we used the ESM-HDAC528 in an atherosclerosis model. We found that ESM-HDAC528, despite the impairment of differentiation of peritoneal macrophages and its pro-inflammatory response, did not have a major effect on atherosclerosis outcome. In **chapter 7**, we made use of a small molecule that inhibits BRD9. BRD9 inhibition modified inflammatory responses of primary human macrophages as shown by cytokine production and transcriptional analysis.

Chapter 8 includes the general discussion and future perspectives based on the findings of this thesis.

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Chapter 2

Classic and new mediators for *in vitro* modelling of human macrophages

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Abstract

Macrophages are key immune cells in the activation and regulation of immune responses. These cells are present in all tissues under homeostatic conditions and in many disease settings. Macrophages can exhibit a wide range of phenotypes depending on local and systemic cues that drive the differentiation and activation process. Macrophage heterogeneity is also defined by their ontogeny. Tissue macrophages can either derive from circulating blood monocytes or are seeded as tissue-resident macrophages during embryonic development. In humans, the study of *in vivo* generated macrophages is often difficult with laborious and cell-changing isolation procedures. Therefore, translatable, reproducible, and robust *in vitro* models for human macrophages in health and disease are necessary. Most of the methods for studying monocyte-derived macrophages are based on the use of limited factors to differentiate the monocytes into macrophages. Current knowledge shows that the *in vivo* situation is more complex, and a wide range of molecules in the tissue microenvironment promote and impact on monocyte to macrophage differentiation as well as activation. In this review, macrophage heterogeneity is discussed and the human *in vitro* models that can be applied for research, especially for monocyte derived macrophages. We also focus on new molecules (IL-34, platelet factor 4, etc.) used to generate macrophages expressing different phenotypes

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Introduction

Macrophages are immune cells with heterogeneous phenotypes and complex functions in tissue homeostasis and innate and acquired immunity. These cells belong to the mononuclear phagocyte system (MPS).[1–3] In the original MPS model, macrophages present in the tissues were all thought to be derived from monocytes.[4] In the 2000s this concept started to change when lineage-tracing studies showed that populations of macrophages with different origins were found in tissues. These cells were capable of self-maintenance independently of circulating monocytes.[5–7] Currently, we know that macrophages have different origins: embryonic yolk sac derived, fetal liver derived, and/or bone marrow monocyte derived macrophages (MDMs).[3,8] In terms of function, tissue-resident macrophages act as "controllers" to maintain tissue homeostasis. They perform several functions, for example, removal of dead cells from tissues,[9] sensing changes in oxygen levels, osmolarity, and iron metabolism.[10–12] Besides the homeostatic functions, tissue-resident macrophages drive local and systemic defensive responses to pathogens.[13–15] MDMs have been implicated in a wide range of diseases, not only those that encompass inflammatory conditions that lead to immune activation, such as atherosclerosis, sepsis, rheumatoid arthritis, and systemic lupus erythematosus, but also those that are accompanied by immune suppression, such as tolerance to bacteria, or cancer.[16–20]

Considering the important role of both tissue-resident macrophages and MDMs in homeostasis and disease it has always been key to develop representative *in vitro* models to study these cells. For these models it is clearly relevant to define the *in vitro* settings, which can mimic both homeostatic and disease-associated situations. The closer *in vitro* models resemble *in vivo* macrophages, the better they will dictate our understanding and translation to study human disease. In general, MDM *in vitro* models have been relatively unrepresentative of the tissue environment that a monocyte faces when entering a tissue. The methods of monocyte to macrophage differentiation *in vitro* have been rather simplistic in terms of the factors used, disregarding many relevant molecules or factors found in diseases that can impact on monocyte to macrophage differentiation. The use of different factors gives an opportunity for further study and improvement of the *in vitro* models. The purpose of this review is to provide an overview of the different methods used to study human macrophages *in vitro*, with a brief discussion of tissue-resident macrophages and a deeper review of MDMs.

Origins and *in vitro* models for tissue-resident macrophages

New advances in the field have shown that tissue-resident macrophages have self-renewing capacities in steady state as well as under inflammatory or infectious conditions.[7–22] Most populations are seeded during embryonic development, and emerge in three sequential waves: the primitive, the transient definitive, and the definitive.[23,24]

The primitive wave starts in the blood islands of the yolk sac and produces primitive progenitors of erythroid cells, megakaryocytes, and macrophages. Microglia originate from these cells.[25,26] In

the second wave, termed transient definitive, the erythro-myeloid precursors are generated in the yolk sac and migrate to the fetal liver where they expand and differentiate into fetal liver monocytes.[27] These fetal liver monocytes subsequently migrate into tissues to differentiate into tissue-resident macrophages, such as Langerhans cells in the dermis.[28] The third wave, termed definitive, gives rise to immature hematopoietic stem cells (HSCs) in the aorta-gonads-mesonephros region. These immature HSCs colonize the fetal liver, the main hematopoietic organ during embryonic development, and ultimately seed the bone marrow generating mature HSCs that can differentiate into adult monocytes and maintain monocyte populations throughout life.

The contribution of yolk sac macrophages, fetal liver monocytes, or bone marrow monocytes to the development of tissue macrophages varies over time and it's specific for different tissues. For instance, microglia in the brain are only derived from yolk sac macrophages,[29] whereas Langerhans cells (epidermis) are mainly derived from fetal liver monocytes. The same is true for alveolar macrophages in the lungs and Kupffer cells in the liver. In the case of the pancreas and the heart, the ontogeny is a mix of macrophages differentiated from fetal liver monocytes and a minor contribution from bone marrow monocytes. Finally, in the gut and the dermis most macrophages are bone marrow monocyte derived, although recent studies show a population of macrophages in the gut with self-maintaining capacities that present a different transcriptome from gut MDMs.[30] A common feature for most tissues is that during the early stages of development, there is a contribution of yolk sac macrophages that is gradually replaced by macrophages from other origins with increasing age.[31]

Recent advances have allowed the *in vitro* generation of tissue-resident macrophages from different sources applying induced pluripotent stem cells (iPSC) derived from either stromal cells or embryonic stem cells.[32,33] The iPSCs are more commonly used as the use of embryonic stem cells entails ethical issues. The first protocols for generating iPSC-derived macrophages (iPSDMs) appeared in the early 2010s[34,35] and all commonly used protocols share three main phases.

The first phase of differentiation of iPSCs to iPSDMs consists of specification of iPSCs into hemogenic endothelium. The second phase aims to achieve the endothelial to hematopoietic transition to obtain hematopoietic progenitors. Finally, the third and last phase is the induction of differentiation of progenitors into macrophages by the addition of cytokines such as M-CSF, GM-CSF, or IL-34.[36–41]

For certain applications, it is worthwhile to differentiate the iPSDMs into specific tissue-resident macrophage phenotypes. To generate such specified iPSDMs appropriate combinations of cytokines and growth factors are added, for example, for the generation of iPSDMs microglia a cocktail of M-CSF, IL-34, TGFβ, cluster of differentiation molecule 200 (CD200), and fractalkine (CX_3CL_1) can be used.[42] Another approach to obtain tissue-specialized iPSDMs is by coculturing them with parenchymal cells. This has been shown convincingly for microglia, where coculturing iPSDMs with iPSC-derived neurons gave specification into brain-resident macrophages. Also in

mice, the *in vitro* generation of microglia-like iPSDMs has been successful by coculture with neurons.[43] These cells acquired similar morphology and gene expression patterns as isolated primary microglia. In humans the *in vitro* generation of these cells was validated by comparing the microglia-like iPSDM transcriptomic profile with available transcriptomes of primary microglia, which showed a high degree of similarity.[44] The capacity to generate tissue-specific iPSDMs was also tested *in vivo* by transferring cells into brain and lungs of mice. As a result, microglia iPSDMs and alveolar-iPSDMs developed in these animals.[45] In terms of function, when the response to LPS from iPSDMs and MDMs is compared at the transcriptomic level, the response is largely conserved and only some minor differences are found in antigen presentation and tissue remodeling-related gene expression.[46]

An important advantage of the use of iPSDMs lies in the source to obtain them. Studying the impact of specific mutations is possible using iPSCs obtained from stromal cells from patients carrying the variant, as the genotype will be maintained. For instance, in neurodegeneration, patients showing mutations in triggering receptor expressed on myeloid cells 2 (*TREM2*) present deficits in phagocytosis and responses to pathogenic signals. Microglia generated *in vitro* from iPSCs from patients carrying this mutation retain this phenotype and provide highly relevant models for insights into disease mechanisms.[44] Similar studies have been conducted with cells from patients with pulmonary alveolar proteinosis where a mutation in the gene *CSF2R* makes the alveolar macrophages unable to respond to GM-CSF signalling leading to an inactivation phenotype and an accumulation of nonphagocytosed proteins. Using iPSCs, alveolar macrophages carrying this mutation were generated *in vitro*, which resembled functional characteristics of the patient, giving a tool to study not only disease but also potential treatments.[47] This approach was also conducted with cells from patients suffering from Gaucher disease,[48] and the cells generated from these patients produced higher levels of TNF, IL-1β, and IL-6 than cells from healthy volunteers.

Another great advantage of using iPSDMs vs. MDMs is that iPSDMs are relatively easy to manipulate genetically. Different groups have used CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9) techniques to generate iPSDMs bearing mutations found in patients, allowing studying the consequences of the mutation for cellular function. As an *in vitro* model to investigate reverse cholesterol transport, iPSCs deficient for ATP binding cassette subfamily A member 1 were used to generate macrophages with impaired cholesterol efflux and shown to have a more proinflammatory phenotype.[49] The same was done for the lysosomal acid lipase (*LIPA*) gene to study the role of LIPA in macrophages and lipid metabolism.[50]

Although it is clear that tissue-resident macrophages are important in health and disease, the contribution of MDMs to the tissue macrophage population in homeostasis and disease increases over time and strongly affects the course and outcome of subsequent inflammation, immune activation, and disease development.[51]

Origins of monocyte-derived macrophages: monocytes

Before discussing in detail, the available approaches for *in vitro* studies of human monocyte-derived macrophages, we deem it important to sketch the current views on the origin, heterogeneity and functionality of human monocytes.

The original definition of the MPS describes monocytes as the precursors of macrophages.[4] Circulating blood monocytes in humans represent about 10% of leukocytes. Pools of monocytes can be found in the spleen and this reservoir can be mobilized quickly in case of injury or acute inflammation. In mice, these reservoirs and their prompt response were studied in ischemic myocardial injury[52] and similar mobilization was seen in humans after acute myocardial infarction, where a fast reduction of monocytes in the spleen and increased amounts in the heart implied swift deployment of this reservoir.[53]

In adults, monocytes are constantly generated in the bone marrow from HSCs via intermediate progenitors, including the granulocyte monocyte progenitor, the macrophage and dendritic cell progenitor, and finally the common monocyte progenitor, which differentiate into monocytes **(Figure 1A)**. Although this linear model for monocyte generation is generally accepted, there are other studies that propose a different model for the generation of monocytes from bone-marrow precursor cells.[54–57] These models propose a less linear process, where the progenitor cells are overlapping populations of precommitted.

Circulating monocytes generated in the bone marrow can be separated into three subsets based on differential expression of CD14 and CD16. Approximately 90% of them, termed "classical monocytes," present CD14 but are negative for CD16 ($CD14^{+}CD16^{-}$). The "nonclassical monocytes" are $CD14^{low}CD16^{+}$.[58] Finally, the third subtype termed "intermediate" has been defined as $CD14^{+}CD16^{+}$. However, this latter subtype has recently been under debate as a study by Villani et al. shows that, transcriptionally, only classical and nonclassical subtypes can be distinguished, and the intermediate subset highly resembles a population in transition between the other two subtypes.[59] Yet, others, also applying single cell techniques, showed clear transcriptomic differences between the three subtypes,[60] or identified multiple phenotypic distinctions.[61,62]

The different monocyte subsets also show differences in CD11b, with higher expression by classical monocytes compared to the nonclassical. Also, CD11c and CX_3CR_1 (fractalkine receptor) expressions, involved in monocyte survival, differ with both markers showing highest expression by nonclassical monocytes. Another major monocyte subset marker is CCR2, highly expressed on classical monocytes and a receptor for CCL2. This chemokine plays a role in the mobilization of monocytes from the bone marrow. This molecule is also related to the recruitment of monocytes cells to inflammatory sites such as atherosclerotic plaques or infection sites.[63–65]

The monocyte subtypes possess differences in their capacity to infiltrate tissues **(Figure 1B)** based on the differential expression of chemokine receptors such as CCR2 or CX_3CR_1. "Classical" monocytes tend to be recruited first and at higher levels in inflammatory conditions whereas "nonclassical" monocytes have a patrolling function, monitoring the luminal side of blood vessels for tissue damage in the form of dying endothelial cells and promoting recruitment of other immune cells in case of damage.[66,67] Further clear differences between the subtypes with respect to phenotype, size, morphology, and transcriptome has been extensively described elsewhere.[56,68]

Monocyte-derived macrophages: differentiation and activation

For clarity, we here discriminate two processes that impact on macrophage phenotype: differentiation and activation **(Figure 1B)**. Differentiation involves the process by which a monocyte transitions into a more mature state of a macrophage or a monocyte-derived dendritic cell (mDDC) induced by cytokines, growth factors or other stimuli; monocytes can also differentiate into other cell types, such as osteoclasts. Activation, also sometimes referred to as polarization, refers to the phenotype that mature macrophages acquire upon encountering certain factors such as pathogen-related molecules or cytokines, e.g. LPS or IFNγ, respectively. This terminology is still evolving greatly, and others distinguish activation and polarization into two separated terms. The term "polarization" is sometimes used to describe a general phenotype change of macrophages upon certain stimuli, whereas "activation" describes the responsiveness of a cell to certain triggers.[69]

Classically, macrophage activation or polarization was divided into two simplistic subtypes, denominated M1 or M2. The two states represent opposite characteristics and their nomenclature was originally based on Th1 and Th2 cytokines.[70] M1 was referred to as the "proinflammatory" phenotype where the macrophages produced cytokines that enhance responses of the immune system and promote inflammation in tissues. On the other hand, the M2 macrophages were considered "anti-inflammatory" cells and have for instance wound-healing capacities.[71] M1 macrophages produce specific chemokines to attract more leukocytes to tissue and to activate other immune cells through co-stimulatory molecules such as CD80. Examples of cytokines produced in the M1 response include IL-6, TNF, IL-12, and IL-1β.[72–74] M2 macrophages are induced by stimuli like IL-4/IL-13, IL-10, or corticosteroids[75] and promote not only wound-healing activities, but also fibrosis. Thus, these cells are important in resolution of the inflammatory state as regulators or dampeners of the immune response.[76,77]

This classical separation of macrophage activation has been expanded and changed by many recent studies (see, e.g., Murray[78] for an overview). Macrophage phenotypes are no longer restricted to two extreme phenotypes but resemble a spectrum **(Figure 1B)** from highly proinflammatory to pro-fibrotic, pro-tumoral, anti-inflammatory, and many more. This was particularly well demonstrated by several transcriptome analysis studies where the response of macrophages to a wide range of stimuli led to the induction of a plethora of transcriptional phenotypes.[79,80] This

model of a spectrum of responses found *in vitro* was also observed *in vivo* in human after comparison of, for instance, data obtained from alveolar macrophages from bronchoalveolar lavage in smokers, non-smokers, and chronic obstructive pulmonary disease patients.[81,82] These studies show how specific cues produce unique phenotypes in the macrophages and that not all can be enclosed in simple dichotomy of M1 and M2 activation.

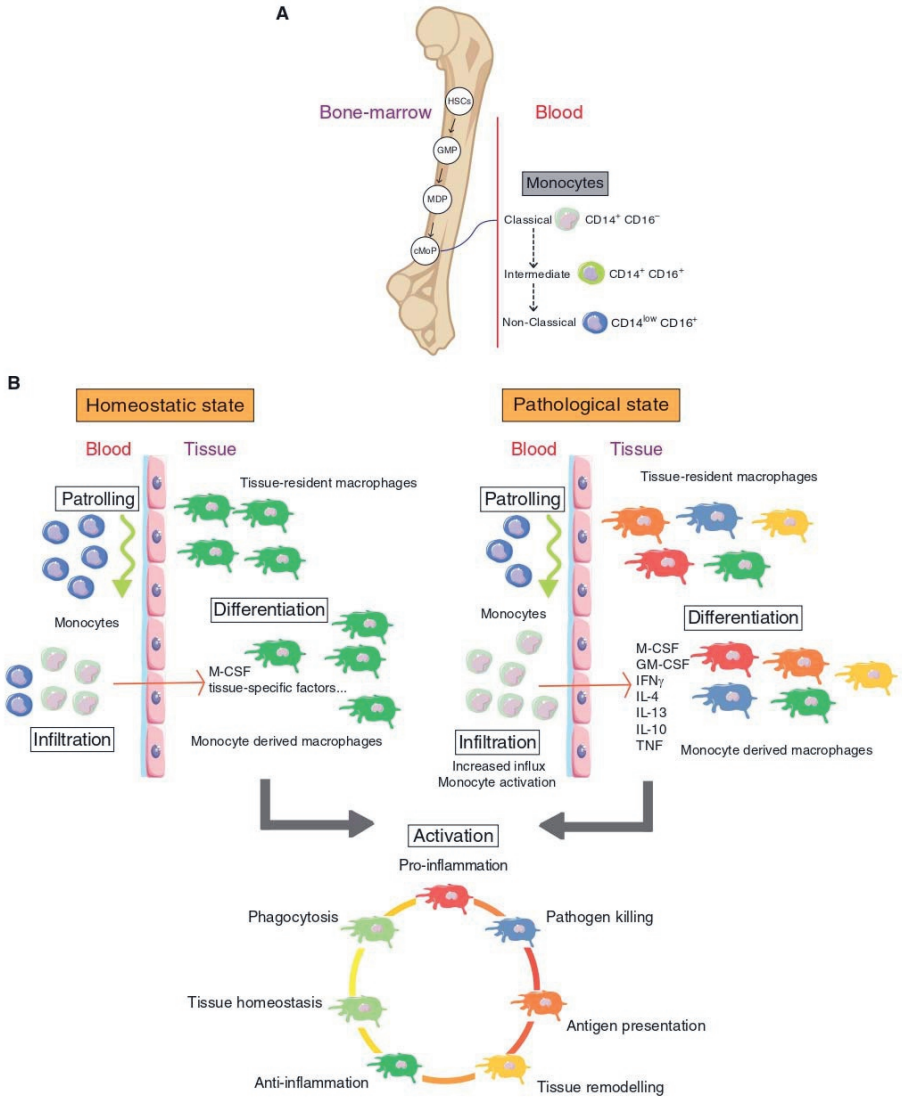

Figure 1. **A**: Cell precursors in the bone marrow that give rise to monocytes. Different subtypes of human monocytes found in circulating blood. **B**: Function of monocytes in homeostatic and pathological situations. Under homeostatic situations, classical, and to a lesser extent, non-classical monocytes infiltrate into tissues. Non-classical monocytes perform patrolling functions on the luminal side of the blood vessel. In pathological conditions there is an increase in recruitment and infiltration of classical monocytes into tissues. Macrophages, either MDMs or tissue-resident, respond to pathological stimuli by acquiring an activated phenotype such as pro-inflammatory, pathogen killing, antigen presenting, anti-inflammatory or tissue remodeling.

M-CSF and GM-CSF: the classical *in vitro* differentiation factors

In vitro differentiation methods of macrophages have also been oversimplified. Historically, macrophages were thought to differentiate mainly from monocytes by the presence of M-CSF or GM-CSF, the latter more under inflammatory conditions.[83] M-CSF is a growth factor readily detected under homeostatic conditions, whereas GM-CSF present in some tissues under homeostatic conditions is not detected systemically unless induced by inflammatory situations.[83] M-CSF is produced by multiple cell types, including macrophages, endothelial cells, and fibroblasts. GM-CSF is produced not only by T cells, mast cells, and natural killer cells but also by macrophages, endothelial cells, and fibroblasts.[84] Endothelial cells produce M-CSF and GM-CSF in response to pro-inflammatory cytokines such as IL-1β, shear stress and also to disease-specific molecules like oxidized LDL in atherosclerosis.[85] Both M-CSF and GM-CSF promote cell survival, monocyte to macrophage differentiation, and enhance monocyte recruitment. The M-CSF and GM-CSF levels produced locally vary depending on the condition; in healthy situations the levels of M-CSF dominate, whereas in pathologic inflammatory conditions, such as rheumatoid arthritis, GM-CSF levels increase.[86–89] An example of a disease where the GM-CSF levels are also increased is multiple sclerosis (MS). In MS patients, CD4+ T cells in the CNS produce GM-CSF, which leads to polarization of macrophages to a more proinflammatory phenotype. These macrophages secrete proinflammatory cytokines such as IL-6, IL-1β, and TNF causing myelin sheath damage in the CNS of the patients. GM-CSF also increases the recruitment of monocytes contributing to disruption of the blood-brain barrier and blood-spinal cord barrier and further demyelination of the neurons in the CNS.[90] In turn, although M-CSF is a key physiologic mediator of macrophage biology, this pathway can become over-active and cause dysregulation as was shown for models of kidney and liver damage[91] and in cancer.[92]

M-CSF and GM-CSF activate cells via distinct signalling pathways. The M-CSF receptor is a homodimer formed by an extracellular domain that contains five immunoglobulin domains, a transmembrane domain and an intracellular domain. This receptor functions via several pathways including PI3K/Akt and MEK-ERK1/2 among others.[93] The GM-CSF receptor is a heterodimeric receptor formed by two subunits: the specific ligand-binding subunit (CSF2Rα) and the common signal-transduction subunit (CSF2Rβ)[84] and activates the JAK2/STAT5 pathway. Transcriptomic analysis of the monocyte differentiation processes by either M-CSF or GM-CSF has shown clear differences between the two cytokines, macrophages differentiated with GM-CSF express higher levels of proinflammatory cytokines genes such as *TNF* or *IL-1*β in response to LPS.[94] Many additional studies have made comparisons between M-CSF and GM-CSF-induced macrophages that were differentiated *in vitro* from monocytes. In general, differentiation protocols involve culturing the cells between 3 and 7 days in culture medium in the presence of either cytokine. In Table 1 different characteristics of the two populations of cells are summarized.

Other mediators used for *in vitro* monocyte to macrophage differentiation

Besides M-CSF and GM-CSF, other cytokines have also been used to differentiate human monocytes into macrophages. The study of alternative differentiation factors is important as monocytes encounter a wide variety of inflammatory mediators that can impact their differentiation whilst entering the tissue micro-environment. These alternative factors can induce a macrophage subtype different from the well-studied phenotypes observed when differentiated with M-CSF or GM-CSF only.

For instance, IL-34 has similar functions to M-CSF but has a more restrictive expression pattern. This cytokine is also key in the development of osteoclasts attached to bone and of microglia in the CNS, and has been related to rheumatoid arthritis.[105] Monocytes differentiated with IL-34 are like M-CSF macrophages as both molecules bind to the CSF-1 receptor and activate the same pathway for differentiation. Even though these macrophages are similar, there are differences in response after activation as IL-34-differentiated macrophages show increased phagocytic capacity and higher IL-10 and CCL-17 production after stimulation. These differences might be explained by the capacity of IL-34 to bind to receptors other than CSF-1R.[106] Macrophages differentiated with IL-34 show a clear anti-inflammatory, immunosuppressive phenotype that in tumors is associated with lower levels of infiltration of cytotoxic CD8 T cells.[107] Another example factor impacting on differentiation is platelet factor 4 (PF4) also known as CXCL4, a chemokine secreted during acute vascular injury. PF4-mediated differentiation of monocytes prevents apoptosis in monocytes and induces a macrophage-like morphology with cells presenting pseudopodia as well as increased expression of macrophage maturation markers, but showed lower expression of HLA.[108] Transcriptomic studies of macrophages differentiated with M-CSF compared to CXCL4[109] showed that the CXCL4 differentiated macrophages acquire macrophage-like morphology and express CD45 and CD68, thus confirming bona fide macrophages. In terms of comparison with M-CSF macrophage differentiation, there is a correlation in genes expressed after M-CSF and CXCL4 differentiation but in terms of function, the CXCL4 differentiated macrophages express higher levels of cytokines such as IL-6 and TNF and can thus be seen as more proinflammatory.[110]

TABLE 1 Comparison of GM-CSF- vs. M-CSF-differentiated macrophages

Type of study	Results	References
Transcriptomic	GM-CSF-induced macrophages express more genes related to the immune/inflammatory response and higher levels of proinflammatory cytokines after stimulation.	80, 94, 95
Transcription factor activation	GM-CSF activates STAT5. IRF4/5 are up-regulated in GM-CSF and IRF4 is down-regulated in M-CSF-differentiated cells.	94, 96
Cytokine production and inflammatory mediator production	M-CSF differentiated macrophages have lower production of proinflammatory cytokines compared to GM-CSF stimulation. Higher levels of IL-10 in M-CSF differentiated macrophages after stimulation. M-CSF derived macrophages produce higher levels of eicosanoids in response to bacteria.	97–100
Morphology	GM-CSF and M-CSF derived macrophages present different morphology.	94, 95
Surface markers	CD163 is expressed higher in M-CSF differentiated macrophages after dexamethasone stimulation. Differential expression of CD14, lower in GM-CSF differentiated macrophages.	96, 101
Lipid metabolism	Differences in expression of genes related to lipid metabolism, higher apolipoprotein E levels in GM-CSF differentiated macrophages. Differential activation of inflammasome, higher in GM-CSF differentiated macrophages.	102, 103
Translation	GM-CSF derived macrophages have a similar phenotype to alveolar macrophages from patients from pulmonary sarcoidosis and pulmonary neoplasia compared to M-CSF.	104

CCL2, also known as MCP-1, is a classical chemokine that drives the recruitment of monocytes to tissues. The expression of CCL-2 and its receptor, CCR2, in macrophages varies depending on the cytokine used for the differentiation, seeing higher levels of CCL-2 expression in M-CSF macrophages. The presence of CCL-2 during polarization leads to the presentation of a less proinflammatory phenotype with reduced levels of IL-6 expression.[111] CCL2 is also important in the tumor microenvironment where CCL-2 in combination with IL-6 promotes de survival of CD11b+ cells by increasing the expression of anti-apoptotic proteins (e.g., cFLIP, Bcl-2, and Bcl-X) and blocking the cleavage of the caspase-8. These cells also show increased expression of anti-inflammatory markers such as the mannose receptor CD206.[112] IL-6 alone is also capable to impact on macrophages in the tumor microenvironment by activation of the STAT3 pathway, increasing the expression of CD206, CD163, and the production of IL-10 and TGFβ.[113] These data suggest that both CCL2 and IL-6 drive macrophages toward a cell-phenotype with reduced inflammatory and increased immunosuppressive characteristics, both relevant in tumor growth.

The IL-32 cytokine also presents differentiation capacities. When monocytes are differentiated in the presence of IL-32 there is an increase in the expression of CD14 and a blockage of the effect of GM-CSF+IL-4 in the differentiation of monocytes toward DC,[114] showing a decrease in CD64. The IL-32-generated cells also show phagocytic capacities. IL-32 promotes monocyte to macrophage differentiation by activation of the p38-MAPK and NF-$\kappa\beta$ pathways.[115] The presence of IL-17 in cultured monocytes increased the expression levels of genes for proinflammatory cytokines, chemokines, and pathways related with leukocyte trans-endothelial migration.[116]

A considerable number of molecules are considered to be able to promote monocyte differentiation by the observation of increased levels of macrophage markers in the cells, for example, CD64 and CD80 after using TNF or IFNγ for the differentiation.[117] The phenotype in terms of function of the obtained macrophages generated in the presence of these two factors has not been characterized in detail. However, it is well known that when used for polarization, IFNγ drives a phenotype that is important in the defence against intracellular pathogens. Both IFNγ and TNF stimulation induce a proinflammatory phenotype with increased expression of IL-1β, IL-12, and reduced IL-10.[118,119] Both TNF and IFNγ induce the Th1 phenotype in T cells as a result of the IL-12 produced inducing the Th1 response.[120,121]

TABLE 2 Inflammatory factors used to differentiate human monocytes into macrophages

Factors	Phenotype relative to M-CSF macrophages	References
IL-4, adiponectin	Macrophages with greater anti-inflammatory phenotype.	122
Microparticles (from platelets)	Microparticles stimulate monocytes to express increased CD11b, CD14, and CD68 after 7 d culture. No comparison against M-CSF macrophages was made.	123
M-CSF or GM-CSF + butylated hidroxyanisole (BHA)	BHA affects the differentiation of M-CSF macrophages by modifying their morphology and reducing CD11b and CD163 expression.	124
M-CSF or GM-CSF, with or without serum-containing media	The absence of serum in the media causes a loss of the M-CSF elongated morphology and decreased CD163 expression.	125
Platelet factor 4 (PF4)	PF4 up-regulates expression of macrophage differentiation markers. Similar capacities of preventing monocyte apoptosis when differentiation is performed with M-CSF or GM-CSF.	108
IL-34	Overall similar phenotype to M-CSF derived macrophages. Less phagocytic capacity than M-CSF after alternative activation. Higher IL-10 and CXCL11 production than M-CSF derived macrophages after activation with LPS + IFNγ.	106
Cathelicidin antimicrobial peptides LL-37 (LL-37) with M-CSF	Lower IL-10 and higher IL-12p70 after LPS stimulation in M-CSF + LL-37 differentiated macrophages.	126
Hemoglobin or IL-4	Different phenotype between macrophages differentiated in the presence of hemoglobin or IL-4. Mannose receptor and CD163 are higher in hemoglobin-differentiated macrophages. Hemoglobin prevents foam cell formation. No comparisons to M-CSF derived macrophages were made.	127
IL-32	Increased expression of macrophage markers (CD14) and phagocytic capacities. Differentiation via NF-κB pathway. No comparisons to M-CSF macrophages were made.	115
IFNγ + GM-CSF + IL-4	IFNγ blocks the GM-CSF + IL-4-mediated dendritic cell differentiation by stimulating M-CSF production and inducing macrophage development.	128
IFNγ, IL-4, IL-10, TNF, dexamethasone, M-CSF, and GM-CSF	Differentiation of macrophages with different mediators induces surface markers at different levels depending on the factor used. Macrophages differentiated with IL-10 present similar levels of CD163 and CD16 compared to M-CSF macrophages.	117
CaOx	Similar morphology to GM-CSF macrophages. CD68 and CD86 expression but no CD163 or CD206. Proinflammatory phenotype with higher levels of IL-12 and TNF and lower IL-10 compare to M-CSF.	129
Lactate	Leads the differentiation of monocytes toward alternative activated macrophages instead of DCs. Increased expression of CD14and lower CD1a. Macrophages with tumor promoting and alternative activated immuno-suppressive phenotype. Increase production of VEGF and some proinflammatory characteristics high IL-1β but absence of Th1-inducing response (low IL-12)	130–134
Hypoxia	Lower phagocytic capacities, CD206, and CD40. Higher production levels of VEGF supporting angiogenesis.	135

Table 2 captures the best-defined examples of molecules with the ability to induce monocyte differentiation, either alone or in combination with M-CSF or GM-CSF and a brief discussion of the phenotypes observed.

It is important also to highlight the role of nonimmune molecules in the monocyte to macrophage differentiation process. Metabolites are widely present and some of them may impact on differentiation. For instance, calcium oxalate (CaOx), a constituent of kidney stones and associated with kidney disease, induces the differentiation of monocytes into proinflammatory macrophages in the kidney. Monocytes differentiated in the presence of CaOx present a macrophage-like morphology similar to GM-CSF derived macrophages, show expression of CD68 and CD86 but not CD163 or CD206. These macrophages also produce higher levels of IL-12, TNF, and lower IL-10[129] compared to M-CSF derived macrophages. Therefore, they seem to have a clear proinflammatory phenotype.

Other nonimmune parameters to consider are the environment where the differentiation takes place. For example, in tumors or inflamed tissues it is common to find hypoxia. During the monocyte differentiation in hypoxia there is an increase in the expression of hypoxia-inducible

factor 1 (HIF-1α and HIF-1β), which give them the ability to respond to hypoxia.[136] If the differentiation takes place in hypoxia conditions the survival rate of the cells it is not affected.[137] Macrophages generated under these conditions compared to normal oxygen levels showed lower phagocytic capacities and lower CD206 and CD40 but higher levels of vascular endothelial growth factor (VEGF).[135]

A major metabolite that is also present in tumor microenvironment and whose production is increased in hypoxia is lactate. This metabolite has not been tested as a sole differentiation factor but has great impact on macrophages. One study has tested the effects of lactate on DC differentiation of DCs from monocytes. When DCs are differentiated in the presence of lactate producer cells, the resulting cells presented an alternative activated macrophage phenotype instead of a DC phenotype. The cells expressed higher levels of CD14 and less CD1a. The cells also induced Th2 responses in T cells. Therefore, this study shows how the presence of lactate is capable to shift monocyte differentiation away from DCs toward an alternatively immuno-suppressive macrophage type.[130] The impact of tumor cell derived lactate on monocyte to macrophage differentiation maybe crucial in cancer situations. It is well established that lactate drives macrophages toward a tumor-promoting phenotype through induction of VEGF and alternatively activated immune-suppressive characteristics.[131–133] Such changes were recently linked to lactate mediated histone modifications.[138] Of note, recent data shows that the shift in the phenotype is not that clear-cut anti-inflammatory. Paolini et al showed that in human macrophages lactate drives a phenotype that has some proinflammatory characteristics (e.g., high IL-1β), but lacks Th1-driving capacity (e.g., low IL-12) and has tumor-promoting activity, for instance by producing of growth and proangiogenic factors.[134]

Conclusions

The macrophage field is evolving rapidly, and the nuance of the M1/M2 paradigm, is a good example of this evolution. Originally in the M1/M2 paradigm only two closed activation states were considered, this idea is changing to understanding the macrophage activation as a spectrum of different activation states. Incorporation of transcriptional studies, and especially those at the single cell level, has revealed the complexity of the MPS and disclosed anatomically specific profiles.[139,140] The diverse cellular ontogeny of macrophages adds an additional layer of functional heterogeneity to these cells. Based on these advances revisions to macrophage nomenclature were proposed based on origin and ontogeny and then on the function, location, and phenotype.[141]

In the same way that the nomenclature is evolving, protocols to generate macrophages have also advanced. *Ex vivo* studies with human macrophages are very difficult to carry out due to logistical or ethical considerations, so developing representative *in vitro* models is necessary. The use of the different models (MDMs, iPSDMs. or cell lines) will vary based on the scientific question and each approach has its merit.

Other *in vitro* methods to study macrophages, which aim to reproduce the *in vivo* settings of macrophages in the context of organs, are 3D cultures and organoids. These methods are very valuable to study the interaction of macrophages with other cell types in a 3D structure and investigate how the organ structure affects their function, morphology, maturation, migration, among others.[142,143] The 3D cultures and organoids are widely used in cancer research where these techniques help to understand how cancer cells modify the macrophage phenotype.[144] For instance, when monocytes are added to a coculture of pancreatic tumor cells and fibroblasts the monocytes added differentiate into macrophages, showing an increase in CD68 expression. These cells present an alternative activated phenotype with high levels of CD163 and CD14 and low levels of CD86 and HLA-DR.[145] Another important aspect that can be studied in cancer by 3D/organoids is how macrophages infiltrate in the tumor; for instance, macrophages can use podosomes with proteolytic capacities that break the extracellular matrix to enter into tissues.[146,147] Besides cancer, 3D cultures and organoids could also be used, for example, to study how macrophage play their role in tissue remodelling or wound healing.[148,149] Another application of 3D cultures is related to microglia in the brain. In this case the organoids used are cells derived from the neuroectodermal lineage, and the microglia generated in this 3D cocultures present phagocytosis capacities and similar morphology and transcriptomic response after inflammatory stimulation compared to post-mortem isolated microglia.[150]

The use of iPSDMs has made great progress in generating protocols for the development of tissue-specific macrophages, to date, and protocols used to generate MDMs have been predominantly based on the use of M-CSF and GM-CSF as differentiation factors. However, many additional inflammatory mediators have the ability to stimulate differentiation of monocytes into macrophages alone or in combination with other factors. Indeed, monocytes infiltrating tissues in health or disease will encounter a range of mediators rather than (G)M-CSF in isolation. Furthermore, application of tissue-specific environments in the induction of MDM differentiation remains an underexplored field, in our view. Therefore, work is needed to advance our knowledge on the impact of multiple mediator-induced macrophage phenotypes with emphasis on tissue-specific factors.

Many of these new possible differentiation molecules are cytokines related to alternative activation of the immune system (IL-4, IL-13, IL-10), the impact of these factors on differentiation is not well understood. These cytokines are for instance important in the pathogenesis of allergic airway disease and asthma. On the other hand, IL-10, an anti-inflammatory cytokine, has been linked to chronic fibroproliferative diseases, such as chronic pancreatitis, pulmonary fibrosis, chronic kidney disease, and others.[151,152] Additional cytokines with the potential to induce monocyte differentiation are IL-32, IFNγ, and TNF, and many of these are key drivers of immune-mediated inflammatory diseases such as IFNγ in rheumatoid arthritis.[153] IL-32 in cardiovascular diseases[154] and TNF in inflammatory bowel disease.[155]

Other molecules besides cytokines also showed differentiation inducing capacities, including adiponectin, butylated hidroxyanisole (BHA), PF4, and hemoglobin. These molecules can be found in tissues under inflammatory conditions, for example, adiponectin in vascular diseases[156] and PF4 in heart failure and lupus nephritis.[157,158] When studying macrophages in these diseases, it would be valuable to include these mediators as part of the microenvironment during monocyte to macrophage differentiation.

Generally, *in vitro* models are restricted in terms of the heterogeneity of the cell populations when compared to those found *in vivo* in disease. However, *in vitro* models continue to provide valuable systems to understand cellular mechanisms and opportunities to modulate these in order to intervene pathogenic processes. However, with the availability of many new technologies it is important to make the next step in *in vitro* modelling of cells and to broaden the way macrophages are generated and activated. Improvement of *in vitro* protocols will provide the much-needed translation to humans and human disease.

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Chapter 3

IFNγ drives human monocyte differentiation into highly pro-inflammatory macrophages that resemble a phenotype relevant to psoriasis

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Abstract

As key cells of the immune system, macrophages coordinate the activation and regulation of the immune response. Macrophages present a complex phenotype that can vary from homeostatic, pro-inflammatory, pro-fibrotic to anti-inflammatory phenotypes. The factors that drive the differentiation from monocyte to macrophage largely define the resultant phenotype as has been shown by the differences found in M-CSF and GM-CSF derived macrophages. We explored alternative inflammatory mediators that could be used for *in vitro* differentiation of human monocytes into macrophages. IFNγ is a potent inflammatory mediator produced by lymphocytes in disease and infections. We used IFNγ to differentiate human monocytes into macrophages and characterized the cells at a functional and proteomic level. IFNγ alone was sufficient to generate macrophages (IFNγ Mϕ) that were phagocytic and responsive to polarisation. We demonstrate that IFNγ Mϕ are potent activators of T lymphocytes which produce IL-17 and IFNγ. We identified potential markers (GBP-1, IP-10, IL-12p70 and IL-23) of IFNγ Mϕ and demonstrate that these markers are enriched in inflamed psoriasis patient skin. Collectively we show that IFNγ can drive human monocyte to macrophage differentiation leading to bona fide macrophages with inflammatory characteristics.

Key points

1. IFNγ alone is capable of inducing monocyte to macrophage differentiation
2. IFNγ derived macrophages present a high pro-inflammatory phenotype.
3. Phenotypic characteristics of IFNγ macrophages are expressed in psoriasis patients.

Introduction

Macrophages are unique cells of the immune system involved in host defence, homeostasis and tissue repair (1). Macrophages can be categorised based on ontogeny into two groups: monocyte derived macrophages (MDMs) or tissue-resident macrophages, which originate from cell progenitors in the yolk sac or fetal liver monocytes (2). The proportion of macrophages from these origins varies depending on the tissue and inflammatory status (2). Core macrophage functions include the phagocytosis of pathogens and dead cells and activation of other immune cells (3). Macrophages are key in the pathogenesis of various diseases where they can either resolve or worsen disease outcomes (1). These cells contribute to excessive inflammation in various immune-mediated diseases and in chronic inflammatory diseases like rheumatoid arthritis (RA), atherosclerosis, IBD, NASH and psoriasis (4-8). Psoriasis is characterized by lesional areas in the skin where there is a thickening of the epidermis, enhanced keratinocyte proliferation, leukocyte infiltration and inflammation (9). Macrophages acquire a pro-inflammatory phenotype and produce cytokines such as IL-23, TNF and IL-1β. The IL-23/IL-17 axis is overactive in psoriasis and biologics targeting these cytokines have proven to be efficacious in severe disease. IL-23, exclusively derived from myeloid cells, activates T cells to produce cytokines such as IL-17 and IFNγ, which further polarise macrophages leading to a continuous inflammatory state (10-12).

Macrophages, as shown by transcriptomic studies, can adopt a spectrum of activation states depending on the environmental stimuli (13-15). The concept of macrophage heterogeneity is not only important for activation of macrophages already within the inflamed tissue but also impacts the differentiation of infiltrating monocytes. The general consensus is that monocyte to macrophage differentiation is driven by either macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (16) and these macrophages present distinct phenotypes, morphology and function (14, 17). GM-CSF derived macrophages are considered to be more pro-inflammatory compared to M-CSF derived macrophages (18-21) and this is in line with the detrimental role of GM-CSF and macrophages across multiple inflammatory diseases (22).

Within the tissue, monocytes and macrophages will also encounter other inflammatory mediators in addition to M-CSF and/or GM-CSF. One important cytokine released in numerous diseases is interferon-γ (IFNγ) which belongs to the type II interferon protein family and is often used to polarise macrophages either alone or in combination with LPS (23). IFNγ is produced by activated lymphocytes, namely CD4 (T_H1) cells, natural killer (NK) cells, and CD8 cytotoxic T cells (24). Elevated circulating levels of IFNγ have been found in patients with psoriasis, sarcoidosis, lupus nephritis and juvenile idiopathic arthritis among other diseases (25-30)

Thus far, most human macrophage models use *in vitro* differentiated macrophages due to the difficulties associated with isolating human macrophages *ex vivo*. Whilst differentiation protocols are usually restricted to M-CSF or GM-CSF with only limited use of alternative differentiation

factors, other factors can yield functional macrophages too (31). Considering the importance of IFNγ as a potent regulator of human disease, we hypothesized that IFNγ would drive monocyte differentiation into pro-inflammatory macrophages. In the present study, we find that IFNγ is sufficient to drive monocyte to macrophage differentiation even in the absence of M-CSF. IFNγ differentiated macrophages have a stronger pro-inflammatory phenotype compared to either M-CSF or GM-CSF derived macrophages. We show that the principal proteins and axis upregulated in IFNγ derived macrophages are also found in psoriasis patients and that these macrophages are distinct from M-CSF/GM-CSF macrophages at the proteomic and gene expression of certain chromatin modifiers. We conclude that the use of IFNγ as a differentiation factor is a useful model to generate human macrophages that resemble a heightened inflammatory disease phenotype, such as that seen in psoriasis.

Materials and methods

Cell culture

In order to culture macrophages, monocytes were isolated from blood of healthy donors. The human biological samples were sourced ethically, and their research use was in accordance with the terms of the informed consents under an IRB/EC approved protocol. Blood from healthy volunteers was collected into tubes containing sodium heparin anti-coagulant. Blood was diluted 1:1 with phosphate buffered saline (PBS) and layered on top of 15 mL of Lymphoprep™ (GE Healthcare). Tubes were spun down at 1500 rpm for 20 minutes without break and acceleration, at room temperature (RT). Peripheral blood mononuclear cells (PBMCs) were collected from the ring fraction. Monocytes were isolated from PBMCs using CD14 positive magnetic beads following manufacturer's instructions (Miltenyi). Cells were cultured in RPMI-1640 (without glutamine and Hepes) (Gibco) with 5% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 mg/mL) (all Gibco).

For the first experiment (Figure 1A) monocytes were cultured for 5 days at 37°C and 5% CO_2 with either M-CSF (50 ng/mL)(R&D System), GM-CSF (5 ng/mL) (R&D System), LPS (100 ng/mL) (*E. coli* 0111:B4, Sigma), IFNγ (31 ng/mL) (R&D System), TNF (50 ng/mL) (R&D system), IL-17 (25 ng/mL) (R&D System) alone or in combination with M-CSF. For the second experiment (Figure 1B, C, E) monocytes were cultured with either 0, 0.1, 0.5, 1, 5, 10, 50 or 100 ng/mL of IFNγ alone or in combination with 50 ng/mL of M-CSF. For all other experiments, monocytes were cultured with 100 ng/mL of M-CSF, 5 ng/mL of GM-CSF or 50 ng/mL of IFNγ. For the stimulations, cells were treated for 24 hours either with LPS (100 ng/mL), IFNγ (50 ng/mL) (alone or in combination with LPS) and IL-4 (10 ng/mL) (R&D Systems). For every experiment, the culture conditions were tightly controlled (using flat bottomed plates, with the same plating density (1 x 10^6 cells/mL), cell culture medium and donor-matched monocytes).

Patient serum

10 mL of human blood from patients (n=10 patients and n=7 healthy volunteers) with either moderate or severe psoriasis were collected into serum gel vacutainers. Blood was supplied by the Clinical Unit Cambridge for GSK-funded research. All donors provided written informed consent for the collection and use of their samples in accordance with the protocol of the local Institutional Review Board (Cambridge Research Ethics Committee). Tubes were centrifuged at 2500 rpm for 5 minutes at RT. The plasma was harvested from each tube and stored in cryopreservation tubes for cytokine measurement.

ICC staining

Monocytes were differentiated into M-CSF, GM-CSF and IFNγ derived macrophages on slide chambers (Nunc Lab-Tek II Chamber slide system) at $4x10^5$ cells/chamber. After 5 days of differentiation, cells were fixed for 15 minutes in 100 µL of 4% Formaldehyde (Pierce, Thermo Fisher). Afterwards cells were washed three times with PBS and blocked for 30 minutes with 200 µL of blocking buffer (1% BSA and 0.1% Tween in PBS). After blocking, cells were incubated with a CD68 antibody (Abcam) (1:100 in blocking buffer) overnight at 4° C. The next day, cells were washed three times with PBS and incubated for 1-2 hours in the dark with a secondary antibody (Goat Anti-Rabbit IgH H&L, Alexa Fluor 488, Abcam, 1:1000 in blocking buffer). The antibody was washed and ProLong Gold antifade mount with DAPI (Thermo Fisher) was added. Microscopic pictures were taken from 3 donors (Leica DFC450) using FITC and Cy5 filters for the CD68 and DAPI with a 10x magnification with the LAS software version 4.0.0. 3. For the analysis, the cells expressing CD68+/DAPI+ were counted as positive cells, the percentage was then calculated from the total number of cells (CD68+/DAPI+ and CD68-/DAPI+).

Cytokine production

After the different stimulations, the supernatant was collected to measure cytokine secretion. To measure IL-1β, IL-12p70, IL-6 & IL-10, a Human Proinflammatory 7-Plex Tissue Culture Kit (Meso Scale Discovery) was used following the manufacturer's instructions. M-CSF was measured using an MSD kit (Meso Scale Discovery) following the manufacturer's instructions with 1:1 diluted sample. A Meso Scale Discovery kit for TNFα was used following the manufacturer's instructions diluting the samples 1:10. This was also done for IP-10 and IL-17 measurements using the V-PLEX Human IP-10 Kit (Meso Scale Discovery) and the MSD 5 PLEX (Meso Scale Discovery N751B) following the manufacturer's instructions. For IP-10 and IL-17 measurement, samples were undiluted. This kit was also used to measure IFNγ concentrations in the co-culture experiments, using undiluted samples. CCL-18 was detected by ELISA (R&D System) following the manufacturer's instructions and M-CSF samples were diluted 1:10. MCP-1 (Meso Scale Discovery) and CCL-1 (Meso Scale Discover) kits were also used following the manufacturer's instructions. For CCL-1, samples were diluted 1:1 and undiluted for MCP-1.

For IL-23, an MSD assay was developed in-house using an MSD Standard Bind assay plate (Meso Scale Discovery). All the antibodies used in the IL-23 MSD assay belong to the IL-23 Human

ELISA kit (Mabtech). Briefly, the plate was coated with 25 µL per well of an IL-23 capture antibody at 1 µg/mL in PBS overnight at 4 °C. The next day the plate was washed three times with washing buffer (0.05% Tween20 in PBS) and incubated with blocking buffer (3% BSA in PBS) for 1 hour at RT on a shaker (700 rpm). After the incubation, the plate was washed and 25 µL of diluted sample (1:10 in culture media) or 25 µL of each of the concentrations of the 8 points standard curve (10.000-0 pg/mL at 1:4 dilution between each point) for IL-23 was added to the plate and incubated for 2 hours at RT on a plate shaker at 700 rpm. The plate was washed and 25 µL of the secondary antibody (1 µg/mL in blocking buffer) was added to the plate for 1.5 hours. The plate was washed and 25 µL of SULFOTAG detection reagent (0.1ug/mL in blocking buffer, Meso Scale Discovery) was added to the plate and incubated for 1 hour at RT on a plate shaker at 700 rpm. Finally, the plate was washed and 150 µL of 2x Read buffer (Meso Scale Discovery) was added. The plate was read by the MSD Sector Imager 6000.

Viability Assay

Viability was determined by measuring total ATP content in monocyte/macrophage cultures via the CellTiter-Glo kit and following manufacturer's instructions. Data are presented as mean and SEM of total luminescence units per condition.

Flow Cytometry

M-CSF, GM-CSF or IFNγ derived macrophages were cultured in 24 well plates at a concentration of $1x10^6$ cells/well. Macrophages were detached using a Cell Dissociation buffer (Sigma-Aldrich), followed by 2 washes in PBS. Cells were stained with a Live/dead stain (1:1000; BD Biosciences) for 15 min at RT in dark. Cells were washed twice with FACS buffer (BioLegend 420201) and incubated with an Fc receptor blocking agent (Human TruStain FcX, BioLegend 422302) for 10 min at RT, prior to incubation with the antibodies. CD14 (V500, BD biosciences 562693), CD16 (BV421, BioLegend 302038), CD80 (PE, BioLegend 305208), CD86 (FITC, BioLegend 374204), CD64 (PerCP Cy5.5, BioLegend 305024) and CD11b (APC, BioLegend 301350) antibodies were all diluted 1:100 in FACS buffer and incubated for 30 min at RT in the dark. Flow cytometric analysis was performed on a BD FACSCanto II flow cytometer. Data was analysed using the FlowJo software v10. Macrophages were gated by removing doublets and afterwards selecting viable cells. Median fluorescence intensity (MFI) was quantified using FMOs as control.

