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Characterization of Gene Regulation During Inflammation in Macrophages

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ABSTRACT

Characterization of Gene Regulation During Inflammation

Mays Mohammed Salih

Inflammation is the host's natural response to protect against infection and maintain homeostasis. The innate immune system is our first line of defense where immune cells such as dendritic cells, macrophages and neutrophils recognize and initiate responses

to the pathogen through pattern recognition receptors (PRRs). Macrophages play an essential role in propagating initial stages of the inflammatory response; hence, it is critical to fully understand their signaling mechanisms to better combat infectious and inflammatory conditions. Macrophages sense danger signals through PRRs -such as TLR4- which recognizes pathogen-associated molecular patterns (PAMPs) on the pathogen surface triggering pro-inflammatory signaling cascades to promote macrophage activation.

Proper propagation of this response is essential to mount sufficient inflammatory activation, however, careful regulation is needed to maintain homeostasis. While we are making tremendous strides in understanding inflammatory signaling pathways and their triggers, the factors responsible for maintaining an effective and balanced response are not well known. This dissertation addresses newly discovered functional regulators of the immune response and stresses the need for developing new and specialized tools to better understand the complexity of the regulatory machinery.

Unraveling the complexity of signaling pathways that govern the magnitude and intensity of response to stimulus continues to progress under the limitations of the tools we use to study it. Development of NGS has propelled a whole field towards better understanding of gene expression changes under inflammatory conditions. However, this powerful technology is limited in detecting subtle and intricate post transcriptional changes in splicing, editing and modifications. In the first chapter, we highlight the evolving capabilities of NGS that allowed for the study of functional lncRNAs in the

immune response as well as splice variants of certain genes that modulate immune activation. We also discuss how long read sequencing allowed for the construction of the complete genome and enabled us to study the role of alternative splicing and RNA modifications in modulating immunity.

In the second chapter we discuss a novel role for HNRNPA2B1 in regulating the macrophage inflammatory response. HNRNPA2B1 is an abundant RNA binding protein that's involved in RNA processing and maturation, in this chapter, we discuss its involvement in promoting IFNG signaling in macrophages to modulate macrophage activation upon exposure to stimulus. HNRNPA2B1 depletion in macrophages resulted in mice being less responsive to endotoxic shock and more sensitive to Salmonella infection due to disruption in the IFNG response signaling causing macrophages to be less efficient in clearing the pathogen. As a result of the disruption in the IFNG response cascade, the macrophage overall inflammatory gene expression was reduced which was represented by lower expression levels of TFs such as JAKs and STATs as well as lower cytokine production in the serum and spleen. Mechanistically, HNRNPA2B1 depletion in macrophages altered splicing outcome of the IFNGR leading to lower levels of the receptor on cell surface. This chapter further highlights the complexity of the immune regulatory machinery represented by the role played by alternative splicing in affecting organism-wide changes in the inflammatory status and disease outcome.

In the third chapter we delve into innate immune modulation because of smoke exposure since it's highly linked to many inflammatory diseases such as chronic

obstructive pulmonary disease (COPD). Here, we showcase smoke exposure novel involvement in immune activation, specifically through modulation in gene expression of a large number of coding and non-coding transcripts in macrophages. Most importantly, smoke exposure led to the activation of an immune regulating lncRNA - *lincRNACox2*- which has been shown to regulate inflammatory gene expression in mice. Through complex mouse models, we show that LincRNACox2 functions in the lung to regulate inflammatory gene expression in mice following smoke exposure. This dissertation showcases significant advances in understanding modes of immune response regulation through an evolving view that extends beyond regulation through gene expression. We highlight non-traditional modes of gene expression regulation through RNA binding proteins, alternative splicing, and non-coding transcripts (e.g., lncRNAs).

DEDICATION

To my two little munchkins, Rami and Maya

You made the hard days better and gave it all a purpose!

I love you.

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sense of humor and presence that lights up rooms, please continue to share that with the world. Maya, you are small but I can already tell that you are incredibly smart, confident and full of love, I hope you continue to grow up with that radiant confidence. I love you!

CHAPTER 1- What sequencing technologies can teach us about innate immunity

1.1 Summary

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For years we have taken a reductionist approach to understanding gene regulation through the study of one gene at a time in one cell at a time. While this approach has been fruitful it is laborious and fails to provide a global picture of what is occurring in complex situations involving tightly coordinated immune responses. The emergence of whole genome techniques provides a systems level view of a response and can provide a plethora of information on events occurring in a cell from gene expression changes to splicing changes and chemical modifications. As with any technology this often results in more questions than answers, but this wealth of knowledge is providing us with an unprecedented view of what occurs inside our cells during an immune response. In this review we will discuss the current RNA-sequencing technologies and what they are helping us learn about the innate immune system.

1.2 Introduction

Over the last four decades there have been many technological advances in high throughput approaches to study gene expression from DNA microarrays to the development of next generation sequencing (NGS) (Roh et al., 2010). NGS provides a wealth of knowledge in terms of biological processes from profiling of gene expression changes to the identification of genetic variants including single nucleotide small nuclear polymorphism (SNPs), to the study of splicing and chemical

modifications. These tools are making a strong impact on a number of fields of research and here we will fo ey have taught us so far and what they could be used for in the future in relation to regulation within the innate immune system.

Our innate immune system provides one of the first lines of defense against infection; It serves as a rapid response involving transient activation of inflammation (Monaco and Robbins, 1973). This is essential to maintaining homeostasis, but if left unchecked can become chronic and result in a host of inflammatory or autoimmune conditions (Chen et al., 2018). Understanding the molecular mechanisms that govern inflammation and drive inflammatory and autoimmune diseases have presented a long-standing challenge due to the combination of genetic and environmental factors in addition to the complexity of the pathways involved (Chen et al., 2018), making it difficult to develop cures or even new drugs for therapeutic intervention. Since the development of NGS it has been used extensively to study inflammatory diseases from efforts such as large genome-wide association studies (GWAS) designed to identify possible disease-causing genes, to the identification of variants as well as studying altered gene expression programs (Petersen et al., 2017). In addition to the complexities surrounding protein regulation in the immune system, NGS has also unveiled the presence of 1000s of non-coding genes. It is now appreciated that the majority of any given genome is transcribed and yet only 3% is protein coding (Derrien et al., 2012). Figuring out the functional and biological significance of these transcripts in relation to innate immunity is only beginning. Finally, NGS has brought a renewed focus on the importance of post-transcriptional events such as splicing,

RNA editing and RNA modifications during an immune response. As expected, inflammatory and autoimmune diseases are complicated; each cell type can be involved to varying degrees in the pathogenesis of any given disease driving the need for the development of single cell sequencing technology. It can be daunting to think about how we unravel such complexity in the immune response. Here we will review what NGS has allowed us to glimpse in terms of regulation within innate immunity. There is still a lot for us to learn and with the speed at which these technologies are developing we continue to get one step closer to producing better therapeutics with the long-term goal of eventually curing inflammatory and autoimmune diseases.

1.3 Evolution of RNA-sequencing

DNA sequencing has evolved rapidly from first generation Sanger sequencing to the so-called next generation sequencing (NGS) which includes second generation short-read sequencing to the more recent third generation long-read sequencing technologies (Bentley, 2006, Goodwin et al., 2016, Slatko et al., 2018). NGS was quickly adopted as a tool to profile the transcriptome by isolating RNA and converting it to cDNA for sequencing (RNA-sequencing or RNA-seq) as an attractive alternative to microarray technology. RNA-seq possesses a number of advantages compared to microarrays including the detection of novel sequences, broad dynamic range, high specificity and sensitivity capable of picking up low abundance transcripts (Govindarajan et al., 2012, Meera Krishna et al., 2019).

The first high-throughput sequencing platform appeared in 2005 (Margulies et al., 2005) and was followed by multiple NGS platforms, the most common of which is the Illumina-based sequencing technology. Rapid growth in NGS use was prompted by its application in the whole genome sequencing project (WGS) and continued to grow as an essential tool due to its biomedical applications, its use in epidemiological studies of infectious diseases, surveillance of foodborne illnesses and viral diversity studies (Besser et al., 2018, Lewis et al., 2010, Beerenwinkel et al., 2012). Massively parallel NGS technology or commonly known as "deep sequencing" refers to sequencing a genomic region multiple times, sometimes hundreds or even thousands of times (referred to as coverage) allowing for the detection of rare clonal cells, or microbes comprising as little as 1% of the original sample (Goldman and Domschke, 2014). Advances in sequencing depth and error reduction elevated the field of biomedical discovery from studying individual genes in order to discover disease variants to whole genome studies. The most commonly used sequencing platforms (second generation technologies) are generally divided into two categories: MiSeq or MiniSeq platforms which are relatively cheap and provide low to medium throughput, while HiSeq, NovaSeq or NextSeq are more expensive but provide high throughput (Table 1.1). All second-generation technologies provide fragmented short reads that require subsequent genome assembly. The low-cost high throughput sequencing technologies allowed for a deeper and more thorough understanding of genetic variation and complexity and allowed us an unprecedented view into novel transcripts and epigenetic regulation.

These advances push us closer to personalized medicine where a patient's genome can be readily sequenced to try and detect disease associated variants. It currently takes less than a few days and costs about $1000 to sequence a human genome (Hayden, 2014). The cost will continue to decrease and soon patients will not just have their genomic DNA sequenced but also their transcriptome to obtain information on post-transcriptional regulatory events that could be dysregulated in a diseased state.

Large scale initiatives that utilized deep sequencing to study genetic variation have shown their effectiveness in covering >91% of the human genome with high confidence and resulted in the discovery of about 150 million single nucleotide variants (SNPs) in the coding and non-coding parts of the genome (Telenti et al., 2016). Thanks to recent advances in sequencing technology, as well as computational pipelines we now have a close to complete reference genome. The first reference genome (GRCh37) published in 2001 covered 90% of the human genome with 15,000 gaps, representing sequences from 13 donors constructed into a mosaic haploid genome (Giani et al., 2020, “E pluribus unum,” 2010). The reference genome is now in its 20th rendition; GRCh38 published in 2013 with merely 738 unclosed gaps (Zhao et al., 2020) with continual advances in NGS holding the promise of closing these gaps in the next iterations. GRCh38 remains limited because it represents genetic sequences from a few individuals and doesn’t begin to cover the complex genetic variability especially in regions with high allelic diversity such as the major histocompatibility complex (MHC). Also, it fails to represent regions where haplotypes are represented in similar frequencies in different populations (“E pluribus

unum," 2010). This paved the way for initiatives such as the "1000 genomes project" which was completed in 2015 and reconstructed the genomes of 2,504 individuals from 26 populations, it characterized over 88 million variants along with 3.6 million short insertions/deletions (indels) covering huge population diversity using multiple sequencing technologies (1000 Genomes Project Consortium et al., 2015). While this work was a heroic undertaking, we need to expand these efforts if we are to appreciate the full genomic diversity of populations across the globe. There are populations that were never even sampled and others such as those of European descent that have been oversampled which has been reviewed in depth in (Popejoy and Fullerton, 2016). Abi-Rached *et al.*, highlight the shortcomings of the 1000s genomes project when it comes to understanding the complexity of the immune system. The human leukocyte antigen (HLA) region is a highly polymorphic and well-studied region that encodes MHC molecules; however, data from the 1000 genomes project failed to detect over 70% of rare and 20% of common HLA variants (Abi-Rached et al., 2018). There are serious immune conditions such as sickle cell anemia as well as autoinflammatory conditions such as systemic lupus erythematosus (SLE) that disproportionately impact African Americans compared to those of European descent (Ramos et al., 2015). Yet, in GWAS studies, Hispanic, African American and indigenous people continue to be under sampled. We need much more inclusive data if we are to fully appreciate how genetic diversity and genome plasticity contribute to disease states (Popejoy and Fullerton, 2016).

NGS provided the depth necessary to detect novel sequences and transcripts, such as long noncoding RNAs (lncRNAs), which we will discuss in depth later. It also allows for the detection of splice isoforms of the same gene, thus providing information about alternative splicing at a specific loci under treatment conditions, as well as alternative promoter usage and premature termination (Han et al., 2015). However, as Illumina short read sequencing arose to become the gold standard in genome profiling, progress continued to be made in developing newer tools to overcome short read technologies shortcomings. Some of these shortcomings include short read length (<300bp), which makes it difficult to detect structural variation, its size bias due to PCR amplification, insensitivity to highly repetitive or GC rich regions or homologous elements (Fig.1.1A) (Logsdon et al., 2020). These limitations have contributed to failed attempts at understanding or identifying causal mutations and or dysregulated pathways in patients suffering from complex inflammatory diseases.

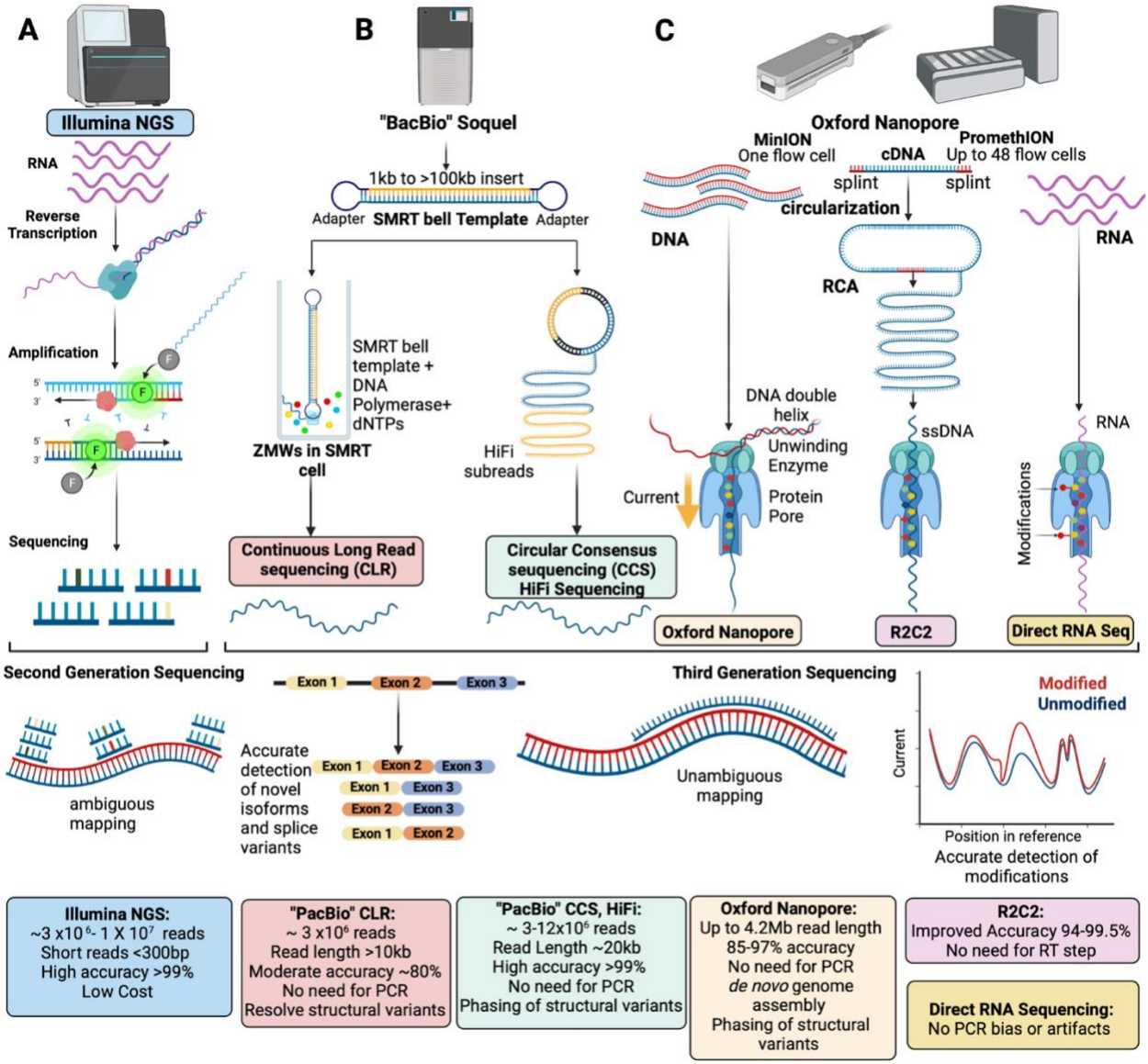

Figure 1.1 Evolution of RNA sequencing technologies. A. Illumina NGS involves RT and amplification steps prior to sequencing. It is high throughput as it can yield anywhere from 3 million to 1 billion short read fragments <300bp in length. It currently provides highest read accuracy >99% in combination with low run cost. However, the short-read length results in ambiguous mapping and inability to resolve genomic variants and GC rich regions. **B.** Long read sequencing using PacBio technology generates up to 3 million long reads and relies on a circular DNA SMRTbell template composed of a double stranded DNA insert flanked by two single

stranded hairpin adapters on both ends. DNA polymerase is attached and the complex is read in a zero mode waveguides (ZMW) SMRT cell where DNA polymerase adds fluorescently labeled dNTPs and allows for base by base readout of the template. The original technology relied on continuous long read (CLR) sequencing that yields reads longer than 10Kb with moderate accuracy ~80%. To increase read accuracy circular consensus sequencing (CCS) was introduced, it produces high fidelity (HiFi) reads through repeated passes of DNA polymerase through the template resulting in multiple error-prone subread, which when compiled produces a highly accurate consensus sequence. It yields a higher number of reads compared to CLR with longer read length and accuracy that exceeds 99%. Both technologies eliminate the need for PCR, and produce reads long enough to detect variants, novel splice isoforms and can be mapped accurately. **C.** Oxford Nanopore Technologies (ONT) uses a linear DNA molecule attached to a sequence adapter loaded with a motor protein that pushes the DNA molecule through a nanopore. As the negatively charged DNA strand travels through the pore, individual bases cause a disruption in the current allowing us to call individual bases in real time. Many platforms were developed such as the MinION and the PromethION that differ in number of flow cells and subsequently in read number output. ONT can generate reads exceeding 1Mb in length with variable base calling accuracy 85-97% which is dependent on the protocol used to generate the reads and the computational program used for base calling. Nevertheless, it generates reads long enough for unambiguous mapping, allows for *de novo* genome assembly and detection of structural variants as well as novel isoforms. The Rolling Circle Amplification to Concatemeric Consensus (R2C2) method relies on introducing 8bp splints to both ends of the reverse transcribed cDNA, only full length-cDNA is then circularized and amplified using rolling circle amplification (RCA). It is used to increase accuracy - can produce >94% read accuracy- and to increase resolution of RNA transcript isoforms. Additionally, ONT allows for direct RNA sequencing which eliminates the need for the RT and PCR steps and allows for detection of native isoforms and RNA modifications.

Platform	**Through put**	**Accuracy**	**Number of Reads per Run**	**Maximum Output**	**Read length**	**Run Time**	**Price**
Sanger	Low	99.99% ‡	1	--	400-600bp [12]	4 hrs	$3/reaction †
Ilumina MiSeq †	Low	>96% §	25 million	15Gb	1x36bp 2x300 bp [12]	4-55hrs	$24/Mb (Besser et al., 2018)

MiniSeq†	Low	>80% high quality bases §	25 million	7.5Gb	1x75bp 2x150 bp [12]	4-24hrs	$24/Mb (Besser et al., 2018)
Illumina HiSeq 4000 RapidRun §	High	>88% bases with high quality scores §	300 million	250GB 750GB 1500GB	1x50bp 2x75bp 2x150 bp	1-3.5 days	$1400-2000/ Lane †
NovaSeq 6000 †	High	>94% bases with high quality scores §	650 – 800M 2 – 2.5B	400 Gb 750 Gb	2 x 250bp 2 x 150bp	38hr 44hr	$8,578/Flow cell $5,590/ Lane
NextSeq 550 †	High	>80% bases with high quality scores §	400 million	120Gb	1x75bp 2x150 bp	12-30hr	20$/Gb †
PacBio Soquel II ŦŦ (HiFi) (Logsdon et al., 2020) (Hon et al., 2020)	High	>99.9%	300K – 12M per SMRT cell	30-50Gb per SMRT cell ŦŦ	~ 10-25Kb	10 hr per SMRT cell	>2000$/flow cell
ONT Minion (DNA) ¶ (Payne et al., 2018)	High	85-97%[29,30]	4-10 million	20-50Gb	Up to 4.2Mb	Up to 72hr	425-900$/flow cell

ONT Promethion ¶ (DNA) (Payne et al., 2018) (Ramos et al., 2015)	High	85-97%	4-10 million	Up to 245Gb	Up to 4.2Mb	Up to 72hr	Up to 2000$/flow cell

Table 1. 1 Developments of evolving sequencing technologies with variable throughput, accuracy and cost.

† Data from Genewiz website

‡ Data from Thermofisher website

§ Data from Illumina Website

¶ Data from Nanopore website

ŦŦ PacBio website

1.4 Exploring the dark matter of the genome.

One of the biggest discoveries emerging from next gen RNA-sequencing studies was the fact that the majority of the genome is actively transcribed, yet only a small percentage <3% is translated into protein (Derrien et al., 2012). The next question was, and continues to be, what is all this RNA doing and is it biologically active? The largest group of non-coding RNA produced in the genome are lncRNAs (Fig.1.2). LncRNAs are defined as transcripts >200bp in length with no protein-coding potential. LncRNAs exhibit low sequence conservation despite some stability in genetic loci conservation (Ulitsky et al., 2011). While there are nearly 18,000 lncRNA transcripts annotated in the human genome (Fig.1.2), the majority of them

remain unstudied and their function remains uncharacterized. While these transcripts were first thought to be transcriptional noise it was clear early on that these are dynamically regulated regions and their exact functions are only beginning to be uncovered (Hon et al., 2017). LncRNAs are often classified based on their orientation or site of transcription relative to their neighboring protein coding gene such as antisense, intronic and intergenic lncRNAs. There are also lncRNAs emerging from enhancer regions (eRNAs) as well as from promoters (Wu et al., 2017). The largest group of lncRNAs are intergenic meaning they lie between two protein coding genes and contain their own independent promoters (Ransohoff et al., 2018). For a thorough review on lncRNA biogenesis and their many modes of post transcriptional regulation we recommend a recent review by Statello et. al, . (Statello et al., 2021).

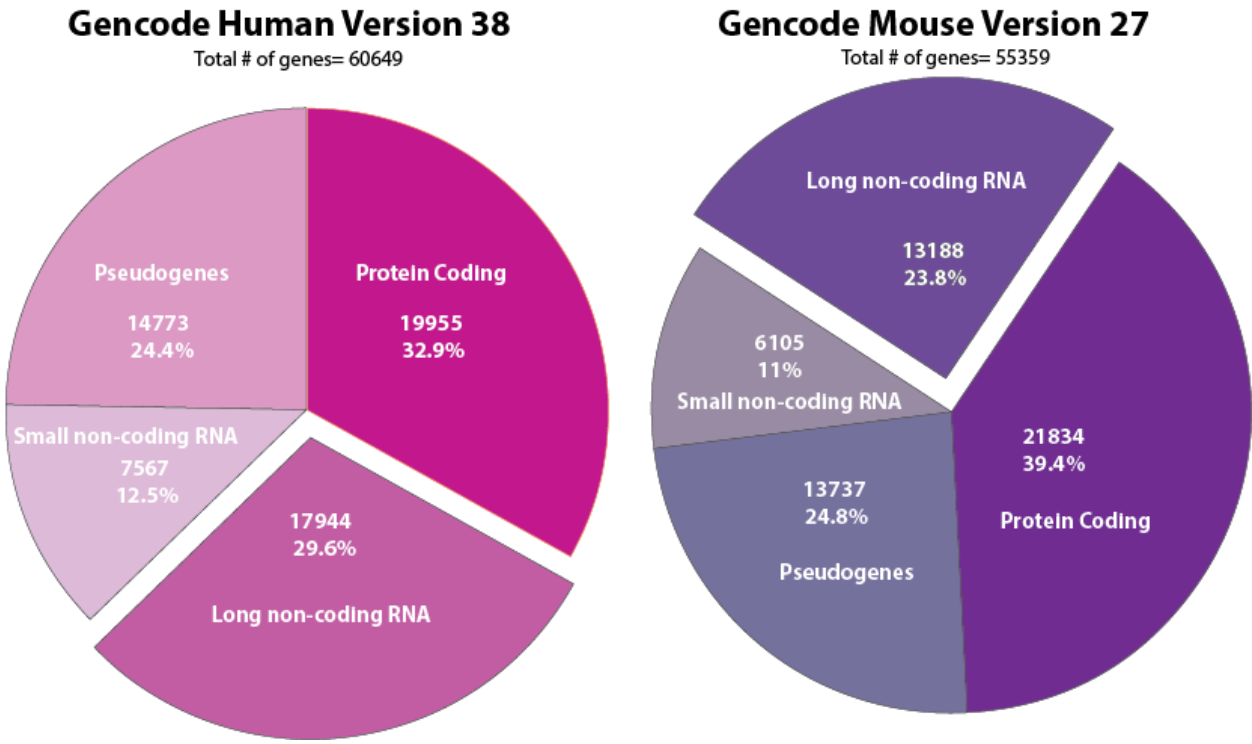

Figure 1.2 Annotated transcripts in Gencode based on the most recent human and mouse release. Pie Charts showing statistics of annotated genes in human GRCh38.p13 (version 38) and mouse GRCm39 (release M27).

LncRNAs can mediate regulation of genes through a wide variety of mechanisms which can broadly be broken into two categories of *cis* or *trans* regulators (Fig. 1.3, 1 and 2). LncRNAs functioning in *cis*, means they regulate are regulating their neighboring genes on the same allele. This form of regulation is perhaps not surprising given the fact that many lncRNAs display a similar expression pattern as their neighboring protein coding genes (Statello et al., 2021). Interestingly, this regulation can be independent of the transcript itself and instead rely on recruitment of the transcriptional and splicing machinery to the neighboring locus (Fig.1.3, 1.A) (Engreitz et al., 2016). LncRNAs can also regulate neighboring genes in *cis* by acting as enhancer lncRNAs (e-lncRNAs) where they recruit mediators and co-activators to the locus and facilitate coordinate activation through chromatin looping between the enhancer and promoter of the neighboring gene (Fig.1.3, 1.B) (Kim et al., 2015; Lai et al., 2013; Xiang et al., 2014; Ballarino et al., 2018; Petermann et al., 2019). An example of *cis* regulation is the innate immune regulatory lncRNA, *Rroid*, that directly interacts with the promoter of its neighboring gene *Id2* in innate lymphoid cells (ILCs), promoting chromatin accessibility and deposition of STAT5 at the promoter of *Id2* prompting the cells to commit to an ILC fate (Mowel et al., 2017). LncRNAs can also repress expression of neighboring genes in *cis* (Fig.1.3, 1.C) including *Morrbid*, which recruits PCR2 complex to *Bcl2l11* promoter through chromatin looping and allows PCR2 to deposit methyl tags at *Bcl2l11*

promoter suppressing its expression (Kotzin et al., 2016; Gil and Ulitsky, 2020). Through this interaction with the promoter of pro-apoptotic *BcL2l11*, *Morrbid* is able to tightly regulate the survival of neutrophils, eosinophils and classical monocytes in response to pro-survival signals by cytokines, thus balancing an appropriate protective immune response against the deleterious consequences of prolonged activation.

