IRF1 is required for chromatin remodeling in bone marrow derived macrophages upon IFN-γ activation

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Abstract

Macrophages are a group of exceptionally diverse phagocytes of the innate immune system that are able to respond to a variety of stimuli, triggering both short and long-term functional adaptations. These responses are driven by specific transcriptional changes which are controlled by complex cellular mechanisms. Among these, changes to the epigenetic landscape by transcriptional mediators, especially at enhancer regions, now arise as key modulators of macrophage activity. IRF1 is a transcription factor whose role in macrophage activation upon distinct pro-inflammatory signals, including IFN-γ, is essential and non-redundant with other IRFs. Recently, genome-wide chromatin accessibility and binding assays have suggested a dependency on IRF1 for chromatin opening in response to IFN-γ. Therefore, I hypothesize that IRF1 is a signal-dependent pioneer transcription factor, involved in macrophage epigenetic reprogramming. In the present study, we characterize for the first-time via ChIP-seq, the early and late binding dynamics of IRF1 in primary macrophages upon IFN-γ. Using ATAC-seq, we have also interrogated chromatin accessibility changes post-stimulation in wild-type and *Irf1* knockout bone marrow derived macrophages to characterize their dependency on IRF1. We found several sites displaying IRF1 binding at basal state, despite being classically considered an inducible factor. Surprisingly, hundreds of new sites were bound by IRF1 just after 15 min of IFN-γ exposure and continued to increase, peaking at 3 – 6 h. Interestingly, genome-wide IRF1 binding appeared to be persisting, with ChIP-seq signal detectable beyond 48 h at most sites. Moreover, we found IRF1 has the ability to bind early to previously closed chromatin regions lacking PU.1 occupancy. Finally, ATAC-seq signal at these sites appeared at 3 h of stimulation and continued to increase throughout the time course in an IRF1 dependent manner. In summary, this thesis reveals that IRF1 can bind to heterochromatin and induce chromatin remodeling independently of PU.1.

Résumé

Les macrophages sont un groupe de phagocytes exceptionnellement diversifiés du système immunitaire inné, étant capables de répondre à divers stimuli qui déclenchent des adaptations fonctionnelles à court et à long terme. Ces réponses sont entraînées par des changements transcriptionnels spécifiques qui sont contrôlés par des mécanismes cellulaires complexes. Parmi ceux-ci, les modifications du paysage épigénétique par des médiateurs transcriptionnels, en particulier au niveau des régions amplificatrices (“enhancers”), apparaissent désormais comme des modulateurs clés de l'activité des macrophages. IRF1 est un facteur de transcription jouant un rôle essentiel et non redondant parmi les facteurs IRF pour l'activation des macrophages par des signaux pro-inflammatoires distincts, y compris par l'IFN-γ. Récemment, des expériences d'accessibilité génomique et de liaison à la chromatine ont révélé une dépendance à IRF1 pour l'ouverture de la chromatine en réponse à des stimuli. J’ai ainsi postulé qu’IRF1 est un nouveau facteur pionnier induisant une reprogrammation épigénétique des macrophages en réponse à des signaux activateurs. Dans la présente étude, j’ai utilisé la méthode de ChIP-seq pour caractériser la dynamique de liaison précoce et tardive d'IRF1 dans les macrophages en réponse à IFN-γ. Parallèlement, j’ai interrogé les changements d'accessibilité de la chromatine par ATAC-seq en réponse à la stimulation IFN-γ dans des macrophages dérivés de souris sauvages ou mutantes pour Irf1, et ainsi identifier les sites dépendants d'IRF1. J’ai identifié plusieurs sites affichant déjà une liaison d'IRF1 à l'état basal, bien qu'ils soient classiquement considérés comme un facteur inductible. Étonnamment, des centaines de nouveaux sites sont liés par IRF1 après seulement 15 minutes d'exposition à l'IFN-γ, recrutement qui continue à augmenter par la suite, atteignant un pic entre 3 et 6 heures. Fait intéressant, la liaison d'IRF1 à l'échelle du génome semble persistante, avec un signal ChIP-seq détectable au-delà de 48 h sur la majorité des sites. Enfin, j’ai démontré qu’IRF1 est capable de se lier tôt à des régions précédemment fermées et dépourvues d'occupation de PU.1. De plus, un signal ATAC-seq est apparu sur ces sites après 3 h de stimulation et a continué à augmenter par la suite, et ce de manière dépendante à IRF1. En sommes, mon mémoire révèle qu'IRF1 est capable de se lier à l'hétérochromatine en réponse à l'IFN-γ et d'induire un remodelage de la chromatine indépendant de PU.1.

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List of Abbreviations

APC	Antigen Presenting Cells
ATAC	Assay for Transposase-Accessible Chromatin
BCG	Bacillus Calmette-Guérin
BM	Bone Marrow
BMDM	Bone marrow derived macrophages
ChIP	Chromatin Immunoprecipitation
CRC	Colorectal cancer
CTL	Cytotoxic T Lymphocytes
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
GAF	Gamma activation factor
GAS	Gamma Activation Site
GFP	Green Fluorescent Protein
GWAS	Genome-wide association study
HSC	Hematopoietic Stem Cells
IAD	Interferon Association Domain
IBD	Inflammatory Bowel Disease
IFN	Interferon
IFN-γ	Interferon gamma
IL	Interleukin
IRF1	Interferon Regulatory Factor 1
ISG	Interferon Stimulated Genes
ISRE	Interferon-Stimulated Response Element
JAK	Janus Kinase

LDTF	Lineage determining transcription factors
M-CSF	Macrophage colony-stimulating factor
NK	Natural Killer
NLS	Nuclear Localization Signal
RBC	Red Blood Cells
RLR	retinoic acid-inducible gene-I-like receptors
SDTF	Signal-dependent transcription factors
SOCS	Suppressor of cytokine signaling
STAT	Signal Transducer and Activator of Transcription
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
TF	Transcription Factor
YS	Yolk Sac

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Acknowledgements and dedication

I would like to thank my supervisor, Dr. Langlais for the above and beyond support and guidance these last two years, which played a key factor in determining my successful and resulted in an extremely pleasing and constructive graduate experience. Since my first start at the lab in 2019, I have been provided with all the necessary tools and counseling to grow into a more mature scientist. I believe it is extremely rare to find this level of quality and attention to detail from a supervisor, for which I am grateful.

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The present work is dedicated to my late father, Jose Javier Ayala Barbosa, whom I miss and love deeply.

Contributions

The design of the experiments in this thesis were by Dr David Langlais and myself. The sample preparation for our two datasets were as follows: ChIP-Seq samples were done by myself and ATAC-Seq samples were prepared by Dr Mathieu Mancini and myself. The sequencing of the ChIP-Seq was done by the Molecular Biology and Functional Genomics Facility at the Institut de Recherches Cliniques de Montréal (IRCM). The sequencing of the ATAC-Seq was done by the McGill Applied Genomics Innovation Core (MAGIC) platform at the McGill Genome Centre. The results from experiments performed by lab members were indicated in the figure captions. Dr Mehdi Emam performed the analysis of flow cytometry data shown in **Supplemental figure 1**. Dr Langlais performed the clustering analysis from which I obtained the genome coordinates to create **Figure 9**. The editorial of the thesis was done by Dr David Langlais. My (M. Ayala) original contributions to this thesis are displayed as methods and results, except for the figures mentioned. In short, I performed the optimization of the IRF1 ChIP antibodies, determined their specificity, solely performed all experiments (except the ATAC-seq), carried out the bioinformatics analyses on the ChIP-Seq and ATAC-seq data sets and created the figures with guidance by Dr David Langlais.

Chapter 1 Introduction

The origins and roles of macrophages, epigenetic regulation, pioneer transcription factors and IRF1.

1.1 Macrophages

Macrophages, originally identified by Ilya Metchnikoff in the late 19th century by their phagocytic activity, are ancient phagocytes of the innate immune system which appeared more than 500 million years ago [1,2]. Their level of conservation throughout mammalian life is perhaps unsurprising considering they are present in nearly all tissues of adult mammals as resident macrophages [3], where they are required for proper organ development [4], maintaining tissue homeostasis [5], regulating inflammation [6] and host defense [7].

1.1.1 Ontogeny of circulating monocytes and tissue resident macrophages

For decades, the prevailing paradigm of the ontogeny of tissue resident macrophages referred to them as the end products from the recruitment and differentiation of circulating adult blood monocytes [8]. These views were further supported by *in vitro* studies showing differentiation into macrophages from either bone marrow cells or circulating monocytes and from *in vivo* adoptive transfer studies which found that labelled monocytes differentiate into tissue resident macrophages [9,10]. However, technical developments surrounding cell lineage tracking studies using transgenic and parabiotic mice have led to findings indicating that many tissue resident macrophage populations are in fact seeded during embryonic development, specifically by fetal hematopoiesis and can be maintained during adulthood by continuous self-renewal, independent from bone marrow-derived monocytes [11,12].

Although the complexity of fetal hematopoiesis has fueled many hypotheses in the field, there appears to be a growing consensus of its occurrence in three main successive waves. The first wave termed, primitive hematopoiesis, occurs during embryonic day 7.5 (E7.5) in mice in the blood islands of the extra-embryonic yolk sac (YS). The progenitors formed here are suspected to give rise to the first observed YS macrophages, as well as primitive erythroblasts and megakaryocytes. The second wave, known as the transient definitive wave, also takes place in the YS (E8.0-8.25), specifically in the developing vascular endothelium, where progenitor cells called erythro-myeloid progenitors (EMPs) develop and are also thought to contribute to the rise of macrophages [10]. After blood circulation is formed (E8.5), these EMPs travel to the fetal liver where they become the

progenitors to multiple cell lineages, including monocytes [13]. At the same time (E8.5), immature hematopoietic stem cells (HSCs) emerge in the embryo's para-aortic splachnopleura region, which then give rise to fetal HSCs in the aorta, gonads, and mesonephros regions [10]. These also begin to colonize the fetal liver at E10.5, establishing definitive hematopoiesis and serving as the major hematopoietic organ during the remainder of embryo development. Lastly, these precursors also seed the fetal bone marrow (BM), which during the perinatal period becomes the primary source of hematopoiesis by producing adult HSCs that give rise to the full complement of immune lineages, including circulating monocytes [14]. These circulating monocytes will be recruited upon inflammation, where they will differentiate into macrophages, further augmenting inflammation processes or aiding in its resolution [15]. Although, some studies have questioned the simplistic *in situ* phenotypic conversion model of monocyte differentiation, suggesting that certain organs have distinct gates of entry, which determines the functional fate of monocytes [16].

The degree to which embryonic versus hematopoietic progenitors contribute to the resident macrophage populations varies by tissue. For example, we now know through fate mapping studies that brain microglia, liver Kupffer cells, lung alveolar macrophages, and splenic macrophages are mainly populated during embryonic development [17]. Whereas populations in the dermis, bone, lymph node and heart are in part maintained by blood monocytes during adulthood, with gut macrophages being exclusively of monocytic origin [18–21]. Of note, is that although the stages of fetal hematopoiesis are known and their contribution in the ontogeny of macrophages accepted, questions persist regarding the degree of contribution from each wave of progenitors to early macrophage establishment in the embryo.

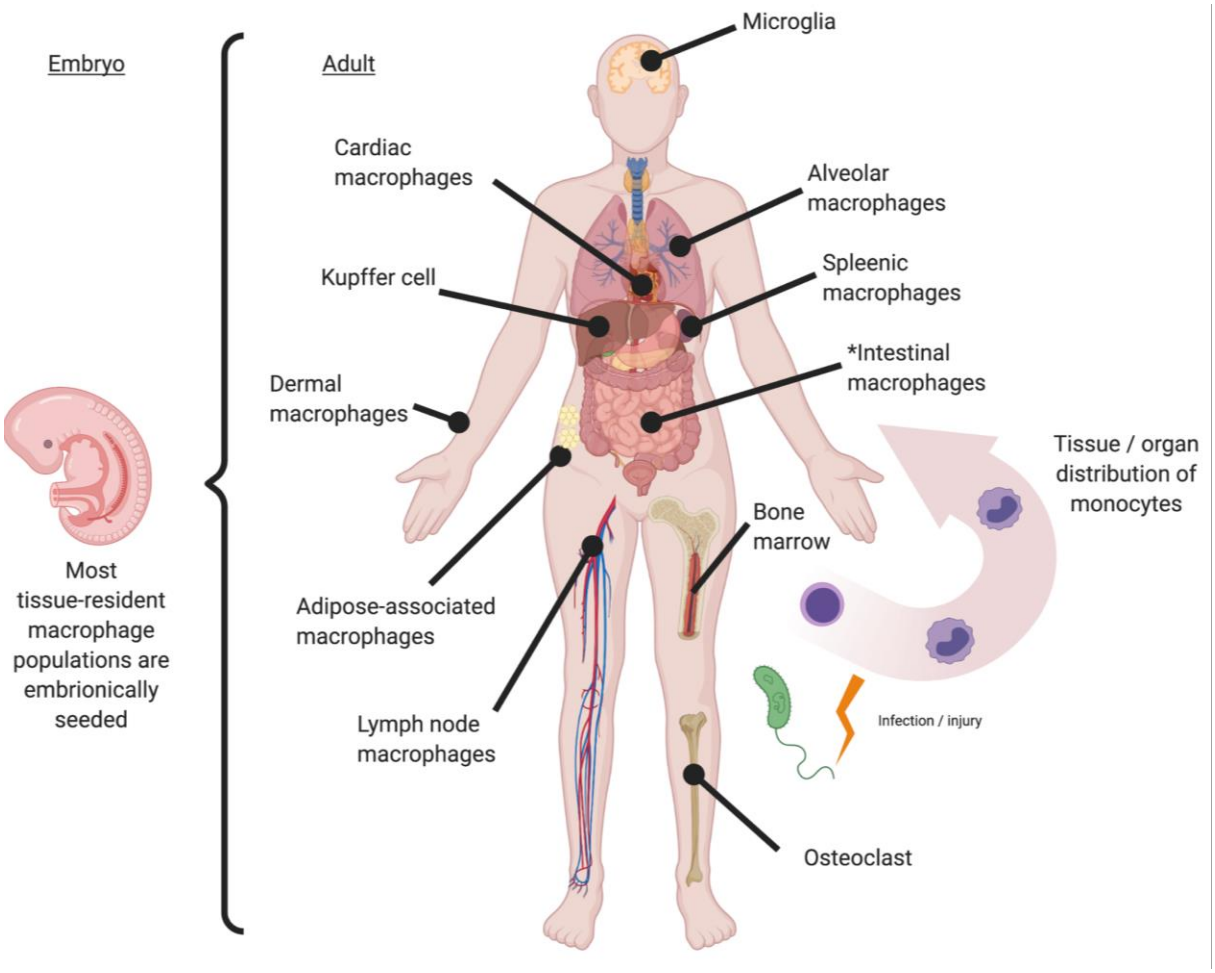

Figure 1. The origins and distribution of tissue-resident macrophages. Intestinal macrophages (*) are the only population without embryonic seeding.

1.1.2 The heterogeneity in macrophage function: from development to disease

Pathogenic microorganisms enter our body through different sites and can cause infection in a wide varied of organs, highlighting the need for global immune surveillance [23–27]. Macrophages contribute considerably to this regard, acting as sentinels with phagocytic capacities, strategically placed throughout our bodies and tissues [28,29]. Once a macrophage senses an invading pathogen, their *modus operandi* consists of two main strategies. The first is by directly eliminating the pathogen via phagocytosis, *i.e.,* recognizing the pathogen by cell-membrane receptors, internalizing it in membrane bound vesicles known as phagosomes, digesting it and presenting the antigen peptides

on its major histocompatibility complex class II. Macrophages present antigens locally, often to CD4+ helper T cells, supporting the adaptive immune response. The second is by releasing antimicrobial molecules (*e.g.,* defensins, nitric oxide and reactive oxygen species) and alarming or recruiting other effectors of the immune system via the production and release of immune regulatory messenger molecules (*e.g.,* IL-1, IL-12, TNFα and CXCL10) [30,31].

As a protective measure against pathologic inflammation, once the infection has been cleared or in the case of an injury, factors within the injured tissue environment will cue macrophages to take an anti-inflammatory role. These include the endotoxin induced tolerization of pro-inflammatory genes [32], inflammatory cytokine suppression via efferocytosis [33], the release of amphiregulin, TGF-β, and IL-10 by regulatory T cells [34] and macrovesicles carrying signaling molecules, such as SOCS2 [35]. Macrophages will return the favor by producing anti-inflammatory cytokines (*e.g.,* IL-10 and TGF-β) and lipid immune mediators (*e.g.,* resolvins and protectins). They will also directly support repair processes by removing obstructing tissue debris [36] and producing growth factors involved in cell proliferation and vascular development to alleviate the hypoxia created in these scenarios (*e.g.,* PDGF, IGF-1, VEGF-α) [37–39].

Much of the attention surrounding macrophages has been focused on their role in immune related functions, such as host defense and repair, often overlooking their crucial role in development and maintenance of homeostasis. Mice lacking *Csf1*$^{op/op}$ (also known as M-CSF), an indispensable growth factor for monocyte/macrophage differentiation and survival, display severe alterations in their distinct macrophage populations [40]. The most visible phenotype of *Csf1*$^{op/op}$ mice is osteopetrosis, where due to a depletion of osteoclasts (*i.e.,* bone macrophages), there is defective bone resorption leading to defective bone remodeling, implicating macrophages directly in bone morphogenesis [41]. In the developing mammary tissue, it's been observed that macrophages synthesise collagen fibres which structure the mammary ducts during growth and branching. They also place themselves at the terminal end buds of the ducts to clear apoptotic epithelial cells as the ducts expand. In the absence of these macrophages, there is a reduction in growth rate and decrease branching complexity [42–44].

With regards to homeostatic functions, intestinal macrophages play key roles in preventing pathological inflammation by maintaining immune tolerance towards the trillions of residing microorganisms, by both responding to and producing IL-10. The secretion of IL-10 forms a positive feedback loop with peripheral regulatory T cells (Tregs) and activates pro-proliferative pathways via WISP-1 induction, important in preserving intestinal barrier integrity [45,46]. Another classic example of the homeostatic function of macrophages are splenic red pulp macrophages and liver Kupffer cells. These are ideally positioned in the sinusoidal space within the capillaries of both the spleen and liver, respectively, where they recognize and digest senescent red blood cells (RBC). Once digested, via heme-binding proteins, they recycle the iron found within the RBCs to be utilized back into erythropoiesis, a process where another type of macrophages is also involved, nurse macrophages [47].