T cell co-cultures

The co-cultured CD4+ T cells and macrophages were isolated from the same donor. Monocyte isolation and macrophage culture were performed as described above. T cells were isolated from the CD14 negative flow-throw using the CD4+ isolation kit (Miltenyi Biotec 130-096-533) following manufacturer's instructions. T cells were kept in culture for six days in RPMI-1640 (without Hepes and L-glutamine) (Gibco) with 10% FCS, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 mg/mL), supplemented with 2 ng/mL of IL-7 (R&D systems 207-IL/CF). Macrophages were cultured for five days as described above. Once the cells had differentiated, they were stimulated for 24 hours with LPS (100 ng/mL) in fresh media. After 24

hours the supernatant was collected (100 µL) and replaced by new media. Supernatant was transferred to an empty 96 well plate. T cells were centrifuged and resuspended at $1x10^6$ cell/mL in media without IL-7. Cells were added to the wells of the plates containing either the macrophages with fresh media (1:1 ratio) or the supernatant from the stimulation. In another plate, the supernatant was not removed following the 24 hour LPS stimulation and T cells (1:1 ratio) were added to the macrophages together with the supernatant. After 3 days of co-culture, supernatant was collected, and cytokines were measured.

Phagocytosis assay

Monocytes ($1x10^5$ cells/well) were differentiated into the different subtypes of macrophages for 5 days in black clear-bottom 96-well tissue culture treated plates (CoStar 3603). To measure phagocytosis, pHrodo Green *E. coli* bioparticles (ThermoFisher P35366) were resuspended in a concentration of 2 mg/mL in 0.9% saline solution. Cells were incubated at 37°C, 5% CO_2 and every 30 minutes, *E. Coli* bioparticles were added at 200 µg/mL. After 3 hours cells were fixed with unbuffered saline solution with 2% glutaraldehyde (Sigma G7651) and 1% parafix (Pioneer Research Chemicals PRC/R/38) for 20 min at RT. After washing, cells were permeabilized with 0.1% TritonX 100 (Sigma T8787) in PBS for 30 minutes. The buffer was removed and Cell mask (Molecular Probes C37608) was added at 1 µg/mL with Hoechst at 2 µg/mL for 30 minutes. Cells were washed and images were taken with an INCell 2200 at a 10x magnification. Images were analysed using the Columbus software (version 2.8.0), identifying individual cells and measuring the MFI per cell.

Gene expression panel

RNA was isolated from the different subtypes of macrophages using RNeasy Mini Kit (Qiagen 74104) following manufacturer's instructions. cDNA was generated using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Thermofisher 11752-250) according to manufacturers' instructions. The 84 genes panel RT² Profiler™ PCR Array Human Epigenetic Chromatin Modification Enzymes (Qiagen PAHS-085ZE) was run according to the manufacturer's instructions. For the analysis, the Software Array Studio (Version 10.1) was used. CT values were converted to abundance and transformed to Log2. Values were normalized to the housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, *RPLP0*) and principal component analysis was performed. The threshold for statistical significance of genes in the comparison, a threshold of Log2FC>0.58<-0.58 and RawPvalue<0.05 was set. ΔΔCt to M-CSF values were calculated and for statistical analysis of these data one way anova with Dunnet's correction was conducted.

Western Blot

Macrophages ($1x10^6$ cells/well), were lysed using MQ water in combination with NuPage LDS non-reducing sample buffer (1x) (ThermoFisher NP0007) and 1x NuPage sample reducing agent (ThermoFisher NP0009). 5 µL of the ladder SeeBlue plus 2 Standard (Invitrogen LC5925) was used and 12 µL of samples per well in a NuPage 4-12% Bis-Tris Mini gel. The gel was run in MOPS buffer (ThermoFisher) at 100-120V for 2-3h. After that, proteins were transferred to a

nitrocellulose membrane using the iBlot™ Gel Transfer system following the manufacturer's instructions. Membranes containing protein were cut above the housekeeping protein (Histone H3) and blocked with 3% milk in PBS (blocking buffer). The different parts were incubated overnight at 4°C with the appropriate antibody, GBP-1 (Abcam ab131255) diluted 1:2000 or H3 (Abcam ab1791) 1:20000 diluted in blocking buffer. Post incubation, membranes were washed (PBS, 0.05% Tween20) and then incubated with a secondary antibody Alexa Fluor 680 donkey anti-rabbit IgG (H+L) (Invitrogen A10043) diluted at 1:8000 in wash buffer for 1 hour in the dark. Membranes were washed and visualized with the Odyssey Infrared Imaging system.

Cell surface proteomics

Surface-protein enrichment followed by quantitative mass spectrometry was performed as previously described (32). In brief: glycosylations of cell surface proteins on live cells were oxidized using sodium-metaperiodate (1 mM, 10 min) and biotinylated using Alkoxyamine-PEG4-biotin (Thermo Fisher Scientific) via an oxime ligation reaction (1 mM, 10 min). Cells were washed, harvested and lysed in 50 mM Tris 4% SDS. Biotinylated proteins were enriched using neutravidin beads (Thermo Fisher Scientific), on-bead digested with trypsin, labelled with tandem mass tags (TMT10, Thermo Fisher Scientific), and subsequently pooled. Samples were derived from four different donors. In each TMT experiment, all samples from one donor (monocytes, M-CSF, M-CSF + LPS, M-CSF + IFNγ, GM-CSF, GM-CSF + LPS, IFNγ, IFNγ + LPS) were combined. Samples were fractionated into 5 fractions using stage-tip based SCX fractionation (33) and measured on a QExactive mass spectrometer, using 120 min gradients. Raw files were processed using an in-house pipeline (34) and spectra were searched against the IPI database using Mascot 2.5 (Matrix Science, Boston, MA). Protein abundance was calculated relative to the M-CSF channel. Proteins utilized for further analysis were filtered for localization to the plasma membrane (manually curated and extended Swissprot annotation) and for at least 3 quantified spectra matches as well as 2 unique peptides to ensure high quality identification and quantification. Differential protein abundance was tested individually for each contrast by a two-sample two-sided t-test with multiple hypothesis testing correction using the Benjamini-Hochberg procedure. Proteins fulfilling the following criteria were regarded as significantly affected: (I) pvalue fulfils the criteria:

$$if(logFC > s)\{\text{-log}_{10}(\text{p-value}) > \frac{1}{(logFC - s)} + P\}$$

$$if(logFC < \text{-s})\{\text{-log}_{10}(\text{p-value}) > \frac{1}{(\text{s - logFC})} + P\}$$

were *logFC* is the log2 ratio of the pair-wise comparison, s the standard deviation of the data set defined as 3x median standard deviation of protein abundance estimations for all proteins between replicates and P the minimal accepted $\log_{10}$ transformed p value for a protein set to p = 0.01, (II) $|logFC| \geq s$, and (III) adjusted p value < 0.1.

Patient dataset analysis

Data sets were obtained from the NCBI Gene Expression Omnibus (GEO) (35), 39 independent human transcriptome-wide datasets for 10 skin diseases were reviewed and selected. All selected

datasets are derived from human skin biopsy samples from published clinical trials and included comparisons of diseased lesional (LS) to non-lesional (NL) and/or normal (NN) skin biopsies). Where comparisons are conducted for patients on treatment, the longitudinal time points are included, and the data are provided as log2 fold change relative to baseline (prior to treatment initiation). Studies were curated to ensure technical quality and comparability across studies and diseases and probes were remapped to allow cross-platform comparisons.

Transcriptomic studies used in the meta-analysis: Psoriasis: GSE2737, GSE6710, GSE13355, GSE14905, GSE26866, GSE30999, GSE34248, GSE41662, GSE41663, GSE50790, GSE51440, GSE52471, GSE53431, GSE54456. Acne: GSE6475, GSE53795. Rosacea: GSE65914. Alopecia Areata: GSE45512, GSE58573. Atopic Dermatitis: GSE5667, GSE16161, GSE27887, GSE32924, GSE36842, GSE58558, GSE59294. Vitiligo: GSE53146, GSE65127. Lichen Planus: GSE38616, GSE52130. Actinic Keratosis: GSE2503, GSE32628. Dermatomyositis: GSE1551, GSE5370, GSE11971, GSE46239. Lupus: GSE52471. Hidradenitis suppurativa: GSE72702. Anti-IL-17 in Psoriasis: GSE31652, GSE53552. Anti-IL-23 in Psoriasis: GSE51440. Anti-TNF in Psoriasis: GSE57376, GSE11903, GSE41663.

The meta-analysis method used here has been described previously in (36). To re-analyse and integrate the available clinical transcriptomics datasets, we obtained the raw mRNA expression data from GEO. We then pre-processed each dataset by applying the robust multi-array average (RMA) algorithm in combination with a method that accounts for evolving transcript definitions by remapping microarray probes to the most current gene annotations (37), as described previously (38). Differentially expressed gene changes (i.e. lesion vs. normal or lesion vs. non-lesion, or time post treatment vs. baseline) were determined by linear model fit accounting for paired study designs where applicable. Additionally, variance estimates were derived by applying an empirical Bayes methodology (39). We then integrated the resulting individual processed data sets by matching the Entrez gene identifiers that correspond to the respective probe set identifiers.

Statistical analysis

Data were analysed using GraphPad Prism version 5.0 (GraphPad software, La Jolla, California). Data represent the mean ± standard error of the mean (SEM). Statistical differences were analysed using a two-way ANOVA with Bonferroni post-hoc test analysis for grouped analysis and one-way anova with Dunnet's post hoc test or t-test for column analysis. *P* values <0.05 were considered statistically significant. * p value < 0.05, ** p value <0.01, *** p value < 0.001, **** p value < 0.0001.

Results

IFNγ alone is sufficient to induce monocyte differentiation into macrophages.

To determine whether factors other than M-CSF or GM-CSF are able to generate genuine and responsive macrophages, we cultured human monocytes for 5 days in the presence of different inflammatory mediators (LPS, IFNγ, TNF or IL-17) alone or in combination with M-CSF **(Figure 1A)**. After 5 days the resultant cells were stimulated for 24 hours with LPS to measure cytokine production. We observed that M-CSF and GM-CSF differentiated macrophages exhibited a strong response to LPS with production of classical inflammatory cytokines **(Figure 1A)**. As expected, monocytes left in culture without any growth factor or stimulation did not survive and acted as an internal control for other conditions that were insufficient for monocyte survival, such as IL-17. In the absence of M-CSF, IFNγ differentiated macrophages exhibited a strong response to LPS by producing the classical pro-inflammatory cytokines, TNF, IL-1β, IL-6 and IL-12p70. Similar effects were observed in macrophages that were differentiated with IFNγ in the presence of M-CSF. Macrophages generated in the presence of LPS alone, responded to a subsequent LPS stimulation with high IL-6 and MCP-1 but not TNF which is in line with a tolerised phenotype (40). Macrophages generated in the presence of TNF exhibited a weak LPS response with IL-12p70, MCP-1 and TNF being detected above the monocyte only culture. Based on these results and the importance of IFNγ in inflammatory diseases (41), we decided to focus on macrophages generated in the presence of IFNγ alone. Firstly, we wanted to identify the optimal concentration of IFNγ needed for differentiation of monocytes into macrophages. We cultured monocytes for 5 days with a range of IFNγ concentrations (0.1-100 ng/ml) in the presence or absence of suboptimal M-CSF concentrations. In the absence of M-CSF, phase bright cells were only observed when 1 ng/ml IFNγ or greater was used for differentiation and these macrophages exhibited a clustered and round morphology **(Figure 1B)**. Whilst M-CSF differentiated macrophages typically display an elongated morphology, the presence of 1 ng/ml IFNγ hindered this morphology, indicating dominance of the IFNγ signalling pathway. Monocytes differentiated with 50-100 ng/ml IFNγ appeared morphologically similar and were found to be distinct from monocytes and other monocyte-derived *in vitro* differentiated macrophages **(Supplementary Figure 1A)**. We measured total cellular ATP content as an orthogonal marker of cell survival and found that ATP levels increased with increasing IFNγ concentrations reaching a plateau at 50-100 ng/ml IFNγ **(Figure 1C)**. Monocytes differentiated with 50-100 ng/ml IFNγ had similar total ATP levels to those generated in the presence of M-CSF + IFNγ or M-CSF alone. Monocytes differentiated with IFNγ alone did not rely on endogenous production of M-CSF as a survival factor **(Supplementary Figure 1B)**.

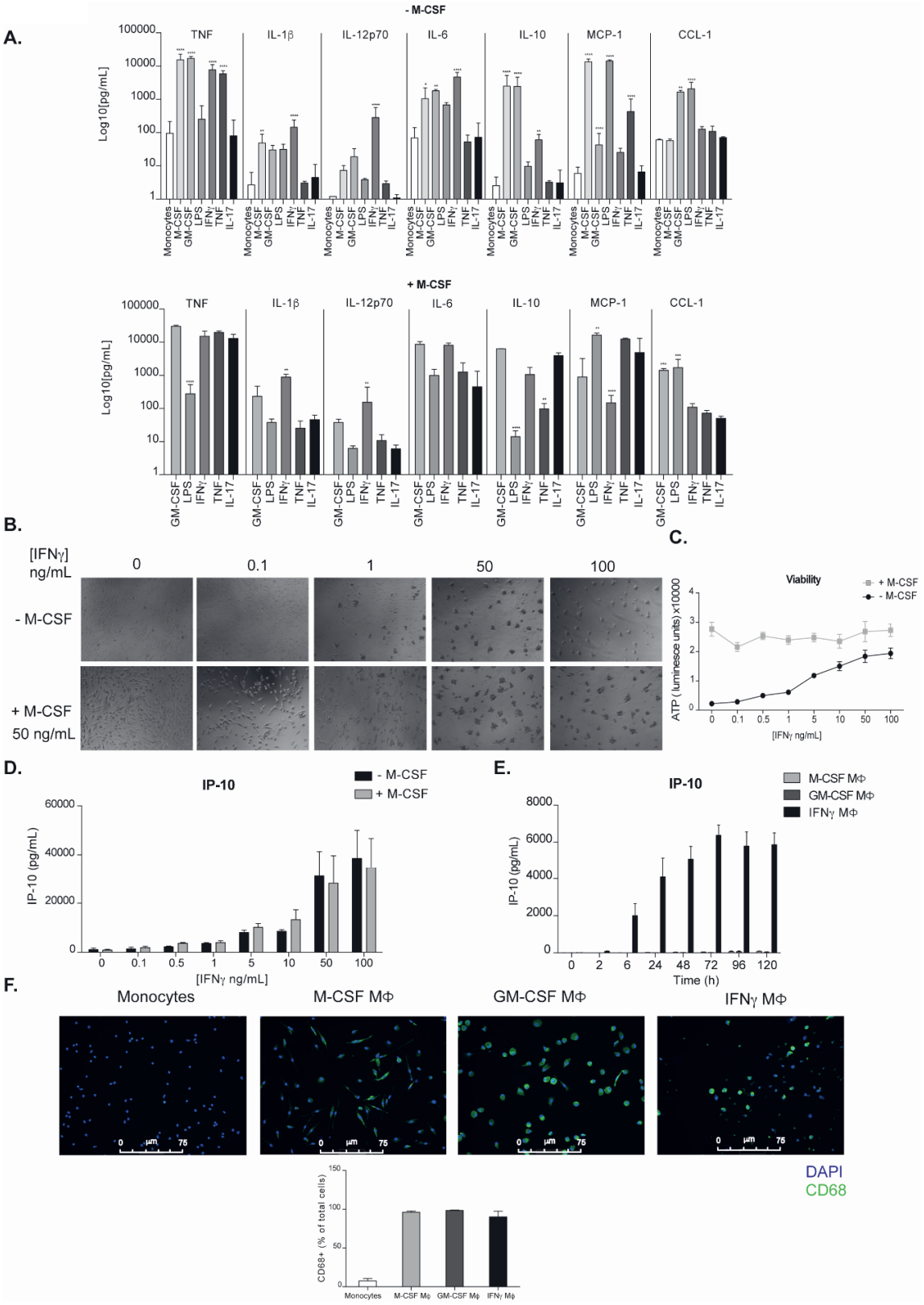
A.
- M-CSF
+ M-CSF
TNF
IL-1β
IL-12p70
IL-6
IL-10
MCP-1
CCL-1
Log10[pg/mL]
Monocytes
M-CSF
GM-CSF
LPS
IFNγ
TNF
IL-17
B.
[IFNγ] ng/mL
0
0.1
1
50
100
- M-CSF
+ M-CSF 50 ng/mL
C.
Viability
+ M-CSF
- M-CSF
ATP (luminesce units) x10000
[IFNγ ng/mL]
D.
IP-10
- M-CSF
+ M-CSF
IP-10 (pg/mL)
[IFNγ ng/mL]
E.
IP-10
M-CSF MΦ
GM-CSF MΦ
IFNγ MΦ
IP-10 (pg/mL)
Time (h)
F.
Monocytes
M-CSF MΦ
GM-CSF MΦ
IFNγ MΦ
DAPI
CD68
CD68+ (% of total cells)

Figure 1: IFNγ is able to differentiate monocytes into macrophages: A. Monocytes were cultured for 5 days in the presence of different factors M-CSF, GM-CSF, LPS, IFNγ, TNF or IL-17 in the absence (top panel) or presence (bottom panel) of M-CSF. After 5 days the resultant cells were stimulated for 24 hours with LPS and the production of indicated cytokines was measured. **B**. Phase contrast images of monocytes differentiated with a range of IFNγ concentrations (0, 0.1 - 100 ng/mL) in the absence or presence of M-CSF for 5 days. **C**. Viability, of the cells generated in B, was assessed by measuring total ATP content. **D.** IP-10 production from the cells differentiated in B. **E.** IP-10 production over the differentiation period for monocytes cultured either with M-CSF, GM-CSF or IFNγ. **F**. Immunocytochemistry staining of CD68 (FITC) and DAPI in monocytes, M-CSF, GM-CSF or IFNγ-derived macrophages from 1 representative donor. Percentage of CD68 positive cells across 3 separate donors is shown. All error bars represent the SEM, n=3 for all experiments. Statistical significance was assessed by 2-way ANOVA test with Bonferroni correction (* p value < 0.05, ** p value <0.01, *** p value < 0.001, **** p value < 0.0001).

Based on these data, we selected 50 ng/ml IFNγ as the optimal concentration to generate these macrophages, herein termed IFNγ Mϕ. Interferon gamma response protein 10 (IP-10) is a well-known IFN response cytokine (42). We wanted to test whether the response to IFNγ was robust and irrespective of the presence of M-CSF over the 5 day differentiation period and therefore, decided to measure IP-10 in the supernatants of these cells. We found enhanced IP-10 levels with increasing IFNγ concentrations irrespective of the presence of M-CSF **(Figure 1D)**. We further tested the selectivity and rapidity of IP-10 production and assessed production throughout the differentiation period and across the three macrophage subtypes. We found that IP-10 was specifically expressed by IFNγ Mϕ and produced as early as 6 hours into differentiation and this further increased over time **(Figure 1E)**. Next, we wanted to confirm that these cells expressed CD68, a prototypical macrophage marker (43). We compared CD68 immunostaining across monocytes, IFNγ Mϕ and the well-characterised M-CSF (M-CSF Mϕ) and GM-CSF (GM-CSF Mϕ) macrophages and found no difference across the three macrophage subtypes **(Figure 1F)**.

These data demonstrate that IFNγ alone is sufficient to induce human monocyte differentiation into macrophages as shown by CD68 staining. These IFNγ Mϕ are viable, morphologically distinct and LPS-responsive.

IFNγ Mϕ exhibit a hyper-inflammatory phenotype whilst retaining phagocytic capacity

As a core macrophage function, we compared the phagocytic capacity of the three Mϕ subtypes using pHrodo green *E. coli* beads. Surprisingly, we observed that IFNγ Mϕ had the same phagocytic function as M-CSF Mϕ and there was no difference in the rate or extent of phagocytosis across the three Mϕ subtypes **(Figure 2A)**.

To better understand and compare the functional capabilities of IFNγ Mϕ, we assessed cytokine production following stimulation with LPS, LPS + IFNγ or IL-4. We found that IFNγ Mϕ produced significantly more IL-12p70 and IL-23 after stimulation (LPS alone of LPS + IFNγ), whereas production of TNF and IL-1β were not statistically different across the three macrophage

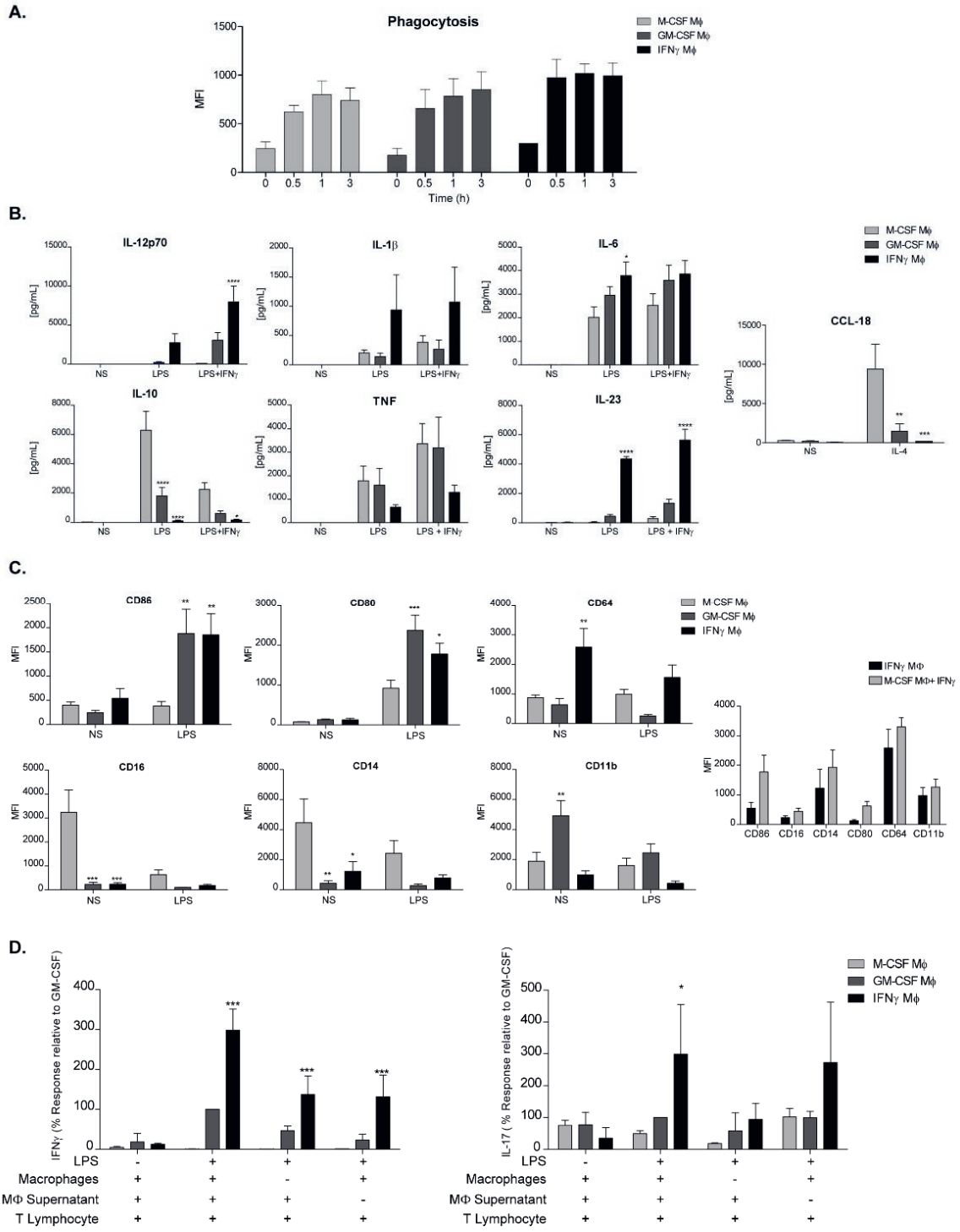
A.
Phagocytosis
M-CSF Mϕ
GM-CSF Mϕ
IFNγ Mϕ
MFI
Time (h)
B.
IL-12p70
IL-1β
IL-6
CCL-18
IL-10
TNF
IL-23
[pg/mL]
NS
LPS
LPS+IFNγ
IL-4
C.
CD86
CD80
CD64
CD16
CD14
CD11b
MFI
IFNγ MΦ
M-CSF MΦ+ IFNγ
D.
IFNγ (% Response relative to GM-CSF)
IL-17 (% Response relative to GM-CSF)
LPS
Macrophages
MΦ Supernatant
T Lymphocyte

Figure 2. IFNγ macrophages exhibit enhanced inflammatory responses but normal phagocytic capacity. A. Macrophages were culture for 3 hours in the presence of pHrodo green *E. coli* bioparticles. MFI was measured at different time points (0, 0.5, 1, 3 hours). **B.** Cytokine response of the three macrophage subtypes was determined following either no stimulation (NS), stimulation with LPS, LPS + IFNγ or IL-4 for 24 hours. Statistical analysis was conducted comparing the data to the M-CSF derived macrophages to each condition. **C.** Cell surface markers were measured by flow cytometry following stimulation for 24 hours. Statistical significance is relative to M-CSF Mϕ expression to each condition. **D.** IFNγ and IL-17 production was measured from autologous T cells co-cultured for 3 days with macrophages stimulated or non-stimulated for 24 h with LPS. For some experiments, the supernatant from LPS-stimulated macrophages was removed and cultured with T cells and for other experiments the activated macrophages alone were cultured with T cells. The data was normalized to the T cell response induced by GM-CSF Mϕ. Statistical analysis was conducted by comparing the data to the T cell response induced by GM-CSF Mϕ. All error bars represent the SEM, n=3 for all experiments. All statistical significance was assessed by 2-way ANOVA test with Bonferroni correction. (* p value < 0.05, ** p value <0.01, *** p value < 0.001, **** p value < 0.0001).

subtypes **(Figure 2B)**. M-CSF Mϕ produced IL-12p70 after stimulation with LPS + IFNγ, although at much lower levels than GM-CSF and IFNγ Mϕ. It is interesting to note that M-CSF Mϕ stimulated with LPS + IFNγ do not produce the same cytokine response as IFNγ Mϕ + LPS demonstrating that the differentiation conditions have produced an inherently differential baseline. In line with this greater pro-inflammatory response, stimulated IFNγ Mϕ did not produce IL-10. IL-4 stimulation of these macrophages evoked a strong CCL-18 response from M-CSF Mϕ whereas this was significantly reduced in GM-CSF Mϕ and absent in IFNγ Mϕ providing evidence that exposure to IFNγ during differentiation has altered the resulting macrophages to be less amenable to resolution signals such as IL-4.

Next, we wanted to study any differences in cell surface marker expression evident at baseline or following stimulation with LPS (for details see **Supplementary Figure 1C and D**). At baseline, IFNγ Mϕ expressed more CD64 in comparison to M-CSF and GM-CSF Mϕ **(Figure 2C)**. CD14 and CD16 are known to be highly expressed in M-CSF Mϕ (44), however both IFNγ and GM-CSF Mϕ exhibit significantly reduced expression in comparison. CD11b, the macrophage adhesion marker (45) whilst expressed across all three Mϕ subtypes was considerably higher in GM-CSF Mϕ. Under inflammatory conditions, both IFNγ and GM-CSF Mϕ induced expression of the co-stimulatory molecules CD80 and CD86. These data indicate that both IFNγ and GM-CSF Mϕ exhibit a large overlap in cell surface markers at baseline and in response to LPS compare to M-CSF Mϕ with a key feature being induction of co-stimulatory molecules CD80 and CD86.

We wanted to follow up on the functional consequences of increased CD80 and CD86 expression and hypothesised that IFNγ and GM-CSF Mϕ may have enhanced T cell activation capacity relative to M-CSF Mϕ. To assess this, we co-cultured macrophages with autologous T cells. We found that naive macrophages co-cultured with T cells for 3 days did not induce T cell activation as assessed by IFNγ and IL-17 production **(Figure 2D)**. LPS stimulation of GM-CSF and IFNγ Mϕ, but not M-CSF Mϕ, led to T cell activation with a strong Th1 response as characterized by IFNγ production. To determine whether T cell activation was driven via macrophage derived secreted

factors, we transferred supernatants from activated macrophages to T cells and found suboptimal IFNγ production. We then assessed whether cell-cell interactions alone were sufficient for T cell activation and used LPS-activated Mϕ in fresh cell culture medium and co-cultured with T cells to find only partial T cell activation as measured by IFNγ production.

We additionally assessed whether other T cell subsets were induced through measuring supernatants for IL-13 and IL-17. Whilst IL-13 was not detectable (data not shown), a strong IL-17 response was induced via IFNγ Mϕ **(Figure 2D)**. IL-17 was induced via IFNγ Mϕ secreted factors together with direct cell-cell interactions and was not evident in M-CSF and GM-CSF Mϕ.

In summary these data show that IFNγ Mϕ are phagocytic, have a similar activation profile to GM-CSF Mϕ but exhibit a hyper-inflammatory phenotype that can induce autologous T cell activation with both TH1 and TH17 responses.

GBP-1 is a potential marker for IFNγ Mϕ

We next set out to identify a selective marker for IFNγ Mϕ using cell surface proteomics analysis. Selective labelling of plasma membrane proteins via an oxime ligation utilizing the N-linked glycosylations terminating in sialic acids is a powerful method to increase the sensitivity for detection for typically low abundant cell surface proteins (32) . Human primary monocytes derived from 4 donors were differentiated into macrophages by M-CSF, GM-CSF or IFNγ and polarised by either LPS or IFNγ. Cell surface proteins were labelled on live cells and enriched glycosylated proteins were analysed by quantitative mass spectrometry. On average we identified and quantified 1200 plasma membrane annotated proteins per individual donor **(Supplementary Table 1)**.

We compared each of the three unstimulated Mϕ subsets to each other and found that the greatest difference was between M-CSF and IFNγ Mϕ **(Figure 3A)**. There were only 6 significantly upregulated proteins but 66 downregulated proteins in IFNγ Mϕ relative to M-CSF Mϕ. This trend was also visible in the comparison between IFNγ and GM-CSF Mϕ in which only 5 (GBP-1, LCK, RDX, STX11 and ITGAL) of the total 25 differential proteins were upregulated in IFNγ Mϕ. Proteins upregulated in IFNγ Mϕ compared to M-CSF Mϕ included, GBP1, IFIT5, STX11, G6PD, HSP90AA1 and SARS. We displayed this data in a heatmap and included an additional condition of M-CSF Mϕ stimulated with IFNγ **(Figure 3B)**. The heatmap shows differences in the cell surface proteome relative to M-CSF Mϕ and clearly demonstrates the overall decrease in several classes of proteins in IFNγ Mϕ. It is important to highlight that the clustering of IFNγ activated M-CSF Mϕ do not exhibit the same pattern as an IFNγ Mϕ, meaning that the differences come from the differentiation process rather than the activation. A list of the results of the proteomic study has been included **(Supplementary Table 1)**.

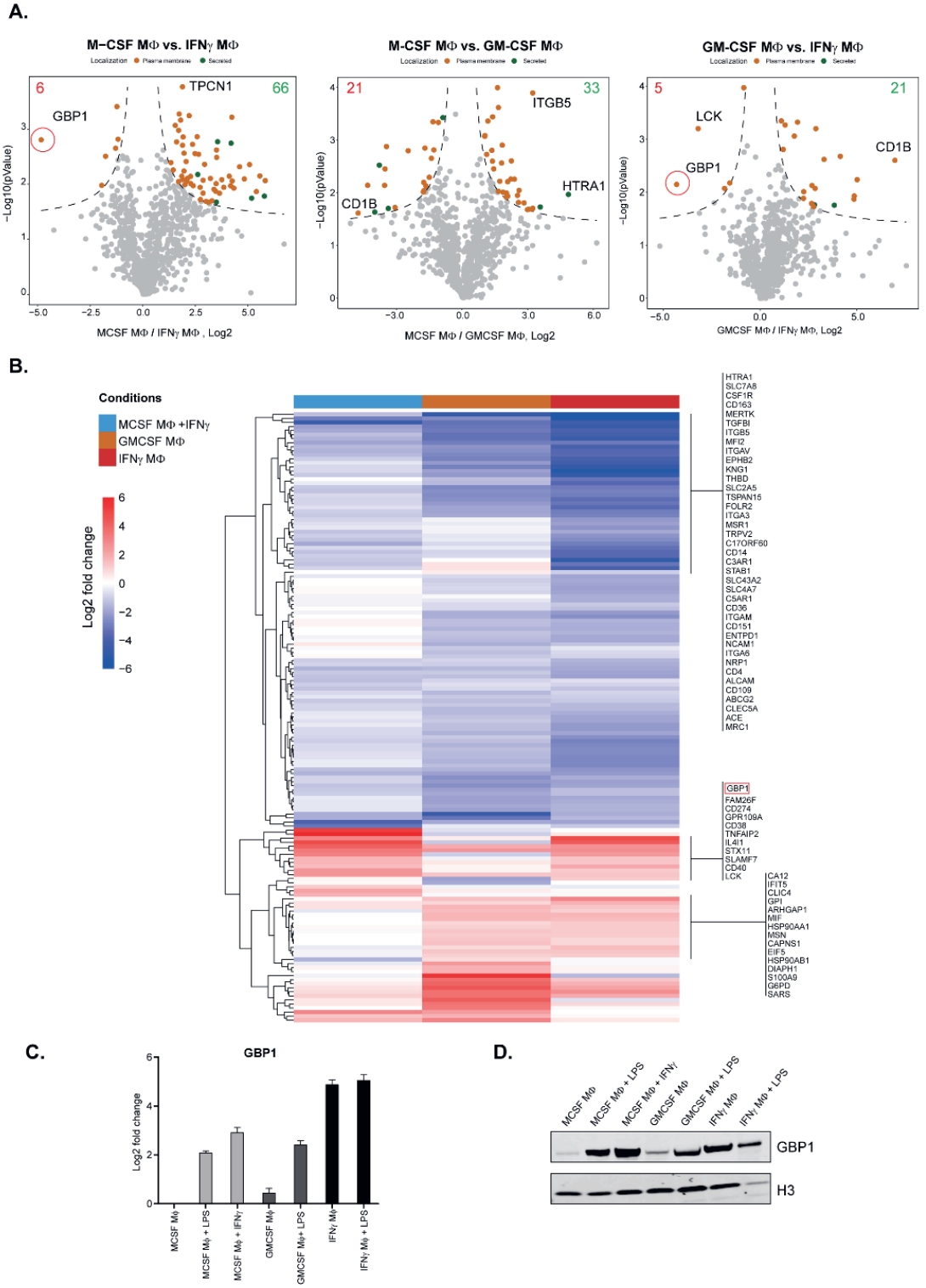
A.
M−CSF MΦ vs. IFNγ MΦ
M-CSF MΦ vs. GM-CSF MΦ
GM-CSF MΦ vs. IFNγ MΦ
Localization
Plasma membrane
Secreted
GBP1
TPCN1
ITGB5
HTRA1
CD1B
LCK
−Log10(pValue)
MCSF MΦ / IFNγ MΦ , Log2
MCSF MΦ / GMCSF MΦ, Log2
GMCSF MΦ / IFNγ MΦ, Log2
B.
Conditions
MCSF MΦ +IFNγ
GMCSF MΦ
IFNγ MΦ
Log2 fold change
HTRA1
SLC7A8
CSF1R
CD163
MERTK
TGFBI
ITGB5
MFI2
ITGAV
EPHB2
KNG1
THBD
SLC2A5
TSPAN15
FOLR2
ITGA3
MSR1
TRPV2
C17ORF60
CD14
C3AR1
STAB1
SLC43A2
SLC4A7
C5AR1
CD36
ITGAM
CD151
ENTPD1
NCAM1
ITGA6
NRP1
CD4
ALCAM
CD109
ABCG2
CLEC5A
ACE
MRC1
GBP1
FAM26F
CD274
GPR109A
CD38
TNFAIP2
IL4I1
STX11
SLAMF7
CD40
LCK
CA12
IFIT5
CLIC4
GPI
ARHGAP1
MIF
HSP90AA1
MSN
CAPNS1
EIF5
HSP90AB1
DIAPH1
S100A8
G6PD
SARS
C.
GBP1
Log2 fold change
MCSF Mϕ
MCSF Mϕ + LPS
MCSF Mϕ + IFNγ
GMCSF Mϕ
GMCSF Mϕ + LPS
IFNγ Mϕ
IFNγ Mϕ + LPS
D.
MCSF MΦ
MCSF MΦ + LPS
MCSF MΦ + IFNγ
GMCSF MΦ
GMCSF MΦ + LPS
IFNγ MΦ
IFNγ MΦ + LPS
GBP1
H3

Figure 3. Cell surface proteomics identifies GBP-1 as a marker of pro-inflammatory macrophages. **A.** The cell surface proteome of the three macrophage subtypes M-CSF Mϕ, GM-CSF Mϕ and IFNγ Mϕ was determined by cell surface labelling via the N-glycosylations followed by MS analysis. All pairwise comparisons for the three macrophage subtypes are visualized by volcano plots. Each dot represents a quantified cell surface protein, dotted lines indicate significance thresholds. Coloured dots indicate proteins with significantly altered cell surface abundance, names indicate proteins referred to in the main text. Numbers of significantly down (red) and up (green) regulated proteins are indicated in the upper corners. **B.** Hierarchical clustering of all cell surface proteins scoring significant in the comparison of each, GM-CSF Mϕ, IFNγ Mϕ and M-CSF Mϕ stimulated with IFNγ for 24 hours versus M-CSF Mϕ. The heatmap displays for each of the proteins the log2 protein abundance difference of the above listed states in comparison to M-CSF Mϕ. Protein names are indicated for selected subclusters where expression is differential in IFNγ Mϕ (up- or down-regulated). **C**. GBP-1 expression relative to M-CSF Mϕ derived from cell surface proteomics analysis across all conditions. **D.** Western blot analysis of GBP-1 expression across all conditions (representative donor). All error bars represent the SEM; n= 4 for all experiments

Next, we wanted to identify proteins that were selectively upregulated in IFNγ Mϕ so that they can be used as markers for these macrophages. We compared the fold change expression of the protein dataset normalized to M-CSF Mϕ across the different conditions. We found one protein that was selectively upregulated in IFNγ Mϕ: interferon-induced guanylate-binding protein 1 (GBP-1). We compared the expression of this potential marker across the three macrophage subtypes under stimulation conditions to determine whether it was sensitive to activation state. We found that GBP-1 was highly expressed under basal conditions in IFNγ Mϕ but it was also induced by activation with LPS or IFNγ in both M-CSF and GM-CSF Mϕ **(Figure 3C).** To independently validate the proteomics data for GBP-1, we generated additional biological replicates for all conditions and used Western blotting to confirm high GBP-1 expression in IFNγ Mϕ under basal conditions **(Figure 3D)**. In these samples, we similarly observed that activation of M-CSF or GM-CSF Mϕ with LPS or IFNγ also led to similar levels of GBP-1 as in IFNγ Mϕ. It is worth noting here, that the proteomics experiment specifically probed cell surface presence, whereas the Western Blot quantified total protein abundance in the cell. Nevertheless, these data suggest that GBP-1 alone can not be used as a reliable marker for IFNγ Mϕ but rather a marker of pro-inflammatory macrophages.

IFNγ Mϕ as a relevant model for psoriasis

We wanted to test the translational relevance of the *in vitro* IFNγ Mϕ model and used GBP-1 expression as a starting point. We conducted a meta-analysis for GBP-1 in a transcriptomics database of inflammatory skin diseases from published clinical studies using whole skin punch biopsies (as described in the methods section). We found that GBP-1 was consistently and significantly upregulated in lesional skin from psoriasis patients **(Supplementary Figure 2A)**. We wanted to explore this further using the functional characteristics identified earlier (high IL-12p70, IL-23 and IP-10 leading to upregulation of T cell production of IFNγ and IL-17) and determined expression in psoriasis patient skin. We further queried the psoriasis dataset and found

that IFNγ Mϕ markers were significantly enriched in lesional areas of psoriasis patient skin **(Figure 4A)**.Whilst expression of *GBP-1*, *IP-10* and *IL-23* were significantly higher, it was reassuring to see that other macrophage markers, *CD68* and *CD14*, were largely unchanged indicating that enhanced expression of *IL-23* was not due to increased numbers of macrophages in lesional skin. We tested whether the secreted mediators IP-10, IFNγ and IL-17 were also elevated in serum from psoriasis patients. IP-10 levels were significantly higher in patient serum versus age-matched controls **(Figure 4B, left panel)** although highly variable. IL-17 **(Figure 4B, right panel)**, IFNγ (data not shown) levels in patient serum were more variable and did not reach statistical significance which may reflect differences in systemic inflammatory mediators versus local production, as the RNA levels in inflamed lesions were more robust. Using the markers identified earlier, we tested whether treatment reversed this signature in patient skin biopsies. Strikingly, GBP-1 was consistently and significantly down-regulated in patients treated with biologics targeting IL-17A, IL-23 or TNF **(Figure 4C)**. A similar effect was seen with *IL23A* and *IP10* but not other macrophage markers *CD14*, *CD68* and *CD16b* suggesting that macrophage populations may not be reducing but the hyper-inflammatory phenotype was being resolved. Additionally, *IFNG* and *IL17A* were also consistently suppressed following treatment indicating the overall axis that we have shown for IFNγ Mϕ is responsive to known efficacious treatments in psoriasis patients. Whilst GBP-1 and IP-10 are not exclusively produced by IFNγ Mϕ, these data indicate that the IFNγ - IL-23 - IL-17 axis observed in this in vitro model could be considered as a relevant model when investigating diseases such as psoriasis where this axis is prominent. Furthermore, GBP-1 expression may provide a valuable marker of hyper-inflammatory macrophages in disease.

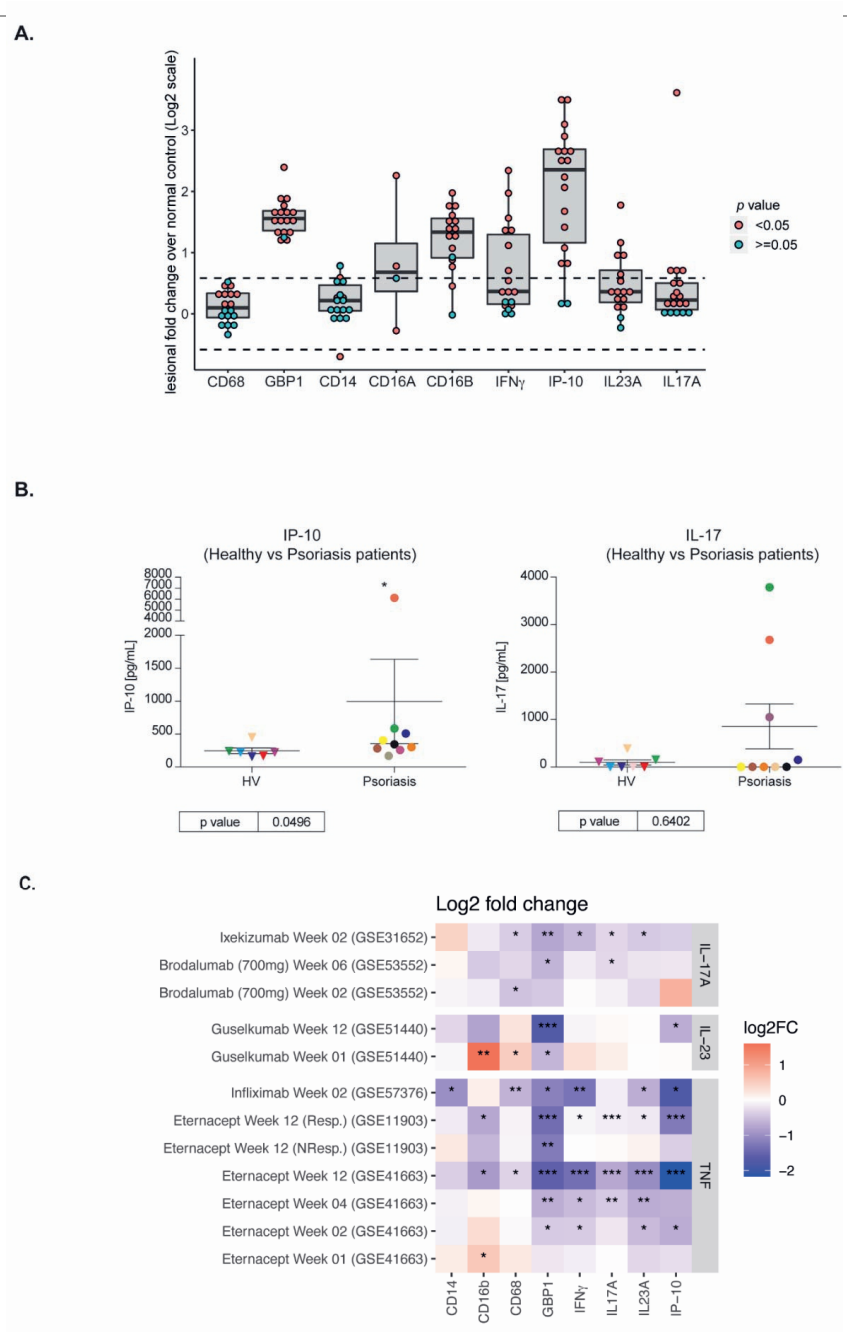

Figure 4. The IFNγ-GBP-1 axis is elevated in lesional skin from psoriasis patients. A. A database of published psoriasis clinical transcriptomics studies was mined for *IFNγ*, *GBP-1*, *IP-10* and macrophage markers. Log2Fold change of mRNA expression in lesional vs non-lesional samples in psoriasis patients; each dot represents a separate patient. **B.** Detection of IP-10 and IL-17 in the serum from psoriasis patients and gender/age-matched healthy volunteers (HV). All error bars represent the SEM of the values, n=6 for healthy volunteers (HV) and n=9 for psoriasis patients. **C.** Published transcriptomics data from skin biopsies taken from psoriasis patients treated with biologics were mined for the identified genes. The directional change in expression post treatment is relative to baseline (prior to treatment initiation) and the longitudinal sampling for each treatment is taken from

the original studies. The statistical significance was determined with Mann-Whitney unpaired *t*-test (* $p<0.05$) in the serum samples; statistical analysis for transcriptomics data are described in the methods section; *p* values indicated in the heatmap represent * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

Differential gene expression of chromatin modifiers

IRF5, is a transcription factor that has been linked to driving pro-inflammatory macrophages (21, 46). We wanted to test whether IRF5 expression was elevated in our model of pro-inflammatory IFNγ Mϕ. We checked basal expression levels after differentiation in the three macrophage subtypes and also after activation. Already under basal conditions, the levels were significantly higher in IFNγ Mϕ compared to M-CSF or GM-CSF Mϕ **(Supplementary Figure 2B)**. In line with their pro-inflammatory phenotype, we found that IFNγ Mϕ expressed higher levels of *IRF5* and this was not further increased upon stimulation with LPS and/or IFNγ. However, *IRF5* was induced in M-CSF and GM-CSF Mϕ following stimulation with IFNγ alone or in combination with LPS to levels similar to those found in unstimulated IFNγ Mϕ.

The chromatin landscape plays a critical role in macrophage activation and phenotype (47-49). Considering this, we wanted to understand whether chromatin modifiers were differentially expressed across the three macrophage subtypes and could account for the strong phenotypic differences. We analysed a standard RT² Profiler PCR 84 gene array panel of chromatin modifiers in M-CSF, GM-CSF and IFNγ Mϕ. When analysing the principal component analysis, the data showed that the samples clustered depending on the differentiation factor used and that M-CSF and GM-CSF Mϕ were more similar than IFNγ Mϕ **(Figure 5A)**. We generated volcano plots that showed significantly upregulated and down regulated genes (threshold of Log2Foldchange -/+ 0.58 and *p* value of 0.05) in the different comparisons **(Figure 5B)**. Gene lists for statistically up and downregulated genes are shown in **Supplementary Table 2**. From the gene lists obtained, Venn diagrams were generated for the genes in the comparison of IFNγ Mϕ to either M-CSF or GM-CSF Mϕ. From this analysis we observed that 6 genes were commonly upregulated in IFNγ Mϕ vs both M-CSF and GM-CSF Mϕ (*CIITA*, *RPS6KA5*, *SETDB2*, *NCOA3*, *KDMC4* and *SETDB1*). Moreover, 6 genes were downregulated in comparison to both M-CSF and GM-CSF Mϕ (*DNMT3A*, *WHSC1*, *AURKA*, *AURKB*, *HDAC9* and *ESCO2*). Interestingly, no genes were specifically upregulated in IFNγ Mϕ compared to M-CSF, while 21 were specifically upregulated when compared to GM-CSF Mϕ. In the downregulated genes, 7 were selectively suppressed when compared to M-CSF Mϕ and only 1 gene was specifically downregulated in IFNγ vs GM-CSF Mϕ (*NEK6*). We checked the ΔΔCt to M-CSF Mϕ values of GM-CSF and IFNγ Mϕ for specific epigenetic genes that have been widely linked to the control of inflammation, such as HDACs (50). Of the HDAC members investigated, only *HDAC5* was upregulated in IFNγ Mϕ. *CIITA* and *SETBD2* were upregulated in IFNγ Mϕ whereas *KAT2A*, an acetyltransferase, and *ESCO2*, *AURKB* and *HDAC9* were downregulated in IFNγ Mϕ **(Figure 5D)**. All these data thus demonstrate that

specific epigenetic modifiers are differentially expressed in IFNγ Mϕ and may be relevant as future targets for inflammatory disease.