LncRNAs can regulate genes on a different allele or different chromosome in *trans*. LncRNAs can function in *trans* through interactions with RNA binding proteins (RBPs) to regulate splicing or stability of transcripts (Fig.1.3, 2.A and B) (Yap et al., 2018; Lee et al., 2016; Tichon et al., 2018). Alternatively, it can occur by binding of a lncRNA to mRNA transcripts through base pairing to promote or suppress stability and translation (Fig.1.3, 2.C and D) (Lee et al., 2016; Kretz et al., 2013; Gong and Maquat, 2011; Carrieri et al., 2012). Others function by sequestering suppressors from the gene promoter to allow transcription factor binding and subsequent gene expression (Fig.1.3, 2.E) (Krawczyk and Emerson, 2014; Imamura et al., 2014). Most of the lncRNAs described to date in the innate immune system regulate genes in *trans*.

1.5 LncRNAs and innate immunity

LncRNAs play various roles in biological processes, including splicing (Gonzalez et al., 2015), protein localization (Munschauer et al., 2018) and cellular proliferation (Li et al., 2020) (Rossi et al., 2019). LncRNAs are highly cell type

specific in their expression patterns which makes them attractive as disease biomarkers (Zou and Xu, 2020). This is something that could be a particularly attractive area of investigation for autoinflammatory conditions such as arthritis and SLE which are notoriously difficult to diagnose quickly in the clinic.

Over the last decade there has been a significant increase in the number of lncRNAs being characterized to function in various ways within the immune system, from immune cell development to gene regulation. We cannot cover in depth all the lncRNAs identified to function in the immune system in this review and therefore we direct readers to the following reviews for in depth analysis of each lncRNA and its specific role in the innate immune system, reviewed in (Atianand et al., 2017; Mowel et al., 2018; Walther and Schulte, 2021; Chen et al., 2017). Here we will focus on lncRNAs that show some common or unique mechanisms of action within the immune system (Table 1.2). One of the first long intergenic non-coding RNAs (lincRNAs) lncRNAs identified in the immune system is *lincRNA-Cox2*; It was first described to be induced ~1000 fold following lipopolysaccharide (LPS) activation by Guttman et. al, (Guttman et al., 2009). They utilized the chromatin signatures of active promoters (trimethylation of lysine 4 on histone 3, H3K4me3) and active transcription (trimethylation of lysine 36 on histone 3 H3K36me3) and performed chromatin immunoprecipitation followed by massively parallel sequencing (ChIP-Seq) to capture all actively transcribed genes including lncRNAs. Since this study we and others have shown that *lincRNA-Cox2* is a highly inflammatory inducible gene that functions broadly to regulate immune genes during the innate immune response

(Carpenter et al., 2013; Hu et al., 2016; Tong et al., 2016; Covarrubias et al., 2017; Elling et al., 2018; Robinson et al., 2021). Interestingly, using multiple genetic mice models we found that *lincRNA-Cox2* can function both in *cis* where it regulates the critical immune gene *Ptgs2 (Cox2)* through an enhancer RNA mechanism as well as in *trans* to regulate a wide variety of immune genes in macrophages (Elling et al., 2018; Robinson et al., 2021). We found that *LincRNA-Cox2* can function in *trans* to negatively regulate basal expression of interferon stimulated genes through interactions with hnRNPA2/B1 and hnRNPA/B (Fig.1.3, 2.B) (Carpenter et al., 2013a). Interestingly, many lncRNAs that function within the immune system appear to do so through interactions with various hnRNP proteins. TNFα and HNhnRNPL HNRNPL related immunoregulatory lincRNA (*THRIL*) was found to regulate expression of the *TNFAα* gene through binding to HNhnHNRNPL forming a complex that binds to TNFAα promoter region (Fig.1.3, 2.B) (Li et al., 2014). *LincRNA-EPS* represses immune response genes by associating with chromatin in the nucleus to create a heterochromatin (repressive) environment. It binds to HNhnHNRNPL through a specialized motif at 3' end forming a complex that represses immune gene expression (Fig.1.3, 2.B) (Atianand et al., 2016).

P50 associated *Cox2* extragenic RNA (*PACER*) is another lncRNA that functions to regulate *Ptgs2* (also known as *Cox2*). *PACER* functions by sequestering the P50 repressive complex of NF-kB away from the *Ptgs2* promoter allowing recruitment of the active dimers of NF-kB and RNA pol II initiation complex to promote the activation of *Ptgs2* (Fig.1.3, 2.D) (Krawczyk and Emerson, 2014). In a

similar mechanism the lncRNA *NEAT1* was found to sequester the *IL8* repressor, splicing factor proline/glutamine rich (SFPQ) from the promoter into a heterochromatin structure "paraspeckle" leading to the transcriptional activation of *IL8* in response to viral infection or toll-like receptor 3 (TLR3) activation (Fig.1.3, 2.D) (Imamura et al., 2014).

IL1B-eRNA is an example of an e-lncRNA in the immune system which acts as an enhancer for *IL1B* gene through binding to the PU.1 transcription factor and the *IL1B* promoter activating *IL1B* gene expression in response to an inflammatory stimulus (Fig.1.3, 1.B) (Ha et al., 2019; IIott et al., 2014).

While GWAS studies have mostly been utilized to study various SNPs arising in protein coding genes it is clear that over 90% of all SNPs lie within the non-coding space in the genome (Kumar et al., 2013). *Lnc13* was identified in a study by Castellanos-Rubio *et al.*, where they showed that the Celiac Disease associated SNP, rs917997, lies within this locus (Castellanos-Rubio et al., 2016). *Lnc13* regulates inflammatory genes and mediates its function *via* hnRNPD (Castellanos-Rubio et al., 2016). They showed that the SNP disrupts the RNA-protein interaction making the lncRNA dysfunctional.

LncRNAs can function within the cytoplasm or the nucleus to mediate their effects on immune genes. *Lethe* is a predominantly nuclear lncRNA that is involved in the negative feedback loop of the NF-κB pathway through direct binding to Rela and inhibiting its interaction and activation of genes within the nucleus (Rapicavoli et al., 2013). *Lnc-DC* is localized to the cytoplasm where it directly binds to signal

transducer and activator of transcription 3 (STAT3) to promote its phosphorylation and induce dendritic cell differentiation (Wang et al., 2014). Recently we characterized the cytoplasmically localized lncRNA, gastric adenocarcinoma predictive long intergenic noncoding RNA (*GAPLINC*), as a conserved lncRNA that functions as a negative regulator of the inflammatory response in human and murine macrophages. *Gaplinc* KO mice are resistant to LPS induced endotoxic shock and mechanistically *GAPLINC* appears to function within the cytoplasm to control expression levels of NF-kKB (Rela) and limit its localization to the cytoplasm during homeostasis.

LncRNA	**Function**	**Mode of regulation**	**Localization**	**Source**
Rroid	Promotes ILC proliferation	*Cis* through direct interaction with neighboring gene promoter	Nuclear	(Mowel et al., 2017)
Morrbid	Represses *Bcl2l11* expression	*Cis* Recruits PCR2 which deposits methyl tags on *Bcl2l11* promoter	Nuclear	(Kotzin et al., 2016)
LincRNA-Cox2	• Required for *ptgs2* expression • Regulates expression of critical immune response genes	• Enhancer RNA mechanism • Inhibits expression of ISGs through interactions with hnRNPA/B and A2/B1 in trans • trans activation via unknown mechanism	Nuclear and Cytoplasmic	(Elling et al., 2018a)

Lnc13	Regulates inflammation and is dysregulated in Celiac disease	*Trans* regulation of immune genes through interactions with hnRNPD	Nuclear	(Castellanos-Rubio et al., 2016)
Lethe	Negative feedback of NF-κB pathway	*Trans* via direct binding to Rela	Nuclear	(Rapicavoli et al., 2013)
THRIL	Induction of TNFAa expression	*Trans* via direct binding to HNRNPL	Nuclear	(Li et al., 2014)
linRNA-EPS	Suppresses immune gene expression	*Trans*; Binds to chromatin and HNRNPL hnRNPLHnrnpl to form complexes	Nuclear	(Atianand et al., 2016)
Lnc-DC	Dendritic cell differentiation	*Trans* via direct binding to STAT3	Cytoplasm	(Wang et al., 2014)
GAPLINC	Inhibits basal activation of NF-kB	Functions in *trans* to regulate to regulate basal levels of NF-kB and limit its localization to the cytoplasm	Cytoplasm	(Vollmers et al., 2021a)
PACER	Promotes *Ptgs/Cox2* expression	Cis; sequesters p50 away from *Ptgs2* promoter allowing for its expression	Nuclear	(Krawczyk and Emerson, 2014)
NEAT1	Promotes *IL8* expression	Trans; sequesters the SFPQ repressor complex to paraspeckles	Nuclear	(Imamura et al., 2014)
IL-1B eRNA	Promotes *IL1B* expression	Binds to PU.1 transcription factor and *IL1B* promoter to activate transcription	Nuclear	(Ha et al., 2019), (IIott et al., 2014)

Table 1.2 lncRNAs involved in inflammatory gene expression regulation.

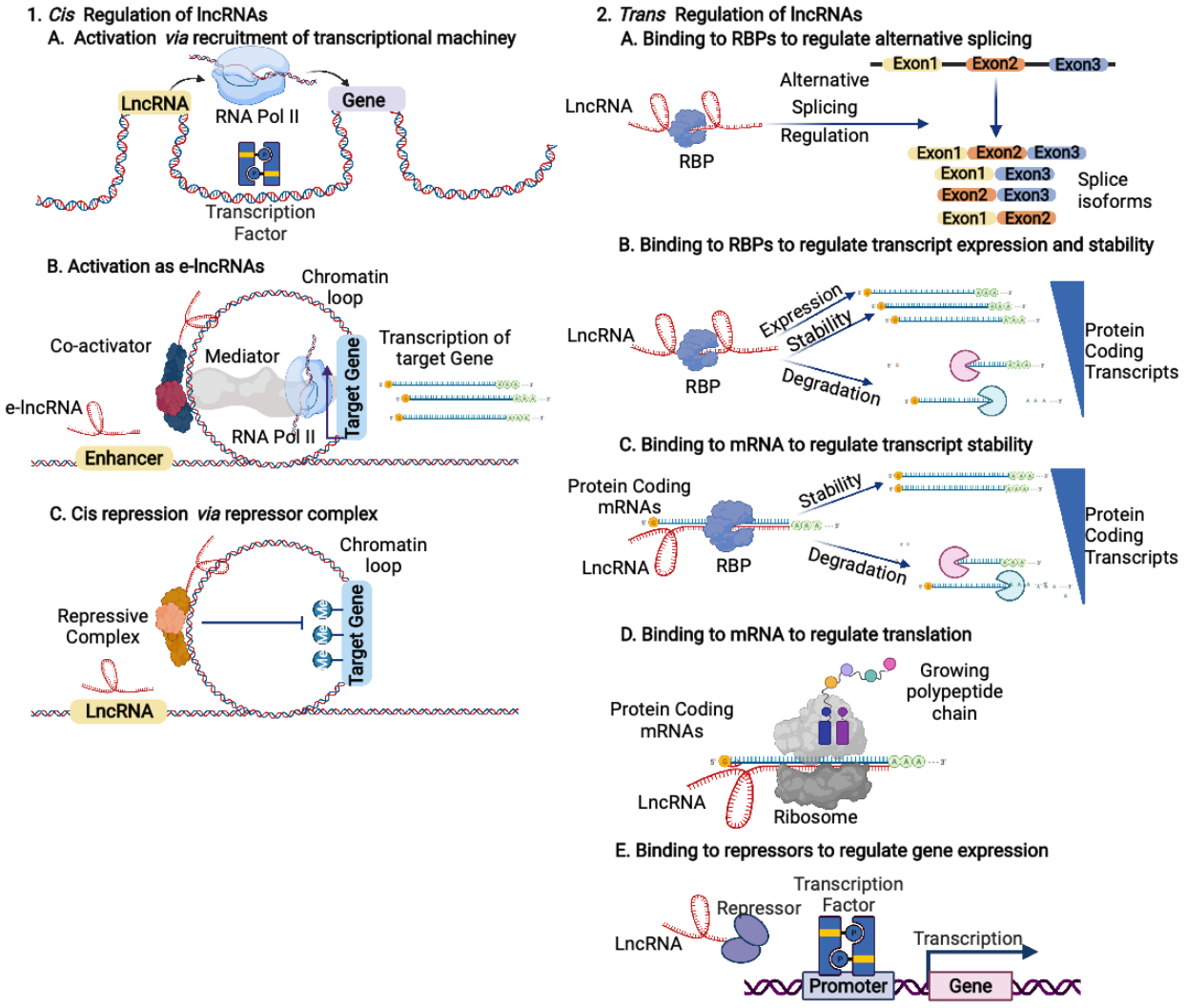

Figure 1.3 Modes of gene regulation by lncRNAs. 1. LncRNAs can regulate neighboring genes in *cis* through a variety of mechanisms. **A:** They can activate gene expression through recruitment of the transcriptional and splicing machinery to the neighboring locus. **B:** They can activate gene expression by acting as enhancer RNAs (e-lncRNAs) where they recruit mediators and co-activators to locus and facilitate coordinate activation through chromatin looping between the enhancer and promoter of the neighboring gene. **C:** They can repress neighboring gene expression by recruiting a repressive complex to the neighboring gene promoter through chromatin looping, leading to methyl tag deposition and gene expression inhibition. 2. LncRNAs can regulate genes on a different allele or different chromosome in *trans* through a variety of mechanisms. **A.** Through interactions with RNA binding proteins (RBPs)

to regulate alternative splicing of the RBP target transcript. **B.** Through interactions with RBPs to regulate target transcript expression, stability or degradation. **C.** It can occur through binding of a lncRNA directly to mRNA transcripts through base pairing impacting transcript stability and degradation. **D.** Binding of a lncRNA directly to mRNA transcripts through base pairing impacting recruitment of the polysome and transcript translation. **E.** LncRNAs can sequester suppressors from the gene promoter to allow transcription factor binding and subsequent gene expression.

1.6 Emergence of long read sequencing technologies:

As we continue to study the transcriptome, we need tools capable of capturing layers of genetic complexity that contribute to gene regulation including detection of strandedness, DNA and RNA modifications and splice variants. In addition, PCR amplification steps involved in the majority of library preparation protocols introduce biases such as large duplicate portions and uneven distribution of read coverage across targeted sequences (Kozarewa et al., 2009). In recent years, Pacific Biosciences (PacBio) and Oxford Nanopore (ONT) have developed multiple new sequencing techniques capable of producing continuous reads longer than 10kb in length directly from DNA or RNA which are helping researchers to answer complex biological questions (Fig 1.1 B and C). These approaches allow for *de novo* transcript assembly which means less reliance on the often error prone reference genome and without the need for PCR thus eliminating PCR bias.

1.7 Pacific Biosciences long read technology (PacBio):

The core technology emerging from PacBio is single molecule, real-time (SMRT) sequencing, where DNA is directly used to produce reads with read length around 10Kb. The initial technology was developed with low accuracy of 70-90%

compared to illumina NGS accuracy of >99%, but increasing read accuracy was possible through read to read correction, despite being computationally intensive (Amarasinghe et al., 2020). PacBio relies on a circular DNA template SMRTbell composed of a double stranded DNA insert flanked by two single stranded hairpin adapters on both ends (Fig.1.1 B) (Logsdon et al., 2020). DNA polymerase is attached and the complex is read through a SMRT cell where DNA polymerase adds fluorescently labeled dNTPs and allows for base by base readout of the template (Logsdon et al., 2020) .

Recent development in PacBio technology allowed for enhanced accuracy (>99%) through the development of high fidelity (HiFi) reads using circular consensus sequencing (CCS) (Hon et al., 2020). The consensus sequence results from repeated passes of DNA polymerase through the template resulting in multiple error-prone subreads. Collectively, these subreads lead to a highly accurate consensus sequence with a high confidence that any detected variability is due to biological variants rather than sequencing errors (Hon et al., 2020). In addition to long read length (average of 20kb) and high accuracy (>99%), PacBio technology provides uniform coverage across the template due to elimination of the amplification step; it can also sequence through regions that are inaccessible to Illumina due to high GC content, complexity, and repetition to achieve unambiguous mapping (Logsdon et al., 2020).

1.8 Oxford Nanopore Technologies (ONT):

Oxford Nanopore Technologies uses a linear DNA molecule attached to a sequence adapter loaded with a motor protein (Fig.1. 1 C) (Logsdon et al., 2020). The motor protein feeds the DNA molecule through a nanopore embedded in a synthetic membrane, as the negatively charged DNA strand travels through the pore, individual bases cause a disruption in the current allowing calling of individual bases in real time (Logsdon et al., 2020). ONT can generate continuous reads exceeding megabases in length, surpassing PacBio read length with a wide range of base calling accuracy (Jain et al., 2018).

ONT is the leading developer of direct RNA sequencing. This is a significant advance as it eliminates the cDNA synthesis step thus reducing errors associated with the reverse transcription step and allows for direct detection of modifications such as N6-methyladenosine (m^6A) and 5-methylcytosine (5-mC) (Liu et al., 2019) which we will cover in more detail in later sections (Soneson et al., 2019). Direct RNA sequencing produced a comparable number of reads that aligned to the transcriptome when compared to Illumina (79%) and slightly less than what was recorded for the cDNA long read dataset (90%). Both cDNA and direct RNA nanopore sequencing yield reads that are similar in length (Garalde et al., 2018). In addition, direct RNA sequencing displayed less of a bias towards transcript length and GC content than Illumina thus allowing for a more uniform coverage across the transcriptome (Garalde et al., 2018). While ONT platforms like the MinION are able to produce more than one million reads per run, there were concerns initially with the higher error rate associated with it (Byrne et al., 2017). In recent years researchers have

applied many protocols and computational changes in an effort to increase accuracy. An example is the 2D sequencing protocol that involves ligating the template and the complementary strand of DNA using a hairpin; this enables both strands to pass through the pore and produce a more accurate consensus sequence (Rang et al., 2018). A more recent approach, ID^2, involves sequencing both strands without the need for physical ligation and yields a high accuracy consensus of ~97% (Rang et al., 2018). The Rolling Circle Amplification to Concatemeric Consensus (R2C2) method was developed by the Vollmers lab at UCSC leading to an increase in read accuracy as well as providing more comprehensive and quantitative analysis of RNA transcript isoforms (Volden et al., 2018). R2C2 relies on introducing 8bp splints to both ends of the reverse transcribed cDNA, only full length-cDNA is then circularized and amplified using rolling circle amplification (RCA) (Volden et al., 2018). Using this protocol and sequencing on a MinION generated more than 400,000 reads with base calling accuracy of 94%, covering the whole cDNA molecule (Volden et al., 2018). In recent studies utilizing R2C2 and enhanced computational base calling programs the accuracy is now reaching 99.45% which rivals all short-read approaches (Vollmers et al., 2021b), with a substantially enhanced ability to resolve transcript isoforms and avoid ambiguous mapping. Another advantage provided by R2C2 is the extremely low concentration input requirement (50ng); which enables accurate, high throughput sequencing from highly limited samples such as patient biopsies or blood samples.

1.9 Advantages to using long read sequencing to study Innate Immunity

While short read RNA-seq has provided us with vast new insights into gene regulation during inflammation we still do not have a complete picture of the key players and mechanisms involved in these complex processes. In this section we will outline the ways that long read technology can help expand our understanding of innate immunity, from better understanding what is being made within the genomes of immune cells, to gaining a picture of the post transcriptional regulatory changes that occur to the genes produced following inflammatory activation.

1.10 Construction of accurate and complete genomes:

While the majority of this review is covering the use of RNA-seq technologies to understand the immune system it is worth noting that we rely on the reference genome to interpret our RNA-seq data. Currently the reference genome is less than ideal for the reasons described earlier. However, there are a number of ways in which researchers have been combining short and long read data to improve accuracy of the reference genome. Short read and long read technologies have their pros and cons. Short-read data is extremely accurate with high depth while long-read data is typically less accurate with shallow depth (Fig.1.1). However, several studies now pair the less accurate long read data with the highly accurate short read data to achieve maximum base calling accuracy (Singh et al., 2019; Zhang et al., 2021). Combining the two technologies means it is possible to obtain correctly mapped genomes covering highly repetitive regions. A recent study has applied this approach to datasets from monocytes and peripheral blood mononuclear cells (PBMCs) to

characterize variations in 8 different immune system genomic loci (Zhang et al., 2021). They were able to construct a *de novo* assembly of the human leukocyte antigen, immunoglobulins, T cell receptors, and killer-cell immunoglobulin-like receptors. This study demonstrates the utility of accurate long read sequencing data in studying complex immune regulation loci and to aid in the discovery of novel structural variants in these regions which we will discuss in more depth later.

Despite continuous efforts and technological advances in NGS, gaps are still present in the latest human genome assembly GRCh38, these gaps are mainly associated with highly repetitive regions. These regions are often found around the centromeres, but they can now be resolved with the use of long accurate reads that resolve the entire region in one continuous read (Staden, 1979). Ultralong nanopore sequencing provides an unprecedented advantage of being able to sequence an entire chromosome telomere to telomere with high base calling accuracy that will enable the gaps in the latest human genome assembly GRCh38 to be filled in. In a recent study, Miga *et al.*, performed the first high-coverage ultra-long-read nanopore sequencing that resulted in the first complete assembly of the human X chromosome (Miga et al., 2020). In this study, they combined ONT ultra-long-reads with complementary technologies such as PacBio sequencing and high coverage Illumina sequencing for quality improvement and validation to sequence the hydatidiform mole CHM13 genome, which is a type of haploid organism and therefore a useful tool that has been used previously to assist with filling in gaps in diploid genomes. This approach allowed them to construct an assembly totaling 2.9Mb with half of the genome

contained in continuous sequences with a continuity that exceeds GRCh38. Through the whole genome assembly in combination with polishing techniques, they were able to manually assemble the complete, gapless X chromosome *de novo* with accuracy that exceeds 99.99%. The continuing advancements in long read sequencing and analysis pipelines open a path for constructing the complete accurate human genome. This will improve mapping accuracy for techniques that rely on mapping to the reference genome such as RNA-seq, ChIP-seq and ATAC-seq.

One clear disadvantage to the hybrid approach just described is the fact that it requires generating different libraries and using multiple sequencing platforms in order to generate a complete genome. Instead, one approach used to assemble bacterial genomes utilizes "consensus polishing" where a subset of the longest reads from long read dataset such as PacBio are utilized as input for the assembly (Loman et al., 2015). Hierarchical genome-assembly process (HGAP) assembler developed by Chin *et al.,* (Chin et al., 2013) uses the entire dataset from one library that consists of both long and shorter read fragments to correct errors in the longest reads and produce an accurate highly polished assembly. While this approach has only been utilized for bacteria so far one can imagine its usefulness in assisting with the assemblies of eukaryotic genomes (Loman et al., 2015; Chin et al., 2013).

1.11 Understanding splicing and its role in immune response regulation

Splicing is the process of intron removal from a pre-mRNA transcript to produce a mature mRNA. Alternative splicing takes a number of forms including use

of alternative start sites, alternative polyadenylation, exclusion or skipping exons, retention of introns, use of alternative start or final exons or use of alternative 5' or 3' splice sites. Alternative splicing (AS) is a highly regulated process enabling a single gene to produce multiple isoforms, thus increasing the complexity of gene function and the proteome (Boudreault et al., 2016; Ivanov and Anderson, 2013; Pai et al., 2016; Wang et al., 2015). Transcriptome profiling performed on many immune cell lineages uncovered that AS affects >60% of expressed genes and that B and T cells differed in AS events of genes that were similarly expressed in both lineages, indicating cell type specificity in the splicing process (Ergun et al., 2013). A study by *Pai et al.*, (Pai et al., 2016) used short reads NGS (SR-NGS) to study mRNA processing changes in macrophages in response to bacterial infections (*Salmonella typhimurium* and *Listeria monocytogenes*) and their impact on the overall immune response. They reported that 6-10% of genes switch their dominant isoform post infection with high enrichment for genes involved in the immune response. Interestingly, they reported that 47% of genes that displayed differential isoform usage were not differentially expressed following infection, which highlights the importance of studying isoform usage in addition to gene expression changes. They found an overall tendency to include skipped exons and to shorten 3'UTRs post infection. The shorter 3'UTRs eliminated target immune associated microRNA (miRNA) binding regions which inhibited their binding and allowed the transcript to escape repression. Their data indicates that the observed splicing changes could be carried out by heterogeneous nuclear ribonucleoproteins and serine and arginine-rich

proteins (HnRNPs hnRNPs and SRs), which belong to splicing gene families and show an increase in expression post infection, in addition to themselves being subject to alternative splicing (Pai et al., 2016).

While short read data can be useful to study isoform usage its major disadvantage is that the reads might not always capture all spliced junctions. Paired end 150bp reads are useful but long read data is a game changer for the isoform profiling field. Advances in long read sequencing allowed for the detection of novel isoforms and quantification of isoform expression under different conditions. Long reads that exceed 1kb in length eliminated the need to assemble short read data to construct isoforms, allowing for a more accurate detection of isoforms and discovery of novel ones. In order to better understand isoform expression and isoform level changes in immune cells in response to inflammatory stimuli, we utilized ONT technology combined with R2C2 to generate an isoform level transcriptome map atlas of macrophage activations (IAMA) following activation with a variety of inflammatory stimuli (Vollmers et al., 2021b). Using this method, we were able to generate 14,961,450 R2C2 reads at a median length 942nt across multiple ONT MinION flow cells. In addition, we were able to achieve an unprecedented increase in base calling accuracy from 97.9 to 99.45%. This enabled us to identify 29,637 high confidence isoforms; they included at least one isoform for 69% of the genes that are differentially expressed in any condition, and one isoform for 80% of the genes that were differentially expressed in all conditions. Of the total number of isoforms, 19%,

were novel with annotated splice sites in unannotated configurations (novel in catalog, NIC) and 7% were isoforms that use at least one unannotated splice site (novel not in catalog, NNC). This data is a reference database for researchers interested in knowing the exact isoform of their gene of interest that is expressed in a primary macrophage at baseline or following inflammatory stimulation. We find this data particularly useful for the study of lncRNAs as it can accurately map their full-length sequence without the need for laborious techniques such as 5'-3' RACE.

A combination of short and long read data is being utilized to help obtain a more accurate picture of the isoforms being produced following splicing. This was utilized by a study that performed Iso-seq (full length isoform sequencing through PacBio long read sequencing) in addition to illumina sequencing to study transcriptional diversity in whole blood samples (Shi et al., 2016). They detected 57 isoforms at 42 loci that do not overlap with any GENCODE transcripts (unannotated) and are missed by short read sequencing (Shi et al., 2016). We recently utilized both illumina short-read and ONT long-read data to study alternative splicing events in human and murine macrophages following inflammatory activation with lipopolysaccharide (LPS) (Elektra K. Robinson et al., 2021a). We showed that alternative first exon usage is the dominant splicing event, making up 50% of all events found in human and mouse macrophages following inflammatory activation. As mentioned earlier, it is known that the reference genome and current annotated transcriptome assemblies are incomplete. Therefore, we utilized the program full-

length alternative isoform analysis of RNA (FLAIR) (Tang et al., 2020; Workman et al., 2019) to combine our long-read data with our short-read data to generate a new reference transcript from which to perform our splicing analysis. This approach enabled the identification of 95 novel alternative first exon (AFE) events in response to LPS, 50% of which were not differentially expressed at the RNA level following stimulation, again highlighting the importance of considering alternative splicing as a key regulatory mechanism during an immune response. We also discovered a novel isoform of cytosolic dsDNA sensor, Aim2, that is induced by an inflammatory stimulus, where an alternative first exon is used through alternative splicing and a new transcription start site (TSS). This novel inflammatory driven isoform is myeloid specific and is shown to be less efficiently translated when compared to the canonical form because this novel isoform possesses an iron specific translational mechanism through an iron-responsive element in its 5′UTR. These studies highlight the power of combining both short and long read sequencing to understand the splicing landscape of immune cells.