While macrophage functions are critical for protection against pathogens, repair and development, its dysregulation can give rise to substantial tissue alteration, and if prolonged, disease. Macrophages have been implicated in a long list of inflammatory diseases, such as atherosclerosis, pathogenic fibrosis, obesity, asthma, multiple sclerosis, rheumatoid arthritis, and cancer [22]. In atherosclerosis for example, macrophages penetrate the intima and subintima of arteries, where they engulf local low-density lipoproteins, becoming foam cells. The accumulation these lipid laden macrophages form atherosclerotic plaques, which are prone to rupture, resulting in cardiac events [48]. They are also involved the appearance of cardiac fibrosis, where after the release of stress signals, myocardial macrophages drive prolonged collagen deposition via myofibroblast activation by secreting IL-10, which leads to myocardial stiffness, and ultimately heart failure [49]. Furthermore, as previously mentioned, macrophages are potent producers of many key inflammatory cytokines, several of which (*e.g.,* TNFα, IL-18, IL-12, and IL-23) have been directly implicated in the development and progression of several autoimmune diseases [22].

Lastly, macrophages are programmed to support normal organ development, which they can also do in the case of malignancies. Solid tumors are often thought of as a random mixture of clones of cancer cells and extracellular matrix, but if anything, their components,

structure, and growth processes can resemble those observed in developing organs [50,51]. Tumor associated macrophages (TAMs) are the main type of tumor-infiltrating cells, and although initially thought of as 'anti-tumor', it is now known that tumors produce cytokines such as CSF-1 or IL-10 to hijack TAMs [52,53]. Macrophages can promote the initiation of tumors by causing chronic inflammation via inflammatory cytokines (*e.g.,* TNFα and IL-6) [54]. They also enable their growth by providing oxygenation and nutrient supply via vascularization factors (*e.g.,* VEGF, EGF, CXCL8 and PDGF) [55,56], as well as interrupting T cell-related antitumor immunity via suppressors (e.g., IL-10, PGE2 and TGF- β) [56]. And finally, they facilitate tumor invasion or metastasis by creating routes of escape for tumor cells via extensive extracellular matrix remodeling, as well as providing key growth factors [57,58].

1.1.3 Bone marrow derived macrophages, a model of study

Models of study, such as animal models, immortal cell lines and primary cultures provide researchers a controlled environment to interrogate biological phenomenon. With regards to functional and genomic studies of macrophages, there's been a preference for the use of cell lines and *in vitro* differentiated cultures over their native *in vivo* differentiated counterparts[59]. This often due to technical incompatibilities, for example, a standard chromatin immunoprecipitation (ChIP) protocol used for genome-wide studies typically requires anywhere from 5–20 x 10^6 cells as starting material [60]. On the contrary, most tissue resident macrophage populations are present in much lower quantities and difficult to recover from complex tissues. For example, you can expect ~3–5 × 10^5 alveolar macrophages cells from one adult mouse [61]. As a result of this low yield, one would have to perform this procedure on groups of >10 mice to achieve cell numbers suitable for certain functional genomic assays.

Macrophage cell lines, such as RAW264.7 and J774A.1, have historically been used in place of primary cells [62,63]. Although their use is now decreasing, primarily due to longstanding concerns from scientist regarding the documented genotypic and phenotypic drift of immortal cell lines, cell line cross-contamination and acquired genetic aberrations. All of which are known to alter their genome integrity, epigenetic landscape, and responsiveness to stimuli [64,65]. In turn, primary macrophages, specifically bone

marrow derived macrophages (BMDMs) have widely adopted. BMDMs are primary macrophage cells, differentiated *in vitro* from bone marrow cells in the presence of M-CSF, driving their differentiation into macrophages. When bone marrow cells are grown under these conditions, progenitors will proliferate and differentiate into a homogenous population of BMDMs [66,67]. First described in the early 1980's, BMDMs offer high yields of 7.5–15 x 10^7 cells per mouse, homogeneity and can be obtained from genetically modified mice, which is of particular importance in functional genomic studies [61,66,68].

Finally, recent studies have highlighted the importance of the local environmental milieu in shaping the chromatin state of macrophages [69], which leads to question if primary cells are too divergent from *in vivo* populations. A recent study explored the differences in transcriptomes and enhancer landscapes of three tissue residing macrophage populations and two cultured macrophage populations, including BMDMs. Their clustering analysis of RNA-seq and ChIP-seq data (H3K27ac and H3K4me2) showed that while differences were found between *in vivo* and cultured macrophages, these differences were no greater than those observed among the three resident macrophage populations [70].

1.2 Mechanisms driving functional diversity and plasticity in macrophages

Both tissue-resident macrophages and tissue-recruited monocyte-derived macrophages are equipped with almost 200 key receptors, including diverse pattern recognition receptors (PRRs, C-type lectins, cytosolic sensors, Toll-like receptors, *i.e.,* TLR), and cytokines and interferon receptors[71,72]. These receptors enable a macrophages' response to a variety of stimuli, such as pro-inflammatory (*e.g.,* interferon gamma [IFN-γ], IL-18, IL-1), anti-inflammatory cytokines, chemokines (*e.g.,* CXCL1, CCL5, CXCL8), and growth factors (*e.g.,* TGF-β)[73]. Importantly, some of these responses trigger both short and long-term functional adaptations, driven by specific transcriptional changes which are controlled by complex cellular mechanisms. Among these, changes to the epigenetic landscape of macrophages, especially at distal cis-regulatory elements, now arise as key modulators of macrophage activity[74].

1.2.1 The interferon gamma signaling pathway

The effects of IFN-γ on macrophage biology were first described by Nathan *et. al*., in 1983 [75]. The group identified a lymphocyte secreted factor capable of enhancing the capacity of human macrophages to release H_2O_2 and to kill *Toxoplasma*. Since then, great advances in elucidating the molecular mechanisms by which it can exert its immunomodulatory functions have been made. IFN-γ, the only known type ll IFN, has now been identified as a pleiotropic cytokine with antiviral, antitumor and immune modulatory functions [76]. It is primarily produced by cells of the immune system such as NK cells, natural killer T (NKT), innate lymphoid cells (ILCs), T helper 1 (Th1) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs)[77]. In these cells, IFN-γ production is induced at early stages of infection by immunomodulators at the site of infection (IL-12, IL-15, IL-18 and IL-21), antigen recognition, and even through a positive feedback loop established by IFN-γ itself [78,79].

Once produced, IFN-γ binds to its target cells via a unique receptor, the type ll IFN receptor, a tetramer composed of two IFNγR1 and two IFNγR2 subunits. Binding of IFN-γ to the high affinity binding receptor IFNgR1 induces the oligomerization of the tetramer to which the protein tyrosine kinases Janus activated kinases (JAKs) JAK1 and JAK2 are associated. This causes a series of auto- and transphosphorylations resulting in the activation of the associated JAKs[80]. Then, the activated JAKs phosphorylate the intracellular compartment of IFNγR1, causing conformational changes and allowing the docking and phosphorylation of the cytosolic signal transducer and activator of transcription 1 (STAT1) proteins in the C-terminus on tyrosine Y701. Such phosphorylation will cause homodimerization of STAT1 subunits, forming the gamma activation factor (GAF)[80,81]. Newly formed STAT1 homodimers will then translocate to the nucleus and bind specifically a gamma activation sites (GAS), containing the consensus sequence 5'-TTC(N)2-4GAA-3' [82].

Once bound to its target sites, STAT1 will activate gene expression in part by recruiting histone acetyltransferases (or HATs, such as p300) and members of the mediator complex[83]. Upon arrival, HATs will acetylate the surrounding nucleosomes to facilitate transcription, and kinases from the mediator complex, specifically CDK8, will phosphorylate STAT1 within its transactivation domain (S727). Phosphorylation of

nuclear STAT1 will activate its full transcriptional potential to drive the expression of Interferon Signature Genes (ISG) [84,85]. STAT1-induced ISG expression represents the first transcriptional wave in response to IFN-γ, among which the Interferon Regulatory Factor 1 (IRF1) is one of the most characterized. The expression of the transcription factor IRF1 will prove to be essential in driving the second transcriptional wave by binding to interferon-stimulated response elements (ISRE). Functionally, IRF1 seems to participate in transcription initiation by triggering enhanceosome formation and facilitating RNA Pol II recruitment to ISGs promoters [86–88] . Additionally, it was recently shown that IRF1 can further promote STAT1 activation via the expression of unknown proteins which can increase JAK phosphorylation, further activating cytosolic STAT1 [89]. Lastly, STAT1 and IRF1 often collaborate by co-binding to ISG promoters and enhancers to regulate their gene expression, although this will be discussed in detail below.

Activation of STAT1 via JAK-STAT is generally transient in nature, with its signalling activity coming back to baseline at 2-4 h. Moreover, most STAT1 targets display peak binding patterns within the first hour of IFN-γ signalling. However, it has been observed by many, that the effects of IFN-γ stimulation extend far beyond the initial signalling period[76]. Evidence is now accumulating of the existence of a multifaceted intracellular amplifier circuit regulating ISG expression and controlling long-term cellular IFN responsiveness [90]. And although, unphosphorylated and phosphorylated STAT1-containing complexes and IRF1 have been suggested to participate, no general mechanistic consensus has been reached. Further adding to this complexity, studies have suggested that under specific circumstances, IFN-γ is able to signal independent of STAT1, via crosstalk with various other TFs[91].

Finally, given that cytokine signalling represents the basis for immune response coordination, regulatory molecules of these signals are often embedded in their pathways. In the case of IFN-γ, the suppressor of cytokine signalling (SOCS) family is one of the most important negative regulators of the JAK/STAT signalling pathway [92]. Lodged into the promoter region of SOCS1 and SOCS3, is a GAS element, which is rapidly bound and activated by STAT1 during the first wave of IFN-γ transcription[82,92]. Upon expression,

these small regulatory proteins will bind to phosphorylated JAK via their SH2 domains, blocking the recruitment of STATs and thus their phosphorylation and activation [93].

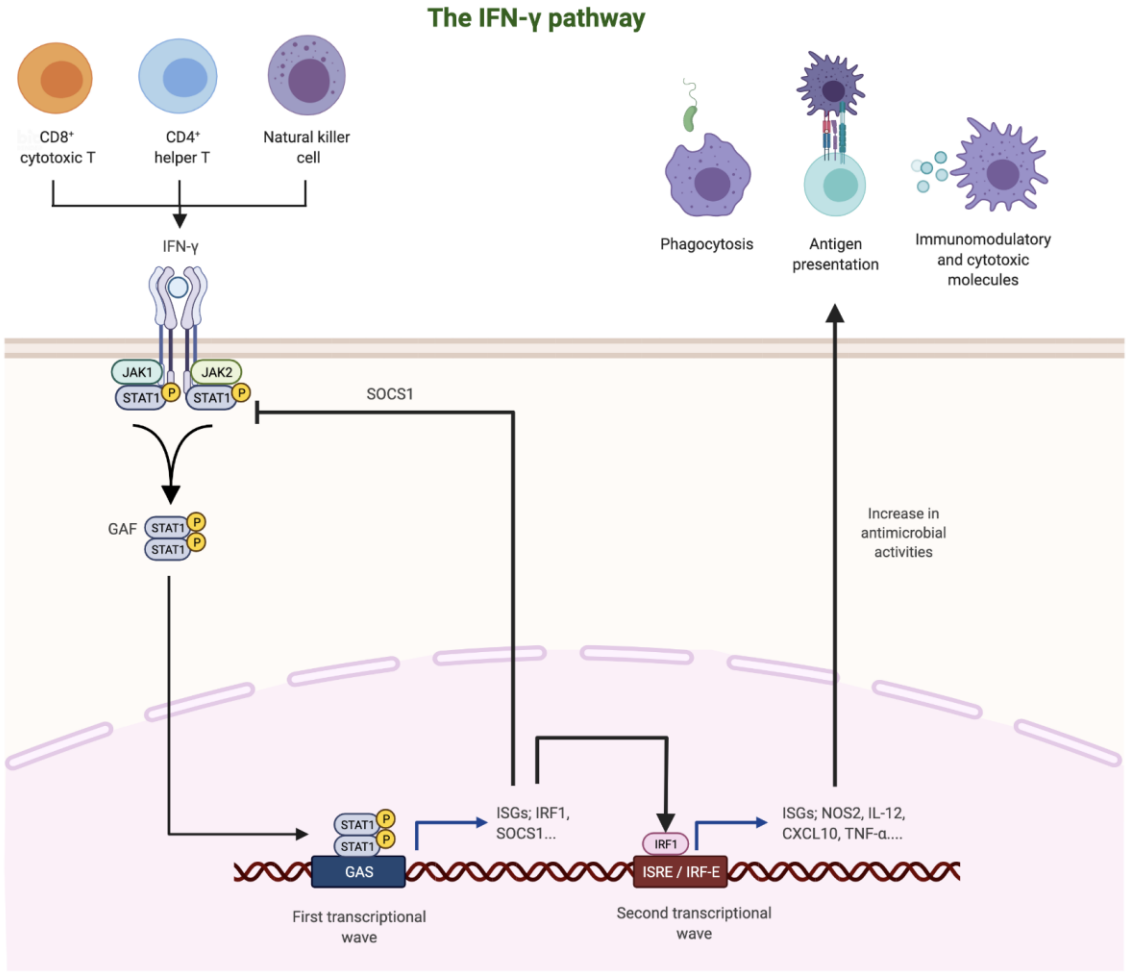

Figure 2. The sources of IFN-γ, its molecular signalling and the transcriptional and functional effects on macrophages.

1.2.2 Epigenetics and gene regulation

Of the trillions of cells that compose our body, from neurons to blood cells, nearly all of them contain the exact same genetic material, encoded in deoxyribonucleic acid (DNA). Despite this genetic redundancy, more than 200 cell types have been identified in the human body [95]. Qualitative and quantitative differences in their gene expression appear to drive their phenotypic and functional diversity [96], demonstrating the necessity to look beyond DNA sequences to fully understand gene regulation processes.

Simplistically, epigenetics takes aim at studying the mechanisms behind gene regulation, which occur without altering the DNA sequence itself [96]. Epigenetic regulation can be sub-divided into three main classes: (1) DNA methylation of the 5'-position of cytosine residues by DNA methyltransferase and is usually associated with gene repression [94]; (2) the expression of non-protein coding RNA species, which have been shown to interact with mRNA molecules, chromatin remodelers, or chromatin itself [95]; and lastly, (3) post-translational histone modifications, which influence chromatin structure, accessibility and function. In the present work, we will focus our attention on chromatin modifications and the effects these have on genetic regulation [99].

1.3 Chromatin structure and histone modifications

Within the nucleus of eukaryotic cells, DNA interacts with histone proteins to form nucleosomes, the basic unit of chromatin. Nucleosomes are consisting of 145-147 bases of DNA wrapped around an octamer composed of two groups of H2A, H2B, H3 and H4 proteins. And although not itself a part of the nucleosome structure, in many cases histone H1 proteins will bind and keep in place inter-nucleosomal linker DNA, playing an important role in stabilizing compact higher-order chromatin structure [100]. How these chromatin structures are modified and organized, will profoundly influence gene transcription. Generally, a more 'relaxed' chromatin structure leads to greater chromatin accessibility, and thus higher transcription levels. Conversely, tightly wound chromatin structures are generally associated with lower transcriptional activity [101].

Histone proteins contain N-terminal tails that are central to the processes that modulate chromatin structure and accessibility. N-terminal histone tails are subjected to a variety of post-translational enzymatic modifications, among which acetylation, phosphorylation and methylation are the most characterized, but others like biotinylation, ubiquitylation, sumyolation, etc. also exist. The effects of these histone modifications on chromatin structure and function are varied and often complex[99]. For example, acetylation of histone tails has been shown to lower the affinity between the positively charged lysine residues of histone tails towards the negatively charged DNA. This leads to a relaxation of the chromatin structure, uncovering a variety of functional elements, such as promoters and enhancers, to which regulatory proteins may bind, including transcription factors (TF) [102].

Histone modifications also play major roles in recruiting chromatin modifying complexes [103], preventing their association, and modulating their level of activity [104].

The arrival of next-generation sequencing allowed for genome-wide large-scale mapping of histone modifications via Chromatin Immunoprecipitation sequencing (ChIP-seq). A comprehensive view of the histone modification landscape began to emerge, making it possible to associate specific histone marks to various genomic features and chromatin states [105]. For instance, active enhancers (*i.e.,* distal regulatory elements) are generally enriched for histone H3 monomethylation of lysine 4 (named H3K4me1) and for acetylation of lysine 27 (H3K27Ac), as well as for the presence of co-activators such as p300 [106,107]. In contrast, active promoters (*i.e.,* proximal regulatory elements) are enriched for trimethylation of lysine 4 (H3K4me3), H3K27ac and H3K9ac [106]. Moreover, the functional status of gene bodies can also be predicted by their associated histone modifications, with inactive genes displaying H3K9me2 and -me3, and active genes displaying H3K36me3 and H3K79me2 [108,109]. However, the epigenetic regulation of these functional elements could be better thought of as a spectrum of activation status, instead of a dichotomy of active versus inactive, where via combinations of distinct histone modifications the activity levels of these genomic elements are rather 'fine-tuned' to meet the transcriptional needs of the cell [105]

Gene expression in eukaryotes is a complex process, involving a multi-step process aimed at ensuring the precise activation of specific transcriptional programs. It involves the docking of TFs onto regulatory elements, such as enhancers and promoters, in order to promote the recruitment and activation of the general transcriptional machinery. Promoters are short sequences which are proximal to the transcription start site (TSS) of genes. Promoters will define where transcription initiation will occur, providing a binding site for RNA polymerase binding[110]. In contrast, enhancers are distant from the TSS, thus they exert their influence via long-range looping interactions with promoters. The extent to which enhancers and their respective promoters interact, will deeply influence gene regulation [88].

In sum, the discovery of a histone 'code' and advances in functional genomic studies have made it possible to interrogate the behavior of these regulatory regions during

development, as well as in response to environmental cues. This will be discussed below in the context of macrophages development and responses.

1.3.1.1 Enhancer establishment during lineage determining and the hierarchical model of transcription factors

More than 35,000 potential transcriptional enhancers have been identified in mouse macrophages, far exceeding that of promoters[111,112]. Also, enhancers present a higher overall density for TF binding motifs than their promoter counterparts[113]. Thus, DNA-TF interactions are not only more likely to occur at enhancer regions, but these also possess a higher potential for combinatorial TF binding, serving as more complex 'hot spots' binding sites than do promoters. Moreover, during the last decade, several studies have highlighted a previously unappreciated role of enhancers in cellular development[69,114–116]. For instance, a study by Lavin *et al*., in 2014 profiled and compared the expression and chromatin landscape, including promoters, active enhancers, and poised enhancers from seven distinct tissue resident macrophage populations [69]. They found that the environment plays a critical role in shaping the unique identity and function of macrophages. And more importantly, via pairwise correlation analysis, they observed that the chromatin state at enhancer regions, and not of promoters, is responsible for driving cell-type-specific gene expression.