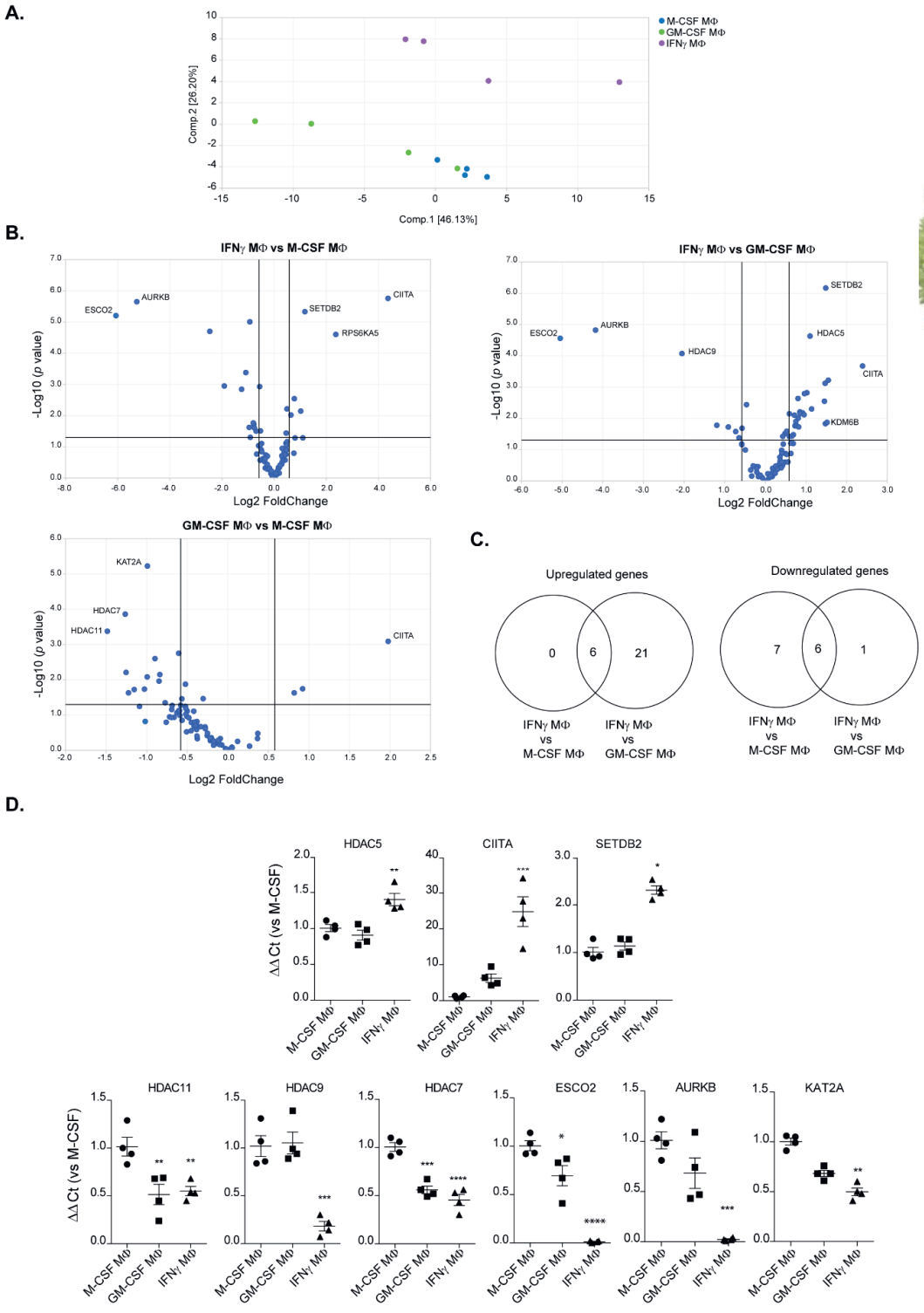

Figure 5. Differential gene expression of chromatin modifiers in M-CSF, GM-CSF and IFNγ derived macrophages. **A.** Principal component analysis of the panel of genes in the 3 subtypes of macrophages. **B.** Volcano plots of chromatin modifying enzymes from the different comparisons between macrophages. **C.** Venn diagram of the statistically significant upregulated or downregulated genes in IFNγ derived macrophages compared with M-CSF or GM-CSF derived macrophages. **D.** ΔΔCt values (to MCSF) of different genes included in the gene panel for GM-CSF, IFNγ and M-CSF derived macrophages n=4. The threshold for differential gene expression was 0.58 of Log2 Fold Change and a p value < 0.05. The statistical analysis used for the ΔΔCt values was one-way ANOVA with Dunnet's correction. All error bars represent the SEM. n=4 for all experiments (* p value < 0.05, ** p value <0.01, *** p value < 0.001, **** p value < 0.0001).

Discussion

The tissue micro-environment that monocytes encounter in disease situations is complex and contains a wide range of inflammatory mediators. Therefore, if we want to generate translatable *in vitro* models that represent this complexity, the use of a wider range of these mediators during the differentiation process is necessary. Knowing the importance of IFNγ in (auto-) immune diseases and its higher levels in the tissue/serum in those conditions we wanted to understand how IFNγ would impact monocyte differentiation and characterize the resultant macrophages. We identified an optimal concentration of IFNγ required for differentiation into viable macrophages and demonstrated that IFNγ signalling was dominant over M-CSF signalling. IP-10 promotes the migration of T cells to inflammatory sites (42) and was rapidly induced during monocyte differentiation induced by IFNγ but not M-CSF or GM-CSF. We further demonstrated that IFNγ Mϕ were not only viable and exhibited macrophage characteristics through CD68 expression (51) but exhibited phagocytic functions to the same extent as M-CSF Mϕ.

Further functional characterization demonstrated that in comparison to M-CSF and GM-CSF Mϕ, IFNγ Mϕ had a stronger pro-inflammatory phenotype together with decreased anti-inflammatory/alternative response. IFNγ Mϕ also expressed higher levels of pro-inflammatory surface markers, CD86, CD80 and CD64 compared with M-CSF Mϕ. Finally, as regulators of the immune system, these macrophages have a higher capacity to activate Th1/Th17 responses in autologous T cells than M-CSF or GM-CSF Mϕ. This may be driven through higher expression of IL-12/IL-23 and CD80/CD86 which are important for T cell activation (52-54).

In the course of this study, we found that IFNγ Mϕ exhibited a more pro-inflammatory phenotype compared to M-CSF Mϕ activated with IFNγ (either alone or in combination with LPS). This suggests that exposure to IFNγ during monocyte differentiation imprints a stronger pro-inflammatory phenotype than with activation alone.

To further characterize these cells, we performed a proteomics study and found that the IFNγ Mϕ are significantly different to both M-CSF and GM-CSF Mϕ. One of the aims of this experiment was to find a protein that could be used as a marker to identify similar macrophages in samples from patients with immune disease. In the proteomics data, we identified the plasma membrane

associated protein, interferon-induced guanylate-binding protein (GBP-1), that was specifically upregulated under basal conditions in IFNγ Mϕ although also present following activation of the other macrophage subtypes. GBP-1 has been associated with inflammatory conditions, where its expression is induced in epithelial cells by pro-inflammatory mediators such as IL-1β, TNF and IFNγ (55), innate responses in defence to pathogens (56) and also elevated in patients with chronic inflammatory diseases like RA, Systemic lupus erythematosus (SLE) and Systemic sclerosis (SSc) (57). In cancer, it has been demonstrated to have immunosuppressor activities in colorectal cancer (58, 59). The conflicting role and broader expression pattern of GBP-1 presents caveats for its use as a single specific identification marker of pro-inflammatory/IFNγ Mϕ in inflammatory disease.

Taking into account the difficulties to define a single specific marker for IFNγ Mϕ, we identified a panel of phenotypic characteristics that were indicative of a strong IFNγ driven effect on monocytes/macrophages and utilised a signature of enhanced GBP-1, IP-10 and IL-23/IL-12 and suppressed CD14 and CD16 levels. We also included increased IFNγ and IL-17 levels in this signature given the well-established link between IL-12/IL-23 activation of T cells to produce IFNγ and IL-17 (54, 60, 61). Searching databases for patients with these signatures, we found that inflamed skin from psoriasis patients exhibited this pattern (high *IP-10*, *IL-23*, *IL-17* and low *CD14*) whilst no change in CD68 suggesting that macrophage numbers were not driving this increase but rather a pro-inflammatory macrophage population. Some of these markers (*GBP1*, *IP10*, *IL23A*, *IFNG* and *IL17A*) were also consistently down-regulated in patients treated with biologics targeting either, IL-17A, IL-23 or TNF. Elevated, although variable, IP-10 levels in serum from psoriasis patients also corroborated this finding, although it seems that elevated *IP-10* expression is more robust locally than systemically. Our results suggest that IFNγ Mϕ could be used as a more relevant *in vitro* macrophage model for the study of inflammatory diseases like psoriasis.

We also investigated potential drivers for this hyper-inflammatory phenotype and focused on chromatin modifiers. Despite PCA plots showing that the differentiation factor was the key driver for differences in gene expression, there were no genes specifically upregulated in IFNγ vs M-CSF Mϕ. However, 21 epigenetic enzymes were differential between IFNγ vs GM-CSF Mϕ, which included the Lysine demethylase 6B (*KDM6B*). This gene has previously reported to be upregulated after IFNγ stimulation (62) and in pro-inflammatory conditions (eg. LPS) and its depletion shows abrogation of pro-inflammatory cytokine production (63, 64). Genes upregulated in IFNγ Mϕ relative to both M-CSF and GM-CSF Mϕ included *CIITA* (MHC class II transactivator known to be induced by IFNγ (65, 66)) and *RPS6KA5* (known to be induced in activated macrophages (67)), a SNP in this gene is associated with superior efficacy of anti-TNF treatment in RA patients (68, 69). *HDAC5* and *SETDB2* were specifically upregulated in IFNγ Mϕ. HDAC5 has been reported to regulate macrophage activation and TNF production via the NF-κb pathway (70-72). *SETDB2* has been reported as an interferon induced gene and expressed

in inflammatory macrophages (73). Unsurprisingly *IRF5* expression was upregulated in IFNγ Mϕ, this transcription factor has been strongly linked to a pro-inflammatory phenotype in macrophages and reported to control expression of *IL-12B* and *IL-23A* amongst others (21, 46). *IRF5* has also been linked to inflammatory diseases, for instance in patients with juvenile idiopathic arthritis and contributes to the pathogenesis of macrophage activation syndrome (74-76)

Genes downregulated in IFNγ Mϕ included *ESCO2* (associated with Robert's syndrome (77)), *AURKB (*upregulated in psoriasis (78, 79) and in RA patients (80, 81)), *HDACs*: *HDAC9*, that is linked to inflammatory macrophages in atherosclerosis (82, 83), *HDAC7*, *HDAC11* (which is reported to block *IL-10* expression in antigen-presenting cells (84)) and *KAT2A* (known to be suppressed by IFNγ (62)).

The development of new *in vitro* models that better recapitulate the human *in vivo* environment is important not only to develop new treatments but also to study disease pathogenesis. Here we show that using IFNγ we can generate pro-inflammatory macrophages that have clear characteristics seen in human inflammatory disease. Thus, this may represent an important model for studying disease. The use of alternate differentiation factors based on their relevance to disease offers a potential translational advantage and could unravel novel features in these *in vitro* models leading to an improved understanding of diseases.

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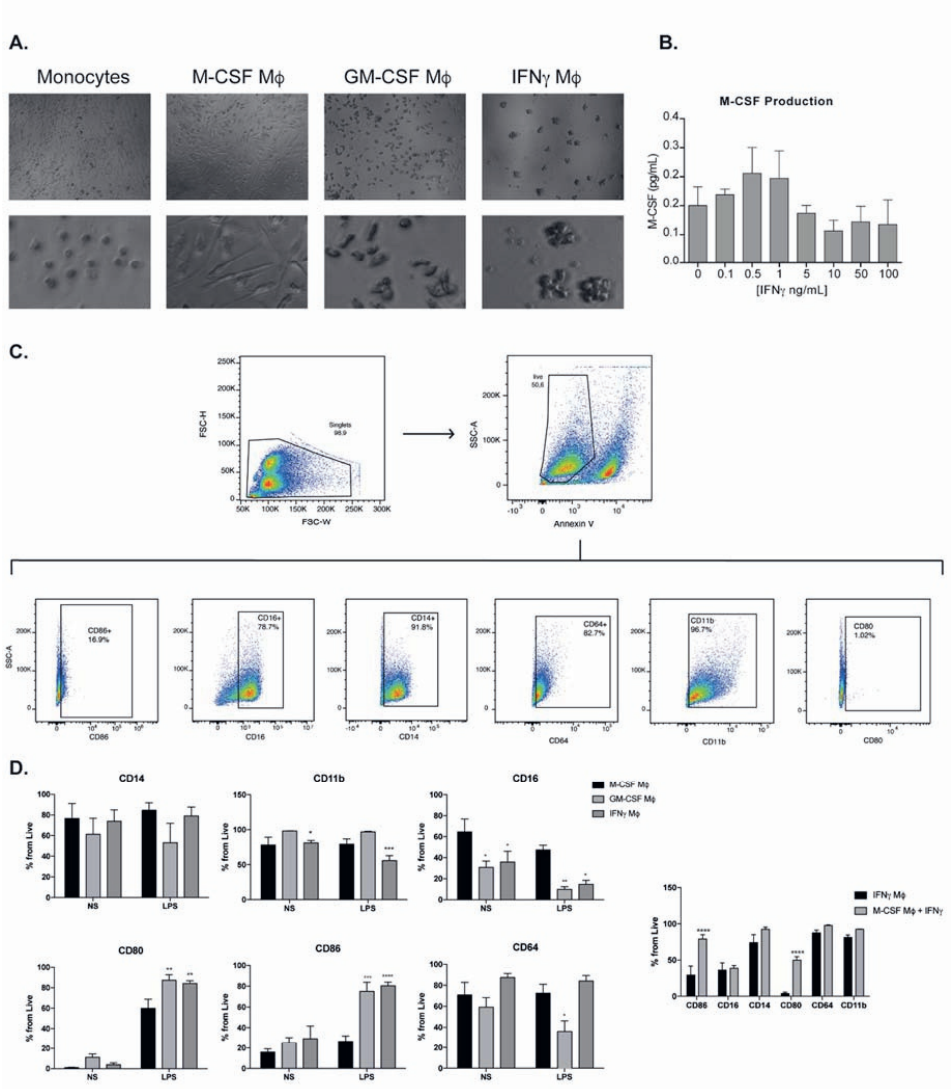
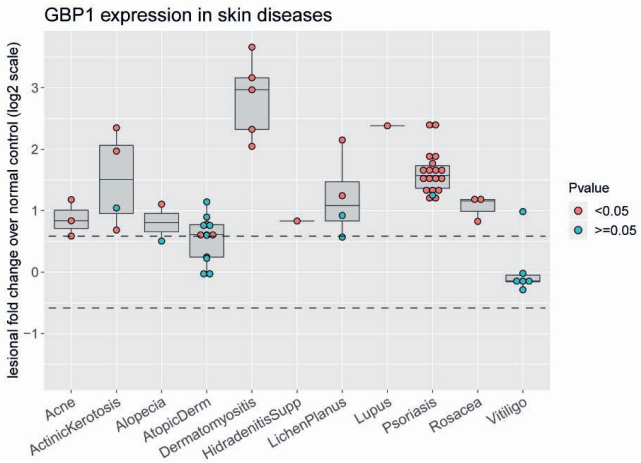
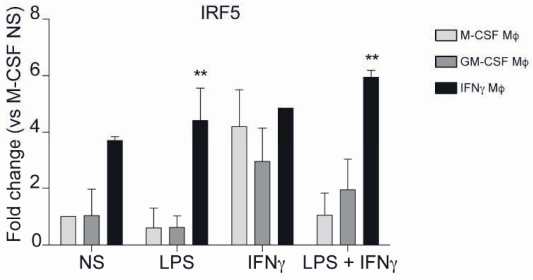


Supplementary Figure 1.
A. Morphology of the macrophages derived from M-CSF, GM-CSF and IFNγ. Images shown are 10x magnification. **B.** M-CSF production by monocytes cultured for 5 days with IFNγ (0, 0.1 - 100 ng/mL). Error bars represent the SEM of the values. **C.** Gating strategy followed for all conditions was the same across all macrophage subtypes as shown here with M-CSF Mf as an example. **D.** Percentage of postive cells for various markers across the three macrophage subtypes after activation. n=3 for all experiments. Statistical significance was assessed by 2-way ANOVA test with Bonferroni correction (* p value < 0.05, ** p value <0.01, *** p value < 0.001, **** p value < 0.0001).

A.



B.



Supplementary Figure 2.
A. Log2Fold change of the mRNA expression of GBP-1 in a database of skin diseases showing lesional area vs healthy skin area. Each dot represents a patient. **B.** Fold change of IRF5 expression in GM-CSF, IFNγ and M-CSF derived macrophages stimulated for 24 hours n=2. Error bars represent the SD of the values. The statistical analysis used was 2-way ANOVA with Bonferroni correction. (* *p* value < 0.05, ** *p* value <0.01).

The supplementary tables (data sets) can be found in the online version of the article or send upon request.

Chapter 4

Pharmacological validation of targets regulating CD14 during macrophage differentiation

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Abstract

The signalling receptor for LPS, CD14, is a key marker of, and facilitator for, pro-inflammatory macrophage function. Pro-inflammatory macrophage differentiation remains a process facilitating a broad array of disease pathologies, and has recently emerged as a potential target against cytokine storm in COVID19. Here, we perform a whole-genome CRISPR screen to identify essential nodes regulating CD14 expression in myeloid cells, using the differentiation of THP-1 cells as a starting point. This strategy uncovers many known pathways required for CD14 expression and regulating macrophage differentiation while additionally providing a list of novel targets either promoting or limiting this process. To speed translation of these results, we have then taken the approach of independently validating hits from the screen using well-curated small molecules. In this manner, we identify pharmacologically tractable hits that can either increase CD14 expression on non-differentiated monocytes or prevent CD14 upregulation during macrophage differentiation. An inhibitor for one of these targets, MAP2K3, translates through to studies on primary human monocytes, where it prevents upregulation of CD14 following M-CSF induced differentiation, and pro-inflammatory cytokine production in response to LPS. Therefore, this screening cascade has rapidly identified pharmacologically tractable nodes regulating a critical disease-relevant process.

Introduction

Macrophages are key players in tissue homeostasis and inflammation but can also contribute to a diverse range of human diseases, including inflammatory, metabolic, and cardiovascular diseases (1, 2). Circulating monocytes can infiltrate inflamed tissues where they differentiate into monocyte-derived macrophages (MDM). Cluster of differentiation 14 (CD14) was described as a monocyte/macrophage differentiation antigen on the surface of myeloid lineages, such as monocytes, macrophages and dendritic cells (DCs) (3). In humans, circulating monocytes generated in the bone marrow have been separated into different subtypes. The first, defined as "classical monocytes" (approximately 85%) express CD14 but are negative for CD16 ($CD14^{+ve}CD16^{-ve}$). The second subset are termed "non-classical monocytes", represent 5-10% of total monocytes, which are defined as $CD14^{low}CD16^{+ve}$ (4). The third subset termed "intermediate" ($CD14^{+ve}$ $CD16^{+ve}$), is currently under debate regarding whether these cells are different, or just in transition between the classical and non-classical subtypes (5, 6). "Classical" monocytes tend to be recruited into tissues first and at higher levels under inflammatory conditions, whilst "non-classical" have a patrolling function (7-10). However the precise role of different monocyte subtypes is still far from clear (11).

CD14 plays a crucial role in the phagocytic clearance of apoptotic cells and in the reactivation and immune recognition of microbial cell wall components from Gram-positive and Gram-negative bacteria (12). Furthermore, CD14 is widely reported to associate with the toll-like receptor 4 (TLR4) by binding to LPS and eliciting a cascade of inflammatory signalling (13) and TLR4 endocytosis (14). CD14 exists in two forms, a 52-55 kDa protein, mCD14, attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor, and the serum soluble 48–56 kDa sCD14, an acute-phase protein (15). In this way, sCD14 can potentiate LPS transfer to trigger TLR4 on cells that do not express mCD14 (16-18). The physiological relevance of CD14 was confirmed in knockout mice which do not respond to low dose LPS in the production of TNF, IL-1β and IP-10 (19), however ingestion of sCD14 restores their capacity to mount this inflammatory response (20).

In pathogenesis where monocytes infiltrate peripheral tissues and differentiate into macrophages, CD14 expression has been markedly upregulated and may contribute to, or aid resolution of disease, depending on context (21). Therefore, inhibitors that prevent upregulation of CD14 may find utility in a variety of inflammatory diseases, but increasing CD14 expression could also potentiate anti-tumor or vaccine responses. Most recently, Martin and colleagues have proposed CD14 as a target to treat cytokine storm in COVID19 (22). The foundation for this is the observation that the plasma concentration of soluble CD14 (sCD14) is increased in severely affected patients (23). Moreover, patients with Acute Respiratory Distress Syndrome (ARDS) have elevated levels of sCD14 in bronchoalveolar lavage (BAL) fluid (24), and there was a small clinical trial (7 treated, 6 controls) of a neutralizing antibody against CD14 which demonstrated

a trend towards reduced neutrophils and cytokine concentrations in BAL fluid (Implicit Bioscience Ltd., data on file for IND12209).

In this study, we wanted to identify regulators of macrophage differentiation and CD14 in human macrophages. To accomplish that, we used Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) with pooled sgRNA screening technology, which allows simultaneous knockout of thousands of individual genes. Here, a whole-genome CRISPR/ Cas9 screen was performed in human THP-1 cells to understand which genes regulate the differentiation of inflammatory macrophages, based on changes in CD14 expression. We identified genes that downregulated CD14 after differentiation of THP-1 cells with PMA and others that promoted differentiation by increasing CD14 expression in cells not treated with PMA. Given the pressing need for drugs that might reduce CD14 to treat cytokine storm in COVID19 patients, we wanted to translate the screen hits as quickly as possible. To this end, we performed validation using existing pharmacological inhibitors, revealing a molecule targeting MAP2K3 which can prevent CD14 upregulation on primary human M-CSF derived macrophages.

Methods

Culture, differentiation and treatment of THP-1 cells

THP-1 cells (ATCC® TIB202™) were cultured using RPMI-1640 (Life Technologies) supplemented with 2 mM L-glutamine, 10% FCS and Penstrep (100 U/ml) at 37 °C, 5% CO_2. Cells were grown to a density of 5×10^5– 1×10^6 cells/ml and used for experiments between passage 5 and 12. For THP-1 compound treatment and differentiation, cells were seeded in plates and incubated with the corresponding GSK compounds **(Supplementary table 1)** or 0.1% DMSO in the controls for 30 minutes. Cells were then left untreated or treated with Phorbol-12-myristate 13-acetate (PMA) (100 ng/ml) (Sigma-Aldrich) for 48 h at 37 °C, 5% CO_2 to allow differentiation. After 48 hours, cells were analysed by flow cytometry.

Primary monocyte differentiation and treatment

Human PBMCs were isolated from whole blood from healthy donors by gradient centrifugation. Monocytes were purified from PBMCs using CD14+ beads (Miltenyi Biotech) according to supplier's protocol. Purified monocytes treated with growth factor M-CSF (100 ng/ml) (R&D Systems) were cultured in RPMI-1640 (Life Technologies) with 2mM L-glutamine, 5% FCS and PenStrep (100 U/ml). Blood monocytes were treated with the corresponding GSK compounds **(Supplementary Table 1)** or 0.1% DMSO for 5 days at 37 °C, 5% CO2 to allow differentiation. On day 5, cells were analysed by flow cytometry or stimulated with LPS (100 ng/ml; Sigma L4391) for 24 h, from which supernatants were collected for cytokine analysis. All human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol.

Cytokine assays

Supernatants were collected and IL-1β, IL-6 and TNF were quantified by Human Pro-Inflammatory 7-Plex Tissue Culture Kit (Mesoscale) in accordance with the supplier's protocol.

Concentration-response study

THP-1 cells were differentiated in the presence of PMA (100 ng/ml) for 48 h in combination with increasing concentrations of the different compounds tested (10 μM, 3.3 μM, 1.1 μM, 370 nM, 123 nM, 41.1 nM, 13.7 nM, 4.5 nM, 0 nM). After 48 h the viability was determined based on the ATP levels of the cells using CellTiter-Glo kit following manufacturer's instructions. Viability is represented in percentage after normalization of the ATP values to the PMA condition without compound. MMP-9 production was measured in the supernatants using MSD MMP-9 kit (Mesoscale) according to supplier's instructions. Data is represented in percentage of response in a non-linear curve for concentration-response data.

Flow cytometry

MDMs and THP-1 cells were detached using a Cell Dissociation buffer (Sigma-Aldrich), followed by 2 washes in PBS and stained with Live/dead stain (Annexin V) (BD Biosciences, #565388) for 15 min at room temperature. Cells were washed twice with FACS buffer (BioLegend) and incubated with an Fc receptor blocking agent (Human TruStain FcX, BioLegend #422302) for 10 min at room temperature, prior to incubation with CD14 antibody (Biolegend, #325604) for 30 min at room temperature. Flow cytometric analysis was performed on a BD FACSCanto II flow cytometer.

Genome wide CRISPR Cas9 screening in THP-1 cells

CRISPR screens were conducted at Horizon Discovery (Cambridge, UK).

Library generation

A whole genome library was developed that exploited informatically optimised guides (25) expressed in tandem with a modified tracrRNA sequence (5′-GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTT-3′) (26). An all-in-one lentivirus plasmid vector was built comprising a selection marker (puromycin resistance), the expression cassette for Cas9 and the sgRNA sequence and cloned by Gibson Assembly (New England BioLabs, NEB #E2611S/L) in accordance with the manufacturer's instructions. Library plasmids were purified using a Qiagen Plasmid Plus purification system in accordance with the manufacturer's instructions.

Lentivirus production

HEK293T cells (ATCC, USA) were grown in DMEM and 10% FBS (Gibco, UK) and transfected with the library plasmids using Lipofectamine 3000 (Invitrogen, USA) and Virapower packaging virus (LifeTechnologies, UK) in accordance with the manufacturer's instructions. After 48 h the medium was removed and centrifuged at 500xg for 10 min at 4°C. The virus was further concentrated using Lenti-X concentrator (Clontech #631232) in accordance with the manufacturer's instructions. The viral supernatant was aliquoted and stored at -80 ºC in DMEM with 10% FBS and 1% BSA.

Cell transduction, staining and screening protocol

Cells were trypsinized, seeded in complete medium supplemented with 8 μg/ml polybrene (Sigma-Aldrich) and seeded into 12 well dishes at $2x10^6$ cells per well and spinfected for 2 hours at 2000 rpm at 37 ºC using virus diluted to achieve a MOI of 0.3. At least $1x10^8$ of THP-1 cells were transduced, resuspended, transferred to a 50 ml falcon and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed, and cells were resuspended in 50 ml fresh media (without polybrene) and after 48 h cells were treated with puromycin at a final concentration of 1 μg/ml. PMA treatment occurred 18 days after transduction (11 days of puromycin selection followed by 7 days of expansion).

Following the completion of antibiotic selection, cells were separated into replicates and treatment groups (DMSO-treated THP-1 monocytes, PMA-treated adherent and suspension macrophages), of at least $3.6x10^7$ cell per condition and grown in continuous culture to enact editing. For staining, cells were diluted to $2x10^6$ cells/ml in FACS buffer (PBS, 2% FBS, 2 mM EDTA). Cells were incubated with an Fc receptor blocking agent (Human TruStain FcX, BioLegend 422302, 1:100) for 10 min at room temperature prior to incubation with primary CD14 antibody (CD14-AF488 HCD14 IgG1, BioLegend 325610, 5 µl/$2x10^6$ cells) for 45 min at 4 °C, followed by 2 washes in PBS. Non-viable cells were stained with a fixable viability dye ZombieNIR (BioLegend 423105, 1:500) for 30 min at room temperature, followed by 2 washes in FACS buffer. Finally, cells were fixed with 4% PFA (BioLegend 420801) for 20 min at room temperature, followed by 2 washes in FACS buffer. All 3 treatment groups were analysed by flow cytometry. DMSO-treated monocytes (stained with isotype control and CD14 antibody) were used to determine the $CD14^{-ve}$ gate. Subsequently, the $CD14^{+ve}$ gate was drawn next to the negative gate, and all screen samples sorted based on CD14 negative and positive gates, so CD14 positive cells were separated from CD14 negative cells. Cell pellets were collected and stored at -80 °C. All samples were then thawed and gDNA extracted using Qiagen Blood Maxi kit. DNA concentration was determined using a Nanodrop spectrophotometer and at least 230µg of genomic DNA for each sample was then amplified with PCR to generate amplicons of the sgRNA cassette using a forward primer: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGU–

[Variable]–TGTGGAAAGGACGAAACACC; and a reverse primer: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATCAATTGCCGACCCCTCC.

These amplicon samples were purified using Agencourt beads (Beckman) and deep sequenced on an Illumina NextSeq platform/system (Microsynth AG, Switzerland).

Data analysis

Statistical analysis

Data represent the mean ± standard error of the mean (SEM). Differences between groups are analysed using an unpaired student's *t*-test using Prism 7 (GraphPad software, La Jolla, California). Differences were considered significant when the *p* value was ≤0.05 (*), 0.01 (**), 0.001 (***), 0.0001 (****).

Flow cytometry

FSC files were exported and analysed in FlowJo software version 10.4.2. For the gating strategy doublets were removed and then live cells were selected. From live cells, $CD14^{+ve}$ cells were gated based on CD14 FMO and the percentage and MFI of CD14 positive cells was measured and exported.

CRISPR screen

Conducted at Horizon Discovery (Cambridge, UK). Raw NGS libraries were evaluated for quality using FASTQC version 0.11.5. (Babraham Institute, Cambridge UK). Guide counts were obtained using an in-house customized version of the MAGeCK workflow version 0.5.56, which took into account guide staggering from the experimental protocol. Briefly, guides were trimmed and mapped with exact string counts from each file to provide raw counts for each guide found in the library. Guide counts were normalised within each group (median-based) and Log2 fold change (LogFC) was calculated to determine the change in abundance of each guide in each sample. RRA values (p-values) were determined using the MAGeCK algorithm (version 0.5.56), as described in Li et al (27). Specific comparison data was extracted and used for volcano plot generation in TIBCO Spotfire v7.11.1.

Stemformatics expression analysis

Median rank values (0= no expression, 1 = highest expression) for each gene were assessed for primary cells collated in the Stemformatics myeloid atlas (28); Hematopoietic Stem and Progenitor Cells (HSPC, n=67), Common Myeloid Progenitors (CMP, n=8), peripheral blood monocytes (n=171) and monocyte-derived macrophages (MDM, n=107). Some genes lack an entry in the atlas compilation.

GO enrichment

A Log2-fold change threshold of 0.58 and FDR p value threshold of 0.05 was applied. The data was imported into MetaCore™ version 19.3 build 69800. Gene ontology (GO processes) enrichment was evaluated. The data for the top ten GO processes by Negative Log p value was exported for the figure. The GO processes table was generated from the genes listed in Supplementary Table 3 and 4.

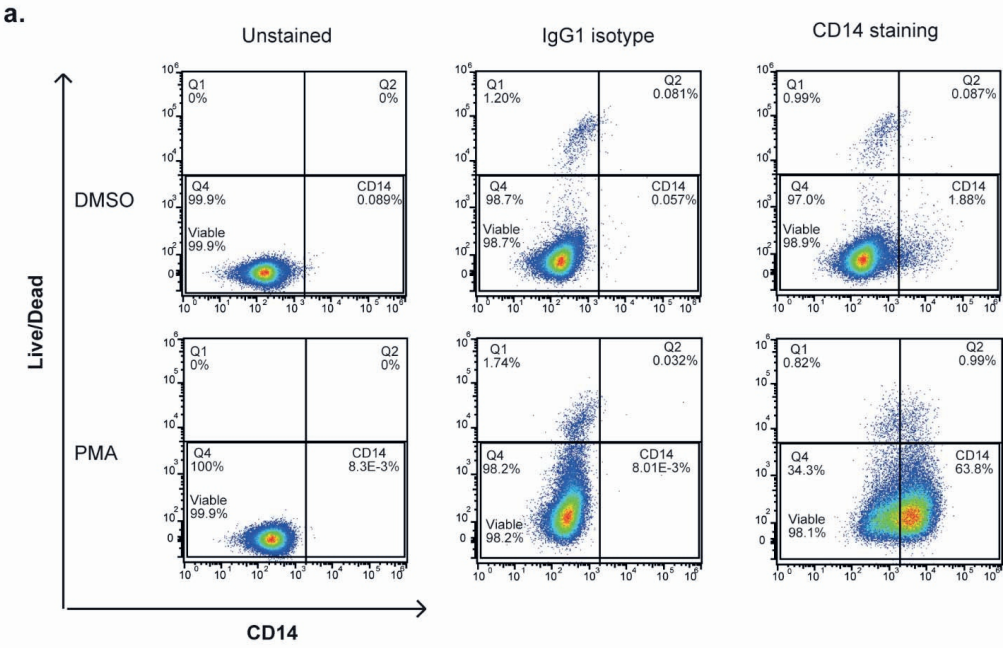
Results

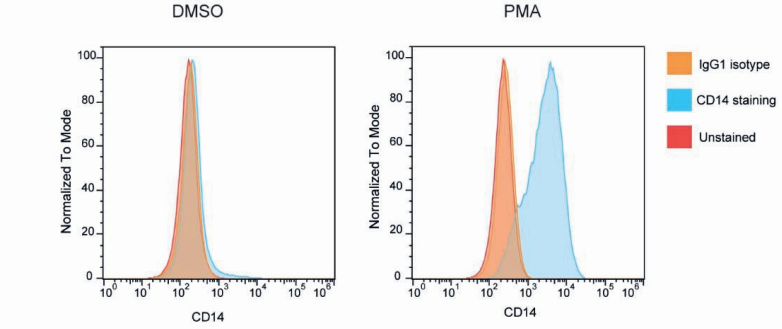
CD14 as a marker of activation during macrophage differentiation

THP-1 monocytic cells can be differentiated into macrophages using PMA (29). We tested a number of cell surface markers of activation and found that CD14 is one that was robustly increased after 48 h of treatment with 100 ng/ml of PMA **(Figure 1a)**. After treatment with PMA both the percentage of $CD14^{+ve}$ cells increased, as well as the mean fluorescence intensity (MFI) of CD14 expression per cell, as compared to the DMSO and isotype controls **(Figure 1a and 1b)**.

Based on these data and the fact that CD14 has been related to a pro-inflammatory phenotype in macrophages, we decided to use it as a readout during a CRISPR/Cas9 whole-genome screen to understand which genes are associated with the differentiation of inflammatory macrophages.

For screening, a library of sgRNAs targeting the whole genome with 6-fold redundancy were constructed in a plasmid containing a puromycin resistance cassette inserted into a lentivirus backbone (see materials and methods). THP-1 cells were transduced with the virus, expanded and selected in puromycin for 18 days **(Figure 1c)**. At this point, the cells were either differentiated into macrophages, by adding PMA for 48 h or kept as monocytes (DMSO vehicle control) for 48 h. After this differentiation step, the cells in each group were sorted by FACS based on CD14 expression and next-generation sequencing performed to identify the sgRNAs present in the individual populations.


a.
Unstained
IgG1 isotype
CD14 staining
DMSO
PMA
Live/Dead
CD14
Q1 0%
Q2 0%
Q4 99.9%
CD14 0.089%
Viable 99.9%
Q1 1.20%
Q2 0.081%
Q4 98.7%
CD14 0.057%
Viable 98.7%
Q1 0.99%
Q2 0.087%
Q4 97.0%
CD14 1.88%
Viable 98.9%
Q1 0%
Q2 0%
Q4 100%
CD14 8.3E-3%
Viable 99.9%
Q1 1.74%
Q2 0.032%
Q4 98.2%
CD14 8.01E-3%
Viable 98.2%
Q1 0.82%
Q2 0.99%
Q4 34.3%
CD14 63.8%
Viable 98.1%


b.
DMSO
PMA
Normalized To Mode
CD14
IgG1 isotype
CD14 staining
Unstained

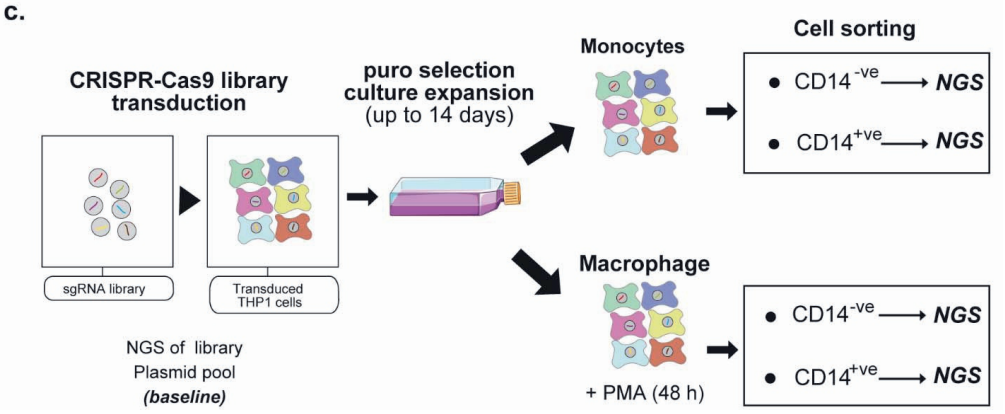

c.
CRISPR-Cas9 library transduction
puro selection culture expansion (up to 14 days)
Monocytes
Cell sorting
CD14 -ve NGS
CD14 +ve NGS
sgRNA library
Transduced THP1 cells
Macrophage
NGS of library Plasmid pool (baseline)
+ PMA (48 h)

Figure 1: THP-1 cells increase CD14 expression during macrophage differentiation. **a.** THP-1 cells increase the expression of CD14 after treatment for 48 h with the monocyte to macrophage differentiation factor PMA (100 ng/ml) in comparison to the DMSO control. **b.** Increased MFI values for CD14 expression after treatment with the differentiation factor PMA (100 ng/ml) for 48 h. **c.** Design of the CRISPR/Cas9 screen in THP-1 cells. A whole-genome library of guide RNAs was built in lentivirus plasmids with a selection marker (resistance to puromycin). THP-1 cells were transduced with lentivirus containing the plasmid, afterwards a culture selection in the presence of puromycin was performed. The remaining cells were treated for 48 h with 100 ng/ml of PMA to induce the differentiation into macrophages or DMSO (undifferentiated cells). Cell sorting was performed based on CD14 expression. The guide RNAs present in either the CD14+ve or CD14-ve cells from both groups were analyzed. n=3 biological replicates for each experiment

Genes regulating CD14 expression

After sorting the cells based on CD14 expression, the sgRNAs present in the different populations were analysed. In PMA differentiated THP-1 macrophages the sgRNAs present in $CD14^{-ve}$ vs $CD14^{+ve}$ were analysed and represented in a volcano plot **(Figure 2a)**. A positive fold change in the volcano plots indicates that more sgRNA targeting a specific gene is present in the $CD14^{-ve}$ population than in the $CD14^{+ve}$. This means that the silencing of those genes reduces CD14 expression in THP-1 PMA differentiated macrophages. A list of the 21 significantly upregulated sgRNAs (L2FC>0.58, FDR<0.05) is presented as **Supplementary Table 3.** Crucially, one of the top hits is, of course, CD14 itself. As expected, there are also a number of genes that regulate phosphatidylinositol glycan (PIG) anchor biosynthesis, which would be required for CD14 adherence to the cell surface. Gene ontology analysis confirmed this observation, with the primary biological process implicated in biosynthetic processes underlying GPI anchors **(Figure 2b)**. Publicly available expression data from the Stemformatics platform reveals that the majority of these hits are highly ranked (top 50%) within the myeloid lineage, and that for some, the rank is increased after differentiation **(Figure 2c)**.

The same analysis was performed for undifferentiated THP-1 monocytes **(Figure 2d).** In this case, we were interested in the sgRNAs with a negative fold change, which means they are enriched in $CD14^{+ve}$ cells, and thus these sgRNAs denote genes that when depleted result in spontaneous upregulation of CD14 expression. A list of the 36 significantly downregulated sgRNAs (L2FC<-0.58, FDR<0.05) is presented as **Supplementary Table 4.** Gene ontology analysis showed significant enrichment of processes related to negative regulation of transcription, which is consistent with the deletion of these genes upregulating CD14 expression **(Figure 2e)**. Stemformatics expression data demonstrates that some of these negative regulators are downregulated during myeloid differentiation, but others are upregulated **(Figure 2f)**. Similar to the positive regulators in **Supplementary Table 3**, the majority of targets we have identified in **Supplementary Table 4** are highly ranked (top 50%) within the myeloid lineage **(Figure 2f)**.

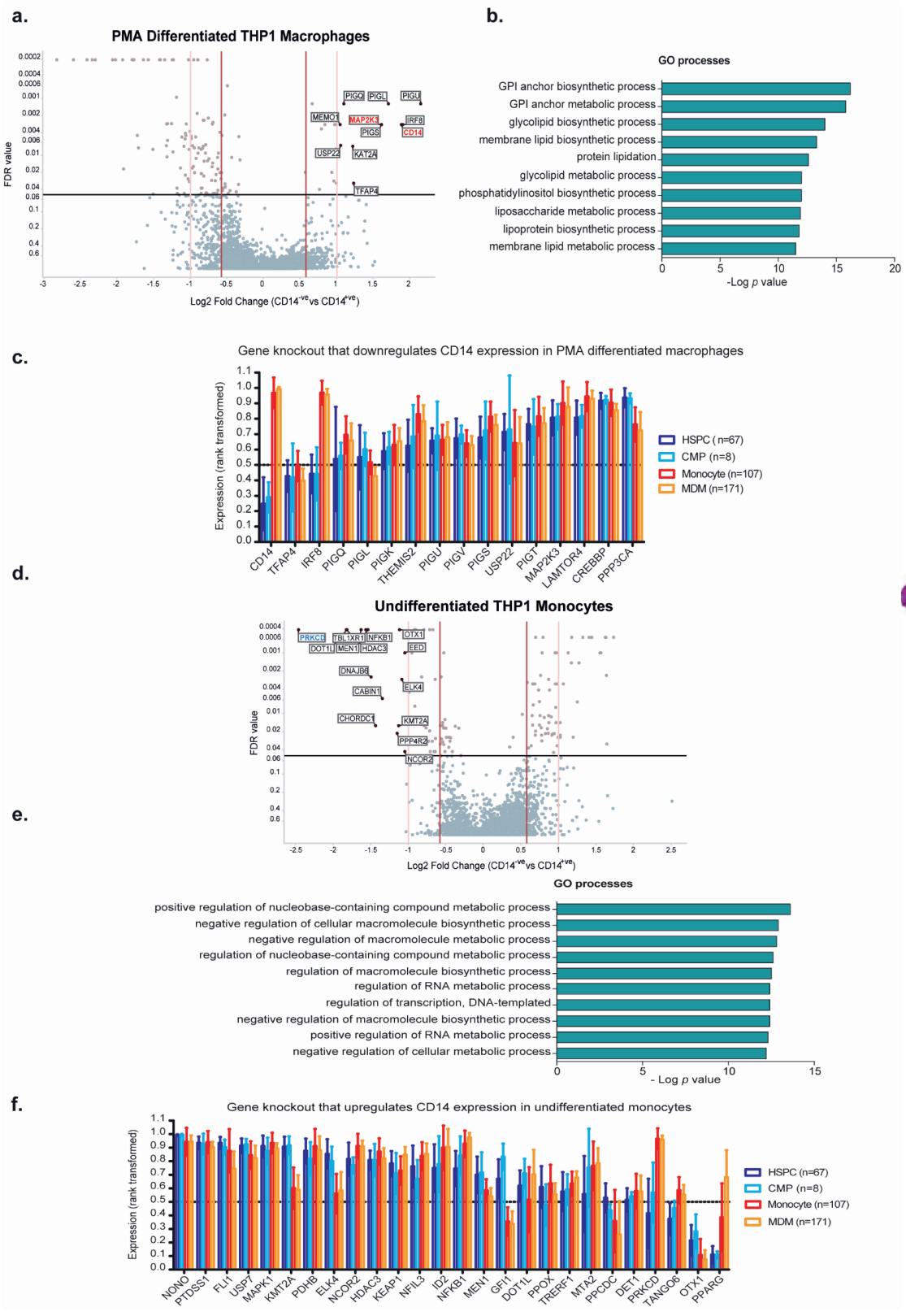

a.
PMA Differentiated THP1 Macrophages
FDR value
Log2 Fold Change (CD14-ve vs CD14+ve)
PIGQ
PIGL
PIGU
MEMO1
MAP2K3
IRF8
PIGS
CD14
USP22
KAT2A
TFAP4
b.
GO processes
GPI anchor biosynthetic process
GPI anchor metabolic process
glycolipid biosynthetic process
membrane lipid biosynthetic process
protein lipidation
glycolipid metabolic process
phosphatidylinositol biosynthetic process
liposaccharide metabolic process
lipoprotein biosynthetic process
membrane lipid metabolic process
-Log p value
c.
Gene knockout that downregulates CD14 expression in PMA differentiated macrophages
Expression (rank transformed)
CD14
TFAP4
IRF8
PIGQ
PIGL
PIGK
THEMIS2
PIGU
PIGV
PIGS
USP22
PIGT
MAP2K3
LAMTOR4
CREBBP
PPP3CA
HSPC (n=67)
CMP (n=8)
Monocyte (n=107)
MDM (n=171)
d.
Undifferentiated THP1 Monocytes
FDR value
PRKCB
BL1XR1
NFKB1
OTX1
DOT1L
MEN1
HDAC3
EED
DNAJB6
ELK4
CABIN1
CHORDC1
KMT2A
PPP4R2
NCOR2
Log2 Fold Change (CD14-ve vs CD14+ve)
e.
GO processes
positive regulation of nucleobase-containing compound metabolic process
negative regulation of cellular macromolecule biosynthetic process
negative regulation of macromolecule metabolic process
regulation of nucleobase-containing compound metabolic process
regulation of macromolecule biosynthetic process
regulation of RNA metabolic process
regulation of transcription, DNA-templated
negative regulation of macromolecule biosynthetic process
positive regulation of RNA metabolic process
negative regulation of cellular metabolic process
- Log p value
f.
Gene knockout that upregulates CD14 expression in undifferentiated monocytes
Expression (rank transformed)
NONO
PTDSS1
FLI1
USP7
MAPK1
KMT2A
PDHB
ELK4
NCOR2
HDAC3
KEAP1
NFIL3
ID2
NFKB1
MEN1
GFI1
DOT1L
PPOX
TRERF1
MTA2
PPCDC
DET1
PRKCD
TANGO6
OTX1
PPARG
HSPC (n=67)
CMP (n=8)
Monocyte (n=107)
MDM (n=171)

Figure 2: Genes that modify CD14 expression in THP-1 cells. **a.** THP-1 monocytic cells transduced with a whole genome sgRNA library were differentiated into macrophages with 100 ng/ml of PMA for 48 h. Cells were sorted by flow cytometry based on CD14 expression. The volcano plots represent fold induction of sgRNAs of genes in the comparison of CD14-ve vs CD14+ve cells. A positive fold change means that the specific sgRNA is greater in CD14-ve cells which means that inhibition of that gene reduces the CD14 expression. Silencing of CD14 and MAP2K3 genes reduces the expression of CD14 in differentiated THP-1 cells. **b**. Top ten gene ontology processes by negative log p value in CD14-ve vs CD14+ve differentiated THP-1 monocytes, using positive Log2 fold change thresholds of 0.58 and a FDR p value threshold of 0.05. **c.** Stemformatics rank-transformed expression data for CRISPR screen hits within the myeloid lineage. Hematopoietic Stem and Progenitor Cells (HSPC, n=67), Common Myeloid Progenitors (CMP, n=8), peripheral blood monocytes (n=171) and monocyte-derived macrophages (MDM, n=107). Some genes lack an entry in the atlas compilation. Mean ± SEM **d.** Transduced cells were left untreated for 48 h. Cells were sorted by flow cytometry based on CD14 expression. The fold induction of sgRNAs of genes in CD14-ve vs CD14+ve cells is presented in the volcano plots. The silencing of PRKCD increases the expression of CD14 in THP-1 undifferentiated cells. n=3 biological replicates for the screen. **e.** Top ten gene ontology processes by negative log p value in CD14-ve vs CD14+ve undifferentiated THP-1 monocytes, using positive Log2 fold change thresholds of 0.58 and a FDR p value threshold of 0.05. **f.** Stemformatics rank-transformed expression data for CRISPR screen hits within the myeloid lineage. Hematopoietic Stem and Progenitor Cells (HSPC, n=67), Common Myeloid Progenitors (CMP, n=8), peripheral blood monocytes (n=171) and monocyte-derived macrophages (MDM, n=107). Some genes lack an entry in the atlas compilation. Mean ± SEM.

Screen validation by small molecule inhibition

As an alternative to conventional validation by repeating individual gene deletions using CRISPR, we searched available internal data and published resources for previously established on-target small molecule inhibitors of the screen hits. From this list we took the top 5 small molecules that target genes found in the screening of CD14 in either differentiated or undifferentiated THP-1 cells **(Supplementary Table 1)**. All of these have literature references to support their on-target effect, with the exception of iPRKCD for which the profiling data is provided here **(Supplementary Table 2)**.

For this validation assay, CD14 expression and sCD14 production were measured in THP-1 cells treated with the different inhibitors (at either 3 μM or 100 nM), in the absence **(Figure 3a)** or presence of PMA (100 ng/ml) for 48 h **(Figure 3b)**. The different concentrations used for the compounds were based on studies of viability and MMP-9 expression **(Supplementary Figure 1)**. MMP-9 is produced by THP-1 as a result of differentiation with PMA (30). We identified one inhibitor that increased CD14 expression in the PMA differentiated cells (iPRKCD) and showed the same trend in undifferentated cells, although did not reach statistical significance (p= 0.06). Similar results were found for sCD14 with this compound, and again the trend in increase was not statistically different. We also identified two compounds that had a significant effect of decreasing CD14 expression in PMA differentiated macrophages (iMAP2K3 and iEIF2AK3). For differentiated cells iMAP2K3 also reduced the production of sCD14, and a similar trend was observed in undifferentiated cells. iPRKCD did not have an overall impact on cell viability but did lead to a small increase in the MFI of CD14 on undifferentiated THP-1 cells **(Figure 3c)**. iMAP2K3 actually led to a small increase in cell viability, but did not influence the MFI of CD14 expression on PMA differentiated THP-1 cells **(Figure 3d)**.