Applying these approaches in disease samples could help provide much needed insights into mechanisms of dysregulation. One of the most important takeaways from all the splicing studies is that many dominant isoform changes seen following inflammation are in proteins where they are not necessarily differentially regulated at the RNA level. These can be missed if one simply focuses on the top most up or down regulated genes. Instead by studying the splice sites it is possible to

uncover disease specific isoforms of genes that could be missed by only focusing on differential expression approaches.

1.12 Identification of Structural Variation

Long-read sequencing is a reliable tool to study native and disease associated structural variations like copy number variations (CNVs), duplications, translocations and inversions (De Coster and Van Broeckhoven, 2019). These variations have been difficult to identify, due to their small size (could be as small as 50bp) leading to inaccurate mapping when using short reads. Long reads enable more accurate mapping and a better understanding of the genetic architecture of the region in healthy and disease conditions. Studies have reported on the advances achieved by using new CCS technology when compared to short read sequencing, where they sequenced the well-characterized human HG002/NA24385 genome and obtained precision and recall rates of ~99.91% for single-nucleotide variants (SNVs), 95.98% for insertions and deletions <50 bp (indels) and 95.99% for structural variants (Wenger et al., 2019). Another study that utilized a SMRT based technology to sequence HX1 was able to construct an assembly that fills 28% of gaps in the reference genome GRCh38 and discover HX-1 specific sequence that has not yet been reported (Shi et al., 2016).

1.13 Studying RNA modifications in innate immunity

1.13.1 *A-to-I editing*

A-to-I editing is a form of post-transcriptional modification that occurs in all classes of eukaryotic RNA (mRNA, tRNA, rRNA and ncRNA) and is considered the most widespread RNA modification in mammals (Nishikura, 2010). It involves the chemical change of an adenosine residue to an inosine by adenosine deaminase that acts on RNA (ADAR) (Singh, 2012), which in turn is recognized as Guanosine (G) by both translational and splicing machinery (Grammeltvedt and Berg, 1976). It serves as an essential mechanism for the immune system to differentiate between self and non-self where A-to-I editing occurs in endogenous double stranded (dsRNA) allowing it to avoid detection by the cytosolic dsRNA receptor MDA5 (Yu et al., 2015). Studies on ADAR1 knockout mice show that loss of ADAR1 results in increased expression of type I IFNs as reviewed by Wang *et al.*, (Wang et al., 2017). ADAR expression is inducible following activation with TNFA, IFNG or LPS in myoblasts (Meltzer et al., 2010), T cells and macrophages (Yang et al., 2003) indicating a possible regulatory role for A-to-I editing during the inflammatory response. When A-to-I editing occurs in coding regions it can lead to changes in protein sequence, however, A-to-I editing is most common in noncoding regions such as introns and UTRs (Yang et al., 2013), which can result in nuclear retention, degradation, alternative splicing, and translation regulation of the mRNA. Dysregulation of editing has been implicated in various inflammatory and autoimmune diseases (Gallo, 2013) (Shallev et al., 2018; Vlachogiannis et al., 2020).

Interestingly, many novel editing sites in lncRNAs have been recorded in glioblastomas (brain cancer) (Silvestris et al., 2020). LncRNAs can form secondary

folds that generate dsRNA making them substrates for ADAR editing (Yang et al., 2013). Editing of lncRNAs can result in nuclear retention or degradation acting as a negative feedback mechanism to regulate lncRNA function. Since ADAR binds to double stranded regions, it could bind a lncRNA and inhibit another RNA binding protein from forming an interaction thereby impacting lncRNA function in both an editing dependent and independent manner. Considering the role that A-to-I plays in the immune system highlights the importance of being able to detect and quantify these changes accurately. Sanger sequencing was able to detect edited sites (Ramaswami and Li, 2016) and revealed that both edited and unedited transcripts can be expressed in the same tissue, and the ratio between the two can vary by tissue type and developmental stage. Next, high throughput NGS proved to be capable of detecting A-to-I editing, however, concerns around NGS biases persisted, including ambiguous mapping, sequencing error and genomic SNPs that could lead to inaccurate identification of edits (Diroma et al., 2019). Therefore, some considerations need to be taken when designing an experiment to estimate differential editing between samples and treatments; sequencing depth and coverage are essential for accurate identification of edited sites. In addition, edits residing in repetitive elements exhibit low abundance and require ultra-high coverage for reliable detection and quantification (Lo Giudice et al., 2020). Improvement in edit identification accuracy can be achieved by increasing sequencing depth, preferentially employing protocols for strand specific-paired end read sequencing (Diroma et al., 2019). This also highlights the need for a complete and accurate reference genome that represents

the vast genetic diversity of the population to enable accurate mapping of detected edits and avoid errors due to sequencing or mapping artifacts. Combining high throughput short with long reads can be extremely beneficial for thorough A-to-I edits detection. Short reads provide the required accuracy to detect edits, while long read sequencing provides high mappability power and ability to map complex repetitive regions (Berbers et al., 2020). Nanopore direct RNA sequencing provides an added advantage by revealing the complexity of mRNA modification in full-length single molecule reads while avoiding the bias associated with PCR and reverse transcription steps (Maitra et al., 2012). In addition, selecting appropriate bioinformatics pipelines is essential for the analysis of NGS data to ensure the correct identification of edited sites, currently available analysis workflows are discussed in detail by Diroma et. al, (Diroma et al., 2019).

1.13.2 ***Methylation***

While there are ~170 chemical modifications that can occur on RNA, the most abundant internal modification in mRNA is N6-methyladenosine (M^6A) (Roundtree et al., 2017), accounting for approximately 50% of methylated ribonucleotides (Wei et al., 1975). These modifications are involved in shaping the fate of the transcript and regulating many aspects of RNA metabolism including transcription, splicing, export, translation and stability (Shi et al., 2019). Studying the importance of these modifications has created the field of "epitranscriptomics" (Boo and Kim, 2020), which has been greatly fueled by the development of NGS allowing for rapid identification and evaluation of these modifications. M^6A is a reversible modification,

which ignited interest in its dynamics and the features that help regulate it (Li et al., 2016). It was discovered that the same position might only be modified in a fraction of transcripts, serving as further indication that m^6A could possibly serve a regulatory role in many biological processes (Horowitz et al., 1984).

There are three methyltransferases, methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14) and Wilms' tumor 1-associating protein (WTAP) primarily responsible for shaping the m^6A RNA landscape by transferring a methyl group to the N-6 position of the adenosine base (Bokar et al., 1997; J. Liu et al., 2014; Xiang et al., 2014). These tags are removed by demethylases "erasers" such as alpha-ketoglutarate-dependent dioxygenase fat mass and obesity-associated protein (FTO) (Jia et al., 2011) and RNA demethylase ALKBH5 (Zheng et al., 2013).

More recently modifications and their roles in regulating lncRNAs have emerged. LncRNAs can function as decoys and scaffolds, which depend on the structure of the RNA and therefore a single modification like m^6A could improve or eradicate these RNA-protein interactions. One of the best studied lncRNAs is X-inactive specific transcript (*XIST*) whose job is to mediate silencing on the inactive X in females. Recently it has been reported that m^6A modifications within XIST are critical to its function (Patil et al., 2016). Patil *et al.*, reported that YTH domain containing 1 (YTHDC1) recognizes m^6A sites on *XIST* and is required for its function such that artificial tethering of this protein to XIST can rescue silencing in the absence of m^6A marks (Patil et al., 2016). m^6A modifications within the lncRNA

THOR are read by YTHDF1 and YTHDF2 and these interactions help stabilize the oncogenic lncRNA (H. Liu et al., 2020).

LncRNAs are also known to play an important role in the stress response; highly repetitive satellite III (HSATIII) l lncRNAs function in forming the stress bodies nSBs in response to thermal stress (Ninomiya et al., 2020). These GGAAU rich lncRNAs sequester serine and arginine rich splicing factors (SRSFs) during thermal stress to suppress splicing of hundreds of introns. In addition, it was found that methylation of the same GGAAU motifs sequesters m^6A reader proteins such as YTHDC1 to repress M^6A dependent splicing during the thermal recovery phase (Ninomiya et al., 2021). Thus, lncRNAs that constitute nSBs serve as gene regulation hubs by serving a dual function as molecular sponges for RNA splicing proteins and m^6A readers to regulate intron splicing events during thermal stress responses.

Recent studies have pointed to RNA modification involvement in immune regulation. A recent study investigated the involvement of m^6A in the inflammatory cycle of dental pulp disease (Feng et al., 2018). They demonstrated that METTL3 depletion resulted in a decrease in inflammatory cytokine expression as well as a decrease in the phosphorylation of IKKα/β, p65 and IκBα in the NF-κB signaling pathway in addition to p38, ERK and JNK in the MAPK signaling pathway in dental pulp cells when treated with LPS. This was facilitated by an increase in production of the myeloid differentiation primary response 88 (MyD88) splice variant (MyD88S). MyD88S exerts a negative effect on TLR signaling pathways and limits the duration of innate immune activation. A separate study that utilized a pooled CRISPR screen

approach showed similar results where they demonstrated that METTL3-deficient macrophages exhibited reduced TNFα production upon LPS stimulation and showed that METTL3 KO mice display susceptibility to bacterial infections and faster tumor growth (Tong et al., 2021). METTL3 depletion resulted in loss of m^6A modifications on the TLR4 negative regulator *Irakm* slowing down its degradation and resulting in suppression of TLR4 activation.

M^6A has been implicated in viral propagation and antiviral immunity by impacting transcript stability. A study showed that viral infection of cells depleted of m^6A writer METTL3 or reader YTHDF2 resulted in an induction of interferon-stimulated gene production which suppressed viral propagation (Winkler et al., 2019).

While NGS has rapidly increased the ability to study RNA modifications there are some limitations to the approaches described thus far. These include the need for the RT step that erases the modifications and renders them indistinguishable from regular RNA bases. Also, the RNA species of interest (mRNA and lncRNAs) are of low abundance which makes sequencing sensitivity a concern, in addition to limited computational tools capable of reliably distinguishing modified bases in sequencing data (Li et al., 2016). Most of the approaches to study m^6A involve use of antibodies to enable m^6A immunoprecipitation and sequencing, such as m^6A-seq and MeRIP-seq (Li et al., 2016; Dominissini et al., 2012). M^6A-seq was used to map the modification on the human and mouse transcriptome to better understand conservation and dynamic changes in response to treatments (Dominissini et al.,

2012). M^6A was shown to be most prevalent around stop codons near 3'UTRs (Meyer et al., 2012) and within long exons and that this pattern is evolutionarily conserved, pointing to a possible functional regulatory role (Dominissini et al., 2012). There are some concerns of non-specific binding of the m^6A antibodies to other methyl tags (Kane and Beemon, 1985; Zeng et al., 2018). For a review and resource on m^6A mapping we direct the readers to the following study by Xiang et. al (Dominissini et al., 2012).

1.14 Direct RNA sequencing

ONT provides the only library preparation to date to probe native RNA, without the need for an RT step, at single nucleotide resolution and with long reads. This approach is capable of detecting changes in the current as the nucleic acid travels through the pore with a sensitivity that allows for direct RNA bases calling with and without modifications (Fig.1.1, C). A variety of computational programs were developed to read the modified bases such as ELIGOS (Jenjaroenpun et al., 2021), MINES (Lorenz et al., 2020) and EpiNano (Liu et al., 2021). MINES (M^6A Identification using Nanopore Sequencing), was able to identify >40,000 m^6A sites at single base and isoform level resolution in primary human epithelial cell line (Lorenz et al., 2020). Using direct RNA sequencing and meRIP-seq revealed that absence of m^6A either by silencing METTL3 or YTHDC1 resulted in an overall decrease in late viral RNAs, viral proteins and infectious progeny of Adenovirus (Price et al., 2020).

This decrease was mainly a result of the decrease in late splicing efficiency indicating that m^6A regulates splicing of viral transcripts.

1.15 Single cell RNA sequencing technologies (scRNA-seq)

Any given cell population within the immune system whether it is macrophages, dendritic cells, neutrophils etc, are all heterogeneous populations. While bulk sequencing provides a systems level view of gene regulation within a given population of cells it fails to describe the variety of responses between individual cells following activation. Single cell RNA-seq (scRNA-seq) allows for the identification and classification of new cell types based on their gene expression profiles as well as providing insights into how cells within a population respond to a given stimulus (Papalexi and Satija, 2018; Yang et al., 2020; Shalek et al., 2013), Shalek *et al.*, produced two of the earliest single cell studies examining the responses of bone marrow derived dendritic cells (BMDCs) to toll-like receptor (TLR) ligand activation (Shalek et al., 2013; Shalek et al., 2014). They demonstrated bimodal activity in both gene expression as well as splicing in BMDCs following stimulation with lipopolysaccharide (LPS) (Shalek et al., 2013). There are a number of reasons why heterogeneity might be evident in a seemingly homogenous population of cells such as differences in cell state including cell cycle, stochastic gene expression differences to name a few. Shalek et. al, showed that there appears to be different maturity states across the BMDCs, and this is accompanied by distinct splicing patterns where on a population level many isoforms of a gene are identified, but there

is a dominant isoform expressed in one cell compared to another (Shalek et al., 2013). They also noted that distinct precocious cells exist that produce large amounts of interferon early in the immune response and could impact neighboring cells in a paracrine manner through the secretion of IFNs (Shalek et al., 2014).

ScRNA-seq has enabled the discovery of distinct classes of human dendritic cells as well as innate lymphoid cells (Breton et al., 2015; Villani et al., 2017; See et al., 2017; Björklund et al., 2016; Yu et al., 2016). ScRNA-seq has also provided insights into macrophage populations as well as their ability to fight infections including *Salmonella* (Avraham et al., 2015) (Saliba et al., 2016). Avraham *et al.*, showed that variation in host cell responses could be related to differences in bacterial factors within the invading bacteria (Avraham et al., 2015). Saliba *et al.*, took a similar approach and noted interestingly that cells harboring non proliferating bacteria are in an M1 pro-inflammatory state while cells containing proliferating bacteria are in an anti-inflammatory (M2) state suggesting the bacteria can alter polarization states of host macrophages (Saliba et al., 2016). The power of scRNA-seq provides insights into the intricacies of immune responses and how much they vary even within what was initially thought to be a homogeneous population of cells.

The human cell atlas project is a consortium wide effect involving scientists across a number of disciplines coming together to map all the cells of the human body (Regev et al., 2017). They will utilize scRNA-seq in addition to techniques such as Mass Cytometry, epigenome sequencing and in situ approaches to provide a complete

picture of the active molecular pathways present in healthy cells. The hope is that by understanding what is occurring in healthy cells and tissues it will provide the framework needed to understand what goes wrong during a diseased state. There are similar efforts underway in Europe by the Lifetime initiative that are utilizing single cell approaches to better understand complex diseases as well as trying to dissect individual cells' response to treatment (Rajewsky et al., 2020).

While these large consortium efforts are exciting and will undoubtedly provide us with enormous amounts of data, there are however limitations to this technology. It is costly and this limits the number of single cells you can study at a given time. This technology is rapidly evolving and with that cost will continue to go down. Kasmia *et al.*, provide a review that covers the scRNA-seq pipelines, as well as the pros and cons of the various techniques (Kashima et al., 2020). 10X genomics is one the most common scRNA-seq pipelines and involves mapping the 5’ or 3’ ends using polyA transcripts and so this approach only gives a glimpse into gene expression and not any information on the whole body of the gene. It lacks isoform and sequence variation information and also fails to capture genes that are not polyadenylated. The average number of reads from any given cell from a chromium platform averages ~10,000 reads, therefore one only gets to study the most abundant genes expressed in a given cell. There are platforms available for in depth analysis of single cells that are capable of getting up to 1 million reads such as the C1 platform, however, this greatly limits the number of cells that can be studied in a single experiment (Kashima et al., 2020). New library prep methods including R2C2 which

was mentioned earlier can be utilized to generate single cell libraries that can be sequenced either using illumina or nanopore sequencing. Volden *et al.*, profiled 3000 peripheral immune cells using R2C2 and were capable of clustering them into their cell types (T, B cell, monocytes etc.) based on their gene expression profiles (Volden and Vollmers, 2020). This is a powerful advance to single cell sequencing as it provides isoform level transcriptomes in addition to gene expression profiles of immune cells. Another disadvantage of all the sequencing technologies discussed thus far is that they require lysing of cells to extract RNA and therefore only provide a snapshot of what was happening at the time the cells were lysed. Live-seq is a new innovative approach designed to allow for sampling of single cells during a live immune response (Chen et al., 2021). It can act as a recorder and allow researchers to evaluate the immune response in the same cell overtime. This technology was used to study macrophages sampled both at baseline and following LPS stimulation over time. They concluded that baseline levels of the protein NFKBIA (IκBα) NFKBIA and cell cycle state as the major determinants of the observed phenotypic changes (Chen et al., 2021). As with all the technologies mentioned here scRNA-seq will undoubtedly continue to develop and become cheaper which will allow for more widespread use. With this will come more information which will be useful in deciphering the complex immune responses that occur in healthy and diseased cells.

1.16 High-throughput Functional Characterization of genes: CRISPR

It is clear that deep sequencing approaches provide us with this unprecedented view of what is being produced from the genome, but it fails to provide insights into the biological relevance of all the transcription that is occurring. In order to make the most of all the emerging genomic sequencing it is necessary to establish rapid functional characterization pipelines. The development of CRISPR-Cas9 has helped revolutionize the field of functional genomics by offering a tool from which we can rapidly functionally characterize genes in our systems of interest. CRISPR/Cas9 is a deoxyribose nuclease (DNase) that can be specifically targeted to genomic regions *via* a guide RNA (gRNA) (Hochstrasser and Doudna, 2015; Sternberg and Doudna, 2015). Classical use of CRISPR in its enzymatically active form is ideal for removing protein coding genes and determining a phenotype. Targeting of Cas9 to such region results in a blunt double-stranded DNA break that is repaired by the imprecise Non-Homologous End-Joining (NHEJ) DNA repair pathway, leading to small deletions or insertions that disrupt the open reading frame and therefore result in loss of the protein. The simplicity of the guide RNA cloning system makes it amenable to high throughput approaches meaning that hundreds to thousands of proteins can be studied in a pooled fashion in any biological context. This is an attractive pairing to high throughput screening in which you identify all the interesting proteins that are turned on or off in your system of choice and now you want to know which of those proteins are actually important in your biology of choice. This has been utilized by a number of groups to try and better understand the genes involved in the immune system.

Genome wide screens have been performed to identify new regulators of TNF, TLR3 signaling as well as the NLRP3 inflammasome (Parnas et al., 2015; Zablocki-Thomas et al., 2020; Schmid-Burgk et al., 2016). There have been many pooled CRISPR screens performed to try and better understand host-viral interactions which is reviewed in (Krey et al., 2020). Perturb-seq is an approach in which CRISPR screening is combined with single cell sequencing readouts allowing for both target identification and mechanistic insights in one experiment. Dixit *et al.*, used this approach to target 24 transcription factors in bone marrow derived dendritic cells and reconstruct the complex interplay between positive and negative regulators within the LPS signaling pathway (Dixit et al., 2016).

We recently performed a pooled high throughput screen in macrophages where we targeted all annotated protein coding genes, microRNAs as well as targeting 3'UTRs of known essential genes. In addition to the inflammatory screen we performed a viability screen which identified all genes required for viability including macrophage specific viability genes such as IRF8 (Covarrubias et al., 2020). We also provided insights into new regulatory elements present in the 3'UTRs of essential genes. We made use of our recently developed NF-kB-GFP reporter system (Covarrubias et al., 2017) and identified 115 novel regulators of NF-kB as well as showing that TNF can act as a negative regulator of the pathway in a cell intrinsic manner (Covarrubias et al., 2020). The majority of the pooled based screens have been performed in cell lines, but the technology is now being utilized to move towards *in vivo* screening as well as screening primary human cells. Lafleur et. al,

have developed CHIME: CHimeric IMmune Editing using CRISPR in the bone marrow to study gene expression *in vivo* (LaFleur et al., 2019). They performed a pooled *in vivo* screen targeting 21 genes (using 110 sgRNAs) specific for T cell biology and identified the Protein Tyrosine Phosphatase Non-Receptor Type 2 (*Ptpn2*) as a negative regulator of CD8+ T cell-mediated responses to LCMV infection.

The Marson lab at UCSF have been pioneering ways to utilize high throughput CRISPR approaches to knockout or knockin genes in primary human T cells with the view to gaining insights into the molecular mechanisms in healthy and diseased T as well as developing tools for therapeutics use in the future (Schumann et al., 2020; Roth et al., 2020). They have shown how amenable their tools are to other immune cells by demonstrating their ability to knockout genes in primary CD14 monocytes, cells that are typically genetically intractable (Hiatt et al., 2021).

While enzymatically active Cas9 is powerful for targeting proteins, it is not so easy to employ to interrogate the function of lncRNAs that do not contain ORFs. Instead CRISPRi has been effectively utilized to target non-coding regions of the genome. CRISPRi involves a catalytically inactivated version of Cas9 fused to the KRAB (Krüppel associated box) chromatin-silencing domain which when targeted to the transcription start of a gene induces heterochromatin formation and silencing (Larson et al., 2013; Ying et al., 2015).

There are many versions of the CRISPRi system that have been used to study lncRNAs and they have been reviewed here (Phelan and Staudt, 2020). More recently the system has been utilized to perform high throughput screens to rapidly determine which lncRNAs are important for viability. Liu *et al.*, (Liu et al., 2017), employed a CRISPRi platform targeting 16,401 lncRNAs in seven different cell lines including human transformed and induced pluripotent stem cells (iPSC) lines. They identified 499 lncRNAs required for cellular growth with cell type specificity, confirming that lncRNAs serve cell type specific functions (Liu et al., 2017). However, it is important to note that there are some technical challenges to the CRISPRi system. Gilbert *et al.*, showed that there is an ideal guide RNA targeting window of -500 to +500 nucleotides surrounding the transcription start site (Gilbert et al., 2013). Therefore, in order to design a library to target all lncRNAs or protein coding genes, it is essential to know exactly where the start sites are. It is well appreciated that lncRNAs in particular are poorly annotated and even in our limited experience using screening tools we have found that many proteins are also incorrectly annotated (Uszczynska-Ratajczak et al., 2018; Salzberg, 2019; Boettcher et al., 2019). Some of this comes down to the nature of the reference genome utilized for the design of the guide RNAs. Since many lncRNAs are cell type specific and even protein coding genes can have alternative start sites that are cell specific (Bertomeu et al., 2018; Reyes and Huber, 2018), it means that it is necessary to obtain sequencing data from your cell type of interest prior to designing a library.

While there are clearly many advantages to the use of CRISPR for functional genetics from the speed at which it can be carried out into the vast number of genes that can be interrogated at once, these are expensive and time-consuming approaches. Screens only work if the read out is amenable to a high throughput system which means these approaches are somewhat limited in the scope of biology, they can provide insights to. However, these functional technologies are continuing to evolve. We need continued innovation in high throughput functional assays if we wish to make sense of all the sequencing data that is being generated.

1.17 Combining CRISPR with long read sequencing

This is an incredibly powerful approach for studying disease causing mutations. A recent study used CRISPR-Cas9 to cut out the known oncogenes BRCA1 and 2 and combined this with long read sequencing to identify new structural variants (Walsh et al., 2020). They studied a family with a history of breast cancer that had negative results for mutations by traditional sequencing methods including whole exome sequencing. They identified a retrotransposon insertion which resulted in the formation of a pseudoexon in the *BRCA1* message and introduced a premature truncation (Walsh et al., 2020). There are many inflammatory diseases for which whole exome sequencing has produced underwhelming results. Perhaps if instead, we focused on possible disease associated genes and sequenced them in individual

patients using this CRISPR-long read approach we might uncover the mechanism at play.

1.18 Conclusions and future directions

Since there are so many sequencing techniques and platforms available, it is important to decide exactly what question you want to answer for any given experiment. It is also important to recognise and utilize the wealth of data that comes from any of these experiments. Many RNA-seq experiments are undertaken to perform differential expression analysis and while this technique is indeed useful for this it is also incredibly expensive and there is so much more you can obtain from the data. These experiments capture not just protein coding genes but also many noncoding RNAs. LncRNAs are known to be more cell type specific in their expression levels compared to proteins so if someone is interested in looking for unique signatures in diseased versus healthy states it is worth analyzing the data for lncRNAs as well as proteins. As mentioned, splicing is a key regulatory mechanism in any immune response, and it also changes in diseased conditions. Many isoforms being used after inflammation are in genes that don't show differential expression levels, instead they display isoform switching and this should not be overlooked especially when studying diseased conditions. We feel that the future lies with long read technology as it offers a wealth of information from isoform identification to RNA modifications. The major focus to date has been only on m^6A, but there remain 169 other modifications that have not been examined to any great extent. A recent consortium has been established and is led by Prof. Angela Brooks called The Long-

read RNA-seq Genome Annotation Assessment Project (LRGASP). They have tasked researchers with comparing library preparation protocols as well as computational approaches in order to help set a standard for long read data capture experiments (https://www.gencodegenes.org/pages/LRGASP/). Long read data will provide a much clearer picture of the splicing and modification landscapes that will allow us to better appreciate disease specific isoforms or modified genes and could allow for more targeted approaches for therapeutic intervention for inflammatory and autoimmune conditions.

1.19 Acknowledgements

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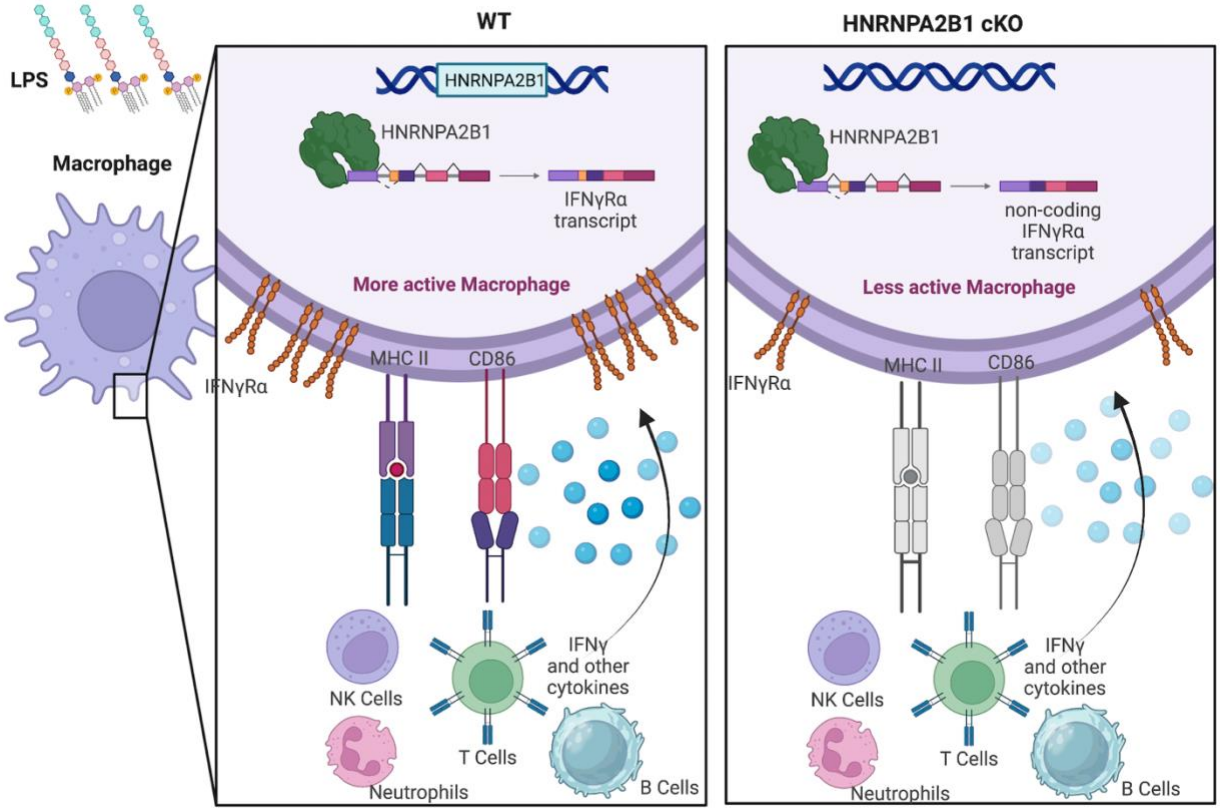
CHAPTER 2- The RNA binding protein, HNRNPA2B1, regulates IFNG signaling in macrophages.