During lineage commitment, an important class of transcription factors, referred to as lineage determining transcription factors (LDTFs) will establish the enhancer repertoire within cells. The enhancer selection will depend on the cooperation from both lineage and tissue specific TFs. A subset of LDTFs function as pioneer transcription factors, meaning they possess the unique ability to induce the opening of previously closed chromatin [117]. PU.1, encoded by the gene *Spi1*, is a hematopoietic-specific factor, considered to be a master regulator of macrophage development [118] and other lineages. ChIP-Seq experiments have shown that nearly all macrophage-specific enhancers are enriched for PU.1, as well as other collaborative LDTFs, such as C/EBPα and AP-1 [111,119]. PU.1 mediates enhancer establishment through its unique ability to recognize and bind to its DNA-binding motif within heterochromatin, promote nucleosome depletion, and the deposition of H3K4me1 [111,120]. Subsequently, these nucleosome-depleted enhancer

regions will be recognized and occupied by a second class of TFs, termed signal dependent transcription factors (SDTFs). In fact, studies have indicated that the majority of SDTFs bind to enhancer sites already pre-occupied by LDTFs [121]. Furthermore, due to the collaborative binding of LDTFs, which vary among different tissues, the established enhancer repertoire is unique to each cell type [111]. This may explain why broadly expressed STDFs can exert cell-type specific functions [122]. Unsurprisingly, *in vitro* studies have demonstrated a dependency of SDTF binding on the presence of LDTF. Specifically, mutations leading to the altered binding of LDTFs, such as PU.1, resulted in a dramatic decrease in STDF binding, such as for NF-κB. On the contrary, when NF-κB binding was altered, PU.1 suffered little to no alterations in its binding patterns [121].

Given the observations that SDTFs reside mostly in LDTF-established regions, and that their binding is dependent of LDTF function, a hierarchical model of transcription factor regulation in macrophages was proposed [123], where LDTFs determine the enhancer and promoter repertoire of macrophages and SDTFs are left to - *mostly* - operate within the confines of this pre-established epigenetic landscape.

1.3.1.2 Pioneer transcription factors

In eukaryotes, nucleosome packaging of DNA presents physical constraints on the ability of TFs to bind to their target regulatory sequences and thus to initiate gene transcription [124]. To this end, a subset of factors holds the remarkable ability to bind to compacted, otherwise inaccessible chromatin, and overcome the constraints of higher order packaging of DNA. This unique subset of TFs are known as pioneer transcription factors. However, the act of their binding alone is insufficient to properly drive gene expression. Consequently, to infer pioneering abilities a TF must: bind to target DNA sequences within heterochromatin, initiate chromatin remodelling processes, permit the recruitment and binding of other non-pioneering TFs, and finally, induce stable long-term structural and epigenetic changes to the surrounding chromatin [125].

Although the mechanistic details behind their ability to induce chromatin remodelling remain poorly understood, the general steps are becoming understood. Initial binding of these factors appears to occur rapidly, with studies suggesting that binding occurs early after their expression and/or activation. For instance, upon induction, ER-Pax7 binding

was detected at both active enhancers and pioneer targets within 30 – 60 min [126] . Once bound, pioneer factors favor the relaxation of the compacted nucleosome structures via several mechanisms. For instance, it was shown via cryogenic electron microscopy, that SOX2 binding locally distorts DNA at superhelical location 2 and repositions the N-terminal tail of histone H4. These alterations led to chromatin opening, as they are presumably incompatible with higher-order nucleosome stacking [127]. Moreover, it was shown that the transcription factor c-Myb's pioneering activity involves the recruitment of HATs, which leads to histone acetylation followed by acetylation-induced chromatin dissociation [128]. Once chromatin opening has occurred, enhancer activation is driven by the recruitment of chromatin modifying complexes, although these may vary. For instance, Pax7 recruits the MLL1/2 complex, while FoxA1 recruits MLL3, both involved in H3K4me1 deposition [126,129,130]. Of note, while pioneer binding to chromatin appears to be quick, the remodelling processes resulting in opening and marking of the chromatin are slower [126]. During the last step, SDTFs will be recruited to these newly accessible regions, further recruiting remodelling and co-activator complexes.

Since the first pioneer factor was identified in the late 1990s, pioneering activity has been identified in an additional ~15 TFs [131]. Interestingly, the gran majority of these are intricately associated to cell fate determination [125]. Raising the question as to whether transcription factor pioneering activity is exclusively reserved for processes such as cell specification and differentiation.

1.3.1.3 Signal-dependent transcription factors and macrophage plasticity

Macrophages are required to rapidly respond to changes in their environment and do so via the expression and/or activation of SDTFs. Steroid hormone receptors, NF-κBs, AP-1, STATs and IRFs are the major signal-induced mediators of macrophages. Upon cue, these mediators will act in a combinatorial manner to regulate gene expression [132–134]. During signal-induced responses, SDTFs will nuclear translocate and bind primarily to pre-existing poised enhancers, established by LDTFs. One of the main purposes for the pre-selection of enhancers, is to allow for rapid bursts of transcription. For example, STAT1 residing in the cytoplasm is phosphorylated, translocated, and bound to pre-

marked enhancers in only a few minutes [116,135]. Indeed, within a few hours of IFN-γ signalling, STAT1 target sites reach their peak expression levels [76].

Moreover, the pre-selection of the enhancer landscape within macrophages is also involved in the homeostatic control of cell-identity. This guarantees that the transient responses of macrophages to both external and internal cues, won't alter cell identity. Thus, the LDTF-established enhancers not only determine cell identity, but also help to keep a balance between a macrophage's ability to respond to stimuli and the maintenance of cellular identity [123,136].

Nonetheless, cellular plasticity has long been appreciated as a hallmark of cells from the monocyte-macrophage lineage, allowing them to react and adapt to their changing environment [137,138]. Cellular plasticity is the ability for cells to functionally diverge from their original terminal differentiation state, without cell division. This divergence is associated with radical changes in their transcriptional programs, often rooted in shifts within a cell's enhancer landscape [122,139]. Moreover, the enhancer repertoire of a cell – at any given time – represents only a fraction of the tens of thousands potential enhancer sites [111,112].

Indeed, several studies have now confirmed that upon stimulation, macrophages undergo a partial reprogramming in their available enhancer regions [69,122,136]. During signal-induced reprogramming, SDTFs will collaborate with PU.1 to activate many 'latent' or *'de novo'* enhancer elements. These are enhancer regions present in differentiated cells, characterized by a lack of TF binding and marks of activation, but that become activated upon certain stimulation [136]. Latent enhancers were first described in 2013 by Ostuni *et al.,* where the authors studied the genome-wide effects of LPS stimulation on PU.1 binding and the enhancer repertoire in macrophages. The authors found that 24 h post LPS stimulation, ~1000 previously inactive regions gained H3K4me1 and H3K27Ac marks, as well as collaborative PU.1/STATs binding. They also noted that while activation of these enhancer regions was slow, once established, many persisted and mediated a faster and stronger response upon re-stimulation. A study later that year found that TLR4 signalling could lead to the appearance of an even greater number of enhancer elements, ~3000 enhancer-like regions [122]. Another aspect of macrophage plasticity involves the

disengagement or decommissioning of enhancers. Upon IFN-γ stimulation, approximately 5,000 enhancer elements lose the activating H3K27Ac mark thereby becoming poised, with a subset of these losing their LDTF binding and chromatin accessibility [140].

The microenvironment appears to also play a critical role in shaping the functional identity and enhancer layout of macrophages, even beyond terminal differentiation. Interestingly, Lavin *et. al.,* found that of the 12,743 macrophage-specific enhancers, less than 2% are shared across all tissue-resident macrophage populations[69]. They observed that upon transferring macrophages from the peritoneal cavity, into alveolar tissue, these were able to switch 70% of their RNA expression profiles to resemble more closely that of lung macrophages. Thus, differentiated macrophages retain a significant portion of their cellular plasticity.

1.4 Interferon Regulatory Factor 1 (IRF1)

The study of interferon regulatory factor 1 (IRF1) dates to 1988, when the Taniguchi laboratory isolated a cDNA clone encoding for a virus-inducible mouse protein that binds to an interferon-β (IFNB) enhancer element. At the time, the novel regulatory factor showed no significant homology to other known proteins and was hence named IRF1 [141]. More than three decades of intensive gene regulation studies have made IRF1's functional diversity stand out among the rest. Highlighting its remarkable ability to drive distinct sets of transcriptional programs involved in host defense, maintaining homeostasis, tumor surveillance and inflammation-mediated diseases [142].

1.4.1 The role of IRF1 in immune cell development, host responses and disease.

The influence of IRF1 on immune system regulation is diverse and begins as early as the development of both the myeloid and lymphoid immune compartments. In mice devoid of IRF1 (*Irf1*$^{-/-}$), impaired myelopoiesis has been observed, resulting in immature macrophages [143], a decrease in CD8^{+} dendritic cells (DCs) with a skewed commitment towards plasmacytoid DCs [144], granulocyte hyperplasia [145], and even altered development of osteoclasts and osteoblasts, resulting in an abnormal bone phenotype [146]. Lymphoid defects have also been observed, for example *Irf1*$^{-/-}$ mice display defective intra-thymic selection of thymocytes, characterized by a marked 10-fold reduction in CD8^{+}

T cells. The functionality of $CD8^+$ T cells is also impaired, as the cytotoxic T lymphocyte (CTL) response to virus-infected cells is significantly reduced in *Irf1*$^{-/-}$ mice [147,148]. Moreover, *Irf1*$^{-/-}$ mice display a reduced number of natural killer cells (NK) with altered cytolytic activity [149]. The former due to a dependency on IRF1 in the induction of IL-15 in bone marrow stroma cells, required for proper NK development [150].

Cellular crosstalk between the innate and adaptive immune system is also impaired in the absence of IRF1 [149,151]. For instance, during the early stages of infection, antigen presenting cells (APCs) such as macrophages and DCs will produce IL-12 [152,153]. IL-12 drives Th1 polarization and stimulates both Th1 and NK cells to secrete IFN-γ. IFN-γ in turn augments the cytolytic and antigen presenting activity of these APCs through activation of type ll IFN signalling (**figure 2**) [154,155]. Proper development of the Th1 immune response has proven critical for the control of intracellular bacterial and parasitic infections [151,154,156]. In *Irf1*$^{-/-}$ mice, this cellular communication is crippled due to an impaired production of the IL-12 p40 subunit, diminished responsiveness of $CD4^+$ T cells to IL-12 itself, and a reduction in the NK cell population [149,151,157]. IRF1 further promotes the Th1 response by binding and suppressing IL-4 expression, a cytokine which drives Th2 responses [158]. In addition to IL-12-p40, IRF1 is also required in the production of other inflammatory molecules involved in activating early immune response, such as IL-18, RANTES and TNF-α [159–161]. Unsurprisingly, *Irf1*$^{-/-}$ mice are highly susceptible to *in vivo* bacterial, parasitic, and even viral pathogens[142,149,151].

IRF1 participates in immune cell development and modulation via its unique ability in driving cell type-specific transcriptional programs in response to stimuli. Specifically, signaling pathways leading to IFN and/or ISG expression via the engagement of a variety of receptors, such as IFNRs, pathogen recognition receptors (RIG-like receptors [RLR] and Toll-like receptors [TLR]) and Tumor necrosis factor receptors. Early studies demonstrated that upon viral challenge, IRF1-depleted mouse embryonic fibroblast cells could induce a normal expression of type I IFNs [162,163]. This placed doubt on IRF1's relevance in the IFN type l responses and granted much of the spotlight on other IRFs, such as IRF3 and IRF7, in the blossoming IFN studies [142]. In the last decade, interest in IRF1 as a regulator of IFN l/lll expression, and in innate immunity in general have been

restored. There is now clear evidence that IRF1 is crucial in host antiviral defense by suppressing the replication in a variety of RNA viruses[164,165]. For example, in human hepatocytes, IRF1 is key in activating the type I and/or III IFN transcriptional responses downstream of RLR signalling, upon infection with Sendai virus and dengue virus[166]. Moreover, IRF1 seems to also participate downstream of TLR signalling, by controlling TLR9-dependent IFNβ production in mouse DCs by interacting with MYD88[167,168]. Specifically, the interaction between these two, licenses the nuclear translocation of newly synthesized IRF1 proteins into the nucleus, where it promotes IFNβ expression, further demonstrating IRF1's involvement in the induction of IFN I responses. But perhaps the most convincing report on the importance of IRF1 in mediating IFN responses was by Forero and colleagues [169]. The authors questioned why despite the similarities in signalling cascades and transcriptional programs between type I and III IFN, the latter seems to have a lower potency and slower kinetics in its inflammatory response. They found that the selective induction of IRF1 by type I IFN signalling, could alone account for the differences in the inflammatory potential of these two pathways. IRF1 induction led to the expression of several chemokines (CXCL9, 10 and 11), key for leukocyte recruitment and inflammation. These studies undoubtedly highlight an unappreciated role for IRF1 in inducing IFN-dependent antiviral responses.

Currently, one of its most exciting roles and the focus of the present study, is IRF1's role in the type II IFN response. As mentioned, early studies on IRF1 noted its involvement in both the development of the IL-12 / IFN-γ axis and the amplification of myeloid cell's response to IFN-γ, via type II IFN signalling [149,151]. And although the dependency on IRF1 was clear, the transcriptional programs it governed, the mechanisms and molecular players involved remained elusive. With the emergence of genome-wide epigenetic techniques, it became possible to determine the global binding patterns of regulatory proteins, identify regulatory elements and interrogate chromatin accessibility statuses. Studies now suggest that IRF1's involvement in the amplification of the myeloid response to IFN-γ is essential and non-redundant with other TFs [170]. By synergizing with other transcription factors, such as STAT1 and IRF8, IRF1 induces the production of a plethora of anti-microbial molecules, (*e.g.,* iNOS, $gp91^{phox}$ and Cox2) and inflammatory cytokines (*e.g.,* IL12-p40, IL18 and TNF-α)[160,161,170–174]. In fact, BMDMs derived from *Irf1*$^{-/-}$ mice

entirely lack iNOS production upon IFN-γ and LPS stimulation [175]. Thus, IRF1 is required for the proper induction of the type ll IFN inflammatory response, critical in activating the full microbicidal potential of macrophages, an important stage of early host defense [149,170].

Interestingly, while most studies have characterized IRF1 as an inducible factor, recent studies have elegantly showcased IRF1's contribution to constitutive viral defense [176]. Specifically, through its low-level basal expression in hepatocytes, IRF1 was found to maintain constitutive transcription of ISRE-dependent antiviral genes. Interestingly via lentivirus depletion experiments it was observed that IRF1 acted independently of MAVS protein and STAT1. Moreover, the depletion of a dozen signalling and regulatory proteins (including IRF3 and IRF7) resulted in relatively small increases in hepatitis A replication. Whereas by depleting IRF1 alone, hepatitis A RNA levels were 30-fold higher than in the control. This level of permanent surveillance by IRF1 is considered necessary due to the frequent targeting of hepatocytes by viruses, as well as the 'stealthy' nature of some, capable of replicating without immediate detection by cytosolic sensing [177,178].

While the inflammatory response driven by IRF1 in myeloid cells is critical for host defense, it could be thought of as a 'double-edge sword' in immunity and disease. A clear example of this dichotomy can be found in inflammatory bowel disease (IBD) and colitis-associated colorectal cancer (CA-CRC). Where large genome-wide association studies (GWAS) have identified IRF1 as a risk factor for the development of IBD, and its expression mechanistically tied to an aggravation of the injury to epithelial cells during intestinal inflammation [179,180]. Yet, on the other hand, a recent study has found that partial or complete loss of IRF1 causes susceptibility to CRC, possibly via alteration of immune cell function and population numbers [181]. Aside from IBD, IRF1 has been shown via association or mechanistic studies to be an important regulator of other inflammation-driven diseases, including atherosclerosis, acute renal ischemia, rheumatoid arthritis, inflammatory demyelination, and experimental cerebral malaria[182–186]. A recent genome-wide transcription factor regulation study identified a set of specific regions for IRF1 and IRF8 binding in macrophages, termed the IRF1/IRF8 regulome [170]. They found a strong concordance between the genes in said regulome and genetic risk loci in a subset of inflammatory diseases.

1.4.2 The structure, binding mechanisms, and regulation of IRF1

IRF1 is one among nine members of the IRF family of transcription factors found in human and mouse. Structurally, all mammalian IRF members are characterized by a unique and conserved N-terminal DNA-binding domain (DBD) containing five tryptophan repeats, a nuclear localization signal (NLS), and a C-terminal IRF-association domain (IAD). Additionally, a subset of IRF members (IRF3, 4, 5, and 7) contain an auto-inhibitory domain (AR) [94]. These elements act in harmony to regulate the activity level and functional role of an IRF.

Crystallography analysis of a purified IRF1-bound to IFN-β promoter sequence revealed that IRF1 possesses an α/β architecture containing three α-helices and four antiparallel β-sheets interrupted by three long loops [187]. Topologically, the 115 amino-acid DBD of IRF1 forms a structure resembling a helix-turn-helix, a structural motif commonly found in proteins regulating gene expression [187,188]. However, its core recognition sequence (5′-GAAA-3′) and mode of interaction is distinct from other HTH-containing regulatory proteins. In short, the third α-helix will position itself within the major groove of the GAAA sequence, leading to extensive contacts between the hydrophobic core of the protein and the DNA backbone. Importantly, three of the five tryptophan (W11, W38 and W58) repeats will position themselves strategically through hydrophobic contacts within the protein and straddle the major groove containing the recognition helix. There, tryptophan residues will create hydrogen bonds and van der Waals contacts, stabilizing the IRF1-DNA complex [94]. Predictably, this ‘tryptophan cluster’ has been evolutionarily conserved within the IRF family, its vertebrate homologues and even in IRF-like genes identified in distant invertebrate organisms [94,142]. Interestingly, after IRF1-DNA coupling has occurred the DNA structure will distort, inclining towards the IRF1 protein. This DNA bending is suggested to have functional implications with how IRF1 interacts with co-transcriptional activators. For example, IRF1-induced DNA bending can bring IRF1 closer to neighbouring regulatory proteins, promote interactions during enhanceosome formation or inhibit the binding of other transcription factors.

In contrast to the DBD-containing N-terminal domain, which is highly conserved among the IRF family, the C-domain is much more structurally diverse. Here lies its association

domain (IAD), for which the structural variation and post-translational modifications (PTMs) will determine its interaction with other transcription factors. The combinatorial interactions between transcription factors will in turn determine the DNA binding motif of the protein complex. IRF1 and IRF2 contain a unique interaction domain (IAD2), while the rest of the IRF members share a conserved IAD1[94,189]. IRF1 has been shown to interact directly with a select few transcription factors, including IRF8, STAT1, BATF2, NF-κB and possibly with itself, forming a homodimer [170,174,190–192]. However, IRF1 homodimers have only been observed a two-hybrid system, yet due to observation that many IRFs form these complexes (IRF3, 5 and 7), it seems likely IRF1 may also do [193]. The implications these interactions have on IRF1's binding patterns and consequently, the gene programs induced will be discussed later in greater detail below [187,194].