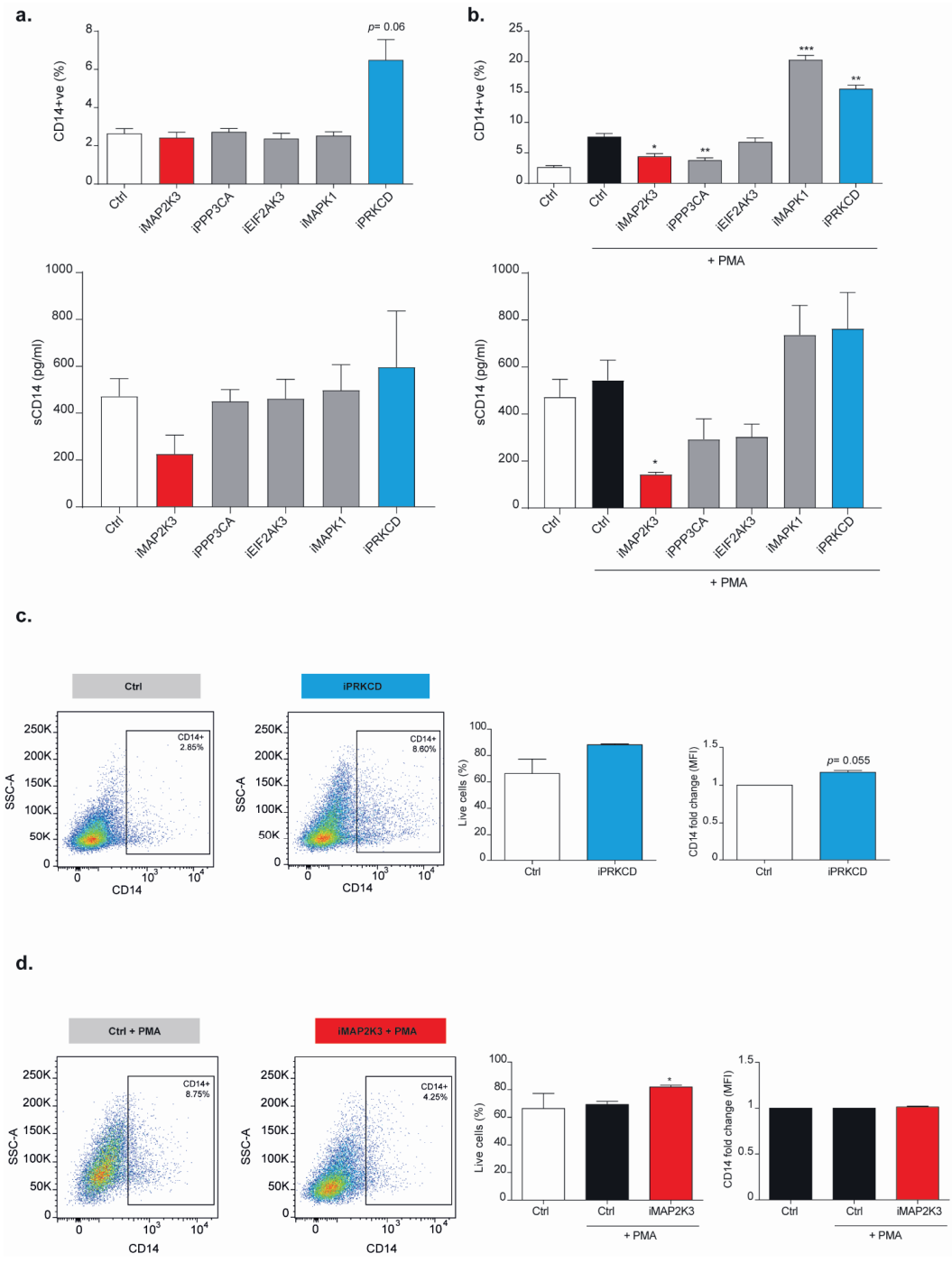


Figure 3: **Small molecules to inhibit targets identified in the screen validate the results based on CD14 expression. a.** THP-1 cells were left untreated for 48 h and were stimulated with either 3 μM or 100 nM of the different compounds. CD14+ve cells were quantified by FACS and levels of sCD14 in the supernatant measured by ELISA **b.** THP-1 cells were differentiated into macrophages with PMA for 48 h (100 ng/ml) in combination with either 3 μM or 100 nM of the different compounds. CD14+ve cells were quantified by FACS

and levels of sCD14 in the supernatant measured by ELISA. **c.** Representative FACS analysis of THP-1 cells left untreated for 48 h in the presence of a PRKCD inhibitor (100 nM) and stained for CD14. The percentage of live cells and MFI fold change of CD14 normalized to DMSO (0.1%) control was then quantified. **d.** Representative FACS analysis of THP-1 cells treated with PMA (100 ng/ml) for 48 h in combination with a MAP2K3 inhibitor (100 nM) and stained for CD14. The percentage of live cells and MFI fold change of CD14 normalized to DMSO (0.1%) control was then quantified. Statistical significance was determined by unpaired t-test ($p<0.05$). All error bars represent the SEM. n=3 biological replicates for each experiment.

Pharmacological modulation of CD14 in primary human macrophages

Although THP-1 are a human cell line, they are an immortalized monocytic leukaemia, and so we looked to translate our results to primary human macrophages, using *in vitro* differentiation of monocytes. Specifically, isolated human monocytes were cultured with M-CSF (100 ng/ml) and DMSO, 100 nM of iMAP2K3 or 100 nM of iPRKCD. After 5 days **(Figure 4a)** we observed differences in the control macrophages compared to the treated macrophages. In the case of iMAP2K3 the macrophages lost the M-CSF elongated morphology and seem undifferentiated. iPRKCD treated MDMs retained the same morphology as the control macrophages.

Flow cytometry revealed that neither of the compounds significantly affected macrophage viability and that there was only a small reduction in the percentage of cells expressing CD14 for MDMs treated with iMAP2K3, which was not statistically significant **(Figure 4b)**. We also quantified CD16 expression, as an alternative readout of macrophage differentiation, and this was very significantly decreased by treatment with iMAP2K3 **(Figure 4b)**. The MFI of CD14 and CD16 expression in the macrophages was significantly decreased by iMAP2K3 but not iPRKCD **(Figure 4c)**. We also measured sCD14 production by these cells. We found a tendency to increase after the treatment with iPRKCD and decrease after iMAP2K3, although it did not reach statistical significance **(Figure 4d)**.

Finally, we wanted to study the effects of identified inhibitors on CD14 inflammatory signalling for the differentiated MDMs, when stimulated with LPS (100 ng/ml) for 24 h. As a readout, we measured the levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF). In agreement with the decreased expression of CD14 due to iMAP2K3, we observed a reduction in the production for all the cytokines tested in response to LPS (**Figure 4e**). Although there was slightly increased cytokine production in iPRKCD differentiated cells treated with LPS, it did not reach statistical significance, which was also expected given that the expression of CD14 was not upregulated with this inhibitor.

Therefore, simply triaging known inhibitors of targets identified in a CRISPR screen of THP-1 differentiation has yielded at least one confirmed hit (MAP2K3) that translates through to effects on human primary pro-inflammatory macrophage function.

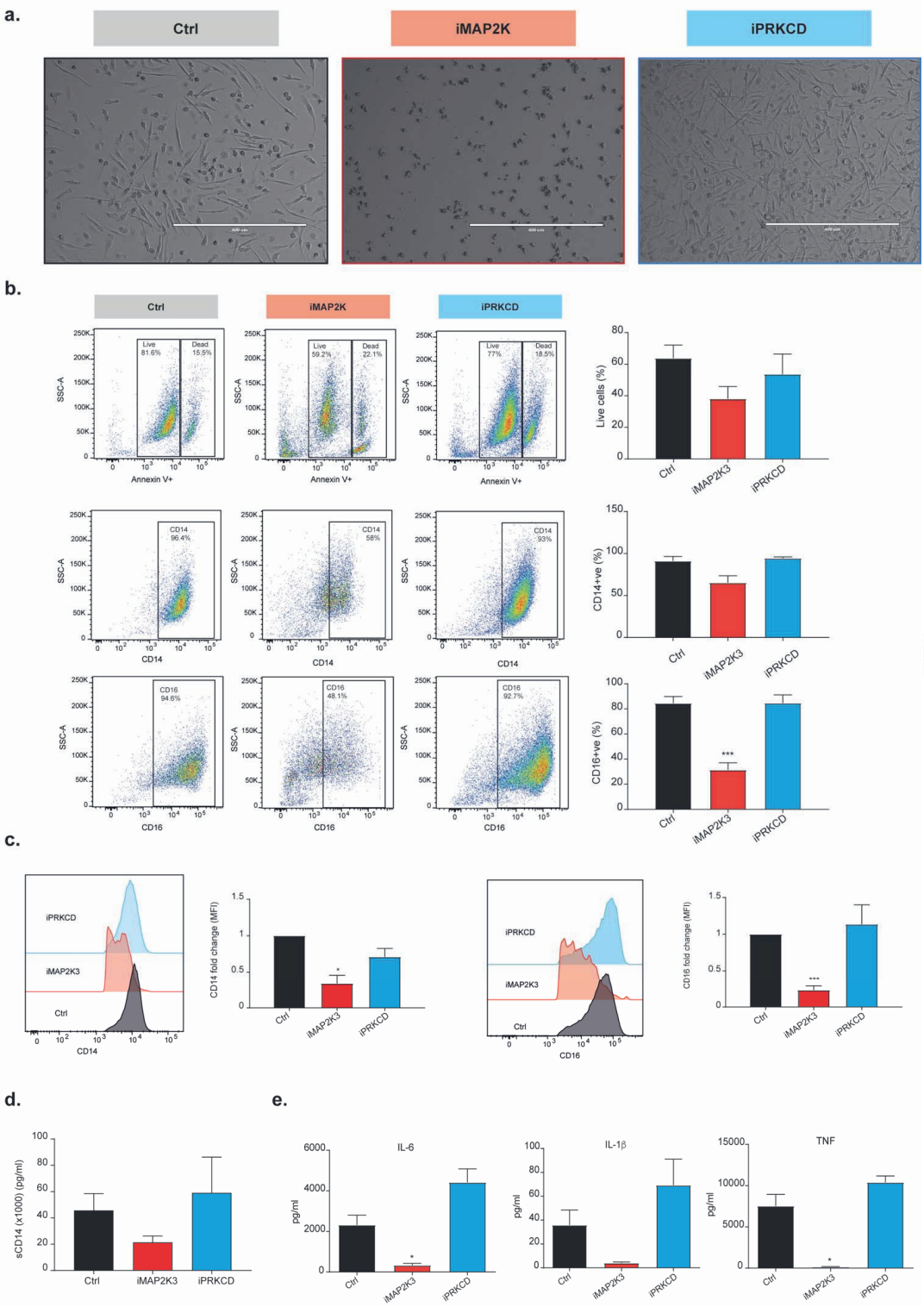

a.
Ctrl
iMAP2K
iPRKCD
b.
Ctrl
iMAP2K
iPRKCD
Live 81.6%
Dead 15.5%
Live 59.2%
Dead 22.1%
Live 77%
Dead 18.5%
SSC-A
Annexin V+
Live cells (%)
CD14 96.4%
CD14 58%
CD14 93%
CD14
CD14+ve (%)
CD16 94.6%
CD16 48.1%
CD16 92.7%
CD16
CD16+ve (%)
Ctrl
iMAP2K3
iPRKCD
c.
iPRKCD
iMAP2K3
Ctrl
CD14
CD14 fold change (MFI)
CD16
CD16 fold change (MFI)
d.
sCD14 (x1000) (pg/ml)
e.
IL-6
IL-1β
TNF
pg/ml

Figure 4: Results from the THP-1 screen partially translate into human primary MCSF macrophages. Effect of the compounds on cytokine production. a. Morphology of human monocytes differentiated with M-CSF (100 ng/ml) into macrophages in the presence of an inhibitor for MAP2K3 (100 nM), PRKCD (100 nM) or DMSO control (0.1%). **b.** Percentage of live, CD14+ve and CD16+ve cells in the cells differentiated in the presence of the different compounds. **c.** MFI peaks and fold change values for CD14 and CD16 after treatment with both compounds and DMSO control. **d.** sCD14 production of MDM differentiated in the presence of MAP2K3 (100 nM), PRKCD (100 nM) or DMSO control (0.1%) was measured by ELISA **e.** Cells were differentiated in the presence of the two compounds, after differentiation cells were stimulated with LPS (100 ng/ml) for 24 h and IL-1β, IL-6, TNF levels were measured. Statistical significance was determined by unpaired t-test ($p<0.05$). All error bars represent the SEM. n=4 biological replicates for flowcytometry experiments and n=3 biological replicates for the stimulation and ELISA experiments.

Discussion

In this work, we have used the human monocytic THP-1 cell line to model macrophage differentiation and CD14 upregulation after treatment with PMA. Immediately, it was possible to see that the screen was successful, with CD14 itself being one of the top hits **(Figure 2a)**. Moreover, many genes that were previously implicated in macrophage differentiation were also observed, providing further validation **(Supplementary Table 3 and 4)**. However, this is not an exhaustive list of all pathways regulating macrophage differentiation and CD14, and certainly, PMA treated THP-1 cells are at best a model system. Therefore, we proceeded to validate hits from the screen with small molecules because these can be validated in primary human monocytes differentiated with M-CSF. As many of the novel targets for CD14 expression and macrophage differentiation from our primary screen do not have well-curated small molecule inhibitors established, they remain unvalidated at this point, and therefore this provides a novel resource to the community for future research. For example, the targets identified in **Supplementary Table 4** could be useful in the context of cancer therapy, to terminally differentiate pro-myelocytic leukemic cell types like THP-1, which resulted from an MLL-AF9 translocation (31). Indeed, not only did we identify the gene encoding MLL (*KMT2A*) but also its upstream activator *Id2* (32), and downstream targets *DOT1L* and *MEN1* (33). Additionally, we suggest that a number of the novel targets identified could facilitate improved responses to infection or be utilised as adjuvants to vaccines.

From the list of selected inhibitors that we attempted to validate for the THP-1 differentiation process, only a few provided results that were in line with the initial genetic screen. This lack of translation could be due to a number of differences. In the genetic screening, many of the gene edits will remove the protein function entirely, however, with a small molecule inhibitor, some residual activity is likely. There could also be off-target effects of the compound, or the pharmacokinetics of inhibition may have been unfavourable in the assay. Additionally, we do not quantify cell death in real time, so the effects of phagocytosis may influence the end-point measurement. Of course, the initial hit from the screen may have been a false positive, so the small molecule inhibitor may not always be at fault.

After the validation in THP-1 cells, we tested if the molecules would show effects on primary MDM and found that only one compound had consistent results: iMAP2K3. Mitogen-activated protein kinase 3 (MAP2K3, MKK3) is a kinase that activates p38 MAPK in response to LPS (34) and a variety of other cell stressors such as cigarette smoke (35), Caerulin (36), TGF-β (37) and TNF (38). Therefore, this pathway regulates inflammatory pathology in models of sepsis (39), lung diseases (40-42), myocardial infarction (43) and diabetic nephropathy (44). Based on our data, either monocyte/macrophage differentiation or regulation of CD14 could account for the effects of MAP2K3 in these conditions, however, it may also have other roles in different cell types. The iMAP2K3 may be particularly effective at preventing inflammatory cytokine production from activated primary macrophages because we found it has a role during macrophage differentiation and CD14 downregulation, additionally it is known to regulate signalling downstream of CD14 (45, 46). For these reasons, one could speculate that MAP2K3 inhibition may improve outcomes for patients suffering from COVID19, especially severe cases associated with inflammatory macrophages and sCD14 (47).

The other inhibitor that we progressed through to studies in primary MDMs was iPRKCD. PRKCD (Protein kinase delta) is a serine/threonine kinase that is activated downstream of diacylglycerol, and participates in a number of cell death/survival pathways (48). Based on the initial screens, iPRKCD should have had the effect to boost CD14 expression and downstream responses to LPS, however, despite a trend in that direction the differences were not statistically significant in differentiated primary MDMs. This was perhaps to be expected because PRKCD levels decrease significantly upon macrophage differentiation (49) and we observed the strongest effects when PRKCD was higher, in undifferentiated THP-1 monocytes **(Figure 3c)**. Overall, an effect of iPRKCD to promote macrophage differentiation and CD14 would be consistent with effects in mouse models of atherosclerosis where genetic deletion from macrophages decreased apoptosis, and increased macrophage number in aortic plaques (50). Whether beneficial effects of iPRKCD to fight infection or act as an adjuvant to vaccination could be harnessed without negative consequences is, therefore, a relevant issue.

In summary, we were able to identify genes that are important for the THP-1 macrophage differentiation process as a result of a CRISPR/Cas9 whole-genome screen. The screen identifies known pathways that validate the methodology, and novel hits that provide a new resource for the community. Based on these results, we were able to rapidly identify small molecules that would target select candidates and then translate our findings to primary human cells. In particular, iMAP2K3 showed the capacity to disrupt macrophage differentiation and CD14 dependent inflammation, and so represents a good candidate for testing in models of inflammatory disease, for example related to pathology due to COVID19.

Contributions

GJD, RLM, MP, CS, NB, CR and SB performed or assisted with experimentation. CAW provided bioinformatics support. GJD, RLM, MP, CS, EK, NB, CR, SB, RC, DA MPJdW, PKM and SLM were involved in experimental analysis and interpretation. All authors contributed to the writing and approved the final version of this manuscript.

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Authors who are GSK employees had a role in paper design, data collection, data analysis, interpretation and writing of the paper. Current affiliation for MP is Technology Transfer, LifeArc, Lynton House, 7-12 Tavistock Square, London, UK, WC1H 9LT, and for RC is Cinnabar Consulting Limited, Bedford, UK.

Declarations of interest:

GJD, EK, SB, CS, NB, CR, NT, DA and PKM are employees and shareholders at GSK. MP and RC contributed to this manuscript whilst employees at GSK. SLM consults for IFM Therapeutics and received funding from GSK. The rest of the authors do not have anything to disclose regarding funding or conflicts of interest with respect to this manuscript. MPJW reports grants from GSK outside the submitted work.

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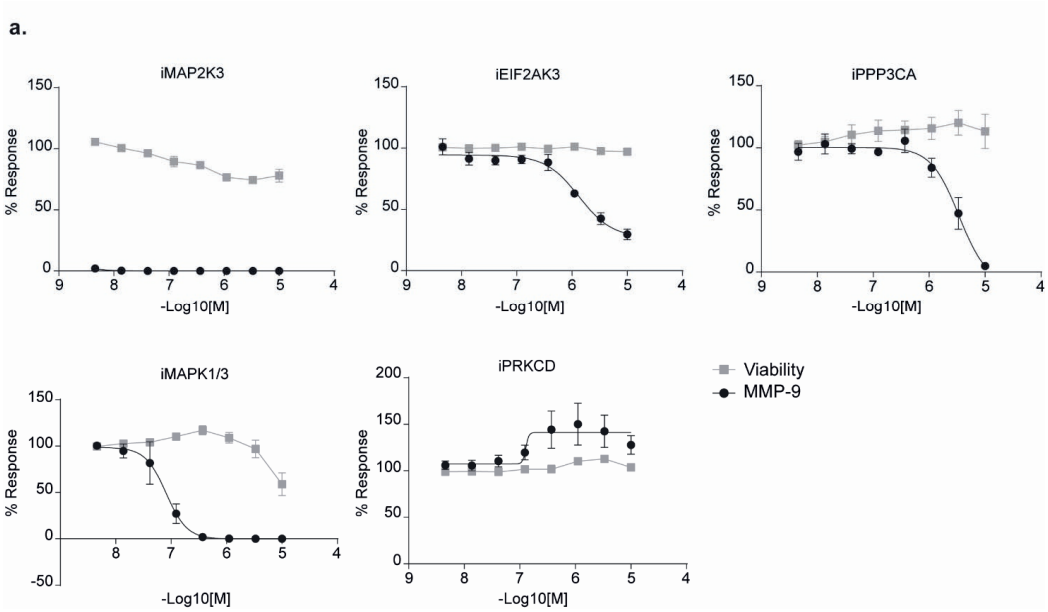

Supplementary Figure 1. **MMP-9 production and viability of THP-1 in a concentration-response study. a.** Percentage of cell viability and production of MMP-9 by THP-1 cells differentiated with PMA (100 ng/ml) in the presence of increasing concentrations of the different compounds (10 μM, 3.3 μM, 1.1 μM, 370 nM, 123 nM, 41.1 nM, 13.7 nM, 4.5 nM). Viability was determined by measuring ATP levels. Data for MMP-9 response represents the non-linear curve for the concentration-response data with SEM. n=3 biological replicates.

Supplementary Table 1: GSK compounds used for CRISPR screen validation in THP-1 and monocyte-derived macrophages.

Target	Registration Nº	Concentration	Compound name	References
PRKCD and ROCK1/ROCK2	GSK269962B	100 nM	GSK269962 hydrochloride	Supplementary table 2 and (Stavenger, Cui et al. 2007)
MAPK1/3	GSK3372091A	100 nM	GDC-0994	(Robarge, Schwarz et al. 2014)
PPP3CA (Calcineurin)	GW295507X	3 μM	PD 144795	(Gualberto, Marquez et al. 1998)
EIF2AK3	GSK2656304A	3 μM	GSK2656304	(Axten, Romeril et al. 2013)
MAP2K3	GSK3360825A	100 nM	TAK-733	(Dong, Dougan et al. 2011)

Supplementary Table 2: GSK269962B compound profiling of PKC isoforms. Geometric mean and standard deviation (SD) of pIC50 values obtained from a 10 point curve for each isoform (n=3), using the Reaction Biology Corp's Kinase HotSpot assay (Anastassiadis, Deacon et al. 2011).

GSK269962B		
Isoform	**Mean pIC50**	**SD pIC50**
PKCα	6.26	0.06
PKCβ1	7.42	0.03
PKCβ2	7.00	0.03
PKCδ	8.29	0.06
PKCε	7.56	0.06
PKCη	8.35	0.04
PKCγ	6.48	0.04
PKCι	6.76	0.17
PKCμ/PRKD1	5.15	0.13
PKCν/PRKD3	5.19	0.05
PKCθ	5.82	0.18
PKCζ	7.39	0.02

Supplementary table 3: pIC50 values obtained from MMP-9 assay

Target	Registration Nº	pIC50
PRKCD and ROCK1/ROCK2	GSK269962B	~ 6.893
MAPK1/3	GSK3372091A	7.091
PPP3CA (Calcineurin)	GW295507X	5.465
EIF2AK3	GSK2656304A	5.874
MAP2K3	GSK3360825A	~ 9.158

Supplementary Table 4: Gene knockout that downregulates CD14 expression in PMA differentiated macrophages.

Gene	Log2 fold change	FDR P value	Known regulation of macrophage differentiation or CD14	References
CERS6	0.66805	0.001238	No	
PIGQ	10.991	0.001238	No	
PIGL	17.101	0.001238	No	
PIGU	21.494	0.001238	No	

CREBBP	0.83885	0.002888	No	
PIGV	0.97403	0.002888	No	
PIGT	0.996	0.002888	No	
MEMO1	10.506	0.002888	No	
PIGS	16.117	0.002888	No	
MAP2K3	16.158	0.002888	No	
CD14	1.887	0.002888	Yes	(Ferrero and Goyert 1988)
IRF8	19.072	0.002888	Yes	(Günthner and Anders 2013)
PPP3CA	0.79489	0.003427	No	
USP22	10.569	0.006719	No	
KAT2A	12.222	0.006931	Yes	(Moris, Edri et al. 2018)
PIGK	0.99707	0.007735	No	
RUNX1	0.93419	0.020077	Yes	(Zaidi, Dowdy et al. 2009)
LAMTOR4	0.96305	0.0284	No	
PIGF	0.99626	0.031353	No	
TFAP4	12.328	0.031353	No	
THEMIS2	0.76571	0.040279	Yes	(Peirce, Brook et al. 2010)

Supplementary Table 5: Gene knockout that upregulates CD14 expression in undifferentiated monocytes.

Gene	Log2 fold	FDR P value	Known regulation of macrophage	References
PRKCD	-2.46	0.000413	Yes	(Su, Lin et al. 2015)
HDAC3	-18.244	0.000413	Yes	(Schulthess, Pandey et al. 2019)
DOT1L	-18.135	0.000413	Yes	(Daigle, Olhava et al. 2011)
MEN1	-16.276	0.000413	No	
NFKB1	-15.637	0.000413	Yes	(Chang, Su et al. 2013)
TBL1XR1	-15.368	0.000413	No	
OTX1	-11.163	0.000413	No	
DET1	-0.91435	0.000413	No	
RBPJ	-0.90019	0.000413	Yes	(Foldi, Shang et al. 2016)
MTA2	-0.78715	0.000413	Yes	(Lu, Chu et al. 2019)
TRERF1	-0.71158	0.000413	No	
PDHB	-0.67079	0.000413	No	
EED	-10.447	0.00099	Yes	(Graffmann, Brands et al. 2015)
MAPK1	-0.95067	0.00099	Yes	(Han, Lee et al. 1994)
DNAJB6	-14.909	0.002475	Yes	(Fagone, Di Rosa et al. 2012)
NFIL3	-0.81952	0.002475	Yes	(Baek, Haas et al. 2009)

ELK4	-10.846	0.002723	No	
PPARG	-0.64026	0.002723	Yes	(Bouhlel, Derudas et al. 2007)
GFI1	-0.98957	0.004479	Yes	(Zhao, Ye et al. 2014)
CABIN1	-13.418	0.005626	No	
INTS12	-0.90872	0.007533	No	
CHORDC1	-14.323	0.01581	No	
KMT2A	-11.245	0.01581	No	
PPOX	-0.78907	0.01581	No	
PPCDC	-0.76066	0.017252	No	
ID2	-0.71229	0.019401	Yes	(Ishiguro, Spirin et al. 1996)
PPP4R2	-11.469	0.021235	Yes	(Herzig, Bullinger et al. 2017)
HIRA	-0.96286	0.024994	No	
TANGO6	-0.63654	0.025673	No	
FLI1	-0.73031	0.028519	Yes	(Klemsz, Maki et al. 1993)
PTDSS1	-0.67634	0.028519	No	
NCOR2	-1.042	0.042296	Yes	(Sander, Schmidt et al. 2017)
KEAP1	-0.70388	0.042296	No	
NONO	-0.65458	0.042296	No	
ZFP36L2	-0.63881	0.042296	Yes	(Chen, Dong et al. 2015)
USP7	-0.7008	0.049257	No	

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Chapter 5

BET inhibition skews monocyte differentiation towards a homeostatic macrophage phenotype that resolves joint inflammation

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Abstract

Monocyte differentiation into macrophages is dictated by the microenvironment of the tissue and determines the function and phenotype of these resulting cells. Here we characterise the differentiation of human monocytes into Mϕ GM-CSF and Mϕ M-CSF and demonstrate that BET proteins play a critical role in differentiation into Mϕ GM-CSF only. Monocytes differentiated in the presence of both M-CSF and GM-CSF exhibit a Mϕ GM-CSF phenotype, however in the presence of I-BET151 monocytes skew towards the Mϕ M-CSF phenotype. This phenotypic switch is stable even days after removing the BET inhibitor as the resultant macrophages are no longer able to activate autologous T lymphocytes (T_H1 response). Transcriptomics and ChIPseq analyses in monocytes identified a small cluster of genes that are BET-sensitive and crucial for GM-CSF, but not M-CSF, induced monocyte differentiation. The translational relevance of the long-lived phenotypic switch in macrophages was evaluated in a rodent model of arthritis and found to be sufficient for efficacy with changes in synovial macrophages indicative of restoring a homeostatic phenotype. These data provide evidence that transient exposure to a BET inhibitor has the ability to provide efficacy that is long-lived and is at least partly due to restoring macrophage homeostasis, which offers exciting opportunities for use of BET inhibitors beyond oncology patients.

Introduction

Macrophages are innate immune cells that function to maintain tissue homeostasis through phagocytosis, tissue surveillance and secretion of growth factors [1-3]. These cells are heterogenic and their highly diverse and plastic nature is evident when studying tissue resident macrophages, as they exhibit distinct functions adapted to suit the environment. Macrophages can arise from progenitor cells or from infiltrating monocytes [4, 5], and this latter mechanism is thought to be most prominent during infections and inflammation. Monocytes leaving the circulation begin to differentiate into macrophages as they encounter growth factors and/or inflammatory mediators and it is this cytokine milieu which determines the macrophage phenotype.

Human macrophage literature is primarily focussed on *in vitro* differentiation of human monocytes into macrophages (MDMs) using colony-stimulating factors (CSFs), with emerging data from induced pluripotent stem cell derived macrophages (iPSDMs) becoming more evident in recent years [6, 7]. Deep transcriptional characterisation of MDMs generated with either M-CSF or GM-CSF have shown the differences between these macrophage subtypes [8, 9] however the function and molecular mechanism leading to these phenotypes is less well characterised [10-12].

Epigenetic enzymes and modulators have emerged as critical points of control in cell activation and polarisation states [13-17]. Approximately 40 proteins contain bromodomains which bind to acetylated lysines in histone and non-histone proteins and these bromodomain-containing proteins collectively form a class of epigenetic readers. Within this class, the bromodomain and extra-terminal (BET) proteins, consisting of BRD2, BRD3, BRD4 and BRDT, are the most widely studied [18-20]. I-BET151 is one of multiple small molecule inhibitors of the BET proteins have been developed [15, 21-23]. I-BET151 has shown anti-inflammatory and immunomodulatory activity across various models and cellular systems [24-27]. The role of BET inhibitors on monocyte differentiation into MDMs has not yet been explored.

We investigated the effect of BET inhibition on human monocyte differentiation into macrophage subtypes. Here, we characterise two commonly used macrophage subtypes and use our newly identified subtype-specific biomarkers to demonstrate the selectivity of our inhibitors for only disrupting the pro-inflammatory MΦ GM-CSF phenotype. We identify the molecular mechanisms involved in the selective impairment of pro-inflammatory macrophages and the functional switch in macrophage-phenotype resulting in deficiency in autologous T-lymphocyte activation. Finally, we show that abrogation of monocyte differentiation into pro-inflammatory macrophages translates *in vivo* in a model of arthritis where a short duration of I-BET151 dosing induces long-lived efficacy and disease reduction.

Materials and Methods

Cell culture and stimulations

All donors provided written informed consent for use of their samples, and the collection and use of the samples received Institutional Review Board approval. Blood from healthy volunteer donors was collected into tubes containing sodium heparin anti-coagulant in order to isolate the monocytes and then culture them into macrophages. Blood was mixed 1:1 with PBS and then layer on top of 15 ml of Ficoll (GE Healthcare). The tube was spin at 1500 rpm for 20 minutes

without break and acceleration at room temperature (RT), after this spin, all following spins were done at 1500 rpm for 5 minutes at RT. After that the layer of PBMCs is collected. PBMCs were wash twice with PBS, after the second spin cells were resuspended in 1 mL of MACs buffer (2mM EDTA, 0.5% BSA in PBS) per 50 mL of the initial volume of whole blood. 1 µL of CD14 positive beads (Miltenyi Biotec) were added per 50 mL of the initial volume of whole blood and then cells were incubated for 15 minutes at 4°C. After this incubation, the cells were washed 2 times with MACs buffer. Afterwards LS Miltenyi columns were place in a magnet and CD14+ monocytes were isolated by passing 1 mL of PBMCs in the columns, washing them 3 times with MACS buffer and then collecting the elute from the columns by removing the columns from the magnet and adding 5 mL of MACS buffer. Cell were washed with MACs buffer and resuspended at $1x10^6$ cell/mL in RPMI-1640 (Gibco) with 5% FCS, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 mg/mL). Monocytes were culture for 5 days in an incubator at 37°C and 5% CO_2 with either, M-CSF (50 ng/mL) or GM-CSF (5 ng/mL). Samples for Western Blot and qPCR were collected at different time points during the differentiation (0 h, 2 h, 24 h, 48 h, 72 h, 96 h, 120 h). Unless specified in a particular experiment I-BET was used at 100 nM in all experiments.

T cell co-cultures

The co-cultured CD4+ T cells and macrophages were isolated from the same donor. Monocyte isolation and macrophage culture were performed as described above. T cells were isolated from the CD14 negative flow-throw using the CD4+ isolation kit (Miltenyi Biotec 130-096-533) following manufacturer's instructions. T cells were kept in culture for six days in RPMI-1640 (without Hepes and L-glutamine) (Gibco) with 10% FCS, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 mg/mL), supplemented with 2 ng/mL of IL-7 (R&D systems 207-IL/CF). Macrophages were cultured for five days as described above. Once the cells had differentiated, they were stimulated for 24 hours with LPS (100 ng/mL) in fresh media. After 24 hours the supernatant was collected (100 µL) and replaced by new media. Supernatant was transferred to an empty 96 well plate. T cells were centrifuged and resuspended at $1x10^6$ cell/mL in media without IL-7. Cells were added to the wells of the plates containing either the macrophages with fresh media (1:1 ratio) or the supernatant from the stimulation. In another plate, the supernatant was not removed following the 24 hour LPS stimulation and T cells (1:1 ratio) were added to the macrophages together with the supernatant. After 3 days of co-culture, supernatant was collected, and cytokines were measured.

Flow cytometry

For CD14 and CD16 surface expression, after differentiation with M-CSF or GM-CSF cells were stimulated for 24 h with I-BET151 (100 nM) or left unstimulated. After stimulation cells were detached using a Cell Dissociation buffer (Sigma-Aldrich), followed by 2 washes in PBS and stained with Live/dead stain (Annexin V) (BD Biosciences, #565388) for 15 min at room temperature. Cells were washed twice with FACS buffer (BioLegend) and incubated with an Fc receptor blocking agent (Human TruStain FcX, BioLegend #422302) for 10 min at room temperature, prior to incubation with CD14 antibody (Biolegend, #325604) or CD16 (Biolegend #302038)

for 30 min at room temperature. Flow cytometric analysis was performed on a BD FACSCanto II flow cytometer.

FSC files were exported and analysed in FlowJo software version 10.4.2. For the gating strategy doublets were removed and then live cells were selected. From live cells, CD14+ and CD16+ cells were gated based on CD14 FMO and the percentage and MFI of positive cells was measured and exported.

Western blot

Cells were plate at the same concentration ($1x10^6$ cells/well), cells were lysate using MQ water with NuPage LDS sample buffer (1x) (ThermoFisher NP0007) and 1x NuPage sample reducing agent (ThermoFisher NP0009). 5 µL of the ladder SeeBlue plus 2 Standard (Invitrogen LC5925) in one well and 12 µL of samples per well were in a NuPage 4-12% Bis-Tris Mini gel with MOPS buffer at 100-120V for 2-3h. After that proteins were transfer to a nitrocellulose membrane using the iBlot™ Gel Transfer system following manufacturer's instructions. After the transfer membranes were cut above the housekeeping and block with 3% milk in PBS (blocking buffer). The different parts were incubated overnight at 4°C with the appropriate antibody BRD2 (Bethyl A302-583A), BRD3 (Bethyl A302-368A) or BRD4 (Bethyl A301-985A) 1:2000, or H3 (Abcam ab1791) 1:20000 in blocking buffer. Post incubation, membranes were washed (PBS, 0.05% Tween20) and then incubated with a secondary antibody Alexa Fluor 680 donkey anti-rabbit IgG (H+L) (Invitrogen A10043) at 1:8000 in wash buffer for 1 hour in the dark. Membranes were washed and visualised, and images were taken using the Odyssey Infrared Imaging system.

Cytokine measurements

TNF, IL-6, IL-10 and IL-12p70 were measured using a Human Proinflammatory 7-Plex Tissue Culture Kit (Meso Scale Discovery; MSD) following the manufacturer's instructions. For IFNγ the MSD 5 PLEX (Meso Scale Discovery) following the manufacturer's instructions was used. CCL-1 and MCP-1 were also measured using MSD kits for those specific cytokines following manufacturer's instructions. Activin A was measured by ELISA (R&D Systems, DAC00B) following manufacturer's instructions.

Viability Assay

Viability was determined by measuring total ATP content in monocyte/macrophage cultures via the CellTiter-Glo kit and following manufacturer's instructions.

ChIPseq

CD14 monocytes were extracted from human PBMCs as described above and differentiated with 5ng/mL GM-CSF or 50ng/mL M-CSF for one hour in the presence of 0.1% DMSO or 100nM I-BET151. Cells were cross-linked with 1% formaldehyde for 10 minutes at RT, quenched with 0.125M glycine and harvested. Cell pellets were frozen on dry ice and shipped to Active Motif for HistonePath™ ChIPseq (for H3 K27Ac and H3 K4me3) and FactorPath™ ChIPseq (for BRD2, BRD4 and RNA PolII S2P) . Chromatin was isolated by the addition of lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated and the DNA sheared to an

average length of 300-500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantitation of the total chromatin yield.

An aliquot of chromatin (10ug for HistonePath™ CHIPseq and 20 ug for FactorPath™ ChIPseq) was precleared with protein A (G for Pol2) agarose beads (Invitrogen). Genomic DNA regions of interest were isolated using antibodies against H3 K27Ac (Active Motif 39133), H3 K4me3 (Active Motif 39159), BRD2 (Bethyl A302-582A), BRD4 (Bethyl A301-985A) and RNA PolII S2P (Active Motif 91115). Complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65 °C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation.

Quantitative PCR (qPCR) reactions were carried out in triplicate on specific genomic regions using SYBR Green Supermix (Bio-Rad). The resulting signals were normalized for primer efficiency by carrying out qPCR for each primer pair using Input DNA.

ChIP Sequencing (Illumina)

Illumina sequencing libraries were prepared from the ChIP and Input DNAs by the standard consecutive enzymatic steps of end-polishing, dA-addition, and adaptor ligation. After a final PCR amplification step, the resulting DNA libraries were quantified and sequenced on Illumina's NextSeq 500 (75 nt reads, single end). Reads were aligned to the human genome (hg19) using the BWA algorithm [28](default settings). Duplicate reads were removed, and only uniquely mapped reads (mapping quality >= 25) were used for further analysis. Alignments were extended in silico at their 3'-ends to a length of 200 bp, which is the average genomic fragment length in the size-selected library, and assigned to 32-nt bins along the genome. The resulting histograms (genomic "signal maps") were stored in bigWig files. BRD4/2 and HistoneMarks peak locations were determined using the MACS algorithm [29] (v1.4.2) with a cutoff of p-value = 1e-7. RNA Pol2-enriched regions were identified using the SICER algorithm [30](cutoff FDR 1E-10, MaxGap = 600 bp). Signal maps and peak locations were used as input data to Active Motifs proprietary analysis program, which creates Excel tables containing detailed information on sample comparison, peak metrics, peak locations and gene annotations.

qPCR

RNA was isolated using RNeasy mini kit (Qiagen) following the manufacturer's protocol. For human samples, cDNA was synthesized using cDNA synthesis kit (Qiagen) following the manufacturer's protocol. Quantitative PCR was performed using SYBR green mastermix or Taqman gene expression assay depending on the primer. The reactions were performed on a QuantStudio Flex 7 (Applied Biosystems) to analyse expression levels of human *BRD2, BRD3*, *BRD4, CCL2 (MCP-1)* and *CCL1,* QuantStudio real time PCR software was used. For normalization the gene used was *UBB*.

The analysis was performed based on a standard curve where the quantity was extrapolated. The number of copies per ng was calculated. Afterwards the log to this quantity was done. To calculate

the NF: From the house keeping gene the average of the log of the number of copies was calculated. Then to every log of the number of copies from the house keeping gene the average was deducted. This gives the NF number that you can use to normalize the other genes to the house keeping gene.For the other genes the NF was added to the log of the number of copies, then the quantity was unlog and is was calculate the number of copies per 50ng.

Primer	Sequence (5'-3')
BRD2_FW	GAAGCTGGGCCGAGTTGTG
BRD2_RV	CGTAGGCAGGAAAGGACATAGC
BRD2_probe	CCAAGCCAGGGAGCCCTCTTTACGTGA
BRD3_FW	AGCAGCTCCTCCGAGTCTGG
BRD3_RV	TCCGAAGCCAGTTCATTCTGAG
BRD3_probe	AGCGGGTCCAGCTCTGACAGCAGTGA
BRD4_FW	GCAGCTGAGGATTGCAGAGC
BRD4R_RV	GCTCGTAACAAGGCGTGTGC
MCP-1_FW	CCCAAGAATCTGCAGCTAAC
MCP-1_RV	GGGTAGAACTGTGGTTCAAGAGG
CCL-1_FW	GGACACAGTTGGATGGGTTC
CCL-1_RV	AAAAGCAGGGCAGAAGGAAT
UBB_FW	CGGCAAGACCATCACTCTGG
UBB_RV	AAAGAGTGCGGCCATCTTCC
UBB_probe	TGGAGCCCAGTGACACCATCGAAAATG

Transcriptomic

RNA samples were processed and analysed for transcriptomic expression on an Affymetrix U133 plus 2.0 microarray platform (CEL file generation). The resulting CEL files were normalised by RMA in Affymetrix Bioconductor package in R 2.15. Normalised data was assessed for quality by 'arrayQualityMetrics' Bioconductor package in R2.15 and then Principal Component Analysis (PCA) in ArrayStudio v7.0. Differential expression analysis to find probes altered by compound at each timepoint was carried out using the General Linear Model functionality in ArrayStudio v7.0. For the selection of a single probe per gene, least square means profiles of each gene were manually viewed to identify the probe with the lowest variability (best confidence intervals). Terms for donor, treatment and time were included in the fitted model. A multiplcity adjustment of the raw p-values was done using the FDR-BH (False Discovery Rate Benjamini & Hochberg) method. Both raw and FDR p-values were made available in the analysed data. For the evaluation of the biology a significance threshold of differentially expressed probes was set as fold change of >1.5 or <-1.5 and raw p-value <0.05.

Collagen Induced Arthritis model

I-BET151, provided by GlaxoSmithKline (Stevenage, United Kingdom), was suspended in 1% methylcellulose (Sigma, M0430) and dosed orally at either 3 mg/kg or 10 mg/kg, as indicated. Female Lewis rats (Charles River, UK), aged 9–11 weeks, were used. Rats were housed in groups of 4 in a room maintained at a temperature of 23+/- 2°C with a 12 h light/dark cycle. Food and water was freely available, and the animals were acclimatised to the environment for 14 days prior to commencement of experiments. All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and GlaxoSmithKline (GSK) internal Policy on the Care, Welfare and Treatment of Animals. Arthritis was induced using a two-step immunisation protocol, similar to that previously described [31]. For the primary sensitisation, bovine type II collagen (MD Bioproducts, CH) was diluted with 0.05 M glacial acetic acid (Alfa Aesar, UK) to make a final concentration of 4 mg/mL and this was stored at 4°C overnight. An emulsion was prepared the following morning with an equal volume of Incomplete Freund's Adjuvant (IFA; Sigma, UK) and collagen using a PRO 200 Turrax Homogenisor (PRO Scientific, USA). The emulsion was administered intradermally (i.d.) to the base of the tail using a dose volume of 0.1 mL. For the booster injection on Day 7, an emulsion containing collagen, IFA and Muramyl dipeptide (N-Acetylmuramyll-Alanyl-D-Isoglutamine; MDP; Sigma, UK) was used. Bovine type II collagen was diluted with 0.05M glacial acetic acid to make a final concentration of 8 mg/mL and MDP was diluted with distilled water to make a final concentration of 120 µg/mL. Both solutions were stored at 4°C overnight. The following day, collagen and MDP were combined at a ratio of 1:1 and then an emulsion was prepared as described previously, between this solution and an equal volume of FIA just prior to dosing. The boost emulsion was also administered to the base of the tail as described previously. The solutions and emulsion were kept on ice at all times. Rats in the control group received an intradermal administration of phosphate-buffered saline (PBS, UK media services) on days of primary sensitisation and booster using a dose volume of 0.1 mL.

Disease onset occurred in the hind paws and typically observed approx. 5 days following boost (D12) and is defined as the first day that swelling or erythema of the paws is observed. Arthritis was assessed by measurement of paw volume and visually as a clinical score of hind limbs on days 6, 10-21, defined as efficacy endpoints. Paw swelling was recorded as the mean paw volume of both hind paws, measured using a plethysmometer (Linton Instrumentation, UK). Clinical score was recorded on a 0 - 4 scale according to the following criteria (Larsson, 1990): 0 = no signs of arthritis; 1 = slight oedema and erythema on the foot or ankle; 2 = slight oedema and erythema to the entire paw; 3 = moderate oedema and erythema to the entire paw; 4 = severe oedema and ankylosis. As only the hind paws tended to be affected, the maximum clinical score attainable per animal was 8, and a clinical score of 2 or more in 1 or more paws was the criteria used for determining a positive incidence, i.e. successful induction of arthritis.

Quantigene Analysis

Slides were prepared from formalin-fixed paraffin-embedded (FFPE) rat hind joints and deparaffinised with xylene (Sigma, cat# 247642). RNA was extracted according using Quantigene Sample Processing Kit for FFPE tissues (Invitrogen, cat# QS0109). Relative gene expression of *inhba*, *nos2* and *ldha* mRNA were determined with an Affymetrix Quantigene plex assay (Invitrogen cat# QP1013), according to manufacturer's instructions, using custom primer/probe

sets designed by the manufacturer. Samples were acquired using a Luminex Flexmap 3D. Data for *Nos*2 and *Inhba* were normalised to *Ldha* expression. Statistical differences between groups were determined using one-way ANOVA with Dunnett's post-test (GraphPad Prism 7.0).

Accession Number	Symbol
NM_012611	*nos2*
NM_017128	*inhba*
NM_017025	*ldha*

CD68 Immunohistochemistry

Slides were prepared from FFPE rat hind joints. Sections were stained with rabbit polyclonal anti-rat CD68 antibody (Abcam, cat# ab125212) followed by HRP-conjugated goat anti-rabbit immunoglobulins (Dako, P044801-2). Staining was revealed using DAB peroxide substrate solution, and slides were counterstained with haematoxylin. Macrophages infiltrates were scored in a semiquantitative manner. Statistical differences between groups were determined using one-way ANOVA with Dunnett's post-test (GraphPad Prism 7.0).

Results

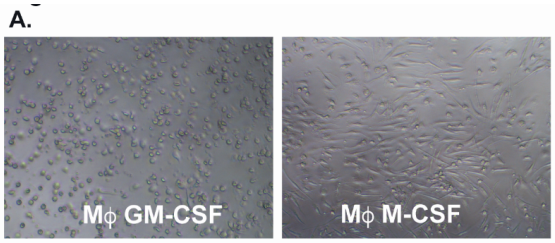
CCL-1 and MCP-1 are activation-independent biomarkers for the functionally distinct human Mϕ GM-CSF and Mϕ M-CSF, respectively

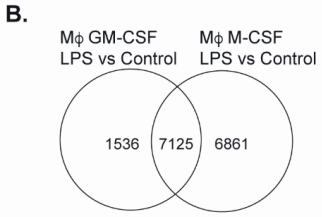
Human macrophages can be generated into distinct populations through the isolation of CD14-positive monocytes from blood and *in vitro* differentiation with either GM-CSF (Mϕ GM-CSF) or M-CSF (Mϕ M-CSF) for 5 days [32, 33]. This method produces morphologically distinct macrophages **(Figure 1A)**. We carried out a full transcriptomics analysis of Mϕ GM-CSF and Mϕ M-CSF in the presence and absence of LPS-activation to test whether functional distinction was evident too. Our data revealed that 15,522 probes were differentially expressed following LPS stimulation, and just under half of these were common to both macrophage subtypes **(Figure 1B)**. Interestingly, there were a much greater number of LPS-responsive changes in Mϕ M-CSF (termed LPS- Mϕ M-CSF probes) than in Mϕ GM-CSF which led us to explore whether these changes were uniquely expressed in Mϕ M-CSF or if they did not meet the significance threshold. LPS- Mϕ M-CSF probes were identified, and the expression was plotted in the presence and absence of LPS across both macrophage types, which showed that these probes were already highly expressed in Mϕ GM-CSF even in the absence of LPS activation **(Figure 1C)**. This strongly suggests that Mϕ GM-CSF are more pro-inflammatory than Mϕ M-CSF and is in line with literature reports [33-37]. Cytokine responses are often reported to be reliable measures of macrophage subtypes. To test whether the LPS response was sufficient to differentiate between these two macrophage subtypes we measured LPS induced TNF, IL-6, IL-10 and IL-12p70 in donor-matched Mϕ GM-CSF and Mϕ M-CSF and found that only IL-12p70 and IL-6 were significantly and categorically different **(Figure 1D)**. IL-12p70 is a well-known pro-inflammatory cytokine that provides Signal 2 for T lymphocyte activation so we hypothesised that Mϕ GM-CSF would be superior to Mϕ M-CSF in activating autologous T lymphocytes. We set up co-cultures with both macrophage subtypes and in the absence of LPS activation we could not

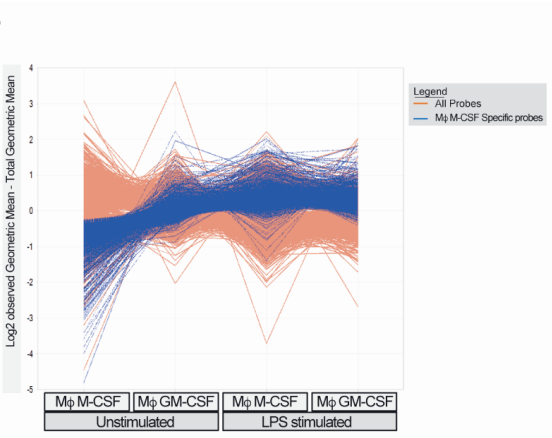
detect any significant production of IFNγ **(Figure 1E)**. However, macrophages activated with LPS overnight and then co-cultured with autologous T lymphocytes exhibited substantial levels of IFNγ from Mϕ GM-CSF but not Mϕ M-CSF. This T lymphocyte activation involved some cell-cell contact but also some secreted factors that were only present after macrophage activation, but LPS-activated macrophages cultured in the absence of T lymphocytes clearly demonstrates the source of IFNγ is not macrophages or any potentially contaminating cells **(Figure 1E)**.

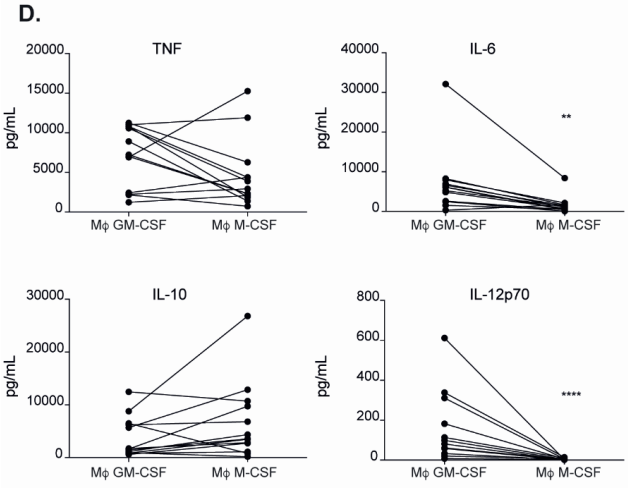
We wanted to identify biomarkers that could be used to distinguish between these two macrophage subtypes in the absence of LPS activation. Flow cytometry was used to assess the expression of commonly used cell surface markers in donor-matched macrophages **(Figure 1F)**. Whilst the majority of the markers evaluated exhibited some differences between the two macrophage subtypes, only CD14 and CD16 were categorically differential, both being highly expressed in Mϕ M-CSF but not Mϕ GM-CSF. However, we were unable to identify cell surface markers that were exclusively expressed in Mϕ GM-CSF but not Mϕ M-CSF. We characterised MCP-1 expression in monocytes and during differentiation [38] into the two macrophage subtypes and found that MCP-1 was indeed a robust and spontaneous biomarker for Mϕ M-CSF and absent in Mϕ GM-CSF supernatants after 5 days **(Figure 1G)**. We found transcriptomics data generated after just 16 hours into the differentiation process that CCL-1 was 129-fold up-regulated in GM-CSF vs M-CSF treated cells [8]. We assessed the utilisation of CCL-1 as a potential biomarker and found it to be highly expressed in Mϕ GM-CSF in comparison to Mϕ M-CSF **(Figure 1H)**. Not only was CCL-1 very high in supernatants at 5 days from Mϕ GM-CSF (and absent in Mϕ M-CSF supernatants) but we were also able to corroborate the literature report for an early timepoint of CCL-1 induction.