2.1 Summary

Heterogeneous nuclear ribonucleoprotein A2B1 (HNRNPA2B1) is a well known RNA binding protein but the mechanisms by which it contributes to innate immune gene regulation are poorly understood. Here we report that HNRNPA2B1 functions in macrophages to regulate IFNG (IFN-γ) signaling through alternative splicing of the IFNG receptor. Specific deletion of HNRNPA2B1 in macrophages resulted in altered cytokine responses in both an endotoxic shock model and following *Salmonella* infection. Interestingly, while HNRNPA2B1 can function as a viability gene, we observed increased macrophage and neutrophil numbers in the KO mice following LPS induced endotoxic shock. We also discovered that HNRNPA2B1 restricts replication of *Salmonella enterica in vivo*. Mechanistically, loss of HNRNPA2B1 resulted in an increase in NGO transcripts, which lack a start codon, of the IFNG receptor (*Ifngr*) leading to lower expression of the receptor at the cell surface impacting the downstream IFNG signaling cascade. Collectively, our data highlight an important role for HNRNPA2B1 in regulating IFNG signaling and restricting intracellular bacterial pathogens in macrophages.



2.2 Introduction

Inflammation is the host's natural response to protect against infection and maintain homeostasis. Macrophages are crucial components of the innate immune system required for mediating the magnitude and outcome of the inflammatory response. By illuminating the mechanisms through which macrophages regulate this response, we will be better equipped to combat infection and inflammatory diseases. Macrophages sense danger signals through PRRs (Franken et al., 2016; Hirayama et al., 2017) -such as TLR4- which recognizes lipopolysaccharide (LPS) triggering complex signaling cascades culminating in the production of pro-inflammatory

cytokines (Franken et al., 2016; Swanson et al., 2020; Tucureanu et al., 2018; Shaughnessy and Swanson, 2007). Due to the complex nature of these responses, there are regulatory steps throughout including at the level of transcription, pre-mRNA splicing, RNA modification, RNA export, and translation (Mohammed Salih and Carpenter, 2022). In recent years a small number of studies have been carried out to investigate the role that HNRNP proteins play in regulating innate immune responses. It is well documented that HNRNPs represent ubiquitously expressed proteins critical for RNA processing and yet some members of the family play highly specific roles in certain cell types (Geuens et al., 2016). HNRNPM regulates gene expression through repression of splicing in macrophages. Repression is only relieved on target genes such as *Il6* following phosphorylation of HNRNPM (West et al., 2019). HNRNPU has been reported to translocate from the nucleus to the cytosol following LPS stimulation in macrophages leading to the stabilization of target mRNA including *Tnf* and *Il6* (Zhao et al., 2012). HNRNPA0 has also been implicated in regulating mRNA within the cytosol of macrophages following LPS stimulation, by interacting with AU rich elements in target genes such as TNF influencing their expression (Rousseau, 2002).

HNRNPA2B1 is a highly conserved, abundant member of the HNRNPA/B subfamily which is involved in all aspects of RNA metabolism from biogenesis to degradation (e.g. processing and splicing, trafficking, mRNA translation and stability) (Lu et al., 2022; Thibault et al., 2021). HNRNPA2B1 is localized to the nucleus (Thibault et al., 2021; Zhang et al., 2019) allowing it to mediate gene expression

regulation through its control over transcription initiation (Barrandon et al., 2007; Carpenter et al., 2013b; Guha et al., 2009), alternative splicing (Clarke et al., 2021; Cáceres et al., 1994; van der Houven van Oordt et al., 2000; Fang et al., 2017; Peng et al., 2021; Makhafola et al., 2020; Clower et al., 2010; Guo et al., 2013) and RNA export (Ryan et al., 2021; Smith et al., 2014; Villarroya-Beltri et al., 2013). HNRNPA2B1 plays a central role in RNA metabolism and dysregulation and mutations in this protein are associated with a range of metabolic and neurodegenerative diseases as well as cancers (Clarke et al., 2021; Cui et al., 2010; Zech et al., 2006; Berson et al., 2012; Jia et al., 2022). Recently HNRNPA2B1 has been reported to function as a DNA sensor within the nucleus facilitating interferon signaling downstream of HSV-1 infection (Wang et al., 2019). Importantly, loss of tolerance to HNRNPA2B1 is recognized as a hallmark of several systemic autoimmune rheumatic diseases (SARDs) such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Maslyanskiy et al., 2014; Isenberg et al., 1994; Hassfeld et al., 1995; Steiner et al., 1996; Hassfeld et al., 1993).

While our group and others have previously shown that HNRNPA2B1 can play a role in regulating the innate immune response downstream of TLR signaling *ex vivo* (Carpenter et al., 2013b; Meng et al., 2023), we do not understand the mechanisms by which HNRNPA2B1 regulates gene expression *in vivo*. To answer this question, we generated a conditional mouse and depleted HNRNPA2B1 in myeloid cells by crossing to *LysM*Cre. RNA-seq was carried out following LPS stimulation comparing CTL ($HNRNPA2B1^{fl/fl}$) and KO ($HNRNPA2B1^{fl/fl}$

*LysM*Cre$^{+/+}$) macrophages. While many genes were altered in expression at baseline and following stimulation in the knockout, one of the most impacted signaling cascades was that of interferon gamma (IFNG) with reduced expression of key components from receptors (IFNGR) to signaling components (STATs), as well as transcription factors (IRFs). Interestingly the dampened IFNG response resulted in mice being less responsive to LPS induced endotoxic shock but more susceptible to *Salmonella* infection, wherein loss of HNRNPA2B1 led to uncontrolled bacterial replication. Mechanistically, loss of HNRNPA2B1 resulted in an increase in NGO transcripts of the IFNG receptor1 (*IfngrI*) leading to lower expression of the receptor at the cell surface impacting the entire downstream IFNG signaling cascade. Collectively our work provides insights into HNRNPA2B1 as a specific regulator of the IFNG signaling cascade in macrophages.

2.3 Results

2.3.1 The immune cell repertoires of HNRNPA2B1 knockout mice are phenotypically normal at steady state.

Due to HNRNPA2B1's wide ranging roles in RNA metabolism it is considered a viability gene, consistent with our failed attempts to generate a full body knockout of HNRNPA2B1. Therefore, to investigate HNRNPA2B1 function in innate immune cells, we generated a HNRNPA2B1 conditional knockout (KO) mouse using a *LysM*Cre system (Fig.2.1, A) (SFig. 2.1). We inserted two loxP sites flanking exons 2 and 7 of the HNRNPA2B1 locus using CRISPR and crossed to *LysM*Cre to

homozygosity to selectively delete HNRNPA2B1 from myeloid cells. Western blot analysis confirmed complete knockout of HNRNPA2B1 in the deficient bone marrow derived macrophages (BMDMs) (Fig.2.1, B). Mice appeared normal and bred at expected mendelian ratios. Immune populations were profiled at baseline in blood and spleen and no difference was observed in immune cell numbers for monocytes, macrophages, eosinophils, T or B cells (Fig.2.1, C and D). A small increase in neutrophils was recorded in the spleen (Fig.2.1D). HNRNPA2B1 deletion impaired macrophage *ex vivo* proliferation at day 7 post differentiation consistent with our previous CRISPR screen findings showing it acting as a viability gene in macrophages *ex vivo* (Covarrubias et al., 2020), however, it did not impact macrophage phagocytic function as measured by pHrodo Green *E. coli* particle uptake (Fig.2.1, E and F).

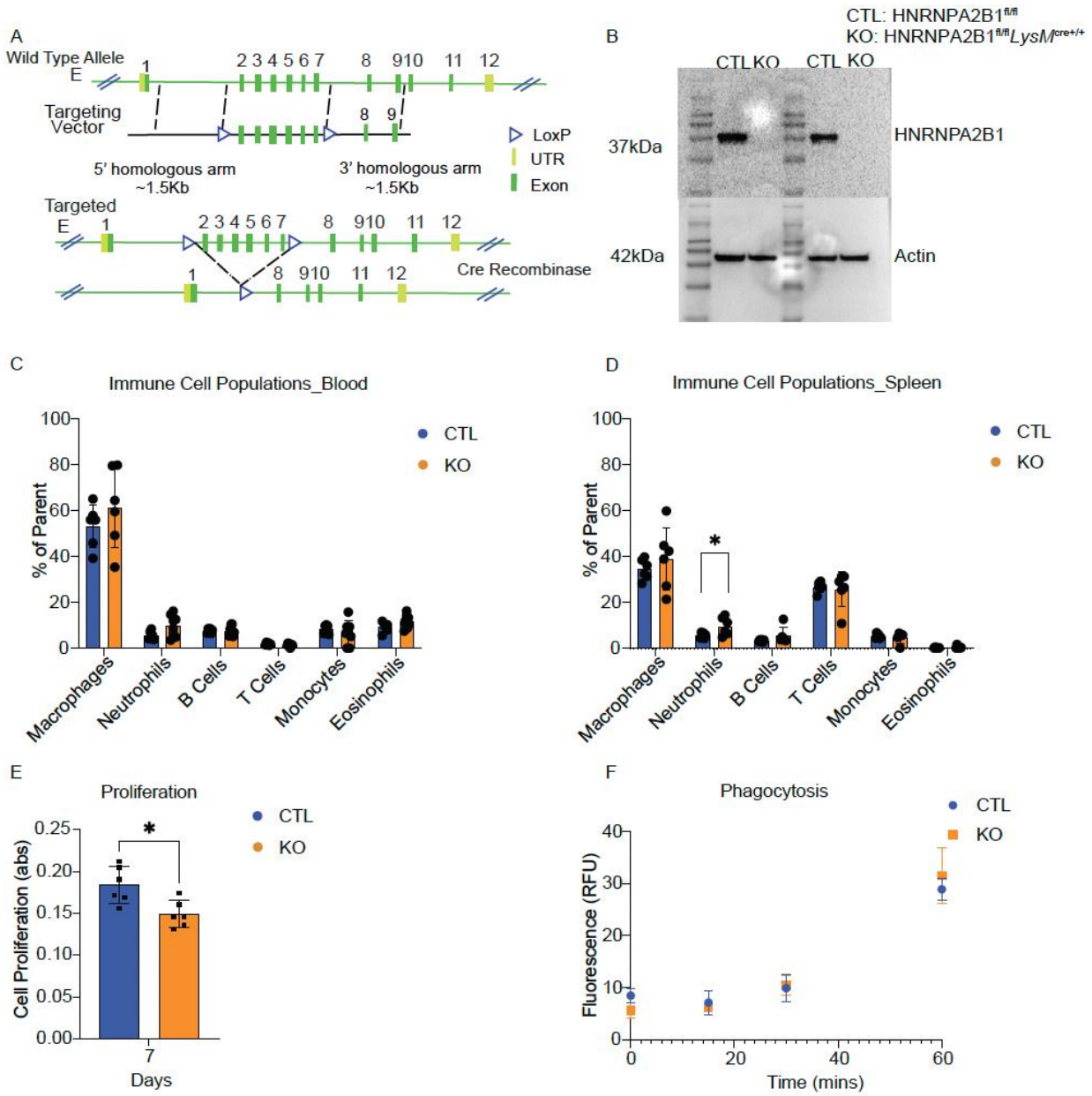
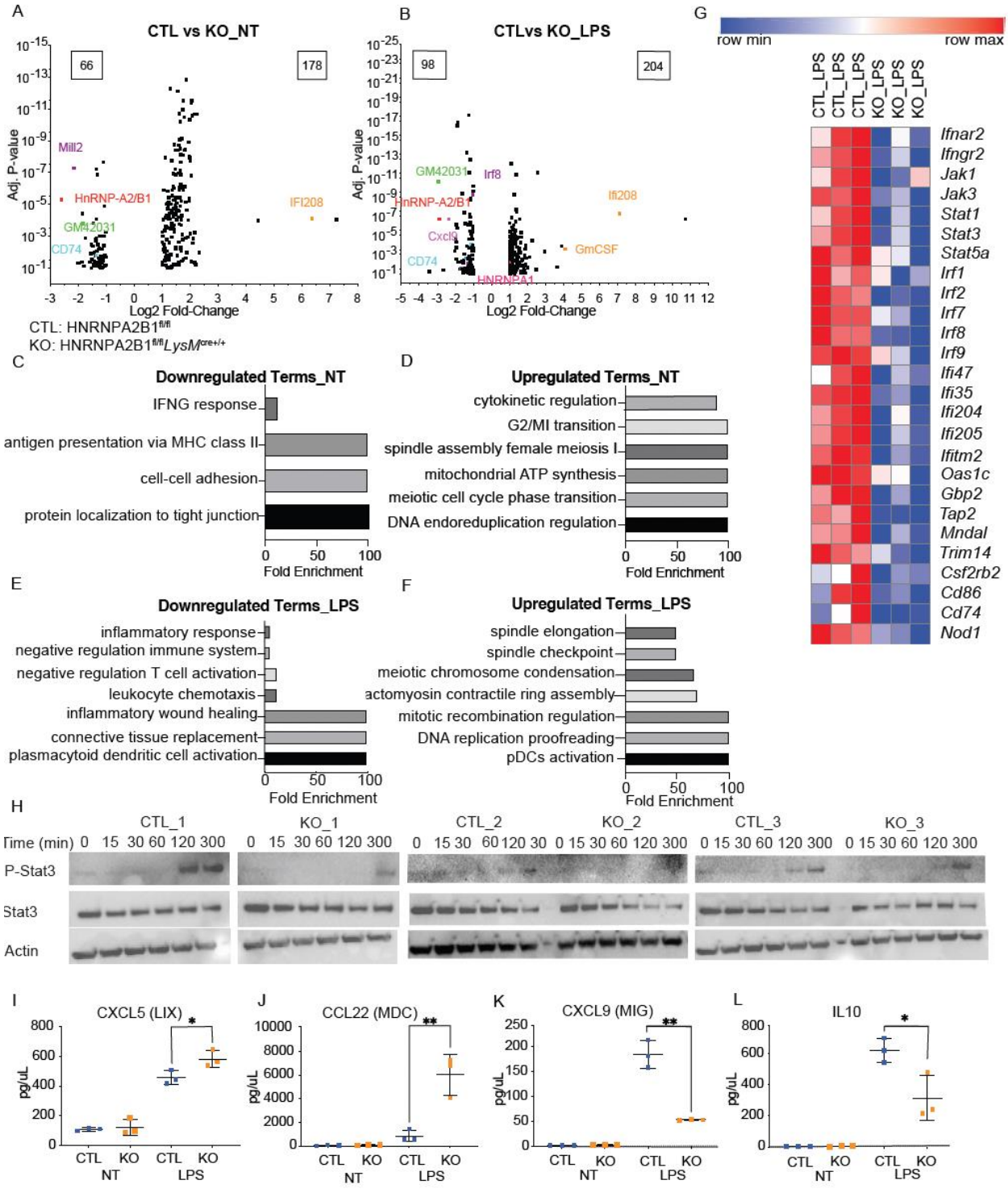


Figure2.1 Generation and characterization of the HNRNPA2B1 conditional knockout mouse. A. CRISPR was used to add loxP sites on the 5' and 3' ends around exons 2 and 7 of the HNRNPA2B1 locus. **B.** Western blot analysis of HNRNPA2B1 levels in murine BMDMs. **C.** Profiling of the immune cell repertoire in the blood of HNRNPA2B1 KO mice. **D.** Profiling of the immune cell repertoire in the spleen of HNRNPA2B1 KO mice. **E.** MTT proliferation assay was performed to assess BMDM proliferation rate in HNRNPA2B1 KO BMDMs. **F.** PHrodo green *E. coli* bioparticle assay was used to assess BMDM phagocytic function.

2.3.2 IFN signaling cascades are downregulated in HNRNPA2B1 KO macrophages in response to LPS.

In order to determine what role HNRNPA2B1 plays in regulating gene expression in macrophages we first performed RNA sequencing on KO BMDMs compared to CTL at baseline and following 5 h of LPS stimulation. Differential expression analysis (DESeq2) revealed a large number of upregulated (178) and downregulated (66) genes in macrophages at baseline, as well as 204 upregulated and 98 downregulated genes under inflammatory conditions when HNRNPA2B1 is knocked out (Fig.2.2, A and B). Gene ontology (GO-term) analysis of differentially expressed genes revealed that HNRNPA2B1 loss led to an overall downregulation of inflammatory response genes such as *Cd74* and *Mill2* at baseline, as well as genes such as *Irf8,* and *Cxcl9* after LPS treatment (Fig.2.2, A, B, C and E). Upregulated GO-terms at baseline consisted mostly of genes involved in cell cycle and cell division regulation as well as DNA replication (Fig.2.2, D and F). Among a large number of downregulated genes in LPS-treated BMDMs, there was a strong enrichment of interferon response genes, specifically those involved in the IFNG (IFN-γ) response signaling pathway such as *IfngrII, Gbp2* and *Cd74* (Fig.2.2, G). Using RT-qPCR, we confirmed lower expression levels in KO BMDMs of several major IFN response genes (*Irf7, Irf8, Stat3,* and *Oas1c*) and an increase in *Ifi208* (SFig.2.2, A-E). To determine the impact these transcriptional changes, have on the downstream IFNG cascade we utilized western blot analysis to assess changes in phosphorylation of STAT3, which is a major transcription factor (TF)

in the IFNG signaling pathway. Our results revealed that phosphorylated STAT3 (pSTAT3) was activated 2 h and 5 h post LPS stimulation in CTL mice, but this was significantly reduced in the KO BMDMs (Fig.2.2, H). Total STAT3 was similar between CTL and KOs (Fig.2.2, H). Using multiplex ELISA, we measured altered expression for many key inflammatory cytokines in HNRNPA2B1 KO BMDMs, with some showing increased expression (CXCL5, CCL22, CSF1, CSF3, CCL17, IL1B, IL5, IL12, and IL15), others showing decreased expression (e.g. CXCL9, IL10, and TIMP1) post-stimulation (Fig.2.2, I-L, SFig.2.3, A-H), and a number of proteins remained unchanged (SFig.2.3, I-M). Collectively, our data indicate that in macrophages LPS induced cytokine responses are altered when HNRNPA2B1 is removed and many components of the IFN signaling pathway are downregulated.


A
CTL vs KO_NT
66
178
Mll2
HnRNP-A2/B1
GM42031
CD74
IFI208
Adj. P-value
Log2 Fold-Change
B
CTLvs KO_LPS
98
204
GM42031
Irf8
HnRNP-A2/B1
CxcI9
CD74
HNRNPA1
Ifi208
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Ifnar2
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Jak1
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Stat1
Stat3
Stat5a
Irf1
Irf2
Irf7
Irf8
Irf9
Ifi47
Ifi35
Ifi204
Ifi205
Ifitm2
Oas1c
Gbp2
Tap2
Mndal
Trim14
Csf2rb2
Cd86
Cd74
Nod1
C
Downregulated Terms_NT
IFNG response
antigen presentation via MHC class II
cell-cell adhesion
protein localization to tight junction
Fold Enrichment
D
Upregulated Terms_NT
cytokinetic regulation
G2/MI transition
spindle assembly female meiosis I
mitochondrial ATP synthesis
meiotic cell cycle phase transition
DNA endoreduplication regulation
Fold Enrichment
E
Downregulated Terms_LPS
inflammatory response
negative regulation immune system
negative regulation T cell activation
leukocyte chemotaxis
inflammatory wound healing
connective tissue replacement
plasmacytoid dendritic cell activation
Fold Enrichment
F
Upregulated Terms_LPS
spindle elongation
spindle checkpoint
meiotic chromosome condensation
actomyosin contractile ring assembly
mitotic recombination regulation
DNA replication proofreading
pDCs activation
Fold Enrichment
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Actin
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CXCL5 (LIX)
pg/uL
CTL KO CTL KO
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CCL22 (MDC)
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IL10
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Figure 2.2 HNRNPA2B1 KO macrophages have altered IFN signaling in response to LPS. A. Volcano plot of differentially expressed genes in HNRNPA2B1 KO BMDMs at baseline. **B.** Volcano plot of differentially expressed genes in HNRNPA2B1 KO BMDMs under LPS stimulation. **C.** Gene ontology analysis of downregulated genes at baseline.
D. Gene ontology analysis of upregulated genes at baseline. **E.** Gene ontology analysis of downregulated genes post LPS stimulation. **F.** Gene ontology analysis of upregulated genes post LPS stimulation. **G**. Heat map of IFNG response genes normalized counts in CTL and KO cells post LPS stimulation. **H.** Western blot analysis of pSTAT3 and STAT3 levels in HNRNPA2B1 KO BMDMs under stimulus (right panel). **I-L.** Cytokine levels as measured by ELISA from BMDM supernatant, the supernatant was harvested from CTL and KO cultured BMDMs and multiplex cytokine analysis was performed for (I) CXCL5 (LIX), (J) CCL22 (MDC), (K) CXCL9 (MIG), (L) IL10. Each dot represents BMDMs from an individual animal. Error bars represent the standard deviation of biological triplicates. Student's t-tests were performed using GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines (*$P \leq 0.05$, **$P \leq 0.01$).

2.3.3 HNRNPA2B1 KO mice display altered immune responses following endotoxic shock *in vivo*.

Next, we investigated whether the observed disruption in macrophage responses would lead to altered responses to endotoxic shock *in vivo* in the KO mice. One early clinical feature of endotoxic shock in mice is the rapid decrease in body temperature. Here we recorded an average temperature of 25ºC for the CTL mice while the HNRNPA2B1 KO mice were at ~32ºC following 5 mg/kg intraperitoneal injection of LPS for 18 h (Fig.2.3, A). In addition, cytokine analysis of serum, spleen and liver revealed an impaired cytokine production represented by the lower inflammatory cytokine levels in the KO mice (Fig.2.3, B-N). Most importantly we observed reduced levels of IFNG in the serum of KO mice (Fig.2.3, B) consistent with what we observed in the macrophage *ex vivo* experiment in Fig. 2. Some impaired cytokines were common in serum and spleen (e.g. CCL2 and CCL3) (Fig.2.3, C, D, H and I), while

others were reduced in serum only (IFNG, CSF1 and CCL5) (Fig.2.3, B, E and F) or were restricted to the spleen (IL6, CSF2 and CXCL1) (Fig.2.3, G, J and K). Only IL20 was reduced in the KO livers compared to CTL while VEGF and TIMP1 were increased (Fig.2.3, M, and N). From these data we can conclude that specific loss of HNRNPA2B1 from myeloid cells resulted in significant changes in the pro-inflammatory cytokine levels throughout the tissues of the knockout mice indicating that HNRNPA2B1 plays an important and specific role in regulating gene expression in these cells.

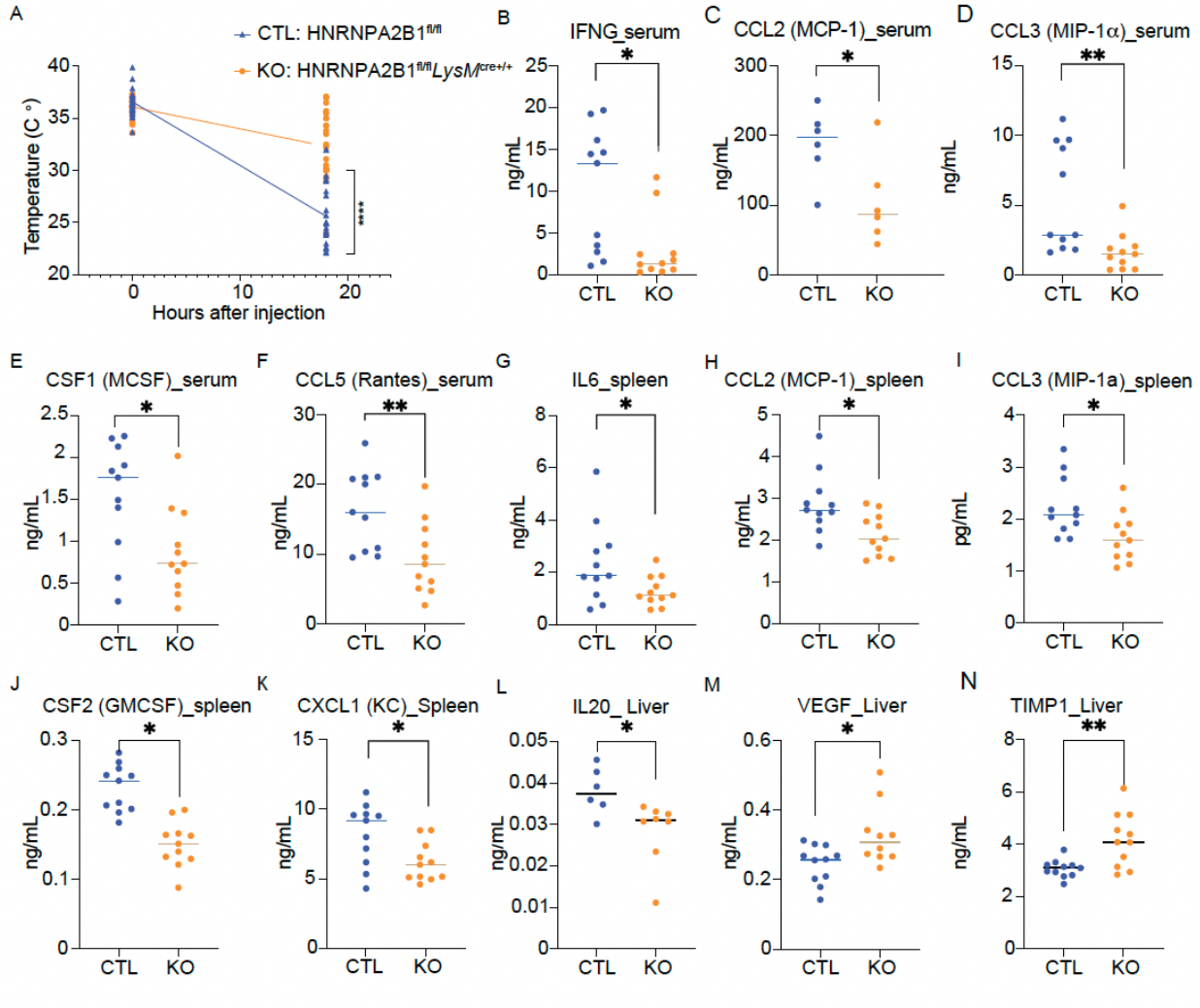

Figure 2.3 HNRNPA2B1 KO mice show altered immune responses following endotoxic shock *in vivo*. A. Temperature change in CTL and HNRNPA2B1 KO mice after i.p. Injection with 5 mg/kg LPS. N= 25 CTL, 26 KO. **B-N.** Cytokine levels in serum, spleen and liver of mice treated with 5 mg/kg LPS 6 hrs post injection. **(B)** IFNG_serum, **(C)** CCL2_serum, **(D)** CCL3_serum, **(E)** CSF1_serum, **(F)** CCL5_serum, **(G)** IL6_spleen, **(H)** CCL2_spleen, **(I)** CCL3_spleen, **(J)** CSF2_spleen, **(K)** CXCL1_spleen, **(L)** IL20_Liver, **(M)** VEGF_Liver, **(N)** TIMP1_Liver. Student's t-tests were performed using GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines (*P ≤ 0.05, **P ≤ 0.01).