IRF1 is an unrivaled mediator of inflammation via its induction of IFN and/or ISG expression [169]. Therefore, its expression, presence and activity levels must be precisely regulated to allow for proper host defense and avoid pathologies. In terms of transcription, IRF1 is strongly induced by a variety of classical pro-inflammatory stimuli, including IFNs (-α, -β, -γ), TNF-α, IL-1, IL-6, cytosolic nucleotide sensing and more generally by viral / bacterial infections [143,169,195,196]. *In vivo* footprinting analysis of the IRF1 promoter have identified a GAS element (5′-TTCN$_3$GAA-3′) and a putative NF-κB region, binding sites for STATs and NF-κBs, respectively [197,198]. Unsurprisingly, *Stat1*$^{-/-}$ mice show a severe lack of IRF1 production at 2 h post IFN-γ activation [199]. Moreover, the lack of an ISRE site (5′-AGTTTCN$_2$TTTC-3′) at the IRF1 promoter may explain why IRF1 expression is higher with type ll IFN stimulus, than IFN l or lll [169]. After appropriate signalling, IRF1 can be expressed and translated as early as 15 minutes, as noted in proximal tubular cells after ischemia-reperfusion injury[184]. This rapid production seems unique to mammals, suggesting IRF1 has continued to evolve functionally since the divergence of vertebrates and invertebrates, to provide a more robust response to infections [142]. For instance, the appearance of *de novo* IRF1 transcripts in mammals occurs at an average of 3 h post-stimulation[200], compared to 12 h for bony fish [201] and 48 h for Mollusca [202].

Once expressed and transcribed, IRF1 proteins are highly susceptible to degradation by the 26S proteasome [203], making them among the shortest-lived IRFs. For example, the

average half-life of IRF1 is 0.5 h, whereas IRF2 or IRF3 are more stable at 8 h and 20-60 h, respectively [204,205]. This short lifespan allows for a more dynamic regulation of IRF1 levels transcriptional activity in response to environmental changes. To date, the precise mechanisms governing IRF1 protein degradation and activity remain elusive. However, its interaction with cytosolic regulatory proteins and post-translational modifications seem to play important roles. In addition to Myd88, several IRF1-regulating proteins have been described in the literature, such as Hsp70, YB-1, NPM1 and TRIM28[206,207]. These interactions are mainly mediated by IRF1's C-terminal multifunctional domain 1 (Mf1), which serves both as site for its modification and interactions with other proteins. For example, in the absence of Mf1, defective ubiquitination and degradation of IRF1 has been observed [208].

In terms of modifications, K48-linked ubiquitination of the 39-residue C-terminal region of IRF1 is known to signal proteasome-dependent degradation[203]. In contrast, K63-linked ubiquitination at the same region appears to activate its transcriptional activity [209]. During sterile inflammation, K63-linked polyubiquitination of IRF1 was found to be essential for IL-1-induced production of chemokines CXCL10 and CCL5[209]. A similar positive regulation of IRF1 is observed upon TLR7/8 engagement, leading to its accumulation in monocytes and B cells[210]. Cellular stress can also influence the appearance of these modifications, for instance, in unstressed cells IRF1 is chaperoned by CHIP (C terminus of Hsc70-interacting protein), preventing its ubiquitination. Oddly enough, under specific stress conditions, a CHIP-IRF1 complex will form leading to an increase in ubiquitination and degradation of IRF1[211]. SUMOylation, phosphorylation and acetylation have also been reported to occur, although the impact these have on the functionality of IRF1 are under debate[212,213].

1.4.3 The transcriptional programs, binding patterns, and co-acting transcription factors of IRF1

IRF1 expression can be induced by a variety of stimuli in practically all human tissues, presenting a relatively low cell type specificity. However, within these tissues, it displays a remarkable functional diversity in the regulation of cellular responses to a variety of stimuli [149,214]. And while the various mechanisms regulating its activity levels surely play

a role, they're unable to account for the spectrum of transcriptional programs IRF1 can induce. It later became clear that factors such as collaborative TF binding and the chromatin landscape surrounding target sites were likely playing major roles in fine-tuning IRF1-induced transcription [170,215].

Through several early single-gene studies, we knew IRF1 could target genes involved in immune responses (*Ciita*, *Nos2* and *Il12b*) [175,216,217], DNA damage (p21 and caspase1) [218,219], and apoptosis (caspase-8, TRAIL and XAFI)[184,220]. High-throughput studies began to emerge in the field, with a study using microarray hybridization (ChIP-chip) on IFN-γ treated breast cancer cells identifying 202 new sites bound by IRF1 [221]. Although known to regulate to the expression of a few DNA repair genes at the time, strikingly close to 10% of the nearby genes were related to DNA damage response, a key finding in the cancer field at the time.

Next-generation sequencing coupled to ChIP allowed for the true genome-wide study of gene regulation and epigenetics surrounding TFs. In 2011, the first ChIP-seq study of IRF1 was published on unstimulated human monocytes [222]. Despite using non-stimulated cells, the authors noted 52 bound regions associated with target genes mainly mapping to immune responses such as AIM2 and IFIT3. These results not only demonstrated considerable IRF1 binding activity but hinted towards a role in basal host defense. A refined 18-bp binding motif (RAAASNGAAAGTGAAASY) was also identified, which appeared to better predict IRF1 binding *in vivo.* Within the growing interest in IRF1 as a regulator of cancer progression, a study in 2015 by Retino and Clarke, identified 17,416 regions bound by IRF1 in breast cancer cells stimulated with IFN-γ [223]. Interestingly, most sites occurred at remote regions from the transcription start site (TSS), suggesting remote gene regulation as a primary mode of action for IRF1. Also, the gene ontology analysis of the closest neighbouring gene indicated apoptosis or cell death as the major target processes, cementing IRF1's role as a tumor suppressor.

Attention was later turned towards the extent and transcriptional impact of collaborative TF interactions with IRF1. A study by Langlais *et. al*., in 2016 using ChIP-seq data from both wild type and genetically altered BMDMs (from *Irf8* mutant and *Irf1*$^{-/-}$ mice), revealed the genomic binding schemes and associated chromatin status of IRF1, IRF8, STAT1

and PU.1 at steady state and in response to IFN-γ [170]. As expected, many sites were preoccupied by PU.1, having little-to-no increase of signal post stimulation. In contrast, IRF1 presented the highest induction of chromatin binding by IFN-γ, with moderate increases for IRF8 and STAT1. Moreover, IRF1 and STAT1 bound the most extensively to remote locations, with about 25 and 30% of their binding peaks occurring >10kb relative to the closest annotated gene, respectively. In total, 7 binding clusters were identified, containing distinct flavors of TF binding combinations, with specific transcriptional functions. For instance, cluster 1 [IRF8/IRF1/STAT1/PU.1] and cluster 5 [IRF1/STAT1/PU.1], were found to have a major role in macrophage's inflammatory transcriptional response to IFN-γ. Furthermore, the analysis also showed a considerable number of regions which seemed to display IRF1 binding independent of PU.1. This study demonstrated the extensive TF interaction occurring in macrophages upon stimulation and highlights the genome-wide importance of IRF1 in macrophage activation.

Other studies characterizing TF collaborations of IRF1, specifically with STAT1, have been published since. Although not genome-wide, Hassan *et al.,* in 2017 studied the binding patterns of STAT1 and IRF1 in HeLa cells via ChIP coupled to a microarray carrying 10% of all known ISGs [89]. IRF1 seemed to outnumber STAT1 binding 2 to 1 at ISG regions after IFN-γ stimulation. Moreover, STAT1 is almost always co-bound to IRF1 (dual binding), but IRF1 binding occurs frequently as isolated events [89]. This appears to disagree with previous studies, where in macrophages most IRF1 sites, were dual binding with STAT1 [170]. Despite the extensive isolated binding IRF1, dual binding sites were linked to ISG responsiveness, while isolated IRF1 sites were mostly non-responsive. A follow up study utilized a custom ChIP tiling array to map chromatin modifications at ISG regions in HeLa cells. The authors found that 86% of sites presenting dual binding of STAT1 and IRF1, underwent induced histone acetylation. In contrast, only 25% of isolated IRF1 binding sites underwent inducible histone acetylation, 33% were constitutively acetylated and 44% showed repressive marks for H3K27me3 and were termed "orphan sites". These orphan IRF1 sites skewed to remote sites and were detected in different cell types, appearing to be of biological relevance [215]. This further highlights the degree of cooperation between IRF1 and STAT1, in driving chromatin remodelling at ISG regions and dictating their responsiveness.

Lastly, despite the essential role of IRF1 on macrophage activation, most studies have focused on its role in cancer, using cell models with little immune relevance. Those who have looked at IRF1 in the context of IFN activation, have not done so on a genome-wide level. There has also been an over reliance on cell lines in IRF1 functional studies, which are known to harbor significant genomic and epigenomic alterations. Furthermore, while the functional importance of IRF1 and STAT1 dual binding has been described, if and how IRF1 may be able to act alone in driving expression has yet to be investigated properly.

1.4.4 Involvement of IRF1 in chromatin remodelling in both myeloid and lymphoid lineages

Paradoxically, IRF1 binding is both influenced by and influences the chromatin landscape [215,224]. For instance, it has been shown that IRF1 can directly interact with chromatin modifying proteins. Moreover, IRF1 seems to be responsible in the recruitment of these complexes to its genomic targets, with IRF1 deficiency resulting in considerably less p300 recruitment and binding to known IRF1 targets in D54MG cells[225]. Similarly, it has been observed that the formation of several 3D looping structures is established pre-stimulation at the CIITA and SOCS1 locus. Despite the absence of BRG1, an important chromatin remodelling enzyme, these loop formations remained stable at the CIITA locus. It's hypothesized that a group of pioneer transcription factors are binding to these sites at basal state, to unwind the chromatin and establish enhancer loop formation [88]. Moreover, both the CIITA and SOCS1 locus are bound by IRF1 and STAT1, which leads to question whether either TF could be displaying low-undetectable levels of binding at basal state which de-stabilizes higher order chromatin structures [89].

As previously mentioned, a study by Langlais and colleagues characterized the genome-wide combinatorial binding of IRF1, STAT1, IRF8 and PU.1 in primary macrophages, before and after IFN-γ stimulation[224]. The authors noted the importance of IRF1 in mediating macrophage activation, showing that loss of IRF1 deeply abrogated IFN-γ–dependent transcriptional activation. This included several key macrophage functions, such as production of costimulatory molecules, cytokines, and chemokines, TLRs and other signaling receptors, antigen processing and presentation machinery, and most

small antiviral GTPases. Additionally, upon further examination it appears that after 3 hours of IFN-γ stimulation, IRF1 had the ability to bind sites not previously bound by the pioneer PU.1 or other transcription factors (*e.g.,* STAT1 or IRF8) and induce the deposition of H3K4me1 and H3K27Ac around these previously closed chromatin sites in BMDMs.

Moreover, this enhancer establishment and activation was abrogated in *Irf1 -/-* macrophages. In a similar manner, a study this year examined the transcriptional and epigenetic response of human myeloid cells to several viral and bacterial ligands[226]. The authors found that upon LPS stimulation, macrophage-like IRF1 KO THP-1 cells displayed an impaired expression and chromatin accessibility of ISGs with regards to the control. Of note, the authors did not prove IRF1 binding occurred at these sites, nor characterized the binding of PU.1, a known pioneer factor in macrophages development and plasticity. Altogether, these studies suggest IRF1 could be binding and mediating chromatin remodeling at these sites, in a pioneer transcription factor-like manner.

Pioneering activity by IRF1 has also been suggested in the lymphoid lineage. A recent study by Karwacz *et. al.,* suggested a role for IRF1 and BATF as pioneering factors during Tr1 differentiation[227]. Deficiencies of either IRF1 or BATF led to reduced numbers of Tr1 cells, with altered function. ATAC-seq data on Tr1 cells indicated that the absence of IRF1 led to a decrease accessibility at 1100 regions. BATF deficiency displayed a much more remarkable alteration, with more than 10,000 regions decreasing in accessibility. However, while the dependency on IRF1 and BATF for chromatin opening was shown, further experiments are required for *bona fide* pioneering activity to be demonstrated.

Of particular interest, IRF1 appears to play little to no role in enhancer establishment during differentiation, however its role in driving signal-dependent transcriptional responses is unique and non-redundant among other SDTFs [224,228]. Moreover, enhancer reprogramming during macrophage adaptation, or plasticity, has been shown to mainly rely on SDTF and LDTF collaborations. In fact, almost all known pioneer transcription factors are intrinsically related to cell fate programming[125], with the notable exception of GR/AR [229]. Thus, in macrophages, there is a lack of knowledge as to whether SDTFs can shape the epigenetic landscape, independently of LDTFs.

1.5 Rationale and hypothesis

IRF1 is a transcription factor with a remarkable capacity to drive distinct transcriptional programs and is thus involved in several biological processes. IRF1's role in macrophage activation upon distinct pro-inflammatory cues, including IFN-γ, is essential and non-redundant with other IRFs. Mice which lack IRF1 are unable to mount appropriate immune responses and are therefore severely susceptible to infections. As a mediator of transcription, IRF1 displays strong intrinsic DNA binding to its target sites, which often leads to the recruitment chromatin remodelling complexes. Recently, genome-wide chromatin accessibility and binding assays have revealed a dependency on IRF1 for chromatin opening in response to stimuli. Which may suggest that IRF1 possesses pioneer factor properties, despite displaying a minor role in shaping chromatin during development. Yet, several questions remain regarding the manner in how IRF1-dependent chromatin remodelling occurs. For instance, whether IRF1 chromatin remodelling is independent of PU.1, a LDTF in hematopoietic lineages with known pioneer factor activity. Also, the early and prolonged chromatin-binding kinetics of IRF1 upon IFN-γ stimulation in macrophages have not been characterized. Thus, interrogating IRF1 binding is required to better understand its unique role in driving inflammatory responses in macrophages, which have been implicated in both host defense and the appearance of immune-driven pathologies. We hypothesized that IRF1 can bind to previously closed chromatin regions and mediate their opening upon IFN-γ stimulation in BMDMs, independently of PU.1.

1.6 Objectives

Aim 1 – Select an IRF1 antibody with adequate target specificity and optimize the chromatin immunoprecipitation protocol to obtain a greater target enrichment.

Aim 2 – Design and execute an IRF1 ChIP-seq time course experiment in BMDM, to characterize early and prolonged chromatin-binding kinetics of IRF1 upon IFN-γ stimulation.

Aim 3 – Design and execute an ATAC-seq time course experiment on WT and *Irf1*$^{-/-}$ BMDM, to characterize how chromatin accessibility evolves globally throughout and beyond early IFN-γ activation, and its dependency on

Chapter 2 Materials and Methods

2.1 Ethics statement

All mice were kept under specific pathogen-free conditions and handled according to the guidelines and regulations of the Canadian Council on Animal Care. Experimental protocols were approved by the McGill University Institutional Animal Care Committee (protocol number 2018-8014).

2.2 Cell culture and transfection

The mouse lines used during our experiments belonged to either C57BL/6 (B6) or *Irf1*$^{-/-}$ mice (The Jackson Laboratory). Briefly, *Irf1*$^{-/-}$ mice were generated, as stated by the distributer, via the disruption of one *Irf1* allele in embryonic stem cells by homologous recombination using a targeting vector which replaced exons 4, 5 and 6 from the *Irf1* gene. ES cells (*Irf1*$^{o/+}$) were then injected into blastocysts which gave rise to chimeric males, which upon mating of their heterozygous offspring yielded *Irf1*$^{-/-}$ pups.

BMDMs were differentiated from bone marrow (BM) isolated from femurs and tibias of C57BL/6 (B6) or *Irf1*$^{-/-}$ mice. In brief, all mice used for BM harvest and experimental procedures were female between 8–16 weeks old. Animals were humanely sacrificed by carbon dioxide asphyxiation followed by cervical dislocation and BM harvest. BM cells were cultured in DMEM (Wisent) containing 10% heat-inactivated FBS (Multicell, Wisent), 1X Penicillin-Streptomycin Solution (Corning), and 20% L cell–conditioned medium (LCCM) as a source of M-CSF, using 15 cm non-treated culture dishes. The BM cells were supplemented with an additional 10% of LCCM 4 days later. On day 7, cells were harvested by gentle washing with a monolayer of PBS-EDTA 10 mM and frozen in 90% FBS and 10% dimethyl sulfoxide (DMSO). Before each experiment, cells were plated in tissue treated culture dishes in DMEM containing 10% FBS, 20% LCCM, and 1X Penicillin-Streptomycin Solution and used the following day.

RAW264.7 and HEK293T/17 (P10) cells were cultured in DMEM containing 10% FBS, and 1X Penicillin-Streptomycin using 6-well TC treated plates. The cells were transfected the following day with a mouse *Irf1* expression and a GFP control plasmid using Lipofectamine 3000 reagent, following the manufacturer protocol. Expression of the reporter GFP from the control plasmid was visualized 48 h later in the transfected cells

using the ZOE™ Fluorescent Cell Imager. The cells were harvested and cryopreserved until they were used

2.3 Flow cytometry purity assessment

Purity assessment was carried out to monitor the *in vitro* differentiation efficiency of BM cells into BMDMS. Cells were analyzed using flow cytometry (FACS Calibur; BD) for expression of CD11b (eFIuor 450; eBioscience), F4/80 (PE-Cy7; eBioscience), and Ly6G (PE, BioLegend) (**Table 1**). More than 84% of cells were positive for F4/80 (**Figure S1**) and CD11b (not depicted), and they were negative for the neutrophil marker Ly6G (not depicted), confirming their differentiation into macrophages.

Table 1. Flow cytometry antibodies utilized for purity assessment of macrophages derived from bone marrow, with their supplier, catalog number and their associated cell type.

Surface marker	***Supplier***	***Catalog #***	***Associated cell type***
F4/80	eBioscience	123114	Murine macrophage marker
CD11b	eBioscience	46-0112-80	Pan-myeloid marker
Ly6G	BioLegend	128044	Neutrophil marker

2.4 Western blotting

BMDMs from both B6 and *Irf1*$^{-/-}$ mice were plated as previously mentioned and stimulated and non-stimulated with IFN-γ at 400 U/ml for 6 h. BMDMs and HEK293T cells were washed with ice cold PBS and lysed in RIPA lysis buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS and 140mM NaCl) supplemented with a protease inhibitory cocktail (Aprotinin, Leupeptin and Pepstatin A at 0.1mg/ml each). Samples were mixed by pipetting and incubated on ice for 30 min and cleared by centrifugation at 20,000 × g for 15 min at 4°C. The protein lysates were recovered and quantified using the DC Protein Assay (Bio-rad), following the manufacturer protocol.