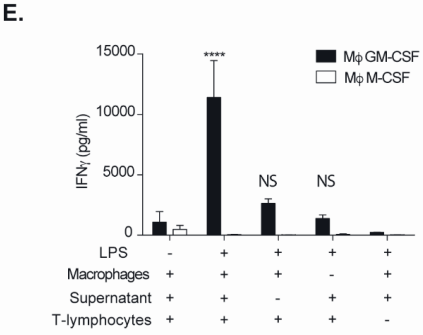
Our data confirms that Mϕ GM-CSF and Mϕ M-CSF are functionally distinct, and that the former are more pro-inflammatory with the ability to activate autologous T lymphocytes. We identify that CCL-1 and MCP-1 can be used as *in vitro* biomarkers for Mϕ GM-CSF and Mϕ M-CSF, respectively by simply using the cell supernatant that is ordinarily discarded after 5 days of differentiation. These biomarkers are valuable in distinguishing between the two macrophage subtypes, without the need to further activate the cells or use flow cytometry.





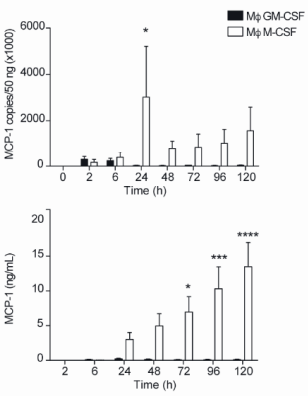






F.

Marker	Mϕ GM-CSF Mean (%)	Mϕ GM-CSF SD	Mϕ M-CSF Mean (%)	Mϕ M-CSF SD	n
CD11a	84.4	6.2	84.6	14.8	4
CD11b	97.7	1.5	89.9	13.8	4
CD14	8.1	5.2	91.2	11.3	6
CD16	12.5	5.9	89.8	7.2	6
CD32	75.5	19.5	93.4	10.5	6
CD33	57.7	18.5	85.8	15.5	6
CD44	99.6	0.5	99.5	0.5	6
CD54	95.5	3.1	92.1	5.3	4
CD64	67.1	23.9	88.1	13.1	6
CD68	26.5	34.6	48.9	27.3	4
CD71	79.2	19.7	71.7	17.9	7
CD86	69.1	24.3	87.2	20.8	4
CD89	62.3	23.8	92.9	4.7	6
CD115	6.3	4.4	72.4	26.5	6
CD116	52.2	13.5	66.4	30.1	6
CD119	60.3	24.0	40.9	32.7	6
CD163	0.4	0.4	27.9	35.4	6
CD181	6.9	14.7	12.3	20.2	6
CD182	3.6	2.2	50.6	36.0	6
CD183	9.3	12.0	11.7	14.8	6
CD282	47.2	19.2	89.5	9.3	6
CD283	63.3	24.0	55.8	37.6	6
CD284	0.6	0.6	2.4	2.5	6
HLA-DR	98.4	1.5	97.0	2.9	6



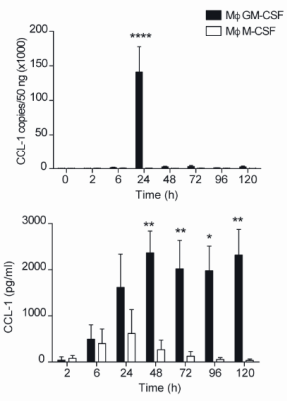


Figure 1. Human Mϕ GM-CSF and Mϕ M-CSF are functionally distinct macrophages
A. Representative phase contrast images taken of CD14-positive monocytes cultured with 5 ng/ml GM-CSF (Mϕ GM-CSF) or 100 ng/ml M-CSF (Mϕ M-CSF) for 5 days. **B.** Transcriptomics data (Affymetrix probes) showing overlap in the LPS response after 18 hours from Mϕ GM-CSF and Mϕ M-CSF. **C.** Line plot showing expression profile of all probes (orange lines) or LPS-sensitive probes in Mϕ M-CSF only (blue lines) from the Affymetrix dataset. **D.** Cytokine production following LPS activation (18 hours) of Mϕ GM-CSF and Mϕ M-CSF generated from the same donors Unpaired t test (* p value < 0.05, ** p value <0.01, *** p value < 0.001, **** p value < 0.0001). **E.** IFNγ production by T-lymphocytes co-cultured for 3 days with LPS-activated autologous macrophages. **F.** Cell surface marker expression determined by flow cytometry in resting donor-matched Mϕ GM-CSF and Mϕ M-CSF. **G.** Time profile of MCP-1 expression in monocytes differentiating into Mϕ GM-CSF or Mϕ M-CSF determined by RT-PCR (top panel) or by MSD analysis of supernatant (bottom panel). **H.** Same as (g) measuring CCL-1 expression over time. Data shown are the Mean ± SEM from 3 or more separate donors; 2-way ANOVA test with Bonferroni correction (* p value < 0.05, ** p value <0.01, *** p value < 0.001, **** p value < 0.0001).

Critical early role for BET proteins in human Mϕ GM-CSF differentiation

A role for BET proteins in macrophage activation has been established in mouse models [39] and we wanted to test the impact of BET inhibition in our human macrophage models. Using our newly identified *in vitro* biomarkers we tested the effect of I-BET151, a selective inhibitor of both bromodomains of the BET family of proteins (BRD2, BRD3, BRD4). I-BET151 (0.0001-1 μM) was added to freshly isolated monocytes which were stimulated with either GM-CSF or M-CSF to initiate the differentiation process over 5 days. Supernatants were removed and assessed for CCL-1 and MCP-1 production whereas the cells were assessed for ATP levels as an indicator of viability. Mϕ GM-CSF were more sensitive to I-BET151 with reduction in CCL-1 production from as low as 1 nM but incubations and a significant impact on viability from 100 nM onwards **(Figure 2A)**. The viability of Mϕ M-CSF was only impaired at concentrations beyond 300 nM and a similar impact was seen on the corresponding biomarker of MCP-1. We used light microscope images to verify this selective impact; macrophages generated in the presence of 100 nM I-BET151 or DMSO portrayed this clearly where the globular morphology of Mϕ GM-CSF was severely disrupted, whereas the elongated spindly morphology of Mϕ M-CSF remained intact **(Figure 2B)**. Furthermore, we tested whether I-BET151 regulated additional Mϕ M-CSF biomarkers, CD14 or CD16, and found no significant impact **(Supplementary Figure 1)**. We used 100 nM I-BET151 in all subsequent studies to determine the reason for differential impairment of monocyte differentiation into GM-CSF but not M-CSF macrophages. We were intrigued by the lower effect of I-BET151 on Mϕ M-CSF and wondered whether this was simply due to the absence or differences in expression of BET proteins, so we assessed this by both RT-PCR and immunoblotting for BRD2, BRD3 and BRD4 and found no significant differences either over time or between the two macrophage subtypes **(Supplementary Figure 2)**.

As I-BET151 was added to monocytes and remained in the culture medium for the full duration of 5 days it was unclear whether there was a continual role for BET proteins in the differentiation process or if there was a critical point of action underlying the effect. We devised a staggered addition of I-BET151 or DMSO to determine this **(Figure 2C)**. I-BET151 added within the first 6 hours of monocytes differentiating with GM-CSF perturbed the phenotype, whereas DMSO or compound added at later timepoints had no effect **(Figure 2D; Supplementary Figure 3)**. In line with the impact on CCL-1, the viability of these macrophages was also only impacted at earlier timepoints.

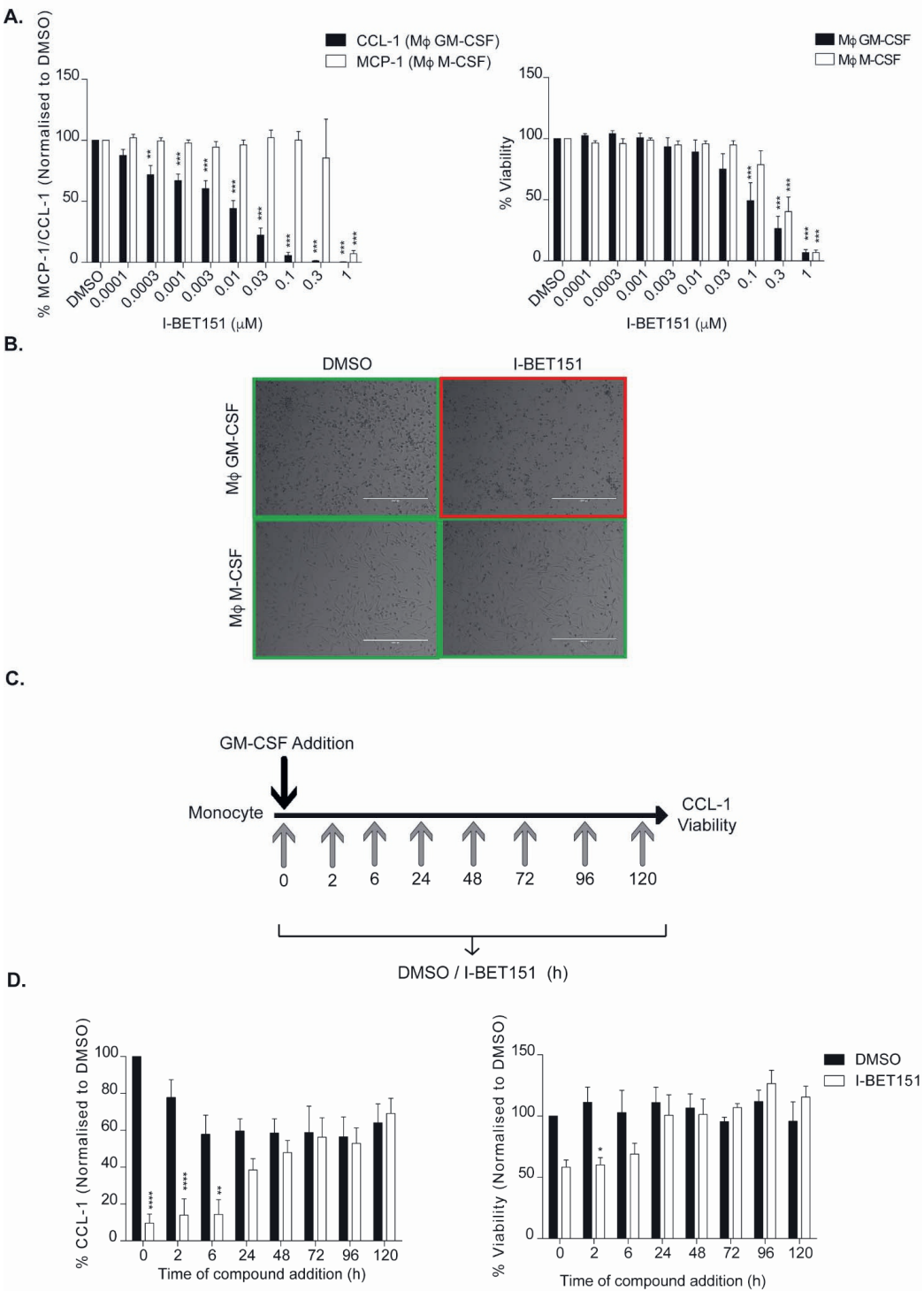

A.
CCL-1 (Mϕ GM-CSF)
MCP-1 (Mϕ M-CSF)
% MCP-1/CCL-1 (Normalised to DMSO)
I-BET151 (μM)
Mϕ GM-CSF
Mϕ M-CSF
% Viability
B.
DMSO
I-BET151
Mϕ GM-CSF
Mϕ M-CSF
C.
GM-CSF Addition
Monocyte
CCL-1
Viability
DMSO / I-BET151 (h)
D.
% CCL-1 (Normalised to DMSO)
Time of compound addition (h)
% Viability (Normalised to DMSO)
DMSO
I-BET151

Figure 2. I-BET151 selectively impairs Mϕ GM-CSF but not Mϕ M-CSF differentiation
A. I-BET151 displays a concentration-dependent inhibition of human monocyte differentiation into Mϕ GM-CSF but not Mϕ M-CSF as seen by biomarker production (CCL-1 and MCP-1, respectively; top panel) or viability (bottom panel) after 5 days. **B.** Representative phase contrast images of macrophages generated in the presence of DMSO or 100 nM I-BET151. **C.** Schematic showing design of experiment to determine timing for critical involvement of BET proteins in differentiation. **D.** Data from experiment in (c) showing CCL-1 production (left panel) and viability (right panel) from monocytes following GM-CSF induced differentiation in the presence of DMSO or I-BET151. Data shown are the Mean ± SEM of 3 or more independent donors. 2-way ANOVA test with Bonferroni correction (* p value < 0.05, ** p value <0.01, *** p value < 0.001, **** p value < 0.0001).

These data demonstrate that BET protein inhibition results in selectively disrupting monocyte differentiation towards Mϕ GM-CSF. This differential effect is even more remarkable given that the expression of the BET proteins is not dissimilar during differentiation between both subtypes. We identify an early critical role during which I-BET151 can disrupt this process.

BET regulation of genes essential for GM-CSF induced differentiation

As BET protein expression was similar for both macrophage subtypes, we hypothesised that it was the GM-CSF specific induction of factors that were critically disrupted by I-BET151. In the hope to identify possible mediators crucial to this process, we ran an Affymetrix transcriptomics study on monocytes undergoing differentiation into Mϕ GM-CSF or Mϕ M-CSF in the presence or absence (DMSO) of I-BET151 after just 1 and 6 hours. Principle Component Analysis of the data revealed that the main separation in the data was time **(Figure 3A)**. We looked at I-BET151 effects across both subtypes and timepoints and identified that the number of differentially expressed probes was not dissimilar between GM-CSF and M-CSF differentiated monocytes at both time points **(Figure 3B; first panel)**. There were 1497 probes which were expressed by both GM-CSF and M-CSF and were also regulated by I-BET151 and we reasoned that these were non-essential differentiation genes as Mϕ M-CSF generated in the presence of I-BET151 were viable and unaffected. We identified all probes that showed significantly higher expression following GM-CSF treatment than M-CSF at either one or both timepoints and checked to see which of these were then reversed by I-BET151 **(Figure 3B, second panel)**. Approximately half of these probes (369 genes) showed significant reversal in expression with I-BET151 and are listed in **Supplementary Table 1**.

With these identified genes, we wanted to determine which were directly regulated by I-BET151 as opposed to a downstream consequence. In order to do this directly, we conducted ChIPseq analysis using the same conditions as the transcriptomics study but only assessed effects at 1 hour, as chromatin events precede transcriptional changes. We determined the impact of I-BET151 on BRD2, BRD4, transcriptionally active RNA Polymerase (RNA Pol II S2P), H3K4Me3 and H3K27Ac in monocytes treated with GM-CSF or M-CSF for 1 hour. The total number of genome-wide binding peaks identified varied with the highest number of peaks for markers of active transcription H3K4Me3 and H3K27Ac **(Figure 3C)**. The number of binding events for BRD2, BRD4 and RNA Pol II S2P were similar to each other but also more dynamic across the treatment groups. H3K27Ac has previously been shown to be highly responsive to changes with BET protein binding [40]. We assessed whether global binding of H3K27Ac, BRD2 and BRD4 was predominantly within gene bodies or at TSS and found that H3K27Ac and BRD2 binding was slightly more prevalent within TSS regions **(Figure 3D)** Interestingly at the genome-wide level BRD2 binding was diminished in the presence of I-BET151 in M-CSF treated monocytes whilst

BRD4 binding was diminished in the presence of I-BET151 in GM-CSF treated monocytes though in both cases the reduction in binding was slight. In line with H3K27Ac sensitivity to I-BET151 and the observation that GM-CSF macrophage differentiation and not M-CSF differentiation is affected by loss of BET protein binding we observed a noticeable decrease in BRD2, BRD4 and H3K27Ac peaks in I-BET151 treated GM-CSF monocytes relative to the DMSO control. Next, we wanted to use our 'essential differentiation genes' list and interrogate the presence of BRD2, BRD4 and H3K27Ac peaks. We found that chromatin changes aligned with this gene list and H3K27Ac binding was the most sensitive to I-BET151 at these loci **(Figure 3E)**. *INHBA* ranked highly across both datasets and exhibited one of the most impressive signals for selective BRD4 recruitment in GM-CSF treated monocytes and concomitant loss of these peaks with I-BET151 treatment **(Figure 3F)**. Additional loci at which similar patterns of recruitment and displacement were seen include *IL-19, MAF and ADRPH* **(Supplementary Figure 4)**.

These data demonstrate that GM-CSF induced differentiation rapidly induces the recruitment of BRD2 and BRD4 to selective loci which leads to transcription of these genes. Not all of these genes are exclusive to GM-CSF signalling and furthermore not all are essential for differentiation as shown by disruption of BET protein recruitment to these chromatin regions does not lead to impairment of M-CSF induced gene expression.

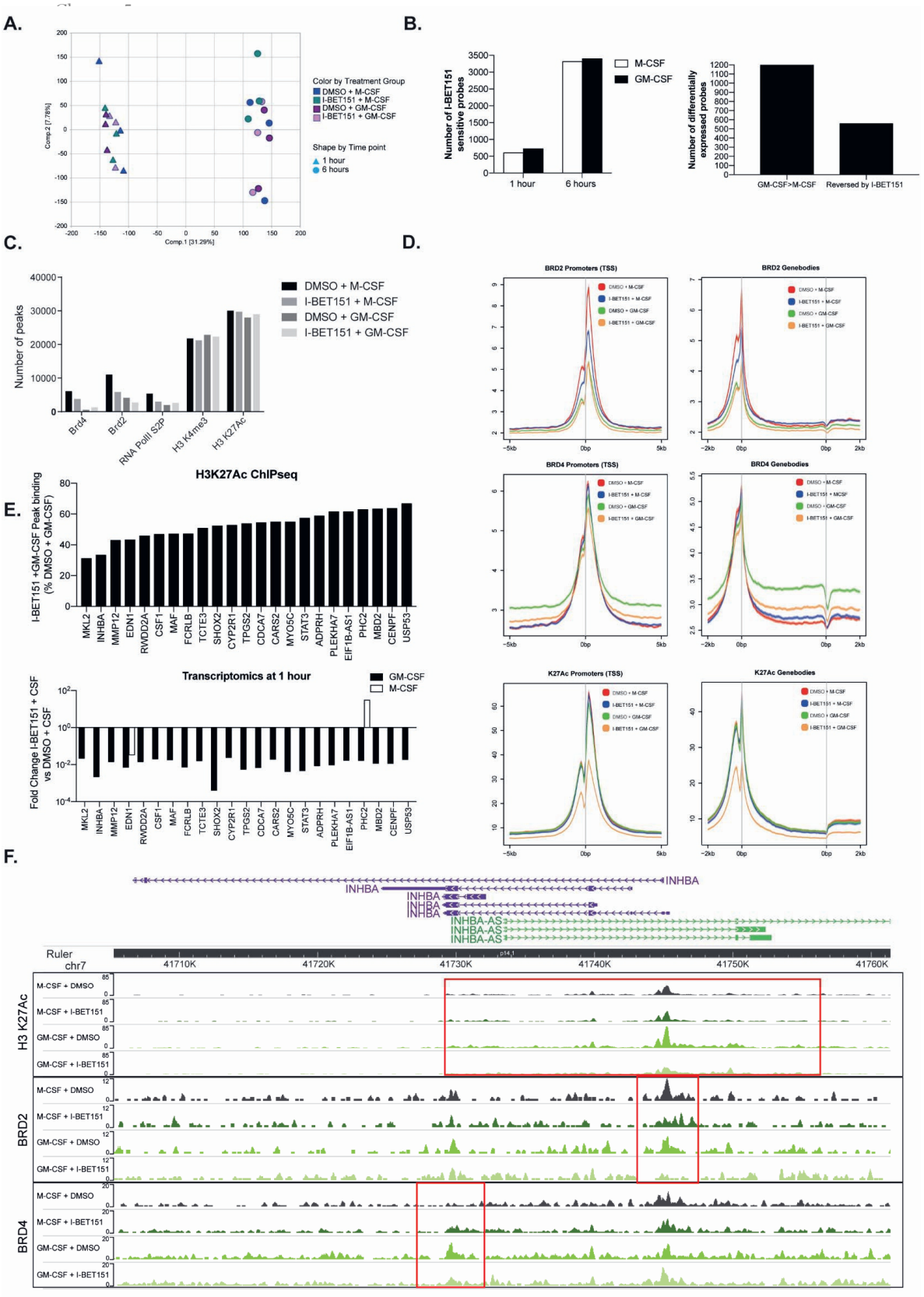

A.
Color by Treatment Group
DMSO + M-CSF
I-BET151 + M-CSF
DMSO + GM-CSF
I-BET151 + GM-CSF
Shape by Time point
1 hour
6 hours
Comp.1 [31.29%]
Comp.2 [7.78%]
B.
Number of I-BET151 sensitive probes
M-CSF
GM-CSF
1 hour
6 hours
Number of differentially expressed probes
GM-CSF>M-CSF
Reversed by I-BET151
C.
Number of peaks
DMSO + M-CSF
I-BET151 + M-CSF
DMSO + GM-CSF
I-BET151 + GM-CSF
Brd4
Brd2
RNA PolII S2P
H3 K4me3
H3 K27Ac
D.
BRD2 Promoters (TSS)
BRD2 Genebodies
BRD4 Promoters (TSS)
BRD4 Genebodies
K27Ac Promoters (TSS)
K27Ac Genebodies
E.
H3K27Ac ChIPseq
I-BET151 +GM-CSF Peak binding (% DMSO + GM-CSF)
MKL2
INHBA
MMP12
EDN1
RWDD2A
CSF1
MAF
FCRLB
TCTE3
SHOX2
CYP2R1
TPGS2
CDCA7
CARS2
MYO5C
STAT3
ADPRH
PLEKHA7
EIF1B-AS1
PHC2
MBD2
CENPF
USP53
Transcriptomics at 1 hour
GM-CSF
M-CSF
Fold Change I-BET151 + CSF vs DMSO + CSF
F.
INHBA
INHBA-AS
Ruler
chr7
41710K
41720K
41730K
41740K
41750K
41760K
H3 K27Ac
BRD2
BRD4
M-CSF + DMSO
M-CSF + I-BET151
GM-CSF + DMSO
GM-CSF + I-BET151

Figure 3. I-BET151 regulates GM-CSF induced genes that are crucial for differentiation
A. PCA plot showing separation of transcriptomics data from monocytes stimulated with either M-CSF or GM-CSF in the presence of 100 nM I-BET151 or DMSO for 1 and 6 hours. **B.** Differentially expressed probes from the transcriptomics study categorised by time and treatment (left graph) followed by exclusively regulated by I-BET151+ GM-CSF (right graph). Differential expression was determined as a fold change of >1.5 or <-1.5 and raw *p* value <0.05 as assessed with a general linear model in 3 separate donors. **C.** Signal peaks from ChIPseq in monocytes stimulated with M-CSF or GM-CSF for 1 hour in the presence of 100 nM I-BET151 or DMSO. **D.** Genome-wide signal for BRD2, BRD4 and H3K27Ac peaks at promoters (transcription start sites; TSS) or within gene bodies across the experimental conditions. **E.** Genes ranked by selective reduction in H3K27Ac binding by I-BET151 in GM-CSF (cf. M-CSF; top panel). I-BET151 induced fold change of the identified genes at one hour post CSF addition (bottom panel). All values shown in the bottom panel are statistically significant (raw *p* value <0.05). **F.** UCSC gene browser view illustrating the effect of I-BET151 on BRD2 and BRD4 binding and acetylation of H3K27 histone mark at the *INHBA* locus in monocytes stimulated with M-CSF or GM-CSF for one hour. Transcriptomic studies were conducted in 3 separate donors whereas the ChIPseq data is from 1 donor.

I-BET151 increases the threshold for monocyte differentiation into pro-inflammatory Mϕ GM-CSF and skews monocytes towards homeostatic Mϕ M-CSF

Having identified the underlying mechanism for the differential effect of I-BET151 on monocyte differentiation, we wanted to understand the pathophysiological significance. M-CSF is constitutively expressed in humans and can be readily measured in the circulation, however GM-CSF is only systemically detected during inflammation or infection [41-44]. We aimed to determine the effect of I-BET151 on monocyte differentiation under heterogeneic (both GM-CSF and M-CSF) conditions. We found that GM-CSF signalling was dominant over M-CSF and significant titrations in GM-CSF concentrations were needed to see a Mϕ M-CSF phenotype, which is in agreement with other reports [8]. Monocytes treated with I-BET151 and differentiated with M-CSF and GM-CSF retained a Mϕ M-CSF phenotype despite increasing amounts of GM-CSF as depicted by high MCP-1 and reduced CCL-1 production **(Figure 4A)**. As expected, macrophages generated in the presence of DMSO under these conditions produced more CCL-1 and less MCP-1 as GM-CSF concentrations increased, demonstrating the utility of these biomarkers. As Activin A (the protein heterodimer of *INHBA*) was identified as one of the critically regulated I-BET151 genes, we assessed production in supernatants. Activin A was selectively produced by Mϕ GM-CSF and this was abrogated in the macrophages generated in the presence of I-BET151 even at higher GM-CSF concentrations **(Figure 4B)**. Next, we aimed to understand how stable these phenotypes were, so we removed the compound and added fresh medium containing LPS and activated the macrophages overnight and assayed for IL-12p70 which we previously showed **(Figure 1D)** was only produced by Mϕ GM-CSF. IL-12p70 was significantly suppressed in heterogeneic macrophages generated in the presence of I-BET151 relative to macrophages generated in the presence of DMSO **(Figure 4C)** demonstrating that even in the absence of compound the phenotypic switch to Mϕ M-CSF is maintained. We wanted to test this further and used these heterogeneic macrophages in a co-culture with autologous T lymphocytes. Despite the compound not being present during LPS activation or the 3 days of co-culture, macrophages generated in the presence of I-BET151 were less capable of activating T lymphocytes **(Figure 4D)**. This was true when using macrophages with or without secreted factors following LPS activation implying that IL-12p70 was not the only contributing factor in this functional assay but cell to cell interactions were also involved.

These data show that under heterogeneic conditions, I-BET151 skews monocyte differentiation towards Mϕ M-CSF and increases the threshold to become pro-inflammatory Mϕ GM-CSF and

this phenotype is long-lived and functionally recapitulates the homeostatic MΦ M-CSF phenotype of low IL-12p70 and lack of autologous T lymphocyte activation.

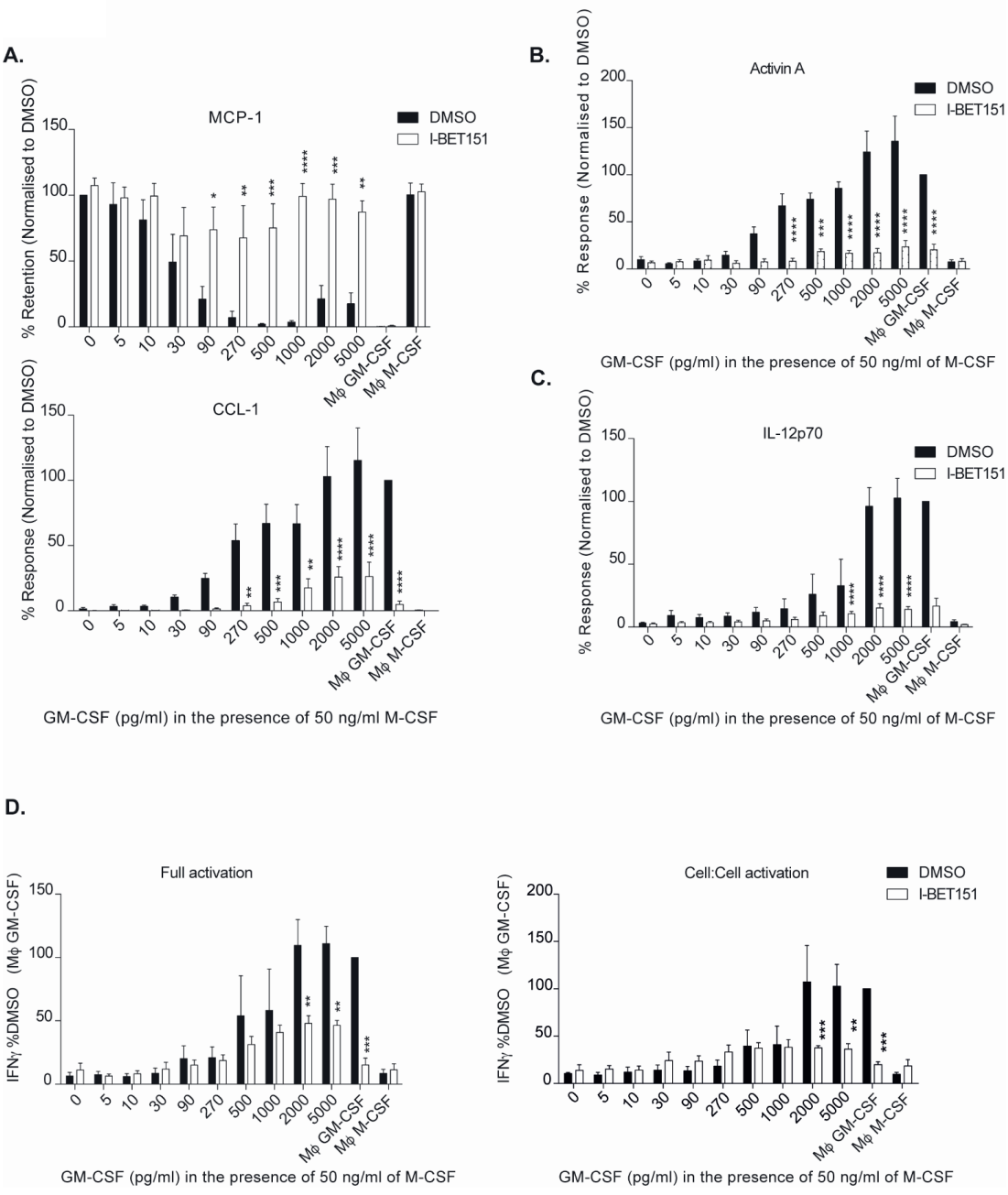


Figure 4. I-BET151 skews monocyte differentiation towards homeostatic Mϕ M-CSF that do not activate autologous T lymphocytes

A. MCP-1 (Mϕ M-CSF biomarker; top panel) and CCL-1 (Mϕ GM-CSF biomarker; bottom panel) production at 5 days after monocytes differentiated in the presence of 100 nM I-BET151 or DMSO, under heterogeneic conditions. **B.** Activin A measured from the same supernatants. **C.** Fresh supernatant added to macrophage cultures from (A) without re-addition of compound, were activated with LPS overnight and IL-12p70 measured. **D.** IFNγ production by autologous T-lymphocytes co-cultured with macrophages from (C) for 3 days. Co-culture experiments were conducted in the presence of supernatant following LPS activation (left panel) or where following LPS activation the supernatant was removed and replaced with fresh medium prior to T lymphocyte addition (right panel). All cytokine levels have been expressed as a percentage of levels induced by Mϕ GM-CSF (DMSO + 5 ng/ml GM-CSF only) with the exception of MCP-1 production (A; top panel) where levels have been normalised to Mϕ M-CSF (DMSO + 100 ng/ml M-CSF only). Data shown are Mean ± SEM from 4 separate donors. 2-way ANOVA test with Bonferroni correction (* p value < 0.05, ** p value <0.01, *** p value < 0.001, **** p value < 0.0001).

Short term I-BET151 abrogates arthritis via skewing macrophage phenotype

The role of macrophages in rheumatoid arthritis (RA) is well precedented [45, 46]. More recently, the clinical data supporting the pathogenic role of GM-CSF in RA has become available [47-49]. We wanted to use a model of arthritis to understand whether macrophage skewing can impact disease progression. The efficacious effect of BET inhibitors in multiple disease models is well known [23, 50] and indeed in models of arthritis [24, 51, 52]. We selected the collagen-induced arthritis (CIA) model to test our hypothesis that I-BET151 leads to efficacy via skewing macrophages towards a more homeostatic phenotype. Multiple measures of joint inflammation and prophylactic and peri-onset dosing regimes were incorporated into the study design **(Figure 5A)**. Continuous I-BET151 dosing resulted in significant suppression of paw volume induced in the CIA model **(Figure 5B)**. Interestingly, either prophylactic or peri-onset dosing regimes also resulted in the same level of efficacy **(Figure 5C)**. Daily I-BET151 dosing throughout the study was also efficacious although not to the same extent and there was a time-dependent increase in the clinical score. Peri-onset dosing resulted in greater efficacy than daily dosing with only mild signs of arthritis seen throughout the study duration. The joints were used for histology and the number of macrophages (CD68+ staining) seen over and above non-inflamed joint tissue were scored in a treatment-blinded fashion. The number of macrophages in I-BET151 treated animals were significantly lower than those in the vehicle group **(Figure 5D)**. We isolated RNA from the joints and assessed expression of *Inhba* and the well-established M1 marker, *Nos2*. *Inhba* and *Nos2* expression were significantly lower across all I-BET151 dosed groups **(Figure 5D**, right panels**)**. Whilst the number of CD68+ cells are lower than vehicle, both D10-22 and D0-22 groups exhibit an increase in macrophage numbers relative to the prophylactic I-BET151 dosed group. This is indicative that in this *in vivo* study, I-BET151 regulates macrophage phenotype (either differentiation or activation), and not just macrophage numbers, to induce anti-arthritic activity. These data demonstrate that a short duration of I-BET151 treatment, either prophylactic or peri-onset, can lead to efficacy in a model of arthritis and this is at least in part driven by suppressing pro-inflammatory macrophages.

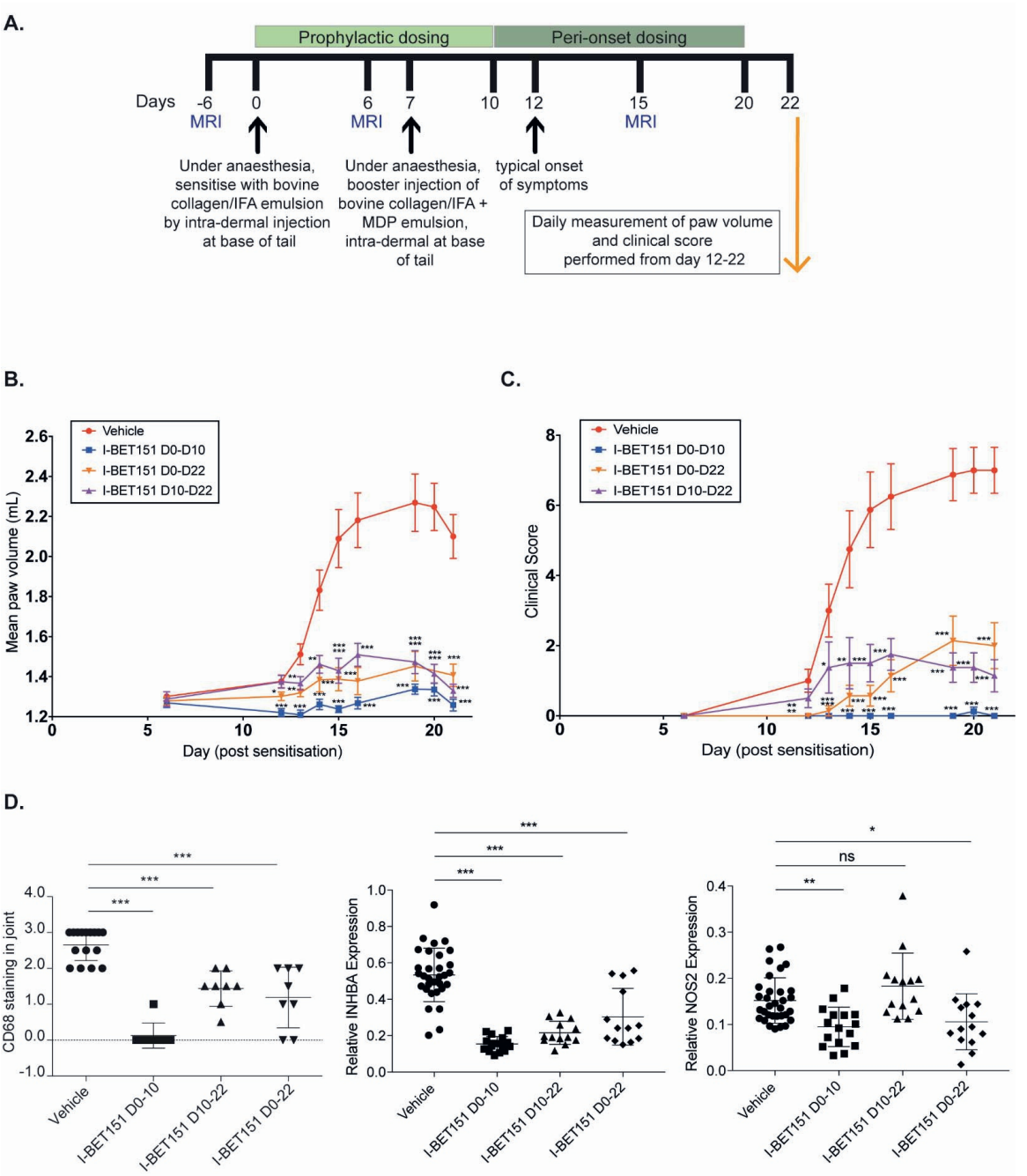


Figure 5. Short duration of I-BET151 dosing abrogates arthritis via macrophage skewing
A. Schematic showing the design and measurements in a rat Collagen Induced Arthritis (CIA) study with I-BET151 treatment. Four treatment groups with daily dosing of Vehicle or I-BET151 (Day 0-22, or Day 0-10 or Day 11-22) **B.** Average volume of both hind paws measured over the duration of the study. **C.** Clinical arthritic Score assessed in all animals over the duration of the study. Each datapoint represents the mean ± sem (n = 7 - 8/ group) expressed as a total clinical score of both hind limbs (maximum score of 8). **D.** Quantification of CD68

staining in joints from front limbs of all animals by IHC (left panel) and qPCR analysis of *Inhba* (middle panel) and *Nos2* (right panel) from the same joints. Statistical analysis was carried out using one-way ANOVA and Dunnett's post hoc test, where $*p < 0.05$, $**p < 0.01$ and $*** p < 0.001$ comparing treatment groups to vehicle control.

Discussion

Activated macrophages are known to produce high levels of pro-inflammatory cytokines, which can contribute to the pathology of multiple immune-mediated diseases, such as rheumatoid arthritis, Crohn's disease, osteoarthritis and multiple sclerosis [53-58]. Macrophage numbers associate closely with disease severity in RA and efficacy of a range of therapeutics is associated with reduction in synovial macrophages [59-62] GM-CSF has long been associated with the aetiology of RA [63-65] and clinical data are emerging showing efficacy in patients through blockade of GM-CSF [66, 67]. Our data show that I-BET151 selectively impairs monocyte differentiation into pro-inflammatory (GM-CSF), but not homeostatic (M-CSF), macrophages that exhibit a less inflammatory phenotype even in the presence of high GM-CSF levels. Strikingly, I-BET151 disrupts GM-CSF signalling very early in the GM-CSF differentiation process but allows the cells to revert to M-CSF macrophages when cultured under heterogeneic conditions. This switch in macrophage phenotype is a long-lived stable effect as activation and co-culturing in the absence of the inhibitor renders the cells less able to activate T lymphocytes. BET inhibitors have already been shown to inhibit secondary response genes in mouse macrophages [39] and IFNγ activation in human monocytes [68, 69] but our data here reveals the impact on cell fate with the ability to skew monocyte-macrophage phenotype and function, akin to that seen previously with BET inhibitors in T cells [70-72].

We proceeded to determine the mediators critical to GM-CSF differentiation which were uniquely sensitive to disruption by I-BET. A group of 369 genes were found to be robustly upregulated upon GM-CSF differentiation and reversed in response to BET protein inhibition, 23 of these genes were directly regulated by BET protein binding, exhibiting H3K27Ac binding at 1 hour of differentiation and differential expression in the presence of I-BET. In line with literature [37], the H3 K27Ac histone mark was found to be most sensitive to BET inhibition at these loci and when ranked in order of percent reduction in acetylation *INHBA* was identified as a key mediator. Examination of the *INHBA* gene locus determined that I-BET treatment of pro-inflammatory macrophages reduced BRD4 protein binding and K27 acetylation to levels comparable to those observed in homeostatic M-CSF monocytes. The effect of BET inhibition at the chromatin level was borne out at the protein level as Activin A was selectively expressed by Mϕ GM-CSF, expression that was abrogated upon I-BET treatment.

I-BET151 attenuated arthritis in the rat CIA and this is in line with literature reports. For instance, I-BET151 inhibited the MYC-NFATC1 axis, that promotes the differentiation of osteoclasts, leading to a reduction of osteoclastogenesis in TNF-induced inflammatory osteolysis and inflammatory arthritis [73]. I-BET151 also potently blocked cytokine production and secretion of MMP1, MMP3, IL-6 and IL-8 in synovial fibroblasts isolated from patients with RA [24].

BET inhibitors and the role of BET proteins have been explored in multiple models of RA and shown pleiotropic effects [74-76]. SNPs in BRD2 have been associated with a subtype of RA patients [77]. Epigenomic studies in fibroblast-like synoviocytes (FLS) from RA patients showed increased levels of acetylation of H3K27 in inflammatory pathways. BET inhibition changed the

chromatin landscape and blocked the induction of fibroblast-sustained genes, that are enriched in binding motifs for NF-κB, IRFs and AP-1 [78]. Studies with other pan BET inhibitors, such as JQ1 show blockade of the activation of super-enhancers genes for inflammation, and in RA FLS block the expression of pro-inflammatory pathways and alters the genome occupancy of transcription factors associated to inflammation [52]. Furthermore, JQ1 also exhibits efficacy in the CIA model through inhibition of IL-1β, IL-6, IL-18 and other cytokines [79].

BET inhibitors have shown broad anti-inflammatory and anti-fibrotic activity [39, 80-88] but progression to clinical trials has been mostly limited to oncology patients [89-91]. The notable exception outside of oncology is Apabetalone, a BD2 selective inhibitor, which has advanced to Phase III trials for cardiovascular disease alone and in combination with type 2 diabetes [92]. BD1 and BD2 domains form two separate subfamilies, and compounds able to selectively bind to BD2 have been reported [13, 93, 94], with RVX-208 (Apabetalone) having shown reduction in major cardiovascular events and lower levels of CRP in patients [95]. Apabetalone is also being evaluated in patients with vascular dementia or chronic kidney disease [96]. Clinical trials with pan BET inhibitors have exhibited dose-limiting toxicities likely preventing exposures needed for anti-tumor activity [97].

Our data with I-BET151 provides an interesting paradigm where a short duration of exposure in an *in vivo* model produced the same level of efficacy as when dosed continuously. These data are suggestive that BET inhibitors may have the potential to induce efficacy after short periods of dosing which may mitigate some of the potential tolerability and dose-limiting toxicities.

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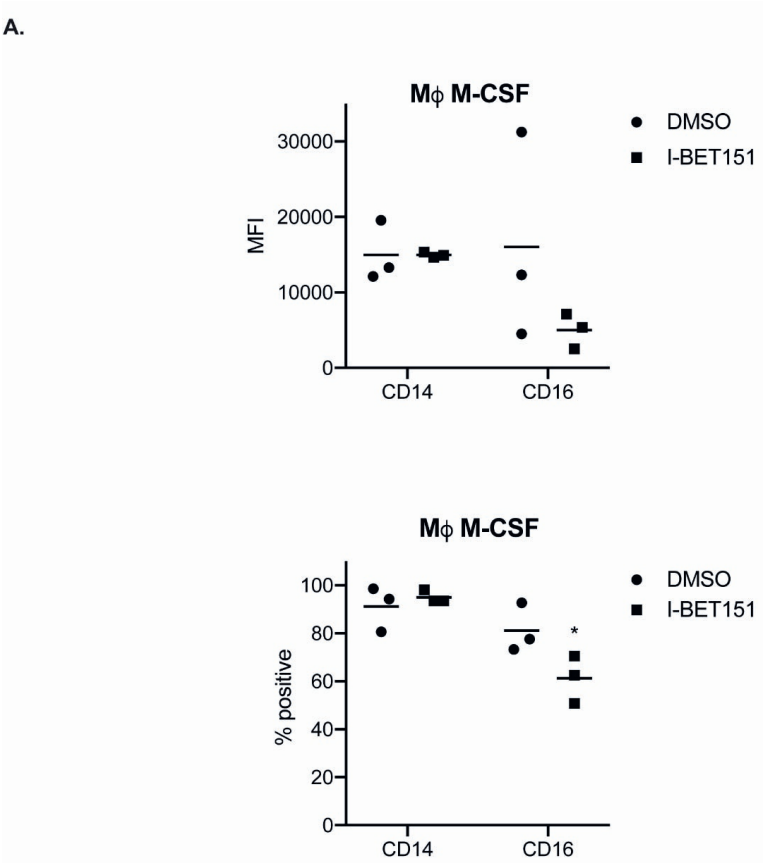
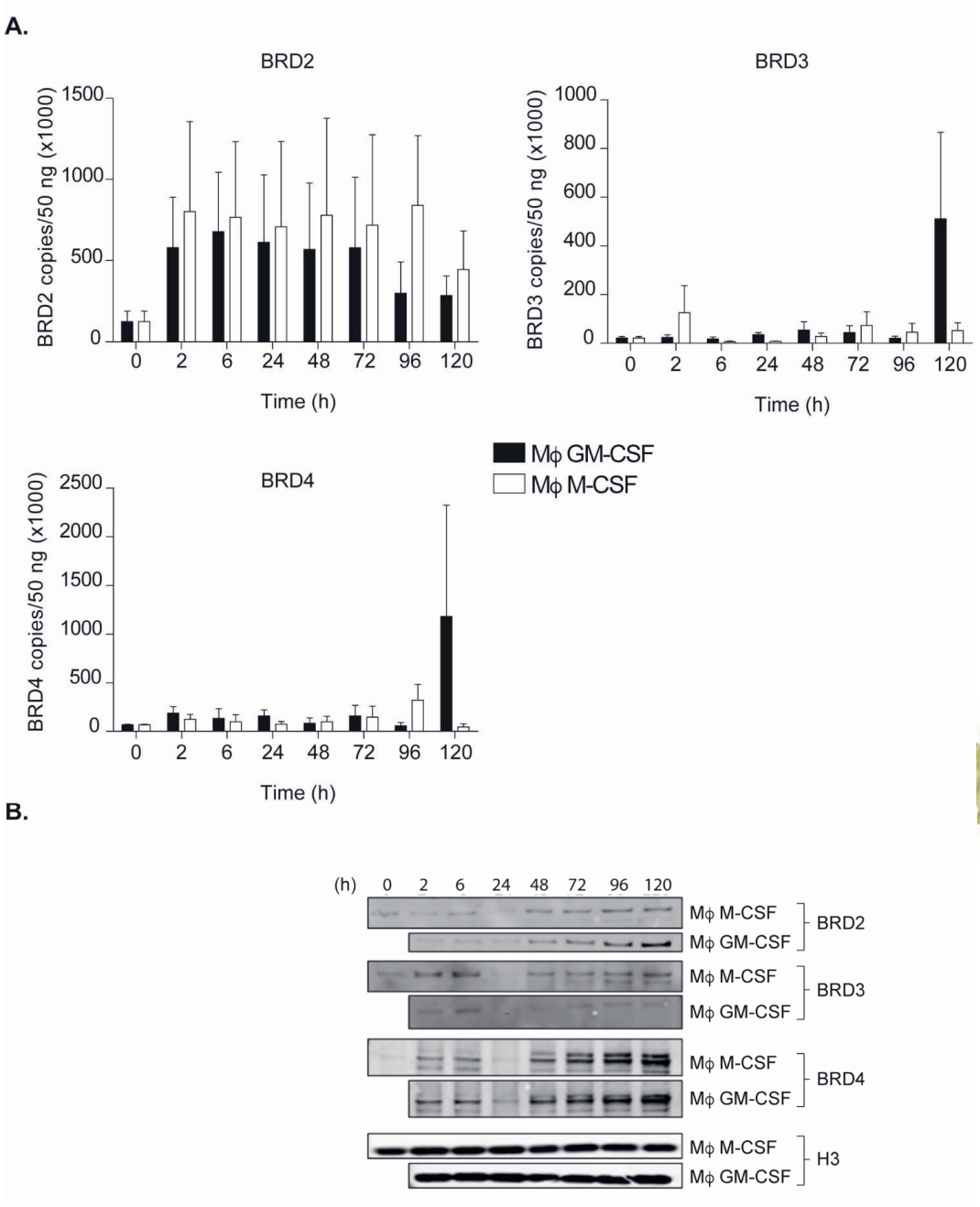


Supplementary Figure 1

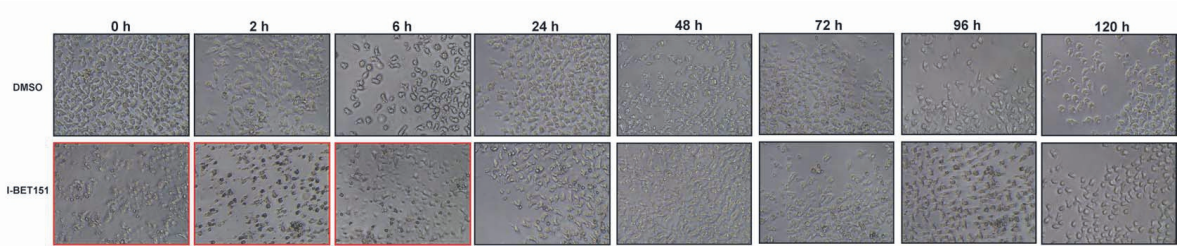
A. CD14 and CD16 expression assesed by flow cytometry in Mϕ M-CSF generated in the presence of 0.1% DMSO or 100 nM I-BET151 over 5 days. MFI for CD14 and CD16 are shown across the two treatment groups (top panel) and the percentage of macrophages expressing these markers (bottom panel). Data shown are from three independent donors and all conditions are donor-matched.