2.3.4 Macrophage and neutrophil numbers are elevated in the HNRNPA2B1 KO mice and macrophages display altered costimulatory molecule expression.

Since HNRNPA2B1 is a known viability gene we speculated that the downregulated responses could be simply due to less macrophages and neutrophils in the KO mice. Interestingly we found the opposite to be true with the KOs showing increased macrophage and neutrophil counts in the spleen and blood following endotoxic shock (Fig.2.4, A-D). There was no difference in other immune cells such as B cells, monocytes and eosinophils in the KO mouse, except for T cells that displayed a small increase in KO mouse spleen (SFig.2.4, A-H). We also investigated whether the observed elevation in macrophage numbers was extended to other macrophage subsets such as peritoneal macrophages (PMs) since they are directly exposed to LPS upon injection, but we observed no changes in PM levels post LPS introduction (SFig.2.4, I and J).

Macrophage activation is an important indicator of macrophage function and ability to respond to challenges. Thus, we assessed macrophage activation markers and found MHC Class II expression to be lower in the spleen, which we confirmed in macrophages *ex vivo* (Fig.2.4, E and F). In addition, we found CD86 levels to be reduced while CD80 levels are higher in BMDMs following LPS stimulation (Fig.2.4, G and H). Thus, HNRNPA2B1 KO macrophages are more abundant in LPS-exposed mice, however, they are less proinflammatory and display attenuated responses following inflammatory activation with LPS.

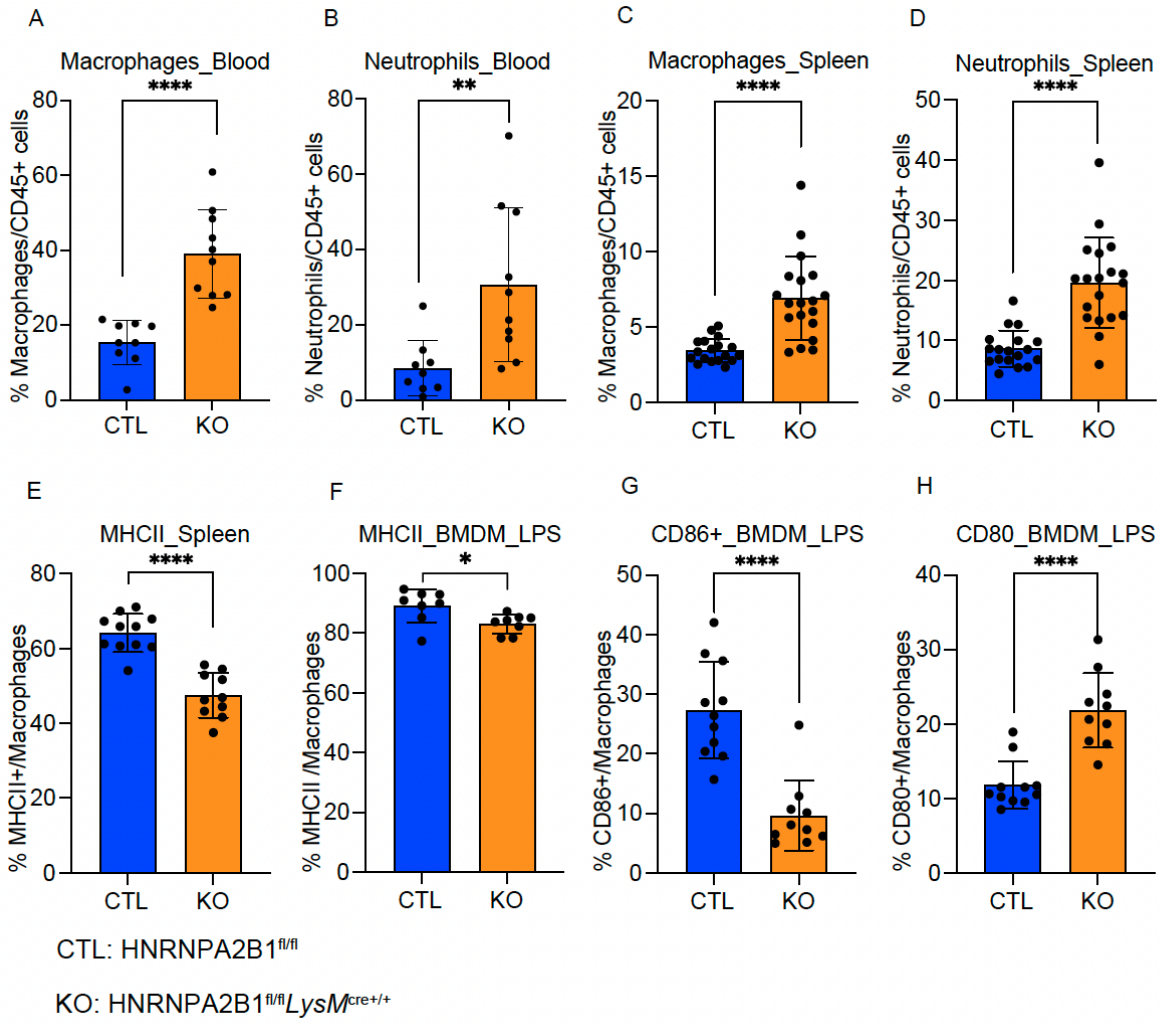

Figure 2.4 Macrophage and neutrophil levels are elevated in the HNRNPA2B1 KO mice and show altered macrophage activation following endotoxic shock. A. Macrophage levels from CTL and HNRNPA2B1 KO mice blood, measured using a flow cytometry panel. **B.** Neutrophil levels from CTL and HNRNPA2B1 KO mice blood, measured using a flow cytometry panel. **C.** Macrophage levels from CTL and HNRNPA2B1 KO mice spleen, measured using a flow cytometry panel. **D.** Neutrophil levels from CTL and HNRNPA2B1 KO mice spleen, measured using a flow cytometry panel. **E.** Level of MHCII marker on macrophage surface in the spleen, analysis performed using flow cytometry. **F-H** Level of activation markers on BMDMs *ex vivo* , analysis performed using flow cytometry **(F)** MHCII, **(G)** CD86, **(I)** CD80. Student's t-tests were performed using GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines (*$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$).

2.3.5 HNRNPA2B1 regulates IFNG signaling through alternative splicing.

HNRNPA2B1 is a well-recognized regulator of alternative splicing (Peng et al., 2021; Makhafola et al., 2020; Clower et al., 2010; Guo et al., 2013). Having confirmed strict nuclear localization of HNRNPA2B1 under a variety of inflammatory stimuli (Fig.2.5, A) (SFig.2.5, A), we hypothesized that it regulates interferon response genes through alternative splicing. We employed Isoform Usage Two-step Analysis (IUTA) (Niu et al., 2014) to detect differential usage of gene isoforms in the KO BMDMs after LPS treatment. We found that only a small portion of the downregulated (DE) genes were alternatively spliced (213 out of 1389) in the KO macrophage. Likewise, only 43 out of 1024 upregulated DE genes were alternatively spliced in HNRNPA2B1 KO macrophages (Fig.2.5, B). GO-term analysis revealed that downregulated alternatively spliced genes were involved in the inflammatory response, specifically, IFN response (Fig.2.5, C). Taking into consideration the changes in IFNG receptor (*Ifngr)* gene expression in the KO BMDMs (Fig. 2.2, G) and the lower level of IFNG cytokine production in the KO mouse (Fig.2.3, B), we speculated that these changes are modulated by alternative splicing events in the *Ifngr* locus. Our IUTA analysis revealed differential isoform expression in the *IfngrI* locus as a result of HNRNPA2B1 deletion, leading to increased expression of a NGO transcript, which we identified using FLAIR (Elektra K. Robinson et al., 2021b), that lacks a start codon and therefore is not translated (Fig.2.5, D). We quantified both IFNGRI and II levels in macrophages from

mice post LPS injection (Fig.2.5, E and G), and on BMDMs treated with LPS *ex vivo* (Fig.2.5, F) and confirmed lower levels of the receptors on the cell surface. We also identified similar alternative splicing events leading to an increase in NGO transcripts of major IFN response transcription factors (TFs) and genes such as (*Stat3*, *Stat1*, *Irf7* and *Oas1c*) (SFig.2.6, A-D). Our results indicate that HNRNPA2B1 modulates IFNG signaling through alternative splicing of the IFNGR transcript, which dampens IFNGR levels on the macrophage surface.

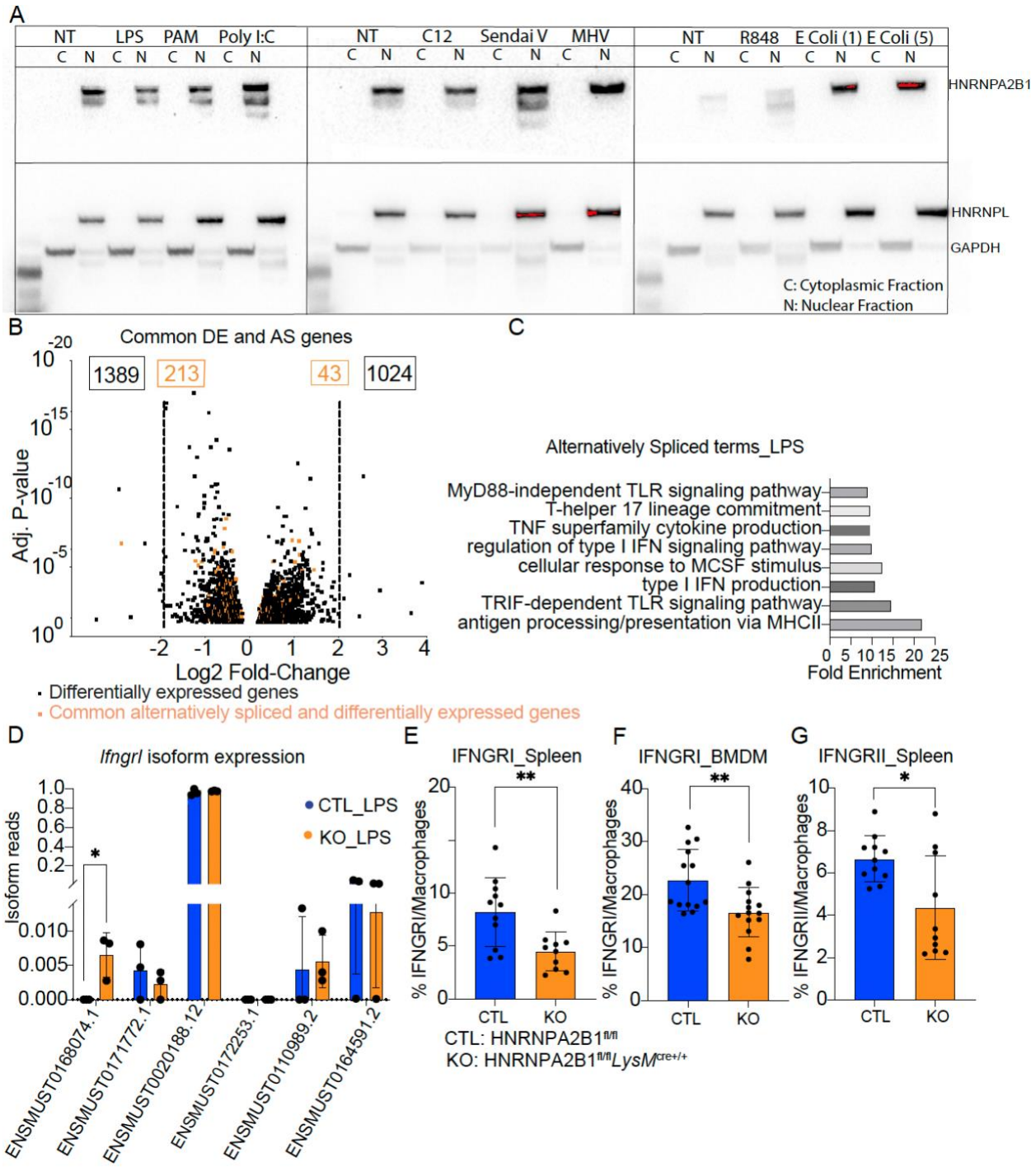

Figure 2.5 HNRNPA2B1 regulates IFNG signaling through alternative splicing. **A.** CTL BMDM nuclear and cytoplasmic fractions were analyzed using western blots after treatment with a panel of immune stimuli to assess changes in HNRNPA2B1 localization. **B.** Volcano plot of DE genes (black) and common DE and alternatively spliced genes in orange in KO BMDMs. **C.** Gene ontology analysis of genes that are

differentially expressed as well as alternatively spliced in KO BMDMs. **D.** *Ifngr1* isoform expression analysis performed using IUTA in CTL and KO BMDMs. **E.** IFNGRI levels on macrophages in the spleen using flow cytometry. **F.** IFNGRI levels on macrophages *ex vivo* using flow cytometry. **G.** IFNGRII levels on macrophages in the spleen using flow cytometry. Student's t-tests were performed using GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines (*P ≤ 0.05, **P ≤ 0.01).

2.3.6 HNRNPA2B1 KO mice are susceptible to *Salmonella* infections due to a failure to clear the pathogen.

Given the altered inflammatory responses observed in the HNRNPA2B1 KO mice during endotoxic shock, we wanted to investigate the role of HNRNPA2B1 in controlling the innate immune response to pathogens. To this end, we chose *Salmonella enterica* serovar Typhimurium as it utilizes macrophages as a replicative niche and is controlled in macrophages via IFNG (Ingram et al., 2017). We asked whether loss of HNRNPA2B1 in macrophages impacted susceptibility to bacterial infection in an *in vivo* model. Briefly, we infected CTL and HNRNPA2B1 KO mice by IP delivery of *Salmonella* ($2.5x10^4$) and mice were followed for signs of imminent morbidity (e.g. hunched posture, ruffled coat, lethargy as described in (Burkholder et al., 2012)) over the course of 6 days. We observed that HNRNPA2B1 KO mice succumbed to infection earlier than controls (Fig.2.6A) and experienced significantly higher bacterial burdens in the spleen and mesenteric lymph nodes (mLNs) (Fig.2.6, B and C). Mouse susceptibility to infection was also represented by reduced expression of IL12, IL13, CXCL5, and CCL11 (Fig.2.6, D-G). Our data indicate that HNRNPA2B1 KO mice are

more susceptible to *Salmonella* infection and fail to produce sufficient levels of key pro-inflammatory cytokines in response to bacterial infection.

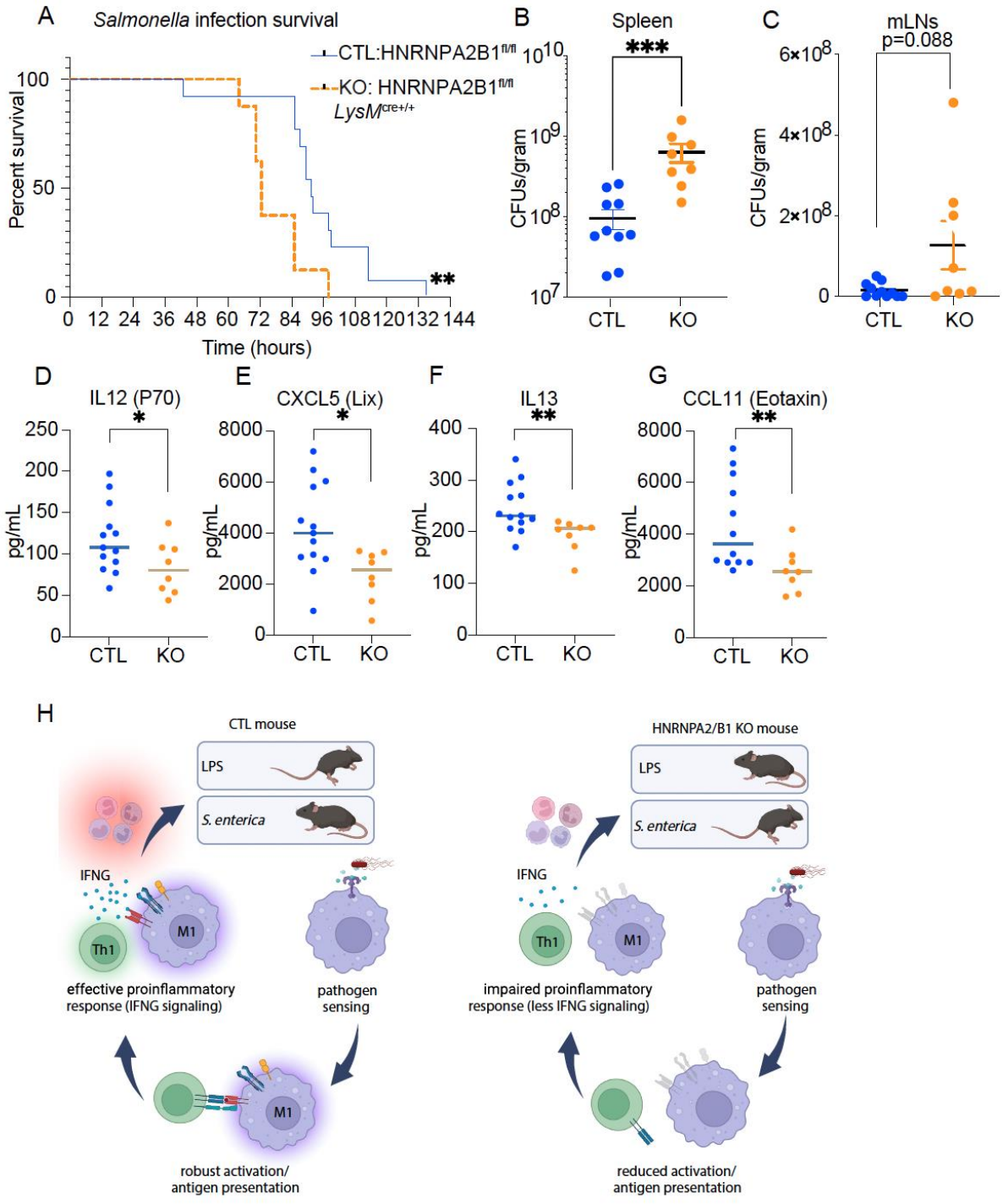

Figure 2.6. HNRNPA2B1 KO mice are susceptible to macrophage specific infection due to KO macrophage's failure to clear the pathogen. A. Survival of CTL and HNRNPA2B1 KO mice after i.p. *Salmonella* infection. **B.** CFU measurement of *Salmonella* bacterial load in mouse spleen. **C.** CFU measurement of *Salmonella* bacterial load in mouse mesenteric lymph nodes. **D-G.** Cytokine levels in serum of CTL and KO mice infected with *Salmonella*. **(D)** Il12 (P70), **(E)** CXCL5, **(F)** IL13, **(G)** CCL11. **(H)** Illustration the change is response to Salmonella infection when HNRNPA2B1 is removed. Student's t-tests were performed using GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines (*P $\leq$ 0.05, **P $\leq$ 0.01).

2.3.7 Nanopore Sequencing reveals transcriptome wide isoform changes

In order to get a better understanding of HNRNPA2B1 involvement in alternative splicing of inflammatory genes, we conducted long read nanopore sequencing on murine BMDMs at baseline and under inflammatory stimulus (Fig. 2.7). We observe >100 differentially expressed isoforms of genes involved in inflammatory signaling in addition to many other cellular and biological processes. Most striking isoform changes are ones in the *Hnrnpa2b1 gene* at baseline and under inflammatory stimulus (Fig. 2.7 A and B). Interestingly, we observe many DE isoforms of inflammatory and IFN regulated genes such as Irf7, Ifi208, Ifi27, and Ly6a (Fig. 2.7 A and B). GO term analysis of DE isoforms at baseline revealed a high enrichment for genes involved in macrophage chemotaxis, regulation of antigen processing and presentation, inflammatory wound healing, and dendritic cell differentiation (Fig. 2.7 C). Interestingly, GO term analysis of DE isoforms under inflammatory stimulus did not yield any enriched biological process. Our nanopore sequencing data reveals HNRNPA2B1 involvement in differential isoform expression of genes involved in the inflammatory response and specifically IFN response genes.

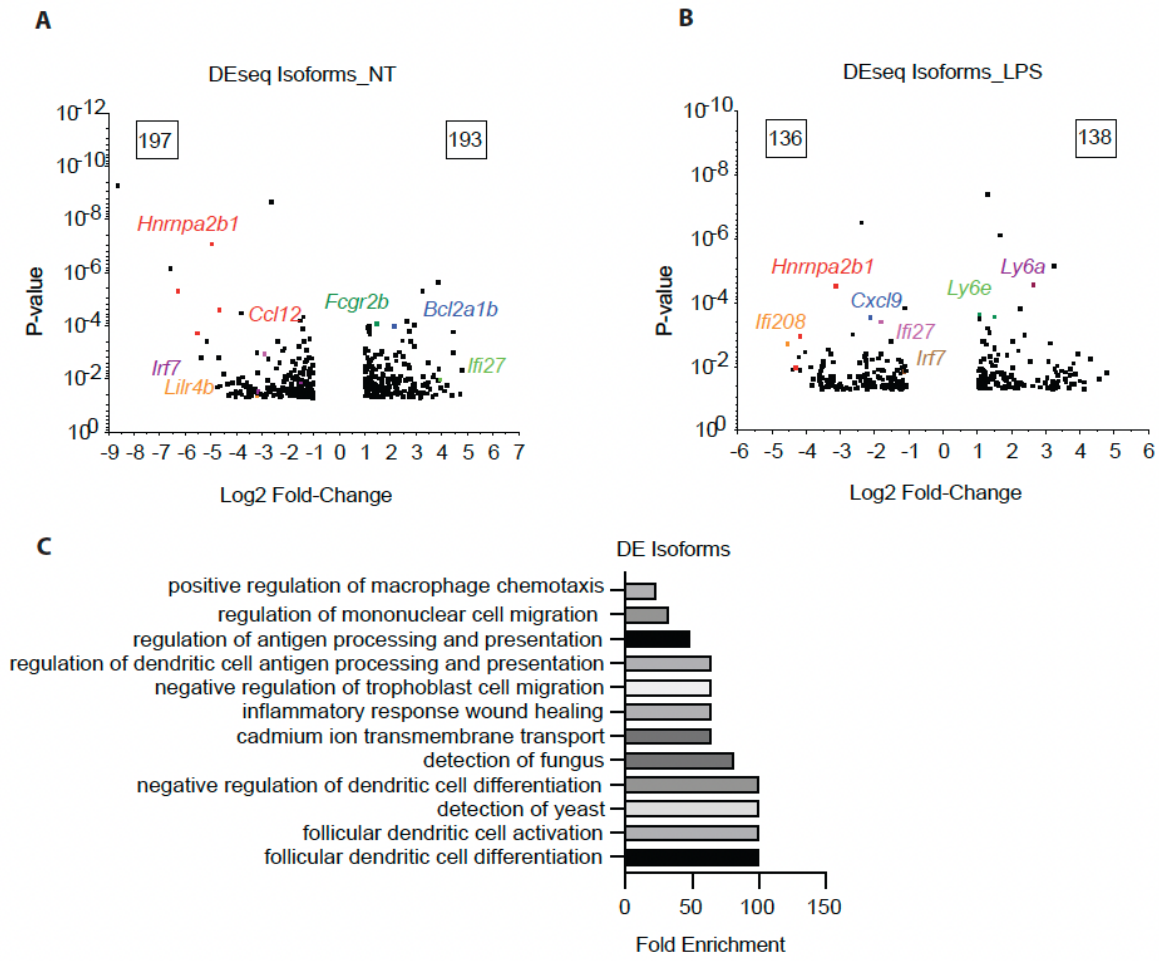

Figure 2.7 HNRNPA2B1 KO macrophages display differential isoform expression at baseline and under stimulus. A. Volcano plot of differentially expressed isoform in HNRNPA2B1 KO BMDMs at baseline, data generated via Nanopore sequencing. **B.** Volcano plot of differentially expressed isoforms in HNRNPA2B1 KO BMDMs under LPS stimulation, data generated via Nanopore sequencing. **C.** GO term analysis of DE isoforms in HNRNPA2B1 KO BMDMs at baseline.

2.4 Discussion:

HNRNPA2B1 is known to play critical roles in processes involving transcription, pre-mRNA splicing and translation (Carpenter et al., 2013b; Peng et al., 2021; Makhafola et al., 2020; Clower et al., 2010; Guo et al., 2013; Ryan et al., 2021; Dai et al., 2017; Kwon et al., 2019; Y. Liu et al., 2020) yet we find it is not essential for viability in macrophages or neutrophils. Using *LysM*Cre we generated a complete knockout in macrophages with only a slight decreased proliferative capacity observed *ex vivo*. HNRNPs have been shown to compensate for each other which is possibly occurring in the KO macrophages as our RNA-seq data indicated increased levels of HNRNPA1 in the HNRNPA2B1 deficient cells. It is possible that the importance of HNRNPA2B1 for macrophage fitness is also more important as cells age which could be the basis of future studies to determine. We were unsuccessful in our attempts to generate a HNRNPA2B1 knockout immortalized cell line using the CreJ2 method which supports the idea that over time HNRNPA2B1 is important for fitness as it takes upwards of 3 months of growth for cells to fully immortalize (data not shown), (Blasi et al., 1989). We observed a widespread defect in cytokine production in the deficient mice following endotoxic shock. While we initially hypothesized that lower cytokine expression *in vivo* could be explained by reduced myeloid cell numbers given the lower *ex vivo* proliferative rate, surprisingly, macrophage numbers were higher in HNRNPA2B1 deficient mice. Elevated macrophage numbers were unexpected given that suppression of proliferation classically occurs upon exposure to proinflammatory

stimuli such as LPS in conjunction with a switch to glycolysis in order to conserve the cell's metabolic capacity (Liu et al., 2016; Vadiveloo et al., 2001). Despite being more abundant, KO macrophages were less active as evidenced by lower levels of M1 polarization markers (CD86 and MHCII) (Ahmed and Ismail, 2020; Zhao et al., 2017; McCauley et al., 2020; Y.-C. Liu et al., 2014) in response to LPS. The increase in macrophage and neutrophil population numbers in the KOs could be a direct result of the weakened response to stimulus and failure to reach a terminally polarized state, prompting the cells to proliferate at a faster rate in order to attempt to compensate for the attenuated response.