Protein lysates were then denatured with Laemmli sample buffer (Bio-rad) supplemented with %5 of β-mercaptoethanol (BME) for 5 min at 95°C. Using stain-free 10% polyacrilamide gels (Bio-rad), 40µg of proteins were loaded alongside 2µL of Precision

Plus Protein™ Dual Color Standard (Bio-rad) and ran at 50V for 70 min, then 100V for 70 min. Protein gels were activated via UV exposure using a ChemiDoc Imaging System, then transferred onto nitrocellulose membranes using the Trans-Blot Turbo transfer system at 15 min transfer at 25V 1.3 A and following the manufacturer's protocols. After transfer, the membranes were briefly washed in PBS with 0.1% Tween-20 (PBST) and blocked overnight at 4^{o}C in 5% milk in PBST (wt/vol). Membranes were then incubated 2 h at room temperature (RT) with primary antibodies (shown below) diluted in 5% milk in PBST (wt/vol). Afterward, the membranes were washed with PBST three times, 5 min each, and incubated for 1 h with secondary antibody diluted with 5% milk in PBST at RT. The membranes were then washed four times, 5 min each, then developed using the Bio-Rad ECL substrate and revealed using Vilber FX7 Gel Documentation Imaging System.

Table 2. Antibodies used in Western blotting, their product information, target protein and region, molecular characteristics and dilutions used.

Target	***Supplier***	***Catalog #***	***Origin, isotype and clonality***	***Dilution***	***Immunogen***
IRF1	Santa-Cruz	Sc-640x	Rabbit / IgG / pAb	1:2,000	M20
IRF1	Abcam	ab186384	Rabbit / IgG / mAb	1:1,000	Synthetic peptide corresponding to Human IRF1 aa 169-183
IRF1	Cell signaling	8478	Rabbit / IgG / mAb	1:250	Residues surrounding Pro261
IRF1	R&D systems	AF4715	Goat / IgG / pAb	1:200	Mouse Thr147--Pro329
IRF1	Novus	NBP2-67330	Rabbit / IgG / mAb	1:1000	Synthetic peptide corresponding to Human IRF1 aa 100-325
Goat anti-rabbit HRP conj.	Bio-rad	1708046	-	1:10,000	-
Donkey anti-goat HRP conj.	Santa-Cruz	SC-2020	-	1:5000	-

2.5 Chromatin immunoprecipitation and sequencing (ChIP-seq)

Twenty million BMDMs from B6 and *Irf1*$^{-/-}$ mice were plated in 15cm tissue culture treated dishes and the next day were stimulated or not stimulated with IFN-γ at 400 U/ml as seen in **figure 3**. ChIP was performed as previously described[134], using distinct IRF1 antibodies for optimization (displayed in **Table 3**). Briefly, BMDMs were cross-linked for 10 min at room temperature with 1% formaldehyde added in the culture medium, after which the medium was removed and cross-link was stopped with ice-cold PBS containing 0.125-M

glycine for 5 min. Nuclei were prepared by sequential incubation on ice for 5 min in buffer A (10-mM Tris-HCl, pH 8, 10-mM EDTA, and 0.25% Triton X-100) and for 30 min in buffer B (10-mM Tris-HCl, pH 8, 1-mM EDTA, and 200-mM NaCl; all buffers included protease inhibitors). Nuclei were resuspended in a sonication buffer (10-mM Tris-HCl, pH 8, 1-mM EDTA, 0.5% SDS, 0.5% Triton X-100, 0.05% NaDOC, and 140-mM NaCl) and sonicated using digital sonifier (Branson Ultrasonics) at 80% amplitude, 7 minutes, ON / OFF 30 seconds, to a size of 100-500bp. Sonicated chromatin was diluted in ChIP buffer 3 : 1 and incubated overnight on a rotating platform at 4°C with a mixture of 40 µl protein G Dynabeads (Invitrogen) pre-bound with 3 µg of control IgG (02-6202) or IRF1 antibodies. Chromatin-antibody-bead complexes were washed sequentially for 2 min at room temperature with 1 ml of the following buffers: wash B (1% Triton X-100, 0.1% SDS, 150-mM NaCl, 2-mM EDTA, and 20-mM Tris-HCl, pH 8), wash C (1% Triton X-100, 0.1% SDS, 500-mM NaCl, 2-mM EDTA, and 20-mM Tris-HCl, pH 8), wash D (1% NP-40, 250-mM LiCl, 1-mM EDTA, and 10-mM Tris-HCl, pH 8), and TEN buffer (50-mM NaCl, 10-mM Tris-HCl, pH 8, and 1-mM EDTA). After de–crosslinking by overnight incubation at 65°C in buffer E (1% SDS, 50mM Tris pH8 and 10mM EDTA), the DNA was purified with Magnetic PCR Clean Up kit (Galenvs) according to the manufacturer's instructions.

ChIP enrichment was quantified by quantitative PCR (qPCR) using the Luna® Universal qPCR Master Mix (New England Biolabs). All C_T values for known IRF1 binding sites (Tlr4 and Cd40) were normalized to those of the negative control region, the pro-opiomelanocortin (*Pomc*) gene (**Table 4**). Enrichment was then calculated in comparison to the Ct values obtained in the IgG control. Once optimized, a kinetic ChIP time course was executed using the control IgG and the selected IRF1 antibody (AF4715, R&D Systems) and sequenced with input DNA from the same cells sequenced as negative control. The ChIP-seq libraries were prepared using the Kapa Hyperprep ChIP library kit (Roche Molecular Systems) and sequenced on a S4 flowcell on a NovaSeq 6000 sequencer in a paired-end 50-bp configuration.

Table 3. Antibodies used during ChIP optimization, alongside their product information, molecular characteristics, bead affinity and quantities used.

Target	Supplier	Catalog #	Origin, isotype and clonality	Concentration and quantity used	Bead affinity
IgG	Invitrogen	02-6202	Goat / IgG / -	1 µg/µl and 3 µl	G
IRF1	Santa-Cruz	Sc-640x	Rabbit / - / pAb	0.5µg/µl and 6 µl	G, A
IRF1	Abcam	ab186384	Rabbit / IgG / mAb	[1.27mg/ml] 5 µl	G, A
IRF1	Cell signaling	8478	Rabbit / IgG / mAb	[Unknown] 5 µl	G, A
IRF1	R&D systems	AF4715	Goat / IgG / pAb	[0.4ug/µl] 12.5 µl	G
IRF1	Novus	NBP2-67330	Rabbit / IgG / mAb	[1ug/µl] 5 µl	G, A
IRF1	Santa Cruz	B0320	Mouse / IgM / MAb	[200µg/0.1ml] 2.5 µl	L
IRF1	Santa Cruz	A0820	Mouse / IgG2a / MAb	[200µg/0.1ml] 2.5 µl	L, G, A
IRF-1	Elabscience	AF0764	Rabbit / IgG / pAb	[Unknown] 5 µl	G, A
IRF-1	Biolegend	B271557	Mouse / IgG2a / MAb	[Unknown] 10 µl	L, G, A

Table 4. List of ChIP-qPCR primers used to assess IRF1 ChIP enrichment.

Target Region	*Forward Sequence*	*Reverse Sequence*
Cd40	CTTCAGCTGTGGTCTTTCCCGTTT	ATCTCTGCAGAACCGAAAGCGTCT
Tlr4	GTCAGCAAACGCCTTCTTCCTGTT	AGAGGAAGTGAGAGTGCCAACCTT
Pomc	AGGCAGATGGACGCACATAGGTAA	TCCACTTAGAACTGGACAGAGGCT

2.6 Assay for Transposase Accessible Chromatin and sequencing (ATAC-seq)

One million BMDMs were plated in 6-well non-tissue treated plates and were either stimulated or not stimulated with IFN-γ at 400 U/ml, as seen in **figure 3**. ATAC-seq was performed as previously described[230]. Briefly, cells were washed with PBS and 25,000 cells were lysed with Lysis Buffer (Tween-20 10%, NP-40 10% and Digitonin 1%) and incubated on ice for 3 min. Cell lysates were then centrifuged to remove the cytoplasm and preserve the nuclei, then incubated in Transposition buffer (TD Illumina buffer, 1x PBS, Tween-20 10%, Digitonin 1% and Tn5 Transposase) at 37ºC for 30 min. DNA was isolated using Favorgen MicroElute Gel/PCR Purification Kit and used in PCR amplification for library generation and qPCR amplification to determine additional PCR cycles. DNA was isolated using Favorgen MicroElute Gel/PCR Purification Kit and library

quality was assessed using Agilent High Sensitivity DNA Bioanalysis chip. Samples were sequenced on a S4 flowcell of the NovaSeq 6000 in a paired-end 100-bp configuration.

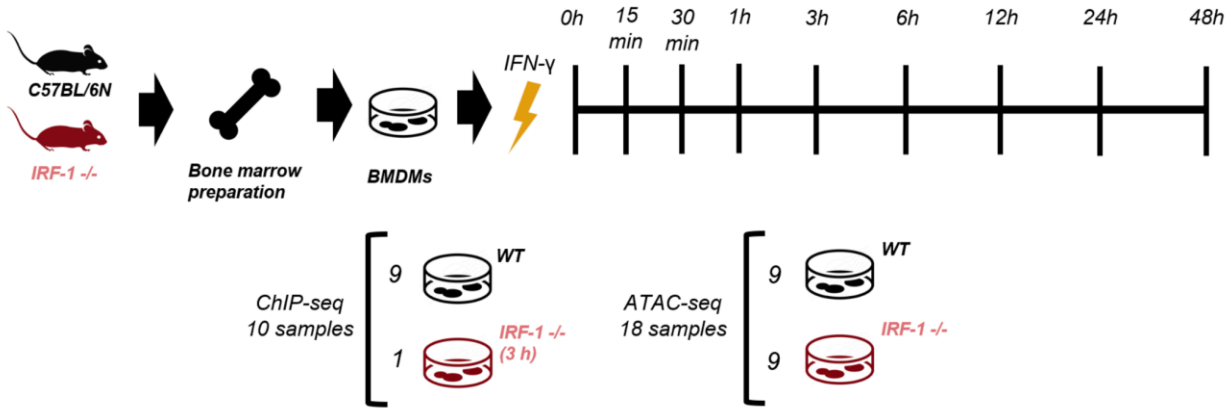

Figure 3. General experimental design for ChIP and ATAC-seq sample preparation, including mouse strains, time course and number of samples.

2.7 ChIP-seq and ATAC-seq data processing and analysis

Sequence quality was assessed using FastQC for both ATAC and ChIP-seq. Nextera adaptor sequences were then removed in the ATAC dataset with Trimmomatic 0.36 [231] using the following function: Illuminaclip:NexteraPE-PE. Both datasets were mapped to the UCSC mouse mm10 reference genome with Bowtie 2.3.5 [232]. Picard and Samtools [233]were used to mark and remove duplicated reads, respectively. Tag directories were generated using the makeTagDirectory function by HOMER [123] and were used to create Bigwig files using the makeUCSCfile function by HOMER for visual exploration of peak intensities on IGV [234]. Peak calling for the ChIP dataset was carri+ed with MACS2 2.1.1.2 [235] by using the -f BAMPE -g mm -q 0.01 functions. IRF1 ChIP peaks were verified by annotating each sample summit BED file with the corresponding tag directories from the sample and input, calculated the fold change and filtering based on fold change (>6 FC) and raw count per million (>6 CPM). De novo motif analysis was carried on all the significant IRF1 ChIP-seq peaks found in at least one of our 9 IRF1 time points using the function findMotifsGenome.pl on HOMER. ChIP and ATAC density heatmaps were generated by re-coordinating previously published clustering data [170] to the mm10

reference genome. New mm10 coordinates were annotated with our ChIP and ATAC datasets using annotatepeaks.pl by HOMER. The output files were then later visualized on Java TreeView [236]. A functional GO classification of the proximal genes to our verified IRF1 binding peaks (only including those < 20kb from the nearest TSS) was carried out using Pantherdb and displayed on GraphPad PRISM.

Chapter 3 Results

3.1 Sodium dodecyl sulfate (SDS) concentration impacts ChIP target enrichment

Our legacy IRF1 antibody (sc-640X, Santa-Cruz) that we previously use to characterize the IRF1-IRF8 transcriptional networks has been discontinued, hence we needed to find a replacement. Moreover, to identify sites with low IRF1 chromatin occupancy, what we expect at the early time points in the ChIP-seq time course, our ChIP protocol was also in need of optimization. However, after comparing 3 new antibodies with the sc-640X, we obtained fold enrichments that were 3-10-fold lower (**Table 5**). We refer to enrichment as the comparison in Ct values derived from our target antibody and a control immunoglobulin (IgG), then its normalization to a control genomic region (POMC). Hence, I have tested a series of modifications using several IRF1 antibodies with the aim to increase IRF1 target enrichment by qPCR. Modifications focused on modifying the wash stringency of the chromatin-antibody complex, increasing antibody concentration, using distinct magnetic beads for immunoglobulin capture and more. Most modification strategies resulted in a <10-fold target enrichment, except for SDS concentrations during sonication and incubation. Lower SDS concentrations during sonication appeared to produce more favorable enrichment profiles in IRF1 ChIP (**Table 5**), but also led to inefficient chromatin fragmentation (**Figure S2**). However, when the SDS concentration was kept to 0.5% during sonication but diluted to 0.125% during the incubation with the bead immuno-complexes, a several fold increase in *Tlr4* and *Cd40* enrichment was obtained.

Table 5. List of IRF1 modification strategies used to optimize the IRF1 antibodies for ChIP-seq, their product information and qPCR fold enrichment obtained during testing.

Supplier	Catalog #	Modification strategy	Fold enrichment of TLR4/CD40
Comparison of different IRF1 antibody using standard conditions			
Santa-Cruz	Sc-640x	Standard ChIP w/ medium washes	30.70 / 39.4
Elabscience	AF0764	Standard ChIP w/ medium washes	1.42 / 0.85
Cell signaling	8478	Standard ChIP w/ medium washes	6.25 / 8.94
Biolegend	B271557	Standard ChIP w/ medium washes	8.51 / 1.44

Increase the quantity of antibody			
Santa-Cruz	Sc-640x	Standard concentration	25.99 / 22.47
Abcam	ab186384	2X antibody concentration	7.31 / 8.94
Cell signaling	8478	2X antibody concentration	0.84 / 8.94
Testing different washing conditions			
Cell signaling	8478	Standard medium washes	5.70 / 13.88
Cell signaling	8478	Mild washes (w/NADOC)	1.38 / 4.84
Cell signaling	8478	4 washes w/ChIP dilution buffer and 1 w/TEN	1.19 / 2.99
Effect of NaDOC in the washes			
Cell signaling	8478	Standard medium washes	5.39 / 13.93
Cell signaling	8478	Medium washes + 0.1% NADOC in wash #2	2.74 / 12.73
Cell signaling	8478	Medium washes + 0.1% NADOC in wash #2 + extra wash w/#2	1.82 / 10.45
Cell signaling	8478	Medium washes + 0.2% NADOC in wash #2	2.71 / 14.67
SDS reduction during sonication			
Cell signaling	8478	ChIP sonication buffer at .5%	6.15 / 9.3
Cell signaling	8478	ChIP sonication buffer at .4%	4.94 / 10.20
Cell signaling	8478	ChIP sonication buffer at .3%	4.81 / 12.04
Cell signaling	8478	ChIP sonication buffer at .2%	4.36 / 32.67
Cell signaling	8478	ChIP sonication buffer at .1%	2.03 / 3.47
SDS reduction in chromatin-antibody incubation			
Santa Cruz	Sc-640x	½ 750 ul ChIP + 250 ul chromatin	96.67 / 102.8
Abcam	ab186384	½	140.07 / 172.45
Cell signaling	8478	½	89.57 / 134.83
SC+Ab	-	½	119.84 / 194.01
SC+CS	-	½	29.55 / 40.93
CS+Ab	-	½	38.99 / 140.07
Santa Cruz	Sc-640x	1/3	95.01 / 716.59

From the newly optimized ChIP protocol, the Cell Signaling Technology (CST) and Abcam IRF1 antibodies showed the most promising enrichments by ChIP-qPCR in IFN-γ stimulated RAW 264.7, a macrophage cell line. These, alongside two new IRF1 antibodies from R&D and Novus, were then tested on IFN-γ stimulated primary macrophages. All 4 antibodies performed extremely well (**Figure 4**), obtaining >50-fold enrichment after normalization; the Novus and R&D antibodies showed the highest enrichment.

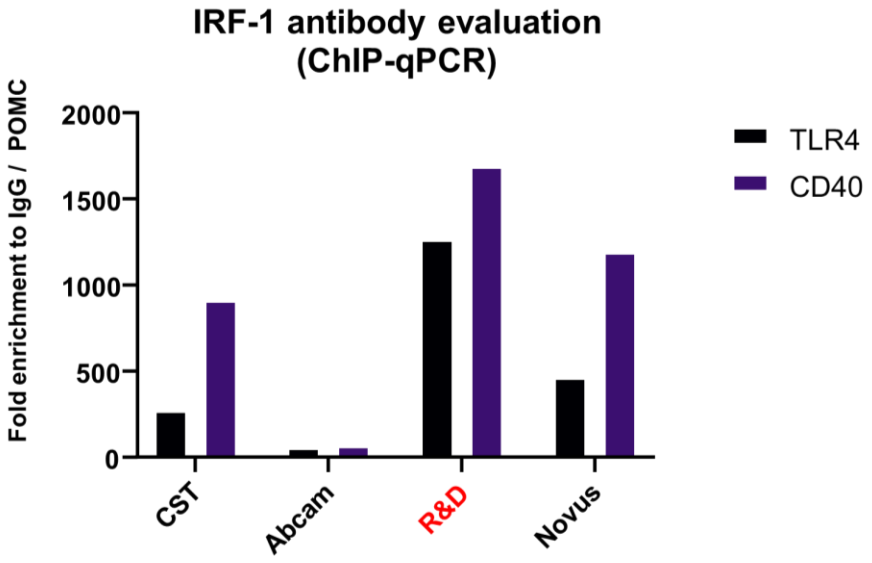

Figure 4. ChIP-qPCR results from 4 different IRF1 antibodies using 3h IFN-γ stimulated WT BMDMs. Fold enrichments were measured by comparing average CT cycles from *Tlr4* and *Cd40* (known targets of IRF1) to those from an IgG control and normalized against the *Pomc* promoter (control inactive gene).