Supplementary Figure 2

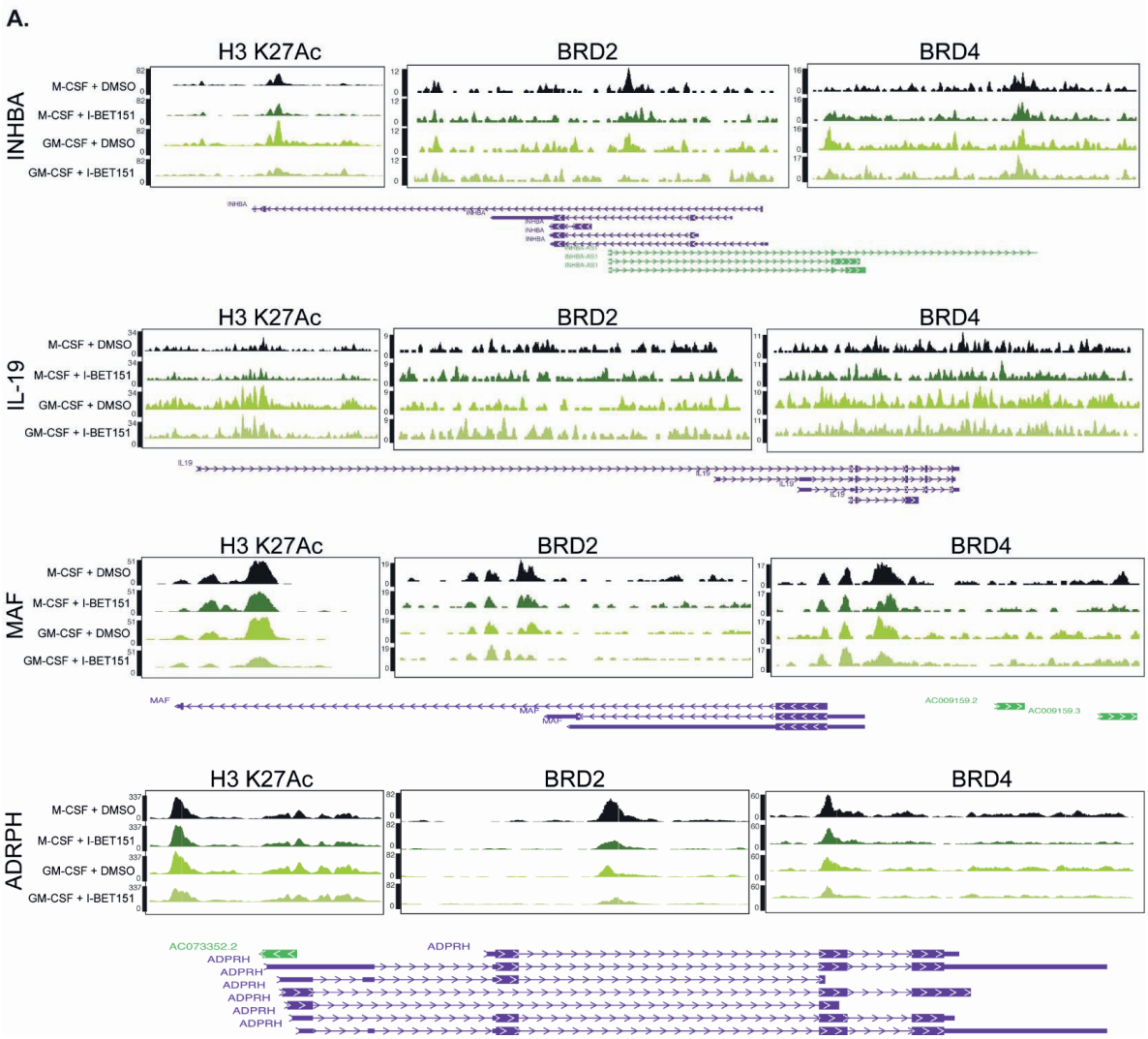
A. RT-PCR analysis of BRD2, BRD3 and BRD4 mRNA expression (normalised to UBB) in monocytes (t=0) or monocytes differentiating into Mϕ GM-CSF or Mϕ M-CSF over 5 days. **B.** Immunoblotting of BRD2, BRD3 and BRD4 in monocytes (t=0) or monocytes differentiating into Mϕ GM-CSF or Mϕ M-CSF over 5 days. Histone 3

(H3) immunoblotting is shown as a loading control for all samples. Data shown are Mean ± SEM from 3 separate donors or images from a representative donor. 2-way ANOVA test with Bonferroni correction (* p value < 0.05, ** p value <0.01, *** p value < 0.001, **** p value < 0.0001).



Supplementary Figure 3

Morphology of MΦ GM-CSF at 5 days where 100 nM I-BET151 or DMSO were added to differentiating cells at different time points (0 h, 2 h, 6 h, 24 h, 48 h, 72 h, 96 h, 120 h). Images shown from one representative donor.



Supplementary Figure 4
UCSC gene browser view illustrating the effect of I-BET151 on BRD2 and BRD4 binding and acetylation of H3K27 histone mark at the *IL-19*, *MAF* and *ADRPH* loci in monocytes stimulated with M-CSF or GM-CSF for one hour. The *INHBA* locus is shown for comparison.

The Supplementary Table I (data set) can be send upon request.

Chapter 6

Targeting histone deacetylases in myeloid cells inhibits their maturation and inflammatory function with limited effects on atherosclerosis

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*Equal contribution

Frontiers in pharmacology, 2019. 10, 1242.

Abstract

Monocytes and macrophages are key drivers in the pathogenesis of inflammatory diseases. Epigenetic targets have been shown to control the transcriptional profile and phenotype of these cells. Since histone deacetylase protein inhibitors demonstrate profound anti-inflammatory activity, we wanted to test whether HDAC inhibition within monocytes and macrophages could be applied to suppress inflammation *in vivo*. ESM technology conjugates an esterase-sensitive motif (ESM) onto small molecules to allow targeting of cells that express carboxylesterase 1 (CES1), such as mononuclear myeloid cells. This study utilised an ESM-HDAC inhibitor to target monocytes and macrophages in mice in both an acute response model and an atherosclerosis model. We demonstrate that the molecule blocks the maturation of peritoneal macrophages and inhibits pro-inflammatory cytokine production in both models but to a lesser extent in the atherosclerosis model. Despite regulating the inflammatory response, ESM-HDAC528 did not significantly affect plaque size or phenotype, although histological classification of the plaques demonstrated a significant shift to a less severe phenotype. We hereby show that HDAC inhibition in myeloid cells impairs the maturation and activation of peritoneal macrophages but shows limited efficacy in a model of atherosclerosis.

Introduction

Emerging evidence suggests that epigenetics play a crucial role in regulating immune cell function and may therefore offer many potential therapeutic opportunities for immune-mediated inflammatory diseases. In recent years, the identification of selective inhibitors of epigenetic enzymes and reader proteins has advanced our understanding of chromatin regulation of gene expression leading to renewed therapeutic efforts to reduce disease progression [1, 2].

Histone deacetylase proteins (HDAC) are a family of proteins that remove acetyl groups from lysine residues on histone tails and other proteins. The removal of these acetyl groups from histones causes DNA to be more compact, leading to a decrease in gene expression. There are 18 HDACs subtypes within the HDAC family that are sub-divided into 4 classes (I, II, III, IV) based on their homology to yeast proteins [3]. HDACs in monocytes and macrophages are involved in multiple processes, from maturation to inflammatory response [4]. The classical inhibitors for these proteins broadly target classes I, II and IV, which include 11 HDACs [5].

Currently, the use of inhibitors in the clinic is limited to oncology patients due to limiting side effects [6-9]. Since the inhibition of HDACs offers great potential in several immune-mediated inflammatory diseases [10-12], the specific targeting of immune cells with inhibitors of epigenetic enzymes may be key to success in non-oncology patients.

Carboxylesterase (CES) enzymes transform membrane-permeable esters into charged acids that are less able to cross the membrane [13]. CES1 expression in humans is restricted to hepatocytes and cells of the mononuclear myeloid lineage, such as monocytes and macrophages [14, 15]. Based on this expression pattern, small molecules with an esterase-sensitive motif (ESM) are selectively hydrolysed by CES1 enabling specific targeting of these cells. The ester-drug leads to the generation and retention of the charged acid, which is also pharmacologically active, within CES1-expressing cells. For instance, the combination of ESM technology with an HDAC inhibitor results in an increase of acetylation levels specifically in monocytes [16].

The inhibition of HDAC enzymes has shown wide-ranging anti-inflammatory effects [8, 17] with demonstrated efficacy in mouse models of inflammatory diseases [18-21]. Furthermore, monocytes and macrophages have an important role in the development and initiation of atherosclerosis [22-24]. Atherosclerosis is a lipid-driven disease that involves chronic inflammation. Monocytes and macrophages detect and phagocytose oxidized low density lipoproteins (OxLDL), becoming foam cells and acquiring a pro-inflammatory phenotype [25, 26]. Modulating this phenotype should have beneficial effects in the outcome of the disease.

Based on the importance of myeloid cells in atherosclerosis and the efficacy seen with HDAC inhibitors in models of inflammatory diseases, we wished to evaluate whether HDAC inhibition in myeloid cells would be sufficient to drive efficacy. In our studies we used a previously

characterized molecule, CHR-4487 (ESM-HDAC528) [16]. A related HDAC inhibitor also using ESM technology (Tefinostat) is being evaluated for efficacy in myeloid oncology indications [27, 28]. However, the application of ESM technology outside of oncology therapies has not been fully explored. In our studies, we tested whether the ESM-HDAC528 targeting would deliver efficacy in a model of atherosclerosis. We found that compound modulated the pro-inflammatory phenotype and maturation of macrophages, with limited effect on reducing severity of plaques in atherosclerosis but no significant improvement in other disease parameters.

Materials and methods

Compounds

The compound used in the studies was ESM-HDAC528 (also termed CHR-4487) described in [16]. The structure of the compound is shown in **Supplementary Figure 1A**. For *in vitro* work the compound was dissolved in DMSO and used at a range of concentrations: 10 nM, 50 nM and 100 nM, and also 1000 nM and 10,000 nM for viability studies. For the *in vivo* studies the compound was used at 3 mg/kg. The compound was dissolved in PBS without calcium and magnesium (PBS -/-), 5% DMSO and 11.25% cyclodextrin. 100 µL of either vehicle control or compound was injected intraperitoneally (i.p.) daily for 4 days for the thioglycollate model and 4 weeks for the atherosclerosis model.

Animals

The human CES1 transgenic mouse (*CES1/Es1e^{lo}*) was generated by Genoway (Lyon, France) from C57BL/6 mice by targeted insertion of the expression cassette into the expression permissive *hprt* locus on the X chromosome by homologous recombination. Expression of the *CES1* transgene was driven by the human CD68 promoter, which has previously been shown to direct transgene expression in macrophages of transgenic mice [29]. These mice were then cross-bred with a naturally plasma esterase-low *Es1e^{lo}* mouse (obtained from Jackson Labs USA: strain 000785 - B6;D2-a *Ces1c^{e}*/EiJ) at Charles River (Margate, UK). From here on, these animals will be referred to as "transgenic mice" or "*CES1/Es1e^{lo}*". Control C57BL/6 mice wild type (WT) were used in the *in vitro* experiments. In the acute study, twelve 10-week male *CES1/Es1e^{lo}* mice were divided in filter-top cages and injected with thioglycolate. Mice were divided in two groups (n=6 per group) and injected either with 3 mg/kg ESM-HDAC528 or vehicle via intraperitoneal (i.p.) injection daily from the day of the thioglycolate injection. On day 3, blood was collected 3 h after i.p. injection and on day 4, mice were sacrificed 24 h after the last injection for collection of blood and peritoneal cells.

For atherosclerosis experiments, we made use of low-density lipoprotein receptor knock-out mice (*ldlr^{-}/$^{-}$*) which are prone to develop atherosclerosis. *Ldlr^{-}/$^{-}$* mice (C57BL/6 non *Es1e^{lo}*) were obtained from Jackson laboratories. A bone marrow transplantation (BMT) was performed by

transferring bone marrow from *CES1/Es1e*^{lo} mice provided by GlaxoSmithKline into the *ldlr*$^{-/-}$ mice. Forty 10-week old female *ldlr*$^{-/-}$ mice were allocated to filter-top cages and provided with water containing neomycin (100 mg/L, Sigma, Zwijndrecht, the Netherlands) and polymyxin B sulphate (60,000 U/L, Invitrogen, Bleiswijk, The Netherlands) from 1-week pre-BMT until 5 weeks post-BMT. The animals received 2x6 Gy total body irradiation on two consecutive days. Bone marrow from *CES1/Es1e*^{lo} mice was resuspended in RPMI-1640 (Gibco, Breda, The Netherlands) with 5 U/mL heparin and 2% heat inactivated FCS (Gibco, Breda, the Netherlands) and 10^7 cells were injected intravenously per irradiated mouse. Bone marrow transplantation efficiency was determined by qPCR for relative presence of the LDL receptor on DNA isolated from blood (GE Healthcare, Eindhoven, the Netherlands). One mouse was excluded from the analysis due to inefficient bone marrow transplantation (<80%). Five weeks after the BMT, the mice were put on a high fat diet (0.15% cholesterol, 16% fat, Arie Blok Diets, The Netherlands) for 10 weeks. In week 5, mice were divided in two equal groups by randomisation based on weight, cholesterol and triglyceride levels. One group received 3 mg/kg ESM-HDAC528 and the other received vehicle daily via i.p. dosing for 4 weeks. On week 9, 7 days prior sacrifice, blood was taken 3 h after i.p. injection of ESM-HDAC528 and on week 10, on the day of the sacrifice, 24 h after i.p. injection of the compound to perform flow cytometry analysis on the blood. After sacrifice, each animal's heart was excised and frozen in Tissue-Tek (DAKO, Eindhoven, The Netherlands) for histology. Two mice were sacrificed before the end of experiment as they reached humane endpoints. One additional mouse was excluded from the analysis due to insufficient tissue quality. A total of 17 mice from ESM-HDAC528 group were compared to 19 mice from the vehicle group for the histological analyses and 18 versus 19 for the flow cytometry experiments, where mice with low number of total events were also excluded.

All animal experiments were conducted at the University of Amsterdam and approved by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam (permits: DBC242 and 103169-2). All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EEC and the GSK Policy on the Care, Welfare and Treatment of Animals.

Bone marrow-derived macrophage culture and functional study

Bone marrow was isolated from femurs and tibia of *CES1/Es1e*^{lo} and WT mice by flushing with RPMI-1640. The cells were cultured in RPMI-1640 with 25 mM HEPES and 2 mM L-glutamine, which was supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100 mg/mL) and 15% L929-conditioned medium as a source of M-CSF for 8 days. On day 8, cells were stimulated with LPS alone (10 ng/mL) or LPS (10 ng/mL) plus IFN-γ (100 U/mL) or left unstimulated for 24 h. Supernatants were collected and IL-6, IL-12(p40), and TNF were quantified by ELISA in accordance with the supplier's protocols (Life Technologies). Nitric oxide (NO) production was measured by NO_2^- quantification by the Griess reaction. To measure viability, the BMDMs from transgenic mice were pre-treated for 30 min with ESM-HDAC528 at 10 nM, 100 nM, 1000 nM

or 10000 nM). Afterwards BMDMs were left untreated or stimulated overnight with 20 μg/ml 7-ketocholesterol (7KC; Sigma), 50 μg/ml oxLDL or 10 μg/ml 25-hydroxycholesterol (25OHC; Sigma) and stained with propidium iodide (PI)/Annexin V-Alexa-Fluor647 according the manufacturer's instructions (Invitrogen). The percentage of viable macrophages (Annexin V-/PI-) was measured using a FACS Canto II.

After overnight ESM-HDAC528 pretreatment at 10 nM or 100 nM and DiI-oxLDL (Biotrend) treatment (3 h, 10 μg/ml), Dil-oxLDL uptake was measured by flowcytometry. Oxidized LDL uptake by BMDMs from transgenic mice was measured by flow cytometry. For lipid staining, BMDMs were pretreated with the inhibitors for 30 min, stimulated with 50 μg/ml oxLDL (BTI) for 24 h and stained with LipidTOX Red (Invitrogen) according to the manufacturer's instructions. The median fluorescence intensities (MFI) were calculated with FlowJo software version 10.4.2.

Peritoneal macrophages

Four days prior to the sacrifice, mice were injected intraperitoneally with 1 mL thioglycolate medium (3%, Fisher, Bleiswijk, The Netherlands). Upon sacrifice, the peritoneum was flushed with 10 mL ice cold PBS and peritoneal cells (PECs) were collected as described previously [30]. Flushed thioglycolate-elicited cells were cultured at a density of 100,000 cells/well in 100 μL in 96-well tissue culture plates (Greiner Bio-One, alphen a/d Rijn, The Netherlands) in RPMI-1640 containing 25mM HEPES, 2mM L-glutamine, 100 U/mL penicillin and 10% FCS (all Gibco, Breda, the Netherlands). After 3 h adherence, non-adherent cells were washed away and the adherent cells were left either unstimulated or stimulated for 24 h with LPS (10 ng/mL) alone, LPS (10 ng/mL) plus IFN-γ (10 U/mL) or 24 h with IL-4 (20 ng/mL). Supernatants were collected and IL-6, IL-12(p40) / IL-12(p70), and TNF were quantified by ELISA in accordance with the supplier's protocols (Life Technologies). NO production was measured by NO_2^- quantification in a Griess reaction. Cells were harvested using 1x Citrate from a 10X stock solution (1.35M potassium chloride (KCl), 0.15M sodium citrate, dilute in 100 mL milliQ and autoclaved) for 5 min at 37°C; the reaction was stopped by adding PBS-/- and cells were detached and washed twice with FACS buffer. Fc receptors were blocked with CD16/CD32 blocking antibody (1:100, eBioscence) in FACS buffer and cells were stained with appropriate antibodies **(Supplementary table 2)** for 30 min at RT. Cells were then washed with FACS buffer and fluorescence was measured with a CytoFLEX flow cytometer and analysed with FlowJo software version 10.4.2. Cells were gated by excluding doublets, then selecting the macrophages based on FSC-A/SSC-A parameters. Positive peaks for markers were defined based on isotype control antibodies and the median fluorescence intensity (MFI) was determined. This method was also used to measure the expression of alternative activation markers (PDL2, CD71, CD206, CD301) *in vitro* in BMDMs from transgenic mice following treatment with ESM-HDAC528 at concentrations of 10 nM, 50 nM and 100 nM and with IL-4 (20 ng/mL) for 24 h.

PECs were used immediately post-isolation to quantify mature peritoneal macrophages (PEMs) and intracellular lysine acetylation levels within those cells. Lysine acetylation was determined using the same protocol as for blood minus for the red blood lysis step. To evaluate maturation markers, cells were washed with FACS buffer and then stained with appropriate antibodies **(Supplementary table 3)** for 30 min at RT and Fc receptors were blocked with CD16/CD32 blocking antibody (1:100, eBioscence) in FACS buffer. Cells were then washed with FACS buffer and fluorescence was measured with a CytoFLEX flow cytometer and analysed with FlowJo software version 10.4.2. After removing the doublets, macrophages were defined as $CD11b^+$ and $F4/80^+$ and then maturation markers Ly6C and CD64 were measured in these populations.

Intracellular acetylation flow cytometry and triglyceride/cholesterol measurement

100 µL of blood was withdrawn from mice at 3 h and 24 h after i.p. injection of ESM-HDAC528. The blood was collected in tubes containing sodium heparin. For the 3 h time point mice were injected with 3 mg/kg ESM-HDAC528 and their food was restricted for 3 h in order to get an accurate measurement of triglycerides and cholesterol. 50 µL of blood was centrifuged (10 min, 4°C, 2000 rpm) to separate the plasma from blood cells. Plasma cholesterol and triglyceride levels were enzymatically measured according to the manufacturer's protocol (Roche, Woerden, The Netherlands). 50 µL of blood was further used for flow cytometry to measure intracellular acetylation at 3 h and 24 h. The blood was mixed 1:1 with PBS -/- and stained with cell surface marker antibodies for 30 min on ice (**Supplemental Table 1**). Red blood cells were lysed and cells were fixed by using BD FACS Lyse/Fix solution following the manufacturer's instructions (BD Pharmingen). After washing the cells twice with FACS buffer (0.5% BSA, 0.01% NaN_3 in PBS), cells were permeabilised using Human FoxP3 buffer following manufacturer's instructions (BD Pharmingen) and stained with an antibody for acetylated lysine (PanAck, Biolegend) for 30 min at RT. Cells were washed twice and resuspended in FACS buffer. Data were acquired using a BD Canto II and analysed with FlowJo software version 10.4.2. The cells were gated by excluding doublets, then $Ly6G^+$ neutrophils were distinguished from monocytes, B and T cells. Monocytes ($CD11b^+/CD115^+$) were distinguished from lymphocytes. Lymphocytes were further separated in B cells ($B220^+/CD3^-$) and T cells ($B220^-/CD3^+$). The MFI was determined from the positively stained cells (following FMO and isotype control corrections).

Histochemistry

Atherosclerotic lesions from the heart were cut into 7 mm sections on a Leica 3050 cryostat at -25°C. Cross area sections of 42 mm were stained with toluidine blue (0.2% in PBS, Sigma-Aldrich, Gillingham, UK) to determine lesion size. Total lesion size per section was measured using Adobe Photoshop CS4. Lesion severity was scored (0,1,2,3,4,5) by an experienced pathologist as no lesion (score 0) early (intimal xanthoma, scores 1,2), moderate (pathological intimal thickening, score 3) and advanced (fibrous cap atheroma, score 4,5), as described

elsewhere [31]. Sirius red staining was performed for 30 min to measure collagen content (0.05% Direct Red in saturated picric acid, Sigma, Zwijndrecht, the Netherlands). Images were obtained using a Leica DM3000 microscope and quantified with Adobe Photoshop CS4 where collagen was quantified as the percentage of total lesion size. For immunohistochemistry, slides were fixed in acetone and blocked with Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, USA). Hereafter, cells were incubated with MOMA-2 (1:4000, AbD Serotec, Uden, The Netherlands) to stain for macrophages, ER-MP58 (1:200, AbD serotec, Uden, The Netherlands) for infiltrating monocytes. Necrosis area was measured based on Toluidine Blue staining by a pathologist and corrected for total plaque size.

Human whole blood intracellular acetylation measurement

All donors provided written informed consent for use of their samples, and the collection and use of the samples received Institutional Review Board approval. Blood from healthy volunteer donors was collected into tubes containing sodium heparin anti-coagulant. 140 µL of blood was treated with compound for 4 h at 37°C after which samples were fixed and lysed for 15 min using FACS lysing solution (BD Pharmingen). Cells were washed with FACS buffer and Fc receptors were blocked using human IgG (Sigma) for 15 min at RT. Samples were stained at RT for 30 min with anti-CD66 (BD Pharmingen 551479) and anti-CD14 (BD Pharmingen 555399), to identify neutrophils and monocytes, after which samples were washed twice in FACS buffer and permeabilized for 30 min at RT using nuclear permeabilization buffer (Biolegend). Samples were then washed once and resuspended in nuclear permeabilization buffer containing anti-acetylated lysine antibody (Biolegend 623404) or a matched isotype control (R&D Systems IC0041P) and incubated at RT for 30 min **(Supplementary Table 4)**. Samples were washed twice in PBS and sample data were acquired using the BD FACS Canto II Flow Cytometer with FACS Diva (BD BioSciences software version 6.1.3.). Cells were gated by excluding doublets and neutrophils and monocytes identified. The remainder of non-stained, viable cells were defined as lymphocytes. The MFI (median fluorescent intensity) of acetylated lysine within each population was determined.

Statistical analysis

Data represent the mean ± standard error of the mean (SEM). Differences between groups were analysed using an unpaired student's *t*-test, two-way ANOVA using Bonferroni post-hoc test analysis for grouped analysis or Chi-Squared test. P values < 0.05 were considered statistically significant. Non-linear curves for concentration-response studies for the data from human whole blood intracellular acetylation experiments were also generated. Data were analysed using GraphPad Prism version 5.0 (GraphPad software, La Jolla, California).

To assess plaque severity (ranked 0,1,2,3,4,5) an average severity score (based on 2-3 sections per animal) was calculated to give a single value for each animal. A non-parametric Mann-Whitney

test was applied using Prism version 5.0 (GraphPad software, La Jolla, California) to determine whether the median score differed significantly between the treatments.

Results

ESM-HDAC528 reduces pro-inflammatory cytokine production

The mouse orthologue of CES1 significantly differs in distribution of expression and substrate specificity [32]. Therefore, we utilised transgenic mice containing the human *CES1* gene under the control of the *CD68* promoter. Bone marrow-derived macrophages (BMDMs) from transgenic or wild type (WT) mice were activated with LPS or LPS/IFNγ in the presence of the targeted HDAC inhibitor (ESM-HDAC528). Structurally**,** the ESM-HDAC528 compound is comprised of an HDAC inhibitor conjugated to an ester group. When the ester group is cleaved from the HDAC inhibitor, by the enzyme CES1, in the myeloid cells of the transgenic mice it gets accumulated within those specific cells **(Supplementary Figure 1A)**.

After stimulation, we observed a concentration-dependent inhibition of the production of pro-inflammatory mediators (IL-6, IL-12p70, NO) but not for TNF **(Figure 1A)**. No inhibition was observed with compound in WT macrophages at these concentrations, likely due to the lack of expression of the human enzyme. Further characterization of functions related to atherosclerosis of BMDMs from the transgenic mice after treatment with ESM-HDAC528 was also performed. First viability was assessed **(Supplementary Figure 1B)**. No effects on viability were observed in the cells after treatment with 10 nM and 100 nM of ESM-HDAC528. At higher concentrations (1,000 nM and 10,000 nM) the viability was reduced.

Based on these results, subsequent experiments were performed at 10 nM, 50 nM and 100 nM. The expression of alternative activation surface marker **(Supplementary Figure 1C)** after IL-4 stimulation was determined. No significant changes were observed except for a trend to reduction of expression at higher concentrations in CD206. Another important function is lipid uptake **(Supplementary Figure 1D)**; in this case no effects were observed in uptake of oxidized LDL after treatment with ESM-HDAC528.

Prior to *in vivo* studies, intracellular acetylation of white blood cells was determined in human whole blood treated with either the non-targeted, conventional HDAC inhibitor SAHA (suberanilohydroxamic acid) or ESM-HDAC528 **(Supplementary Figures 1E and 1F)**. ESM-HDAC528 is more potent than SAHA at increasing intracellular acetylation levels and this phenomenon was selectively observed in monocytes.

To assess if a suppressed macrophage response also manifested *in vivo*, an inflammatory response was initiated in *CES1/Es1e*^{lo} by a a single i.p. thioglycolate injection **(Figure 1B)**. It has previously been demonstrated that ESM-HDAC528 specifically targets circulating monocytes [16] in these

mice, and we wanted to extend this observation to peritoneal macrophages. In this study, mice were injected i.p. daily for 4 days with 3 mg/kg ESM-HDAC528 or vehicle and on day 4 peritoneal cells (PECs) were isolated. The total number of cells isolated from the ESM-HDAC528 group was significantly reduced compared to the vehicle group **(Figure 1C)**.

After 3 h attachment (to enrich for peritoneal macrophages; PEMs), cells isolated from both groups were stimulated *in vitro* with LPS or LPS/IFNγ. Interestingly, PEMs from the ESM-HDAC528 group produced lower levels of pro-inflammatory mediators after activation compared to equal numbers of plated PEMs from the vehicle group **(Figure 1D)**. These data indicate that ESM-HDAC528 reduces macrophage activation both *in vitro* and *in vivo*.

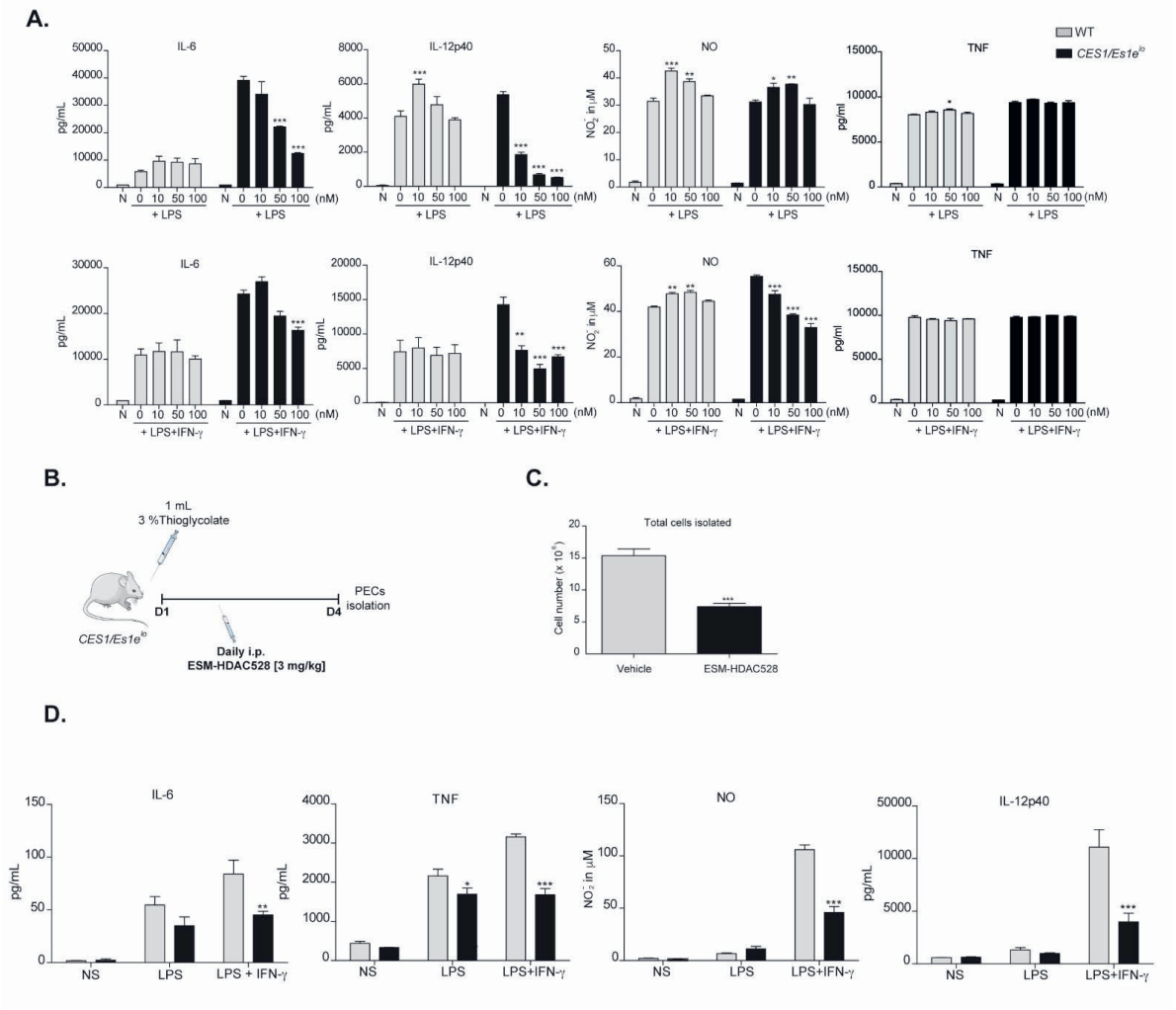

Figure 1: **ESM-HDAC528 reduces pro-inflammatory cytokine production both *in vitro* and *in vivo*.**
A. Cytokine production by BMDMs from CES-1/Es1e^{lo} mice and WT mice after stimulation with LPS (10 ng/mL) or LPS (10 ng/mL) + IFN-γ (10 U/mL) in the presence of increasing concentrations of ESM-HDAC528

for 24 h. n=3 **B**. Design of acute thioglycolate model. Transgenic mice were treated for 4 days with daily i.p. injection of 3 mg/kg ESM-HDAC528 (n=6) or vehicle (n=6), on day 4, 24 h after i.p. injection peritoneal cells were isolated. **C.** Total number of cells isolated from the peritoneal lavage in each group n=6 per group. **D.** tokine production by PEMs isolated from the mice (n=6) of each group attached and then stimulated for 24 h with LPS (10 ng/mL) or LPS (10 ng/mL) + IFN-γ (10 U/mL). Statistical significance was determined by unpaired t-test (C) or 2 way ANOVA with Bonferroni correction (A,D) ($p<0.05$). All error bars represent the SEM.

ESM-HDAC528 modulates the maturation of freshly isolated PEMs and the expression of macrophage activation markers on cultured PEMs.

We next wanted to understand whether the effects of an ESM-HDAC inhibitor on cytokine production was due to a change in polarisation or maturation. The maturation status was measured in freshly isolated PEMs. Mature PEMs can be defined as a $CD11b^+$ and $F4/80^+$ population [33, 34] **(Figure 2A)**. The percentage of this population was different between groups, with a reduction of 28% in the ESM-HDAC528 group compared to vehicle-treated mice. Other markers (Ly6C, CD64) were also measured within the macrophage population ($CD11b^+$ $F4/80^+$). Ly6C is a monocyte marker expected to be higher in immature macrophages [35] while CD64 is expressed in mature macrophages rather than in monocytes [36]. The population showed an increased percentage of $Ly6C^+$ cells and reduction in $CD64^+$ cells which indicates reduced maturation in the PEMs of the ESM-HDAC528 treatment group **(Figure 2A)**. Additionally, in cells that were mature ($CD11b^+$, $F4/80^+$) the MFI for these maturation markers (CD11b and F4/80) was lower in the ESM-HDAC528 group **(Figure 2B)**.

Next, we measured the expression of pro-inflammatory and alternative activation surface markers. The gating strategy used for stimulated cells is shown **(Figure 2C)**. After attachment, the cells are expected to be predominantly PEMs. After stimulation, cells were harvested, doublets excluded, and the PEMs were gated based on FSC-A/SSC-A gating. Surface markers were detected using either PE- or APC-conjugated antibodies. The positive peaks of those markers were defined using an isotype control and the MFI of the markers was determined from the positive population **(Figure 2C)**. CD80 expression was significantly decreased on both unstimulated and stimulated PEMs from ESM-HDAC528 treated mice compared to vehicle controls. Furthermore, CD86 expression was significantly lower in LPS-treated PEMs **(Figure 2D)**. No effects were observed on PDL2 and CD71, IL-4-induced markers of alternatively activated macrophages **(Figure 2E)**. We conclude that ESM-HDAC528 blocks PEM maturation and inhibits the expression of pro-inflammatory markers.

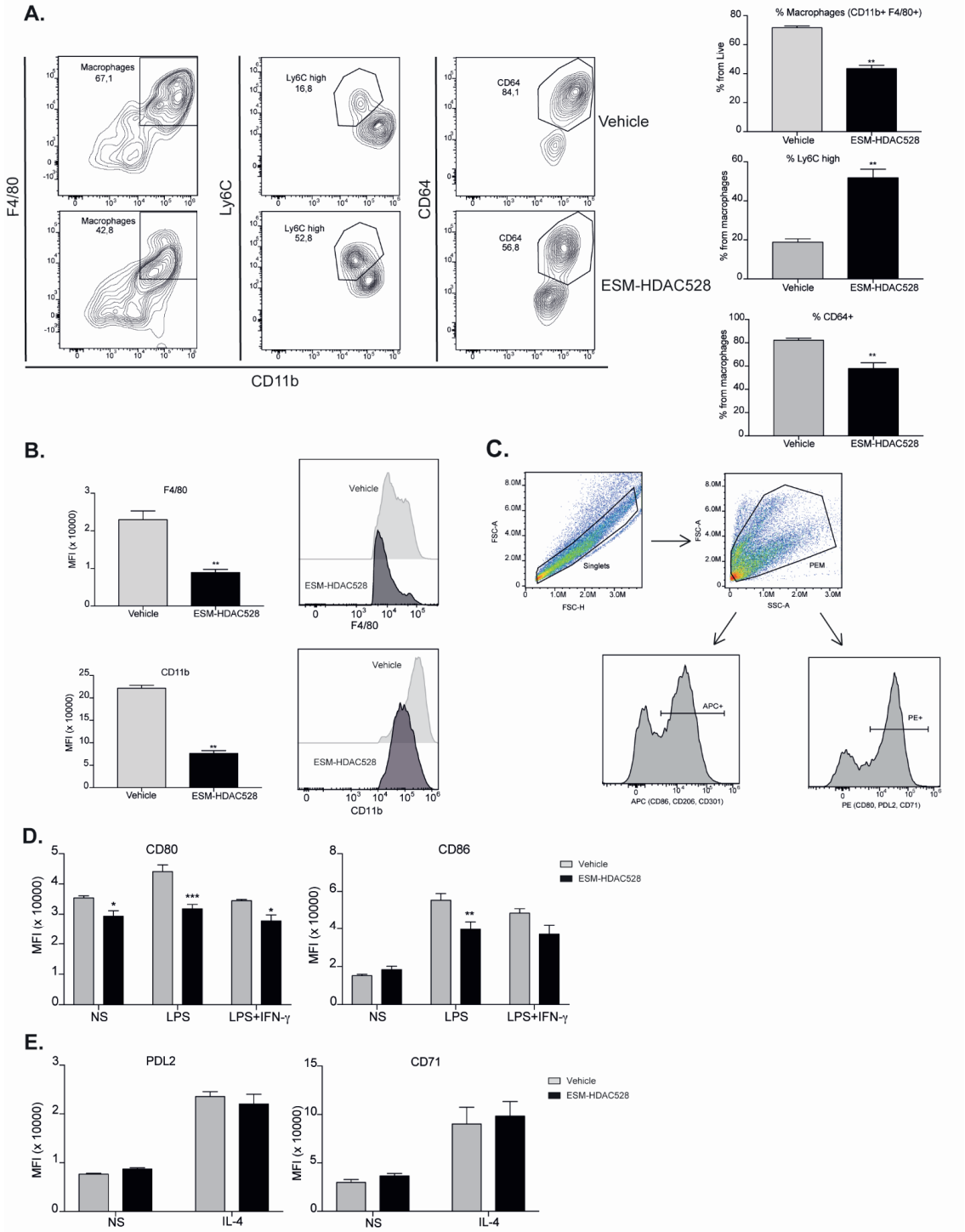
A.
F4/80
CD11b
Ly6C
CD64
Macrophages 67,1
Macrophages 42,8
Ly6C high 16,8
Ly6C high 52,8
CD64 84,1
CD64 56,8
Vehicle
ESM-HDAC528
% Macrophages (CD11b+ F4/80+)
% from Live
% Ly6C high
% from macrophages
% CD64+
B.
F4/80
MFI (x 10000)
CD11b
C.
FSC-A
FSC-H
Singlets
SSC-A
PEM
APC+
PE+
APC (CD86, CD206, CD301)
PE (CD80, PDL2, CD71)
D.
CD80
CD86
NS
LPS
LPS+IFN-γ
E.
PDL2
CD71
IL-4

Figure 2: **ESM-HDAC528 treatment modulates PEM maturation and surface marker expression**. **A.** Percentage of mature macrophages (CD11b+ and F4/80+), and the maturation markers Ly6C and CD64 within the mature macrophages in the freshly isolated cells 24 h after injection n=6 per group. **B**. MFI of F4/80 and CD11b in the mature macrophages. n=6 per group. **C**. General gating strategy for activation marker expression on PEMs after attachment and 24 h stimulation for activation. Antibodies were conjugated to either APC or PE depending on the panel. **D**. MFI of the positive peaks for the pro-inflammatory surface markers in PEMs isolated attached and stimulated for 24 h with LPS (10 ng/mL) or LPS (10 ng/mL) + IFN-γ (10 U/mL). n=6 per group **E.** MFI of the positive peaks for the alternative activation surface markers in PEMs attached and stimulated for 24 h with IL-4 (20 ng/mL). n=6 per group. Statistical significance was determined by unpaired t-test (A, B) or 2 way ANOVA with Bonferroni correction (D,E) ($p<0.05$) All error bars represent the SEM.

ESM-HDAC528 treatment does not affect lipid levels in an atherosclerosis model

The preceding experiments demonstrated that ESM-HDAC528 affects the pro-inflammatory and maturation status of macrophages. We next wanted to test if this would be of benefit in a model of atherosclerosis. In this *in vivo* atherosclerosis study *ldlr* $^{-/-}$ mice were irradiated and transplanted with bone marrow from *CES1/Es1e*^{lo} mice. Mice were fed for 10 weeks on a high fat diet (HFD) and treated from week 5 either with 3 mg/kg ESM-HDAC528 or vehicle **(Figure 3A)**.

The efficiency of the bone marrow transplantation, measured by chimerism, was equal and above the threshold of 85% for the mice divided between both analysis groups **(Figure 3B)**. The mean weight remained similar across the study in both treatment groups **(Figure 3C)**. As expected, triglycerides and cholesterol levels increased over the study duration. However, levels of both analytes remained similar in both groups **(Figure 3D)**.

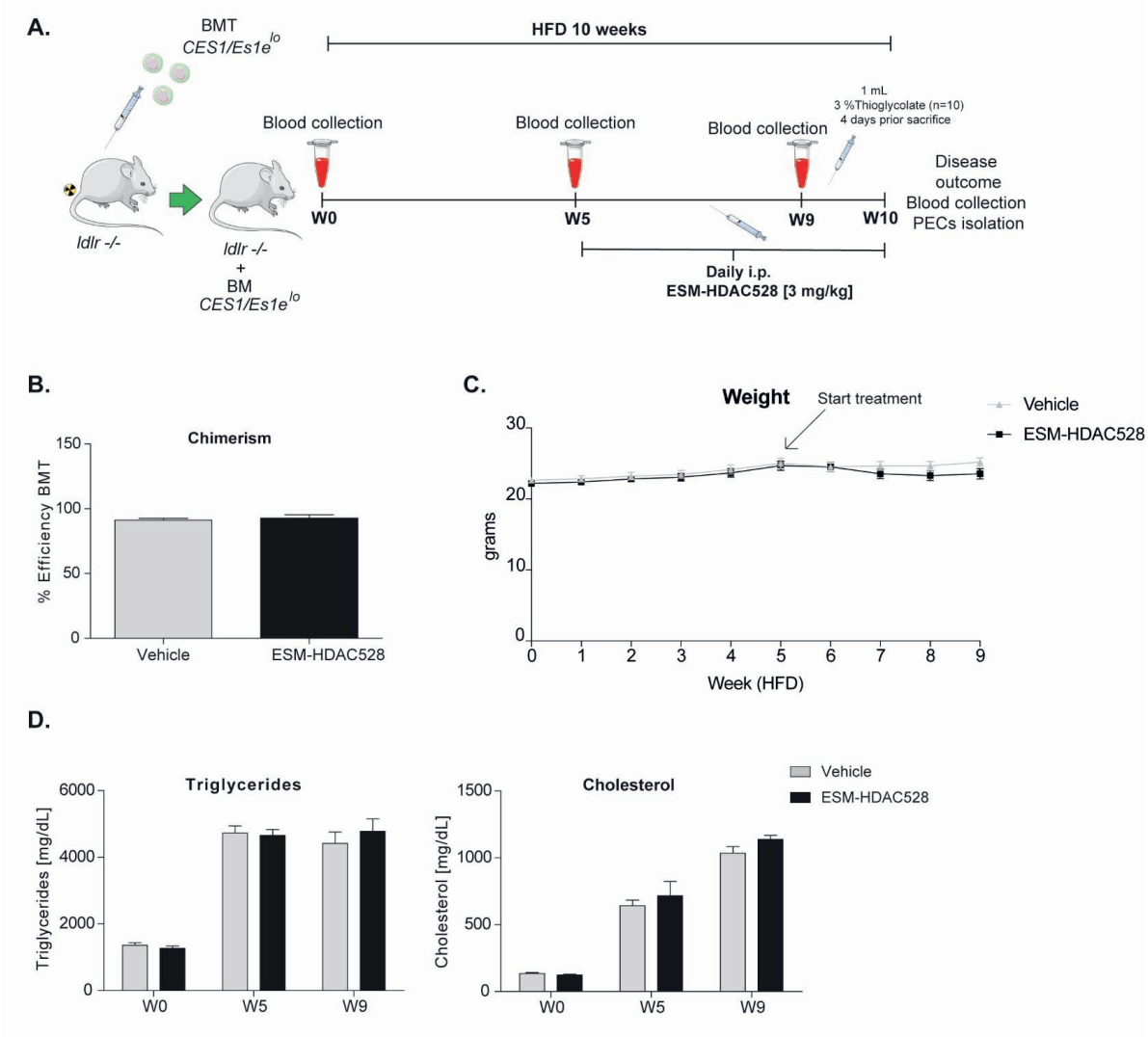

Figure 3: **ESM-HDAC528 treatment does not affect clinical features in a model of atherosclerosis.** **A.** Study design for the atherosclerosis study. The ldlr knockout mice were transplanted with bone marrow from CES-1/Es1elo mice. The mice were divided in 2 groups ESM-HDAC528 (n=19) and vehicle (n=19). Mice were on high fat diet (HFD) for 10 weeks. On week 5 the mice were treated with 3 mg/kg ESM-HDAC528 or vehicle by daily i.p. injection. Blood was collected on week 0, 5, 9 and 10. On week 10 mice were sacrificed, the disease outcome was assessed and PECs were isolated. **B.** Efficiency of the bone marrow transplantation in both groups. n=19 vehicle vs n=18 ESM-HDAC528. **C.** Weight of the mice from both groups during the study. n=19 vehicle vs n=18 ESM-HDAC528 D. Triglycerides and cholesterol levels of the groups on week 0,5 and 9. n=19 vehicle vs n=18 ESM-HDAC528. Statistical significance was determined by unpaired t-test (B) or 2 way ANOVA with Bonferroni correction (C, D) ($p<0.05$). All error bars represent the SEM.

ESM-HDAC528 increases acetylation specifically in murine myeloid cells

To confirm the targeted activity of ESM-HDAC528 we measured the levels of acetylation in circulating white blood cells using the gating strategy described **(Figure 4A).** In blood samples collected 3 h after ESM-HDAC528 treatment there was a significant increase in acetylation in monocytes. The acetylation levels in other immune cells was unchanged compared to the vehicle control, showing the specific targeting of this compound to mononuclear myeloid cells **(Figure 4B)**. After 24 h, when compound was no longer systemically detectable, acetylation was modestly increased in neutrophils in this study, although monocyte acetylation had reverted to similar levels in both groups **(Figure 4C)**.

We also determined acetylation in freshly isolated PEMs and found higher levels in the compound group compared to the vehicle, demonstrating specificity of the compound not only in monocytes but also in macrophages in this mouse model **(Figure 4D)**.

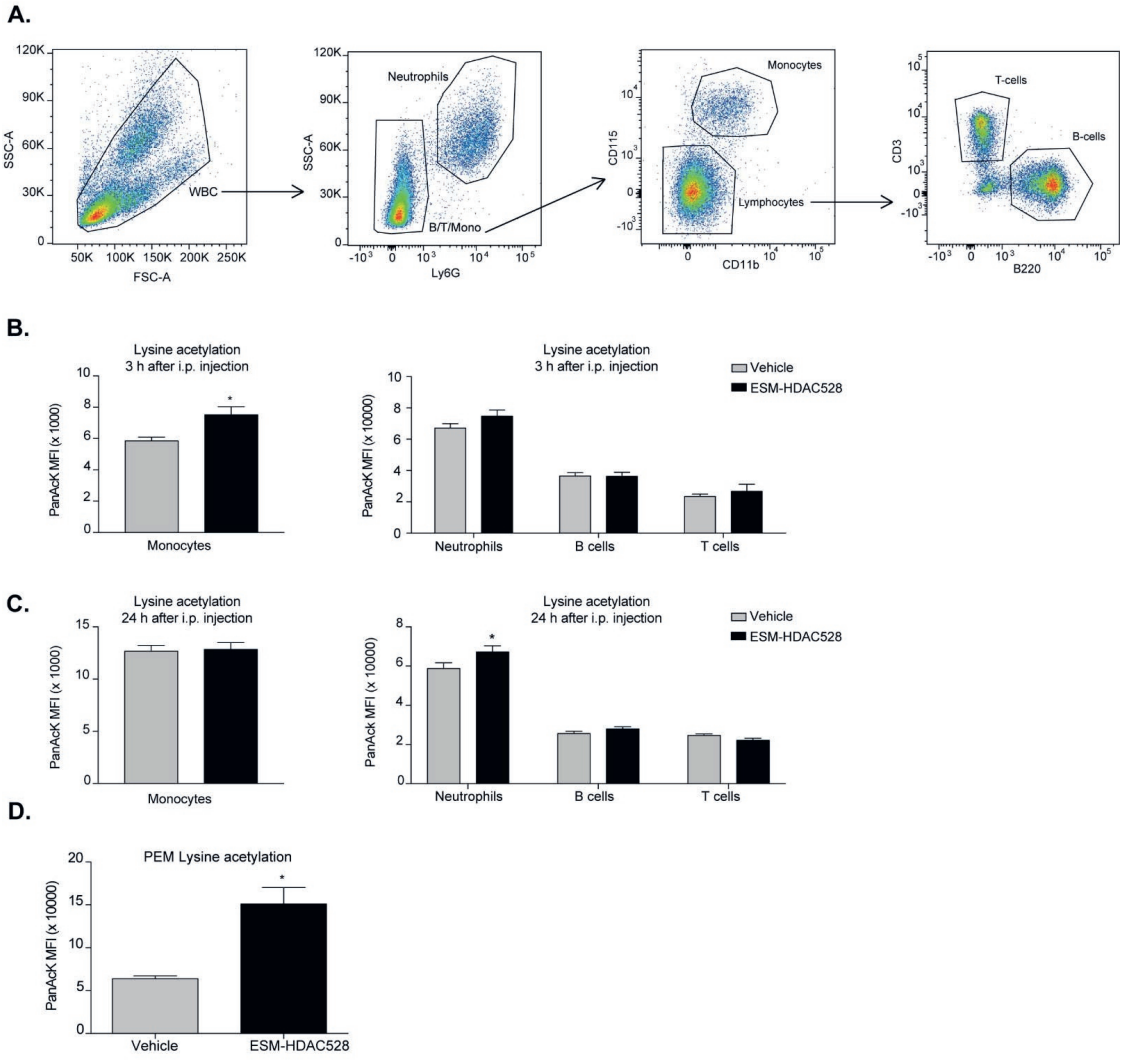

Figure 4: **ESM-HDAC528 selectively increases acetylation in myeloid cells**. **A**. Gating strategy for white blood cells after doublet exclusion. **B**. MFI of intracellular acetylation levels in monocytes and other WBC 3 h after i.p. injection. n=19 vehicle vs n=18 ESM-HDAC528 **C**. MFI of intracellular acetylation levels in monocytes and other WBC 24 h after i.p. injection. n=19 vehicle vs n=18 ESM-HDAC528 **D**. MFI for intracellular acetylation levels in fresh PEMs isolated 24 h after i.p. injection. n=5 per group. Statistical significance was determined by unpaired t-test (B, C) or 2 way ANOVA with Bonferroni correction (B, C, D) ($p<0.05$). All error bars represent the SEM.