We previously reported that HNRNPA2B1 regulates immune gene expression using shRNAs in macrophages *ex vivo* (Carpenter et al., 2013), where HNRNPA2B1 knockdown led to increased expression of a subset of IFN response genes. Here we performed RNA sequencing analysis comparing HNRNPA2B1 KOs to CTL and similar to shRNA mediated silencing, we observed widespread changes in the macrophage transcriptome. However, the effects we observed in the HNRNPA2B1 knockouts are more pronounced compared to the shRNA results likely due to more efficient removal of the gene *in vivo* and the use of primary macrophages rather than immortalized cells. While some genes were upregulated, the dominant phenotype was an overall dampening of the IFN response supported by down regulation in *Cd74* (54-56), *Mill2* (Tanaka and Kasahara, 1998), *MhcII* (H2Aa) (Wijdeven et al., 2018; Muhlethaler-Mottet et al., 1998; Steimle et al., 1994), *Ciita* (Muhlethaler-Mottet et al., 1998; Steimle et al., 1994; Lee et al., 2008) and *Cxcl12* (Han et al., 2018). Interestingly,

we found IFI208, an interferon inducible gene, to be one of the most highly upregulated genes when HNRNPA2B1 was removed despite the overall dampening of IFN response signaling, suggesting a more complex role with a possible negative regulatory aspect for HNRNPA2B1. Likewise, we observed a downregulation of the costimulatory molecule CD86 both at the RNA and protein levels yet CD80, a costimulatory molecule with semi overlapping function, was increased suggesting specificity and complexity to the mechanisms by which HNRNPA2B1 regulates gene expression. It has been previously shown in an alternative septic shock model that macrophage CD80 plays a greater role in promoting inflammation than CD86 (Nolan et al., 2009). Consistent with an alternative driver of protection, HNRNPA2B1 transcriptionally regulated expression of major components of the IFNG signaling pathway from the receptor *Ifngr* to the adaptor proteins, *JAK1,3* and *STAT1,3* as well as transcription factors (*IRF1,2,7,8,9*). Effects were not limited to the level of transcription and extended to reduced pathway activation through reduction in phosphorylation of STAT3 and the total STAT3 protein.

Given the changes we observed to IFN signaling and cytokine responses *ex vivo* upon loss of HNRNPA2B1, we chose to correlate those findings to *in vivo* challenge with LPS. Mice deficient in HNRNPA2B1 produced lower levels of proinflammatory cytokines including many produced predominantly by macrophages such as CCL2 (MCP1), CCL3 (MIP1a), CCL5 (Rantes), CXCL1 (KC) and IL6 in serum and spleen. This disruption in the inflammatory response proved advantageous to KO mice which did not become as hypothermic as the CTL mice following challenge. However, this

attenuated inflammatory response proved detrimental to mice deficient in HNRNPA2B1 when infected with *Salmonella*. We chose *Salmonella* as it readily infects macrophages which provide a replicative niche that is abolished upon IFNG signaling and polarization of macrophages to an M1 state for pathogen clearance (Ingram et al., 2017; Rupper and Cardelli, 2008; Shenoy et al., 2012). Following *Salmonella* infection, the HNRNPA2B1 deficient mice succumbed earlier to infection and displayed higher bacterial burden, indicative of failure to clear the pathogen. This phenotype can be explained by the fact that the knockout mice have lower levels of the IFNG receptors, downstream adaptors, transcription factors, and co-stimulatory molecules. With all these pathways dampened, IFNG is no longer capable of coordinating cytokine activation (Kawa et al., 2010; Freudenberg et al., 1993; Wysocka et al., 1995; Zha et al., 2017) and sensitization of macrophages to potentiate a strong inflammatory response resulting in reduced effector cell (T cells and NK cells) activation and an inability to clear the pathogen.

Mechanistically, HNRNPA2B1 -similar to other HNRNPs- is involved in RNA metabolism, specifically alternative splicing. We report its strict localization in the nucleus of macrophages at steady state and following stimulation with a variety of PRR ligands as well as Sendai and murine gamma herpesvirus (MHV) (Fig.2.5). A recent study by Wang et al. showed that HNRNPA2B1 acts as a DNA receptor and can translocate to the cytosol following HSV-1 infection, influencing IFN signaling through activation of TBK1-IRF3 (Wang et al., 2019). It is possible that movement of HNRNPA2B1 is stimulus dependent. Using RNA-seq we found that HNRNPA2B1

plays a role in the alternative splicing of the *Ifngr* locus. Macrophages deficient in HNRNPA2B1 showed an increase in an NGO isoform of *Ifngr* which lacks a start codon and is therefore not translated, and this was confirmed by lower expression of the IFNG receptor on the cell surface of HNRNPA2B1 knockout mice using flow cytometry. This downregulation of the IFNGR could explain the observed *in vivo* phenotypes as discussed earlier as lower expression of the receptor will dampen all the downstream responses following IFNG production impacting the overall adaptive immune response. Other IFN response genes such as *Stat1*, *Stat3*, *Irf7* and *Oas3* showed similar upregulation of an NGO transcript in the knockout macrophages, indicating HNRNPA2B1's involvement in alternative splicing of other IFN response genes in the IFN pathway in a similar manner, thus regulating their expression pattern. Alternatively, some genes such as *Cd86* that are downregulated at the RNA and protein levels in the HNRNPA2B1 deficient mice could also be targets of alternative splicing, but they did not appear in our splicing analysis. It is technically difficult to capture transcripts such as NGO and other isoforms as they could be degraded more rapidly. Therefore, we do not know how many of the genes downregulated in the HNRNPA2B1 knockouts are controlled through splicing, transcription control or an alternative mechanism.

In conclusion, our findings highlight HNRNPA2B1's integral role in promoting IFNG inflammatory responses in macrophages *in vivo*. Mechanistically HNRNPA2B1 ensures appropriate processing of the *IfngrI* transcript allowing adequate activation upon IFNG binding, driving expression of JAKs, STATs and IRF proteins as well as a

large number of ISGs. This initiates macrophage activation and facilitates downstream activation of adaptive immunity. This work highlights HNRNPA2B1 as a key regulator of innate immunity providing new insights into how a ubiquitously expressed RNA binding protein can play unique roles in regulating IFN gene expression in macrophages.

2.5 Materials and Methods

2.5.1 Mice

Heterozygous floxed mice for the HNRNPA2B1 locus were generated using CRISPR to insert two loxP sites flanking exons 2 and 7 in the HNRNPA2B1 locus. Mice homozygous for the loxP sites were used as controls in these experiments. Heterozygous loxP mice were generated by Biocytogen where CRISPR/Cas9 was used to insert two loxP fragments in introns 1 and 7 of the HNRNPA2B1 locus. When exons 2-7 are removed a protein reading frame shift will occur which results in the production of a 100aa protein that eventually undergoes NMD.

2.5.2 CRISPR/Cas9 sgRNA

sgRNA ID	Sequence name	Sequence (5'-3')

EGE-ZY-021-B-T7-sgRNA6-A	**target sequence**	GTCTTCATACCGTTTCGAGG **TGG**
	EGE-ZY-021-B-T7-sg6-A-dn	CTATTTCTAGCTCTAAAACCCTCGAAACGGTATGAAGACCTATAGTGAGTCGTATTA
EGE-ZY-021-B-T7-sgRNA10	**target sequence**	AGTAATTGGTAACAAGCTGC **AGG**
	EGE-ZY-021-B-T7-sg10-dn	CTATTTCTAGCTCTAAAACGCAGCTTGTTACCAATTACCTATAGTGAGTCGTATTA

2.5.3 LoxP site integration detection primers

Primer	Sequence (5'-3')	Tm(℃)	Product size (bp)
EGE-ZY-021-B-5'loxP-F1	CCGGATTTGGCGGCCGCCATTTTC	60	WT:342 Mut:433

EGE-ZY-021-B-5'loxP-R1	CCAGGCCTCGGTTGTACTACGTTC	60	
EGE-ZY-021-B-3'loxP-F2	GCATAGGCCTGAGCTCTCAGCATTCTG	58	WT: 573 Mut:664
EGE-ZY-021-B-3'loxP-R1	GTTGATTTGTTGGGGACATTGAGGG	52	

*LysMC*re mice were purchased from the Jackson Laboratory (Bar Harbor, ME), reference ID: RRID:IMSR_JAX:004781. Conditional Ko mice were homozygous for both the loxP sites and *LysM*Cre site. All mouse strains were bred at the University of California, Santa Cruz (UCSC) and maintained under specific pathogen-free conditions in the animal facilities of UCSC. All protocols were performed in accordance with the guidelines set forth by UCSC and Texas A&M Institutional Animal Care and Use Committees.

2.5.4 Cell culture

BMDMs were generated by culturing erythrocyte-depleted BM cells in DMEM supplemented with 10% FCS, 5 mL pen/strep (100×), 500 μL ciprofloxacin (10 mg/mL), and 10% L929 supernatant for 7 to 14 d, with the replacement of culture medium every 2 to 3 d.

2.5.5 *Ex vivo* stimulation of macrophages and inflammasome activation

Bone marrow derived macrophage cells were stimulated with Lipopolysaccharide (LPS) at 200 ng/ml (TLR4) for 5 h for RNA extraction and 18 h for ELISA and western blots. For inflammasome activation assay, cells were primed with either LPS at 20ng/mL or IFNγ at 50 ng/mL overnight followed by LPS for 3 h. Inflammasome was activated by ATP at 5mM for 2 h or dA:dT at 1ug/mL for 6 h or Nigericin at 25uM for 0.5hrs. For RNA and protein isolation, 1-2x10^6cells were seeded in 12-well format or 10x10^6 cells were seeded in 10cm plates.

2.5.6 RNA isolation, cDNA synthesis and RT-qPCR

Total RNA was purified from cells or tissues using Direct-zol RNA MiniPrep Kit (Zymo Research, R2072) and TRIzol reagent (Ambion, T9424) according to the manufacturer's instructions. RNA was quantified and assessed for purity using a nanodrop spectrometer (Thermo Fisher). Equal amounts of RNA (500 to 1,000 ng) were reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, 1708841), followed by qPCR using iQ SYBR Green Supermix reagent (Bio-Rad, 1725122) with the following parameters: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72°C for 30s, followed by melt-curve analysis to control for nonspecific PCR amplifications. Oligos used in qPCR analysis were designed using Primer3 Input version 0.4.0.

Gene expression levels were normalized to *Actin* or *Hprt* as housekeeping genes as inidicated.

2.5.7 Cells Supernatant Collection for ELISA

Supernatant was collected from cultured and treated BMDMs, centrifuged at 12000xg, 5mins at RT and submitted for cytokine analysis.

2.5.8 Serum Harvest

Mice were humanely sacrificed; blood was collected immediately postmortem by cardiac puncture. Blood was allowed to clot and centrifuged, serum was stored at −70°C, then sent to EVE for measurements of cytokines/chemokines.

2.5.9 Spleen Tissue Harvesting for cytokine measurement

Mice were humanely sacrificed, and their spleens were excised. The whole spleens were snap frozen and homogenized, and the resulting homogenates were incubated on ice for 30 min and then centrifuged at 300 × g for 20 min. The supernatants were harvested, passed through a 0.45-μm-pore-size filter, and used immediately or stored at −70°C, then sent to EVE for measurements of cytokines/chemokines.

2.5.10 RNA sequencing libraries

RNA-Seq was performed in BMDMs with no treatment or treated with LPS for 5 hrs. The data are accessible at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database, accession: GSE243269.

RNA-Seq was performed in biological triplicates in fl/fl BMDMs used as control and KO BMDMs at 0 and 5 h after LPS treatment (200 ng/mL). RNA-Seq libraries were generated from total RNA (1 μg) using the Bioo kit, quality was assessed, and samples were read on a High-SEq 4000 as paired-end 150-bp reads. Sequencing reads were aligned to the mouse genome (assembly GRCm38/mm10) using STAR. Differential

gene-expression analyses were conducted using DESeq2. GO enrichment analysis was performed using PANTHER. Data was submitted to GEO, accession: GSE243269.

2.5.11 Alternative Splicing Analysis

Alignment files from RNA-Seq analysis were filtered to include only canonical chromosomes and were passed to IUTA to test differential isoform usage. Gene isoforms from the Gencode M25 Comprehensive gene annotation file were used. Family wise error rate (FWER) was accounted for with Bonferroni correction and changes were called significant at FWER < 0.01.

2.5.12 Cell Extracts and Western Blots

Cell lysates were prepared in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH 7.4], and 1.0 mM EDTA) containing protease-inhibitor mixture (Roche, 5892791001) and quantified using Pierce Bicinchoninic Acid Assay assay (Thermo Fisher, 23225). When indicated, the NEPER kit (Thermo Fisher Scientific, 78833) supplemented with protease inhibitor mixture (Roche) or 100 U/mL SUPERase-In (Ambion, AM2694) was used for cellular fractionation prior to Western blotting. Equivalent amounts (15 μg) of each sample were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes using Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with PBS, supplemented with 5% (wt/vol) nonfat dry milk for 1 h, and probed with primary antibodies overnight with either HNRNPA2B1 (1:1000, Santa Cruz Biotech, Sc-374053), pSTAT3 (1:500, Cell Signaling, 9138S), STAT3 (1:500, Cell Signaling, 9139S), pJAK3 (1:500, Cell Signaling, 5031S), or JAK3 (1:500, Cell Signaling,

8863S). Horseradish peroxidase-conjugated β-actin (1:500, Santa Cruz Biotechnology, sc-47778), HNRNPL (1:1000, Santa Cruz Biotech, Sc-32317) or GAPDH (1:1000, Santa Cruz Biotech, Sc-32233) were used as loading controls. Horseradish peroxidase-conjugated goat anti-mouse (1:10,000, Bio-Rad, #1721011) or anti-rabbit (1:2,000, Bio-Rad, #1706515) secondary antibodies were used. Western blots were developed using Amersham enhanced chemiluminescence (ECL) Prime chemiluminescent substrate (GE Healthcare, 45-002-401) or Pierce ECL (Life Technologies, 32106).

2.5.13 *In Vivo* LPS-Induced Endotoxic Shock Assay.

Age- and sex-matched CTL and KO mice (10 to 12 wk old) were i.p. injected with PBS as a control or *E. coli* LPS (5 mg/kg/animal). For gene expression and cytokine analysis, mice were euthanized 6 h or 18 h post injection. Blood was collected immediately postmortem by cardiac puncture. Serum was submitted to Eve Technologies for cytokine analysis.

2.5.14 Assessment of Immune Cell Populations Using Flow Cytometry

Blood was collected immediately postmortem by cardiac puncture, and single-cell suspensions prepared from the spleen of CTL and HNRNPA2B1 KO mice were depleted of red blood cells (RBCs) prior to staining. Whole spleens were collected, homogenized and depleted of RBCs.

Fragment, crystallizable (Fc) receptors were blocked (anti-CD16/32, BD Pharmingen) prior to staining with LIVE/DEAD Fixable near IR Dead Cell Stain (Thermo Fisher), anti–CD11b-AlexaFluor 488, anti–LY6G-BV421, anti–LY6C-PE-cy7, anti–CD19 BV786, anti–CSF-1R APC, anti–CD3-PE, anti–SiglecF Super Bright 645, and anti–

F4/80 PE-eFluor 610, MHCII BV421, CD86 Super Bright 645, CD80 PE-Cy7, IFNGRI BV605, IFNGRII PE, all by Thermo Fisher.

Flow cytometry analysis of BMDMs harvested from CTL and HNRNPA2B1 KO mice was performed; cells were stained with LIVE/DEAD Fixable near IR Dead Cell Stain (Thermo Fisher),anti–CD11b-AlexaFluor 488, anti–F4/80 PE-eFluor 610, MHCII BV421, CD86 Super Bright 645, CD80 PE-Cy7, IFNGRI BV605, IFNGRII PE, all by Thermo Fisher.

Data acquisition was performed using Attune NxT (Thermo Fisher). Analysis was performed using FlowJo analysis software (BD Biosciences).

2.5.15 Proliferation Assay

CTL and KO BMDMs were cultured as described above. Cell proliferation kit (MTT) by Millipore Sigma was used to assess cell proliferation. Solubilization of the purple formazan crystals was measured as an indicator of metabolically active cells through spectrophotometric absorbance of the samples using a microplate (ELISA) reader.

2.5.16 Phagocytosis Assay

CTL and KO BMDMs were cultured as described above. Cells were then administered with pHrodo Green *E. coli* BioParticles (Invitrogen) conjugates for 0, 15, 30, and 60 min at 37 °C. pHrodo green fluorescence, a measure of *E. coli* in an acidified phagosome, was examined by flow cytometry.

2.5.17 *S. enterica* (ser. Typhimirium)

Salmonella enterica serovar Typhimurium (SL1344) was obtained from Dr. Denise Monack, Stanford. S. T. stocks were streaked out on LB agar plates and incubated at

37 °C overnight. For S. Typhimurium infection, overnight cultures of bacteria were grown in LB broth containing 0.3 M NaCl and grown at 37 °C until they reached an OD600 of 0.9. On the day of infection cultures were diluted 1:20. Once cultures had reached mid-log phase (OD600 0.6-0.8) 2-3 h, 1 mL of bacteria were pelleted at 5000 rpm for 3 min and washed twice with 2X PBS.

2.5.18 *In vivo* S. enterica (ser. Typhimurium) infection

Age- and sex-matched CTL and KO mice (10 to 12 wk old) were i.p. injected with *Salmonella enterica* serovar Typhimurium 2.5 x 10^4 bugs per animal. For CFUs and cytokine analysis, mice were euthanized 3 days post infection. Blood was collected immediately postmortem by cardiac puncture. Serum was submitted to Eve Technologies for cytokine analysis. For time to death mice were monitored for signs of imminent morbidity (e.g. hunched posture, ruffled coat, lethargy as described in 43) while blinded to genotype.

2.5.19 KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HNRNPA2B1 mouse monoclonal ab	Santa Cruz Biotech	Sc-374053
pSTAT3	Cell Signaling	9138S
STAT3	Cell Signaling	9139S
pJAK3	Cell Signaling	5031S

JAK3	Cell Signaling	8863S
Horseradish peroxidase-conjugated β-actin	Santa Cruz Biotech	Sc-47778
HNRNPL	Santa Cruz Biotech	Sc-32317
GAPDH	Santa Cruz Biotech	Sc-32233
Horseradish peroxidase-conjugated goat anti-mouse	Bio-Rad	#1721011
Horseradish peroxidase-conjugated goat anti-rabbit	Bio-Rad	#1706515
Annexin V	Biolegend	640920
anti-CD16/32	BD Pharmingen	
LIVE/DEAD Fixable near IR Dead Cell Stain	Thermo Fisher	L34992
anti–CD11b-AlexaFluor 488	Thermo Fisher	53-0112-82
anti–LY6G-BV421	Thermo Fisher	404-9668-80
anti–LY6C-PE-cy7	Thermo Fisher	25-5932-80
anti–CD19 BV786	Thermo Fisher	417-0193-80
anti–CSF-1R APC	Thermo Fisher	17-1152-80
anti–CD3-PE	Thermo Fisher	12-0031-81

anti–SiglecF Super Bright 645	Thermo Fisher	64-1702-82
anti–F4/80 PE-eFluor 610	Thermo Fisher	61-4801-82
MHCII BV421	Thermo Fisher	404-5321-80
CD86 Super Bright 645	Thermo Fisher	64-0862-80
CD80 PE-Cy7	Thermo Fisher	25-0801-80
IFNGRI BV605	Thermo Fisher	745111
IFNGRII PE	Thermo Fisher	113604
Chemicals, Peptides, and Recombinant Proteins		
Lipopolysaccharide E.coli	Sigma-Aldrich	L2630-25MG
IFNG	Biolegend	575304
ATP	Sigma	A6419
Nigericin	Sigma	N7143-5MG
Critical Commercial Assays		
NEXTflex Rapid Illumina RNA-Seq Library Prep Kit	Bioo Scientific	
Cell Proliferation Kit I (MTT)	Miilipore Sigma	11465007001
IL1b Mouse DuoSet ELISA Kit	R&D	DY401

pHrodo™ Green E. coli BioParticles™ Conjugate for Phagocytosis	Thermo Fisher	P35366
Oligonucleotides		
5‘ loxp_F	CCGGATTTGGCGGCCATTTTC	
5‘ loxp_R	CCAGGCCTCGGTTGTACTACGTTC	
3‘ loxp_F	GCATAGGCCTGAGCTCTCAGCATTCTG	
3‘ loxp_R	GTTGATTTGTTGGGGACATTGAGGG	
Cre_Rxn_A	CCCAGAAATGCCAGATTACG	
Cre_Rxn_B	TTACAGTCGGCCAGGCTGAC	
Cre_Rxn_common	CTTGGGCTGCCAGAATTTCTC	
Irf7 F	GACTGGGAAAGATCACCGGC	
Irf7 R	TTGCGCCAAGACAATTCAGG	
Irf8 F	CAATCAGGAGGTGGATGCTTCC	
Irf8 R	GTTCAGAGCACAGCGTAACCTC	
STAT3 F	AGGAGTCTAACAACGGCAGCCT	
STAT3 R	GTGGTACACCTCAGTCTCGAAG	
Oac1c F	GACTTCCGACATCAAGAGGTCTG	
Oac1c R	ATCCAGGTCTGAGCCTCCTTTG	
Ifi208 F	GCCACTCAAGGCAAAGATAGGATCTC	

Ifi208 R	CTGGGGATTCTGCATTTCATTGTCCTC

2.6 Acknowledgements:

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CHAPTER 3: *HNRNPA2B1* silencing reduces inflammation in human RA FLS cells

3.1 Abstract

Available Rheumatoid Arthritis (RA) therapies in the market are only effective in a portion of patients and often result in harmful side effects. There is a great need for development of targeted therapies that silence genes directly involved in RA pathology. In this chapter we target HNRNPA2B1 in fibroblast-like synoviocytes (FLS) cells, which is a regulator of IFN signaling through siRNA. Silencing HNRNPA2B1 attenuated the RA FLS inflammatory profile and disrupted IFN gene expression. This novel approach is effective in downregulating IFN signaling, especially IFNG which is responsible for FLS activation without completely abrogating this response. This can be effective in alleviating disease symptoms and lowering overall RA inflammation.

3.2 Introduction

HNRNPA2B1 is emerging as a critical player in a variety of inflammatory diseases including cancer, amyotrophic lateral sclerosis (ALS) and rheumatoid arthritis (RA) (Guo et al., 2013; Maslyanskiy et al., 2014; Fritsch et al., 2002; Kim et al., 2013). RA is a chronic systemic autoimmune disease, characterized by joint erosion and abundance of autoantibodies in the serum and synovial fluid (Maslyanskiy et al., 2014). Autoantibodies against the native and citrullinated form of HNRNPA2B1 were found in the synovium of RA patients (Fritsch et al., 2002) Where they targeted HNRNPA2B1 (RA33) in 15-35% of patients (Fritsch et al., 2002). Autoantibodies against the citrullinated form were also correlated with disease duration and erosion

(Maslyanskiy et al., 2014), implicating both the native and citrullinated form of HNRNPA2B1 in RA disease phenotype.

Despite what we know about HNRNPA2B1 protein and autoantibodies association with RA disease phenotype, it is unknown whether HNRNPA2B1 contributes to disease pathogenesis. Given our findings that support HNRNPA2B1's involvement in promoting a major inflammatory pathway (IFNG signaling) in macrophages, we were intrigued to study whether HNRNPA2B1 is involved in driving the RA inflammatory circuit. This is especially interesting given the interferon signature typically displayed in RA patients (Muskardin and Niewold, 2018; Conigliaro et al., 2010) where Type I IFNs are elevated. This is also accompanied by elevated levels of IFNG which plays a central role in RA synovial biology and bone metabolism (Kato, 2020). IFNG is produced by activated T cells driving their maturation into CD4+T (Dolhain et al., 1996), in addition to its direct involvement in activation of antigen presenting cells such as macrophages, thus driving autoimmunity (Kato, 2020). Elevated levels of IFNG in RA is accompanied by a shift towards T helper 1 (Th1) cells which are considered effector cells in RA where they are believed to drive the disease inflammatory phenotype (Dolhain et al., 1996). This evidence points to a possible involvement of HNRNPA2B1 in RA inflammatory activation through its function in promoting IFNG signaling in macrophages (Chapter 2).

In order to study HNRNPA2B1 involvement in driving inflammation in human disease, we have to investigate whether its function in driving IFN signaling in mice is conserved in humans. We utilized fibroblast-like synoviocytes (FLS) which constitute a major cell type in the synovial membrane (Bottini and Firestein, 2013) to study HNRNPA2B1 role in driving RA inflammation. FLS cells are known to take on both an aggressive and invasive phenotype in patients' joints where they contribute to inflammatory cell recruitment, pannus angiogenesis, cartilage degradation and bone erosion (Bottini and Firestein, 2013; Bustamante et al., 2017). Their contribution to pathogenesis is achieved through increased production of inflammatory cytokines, adhesion molecules and matrix-modeling enzymes in RA (Bottini and Firestein, 2013). FLS cells activation in RA is carried out via IFNG which is produced by activated T cells which in turn activates antigen presenting cells such as dendritic cells and macrophages leading to overproduction of cytokines (van Holten et al., 2002; Nocturne and Mariette, 2022; Kato, 2020). We used FLS extracted from arthritic joints to study HNRNPA2B1 role in driving RA inflammation through silencing this protein and assessing the impact of its KD on the FLS inflammatory gene expression. HNRNPA2B1 KD impacted inflammatory gene expression in RA FLS with major IFN response genes downregulated, specifically IFN response genes. Thus, we concluded that HNRNPA2B1 promotes the inflammatory circuit in RA and its silencing attenuates the RA inflammatory overactivation. Our results illustrate a potential benefit in eliminating HNRNPA2B1 in RA joints which could help attenuate the disease phenotype, however, more extensive studies are required.

3.3 Results

To study HNRNPA2B1 role in RA inflammation we collected FLS cells from RA patients and silenced HNRNPA2B1using siRNAs designed to target the HNRNPA2B1 locus (Fig. 3.1. A and B). Western blot analysis indicated the siRNAs against HNRNPA2B1 were successful in achieving >80% KD in both patient's samples (Fig. 3.1. C). RNA sequencing was conducted for RA FLS treated with siRNA against HNRNPA2B1 compared to RA FLS treated with non-targeting siRNA (CTL) to assess change in gene expression after HNRNPA2B1 KD. In HNRNPA2B1 KD FLS we observed more genes showing decreased expression (325) compared to genes where expression increased (72) (Fig. 3.1. D). Interestingly, a large majority of the downregulated genes were involved in the inflammatory response, with a large portion of them involved in IFN response (Fig. 3.1. E). This was confirmed using Go-term analysis where enriched downregulated pathways included regulation of type II and III interferons, production of IP10 and MCP1, as well as T cell proliferation (Fig. 3.1. F). RNA sequencing showed that HNRNPA2B1 KD in RA FLS dampened IFN gene expression and weakened RA inflammatory overactivation.