3.2 High specificity for R&D and Cell signaling IRF1 antibodies.

After verifying their efficiency in ChIP, the 4 antibodies were tested for specificity using western blotting (**Figure 5**), including the M-20 Santa Cruz. All 5 of the antibodies tested were able to detect IRF1 in both IFN-γ stimulated WT BMDMs and HEK293T cells overexpressing IRF1. In terms of specificity, the Abcam and Novus seemed to suffer from the same non-specific bands, most likely since they target the same immunogen (**Table 2**). On the other hand, R&D and CST were found to be specific to IRF1.

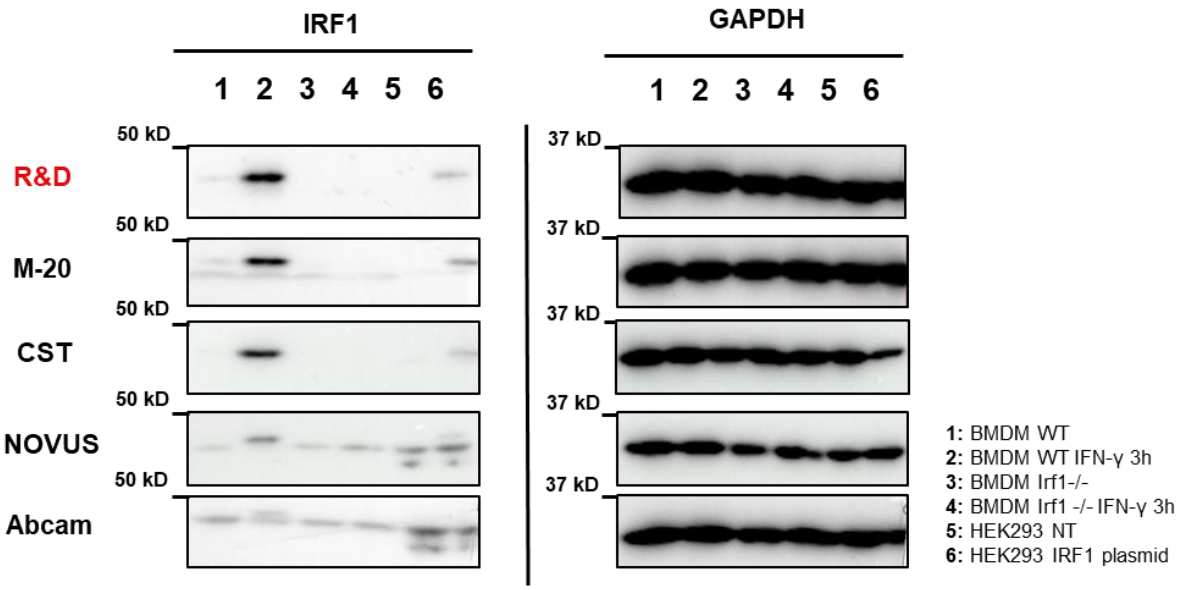

Figure 5. Western blot results from total protein extracts of WT and *Irf1*$^{-/-}$ BMDMs, both stimulated and non-stimulated with IFN-γ and HEK293T cells transfected and non-transfected with an IRF1 expression plasmid. The membranes were blotted against IRF1 protein and imaged via chemiluminescence using the Vilber FX7 imaging system.

For the ChIP-seq studies, we decided on using the R&D systems IRF1 antibody based on its specificity (**Figure 5**) and strongest enrichment profile in ChIP-qPCR (**Figure 4**). Then, we ran a small 5 time point kinetic trail (**Figure 6**) to evaluate if we obtain sufficient enrichment at very early time points following IFN-γ stimulation. The results showed, in comparison with the 0h time point, that even after 15 minutes of stimulation, enrichment could be detected, and at the 1h time point > 140-fold enrichment was observed. As previously mentioned, fold enrichment refers to the comparison of Ct values of our target genes obtained by our IRF1 antibody vs an IgG antibody, normalized to POMC. This provided sufficient rationale to go forward with the execution of a 9-time point IRF1 ChIP-seq experiment (as shown in **Figure 3**).

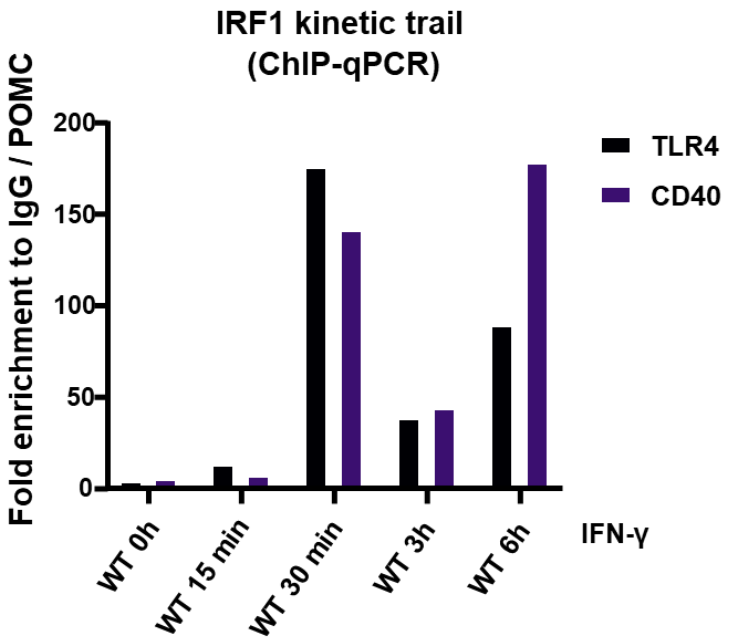

Figure 6. ChIP-qPCR results from the R&D systems IRF1 antibody using WT BMDMs stimulated with IFN-γ at 5 different time points. Fold enrichments were measured by comparing average CT cycles from *Tlr4* and *Cd40* to those from *Pomc* and the IgG control antibody.

3.3 IRF1 rapidly binds to chromatin upon stimulation and remains bound at most sites.

Upon IFN-γ stimulation, *Irf1* transcription occurs downstream of the JAK-STAT1 signalling cascade. Early biochemical signalling studies revealed that JAK 1 and 2 phosphorylation occurs as soon as 5 minutes after IFN-γ receptor activation, with similar results for STAT1[237] . *Irf1* expression and protein production have been documented to occur as early as 15 minutes post ischemic injury [184]. Here, we investigated the early and late IRF1 binding via ChIP-seq in IFN-γ stimulated primary macrophages at 9 different time points (as shown in **Figure 3**).

ChIP-seq peak calling analysis revealed a time-dependent increase in IRF1 binding sites, going from 83 peaks at steady state, with a rapid increase to 1,583 peaks only 15min following IFN-γ stimulation, reaching a maximum of 23,916 peaks at 6h and decreasing

thereafter (**Figure 7A**). These results are in concordance with what we could expect to see from a SDTF upon stimulation. Furthermore, de novo motif analysis carried out on all 37,357 IRF1 ChIP-seq peaks (**Figure 7B**), indicated that the majority of sites (61.76%) are enriched for an ISRE binding motif, followed by IRF4 and PU.1. The abundance of the ISRE binding motif adds further confidence in our results as this binding motif belongs to IRF1, and PU.1 binding sites are often proximal to IRF1. Of note is that our 24 h ChIP time point seemed to have failed due the divertingly low number of detected peaks, as well as follow up analysis focusing on the enrichment of the peaks and their locations (not shown).

Strikingly, peak analysis also revealed a significant increase in peak binding intensity just 15 minutes post-stimulation (**Figure 7A, 8A**). Visualization of ChIP-seq data demonstrated a strong early increase in IRF1 signal at the *Nos2* enhancer region (**Figure 7D**), a gene crucial for early host defense[173]. Of note, is that although IRF1 displays strong binding at the *Nos2* enhancer, it also appears to be binding inconsistently to an intronic region close to its promoter. Inconsistent IRF1 signal at this site could be the result of multiple scenarios, for instance, it could be part of a transcriptional mechanism to regulate gene expression in a time specific manner. It also could indicate differences in the immunoprecipitation efficiency during our ChIP protocol, affecting some regions more than others. Nonetheless, further ChIP-qPCR validations will be carried to ascertain our observations and discard any artifacts arising from experimental variations, although this last option is less likely given we don't observe this lower binding IRF1 peak intensity at the genome-wide level.

Moreover, despite a decrease in binding sites past 6h, IRF1 remained bound to 6,270 sites well into the 48h time point (**Figure 7A**). Suggesting that IRF1 chromatin occupancy does not return to a baseline or un-stimulated state. Principal component analysis further showed that late time points, 48h and 12h, more closely resemble peak binding time points than those very early or non-stimulated (**Figure 7C**). To our knowledge, our study is the first to demonstrate that upon IFN-γ stimulation in primary macrophages, IRF1 can bind early to chromatin and persist at target sites well beyond the first few hours of induction.

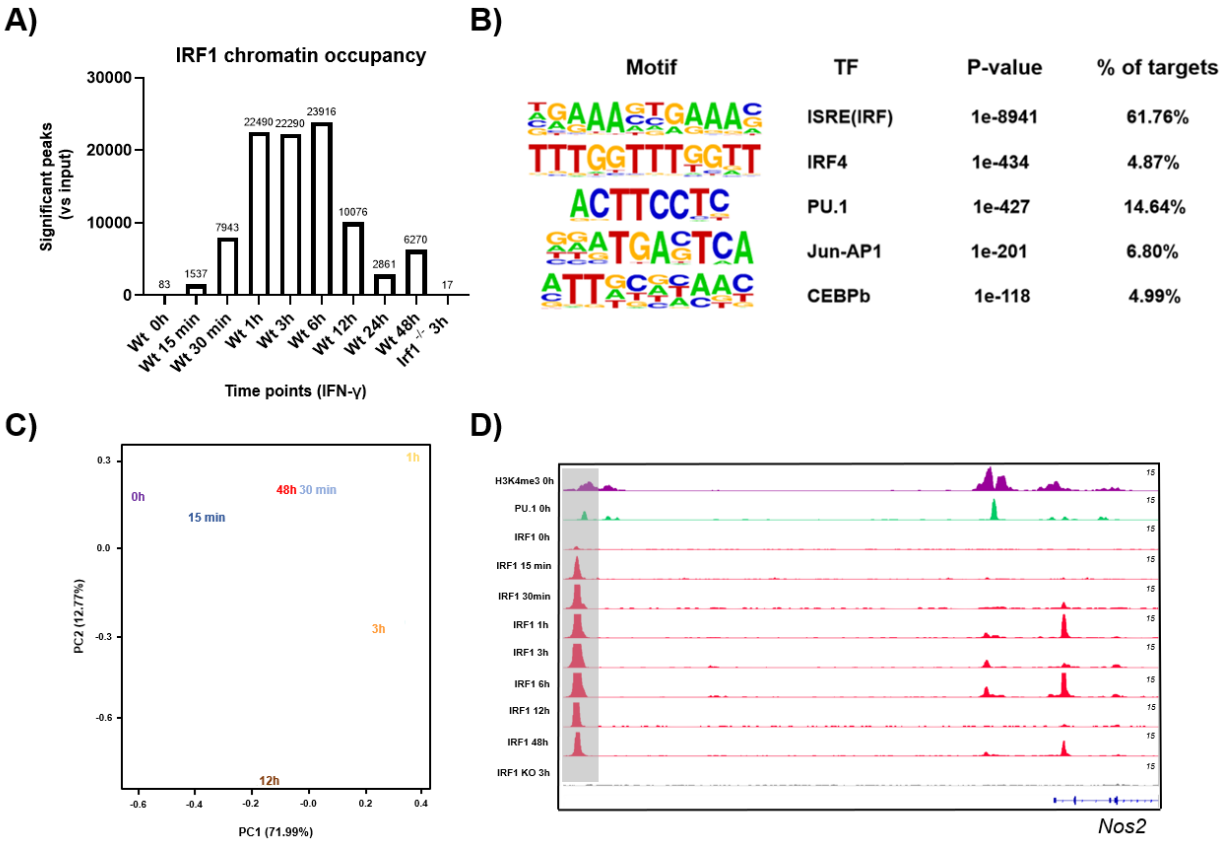

Figure 7. IRF1 ChIP-seq characteristics. **(A)** Boxplot displaying the number of significant peaks from our peak calling analysis of the IRF1 ChIP-seq samples. The peaks were called using macs2, then manually filtered to only include those with an FC of > 6, as well as a CPM of > 6. **(B)** De novo motif analysis was carried on all the significant IRF1 ChIP-seq peaks. **(C)** Principal component analysis based on the IRF1 ChIP-seq signal intensity at sites in common among all ChIP-seq time points, using log2 normalized CPM data. **(D)** Scaled and normalized ChIP-seq genomic tracks of H3K4me3 (purple) and PU.1 (green) [170] at steady state, and our IRF1 ChIP-seq time course (red), surrounding the *Nos2* locus. The binding event of interest is shaded in gray.

Lastly, as an important negative control, we included in the experimental design (**Figure 3**) the sequencing of an IRF1 ChIP carried out on IFN-γ treated *Irf1* $^{-/-}$ primary macrophages. The *Irf1* $^{-/-}$ ChIP-seq track showcased in **Figure 7D** shows no signal at the *Nos2* locus, a known target site of IRF1 and which displays strong signal in the WT

macrophage ChIP-seq samples. Moreover, only 17 peaks (**Figure 7A**) were detected in our *Irf1* $^{-/-}$ ChIP-seq sample, which were later identified as artifacts due to non-specific signal in the input DNA control samples (not shown). These results indicate our IRF1 antibody was highly specific in its protein targeting during immunoprecipitation.

3.4 IRF1 displays chromatin occupancy near ISGs at steady state in primary macrophages

Recent transcriptomic studies on primary hepatocytes and bronchial cell lines have demonstrated IRF1 is the main driver of basal ISG expression. Therefore, despite being classically thought of as an inducible factor, IRF1 contributes significantly to maintaining a constitutive antiviral state [176,238]. Peak calling analysis from our IRF1 ChIP-seq 0 h time point, or resting state, indicate that after manual peak verification there are a total of 83 IRF1 binding peaks (**Figure 7A**). Furthermore, peak height distribution analysis (**figure 8A**) indicates the existence of peaks at resting state with considerable peak signal. A gene ontology (GO) analysis using PANTHER (**Figure 8B**) for the nearest genes to these IRF1 binding sites, located within 20kb of a known TSS, showed a strong enrichment for biological processes such as cellular response to exogenous dsRNA and production and regulation of IFN-α. Among the list of genes there is *Stat1*, *Ifih1*, and *Parp12*, all of which are ISGs involved in viral host defense. For instance, *Ifih1* encodes for MDA5, an important intracellular sensor of viral RNA [239]. Visualization of ChIP-seq data surrounding *Stat1* and *Ifih1* regions show noticeable IRF1 signal at resting state (**Figure 8C-D**). Although resting state expression of these genes cannot be confirmed due to a lack of transcriptional data, our analysis shows for the first time that IRF1 might be involved in maintaining a similar constitutive antiviral state in primary macrophages and contributes to our knowledge on IRF1 function in resting cells.

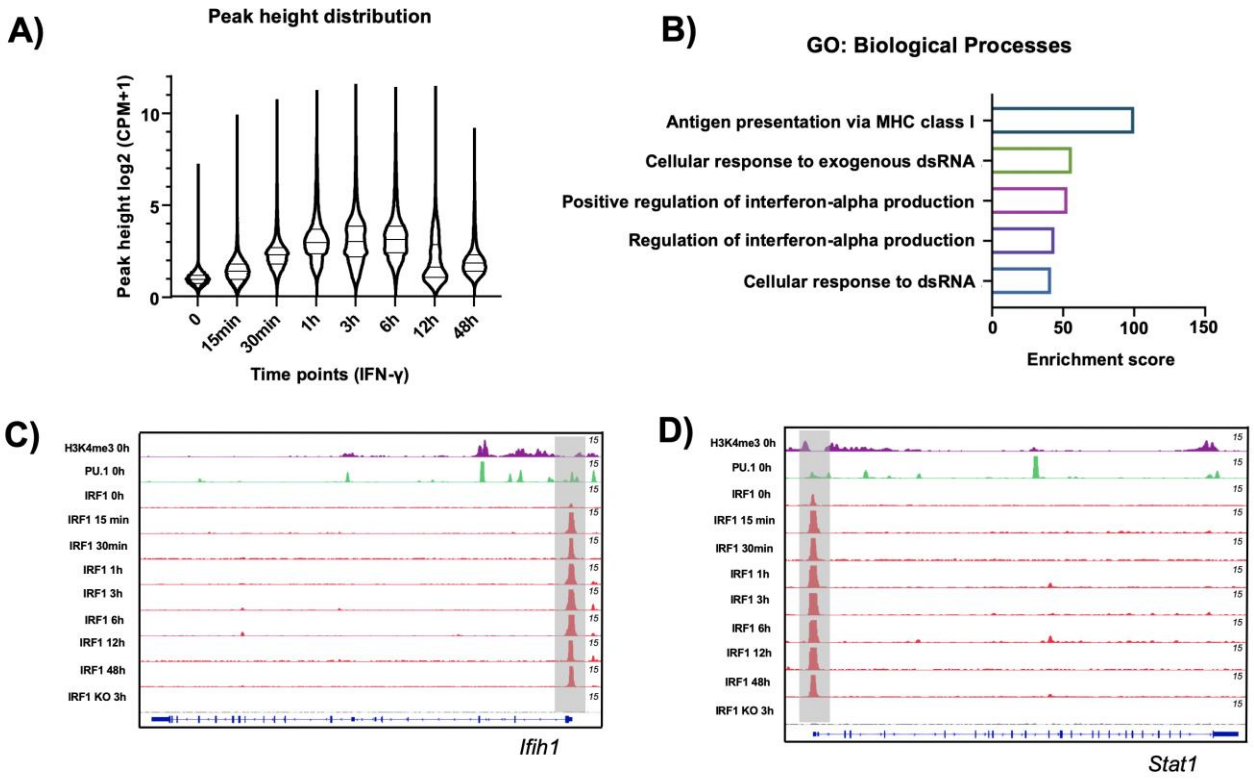

Figure 8. **(A)** Violin plot presenting the peak height distribution for IRF1 binding sites found within at least one time point, with a transformation of its CPM using log2(CPM+1). **(B)** PANTHER GO enrichment results displaying the top 5 biologically processes for the 76 significant IRF1 binding peaks in primary macrophages at resting state (located within 20kb from a TSS). **(C-D)** Normalized ChIP-seq genomic tracks for H3K4me3 and PU.1 at steady state [170], and IRF1 kinetic binding (shaded in gray) at the *Stat1* and *Ifih1* locus.