ESM-HDAC528 modulates PEM maturation and activation to a lesser extent in the HFD atherosclerosis model

We wanted to evaluate whether ESM-HDAC528 dampened macrophage maturation and activation in the atherosclerosis model to a similar extent to that seen in the acute model **(Figure 2)**. The percentage of mature macrophages ($CD11b^+$ $F4/80^+$) following thioglycolate administration, was lower in the compound treated group **(Figure 5A)**. Within this macrophage population, maturation markers also showed the same trend as previously observed, with CD64 expression being significantly lower in the compound group and a trend for increased Ly6C **(Figure 5A)**. The expression levels of the maturation markers CD11b and F4/80 (as assessed by MFI) was significantly lower for CD11b and there was a trend towards a reduction of F4/80 expression within the mature macrophages in the ESM-HDAC528 treatment group **(Figure 5B)**. Overall, we observed an effect of the compound on the maturation of macrophages, although the magnitude of change was generally weaker than observed in the acute model.

The gating strategy for the measurement of activation markers was as previously defined **(Figure 5C)**. Interestingly, in contrast to our previous observation, in PEMs from atherosclerotic mice stimulated with LPS alone or in addition to IFNγ, an increase of CD80 was seen following ESM-HDAC528 treatment. However, for LPS+IFNγ induced expression of CD86 there was a reduction in the ESM-HDAC528 treated animals **(Figure 5D)**. For the alternative activation markers following IL-4 stimulation there was an increase of CD71 and PDL2 and a reduction of CD301 with ESM-HDAC528 **(Figure 5E)**. Pro-inflammatory mediators were also measured, and, except for NO, which was reduced in the ESM-HDAC528 group, there was no significant inhibition of the production of pro-inflammatory mediators by macrophages **(Figure 5F)**. In general, ESM-HDAC528 had a reduced ability to inhibit macrophage maturation in this model. Additionally, the effects on pro-inflammatory mediators were milder and polarisation markers were inconsistent with inhibiting a pro-inflammatory phenotype.

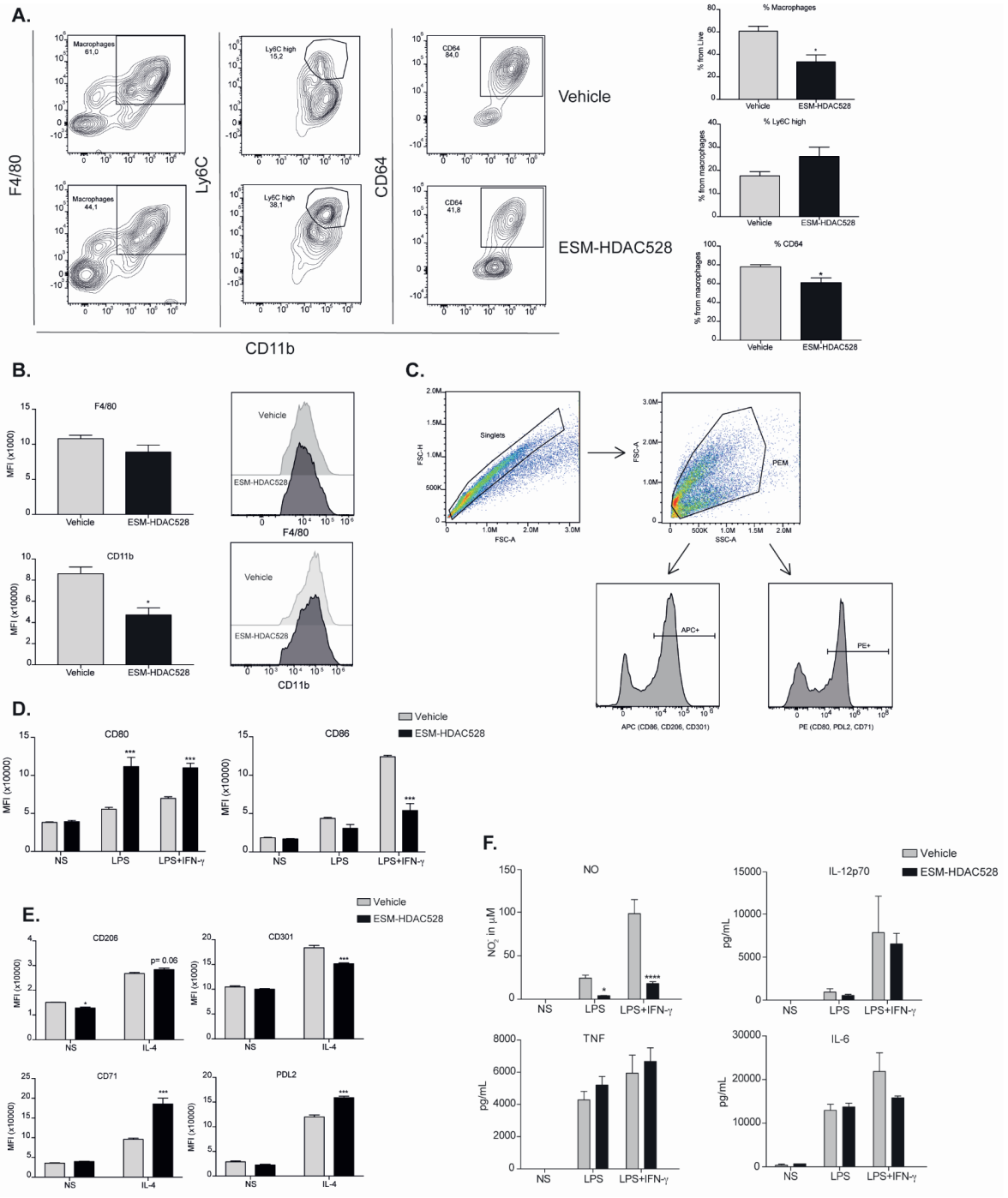
A.
F4/80
Ly6C
CD64
CD11b
Macrophages 61,0
Macrophages 44,1
Ly6C high 15,2
Ly6C high 38,1
CD64 84,0
CD64 41,8
Vehicle
ESM-HDAC528
% Macrophages
% from Live
% Ly6C high
% from macrophages
% CD64
B.
F4/80
MFI (x1000)
CD11b
MFI (x10000)
C.
Singlets
FSC-H
FSC-A
SSC-A
PEM
APC+
PE+
APC (CD86, CD206, CD301)
PE (CD80, PDL2, CD71)
D.
CD80
CD86
NS
LPS
LPS+IFN-γ
E.
CD206
CD301
CD71
PDL2
IL-4
p= 0.06
F.
NO
NO2 in µM
IL-12p70
pg/mL
TNF
IL-6

Figure 5: ESM-HDAC528 modulates PEM maturation and activation to a lesser extend in an atherosclerosis model. A. Percentage of maturate (CD11b+ and F4/80+) macrophages from freshly isolated PECs 24 h after ESM-HDAC528 injection, and of cells expressing Ly6C and CD64 within this population. n=5 per group **B**. MFI of F4/80 and CD11b in the maturate macrophages 24 h after injection. n=5 per group **C**. General gating strategy for activation marker expression on PEMs after attachment and 24 h stimulation for activation. Antibodies were conjugated to either APC or PE dependent on the panel. **D**. MFI of the positive peaks for pro-inflammatory surface markers in PEMs isolated attached and stimulated for 24 h with LPS (10 ng/mL) or LPS (10 ng/mL) + IFN-γ (10 U/mL). n=5 per group **E.** MFI of the positive peak for alternative activation surface markers in PEMs attached and stimulated for 24 h with IL-4 (20 ng/mL). n=5 per group F. Cytokine production by PEMs isolated from the mice (n=5) of each group stimulated 24 h with LPS (10ng/mL) or LPS (10ng/mL) + IFN-γ (10 U/mL). Statistical significance was determined by unpaired t-test (A, B) or 2 way ANOVA with Bonferroni correction (D, E, F) ($p<0.05$). All error bars represent the SEM.

Disease outcome after treatment with ESM-HDAC528

Considering the characteristics of the macrophages after the treatment with ESM-HDAC528, we wanted to understand the impact on the disease outcome. Therefore, the severity of the atherosclerotic lesions in the mice was scored. We observed a reduction in the percentage of the more severe phenotypes of the plaques (pathological intimal thickening and fibrous cap atheroma) in the ESM-HDAC528 treated mice together with an increase of the less severe phenotype (intimal xanthoma) **(Figure 6A)**. The severity scores of all lesions were combined to give a composite score per animal, showing that the animals treated with ESM-HDAC528 had a significantly reduced median severity score upon ESM-HDAC528 treatment compared to vehicle treated mice (ESM-HDAC528 median = 3.00; Vehicle median = 3.67; $p = 0.0163$). A trend for reduction in total plaque area was also observed ESM-HDAC528 treated mice **(Figure 6B)**.

For the rest of the disease parameters, there were no significant differences between the groups. Nevertheless, the necrotic plaque area and monocyte influx also showed a tendency to be reduced in the atherosclerosis model and the increase in collagen could indicate a more beneficial phenotype **(Figure 6C)**. In conclusion, in parallel to effects on maturation of the macrophages, atherosclerotic plaque severity was partially improved by the ESM-HDAC528 treatment in this model.

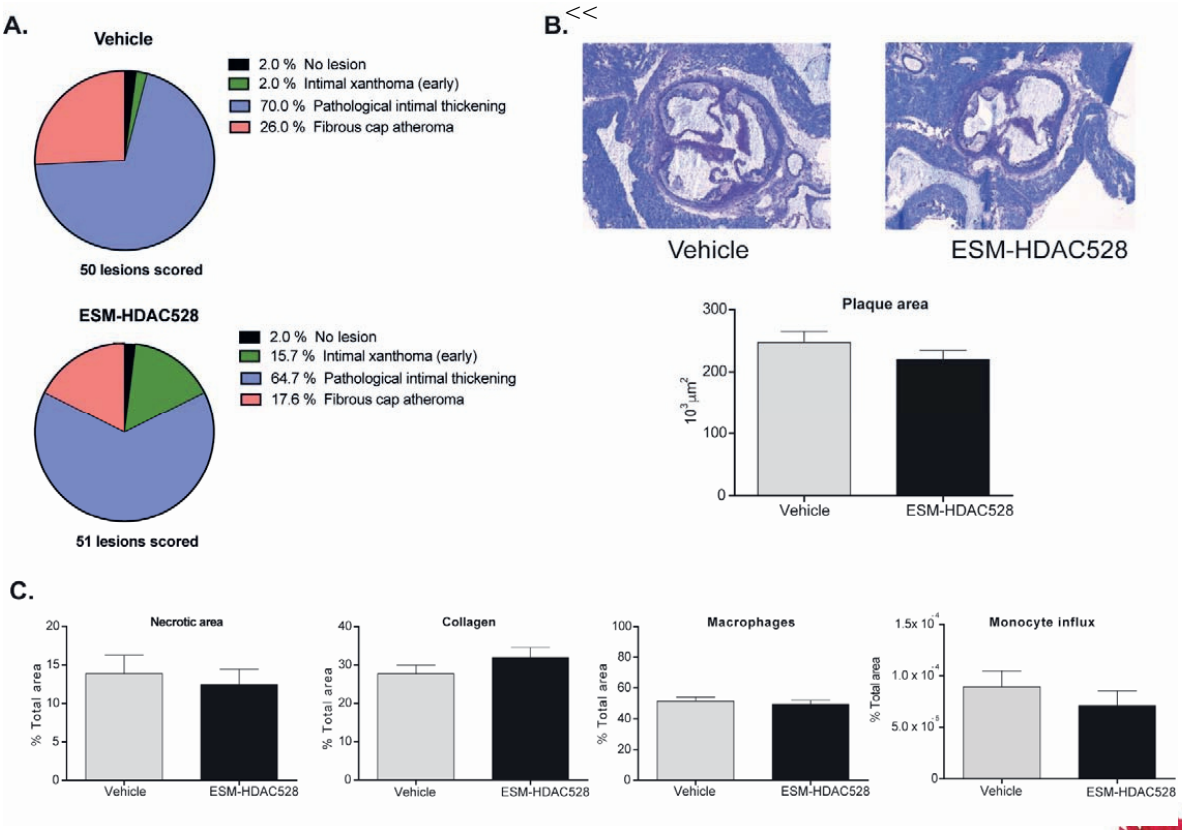

Figure 6: **ESM-HDAC528 does not reduce plaque formation**. **A**. Severity of the plaque for the different groups, the plaque is rated according to the morphology. n=50 lesions in vehicle vs n=51 lesions in ESM-HDAC528 **B**. Plaque size for the different groups. Representative images of the plaque area and plaque area for the different groups. n=19 vehicle vs n=17 ESM-HDAC528 **C.** Different disease characteristics: Necrotic area, percentage of macrophages, collagen and monocyte influx in the plaques of the different treatment groups. n=19 vehicle ns n=17 ESM-HDAC528. Statistical significance was determined by unpaired t-test (B, C) or Chi-square test (A) ($p<0.05$). All error bars represent the SEM.

Discussion

Macrophages play a role in virtually every stage of atherosclerosis and reshaping their deregulated activation is considered to be the holy grail of macrophage therapeutic targeting [37]. The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) recently delivered clinical data demonstrating that inflammation is a key driver of atherosclerosis [38]. Therefore, targeting macrophage-mediated inflammation has emerged as an attractive approach for atherosclerosis therapy. Meanwhile, it has become increasingly clear that epigenetic mechanisms are critical regulators of inflammatory responses. Histone deacetylases that regulate the acetylation status of histones and non-histone proteins are of high interest since broad-spectrum HDAC inhibitors are well documented to decrease inflammation and disease severity in multiple diseases [39]. Moreover, inhibition of HDACs in macrophages has beneficial athero-protective

effects *in vitro* [21] but their progression as a potential atherosclerosis therapy was prevented by the observation that the broad-spectrum HDAC inhibitor Trichostatin A (TSA) unexpectedly increased plaque size in a mouse model of atherosclerosis [40]. However, this could be due to negative effects of TSA on other cell types that are known to affect atherosclerosis such as endothelial cells and smooth muscle cells [41].

Therefore, we reasoned that inhibiting HDACs specifically in macrophages and monocytes would be beneficial in an atherosclerosis setting. To achieve this, we used an ESM-conjugated HDAC inhibitor which is selectively hydrolysed into a charged molecule and retained within monocytes and macrophages by human carboxylesterase-1 (CES1) [16]. Accordingly, this ESM-HDAC528 had no inhibitory effect on bone marrow-derived macrophages from WT mice, but efficiently inhibited inflammatory responses in BMDMs that were derived from transgenic mice that expressed human CES1 driven by the monocyte/macrophage-specific CD68 promoter (*CES1/Es1e^{lo}*). ESM-HDAC528 exhibited monocyte-specific activity (lysine acetylation) in human white blood cells. Moreover, we found that ESM-HDAC528 was more potent than the conventional non-targeted HDAC inhibitor SAHA in these cells.

In vitro data showed decreased levels of cytokine production in transgenic BMDMs in contrast to WT BMDMs, where no decreases were observed. Interestingly, in case of IL-12p40, NO and, to a much lesser extent, TNF, a significant induction was observed in WT cells. This phenomenon is not understood and could be addressed in future work with more extended concentration ranges to explore the potential of biphasic responses.

After validating our approach *in vitro*, we next confirmed the efficacy of ESM-HDAC528 *in vivo* and that i.p. injection of the drug into *CES1/Es1e^{lo}* mice efficiently reduced the LPS (+/- IFNγ)-induced secretion of IL-6, TNF, IL-12 and NO. Interestingly, the total number of peritoneal cells and macrophages isolated from ESM-HDAC528-injected mice was reduced and these cells appeared less mature as evidenced by increased Ly6C, and decreased CD11b, F4/80 and CD64 expression. Since these distinct ESM-HDAC528-mediated effects could potentially dampen atherosclerosis progression, we next assessed the effect of this drug on atherosclerosis in *ldlr$^{-/-}$* mice that were transplanted with *CES1/Es1e^{lo}* bone marrow. Acetylation levels in monocytes and peritoneal macrophages were increased in the ESM-HDAC528-treated group and this was accompanied by reduced macrophage activation. Yet, the effects of HDAC inhibition on inflammatory and maturation endpoints in peritoneal macrophages were less pronounced in these hypercholesterolemic mice. Although ESM-HDAC528 treatment did not significantly affect plaque size, these plaques were classified as less severe histological phenotypes, with the change being statistically significant and consistent with at least a partial impact on an important disease outcome.
One of the outstanding questions from our observations is how HDAC inhibition impairs monocyte to macrophage differentiation and inflammatory responses, and why the latter effect is less pronounced in a hypercholesterolemic environment. It should be noted that in this BMT

model, ESM-HDAC528 would not be targeted to non-bone marrow derived lineages of macrophages which would not express human CES1. This could explain the limited efficacy seen in the atherosclerosis model. Additionally, it is well described that cell fate decisions within the hematopoietic system are regulated by epigenetic mechanisms and distinct HDACs were shown to be implicated in myeloid development (reviewed in [4]). Specific HDACs are differentially regulated and expressed in response to environmental factors and, while HDAC inhibitors mediate anti-inflammatory effects via a wide range of mechanisms, they can also amplify inflammatory responses in macrophages. For example, HDAC6 normally acts as a transcriptional activator of the anti-inflammatory cytokine IL-10 and consequently HDAC6 inhibition or genetic knock-down diminishes IL-10 secretion [42]. As such, ESM-HDAC528 could potentially inhibit distinct HDACs in normal versus hypercholesterolemic mice and this might explain why broad spectrum HDAC inhibition is less beneficial in the context of atherosclerosis.

Together, our data highlight the potential for drugs that selectively target individual HDACs to improve effectiveness in atherosclerosis treatment. In this context, (macrophage-specific) HDAC3 inhibition may be an attractive target for atherosclerosis therapy since its deletion promotes anti-atherogenic macrophage responses, whilst inhibiting inflammatory macrophage cues [43, 44]. Moreover, myeloid HDAC3 deficiency improved collagen deposition and lipid handling in atherosclerotic plaques and induced a more stable plaque phenotype [19]. Inhibitors that preferentially inhibit HDAC3 also exert anti-atherogenic effects *in vitro [21]* but HDAC3-selective drugs (especially macrophage-specific ones) that are applicable *in vivo* are currently not available.

Overall, we demonstrate that targeting HDACs in monocytes and macrophages with ESM drugs inhibits both inflammation and monocyte to macrophage differentiation, while only minimally affecting atherosclerosis endpoints. While this is an improvement in comparison to the previously applied non-targeted broad-spectrum inhibitor TSA, our study supports the need for drugs that selectively inhibit individual HDACs in target cells.

Ethics statement

All animal experiments were conducted at the University of Amsterdam and approved (permits: DBC242 and 103169) by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam. All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EEC and the GSK Policy on the Care, Welfare and Treatment of Animals.

Funding

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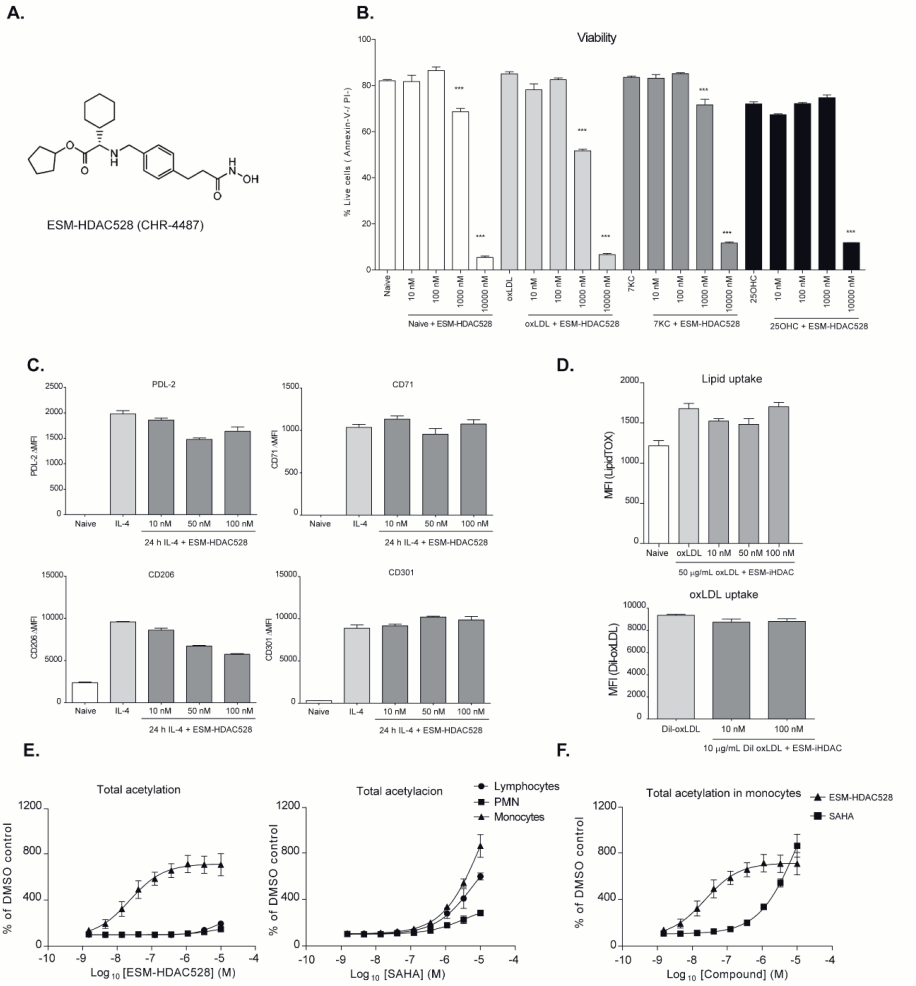
Supplementary Figure 1.



Supplementary Figure 1. A. Chemical structure of ESM-HDAC528 (also termed CHR-4487). The compound is comprised of an HDAC inhibitor bound to an ester group. **B.** Viability in BMDMs from transgenic mice after stimulation with ESM-HDAC528 and oxLDL (50 µg/ml), 7KC (20 µg/ml) or 25OHC (10 µg/ml) n=3 **C.** Alternative activation marker expression in transgenic BMDMs after 24 h stimulation with 20 ng/mL of IL-4 and different concentrations of ESM-HDAC528 n=3 **D.**Lipid uptake by BMDMs from transgenic mice. Stimulated with 50 µg/mL of oxLDL together with different concentrations of ESM-HDAC528 for 24 h n=3**.** Measurement of DiI-oxLDL uptake. Pre-incubation with the compound overnight and stimulation for 3 h with 10 µg/ml of DiI-oxLDL n=3 **E.** Human whole blood measurement of intracellular acetylation in different WBC comparing ESM-HDAC528 with the non-targeted pan-HDAC inhibitor SAHA n=3 **F.** Comparision of intracellular acetylation in monocytes in human whole blood after stimulation with different concentrations of ESM-HDAC528 or SAHA n=3. Statistical significance was determined by unpaired *t*-test (*** p<0.00.1). Bar graphs represent the mean and SEM of the values, acetylation plots show the non-linear curve for the concentration-response data with SEM.

Chapter 7

BRD9 inhibition evokes a pro-inflammatory response in macrophages

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Manuscript in preparation

Abstract

Epigenetic modifications regulate gene expression dictating the activation state and phenotype of cells, including immune cells like macrophages. Epigenetic enzymes add, remove or recognise specific epigenetic modifications. Considering the importance of epigenetics and its regulation by epigenetic enzymes, targeting these enzymes with small molecule inhibitors has become an important field to explore. To modify the phenotype of cells involved in diseases, e.g. macrophages in atherosclerosis, small molecules targeting epigenetic enzymes are being tested and show promising results. In this study, we wanted to determine the effect of BRD9 inhibition on LPS activation of human macrophages. We performed concentration-response and RNA-Sequencing studies. We found that the treatment of LPS activated macrophages with a BRD9 inhibitor (iBRD9) increased the production of pro-inflammatory cytokines like IL-1β and IL-6 and blocked the production of the anti-inflammatory IL-10 in different subtypes of macrophages. After transcriptomic studies of the response of macrophages to iBRD9 treatment and LPS stimulation, we found that the combination of iBRD9 and LPS results in mixed effects on inflammatory genes, with clear suppression of inflammatory pathways by iBRD9 but also promotion of specific inflammatory processes, both in unstimulated as well as under LPS activated conditions. In summary, we found that iBRD9 treatment may increase particular aspects of the pro-inflammatory phenotype of macrophages in response to LPS which could be useful in vaccines, where this inhibitor could be used as an adjuvant to boost immune responses. However, a better definition of the exact effects on macrophages activation first needs to be established.

Introduction

Changes in gene expression caused by epigenetic enzymes drive changes in phenotype in all cell types, including immune cells. Macrophages, innate immune cells, perform heterogeneous and various functions, having a role in initiation and control of immune responses. Similar to other immune cells, epigenetic processes control the differentiation and activation of these cells towards specific phenotypes [1-4]. Considering the importance of epigenetics for macrophages in immune-related diseases, compounds targeting epigenetic enzymes are under development as attractive targets for therapy. Macrophage specific histone deacetylase (HDAC) inhibitors have been shown to block peritoneal macrophage maturation and activation in mice treated with thioglycolate [5] and macrophage specific HDAC3 inhibition in atherosclerosis leads to increased stability of the atherosclerotic plaques [6].

Knowing the importance of epigenetics, the readers of epigenetic modifications have a crucial role in regulating the effects of epigenetic marks. Acetyl residues of histone lysines are read by bromodomain (BRD) proteins. Eight families of human BRD proteins have been defined based on their sequence and structural similarities [7]. All bromodomains are formed by four alpha-helices. The different families can have relatively different functions and they are involved in various diseases like inflammatory diseases and cancer [7-9]. One of these BRD families are the BET proteins (extra terminal bromodomain). Inhibitors for BET proteins like JQ1 have shown beneficial results in cancer, for instance, by reducing angiogenesis and response to hypoxia in breast cancer [10]. JQ1 also showed anti-inflammatory effects, modulating the response to LPS by decreasing the expression of IL-6 and TNF in macrophages and microglia [11, 12], which could be beneficial in diseases like periodontitis and heart failure [13, 14].

BRD9 is a bromodomain protein from the family IV and although the knowledge about its function is limited, we know that it is part of the chromatin remodelling SWI/SNF complex. Mutations in this complex and overexpression of BRD9 have been found in cancer [15, 16]. To gain a better understating of BRD9 and possible implications in other diseases besides cancer, various inhibitors for BRD9 have been developed and tests in cancer are being done [17-19]. For example, a BRD9 inhibitor has been shown to block proliferation of AML cells in mouse and human [20].

Based on the importance of epigenetic modifications on the macrophage phenotype and the lack of knowledge of the role of BRD9 in these cells, we tested the effects of an inhibitor of BRD9 in human macrophages. We used an inhibitor for BRD9 (iBRD9) that is previously described [21]. We observed an increase in pro-inflammatory cytokine production in LPS activated macrophages. To fully understand the possible role of BRD9 in the inflammatory response, we examined the BRD9 protein expression in the cells after LPS stimulation and performed an RNA-Sequencing experiment in macrophages treated with the inhibitor alone or in combination with LPS to understand global changes in gene expression.

Materials and methods

Cell culture and stimulations

All donors provided written informed consent for use of their samples, and the collection and use of the samples received Institutional Review Board approval. Blood from healthy volunteer in buffy coats from Sanquin (The Netherlands) and collected into tubes containing sodium heparin anti-coagulant in GSK (UK) was obtained in order to isolate the monocytes and then culture them into macrophages. Blood was mixed 1:1 with PBS and then layer on top of 15 ml of Ficoll (GE Healthcare). The tube was spun at 1500 rpm for 20 minutes without break and acceleration at room temperature (RT), after this, all following spins were done at 1500 rpm for 5 minutes at RT. PBMCs were collected and washed twice with PBS. Hereafter cells were resuspended in 1 mL of MACs buffer (2mM EDTA, 0.5% BSA in PBS) per 50 mL of the initial volume of whole blood. 1 µL of CD14 positive beads (Miltenyi Biotec) were added per 50 mL of the initial volume of whole blood and then cells were incubated for 15 minutes at 4°C. After this incubation, the cells were washed twice with MACs buffer. Afterwards, LS Miltenyi columns were place in a magnet and CD14+ monocytes were isolated by passing 1 mL of PBMCs in the columns, washing them three times with MACS buffer and then collecting the elute from the columns by removing the columns from the magnet and adding 5 mL of MACS buffer. Cell were washed with MACs buffer and resuspended at $1x10^6$ cell/mL in RPMI-1640 (Gibco) with 5% FCS, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 mg/mL). Monocytes were culture for 5 days in an incubator at 37°C and 5% CO_2 with either, M-CSF (50 ng/mL, R&D systems), GM-CSF (5 ng/mL, R&D systems), or IFNγ (50 ng/mL, R&D systems).

In the concentration-response study macrophages were pre-incubated for 30 minutes with iBRD9 (10 µM, 3.3 µM, 1.1 µM, 370 nM, 123 nM, 41.1 nM, 13.7 nM, 4.5 nM 0 nM) and then, without washing, either LPS (100 ng/mL) or LPS (100 ng/mL) with IFNγ (50 ng/mL) was added for 24 h. For protein measurements, macrophages were stimulated for 24 h either with LPS (100 ng/mL) or LPS (100 ng/mL) with IFNγ (50 ng/mL). For the RNA-Sequencing experiments, M-CSF derived macrophages were untreated or treated with 3 µM of iBRD9 for 6 hours alone or in combination with LPS (100 ng/mL).

Bone marrow was isolated from femurs and tibia of wild type BL6 mice by flushing with PBS. Cells were spun down and resuspended in RPMI-1640 with 25 mM HEPES and 2 mM L-glutamine, which was supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100 mg/mL) and 15% L929-conditioned medium (LCM) as a source of M-CSF and cultured on bacterial plastic plates for 8 days. On day 3, fresh media containing LCM was added to the plates and on day 6 all media was replaced. 100.000 cells were plated in a 96 well suspension culture plate at a concentration of $1x10^6$/ml. On day 8, cells were pre-incubated for 30 minutes with different concentrations of iBRD9 (10 µM, 3.3 µM, 1.1 µM, 370 nM, 123 nM, 41.1 nM, 13.7 nM, 4.5 nM 0 nM) and afterwards stimulated with LPS alone (10 ng/mL, Thermo Fisher), or LPS

(10 ng/mL) plus IFN-γ (100 U/mL R&D systems) or left unstimulated for 24 h in the presence of 2.5 nM of ATP. Supernatants were then collected to measure IL-1β cytokine levels.

Viability

The viability was determined based on the ATP levels of the cells measured using the CellTiter-Glo® kit (Promega) following manufacturer's instructions. Data is represented as percentage normalised to the no compound condition.

Cytokine production

After the different stimulations the supernatant was collected to measure cytokine production. To measure IL-1β, IL-12p70, IL-6, IL-10 and TNF a Human Pro-inflammatory 7-Plex Tissue Culture Kit (Meso Scale Discovery K15008B) was used following manufacturer's instructions. For IL-1β production by BMDMs an ELISA kit was used following manufacturer's instructions (Thermo Scientific).

Western blot

Human macrophages were plated in a concentration of $1x10^6$ cells in a 12 wells plate. After removing the supernatant and washing with PBS, cells were lysed using MQ water in combination with: NuPage LDS sample buffer (ThermoFisher NP0007) and NuPage sample reducing agent (ThermoFisher NP0009), the buffer was added into the wells and incubated for 10 minutes at RT. Afterwards the lysate was boiled at 85 °C for 5 minutes. The whole lysate, including nuclear fraction was used for the western blot procedure. 5 µL of the ladder SeeBlue plus 2 Standard (Invitrogen LC5925) and 12 µL of sample was loaded per well on a NuPage 4-12% Bis-Tris Mini gel with MOPS buffer and samples were ran at 100-120V for 2-3h. After that proteins were transferred to a nitrocellulose membrane using the iBlot™ Gel Transfer system following manufacturer's instructions (20-25V for 7 minutes). After the transfer, membranes were cut above the housekeeping protein and blocked with 3% milk in PBS (blocking buffer). The different parts were incubated overnight at 4°C with the either BRD9 antibody (1:5000; Abcam ab137245), or H3 antibody (1:20000; Abcam ab1791) as a loading control in blocking buffer. Post incubation, membranes were washed (PBS, 0.05% Tween20) and then incubated with a secondary antibody Alexa Fluor 680 donkey anti-rabbit IgG (H+L) (Invitrogen A10043) at 1:8000 in wash buffer for 1 hour in the dark. Membranes were washed and visualised using the Odyssey Infrared Imaging system.

RNA-Sequencing experiments

Cells were stimulated as described above and lysed after 6 hours using Qiagen RLT lysis buffer following manufacture's protocol. RNA isolation and cDNA library preparation were performed

and prepared as previously described [22]. In short, total RNA was isolated with RNeasy Mini Kit (Qiagen) and RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol. cDNA libraries were prepared using the standard protocol of NuGEN Ovation RNA-Seq System V2 kit with input of 100ng RNA per sample. Size-selected cDNA libraries were pooled and sequenced on a HiSeq 4000 sequencer (Illumina) to a depth of 12-18M per sample according to the 50 bp single-end protocol at the Amsterdam UMC, location VUMC. Raw FASTQ files were aligned to the human genome GRCh38 by STAR (v2.5.2b) with default settings. Indexed Binary alignment map (BAM) files were generated and filtered on MAPQ>15 with SAMTools (v1.3.1). Raw tag counts and reads per kilo base million (RPKM) per gene were calculated using HOMER2's analyzeRepeats.pl script with default settings and the -noadj or -rpkm options for raw counts and RPKM reporting for further analyses.

Functional analyses of transcriptomic data

All analyses were performed in the R statistical environment (v3.6.3). Differential expression was assessed using the Bioconductor package edgeR (v3.28.1). Lowly expressed genes were filtered with the filterByExpr function and gene expression called differential with a false discovery rate (FDR) <0.05. Pathway enrichment analyses were performed using the Metascape (http://metascape.org/gp/index.html) on 2020-10-2

Statistical analysis

Data represent the mean ± standard error of the mean (SEM). Non-linear curves for concentration-response studies were also generated. Data were analysed using GraphPad Prism version 5.0 (GraphPad software, La Jolla, California).

Results

iBRD9 induces a pro-inflammatory phenotype in activated human macrophage subtypes.

Macrophages produce pro-inflammatory (IL-1β, IL-6, IL-12p70) and immune regulatory (IL-10) cytokines in response to bacterial stimuli, like LPS. We wanted to test how the treatment of macrophages with an epigenetic reader inhibitor against BRD9 (iBRD9) would affect the induction of this pro-inflammatory phenotype. Macrophages were differentiated either with M-CSF, GM-CSF or IFNγ to obtain different subtypes ranging from more homeostatic macrophages (M-CSF) to more pro-inflammatory ones (GM-CSF and IFNγ). We treated the different subtypes of macrophages with increasing concentrations of iBRD9 together with LPS or LPS + IFNγ for 24 h. The viability **(Figure 1A)** was not affected for M-CSF or GM-CSF macrophages after the treatment for both stimulations. An increase of cell death was observed in IFNγ macrophages only at high concentrations of the compound, in both stimulations. Interestingly, iBRD9 treatment of

M-CSF macrophages **(Figure 1B)** caused an increase in the production of pro-inflammatory cytokines. iBRD9 treatment led to a strong increase in the production of IL-1β and a milder increase of IL-6 in both stimulations. IL-12p70 production was only increased upon LPS + IFNγ stimulation and there was a clear inhibition of IL-10 under both conditions. In GM-CSF macrophages **(Figure 1C)** there was an overall increase in the production of IL-1β, IL-6 production was moderately increased under LPS stimulation and IL-12p70 was increase only after LPS stimulation. A reduction in the production of IL-10 was also observed. For IFNγ macrophages **(Figure 1D)** only IL-1β production was increased, with no changes in the other cytokines measured (data not shown). In all the conditions presented here (i.e. Figures 1B-1D) the viability of the cells was not affected. We also wanted to test the effect on murine macrophages, because mice could be used as potential animal model to test this compound *in vivo*. Bone marrow derived macrophages (BMDMs) differentiated with M-CSF **(Figure 1E)** were stimulated with increasing concentrations of iBRD9 together with LPS or LPS+IFNγ in combination with ATP to induce the release of IL-1β. We also observed an increase in the production of IL-1β by BMDMs after stimulation, although weaker than the changes observed in human cells and in LPS + IFNγ stimulation the response increased only at high compound concentrations.

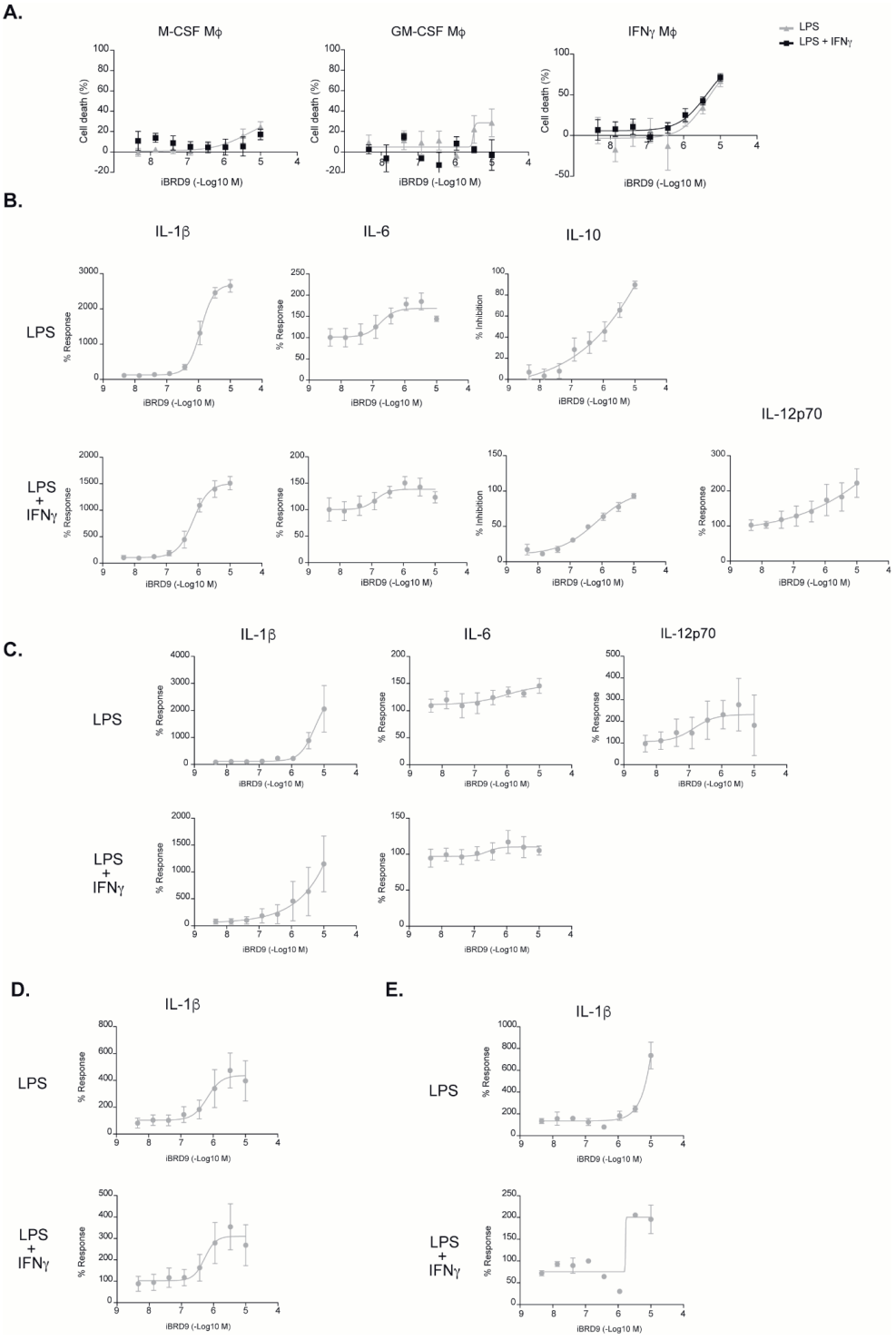

A.
M-CSF Mϕ
GM-CSF Mϕ
IFNγ Mϕ
LPS
LPS + IFNγ
Cell death (%)
iBRD9 (-Log10 M)
B.
IL-1β
IL-6
IL-10
IL-12p70
LPS
LPS + IFNγ
% Response
% Inhibition
C.
D.
E.

Figure 1. A. Viability of M-CSF, G-MCSF and IFNγ macrophages after stimulation with LPS (100 ng/mL) or LPS (100 ng/mL) + IFNγ (50 ng/mL) for 24 h in combination with different concentrations of iBRD9. **B.** Cytokine production of M-CSF macrophages after stimulation with LPS (100 ng/mL) or LPS (100 ng/mL) + IFNγ (50 ng/mL) for 24 h in combination with different concentrations of iBRD9. **C.** Cytokine production of GM-CSF macrophages after stimulation with LPS (100 ng/mL) or LPS (100 ng/mL) + IFNγ (50 ng/mL) for 24 h in combination with different concentrations of iBRD9. **D.** Cytokine production of IFNγ derived macrophages after stimulation with LPS (100 ng/mL) or LPS (100 ng/mL) + IFNγ (50 ng/mL) for 24 h in combination with different concentrations of iBRD9. **E.** IL-1β production by BMDMs after stimulation with LPS (100 ng/mL) or LPS (100 ng/mL) + IFNγ (50 ng/mL) for 24 h in combination with different concentrations of iBRD9. Graph show the non-linear curve for the concentration-response data with SEM. n=4

BRD9 expression is human macrophages is not regulated by activation.

We next wanted to define BRD9 expression in the different subtypes of human macrophages and whether activation impacted on its expression. BRD9 levels were detected by western blot and we measured the protein at different time points over 24 hours with and without stimulation (LPS or LPS + IFNγ) in the 3 subtypes of macrophages. In M-CSF derived macrophages **(Figure 2A)**, GM-CSF derived macrophages **(Figure 2B)** and IFNγ derived macrophages **(Figure 2C)** the expression of BRD9 was the same independently of the activation or the time point. Based on the cytokine production, where M-CSF macrophages showed the most pronounced effects, we decided to further study iBRD9 in M-CSF macrophages at the transcriptomic levels by RNA-Sequencing experiments.

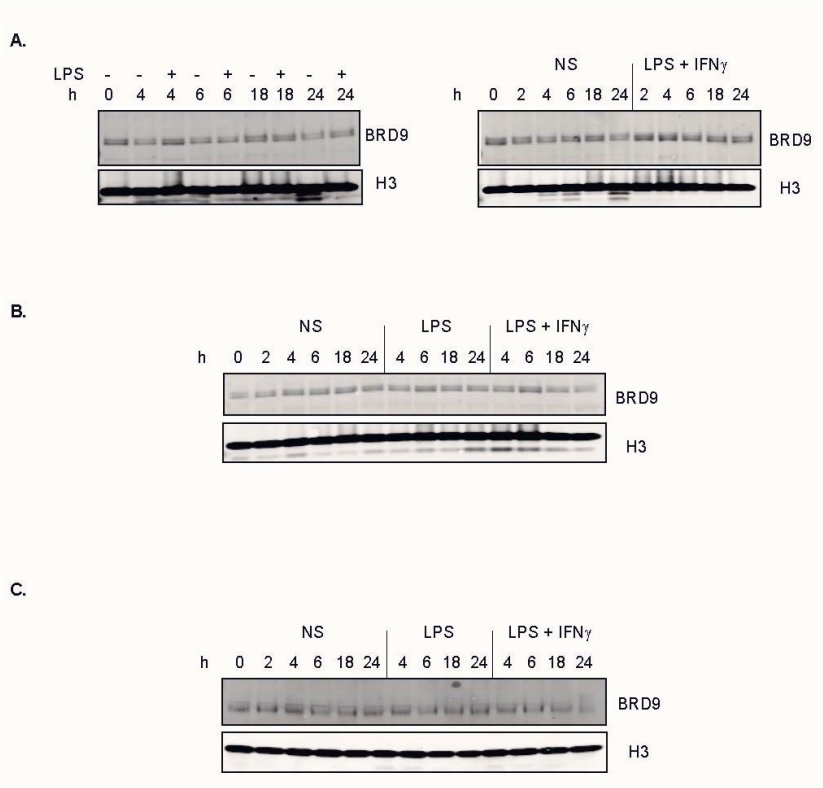


Figure 2. A. BRD9 expression of M-CSF derived macrophages over a time course of 0, 2, 4, 6, 18, 24 h without stimulation or either LPS (100 ng/mL) or LPS (100 ng/mL) + IFNγ (50 ng/mL) stimulation. **B.** BRD9 expression of GM-CSF derived macrophages over a time course of 0, 2, 4, 6, 18, 24 h without stimulation or either LPS (100 ng/mL) or LPS (100 ng/mL) + IFNγ (50 ng/mL) stimulation. **C.** BRD9 expression of IFNγ derived macrophages over a time course of 0, 2, 4, 6, 18, 24 h without stimulation or either LPS (100 ng/mL) or LPS (100 ng/mL) + IFNγ (50 ng/mL) stimulation. N=1

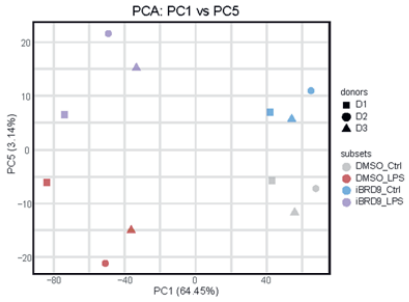
RNA-Sequencing analysis of M-CSF macrophages treated with iBRD9 shows effects on the inflammatory response both under basal conditions and in combination with LPS stimulation.

To gain a better understanding of the effect of the compound, we studied the changes in gene expression of M-CSF differentiated macrophages in response to the treatment with iBRD9 with and without LPS. We performed RNA-Sequencing analysis of M-CSF derived macrophages treated with iBRD9 at 3 μM or DMSO control, with and without LPS for 6 hours. Principal component analysis **(Figure 3A)** showed that the changes in the expression pattern were mainly (64.45%) due to the presence or absence of LPS in principal component (PC) 1 while 3.14% of the variance could be explained by PC5, which showed a clustering based on iBRD9 treatment.

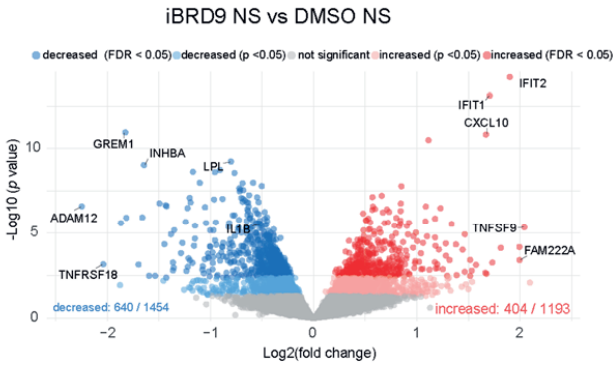
First, we examined the effect of the compound in unstimulated cells. We found 640 significantly downregulated genes and 404 upregulated genes (FDR<0.05) **(Figure 3B)**. As is clear from labelled genes in the volcano plot several genes linked to the interferon (IFN) pathway, including *IFIT1*, *IFIT2* and *CXCL10*, were among the upregulated genes. Also, pathway analysis showed that IFN related pathways were up, including GO-terms like "antiviral mechanism by IFN-stimulated genes" and "Toll-like receptor signalling pathway" **(Figure 3C)**. However, pathway analysis of the downregulated genes also implicated effects on leukocyte activation and interleukin signalling (Figure 3C). Thus, these data suggest that different aspects of the inflammatory profile and IFN response of unstimulated macrophages are impacted differentially by BRD9 inhibition.

Next, we compared iBRD9 treated vs DMSO control macrophages stimulated with LPS**.** We now found 1027 downregulated genes and 746 upregulated genes upon iBRD9 treatment **(Figure 3D)**. *IL1B* and *IL6* were identified as upregulated genes. This is in line with the observed effects of the previous *in vitro* study for cytokine production **(Figure 1)**. Regarding pathway analysis of the upregulated genes, we found that the main upregulated pathways were "TNF□ signalling via NFκB", "cellular responses to external stimuli" and "signalling by interleukins" **(Figure 3E)**. On the other hand, the downregulated genes were enriched for "Inflammatory response" and "Interferon gamma" **(Figure 3E)**, again indicating a mixed response of the LPS-activated macrophages upon BRD9 inhibition.

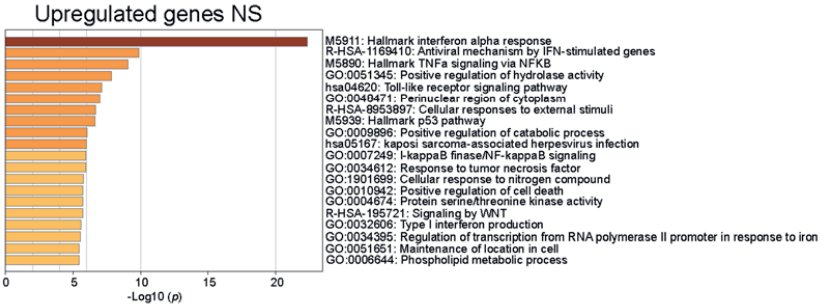
A.

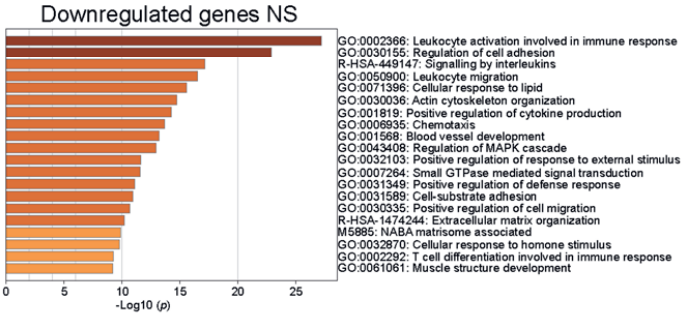

PCA: PC1 vs PC5
PC5 (3.14%)
PC1 (64.45%)
donors
D1
D2
D3
subsets
DMSO_Ctrl
DMSO_LPS
iBRD9_Ctrl
iBRD9_LPS

B.