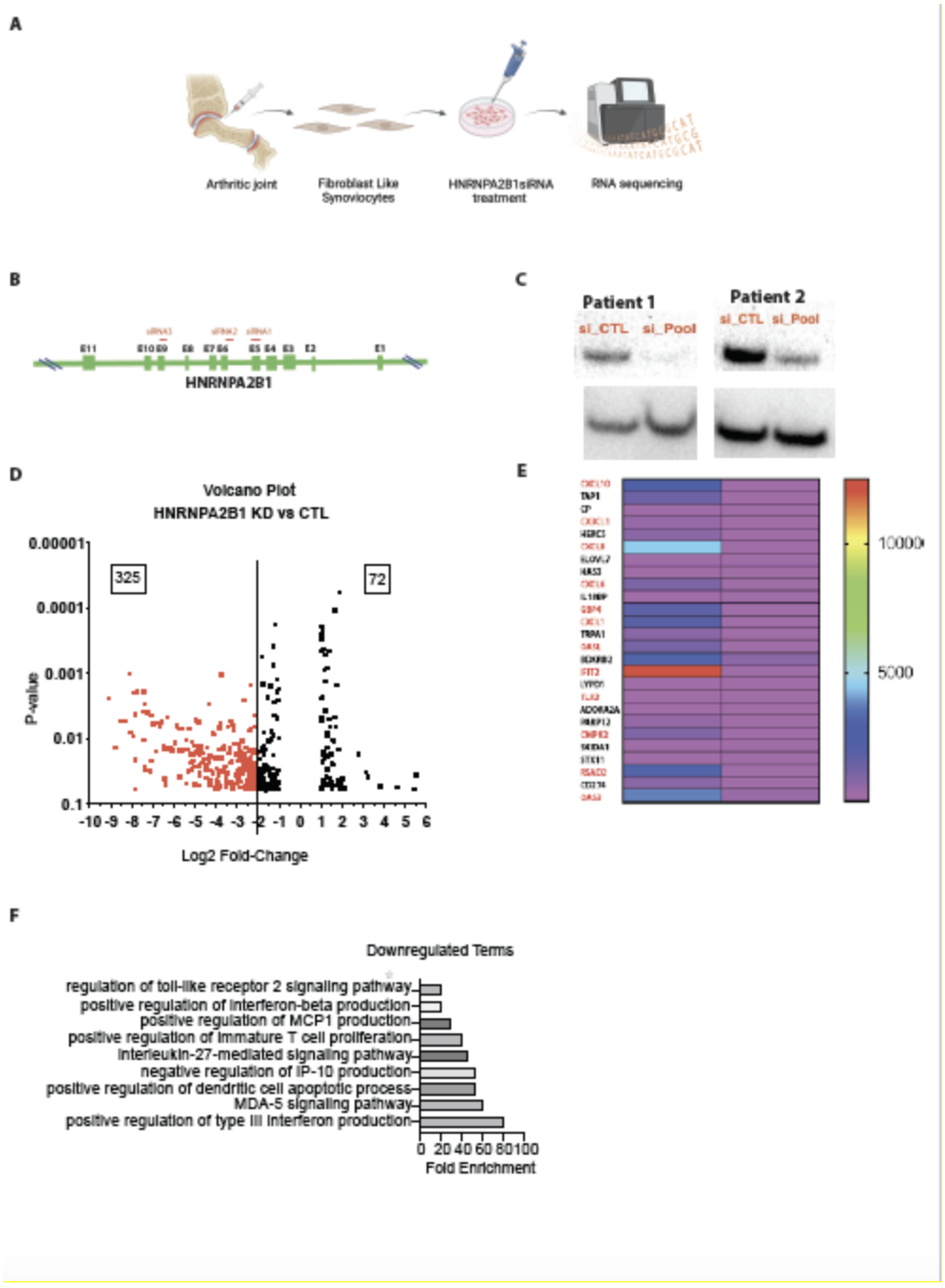


Figure 3.1. HNRNPA2B1 KD dampens IFN signaling in RA in FLS. A. FLS samples were collected from two arthritis patients and HNRNPA2B1 was knocked down using a pool of 3 different siRNAs. **B.** The locations targeted by 3 different siRNAs on the HNRNPA2B1 locus. **C.** HNRNPA2B1 expression analysis using western blotting where samples treated with non-targeting siRNA are compared to samples treated with a pool of HNRNPA2B1 siRNAs. **D.** Volcano plot of

differentially expressed genes post HNRNPA2B1 knock down. **E.** Heat map analysis of genes downregulated by more than 4 fold post HNRNPA2B1 KD with IFN response genes annotated in red. **F.** GO term analysis of downregulated pathways.

3.4 Discussion

Our results showcase HNRNPA2B1's involvement in immune modulation, and more importantly, in promoting IFN gene expression and activation. In this chapter, we confirmed that this function is not limited to murine macrophages as it was conserved in humans as represented by FLS cells. This finding is significant due to the pathogenic and destructive role FLS cells play in RA (Bottini and Firestein, 2013; Bustamante et al., 2017) through production of pro-inflammatory cytokines and matrix metalloproteases (MMPs) which play a role in immune overactivation and destruction of the joint (Bottini and Firestein, 2013; Bustamante et al., 2017). Targeting FLS cells to weaken their inflammatory activation presents a great potential for alleviation of RA symptoms, this can be achieved through reducing IFNG signaling in the synovium.

IFNs in general and IFNG in specific are a family of cytokines that function in immune modulation (van Holten et al., 2002) they enhance macrophage activity and allow it to combat tumor cells, viruses and bacteria (van Holten et al., 2002). Studies have supported a putative role of IFNG in driving the inflammatory state in RA synovium (Kato, 2020) which positions IFNG-FLS axis as a prime target for treatments aimed to dampen RA inflammatory activation.

Current available treatments for RA include anti biologics such as anti IL-6 and anti TNF, both of which have immune modulation properties which exposes patients to infectious diseases (Yang et al., 2018; Nocturne and Mariette, 2022). The other option is disease-modifying antirheumatic drugs (DMARDs) which are only effective in a portion of patients and are not well tolerated (Yang et al., 2018). Thus, new RA treatments are needed to alleviate patient's pain and improve their lifestyle. While clinical trials aimed at targeting IFNG directly did not show positive reduction of inflammation (Kato, 2020), targeting an IFNG regulator which can help dampen IFNG signaling in conjunction with Type I IFN as seen in our HNRNA2B1 KD data may prove more beneficial.

In this study, the IFNG-FLS axis was successfully targeted through silencing HNRNPA2B1 in cultured FLS cells. HNRNPA2B1 depletion weakened IFN gene expression and lowered the RA FLS overall inflammatory profile. This study introduces a new method to target the autoimmune IFN signature through directly manipulating upstream regulators of IFN gene expression which successfully altered the cell's inflammatory profile.

3.5 Methods

3.5.1 Cell Culture

RA and OA FLS cells extracted from two RA and two OA patients were generously provided by Dr. Firestein's lab in UCSD. Cells were cultured for 5 days in RPMI supplemented with 10% FCS, 5 mL pen/strep (100×), 500 μL ciprofloxacin (10

mg/mL). Cells were transfected with a mix of 3 siRNA at 15nM each using Lipofectamine 2000 for 72hrs.

3.5.2 RNA Extraction and RNA sequencing libraries

Total RNA was purified from cells or tissues using Direct-zol RNA MiniPrep Kit (Zymo Research, R2072) and TRIzol reagent (Ambion, T9424) according to the manufacturer's instructions. RNA was quantified and assessed for purity using a nanodrop spectrometer (Thermo Fisher).

RNA-Seq was performed for two RA samples and two OA samples were used as control. RNA-Seq libraries were generated from total RNA (1 μg) using the Bioo kit, quality was assessed, and samples were read on a High-SEq 4000 as paired-end 150-bp reads. Sequencing reads were aligned to the mouse genome (assembly GRCm38/mm10) using STAR. Differential gene-expression analyses were conducted using DESeq2. GO enrichment analysis was performed using PANTHER.

3.5.3 Cell extracts and western blots

Cell lysates were prepared in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH 7.4], and 1.0 mM EDTA) containing protease-inhibitor mixture (Roche, 5892791001) and quantified using Pierce Bicinchoninic Acid Assay assay (Thermo Fisher, 23225).

Equivalent amounts (10 μg) of each sample were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes using Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with PBS, supplemented with 5% (wt/vol) nonfat dry milk for 1 h, and probed with primary antibodies overnight with HNRNPA2B1 (1:1000, Santa Cruz Biotech, Sc-374053) followed by Horseradish peroxidase-conjugated goat anti-mouse (1:10,000, Bio-Rad, #1721011) secondary antibodies. Actin was used as a loading control using Horseradish peroxidase-conjugated B Actin (Santa Cruz Biotech, SC47778). Western blots were developed using Amersham enhanced chemiluminescence (ECL) Prime chemiluminescent substrate (GE Healthcare, 45-002-401) or Pierce ECL (Life Technologies, 32106).

CHAPTER 4: *LincRNA-Cox2* regulates smoke-induced inflammation in murine macrophages

4.1 Abstract:

Cigarette smoke (CS) exposure is a risk factor for many chronic diseases including chronic obstructive pulmonary disease (COPD), however the mechanism by which smoke exposure can alter homeostasis and bring about chronic inflammation is poorly understood. Here, we showcase a novel role for smoke in regulating long noncoding RNAs (lncRNAs), showing that it activates *lincRNA-Cox2*, which we previously characterized as functional in inflammatory regulation. Exposing *lincRNA-Cox2* murine models to smoke *in vivo* confirmed *lincRNA-Cox2* as a regulator of inflammatory gene expression in response to smoke both systemically and within the lung. We also report that *lincRNA-Cox2* negatively regulates genes in smoked bone marrow derived macrophages exposed to LPS stimulation. In addition to the effects on lncRNAs, we also report dysregulated transcription and splicing of inflammatory protein-coding genes in the bone marrow niche following CS exposure *in vivo*. Collectively, this work provides insights into how innate immune signaling from gene expression to splicing is altered following *in vivo* exposure to CS and highlights an important new role for *lincRNA-Cox2* in regulating immune genes following smoke exposure.

4.2 Introduction:

Chronic obstructive pulmonary disease (COPD) is a debilitating inflammatory lung disease associated with tobacco smoking. The pathogenesis of COPD remains poorly understood but is known to involve abnormal inflammatory responses of lung macrophages that are induced by cigarette smoke (CS) exposure (Niewoehner et al.,

1974). Macrophages specialize in the initial response to external signals such as infectious agents, environmental pollutants as well as tissue damage (Fujiwara and Kobayashi, 2005). CS exposure can attenuate macrophage function including altering responses to infectious agents (Fujiwara and Kobayashi, 2005) and suppressing phagocytic rate, (Thomas et al., 1978) in addition to increasing pro-inflammatory cytokine and chemokine production (D'hulst et al., 2005; Botelho et al., 2010). CS-driven lung inflammation is also linked to tissue injury, susceptibility to microbial infections, and poor wound healing (Strzelak et al., 2018; Tura-Ceide et al., 2017). The mechanism by which CS contributes to the heightened inflammation observed in COPD is not well understood.

Alternative splicing is a key regulator of inflammation and a potential mechanism that links CS exposure to abnormal an inflammatory response in macrophages in COPD (Elektra K. Robinson et al., 2021). Alternative splicing is a highly regulated process which allows for multiple isoforms of a protein to be produced from a single gene (Boudreault et al., 2016b; Ivanov and Anderson, 2013b; Pai et al., 2016b; E. T. Wang et al., 2015). Previous work identified important splicing events in genes involved in chemokine signaling and metabolism following lung inflammation (Elektra K. Robinson et al., 2021c; Janssen et al., 2020). With relevance to COPD, comparisons of bronchial biopsies of smokers versus non-smokers suggest that smoke induces alternative splicing of the receptor for advanced glycation end products (AGER or RAGE), which in turn is known to have a role in mediating lung endothelial inflammation in COPD (Faiz et al., 2019). However, a robust understanding of how CS

can alter gene expression and isoform usage specifically in bone marrow-derived macrophages (BMDMs) has yet to be investigated. Here we made use of multiple splicing tools and identified retained intron events as the top alternative splicing events occurring in macrophages following *in vivo* smoke exposure.

To better understand CS induced transcriptional modulation, we also focused on expression changes in long noncoding RNA (LncRNA) genes in addition to coding genes. LncRNAs have emerged as key regulators of gene expression and as effective modulators of immune cell development and function (Robinson et al., 2020; Atianand et al., 2017b). We have previously characterized the function of the inflammatory inducible *lincRNA-Cox2* using both *in vitro* and *in vivo* approaches where it plays a broad regulatory role during inflammation (Elling et al., 2018; Robinson et al., 2022). *LincRNA-Cox2* can function in *cis* through an RNA enhancer mechanism to regulate its neighboring protein Ptgs2 (Cox2), as well as functioning in *trans* to globally regulate expression of innate immune genes (Robinson et al., 2022; Carpenter et al., 2013; Covarrubias et al., 2017; Tong et al., 2016; Hu et al., 2016; Hu et al., 2018; Liao et al., 2020; Xue et al., 2019). In this study, we identified *lincRNA-Cox2* as one of the most upregulated genes in bone marrow derived macrophages following *in vivo* smoke exposure. We then used two different *lincRNA-Cox2* mouse models to determine the effect of modulating *lincRNA-Cox2* expression on CS-induced macrophage inflammatory responses. Our *lincRNA-Cox2* deficient (mutant, Mut) model represents *lincRNA-Cox2* knockdown where the splice sites have been targeted with CRISPR/Cas9 as previously reported (Elling et al., 2018). We specifically focus on the

lincRNA-Cox2 deficient mice as these mice do not have any altered *cis* phenotype on the neighboring protein coding gene Ptgs2 as we have previously described (Elling et al., 2018). Instead, this mouse enables us to focus on the *trans* regulatory functions of *lincRNA-Cox2*. In addition, we used our recently generated rescue transgenic mouse model that involved crossing the *lincRNA-Cox2* transgenic overexpressing line with our *lincRNA-Cox2* deficient (Mut) line labeled MutxTg (Robinson et al., 2022). The two models allow to us to specifically investigate the *trans*-regulatory role for *lincRNA-Cox2* and determine whether ubiquitous overexpression of *lincRNA-Cox2* from a non-native locus could rescue the phenotypes we observed in the *lincRNA-Cox2* deficient mice. Utilizing a physiologically relevant model of *in vivo* smoke exposure, we found that *lincRNA-Cox2* functions in the lung to regulate inflammatory gene expression following CS exposure, and that it can regulate inflammatory genes in *trans* under these conditions.

4.3 Results

4.3.1 *In vivo* CS exposure alters the expression of genes at the RNA and protein levels in BMDMs

To determine the impact of CS exposure on inflammatory pathways, we utilized a well-established CS-induced inflammation model, whereby WT mice were subjected to main and side stream CS for 8 weeks (Vandivier and Ghosh, 2017; Hautamaki et al., 1997; Cloonan et al., 2016; Mizumura et al., 2014). Bone marrow from RA and CS exposed mice was harvested and differentiated into bone marrow-derived macrophages

by culturing for 8 days in L929 media. RNA sequencing (RNA-seq) was performed to investigate the effect of *in vivo* CS exposure on the macrophage transcriptome (**Fig.4.1, A**). Differential expression (DESeq2) analysis revealed a significant number of dysregulated protein-coding genes following CS exposure (**Fig.4.1, B**). Interestingly, most protein coding genes were up-regulated (102 genes) while only 22 protein coding genes were down-regulated following CS exposure. While CS smoke has been shown to robustly increase the expression of matrix metalloproteinases in the lung, we do not see any change in MMPs in macrophages in our model (**SFig.4.1, A**). To investigate if there was a common regulatory mechanism controlling the up-regulated genes, we used "i-cis target" (Herrmann et al., 2012), an analysis software that determines enriched transcription factor (TF) motifs (**Fig.4.1, C**). I-cis target showed high enrichment scores for inflammatory response related TFs, including type I IFN response (*Stat1*, *3,* and *4*) and NF-kB pathway (*Rela*) (**Fig.4.1, C**). Of all the TFs associated with CS exposure, only *Cebpb* and *Cepbd* were found to be significantly upregulated at the transcriptional level (**SFig.4.1, B**). To identify biological processes and pathways impacted by CS, we performed gene ontology (GO-term) analysis on up and down-regulated protein coding genes which, revealed enrichment for immune response associated pathways, including IL1, IFNG and TNFA response biological processes as well as the involvement of NF-kB and JAK-STAT in KEGG pathways (**Fig.4.1, D and E**). We also observed enrichment of KEGG pathway terms associated with hypoxia as well as inflammatory diseases such as rheumatoid arthritis and cancer, both of which are associated with CS-exposure (**Fig.4.1, D**) (Kispert and McHowat,

2017; Chang et al., 2014). GO-term analysis of downregulated protein-coding genes was enriched in the regulation of RHO protein signal transduction (**SFig.4.2**), previously reported to be dysregulated by smoke exposure (Unachukwu et al., 2017; Park and Kim, 2017). To determine whether CS exposure effects extend beyond changes at the level of transcription, we next measured cytokine and chemokine protein production changes secreted from CS-exposed BMDMs by ELISA, finding that IL9, IL16, GCSF, and VEGF were all significantly downregulated by CS (**Fig.4.1, F-I**). These results revealed that *in vivo* CS exposure can shape the bone marrow niche, impacting a newly developed macrophage's baseline levels of RNA and protein expression.

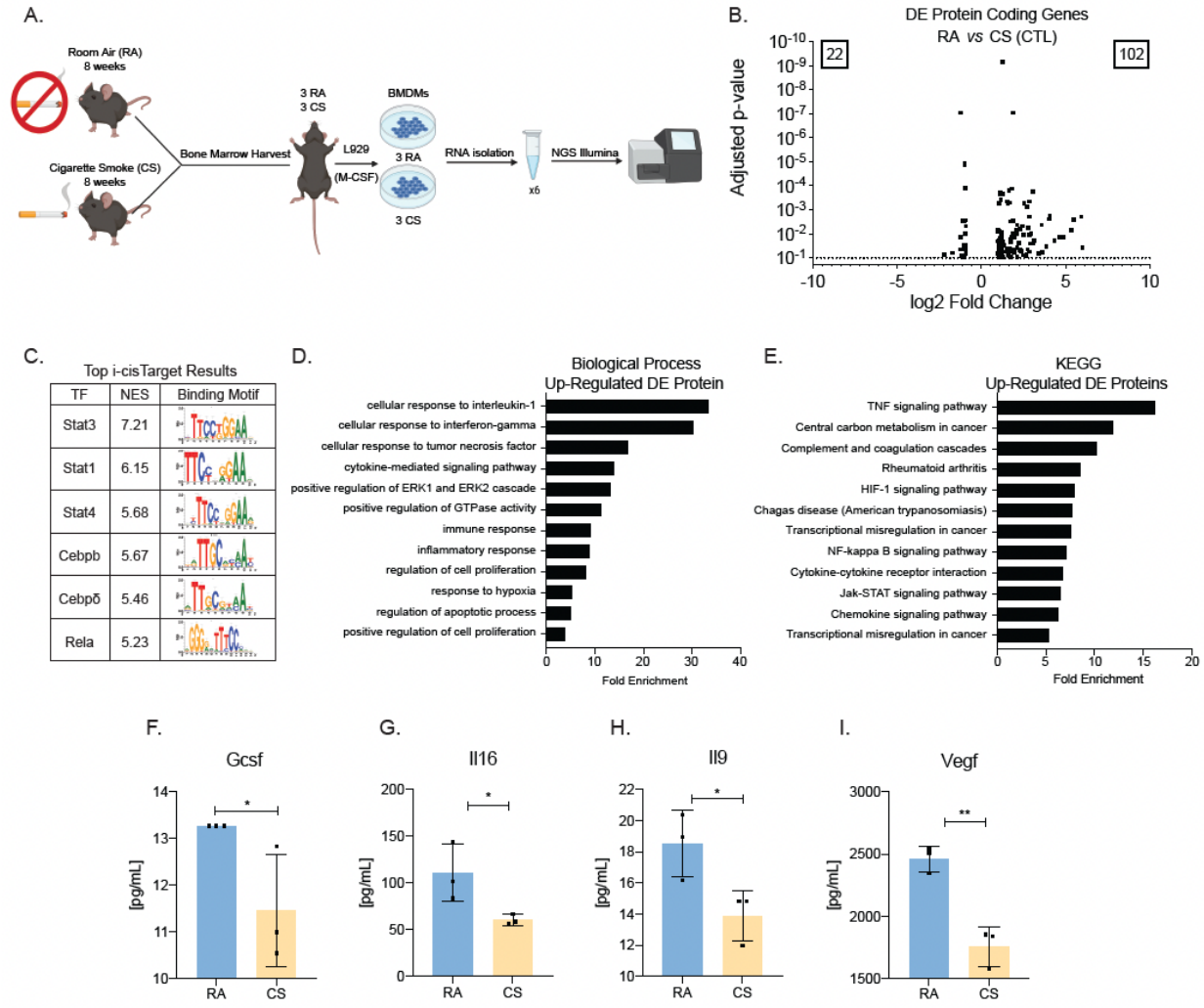


Figure 4.1 Cigarette smoke exposure activates inflammatory protein-coding genes in bone marrow-derived macrophages. (A) Schematic of cigarette smoke exposure and RNA-sequencing experiment L929 (MCSF) induced BMDMs. (B) Volcano plot of all differentially expressed protein-coding genes when comparing BMDMs from room air (RA) to cigarette smoke (CS) mice. (C) All promoters of upregulated protein-coding genes were analyzed using i-cisTarget to assess the top transcription factors regulating genes, sorted by normalized enrichment score (NES) and binding motif. (D) The associated biological process of upregulating differentially expressed protein-coding genes from CS exposure using DAVID tools. (E) The associated KEGG pathways of upregulated differentially expressed protein-coding genes from CS exposure using DAVID tools. The supernatant was harvested from RA and CS cultured BMDMs and multiplex cytokine analysis was performed for (F) GCSF (G) IL16, (H)

IL9, (I) VEGF. Each dot represents an individual animal (N=3 RA and CS). Error bars represent the standard deviation of biological triplicates. Student's t-tests were performed using GraphPad Prism, represented by statistical brackets. Asterisks indicate statistically significant differences between mouse lines using student's t-test (*$p \geq 0.05$, **$p \geq 0.01$).

4.3.2 *In vivo* CS exposure drives alternative splicing events including intron retention in BMDMs

RNA-seq data is commonly used to determine changes in gene expression under different conditions, but it can also be used to profile alternative splicing events and uncover changes in isoform usage. To this end we utilized two different splicing tool pipelines, rMATS and JuncBASE+DRIMSeq (**SFig.4.3).** We incorporated information from both toolsets and performed manual inspection of reported splice events via sashimi plots. Here we showed that most genes undergoing alternative splicing following CS exposure were not differentially expressed (**Fig.4.2, A, SFig.4.4**). In total, rMATS identified 425 splicing events while JuncBASE+DRIMSeq identified 47 that occur in macrophages following *in vivo* CS exposure (padj < 0.1 $|\Delta PSI| > 10$) (**Fig.4.2, B**). The dominant alternative splicing events we identified using both JuncBASE and rMATS were retained intron (RI) events (**Fig.4.2, B**), with a total of six RI events across four genes common to both JuncBASE and rMATS analysis (**Fig.4.2, C**). One prominent RI event was preferential retention in the RA condition of the intron between exons 11 and 12 (as annotated in Gencode vM18 basic) of transcripts coding *Mib2*, a ubiquitin-protein ligase that mediates ubiquitination of proteins in the Notch signaling pathway (**Fig.4.2, D, SFig.4.5**). We validated this event using RT-PCR, confirming a significant decrease in the retained intron isoform under CS conditions

(**Fig.4.2, D-F, SFig.4.6**). These results highlight how CS exposure can modify the macrophage transcriptome by ways other than gene expression changes and showcases alternative splicing as a mechanism for functional modulation post smoke exposure.

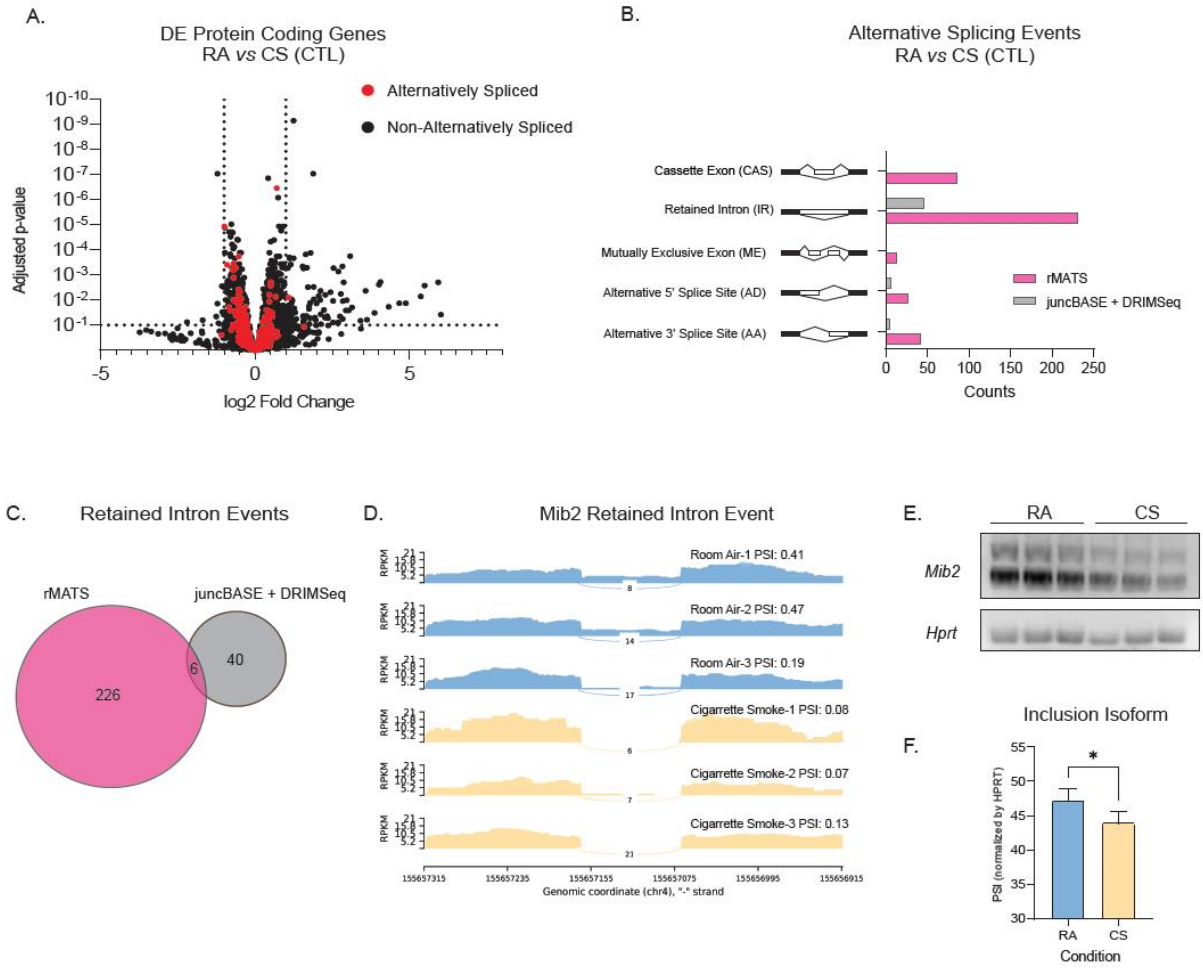


Figure4.2 Cigarette Smoke COPD induces alternative splicing in bone marrow-derived macrophages. (A) Significant event counts from rMATS and JuncBASE+DRIMSeq. Events were considered significant if FDR < 0.1, |PSI| > 10. **(B)** Venn diagram of significant retained intron events w[1]ith shared events determined by Bedtools' intersect function. **(C)** Sashimi plot of the Mib2 retained intron event. **(D)** RT-PCR gel results of Mib2 at the event site and loading control HPRT in biological triplicates and **(E)** percent spliced in (PSI) as determined by the average relative intensity of the inclusion form bands. Error bars represent standard deviation of

biological triplicates. Asterisk indicates statistically significant differences between conditions using Welch's t-tests (*$p < 0.05$).