3.5 Genome-wide analysis reveal IRF1 is required for chromatin remodeling of previously closed regions.

In macrophages, enhancer establishment has typically been thought to be PU.1 dependent, an important LDTF. Recent studies have provided new evidence against this notion [170,226], suggesting a possible pioneering role for IRF1. Although, whether this occurs independently of the known pioneer TF PU.1 and if chromatin remodelling is dependent on IRF1 remains unknown. Moreover, a comprehensive genome-wide catalog of transcription factor cistrome, the genes they regulate and their associated chromatin

status was carried out in 2016 by Langlais and colleagues. The authors evaluated the functional interplay between the genome-wide binding of IRF8, IRF1, STAT1 and PU.1 in BMDMs, before and after pro-inflammatory stimulation with IFN-γ. Their clustering analysis of the IRF8, IRF1, STAT1 and PU.1 containing regions resulted in the appearance of 9 clusters with distinct TF binding combinations. Interestingly, IRF1 appeared to bind independently of PU.1 in 3 of the 9 resulting clusters. Furthermore, these sites appeared to show a modest but detectable increase in H3K27Ac and ATAC-seq signal 3h post stimulation with IFN-γ. Thus, a further interrogation of these sites, to characterize IRF1's binding, independently of PU.1 presence, and the changes in chromatin accessibility it may induce is of great interest.

Using primary macrophages stimulated with IFN-γ at distinct time points, we interrogated chromatin accessibility via ATAC-seq and compared it to our IRF1 ChIP-seq dataset, as well as with previously published PU.1 ChIP-seq data [170]. Peak analysis of our ATAC-seq showed that most peaks genome-wide are not influence by neither the BMDM genotype nor the IFN-γ status (**Figure S4)**. Slight but global variations were observed between samples, which is to be expected in assays which depend on enzymatic activity, as several uncontrollable factors seem to influence the rate at which enzymes are able to catalyze reactions. Nevertheless, we have normalized this effect by randomly selecting 10,000 non-variable peaks, calculating a normalization for each dataset and applying these factors to all peaks in each condition. Despite these variations, all of our ATAC-seq samples appeared to be successful in identifying chromatin accessible regions, as indicated by the often and strong overlap this dataset has with respect to our TF binding peaks, as shown in **figure 10B**, as well as its congruency with previous ATAC datasets (not shown).

By superimposing our newly generated data onto the previously mentioned clustering analysis carried out by Langlais and colleagues, we show that IRF1 is capable of binding to previously closed chromatin regions, devoid of PU.1 binding, and does so as early as 15 minutes post-stimulation (**Figure 9; Clusters 2, 6 and 7**). These clusters, which together total 5,027 genomic regions, begin to demonstrate an increase in ATAC-Seq signal, or chromatin opening at 3h, which continues to increase throughout the entire time

course in the WT ATAC-seq samples, but not in the *Irf1*$^{-/-}$ BMDMs. Therefore, chromatin remodeling at these sites appeared to be dependent on the presence of IRF1. Density graphs of ATAC-seq signal at resting state (0h), 3h, and 48h post IFN stimulation at the regions surrounding cluster 5 (non-pioneer sites) shows that chromatin is already accessible at these sites, while presenting a small increase in signal that appear dependent on IRF1 **(Figure 10A)**. On the contrary, the chromatin is completely closed at cluster 7 sites at resting state and shows a steady increase in ATAC-seq signal post-stimulation with IFN-γ, which is completely abrogated in the *Irf1*$^{-/-}$ BMDMs **(Figure 10A)**. ATAC-seq signal comparisons between resting state (0h) and 48h post-stimulation are displayed in **Figure S5** for a set of 10,000 non-variable peaks, cluster 5, and the 3 clusters with suspected IRF1 pioneering activity. Altogether, these results clearly highlight the dependency on IRF1 for the opening of closed chromatin sites that are devoid of PU.1 binding, supporting a novel pioneer function for IRF1.

Lastly, an example of PU.1-independent chromatin remodelling by IRF1 is shown within the Cluster 2 gene, *Copz2*, a gene known to harbor microRNAs with important tumor suppressing activities [240] (**Figure 10B**). Within the *Copz2* gene, shaded in gray, we can observe a complete lack of PU.1 signal, but a strong IRF1 binding at just 1h post stimulation. Strikingly, IRF1 appears to be able to bind with high intensity even before ATAC-seq signals begin to appear, as these only begin to accumulate after 3h, but not in the *Irf1*$^{-/-}$ samples. Our results confirm IRF1's ability to bind – *early* – to previously closed chromatin in response to IFN-γ induction, and the dependency on IRF1 function for chromatin remodelling at these sites, independently of PU.1. This suggested IRF1 may be leading the opening of ~5,000 sites, in a pioneer transcription factor-like manner.

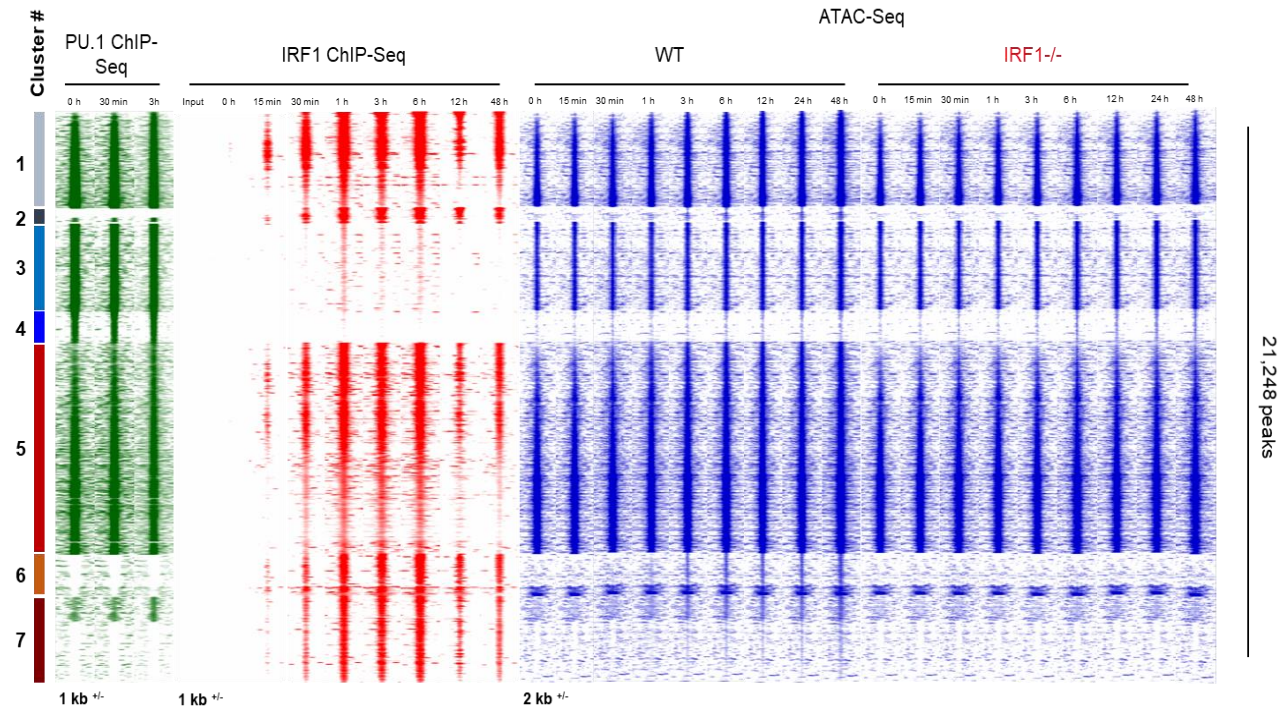

Figure 9. Clustering analysis of 21,248 regions from a multi-TF binding analysis by Langlais et al., 2017, before and after IFN-γ treatment, on which we annotated our new datasets. Each horizontal line presents the read density in a ±1-kb region around a unique position for IRF1 ChIP-seq and PU.1 [170]; ATAC-seq datasets are shown for a ±2-kb region surrounding the cluster peaks.

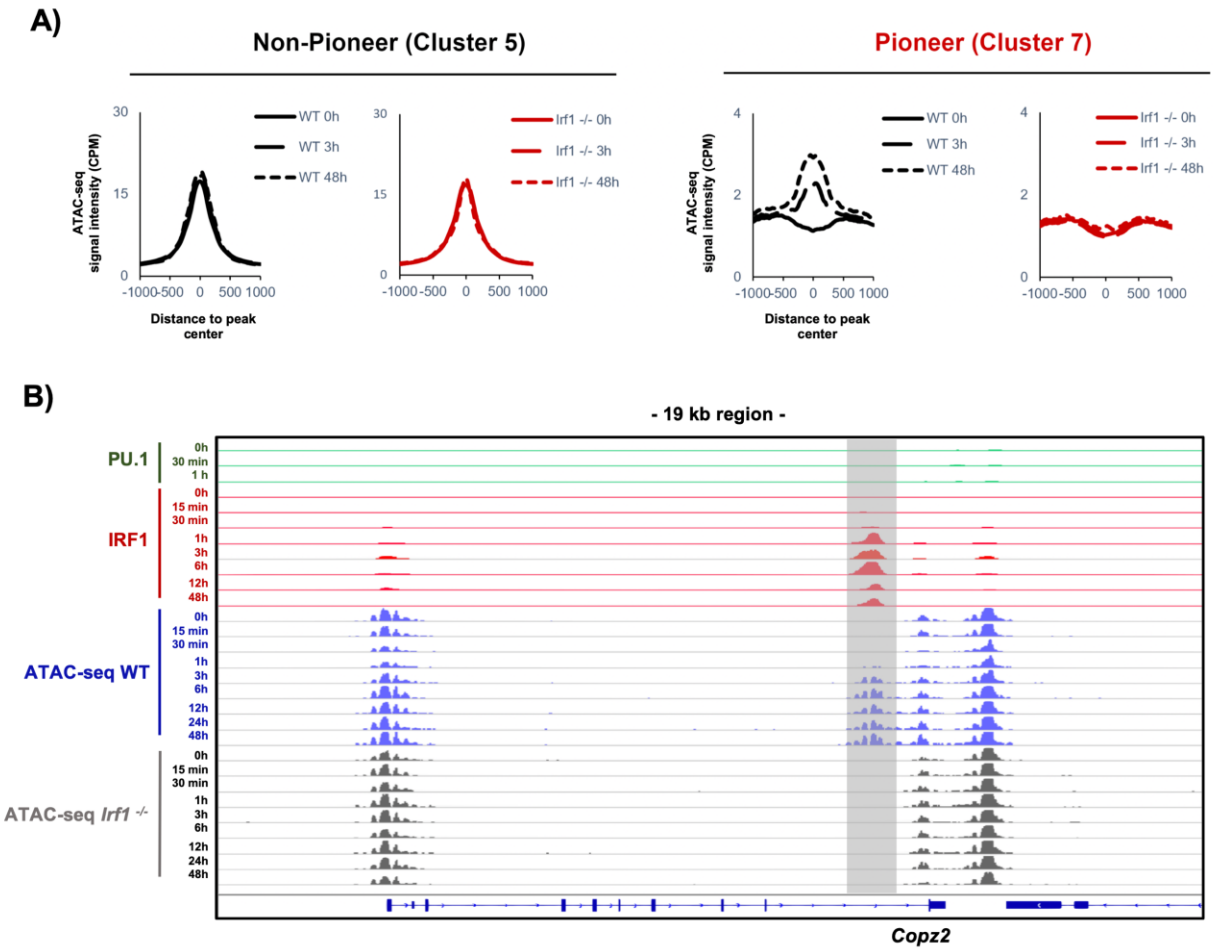

Figure 10. (A) ATAC-Seq signal intensity density graphs surrounding ±1 kb on WT (black) and *Irf1*$^{-/-}$ (red) primary macrophages at steady state (0h), 3h and 48h post IFN-γ stimulation. The histograms were created using peaks coordinates from cluster 5 and 7 genomic sites, showing that IRF1 displays pioneering activities cluster 7 sites. **(B)** Normalized ChIP-seq genomic tracks of PU.1 and IRF1, as well as ATAC-Seq tracks between 0 and 48 h, at the *Copz2* locus (a site found within cluster 2). Shaded in grey is an IRF1 binding site, displaying no PU.1 signal, early IRF1 binding (1h) and chromatin opening in WT, but not in the *Irf1*$^{-/-}$.

Chapter 4 Discussion

In the present body of work, I sought to investigate a putative new role for the transcription factor IRF1 as pioneer factor driving macrophage epigenetic remodeling in response to inflammatory activation signals, in particular to IFN-γ. To this end, I optimized a method for studying the role of IRF1 in cultured primary macrophages (BMDMs) using ChIP-seq and profiled its genomic recruitment at early and late time point following IFN-γ stimulation. I have also interrogated chromatin accessibility changes occurring after IFN-γ treatment in BMDMs. Through multi-omics data integration of publicly available datasets and the ones presented herein, I have furthered our understanding on the kinetic of IRF1 binding, as well as the consequences this has on chromatin structure, adding supplemental evidence for a function as signal dependent pioneer factor.

Rapid IRF1 binding upon IFN-γ stimulation

Experimentally, early time points of IFN-γ stimulation in the present work revealed a strikingly fast response time by IRF1, with binding occurring as early as 15 minutes. Gene expression data of IRF1 collected from several organisms positioned at distinct phylogenetic families [200–202], indicate that the time required for induction of IRF1 expression has continued to lower in vertebrates [200]. Suggesting that pathogens have placed significant selective pressure for more rapid IFN responses. This is perhaps unsurprising since pathogens, in particular viruses, have long plagued mammalian life costing untold deaths over the millennia. Consequently, they have exerted an immense selective pressure on mammalian life, with a recent study indicating that viruses have driven close to 30% of all protein adaptations occurring within ~1,300 mammalian conserved proteins, which included type ll IFN response elements such as STAT1 and IRF1 [241].

Interestingly, apart from phylogenetic positioning, the nature of the stimulus and cellular type seem to also influence IRF1 induction time. For instance, during viral challenge, mouse embryonic fibroblasts begin expression of IRF1 3 h post challenge, while BMDMs display expression at 2 hours [200] and renal cells display higher mRNA expression and protein production just after 15 minutes of injury signalling[184]. Furthermore, due to the apparent complexity of variables determining IRF1 response times, whether early bound

IRF1 corresponds to newly synthesized or to a pre-existing pool of IRF1 molecules awaiting activation remains to be determined.

Steady state IRF1 binding and its biological relevance

Additionally, viral pressure has also shaped the way our bodies achieve immunocompetence, even in their absence. A range of constitutive defense mechanisms been identified which include the production of antimicrobial peptides, basal autophagy activity and proteosomal degradation [242]. Despite its role in antiviral responses often being overshadowed by that of IRF3 and IRF7, recent studies have now placed a IRF1 at the center of constitutive viral defense [176,238]. Similarly, within our 0 h IRF1 ChIP analysis, we found IRF1 bound near several immune response genes, specifically ISGs.

An interesting thought is the function constitutive IRF1-driven gene expression has beyond host defense. STAT1 for instance, a gene pre-bound at steady state by IRF1 in our analysis, is considered crucial for tumor immunosurveillance. STAT1 is involved in the expression of MHC Class I molecules, which are required for antigen presentation towards T effector cells, enabling effective anti-tumor responses [243]. In fact, antigen presentation was the top hit in our GO analysis of the steady state IRF1 binding peaks. MDA5 (*Ifih1*), also pre-bound by IRF1, is an innate immune receptor for double stranded viral RNA which has been demonstrated to be involved in tumor control. MDA5 signalling induces tumor cell apoptosis via activation of the intrinsic pathway, and furthermore sensitizes tumor cells toward extrinsic apoptosis [244]. More generally speaking, the IFN-γ signalling appears to be very important in tumor control, showcased by the fact that many tumors often undergo through mutations/silencing of genes encoding IFNGR1, IFNGR2, JAK1, JAK2 and STAT1, as a mechanism contributing to tumor escape from immune surveillance [245]. Indeed, while no reports have been made in the literature about a putative role for IRF1 in contributing towards steady state tumor surveillance through constitutive gene expression, its recently discovered ability to both bind and induce the expression of several tumor suppressors at steady state certainly hints towards this direction. Indeed, *Irf1*$^{-/-}$ mice seem to display a clear hyper-susceptibility to tumorigenesis in both chemically induced and spontaneous mouse tumor models [246].

IRF1 pioneers the opening of closed chromatin at selected sites upon IFN-γ stimulation

Perhaps our most important finding lies in the discovery that IRF1 can bind to previously closed chromatin, independently of PU.1 presence. Cell-type-specific enhancer repertoires are thought to be established by LDTF, as these display unique heterochromatin binding abilities[69]. And although macrophage plasticity driven by the activation of latent enhancers has now been shown to rely on SDTF, in the case of the myeloid lineage, the recruitment and occupancy of the master regulator PU.1 is thought to be strictly required [136]. Furthermore, despite studies directly suggesting IRF1 dependent chromatin remodelling exist, these present major limitations. Karwacz and colleagues found IRF1 deficiency led to altered chromatin opening during type 1 regulatory T cell differentiation [227]. Yet, direct binding evidence of IRF1 to those chromatin sites which were shown to be dependent on its presence was not investigated. Moreover, a characteristic necessary for *bona fide* pioneer transcription factor activity is the ability to create stable epigenetic changes surrounding target sites, which aside from a 72 h ATAC-seq data, was missing. And lastly, the binding of PU.1 at these sites was not discarded, and as a result, uncertainty lies as to whether PU.1 is the TF mediating chromatin unwinding in their model of Tr1 cells. In a similar manner, Song and colleagues observed that IRF1 function after TLR4 signaling is required for chromatin opening [226]. While the authors did identify an enrichment for IRF1 motif sites at these chromatin opening sites, their over-reliance on cell lines, lack of direct binding evidence or PU.1 binding, represents major limitations. Moreover, an over-representation of IRF1 binding motif doesn't imply IRF1 binding *per se*, and since most IRF factors can bind very similar motifs, it could involve other members of the family. Possibly, the most convincing line of evidence comes from a study by Langlais and colleagues, which consisted of a multi-omics combinatorial genome-wide analysis on transcription factor binding, and its impact on chromatin accessibility and RNA expression [170]. Their data suggested IRF1 could bind to heterochromatin, in a PU.1 and STAT1 independent manner, which led to an increase in H3K27Ac and ATAC-seq signal 3 h post IFN-γ stimulation.

Long-lasting chromatin remodelling induced by IRF1 and its implications

Our ATAC-Seq analysis not only demonstrated IRF1 binds early to previously closed chromatin sites but induces relatively long-term IRF1-dependent chromatin remodelling in primary macrophages upon IFN-γ treatment. Trained immunity, a recent concept in host defense, refers to the capacity for innate immune cells to undergo long term metabolic and epigenetic reprogramming, granting them “memory” and influencing their response to posterior stimulus [247]. Myeloid cell reprogramming has been observed to occur after stimulation with cytokines, PAMPs and whole pathogens [248–250]. IFN-γ has long been known to induce macrophage or monocyte ‘priming’, allowing these to better respond to later proinflammatory challenges [250]. IFN-γ exposure also overrides the effects of other macrophage stimulants, such as endotoxin tolerance induced by LPS exposure, in which the expression of various proinflammatory cytokines is repressed [251]. The molecular mechanisms behind the ability of IFN-γ to prime gene expression and rescue cytokine production remain unclear, but in some instances involve STAT1 and IRF1 binding at enhancer regions which leads to an increase in histone acetylation [250].