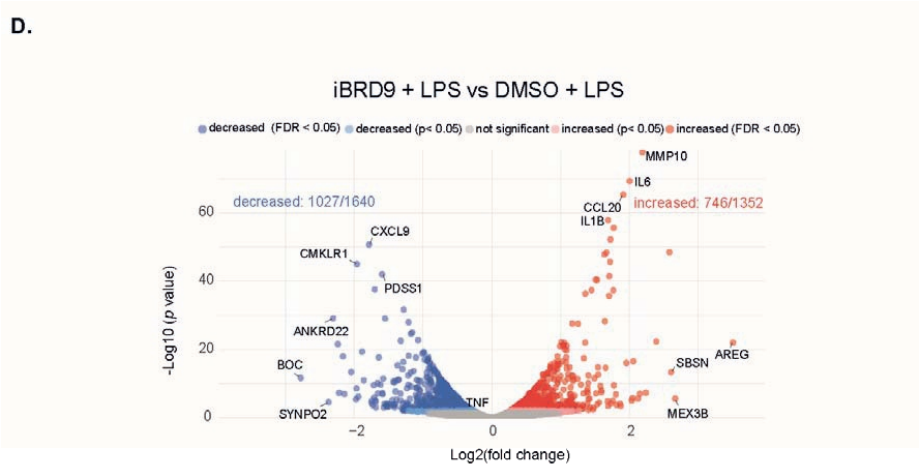

iBRD9 NS vs DMSO NS
decreased (FDR < 0.05)
decreased (p <0.05)
not significant
increased (p <0.05)
increased (FDR < 0.05)
-Log10 (p value)
Log2(fold change)
GREM1
INHBA
LPL
ADAM12
IL1B
TNFRSF18
IFIT2
IFIT1
CXCL10
TNFSF9
FAM222A
decreased: 640 / 1454
increased: 404 / 1193

C.

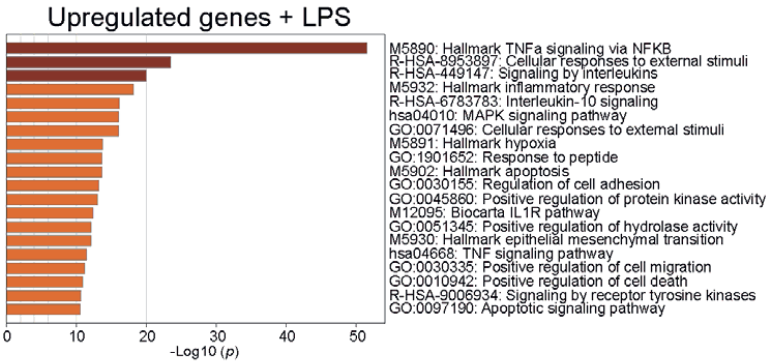

Upregulated genes NS
M5911: Hallmark interferon alpha response
R-HSA-1169410: Antiviral mechanism by IFN-stimulated genes
M5890: Hallmark TNFa signaling via NFKB
GO:0051345: Positive regulation of hydrolase activity
hsa04620: Toll-like receptor signaling pathway
GO:0048471: Perinuclear region of cytoplasm
R-HSA-8953897: Cellular responses to external stimuli
M5939: Hallmark p53 pathway
GO:0009896: Positive regulation of catabolic process
hsa05167: kaposi sarcoma-associated herpesvirus infection
GO:0007249: I-kappaB finase/NF-kappaB signaling
GO:0034612: Response to tumor necrosis factor
GO:1901699: Cellular response to nitrogen compound
GO:0010942: Positive regulation of cell death
GO:0004674: Protein serine/threonine kinase activity
R-HSA-195721: Signaling by WNT
GO:0032606: Type I interferon production
GO:0034395: Regulation of transcription from RNA polymerase II promoter in response to iron
GO:0051651: Maintenance of location in cell
GO:0006644: Phospholipid metabolic process
-Log10 (p)


Downregulated genes NS
GO:0002366: Leukocyte activation involved in immune response
GO:0030155: Regulation of cell adhesion
R-HSA-449147: Signalling by interleukins
GO:0050900: Leukocyte migration
GO:0071396: Cellular response to lipid
GO:0030036: Actin cytoskeleton organization
GO:0031819: Positive regulation of cytokine production
GO:0006935: Chemotaxis
GO:001568: Blood vessel development
GO:0043408: Regulation of MAPK cascade
GO:0032103: Positive regulation of response to external stimulus
GO:0007264: Small GTPase mediated signal transduction
GO:0031349: Positive regulation of defense response
GO:0031589: Cell-substrate adhesion
GO:0030335: Positive regulation of cell migration
R-HSA-1474244: Extracellular matrix organization
M5885: NABA matrisome associated
GO:0032870: Cellular response to homone stimulus
GO:0002292: T cell differentiation involved in immune response
GO:0061061: Muscle structure development
-Log10 (p)

D.


iBRD9 + LPS vs DMSO + LPS
decreased (FDR < 0.05)
decreased (p< 0.05)
not significant
increased (p< 0.05)
increased (FDR < 0.05)
decreased: 1027/1640
increased: 746/1352
MMP10
IL6
CCL20
IL1B
CXCL9
CMKLR1
PDSS1
ANKRD22
BOC
SYNPO2
TNF
SBSN
AREG
MEX3B
-Log10 (p value)
Log2(fold change)

E.


Upregulated genes + LPS
M5890: Hallmark TNFa signaling via NFKB
R-HSA-8953897: Cellular responses to external stimuli
R-HSA-449147: Signaling by interleukins
M5932: Hallmark inflammatory response
R-HSA-6783783: Interleukin-10 signaling
hsa04010: MAPK signaling pathway
GO:0071496: Cellular responses to external stimuli
M5891: Hallmark hypoxia
GO:1901652: Response to peptide
M5902: Hallmark apoptosis
GO:0030155: Regulation of cell adhesion
GO:0045860: Positive regulation of protein kinase activity
M12095: Biocarta IL1R pathway
GO:0051345: Positive regulation of hydrolase activity
M5930: Hallmark epithelial mesenchymal transition
hsa04668: TNF signaling pathway
GO:0030335: Positive regulation of cell migration
GO:0010942: Positive regulation of cell death
R-HSA-9006934: Signaling by receptor tyrosine kinases
GO:0097190: Apoptotic signaling pathway
-Log10 (p)

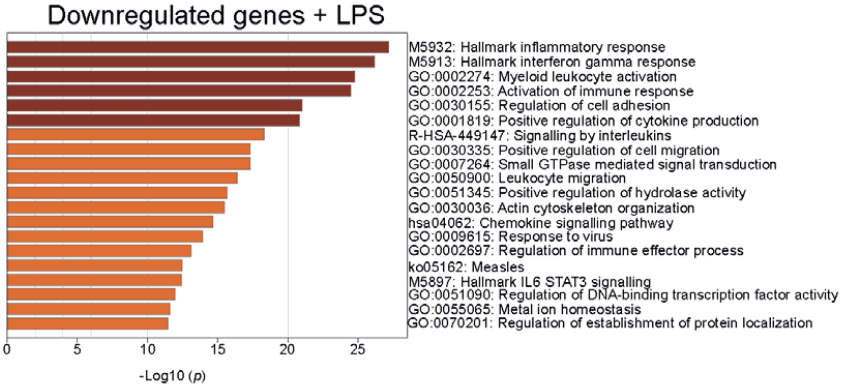

Downregulated genes + LPS
M5932: Hallmark inflammatory response
M5913: Hallmark interferon gamma response
GO:0002274: Myeloid leukocyte activation
GO:0002253: Activation of immune response
GO:0030155: Regulation of cell adhesion
GO:0001819: Positive regulation of cytokine production
R-HSA-449147: Signalling by interleukins
GO:0030335: Positive regulation of cell migration
GO:0007264: Small GTPase mediated signal transduction
GO:0050900: Leukocyte migration
GO:0051345: Positive regulation of hydrolase activity
GO:0030036: Actin cytoskeleton organization
hsa04062: Chemokine signalling pathway
GO:0009615: Response to virus
GO:0002697: Regulation of immune effector process
ko05162: Measles
M5897: Hallmark IL6 STAT3 signalling
GO:0051090: Regulation of DNA-binding transcription factor activity
GO:0055065: Metal ion homeostasis
GO:0070201: Regulation of establishment of protein localization
-Log10 (p)

Figure 3. A. Principal component analysis of RNA-Sequencing data from M-CSF derived macrophages non treated (DMSO) or treated with iBRD9 (3 μM) and non-stimulated (NS) or stimulated with LPS (100 ng/mL) for 6 hours. **B.** Volcano plots of up and downregulated genes for cells treated with iBRD9 (3 μM) vs untreated cells without LPS stimulation. **C.** Gene ontology enrichment pathway for the upregulated and downregulated statistically significant genes for cells treated with iBRD9 (3 μM) vs untreated cells without LPS stimulation. **D**. Volcano plots of up and downregulated genes for cells treated with iBRD9 (3 μM) vs untreated cells with LPS (100 ng/mL) stimulation for 6 h**. E.** Gene ontology enrichment pathway for the upregulated and downregulated statistically significant genes for cells treated with iBRD9 (3 μM) vs untreated cells with LPS (100 ng/mL) stimulation for 6 h. For the statistical selection of genes, a threshold of FDR <+-0.05 and a *p* value 0.05. n=3

Discussion

Epigenetic readers, such as BRD9, impact on epigenetic processes that control gene expression and therefore affect the phenotype of cells [3]. Here, we tested the effects of a BRD9 inhibitor (iBRD9) [21] on the macrophage response to LPS alone or in combination with IFNγ. We observed that inhibition of BRD9 causes an increase in the secretion of pro-inflammatory cytokines. The cytokine production was especially increased in M-CSF macrophages showing increased levels of IL-1β, IL-6, IL-12p70 and a reduction of IL-10. GM-CSF macrophages had increased levels of IL-1β and IL-6 and only IL-1β was increased in IFNγ derived macrophages after iBRD9. Transcriptional profiling showed certain pro-inflammatory effects of iBRD9 but also indicated suppressive effects on specific aspects of macrophage activation.

We measured if the BRD9 protein was present in all the different subtypes of macrophages and how it was affected by activation. Not only to corroborate that the compound was targeting a protein that was present, but also to understand the expression pattern of BRD9 after stimulation. We found that BRD9 protein expression was present in all subtypes of macrophages and no clear differences in BRD9 expression were observed after LPS and LPS + IFNγ stimulation. Targeting the BRD9 expression, by for instance siRNA studies, should be performed to fully corroborate the specificity of the iBRD9 compound and the observed effects, as BRD9 has a close member in its family, BRD7, that also might be targeted by this compound. However, the binding affinity of iBRD9 is lower for BRD7 than for BRD9 [21]. Considering the effect on the different macrophages and possible applications of this molecule we decided to use M-CSF macrophages for further studies.

In addition to cytokine secretion profiling, we wanted to understand the changes at the transcription level caused by iBRD9 and for that we performed a RNA-Sequencing study. When we analysed the data, the main effects at the transcriptional level were due to LPS activation of the cells, as was shown by the principal component analysis. When cells were left unstimulated, suppression of inflammation related pathways was observed. However, there was also an increase in pathways related to type I interferons. Type I interferons are essential in anti-viral responses [23] and have also been shown to regulate inflammatory activation of macrophages [24]. Our studies have previously shown a critical role for type I IFNs in atherosclerosis [25] and it is now well established that they are important regulators of atherosclerotic disease [26]. Interestingly,

when we examined the effect of iBRD9 in LPS activated cells, we observed upregulation of some of the cytokine genes that were also enhanced at the protein level, i.e. *IL1B* and *IL6*. Pathway analysis suggested clear pro-inflammatory effects but studying the suppressed genes indicated that there are also inflammatory pathways supressed. Overall, the transcriptional data seem to confirm some of the cytokine protein data that we obtained but also clearly indicate anti-inflammatory effects of iBRD9. Future studies should better elucidate the exact inflammation modulating effects of BRD9 inhibition in macrophages.

Previously inhibitors of other epigenetic enzymes like JMJD3 and readers like BET proteins have been shown to suppress the LPS response of macrophages [27, 28]. As mentioned previously, BET inhibitors like JQ1 have potential as anti-inflammatory and cancer treatment. The beneficial effects of BET inhibitors have led to get these types of molecules into clinical trials. For example, RVX-208 is a BET inhibitor that has shown anti-inflammatory effects [29]. This molecule is now in clinical trials for cardiovascular diseases, with promising results showing a decrease of high sensitivity C-reactive protein, increased levels of HDL accompanied by a decrease in cardiovascular events [30].

In stark contrast, we here show that iBRD9 impact strongly on the inflammatory activation of macrophages. At the transcriptional level, both suppression and activation of specific inflammatory pathways was observed, while cytokine secretion was clearly enhanced by iBRD9. This capacity of iBRD9 of boosting the inflammatory responses could be useful in settings of vaccines or in promoting host-defence [31]. Vaccination depends on a well-controlled activation of the immune system in order to generate a potent and long-lasting protection. Adjuvants are needed to produce a proper immune response in vaccines with killed organism or purified antigens [32]. iBRD9 may serve as an adjuvant as in the presence of LPS, not a live organism but rather an antigen, the immune response is potentiated including production of molecules like IL-12p70 that will contribute to the activation of adaptive immunity [33]. Furthermore, considering that iBRD9 shows in the transcriptome data downregulation of some inflammatory pathways, meaning that it is possible that the pro-inflammatory response is not excessive and maybe the inflammatory syndrome associated to adjuvants could be avoided [34].

Overall, we show that pharmacological inhibition of BRD9 affects inflammatory activation of macrophages. Future research should focus on more details of the underlying molecular mechanisms and potential applications.

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Chapter 8

General discussion

General discussion

Macrophage differentiation and activation are key processes in disease development. This thesis focused on understanding the process of macrophage differentiation and activation and identifying small molecules that interfere with these processes. The main findings are:

- IFNγ induces monocyte-to-macrophage differentiation resulting in macrophages that have a marked inflammatory phenotype. M-CSF induces a less inflammatory phenotype than GM-CSF or IFNγ. IFNy differentiated macrophages could prove to be a useful *in vitro* tool to study macrophages in models of inflammatory diseases like psoriasis.
- Through the use of a CRISPR/Cas9 screen, we were able to identify that the *MAP2K3* gene is implicated in monocyte to macrophage differentiation based on CD14 expression.
- Pan BET inhibition blocks the GM-CSF induced differentiation of human primary monocytes and abrogates disease activity in a rat model of rheumatoid arthritis (RA).
- Myeloid-specific HDAC inhibition by ESM-HDAC528 impairs peritoneal macrophage maturation and its inflammatory response without affecting atherosclerotic lesion size in a mouse model of atherosclerosis.
- Inhibition of BRD9 by iBRD9 increases the pro-inflammatory response of different subtypes of macrophages after LPS (+/- IFNy) stimulation.

Differentiation as a key process: influence of the use of M-CSF, GM-CSF or IFNγ

Based on the literature reviewed in **chapter 2** it is clear that for monocyte-derived macrophages (MDMs), the mediator used for differentiation greatly affects the phenotype of the macrophage obtained. Considering this, we explored how different molecules, relevant in diseases, shape the phenotype of an MDM.

As shown in **chapter 2, 3** and **4**, differentiation from monocytes to macrophages is accompanied by an increase in expression of macrophage maturation markers on the cells (CD68, CD14, CD16, CD11b, CD64, CD80, CD86, CD40, etc). From these markers, CD68 has been historically used as the gold standard macrophage marker [1]. Macrophages also acquire phagocytic capacity [2] and their morphology is dependent on differentiation mediators **(chapter 2-4)**. Therefore, when investigating the ability of new molecules to induce monocyte to macrophage differentiation many factors need to be considered. Including but not limited to, measurement of surface markers (CD14, CD16, CD11b, etc.), phagocytic capacity and expression of CD68.

Historically, the mediators used for *in vitro* differentiation have been M-CSF or GM-CSF. M-CSF is a cytokine produced by various cells, including macrophages [3]. M-CSF is found at a higher ratio in homeostatic conditions compared to GM-CSF [4]. In **chapter 3** and **5** we functionally compare M-CSF and GM-CSF-derived macrophages. We found that macrophages differentiated by M-CSF present a more homeostatic phenotype compared with GM-CSF induced macrophages.

GM-CSF induced macrophages exhibit an increased pro-inflammatory phenotype in terms of pro-inflammatory cytokine production (high IL-6, IL-12p70, low production of IL-10, etc) and T cell activation towards Th1 phenotype. The literature reviewed in **chapter 2** compiled data which shows similar results in terms of phenotypes obtained in M-CSF and GM-CSF induced macrophages. For instance, comparison of the phenotypes of M-CSF with GM-CSF derived macrophages also showed a higher pro-inflammatory response in GM-CSF derived macrophages [5]. Once the role of GM-CSF and M-CSF has been linked to a specific macrophage phenotype and health or pathological situation, the next step would be to explore less common monocyte to macrophage differentiation mediators.

Based on this idea, in **chapter 2** we performed a literature search to find mediators, besides M-CSF and GM-CSF, used for *in vitro* differentiation of human macrophages. For example, platelet factor 4, also known as CXCL4, induces the acquisition of a macrophage-like morphology, expression of CD45 and CD68 and higher levels of production of IL-6 and TNF compared to M-CSF induced macrophages [6]. It was found that different conditions can also affect the phenotype of macrophages during differentiation. Macrophages obtained under hypoxic conditions exhibit a low phagocytic capacity, low CD206 and CD40 but high production of VEGF [7]. Considering this, we concluded that the use of a specific mediator might greatly alter the phenotype, which opens the door for testing different mediators.

Therefore, we propose that a more extensive study into the role of different mediators (TNF, LPS, IFNγ, IL-17, etc) as inducers of *in vitro* monocyte to macrophage differentiation should be performed. This would allow for a more representative overview of the possible *in vivo* environment that monocytes encounter when entering a tissue in different pathologies. For example, we hypothesised that in sepsis/chronic infections, LPS could affect the differentiation of monocytes into macrophages and perhaps lead to a more tolerant macrophage phenotype [8]. IFNγ could be interesting, as it is present in inflammatory diseases like psoriasis [9, 10]. TNF is present in inflammatory diseases like inflammatory bowel disease (IBD) and testing its effect on the phenotype of MDMs could provide a tool to study macrophages *in vitro* for IBD [11, 12].

To extend our knowledge and confirm our hypothesis regarding the ability of different mediators to affect macrophage differentiation, in **chapter 3** we tested the capacity of IFNy to drive MDM differentiation. IFNγ is a molecule present in a wide range of inflammatory-related diseases like psoriasis, juvenile idiopathic arthritis and others [13, 14]. This molecule is produced by natural killer (NK) cells, CD4 Th1 and CD8 cytotoxic T cells [15]. We show that IFNγ is able to generate viable and functional macrophages when used for *in vitro* differentiation of human monocytes into macrophages. The obtained macrophages expressed CD68 and have phagocytic capacity. Regarding their function, IFNγ macrophages present a higher pro-inflammatory phenotype compared to M-CSF and GM-CSF induced macrophages, with higher capacity to induce a Th1 response and release of pro-inflammatory cytokines (e.g. IL-6, IL-12, IL-1β). We studied these cells at the proteomic level and found differences in the proteome of IFNγ macrophages. Heatmap

comparisons showed different clusters of proteins for the different macrophages. One protein, GBP-1, was specifically upregulated only in IFNγ derived macrophages compared to M-CSF or GM-CSF ones. We also investigated a panel of chromatin modifiers enzymes and found differential expression of some genes (e.g. *HDAC5*, *HDAC9*, *CIITA*, *SETDB2*, etc.) between the 3 subtypes. This suggests that at least part of the epigenetic landscape of these macrophages is different between them. Future experiments involving Chip-Seq or ATAC sequencing would provide further information into this process. Understanding the full epigenetic landscape could be the key, together with the differential protein expression, for the different phenotype of the macrophages.

Together with upregulation of GBP-1, a protein related to inflammatory diseases [16], we also found increased production of IL-23/IL-12 in these IFNy macrophages which leads to increased T cell responses [17, 18]. Interestingly, we found many markers specific for IFNy differentiated macrophages like IP-10, to be upregulated in psoriasis patients, opening a door for the use of IFNγ macrophages as an *in vitro* model for macrophages in psoriasis.

A key point in this study was to differentiate if the phenotypic effects were caused by the presence of IFNγ during the differentiation or were just a product of later activation by IFNγ. In the case of the latter, M-CSF derived macrophages stimulated with IFNγ would show the same phenotype as IFNγ derived macrophages. By comparing the differences in the response of IFNγ differentiated macrophages and M-CSF differentiated macrophages activated with IFNγ (+/- LPS), we found this not to be the case. We also observed that the proteomic clustering of IFNγ derived macrophages and M-CSF derived macrophages stimulated with IFNγ was not the same, for instance, higher expression of GBP-1 in IFNγ derived macrophages. In terms of gene expression, *IRF5* was highly expressed in IFNγ derived macrophages compared to M-CSF macrophages in LPS+IFNγ stimulated conditions. Under the same conditions, we observed differences in production of IL-12p70, IL-23, IL-10. Taking all these measurements into account we showed that an IFNγ derived macrophage is not the same as stimulating a macrophage with IFNγ. Further studies involving RNA-Seq of the 3 subtypes, will elucidate the mechanism behind this phenotype even further.
Since IFNy macrophages show common characteristics with psoriatic macrophages and patient data (high GBP-1 expression, IP-10, activation of the IL-23/IL-12 axis) we believe they could be used as an *in vitro* tool for psoriasis.

The testing of new mediators could be improved by using them in combination with others, like IL-17, IL-6 and TNF to mimic RA conditions [19, 20]. The ultimate goal will be to study and generate human macrophages *in vitro* replicating those *in vivo*.

Small molecules that impact the differentiation process

It has become increasingly clear that macrophage differentiation is a key process with great potential as a target for the treatment of various diseases. As a next step, we wanted to target the differentiation process. In order to do this, we needed to understand which genes were key for differentiation and therefore could be potential targets of small molecules. The function of these molecules would be to block/promote the differentiation of monocytes into macrophages depending on what would be beneficial in a given disease.

In **chapter 4** we performed a CRISPR/Cas9 screen to identify key genes in monocyte-to-macrophage differentiation CD14 is a monocyte/macrophage differentiation marker present on the surface of myeloid cells (monocytes, macrophages) and M-CSF macrophages [21] **(chapter 3, 5)**. We used the expression of CD14 as a read-out for differentiation of THP-1 cells after performing a whole-genome silencing screen. As a result of this, we were able to identify a list of genes that either increased or reduced CD14 expression. Following the screen, we validated if inhibition of the proteins encoded by the identified genes in the screen affected differentiation. We used available small molecule inhibitors that target the differentiation-related proteins in both THP-1 and primary M-CSF human macrophages. We found that, according to the results of the screen, inhibition of MAP2K3 during differentiation reduces the expression of CD14 in THP-1 and primary human M-CSF derived macrophages. We also performed functional macrophage assays, by stimulation with LPS. After the application of the MAP2K3 inhibitor during primary human M-CSF induced macrophages differentiation, we found reduced production of pro-inflammatory cytokines. It would be interesting to conduct more functional assays, like response to other molecules besides LPS e.g. oxidised LDL. This would allow us to understand if these molecules play a role in inflammatory diseases, where the recruitment of monocytes and its maturation into macrophages is important, e.g. atherosclerosis [22].

Although we identified multiple genes important for monocyte-to-macrophage differentiation, small molecule drugs were only available for a limited number of genes. Future studies on the impact of these genes in differentiation and macrophage activation could create an interest in developing drug targets. These drugs could be used to study their effects on macrophage differentiation, allowing us to not only provide information on the quality of the screen but also help aid the development of new treatments for diseases.

Epigenetic targeting molecules

Epigenetics is a key regulator of the macrophage phenotype [23]. As shown in **chapter 3,** epigenetic enzymes coding genes were differentially expressed in macrophages and this was shown to be dependent on the differentiation mediator. This suggests that differences in the expression of epigenetic proteins control the differentiation process and may lead to different macrophage phenotypes as a result of a particular epigenetic landscape.

In the final chapters of the thesis, we used small molecule drugs that target epigenetic proteins and studied how these compounds affect macrophage differentiation or activation.

In **chapter 5** we tested the effect of a pan BET inhibitor (I-BET151) on human monocyte differentiation induced by M-CSF or GM-CSF. We found that the differentiation of GM-CSF derived macrophages was blocked by the BET inhibitor while the M-CSF induced differentiation process was not. We show that iBET displaces Brd2 and Brd4, that regulate GM-CSF-induced genes critical for differentiation. It is known that GM-CSF is detectable in the synovial fluid of RA patients [24, 25] and it leads to the differentiation of the recruited monocytes into a pro-inflammatory phenotype [26], in order to test the effect of I-BET151 we used a rat model of RA. I-BET151 treatment blocked arthritis developing in the rats and a therapeutic dosing regime was almost equally as effective. Analysis of the joints showed a similar number of macrophages in the joint but with significantly reduced pro-inflammatory markers. This data indicates that I-BET151 may have skewed monocyte differentiation towards a homeostatic phenotype which contribute to the resultant reduced inflammation and joint destruction. GM-CSF is not only important in RA but plays a crucial role in many other diseases [27]. Therefore, the effect of BET inhibitors in other GM-CSF driven diseases should also be tested.

In the same way that BET inhibition only affects GM-CSF differentiation, it would be interesting to study if this inhibitor also affects IFNγ monocyte to macrophage differentiation described in **chapter 3** where it could be further tested in a model of psoriasis.

Besides BET proteins, another family of epigenetic proteins that have been linked to the inflammatory phenotype of macrophages are the HDACs [28, 29]. Inhibition of HDACs is being studied as a possible treatment for inflammatory diseases and cancer [30, 31], e.g. inhibition of HDAC3 in a mouse model of atherosclerosis increases the stability of the atherosclerosis plaque [32]. Since the HDAC inhibition results in different outcomes depending on the cell type, in **chapter 6**, we tested if specific targeting of myeloid cells with an HDAC inhibitor (ESM-HDAC528) affects the inflammatory response. For specifically targeting myeloid cells, we used the ESM technology that allows accumulation of a compound specifically in myeloid cells [33]. We observed a reduced pro-inflammatory response of peritoneal macrophages in an acute mouse model of inflammation induced by thioglycolate. We also found that ESM-HDAC528 treatment led to an impairment of the maturation of peritoneal macrophages [34]. Macrophages are important in the initiation and development of atherosclerosis [22, 35]. Therefore, we decided to test ESM-HDAC528 in a mouse model of atherosclerosis. We found that the peritoneal macrophage maturation was impaired while the pro-inflammatory response was mildly affected. We did not find statistically significant effects in the atherosclerosis plaque size, although there was a trend towards an improvement in terms of severity of the disease. In this chapter, we were able to show that the ESM technology works for targeting specific myeloid cells, as we only saw increased acetylation in monocytes in the treated group compared to vehicle. This opens the door for using this technology with compounds targeting different proteins. The compound we used

was a pan-HDAC inhibitor and maybe specific HDAC inhibition would have shown better results. For instance, inhibition of HDAC6 reduces alternative activated phenotype while HDAC3 inhibition promotes plaque stability [32, 36]. These examples show how different HDACs have different effects and how, for example inhibition of HDAC3, is beneficial for atherosclerosis while the opposite is true for HDAC6. In our chapter, the lack of beneficial effects in the atherosclerosis model could be explained as macrophages are not the only source of inflammation and therefore solely targeting these cells may not be enough to improve the atherosclerosis phenotype [37, 38].

Besides differentiation, the effect of inhibition of epigenetic enzymes in macrophage activation is also an interesting field that has been deeply studied [39, 40]. So far, we have mainly described the effect of epigenetic inhibition in differentiation **(chapter 5)** but we also investigated the effect on activation in **chapter 7**. In order to investigate, the effect of epigenetic inhibition in activation we used a BRD9 inhibitor.

We decided to test a BRD9 inhibitor (iBRD9) as a result of a screen of the effect in macrophage activation of different compounds targeting epigenetic enzymes. iBRD9 showed interesting results in terms of macrophage activation. We tested this inhibitor in M-CSF, GM-CSF and IFNγ induced macrophages. In all subtypes of human macrophages, we found that the pro-inflammatory response to LPS or LPS plus IFNγ was boosted with a marked increase of IL-1β in all subtypes. RNA sequencing analysis was used to study the transcriptomic effect of BRD9 inhibition in M-CSF macrophages. This showed regulation of pathways involved in inflammation and activation of the immune system in the presence of LPS, like increased response to external stimuli, upregulation of pro-inflammatory cytokine genes (*IL-6*, *IL-1β*) but also downregulation of certain inflammatory pathways. In the absence of LPS, even though we observed downregulation of pathways related to activation of the immune system, we also found increased expression of genes related to the interferon alpha response.

Based on these results we think that one of the possible uses of this inhibitor would be as an adjuvant to vaccines. iBRD9 has shown by RNA-Seq analysis, to activate the immune system in response to external stimuli in the presence of LPS. In vaccines, boosting the immune response, by the use of adjuvants, is key and therefore iBRD9 presents good characteristics for becoming an adjuvant. Another interesting effect is that the molecule increases the production of cytokines that regulate the adaptive immune response like IL-12p70 [41]. Finally, we also thought of a possible use of this molecule in cancer, with the aim of modifying the tumour associated macrophage phenotype towards a more anti-tumour phenotype. The lack of LPS in the tumour environment could present a problem, so for future studies testing the effect of BRD9 inhibition after activation with other molecules like tumour microenvironment ones (e.g. VEGF, TNF, TFGβ, IL-4) [42, 43] rather than LPS could be of interest for finding uses for this molecule.

Overall conclusions

In this thesis, we aimed to gain an understanding in the differentiation from monocyte to macrophage and find small molecules to impact this process. Throughout the thesis, we were able to further understand how different mediators that initiate monocyte to macrophage differentiation will affect the phenotype of the macrophage obtained. This is very important, as when we generate macrophages *in vitro*, especially from primary material, it is key to obtain macrophages that would resemble as much as possible the *in viv*o MDMs. In chapter 3, we showed the importance of cytokines used in the differentiation process. We found that macrophages have different inflammatory phenotypes when they are differentiated with M-CSF, GM-CSF or IFNγ, from a less pro-inflammatory phenotype to more pro-inflammatory. In this thesis, we also identified small molecules, two of them targeted epigenetic enzymes, that have the ability to affect monocyte to macrophage differentiation (I-BET151, ESM-HDAC528, iMAP2K3), something key in diseases where there is an excess of macrophages. We also tested different molecules including ESM-HDAC528 and iBRD9 in macrophage activation. We found the opposite effect for ESM-HDAC528 with a reduced pro-inflammatory response of peritoneal macrophages, and iBRD9 promoted secretion of inflammatory cytokines and impacted on the regulation of the immune response in human macrophages. With these results, we observed that targeting epigenetics can differentially modulate the immune response depending on the enzyme targeted.

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Appendices

I. Summary

The immune system is our defence from exogenous and endogenous threats, and it is formed by a wide variety of cells. Macrophages are a key part of this system and play a role in nearly every inflammatory disease. Understanding their origin and functions is key for understanding various diseases. The role of macrophages in diseases varies and the dysregulation of macrophage function can lead to the development of diseases. Epigenetic regulation and treatments based on targeting epigenetics and cytokines are a field in evolution. These aspects in function, regulation and therapies regarding macrophages are discussed in chapter 1. The overall aim of this thesis is to understand the differentiation of monocytes into macrophages to further investigate the use of small molecules to impact this process and to modulate their function in disease.

Chapter 2 reviews the origin of the macrophages present in the tissues. It reviews the in vitro phenotypes that can be observed by using different cytokines during the differentiation, e.g., GM-CSF produces macrophages with a higher pro-inflammatory phenotype than M-CSF.

Based on the idea of using new cytokines for differentiation, in chapter 3 we use IFNγ to differentiate monocytes into macrophages and compare their phenotype to macrophages differentiated by M-CSF or GM-CSF. We found that IFNγ alone is capable to produce macrophages from monocytes with a marked pro-inflammatory phenotype compared to other subtypes. To completely understand these cells, we characterise these IFNγ driven macrophages from a functional and proteomic level. We also found markers expressed in these macrophages, e.g., GBP-1, and activation of IL-23/IL-12 axes upregulated in psoriasis patients.

After studying the importance of the differentiation process for the phenotype of macrophages we wanted to use small molecules to interfere in the differentiation. In chapter 4 we use a CRISPR/Cas9 whole genome screen to identify genes that were important to the expression of CD14, a maker classically used as maturation marker for macrophages. We then used small molecules to inhibit the proteins encoded by genes that the screen showed as candidates for regulating CD14 expression. We found that the use of a MAP2K3 inhibitor is capable of decreasing CD14 expression.

With the same aim of altering the differentiation process, in chapter 5 we investigate the effect of a pan BET epigenetic enzyme inhibitor (I-BET151) on the differentiation induced by M-CSF or GM-CSF. We found that this compound blocks the differentiation induced by GM-CSF but not M-CSF. After seeing the effects of epigenetic targeting in macrophage phenotype we wanted to test further compounds in a disease setting.

Therefore, in chapter 6 we used an HDAC inhibitor that targets specifically myeloid cells in a mouse model of atherosclerosis. Here we found a lack of maturation of peritoneal macrophages after induction of acute inflammatory activation in these mice in response to thioglycolate. In most

of the atherosclerosis parameters (e.g. plaque size, monocyte influx, necrosis area), no effects were observed in the treated group, but the lesions were less severe.

We tested the effect of another epigenetic enzyme inhibitor in chapter 7. In this case we used an inhibitor for BRD9 (iBRD9) and tested the effect on activation of macrophages rather than differentiation. We found that iBRD9 increases the production of some pro-inflammatory cytokines like IL1β in LPS stimulated MDMs. We also performed RNA-sequencing analysis to understand the effect of the inhibition. We found that the compound has mixed effects on the response of the cells to LPS. This effect of iBR9 could be interesting in cases where you need increased macrophage activation like in vaccines.

The final chapter, chapter 8, discusses the general findings of the thesis.

II. Nederlandse samenvatting

Het immuunsysteem, ofwel het afweersysteem, is ons verdedigingsapparaat tegen allerlei bedreigingen, zowel vanuit ons lichaam zelf als van buitenaf. Ons afweersysteem wordt gevormd door een grote variëteit aan cellen. Macrofagen, en hun voorloper cellen de monocyten, zijn een onderdeel van het afweersysteem en spelen een rol bij bijna alle ontstekingsziekten. De rol van macrofagen in deze ziekten is zeer divers en verkeerd functioneren van macrofagen kan leiden tot de ontwikkeling en verergering van ziekten. Epigenetische processen en behandelingen gericht op epigenetica en cytokines (signaalstoffen) zijn een veld van onderzoek dat volop in ontwikkeling is. De verschillende aspecten van macrofaagfunctie, regulatie en therapieën worden bediscussieerd in hoofdstuk 1. Het doel van mijn proefschrift was het differentiatieproces van monocyten naar macrofagen beter te begrijpen en te bestuderen hoe farmacologische remmers invloed hebben op dit proces en de functie van de macrofaag beïnvloeden in ziekten.

Hoofdstuk 2 is een overzicht over de oorsprong en het ontstaan van verschillende soorten macrofagen in weefsels. Het geeft inzicht in hoe verschillende soorten macrofagen in kweekschaaltjes (in vitro) kunnen worden gemaakt, gebruik makend van verschillende groeifactoren en signaalstoffen. Zo zijn macrofagen die gekweekt worden met de groeifactor GM-CSF meer ontstekingsbevorderend dan macrofagen die gekweekt worden met de groeifactor M-CSF.

Om nieuwe signaalstoffen te identificeren die een rol spelen bij het differentiatieproces van monocyten naar macrofagen maakten we in hoofdstuk 3 gebruik van interferon-gamma (IFNy) als welbekende signaalstof. We keken of IFNy monocyten kan differentiëren tot macrofagen en vergeleken de eigenschappen van deze macrofagen met macrofagen die zijn gedifferentieerd met de klassieke groeifactoren M-CSF en GM-CSF. We vonden dat IFNy als signaalstof op zichzelf al voldoende is om macrofaagdifferentiatie te induceren en dat dit type macrofaag meer ontstekingsbevorderend is dan de andere macrofaag types. Om het fenotype van deze IFNy gedifferentieerde macrofagen nog beter te begrijpen hebben we deze bestudeerd met functionele experimenten en eiwitanalyses (proteomics) uitgevoerd. Zo identificeerden we GBP-1 als eiwit dat tot expressie komt in deze macrofagen en dat de signaalstoffen IL-23/IL-12 geïnduceerd worden in deze cellen net zoals in patiënten met psoriasis.

Na het belang van het differentieproces op het fenotype van de macrofaag te hebben bestudeerd, hebben we vervolgens tijdens dit proces farmacologische remmers gebruikt die hier mogelijk op aangrijpen. In hoofdstuk 4 deden we een CRISPR/Cas9 genomische screen om genen te identificeren die belangrijk zijn voor de expressie van het oppervlakte eiwit CD14. Dit is een oppervlakte eiwit dat klassiek gebruikt wordt als marker voor gedifferentieerde macrofagen. Vervolgens gebruikten we farmacologische remmers om de eiwitten te remmen die worden gecodeerd door de genen uit de screen, omdat dit kandidaten zijn die CD14 expressie reguleren.

We vonden dat het gebruik van een MAP2K3 remmer in staat is om de expressie van CD14 te verlagen en dus mogelijk monocyt naar macrofaag differentiatie remt.

Als vervolg op hoofdstuk 4 bestudeerden we in hoofdstuk 5 het effect van een breed-spectrum BET epigenetische remmer (genaamd IBET-151) op de differentiatie van macrofagen geïnduceerd door de groeifactoren M-CSF of GM-CSF. We ontdekten dat deze remmer de door GM-CSF geïnduceerde differentiatie remde, maar niet die van M-CSF. Na de effecten van epigenetische remming op het macrofaag fenotype te hebben bestudeerd, onderzochten we vervolgens epigenetische remmers in verschillende ziektemodellen.

In hoofdstuk 6 gebruikten we daarom een epigenetische HDAC-remmer in een muismodel voor slagaderverkalking die specifiek werkt in macrofagen. We vonden dat als we een steriele ontsteking in de buikholte (peritoneum) induceerden met de stof thioglycolaat, er een sterke remming was op maturatie van de macrofagen na behandeling met de HDAC-remmer. Vervolgens bestudeerden we de ontwikkeling van slagaderverkalking door de grootte van de atherosclerotische laesies te meten en het fenotype te bestuderen zoals bijvoorbeeld het binnenkomen van monocyten en necrose. We zagen geen verschillen in slagaderverkalking na behandeling met de HDAC-remmer, alhoewel de laesies minder ernstig waren.

In hoofdstuk 7 onderzochten we het effect van een epigenetische remmer voor BRD9 (iBRD9) op macrofaag activatie. We ontdekten dat iBRD9 de productie van een aantal ontstekingsbevorderende signaalstoffen zoals IL1β verhoogde na activatie met LPS in macrofagen. Daarnaast voerden we ook een RNA-sequencing experiment uit om het effect BRD9 remming op genexpressieniveau te begrijpen. We zagen wisselende effecten van de remmer op genexpressie na LPS-stimulatie. Deze effecten van iBRD9 kunnen mogelijk interessant zijn in situaties waar macrofagen geactiveerd moeten worden zoals bij vaccinaties.

Het laatste hoofdstuk, hoofdstuk 8, is een algemene discussie van dit proefschrift en bespreekt de toekomstige richtingen van het onderzoek.

III. PhD portfolio

PhD student: Rosario Luque Martín

PhD period: June 2015-June 2019

PhD supervisors: prof.dr. Menno P.J. de Winther & prof. dr. Wouter J. de Jonge

PhD co-supervisors: dr. Annette E. Neele & dr. ir. Jan Van den Bossche

Amsterdam UMC, location AMC, Netherlands:

- June 2015 – February 2016
- October 2016 – May 2017
- August 2017 – March 2018
- August 2018 – September 2018
- February 2019 – March 2019

GlaxoSmithKline (GSK), UK:

- March 2016 – September 2016
- June 2017 – July 2017
- April 2018 – July 2018
- October 2018 – January 2019
- April 2019 – June 2019

PhD training	Year	Workload (Hours/ECTS)
General courses		
Basic Laboratory safety	2015	0.3
Laboratory Animal science, article 9	2015	3.9
Management in medical literature	2017	0.3
The AMC World of Science	2017	0.7
Scientific Writing in English for Publication	2017	1.5
Oral presentation in English	2019	1.5
Specific courses		
Advanced immunology	2016	2.9
Translational medicine	2017	2.8
Biobusiness summer school	2018	2.8
Developing a plan for further your career	2019	0.8
Practical Biostatistics	2019	1.4
Seminars, workshops and master classes		
Weekly medical biochemistry department seminars	2015-2019	5
Weekly experimental vascular biology meeting	2015-2019	5
Annual EPIMAC meeting	2015-2019	1
Monthly Journal Club	2015-2019	1

Presentations		
Oral talk & Poster presentation: "A screening approach to identify epigenetic compounds that modulate macrophage activation". Keystone regulation and dysregulation of innate immunity in disease, Vancouver, Canada	2018	0.5
Poster presentation: A screening approach to identify epigenetic compounds that modulate macrophage activation" International Symposium new frontiers in innate immunity and inflammation, Cluj-Napoca, Romania	2018	0.5
(Inter)national conferences		
Rembrandt Symposium, Noordwijkerhout, The Netherlands	2017	0.5
Annual ACS meeting, Amsterdam The Netherlands	2017	0.5
Keystone regulation and dysregulation of innate Immunity in disease, Vancouver, Canada	2018	0.5
International Symposium new frontiers in innate immunity and inflammation, Cluj-Napoca, Romania	2018	0.5

IV. Curriculum vitae

Rosario Luque Martín was born on June 22nd, 1992 in Jerez de la Frontera, Cádiz, Spain. After graduating from high school with honors she obtained her Bachelor in Biochemistry at the University of Seville in 2014. In 2015 she obtained her master's degree in current knowledge in rare diseases at the International University of Andalucía. Rosario's first research internship was in the department of Genetics during her bachelor. There, under the supervision of Dr. Javier Avalos she created plasmids used as tools for identification of specific gene expression. During her master she did her second internship in the department of Human Genetics and Reproduction at the Hospital Virgen del Rocío, under the supervision of Dr. Guillermo Antiñolo Gil, where next generation sequencing was used to diagnose rare ocular dystrophia patients and identify new mutations linked to the disease. In June 2015, Rosario started her PhD under the supervision of prof. dr. Menno P.J. de Winther and prof. dr. Wouter J. de Jonge at the Amsterdam UMC, location AMC, University of Amsterdam in collaboration with GlaxoSmithKline (GSK) in Stevenage UK, under the supervision of Dr. Palwinder Mander and Dr. Imnaculada Rioja Pastor. During her PhD, Rosario investigated the monocyte to macrophage differentiation process and how to interfere in it. In 2019, Rosario finished the PhD work that resulted in this thesis. She now pursues her career as a postdoctoral researcher in the laboratory of prof. dr. Olaf Heidenreich in childhood AML at the University of Newcastle.

V. Publications

Peer reviewed	Year
Van den Bossche J., Baardman J., Otto N.A., van der Velden S., Neele A.E., van den Berg S.M., Luque-Martin R., Chen H.J., Boshuizen M.C., Ahmed M., Hoeksema M.A., de Vos A.F. and Winther M.P.J. "Mitochondrial Dysfunction Prevents Repolarization of Inflammatory Macrophages". Cell Reports. vol. 17, no 3, p. 684-696.	2016
Luque-Martin, R*., Van den Bossche J*., R. C. Furze R.C., Neele A.E., Van Der Velden S., Gijbels M. J., Van Roomen C. P., Bernard. S. G., De Jonge W. J., Rioja I.,Prinjha R.K., Lewis H.D., Mander P.K. and Winther M.P.J. "Targeting histone deacetylases in myeloid cells inhibits their maturation and inflammatory function with limited effects on atherosclerosis". Frontiers in pharmacology. vol. 10, p. 1242. *Equal contribution	2019
Luque-Martin, R., Mander P.K, Leenen P. J., and Winther M.P.J. "Classic and new mediators for in vitro modelling of human macrophages". Journal of Leukocyte Biology. vol. 109, no 3, p. 549-560	2020
Jimenez-Duran G*., Luque-Martin R*., Patel M., Koppe E., Bernard S.G., Sharp C., Buchan N., Rea C., Winther M.P.J., Turan N., Angell D.C., Wells C.A., Cousins R., Mander P.K. and Masters S.L."Pharmacological validation of targets regulating CD14 during macrophage differentiation". EBioMedicine. vol. 61, p. 103039 *Equal contribution.	2020
Luque-Martin R., Angell D.C., Kalxdorf M., Bernard S.G., Thompson W., Eberl C.H., Ashby C., Freudenberg J., Sharp C., Van den Bossche J., de Jonge W.J., Rioja I., Prinjha R.K., Neele A.E, de Winther M.P.J. and Mander P.K." IFNγ drives human monocyte differentiation into highly pro-inflammatory macrophages that resemble a phenotype relevant to psoriasis". The Journal of Immunology. vol. 207, no 2, p. 555-568	2021
Luque-Martin R*., Harker N*., Angell D.C., McCleary S., Sharp C., Slocombe T., Pearse G., Fulleylove M., Wienholz F., Vorlova B., van Eijl A.P.M.R, Winther M.P.J., Haynes A., Tough D.F., Rioja I., Prinjha R.K and Mander P.K. "BET inhibition skews monocyte differentiation towards a homeostatic macrophage phenotype that resolves joint inflammation". *Equal contribution. Manuscript in preparation	2021
Luque-Martin R., Chen H.J., Mander P.K, Winther M.P.J. "BRD9 inhibition evokes a pro-inflammatory response in macrophages". Manuscript in preparation	2021

VI. Author contributions

Chapter 2: Conceptualisation, R.L-M., P.K.M., M.P.J.d.W. and P.J.L.M.; Writing – Original Draft, R.L.M.; Writing – Review & Editing, R.L.M., P.K.M., M.P.J.d.W. and P.J.L.M; Visualization, R.L.M.; Supervision, M.P.J.d.W and P.K.M.; Funding Acquisition, M.P.J.d.W. All authors read and approved the final manuscript.

Chapter 3: R.L-M., D.C.A., S.B., W.T., H.C.E., C.A., performed or assisted with experimentation. M.K., H.C.E., J.F., C.S., provided bioinformatics support. R.L-M., D.C.A., M.K., S.B., W.T., H.C.E., C.A., J.F., C.S., A.E.N., M.P.J.d.W., P.K.M.were involved in experimental analysis and interpretation. All authors contributed to the writing and approved the final version of this manuscript

Chapter 4: G.J.D., R.L-M., M.P., C.S., N.B., C.R. and S.B. performed or assisted with experimentation. C.A.W. provided bioinformatics support. G.J.D., R.L.M., M.P., C.S., E.K., N.B., C.R., S.B., R.C., D.A., M.P.J.d.W., P.K.M and S.L.M. were involved in experimental analysis and interpretation. All authors contributed to the writing and approved the final version of this manuscript.

Chapter 5: R.L-M., N.H., D.A., S.M., T.S., Su.B., G.P., M.F., F.W., B.V., R.A.P.M.vE., V.J.L., S.R., W.T., S.B. and P.K.M. performed or assisted with experimentation. C.S. and N.H. provided bioinformatics support, R.L.M., N.H., D.A., S.M., C.S., T.S., Su.B., G.P., M.F., F.W., B.V., R.A.P.M.vE., V.J.L., S.R., W.T., S.B. and P.K.M were involved in experimental analysis and interpretation. All authors contributed to the writing and approved the final version of this manuscript.

Chapter 6: Conceptualization, J.B., R.L-M., M.W., W.J., P.M. and H.L. Methodology, J.B., R.L-M., R.F., S.B., S.V., M.G., C.R., A.N. Formal Analysis, R.L-M., J.B., A.N., R.F., S.B., S.V., M.G. and C.R. Writing – Original Draft, R.L-M, J.B. Writing – Review & Editing, R.L-M., J.B., R.F., H.L., P.M., and M.W. Visualization, R.L-M, J.B. Supervision, M.W., P.M. and J.B. Funding Acquisition, M.W., W.J., R.P. and I.R. All authors read and approved the final manuscript.

Chapter 7: Conceptualization, R.L-M., M.W., P.M. Methodology, R.L-M. and H-J.C. Formal Analysis, R.L-M. and H-J.C. Writing – Original Draft, R.L-M. Writing – Review & Editing, R.L-M., H-J.C., P.M., and M.W. Visualization, R.L-M., H-J.C. Supervision, M.W., P.M. Funding Acquisition, M.W., P.M. All authors read and approved the final manuscript.

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I also want to thank my thesis supervisors Menno de Winther and Wouter de Jonge. Menno thank you for giving me the opportunity of starting my scientific career, you have been a good supervisor, always with an open door for questions, even if it was for 1-minute questions. I appreciate all you help, support and feedback in the projects, meetings, and presentations. Even though it was difficult sometimes by being in 2 different countries you have always been involved in the projects and keep supervising from a distance. Thanks also for the fun times in borrels and lab days, as well as the UK travels for the annual Epimac meetings (despite being very long and including flight delays). You have created a good group and I only can wish for good things for your future in research and life. Wouter thanks for your valuable feedback in our meetings. You were continuously very excited about the progress of project which was a great encouragement.

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And now all the way across the English Channel to UK, starting at GSK

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Pal, I don't even know how to start…Thank you (more than you can imagine), for being such and incredible supervisor, colleague, friend, macrophage mother, and support. I know I couldn't

have managed it without you. You are an inspirational woman in work and life, I look up to you a lot as an example of what a complete scientist should be. I appreciate every piece of advice you have given me and I know I will use them for the rest of my life. Thanks again and a million times thanks. Thanks for being my paraninfo and friend, and again one my biggest help this whole time.

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En general tengo una familia que vale un universo (o varios), os quiero, seguiremos hablando en los desayunos de la 1 y en las tardes de piscina.

Gallego…uf, ¿te lo digo? ¿te lo cuento? ¿Te lo escribo? Eres una persona increíble y mi mayor apoyo en todo esto…viniste conmigo y sobrevivimos juntos. Hemos pasado tantas historias, tantos viajes, tantos momentos que no puedo ni contarlos y me quedo en blanco porque no soy capaz de decirte lo que te agradezco y lo que te quiero. Estos últimos 5 años juntos has estado siempre ahí, incondicionalmente.

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