The importance of the IFN-γ pathway in immune cell reprogramming has been further highlighted by studies demonstrating that peripheral blood mononuclear cells isolated from patients with chronic mucocutaneous candidiasis (caused by a STAT1 deficiency) were unable to undergo *C. albicans*-induced training [252]. In contrast, mononuclear cells from patients with STAT3 deficiencies challenged with *C. albicans*, showed significantly higher TNF-α and IL-6 production upon secondary exposure. Despite both groups of patients displaying major primary immunodeficiencies, underscored by a chronic susceptibility to infections, mononuclear cells from STAT3 deficient appeared to undergo normal cellular reprogramming. This describes a role for STAT1 in the induction of trained immunity, which could be explained by its unique ability to potently drive IRF1 expression during type ll IFN signalling[199].

In vivo studies have also tied IRF1 function to the transcriptional and epigenetic reprogramming of HSC by Bacillus Calmette-Guérin (BCG) vaccination. A recent study by Kaufman and colleagues found that BM accessing of BCG-iv, educated the residing HSCs, which later gave rise to epigenetically modified macrophages providing enhanced

protection against virulent *M. tuberculosis* [249]. Interestingly, IFNγ-receptor-deficient (IFNγR$^{-/-}$) mice failed to undergo HSC reprogramming in the BM. IRF1 appeared to be one of the most highly differentially expressed genes in response to BCG-iv vaccination, for both HSCs and multipotent progenitors. Moreover, TF motif enrichment analysis on β-glucan-trained neutrophils revealed IRF1 motifs are among the top enriched motifs at differentially accessible regions [253]. Hence, despite innate immune training studies focusing on the STAT1 and more generally, IFN functionality, our results describing epigenetic reprogramming by IRF1 in macrophages, provides sufficient rationale for considering the possibility of IRF1 being a main driver during innate immune cell training.

Chapter 5 Conclusion and future directions

The overarching goal of my master's thesis was to optimize the experimental methods to study the chromatin binding kinetics of IRF1 and to identify chromatin remodelling events dependent on IRF1 in primary mouse macrophages in response to IFN-γ. I have successfully completed the three objectives by optimizing our IRF1 ChIP protocol and carrying out a 2-day time course using IRF1 ChIP-seq and ATAC-Seq on IFN-γ treated primary macrophages. Overall, this work has expanded our knowledge on IRF1's transcriptional dynamics upon macrophage activation, and its influence on chromatin accessibility.

Our data strongly suggests IRF1 to be a transcription factor with pioneering abilities at thousands of regions in IFN-γ treated primary macrophages. Moreover, IRF1 has been previously shown to interact with several chromatin remodeling complexes and alter DNA structure upon binding to its target sites. However, how the chromatin is remodelled at IRF1 pioneering sites and what are the participating factors remains completely unknown. Interrogating the recruitment of remodelling complexes and changes in histone post-translational modifications occurring at these sites via ChIP-Seq would help our understanding of both the time and manner at which IRF1 dependent remodelling occurs. Additionally, longer time points such as 72 – 96 h post IFN-γ stimulation would help confirm if IRF1-induced remodelling produces long lasting histone chromatin marking, indicative of stable cell reprogramming.

Also, despite ATAC-Seq signal increasing at IRF1 pioneering sites, we lack the knowledge as to whether chromatin relaxation influences nearby gene expression. Nascent transcriptomic experiments can provide direct insights into gene regulation changes and enable the highly sensitive detection of short-lived RNA species such as enhancer and long non-coding RNAs. Thus, a nascent RNA-seq experiment ran on the same time course using both WT and *Irf1*$^{-/-}$ IFN-γ stimulated macrophages would (1) measure the activity of these cis-regulatory regions by detecting eRNAs, (2) identify the dynamic gene expression changes driven by IFN-γ, and (3) demonstrate the dependency of enhancer and gene activity on IRF1 function. Also, to functionally connect the reprogrammed cis-regulatory regions to their target genes, methods such as Hi-C or

Omni-C, capturing chromosomal conformation and regulatory regions interactions should be employed.

Lastly, the long-term epigenetic reprogramming surrounding ISGs in monocytes and macrophages is considered an underlying mechanism of innate immune memory or ‘trained immunity’. Since the involvement of IRF1 in chromatin remodelling is now becoming accepted, its role in innate immune cell training is worth investigating. Both *in vitro* and *in vivo* experiments designed to assess the degree of dependency on IRF1 in chromatin remodelling at ISGs and in general the development of a training phenotype, would be of great importance to the field.

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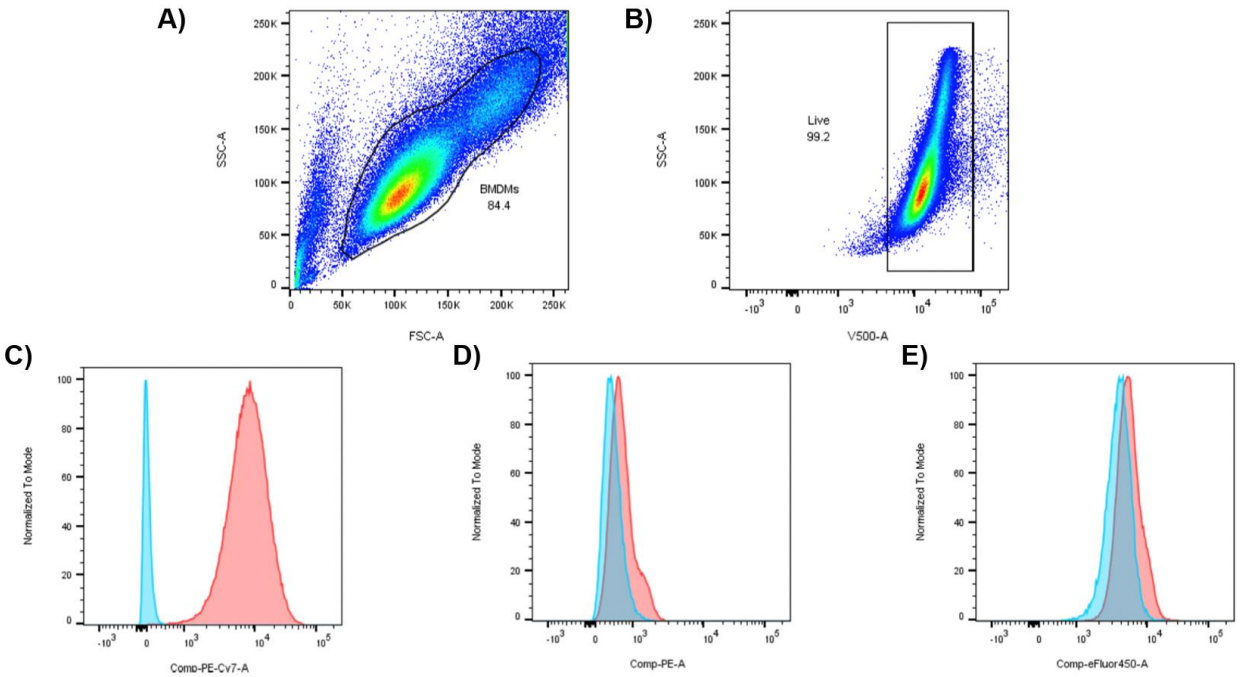
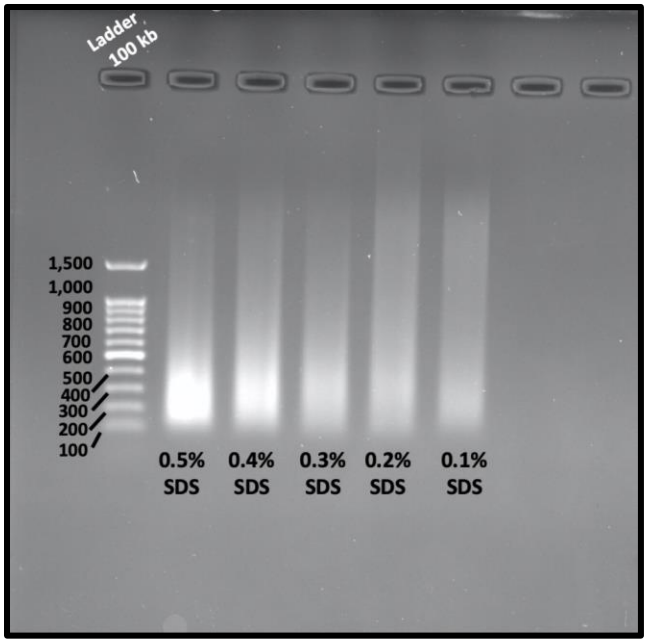
Appendix I: Supplemental Figures and Tables

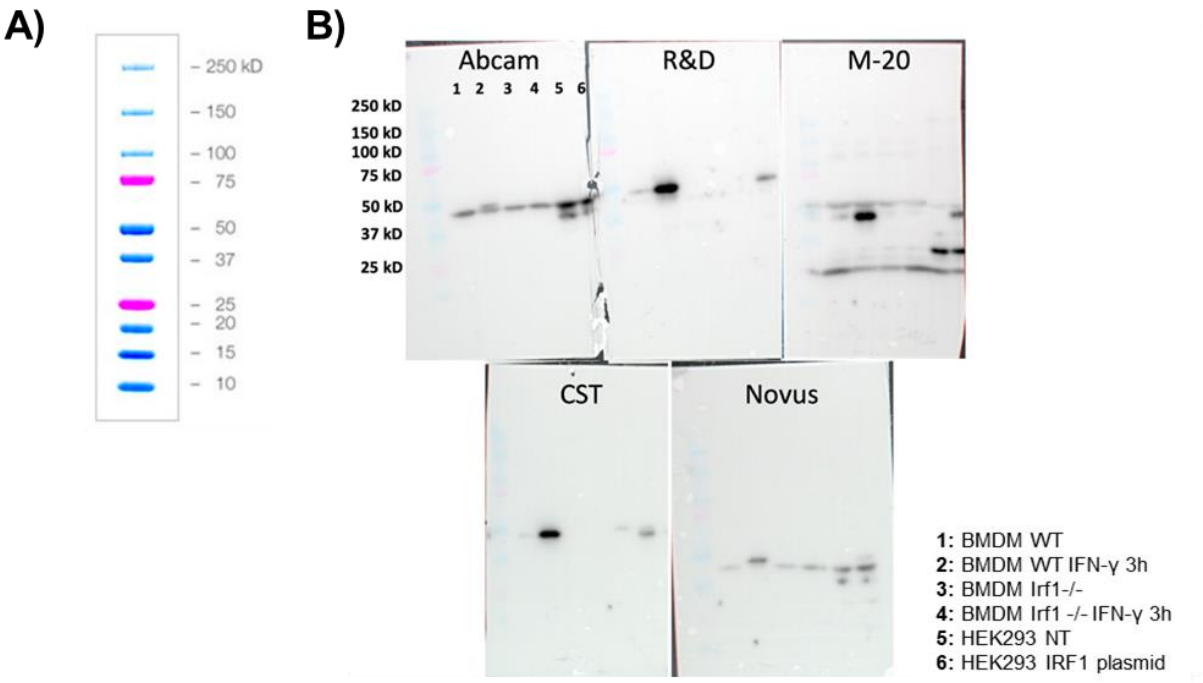


Supplemental Figure 1. Flow cytometry was performed to assess the efficiency of the *in vitro* BMDM differentiation protocol using LCCM. Flow cytometry plot showing the initial gating on cells to exclude debris and cell aggregates in **(A)** and to keep only live cells in **(B)**. **(C-E)** Flow histogram plot showing the signal intensity for F4/80, Ly6G and CD11b, respectively. Blue shaded areas correspond to unstained BMDMs and the red shaded areas to the signal from the stained sample.

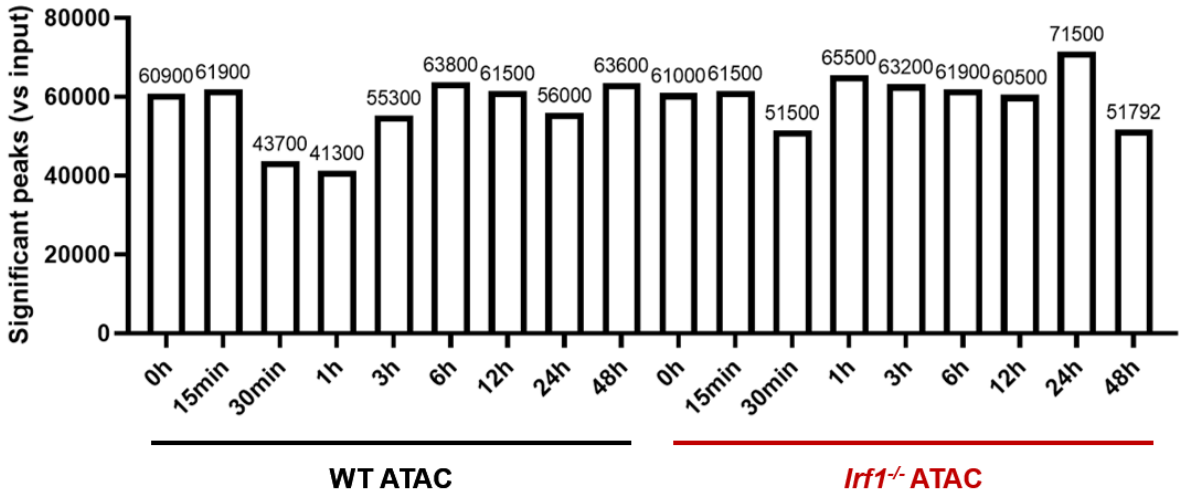


Supplemental Figure 2. Electrophoresis of 5 sheared chromatin samples and DNA ladder, sonicated at distinct SDS concentrations, ran on an agarose gel at 1% concentration at 100 V for 50 min.

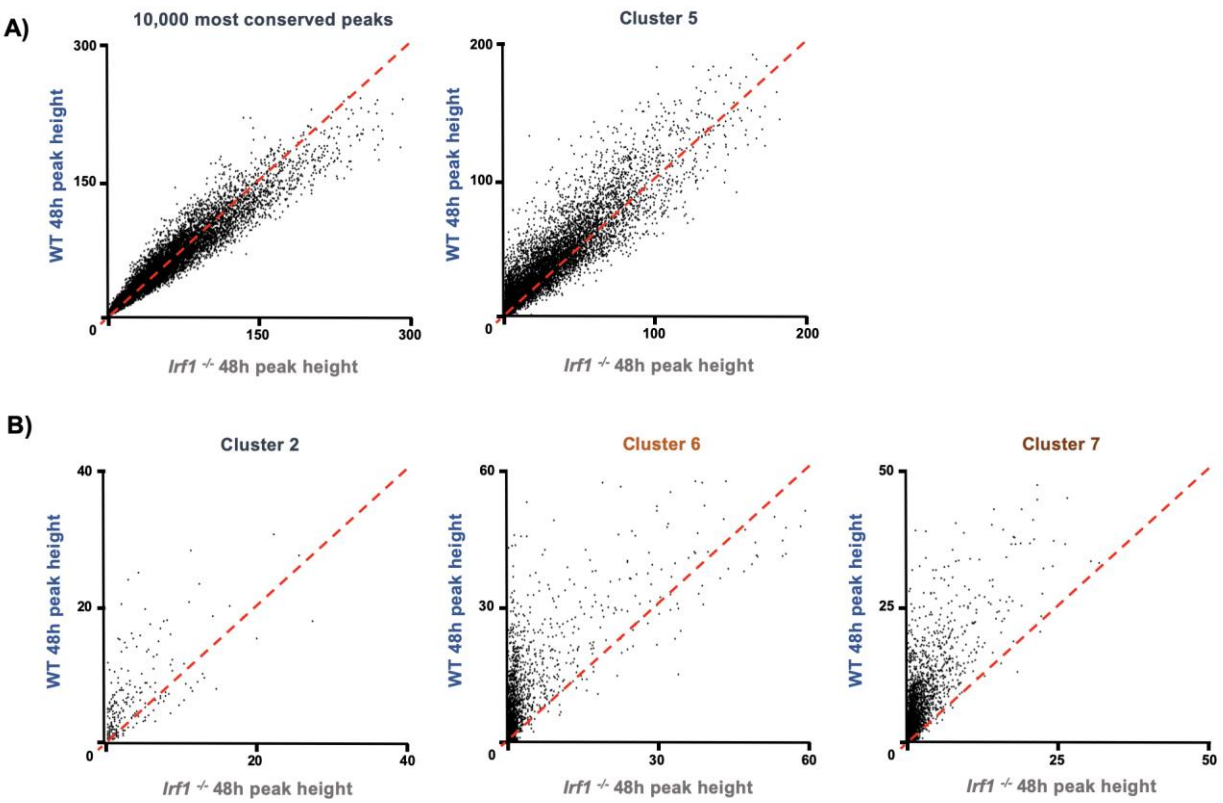
Loss of chromatin sonication efficiency strongly correlates with a reduction in the concentration of SDS detergent in the sonication buffer showcased by an abnormal electrophoretic migration pattern.



Supplemental figure 3. Uncropped western blot results from total protein extracts of WT and *Irf1*$^{-/-}$ BMDMs, both stimulated and non-stimulated with IFN-γ and HEK293T cells transfected and non-transfected with an IRF1 expression plasmid. **(A)** Precision Plus Protein™ Dual Color Standards **(B)** Chemiluminescence imaging of the PVDF membrane blotted against IRF1 resulting from 5 minutes of exposure with colorimetric images overlapped to show the ladder.



Supplemental figure 4. Bar graph displaying the number of significant peaks from our peak calling analysis of the ATAC-seq samples. The peaks were called using macs2, then manually filtered to only include those with an FC of > 3 in comparison with our input IRF1 ChIP-seq, as well as a CPM of > 5.



Supplemental figure 5. X-Y plots comparing ATAC-seq peak height signal intensity (displayed as raw CPM) at 48h post IFN-γ stimulation in WT and *Irf1*$^{-/-}$ BMDMs. **(A)** Peak height comparisons at the 10,000 least variable sites among all ATAC samples according to their ATAC-seq signal intensity, as well as at a non-pioneering cluster (cluster 5) corresponding to **figure 9 (B)** Peak height comparisons at suspected IRF1 pioneering clusters 2, 6 and 7 corresponding to **figure 9**.