4.3.3 LncRNAs are differentially expressed in response to CS exposure in BMDMs

Since CS exposure impacts transcription and splicing of protein-coding genes, we next determined if CS also influenced the expression of lncRNAs, which are themselves often products of alternative splicing. Using DESeq2 we identified 47 lncRNAs that were downregulated and 3 that were upregulated following *in vivo* CS exposure **(Fig.4.3, A**). LncRNAs can function in *cis* to regulate their neighboring genes or in *trans* to regulate genes on different chromosomes (Robinson et al., 2020). To gain insight into the mechanism of action of the dysregulated lncRNAs, we first performed bioinformatic analysis to determine the expression of the proteins neighboring the lncRNAs to ascertain if they are co-regulated which can provide insights into the mechanism of action of the lncRNAs (guilt by association) (Rinn and Chang, 2012). We utilized the genomic regions enrichment of annotation tool (GREAT) (McLean et al., 2010) to identify the neighboring protein-coding genes for all down-regulated lncRNAs based on the genomic location (**Fig.4.3, B**). Gene ontology (GO) analysis of the proximal protein-coding genes are associated with negative regulation of TGFB production and mRNA splicing regulation (**Fig.4.3, C**). To further assess the guilt by association model and the possibility that these lncRNAs might function in a *cis*-regulatory mechanism, we examined the normalized counts of the neighboring protein-coding genes in our RNA-seq data and found that 9 of the protein-coding genes neighboring down-regulated lncRNAs were significantly differentially expressed. Specifically, 7 of the neighboring protein-coding genes were co-regulated while 2 were

anti-correlated in expression (**Fig.4.3, D**). Of the 3 up-regulated lncRNAs all neighboring protein-coding genes were co-regulated (**Fig.4.3, E**). Most importantly, *lincRNA-Cox2* was identified as a significantly induced lncRNA following smoke exposure, similar to its neighboring protein-coding gene *Ptgs2* (*Cox2*), a critical inflammatory response gene (**Fig.4.3, E-F**). These results highlight how CS exposure can impact the expression of lncRNAs that play important roles within the regulatory network that governs the innate immune response in macrophages.

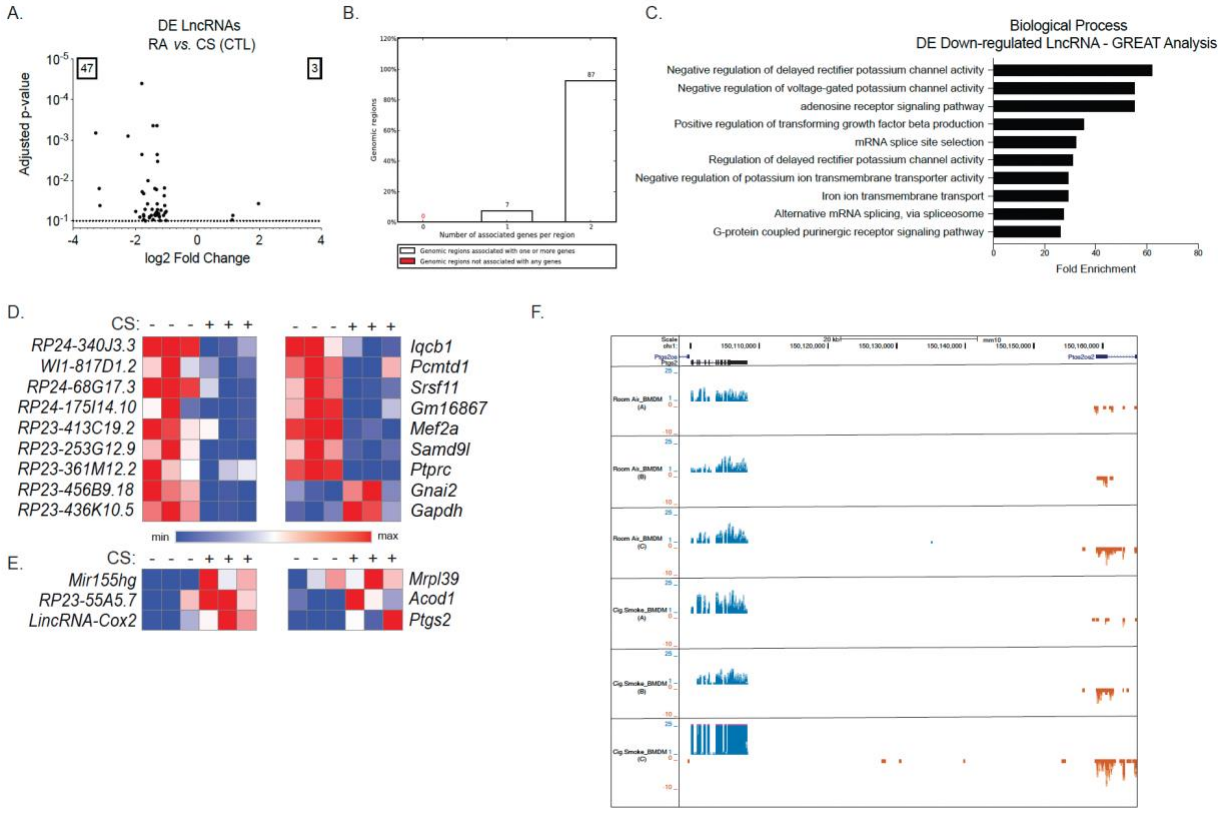


Figure4.3 LncRNA regulated by cigarette smoke in bone-marrow-derived macrophages harvested from COPD mice. (A) Volcano plot of all differentially expressed long non-coding RNA genes when comparing BMDMs from room air (RA) to cigarette smoke (CS) mice. **(B)** Bar-graph of the number of associated genes per region generated using GREAT. **(C)** The neighboring protein-coding genes of upregulated differentially expressed lncRNA genes from CS exposure also determined

the associated biological process using GREAT analysis. **(D)** A heat map of only significantly DE protein-coding and associated lncRNA genes from GREAT was generated with normalized read counts from RNA-seq of BMDMs with and without cigarette smoke (CS). **(E)** A heat map of up-regulated lncRNAs and associated protein-coding genes based on normalized read counts. **(F)** UCSC Genome Browser shot of 7 separate tracks. The top track is of M18 gene annotations, including *Ptgs2* (black) and *lincRNA-Cox2* (blue). Tracks following 2-4 tracks are stranded RNA-Sequencing reads of BMDM from room air (RA) mice. Tracks 5-7 are of stranded RNA-Sequencing reads of BMDM from cigarette smoked (CS) mice. Blue reads are positive stranded and the orange reads are negative stranded.

4.3.4 Loss of *lincRNA-Cox2* results in dysregulated responses to CS exposure *in vivo*

We have recently reported that *lincRNA-Cox2* is a critical regulator of lung homeostasis and acute lung inflammation (Robinson et al., 2022) but have yet to determine the impact of *lincRNA-Cox2* during smoke-induced inflammation. Since *lincRNA-Cox2* is upregulated following CS exposure, we examined WT and *lincRNA-Cox2* deficient (Mut) mice (Elling et al., 2018) in an 8-week CS exposure model to explore the role for *lincRNA-Cox2*-mediated regulation in smoke-induced inflammation (**Fig.4.4, A**). CS exposure is documented to shape the lung environment and promote pathogenesis through increased cell recruitment to the lung, specifically macrophages through infiltration (Niewoehner et al., 1974; Strzelak et al., 2018). We assessed cell numbers in bronchoalveolar lavage fluid (BALF) from room air (RA) and CS exposed WT and *lincRNA-Cox2* deficient mice and found that BALF from CS exposed mice displayed an overall increase in infiltrating leukocytes, predominantly composed of macrophages when compared to RA mice (**SFig.4.7, A-D**). While the cell numbers and macrophages trended upwards in the CS treated *lincRNA-Cox2* mutant mice, there were not statistically significant compared to WT smoke-exposed mice (**SFig.4.7, B-**

C). To assess CS-associated inflammation, we harvested bronchoalveolar lavage fluid (BALF), lung tissue, and serum of mice exposed to 8 weeks of RA or CS and measured cytokine and chemokine levels. These experiments revealed a disruption of cytokine and chemokine expression locally in the lung (from BALF and tissue) and globally from the peripheral serum (**Fig.4.4, B-O**) in *lincRNA-Cox2* deficient mice. At baseline in the room air mice, we see that loss of *lincRNA-Cox2* results in an increase in MIP2, IL1A and LIX in the lung (**Fig.4.4, N-O**) and a decreased in IL16 in the serum (**Fig.4.4, G**). Both GCSF and IL16 are known mediators in COPD pathogenesis (38, 39), and they were significantly downregulated in the BALF (**Fig.4.4, B-C**) and serum (**Fig.4.4, F-G**) while increased in the lung (**Fig.4.4, J-K**) of *lincRNA-Cox2* deficient (Mut) mice. Moreover, we found that some dysregulated cytokines were localized to the BALF (**Fig.4.4, D-E**), serum (**Fig.4.4, H-I, SFig.4.8, B-C**), or lung tissue compartments (**Fig.4.4, L-O, SFig.4.8, D-I**). For instance, we see MIP2 (CXCL2) is downregulated in the *lincRNA-Cox2* deficient mice only in BALF after CS exposure (**Fig.4.4, D**). Fractalkine is downregulated in BALF (**Fig.4.4, E**) and lung (**Fig.4.4, M**), while IL1A is upregulated in the serum (**Fig.4.4, I**) and lung (**Fig.4.4, N**) of *lincRNA-Cox2* deficient mice. These data suggest that *lincRNA-Cox2* exerts different effects within the lung compared to the periphery, and that *lincRNA-Cox2* exert control beyond the transcriptional level and can modulate smoke-induced signaling at the protein level within the lung.

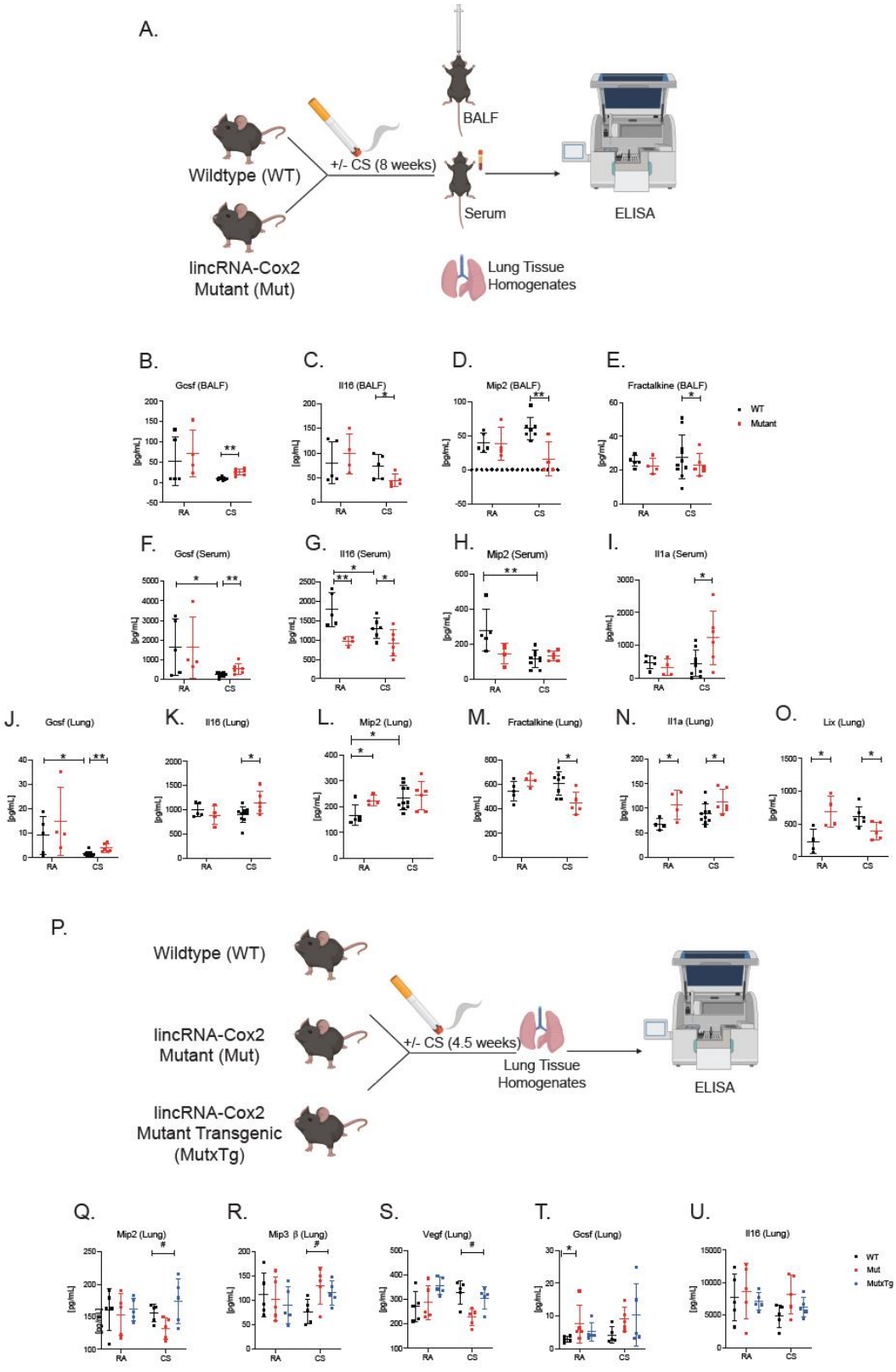

A.
Wildtype (WT)
lincRNA-Cox2 Mutant (Mut)
+/- CS (8 weeks)
BALF
Serum
ELISA
Lung Tissue Homogenates
B. Gcsf (BALF)
C. Il16 (BALF)
D. Mip2 (BALF)
E. Fractalkine (BALF)
WT
Mutant
F. Gcsf (Serum)
G. Il16 (Serum)
H. Mip2 (Serum)
I. Il1a (Serum)
J. Gcsf (Lung)
K. Il16 (Lung)
L. Mip2 (Lung)
M. Fractalkine (Lung)
N. Il1a (Lung)
O. Lix (Lung)
[pg/mL]
RA
CS
P.
Wildtype (WT)
lincRNA-Cox2 Mutant (Mut)
lincRNA-Cox2 Mutant Transgenic (MutxTg)
+/- CS (4.5 weeks)
Lung Tissue Homogenates
ELISA
Q. Mip2 (Lung)
R. Mip3 β (Lung)
S. Vegf (Lung)
T. Gcsf (Lung)
U. Il16 (Lung)
WT
Mut
MutxTg

Figure 4.4 *LincRNA-Cox2* positively and negatively regulates cytokines in a cigarette-smoke COPD model. (A) Experimental schematic of 8-week cigarette smoke COPD model using WT and *lincRNA-Cox2* mutant mice. After 8 weeks BALF, serm and lung tissue were harvested for subsequent cytokine analysis. ELISAs were performed on room air and cigarette-smoked BALF samples for **(B)** GCSF, **(C)** IL16, **(D)** MIP2 and **(E)** Fractalkine. Cytokine analysis was performed on serum for **(F)** GCSF, **(G)** IL16, **(H)** MIP2 and **(I)** IL1A. ELISAs were also performed on normalized lung tissue samples for **(J)** GCSF, **(K)** IL16, **(L)** MIP2, **(M)** Fractalkine, **(N)** IL1A and **(O)** LIX respectively from lung samples. **(P)** Experimental schematic of cigarette smoke COPD 4.5 week model for WT, *lincRNA-Cox2* mutant and *lincRNA-Cox2* MutxTg mice. Lung tissue was harvested for cytokine analysis to measure **(Q)** MIP2, **(R)** MIP3B, **(S)** VEGF, **(T)** GCSF and **(U)** IL16 respectively. Each dot represents an individual animal. Error bars represent standard deviation of biological replicates. Asterisks indicate statistically significant differences between mouse lines using Student's t-tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Student's t tests were performed using GraphPad Prism to obtain p values.

4.3.5 *LincRNA-Cox2* functions *in trans* to regulate gene expression

To gain insights into how *lincRNA-Cox2* might function mechanistically to control these gene expression changes, we wanted to determine if it was mediating its effects in *trans* since many of the affected cytokines are encoded on different chromosomes. To answer this question, we utilized our newly generated Mutant/Transgenic mouse model (MutxTg), whereby a transgenic mouse overexpressing *lincRNA-Cox2* ubiquitously in locus H11 was crossed with our *lincRNA-Cox2* deficient (Mut) mouse model (Robinson et al., 2022). The hypothesis was that if *lincRNA-Cox2* is functioning in *trans* to regulate gene expression then the differences observed in the *lincRNA-Cox2* deficient (Mut) mice would be rescued by the overexpression of *lincRNA-Cox2* in the Mutant/Transgenic mouse model (MutxTg). We performed a 4.5-week CS-exposure model on WT, *lincRNA-Cox2* deficient and *lincRNA-Cox2* MutxTg, lung tissue was harvested to measure cytokine and chemokine expression (**Fig.4.4, P**). Interestingly, we found several cytokines and

chemokines that are dysregulated in the *lincRNA-Cox2* mutant mouse and rescued in the *lincRNA-Cox2* MutxTg, including MIP2, MIP3B and VEGF (**Fig.4.4, Q-S**). Moreover, we found that while GCSF and IL16 are not significantly differentially expressed at the 4.5-week time point between the *lincRNA-Cox2* deficient and the *lincRNA-Cox2* MutxTg mice, they trend upwards in the *lincRNA-Cox2* deficient mice and are unchanged comparing WT and MutxTg mice (**Fig.4.4, S-T**). In total these data suggest that *lincRNA-Cox2* functions *in trans* to regulate immune genes within the lung following CS exposure.

4.3.6 *LincRNA-Cox2* regulates the acute inflammatory response in BMDMs pre-exposed to CS

Given the dysregulation of inflammatory inducible genes observed in BMDMs from CS exposure at resting states (**Fig.4.1**), we next aimed to determine how macrophage inflammatory response to stimulus could be altered following CS exposure. To assess this globally, we isolated and generated BMDMs from CS exposed mice (8 weeks) and compared control to LPS stimulated cells (**Fig.4.5 A**). Overall, we observed a subtle ablated inflammatory response in protein-coding genes as well as an overall decrease in lncRNA genes response following *in vivo* CS exposure (**Fig.4.5, B-C**). For the differentially expressed lncRNAs we did not observe any co-regulation between them and their neighboring protein coding genes (**Fig.4.5, C**). We also performed ELISAs to measure protein production levels in LPS stimulated BMDMs that were exposed to CS *in vivo*, we found that IL16 and IL12 (P40) were downregulated following CS exposure while MIP3B (CCL19) and MCP-5 (CCL12)

were upregulated (**Fig.4.5, D-G**). These data emphasize the complexity of the signaling pathways that are impacted following *in vivo* continual exposure to CS.

LincRNA-Cox2 has been widely studied in macrophages and shown to act as both a positive and negative regulator of immune genes following inflammatory activation genes (Robinson et al., 2022; Carpenter et al., 2013; Covarrubias et al., 2017; Tong et al., 2016; Hu et al., 2016; Hu et al., 2018; Liao et al., 2020; Xue et al., 2019). While it is clear *lincRNA-Cox2* provides a layer of regulation for inflammation driven by CS exposure *in vivo* (**Fig.4.4**), we next aimed to elucidate how *lincRNA-Cox2* impacts the acute inflammatory response in BMDMs, from mice exposed to CS for 4.5 weeks by measuring cytokine and chemokine production using ELISAs (**Fig.4.5, H**). First, we found that BMDMs differentiated from 4.5-week CS exposed WT, *lincRNA-Cox2* deficient and *lincRNA-Cox2* MutxTg mice displayed no significant changes in cytokine levels between the genotypes either after RA or CS (**SFig.4.9, B-J**). However, we found LPS treated BMDMs from *lincRNA-Cox2* deficient mice exposed to CS for 4.5 weeks exhibited higher IL16, GCSF, IL1A, MDC and IL11 in comparison to WT mice (**Fig.4.5, I-N**). Additionally, we found that IP10 (CXCL10) and MCSF (CSF1) were downregulated in BMDMs from *lincRNA-Cox2* deficient mice (**Fig.4.5, O-P**) compared to WT mice. Importantly, we found that all of the affected cytokines were rescued in the MutxTg mice, indicating that *lincRNA-Cox2* regulates these genes in *trans* through a yet unknown mechanism (**Fig.4.5, I-P**). Overall, our data supports *lincRNA-Cox2's* involvement in regulating the macrophage response to smoke

exposure and its contribution to driving an exacerbated inflammatory response to stimulus in macrophages.

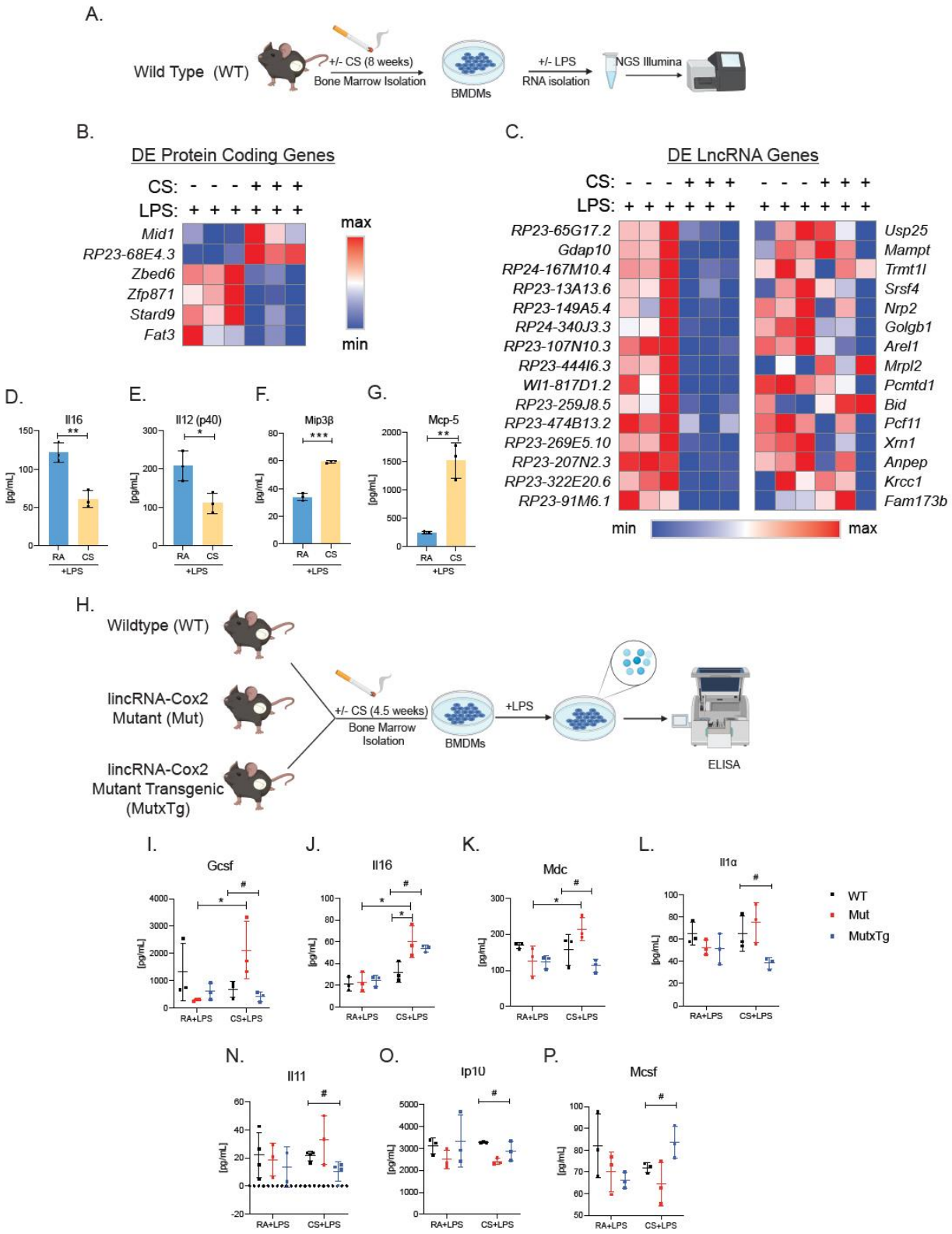

A.
Wild Type (WT)
+/- CS (8 weeks)
Bone Marrow Isolation
BMDMs
+/- LPS
RNA isolation
NGS Illumina
B.
DE Protein Coding Genes
CS: - - - + + +
LPS: + + + + + +
Mid1
RP23-68E4.3
Zbed6
Zfp871
Stard9
Fat3
max
min
C.
DE LncRNA Genes
CS: - - - + + + - - - + + +
LPS: + + + + + + + + + + + +
RP23-65G17.2
Gdap10
RP24-167M10.4
RP23-13A13.6
RP23-149A5.4
RP24-340J3.3
RP23-107N10.3
RP23-444I6.3
WI1-817D1.2
RP23-259J8.5
RP23-474B13.2
RP23-269E5.10
RP23-207N2.3
RP23-322E20.6
RP23-91M6.1
Usp25
Mampt
Trmt1l
Srsf4
Nrp2
Golgb1
Arel1
Mrpl2
Pcmtd1
Bid
Pcf11
Xrn1
Anpep
Krcc1
Fam173b
min
max
D.
Il16
[pg/mL]
RA
CS
+LPS
E.
Il12 (p40)
F.
Mip3β
G.
Mcp-5
H.
Wildtype (WT)
lincRNA-Cox2 Mutant (Mut)
lincRNA-Cox2 Mutant Transgenic (MutxTg)
+/- CS (4.5 weeks)
Bone Marrow Isolation
BMDMs
+LPS
ELISA
I.
Gcsf
RA+LPS
CS+LPS
J.
Il16
K.
Mdc
L.
Il1α
WT
Mut
MutxTg
N.
Il11
O.
Ip10
P.
Mcsf

Figure 4.5 *LincRNA-Cox2* regulates the impact of cigarette smoke during acute inflammation in bone marrow-derived macrophages. (A) RNA-sequencing experimental schematic of BMDM COPD with LPS**. (B)** Heatmap generated for normalized counts of protein-coding genes from room air and cigarette smoked BMDMs post LPS treatment. **(C)** Heatmap of lncRNA genes and their respective neighboring protein-coding genes normalized counts from room air and cigarette smoked BMDMs post LPS treatment. Cytokines measured from supernatants of BMDMs using ELISA technique for **(D)** IL16, **(E)** IL12(P40), **(F)** MIP3B and **(G)** MCP5. **(H)** Experimental schematic for LPS treated BMDM harvest from WT, *lincRNA-Cox2* mutant and *lincRNA-Cox2* MutxTg mice exposed to room air or cigarette-smoked. Supernatants were harvested and ELISAs were performed to measure **(I)** GCSF, **(J)** IL16, **(K)** MDC, **(L)** IL1A, **(N)** IL11, **(O)** IP10 and **(P)** MCSF. Each dot represents an individual animal. Error bars represent standard deviation of biological triplicates. Asterisks indicate statistically significant differences between mouse lines using Student's t-tests (*$p < 0.05$, **$p < 0.01$). Number signs indicate statistically significant differences between mouse lines using one-way ANOVA statistical test (#$p<0.05$). Student's t tests and ANOVA tests were performed using GraphPad Prism to obtain p values.

4.4 Discussion

Cigarette smoking is a major risk factor for COPD which is characterized by chronic inflammation in the airways that results in irreversible airflow obstruction (MacNee, 2005). In this study, we investigated the impact of smoke on the bone marrow (BM) niche using an *in vivo* murine smoking model (Elektra K. Robinson et al., 2021). We were intrigued to find that BMDMs from 8-week CS exposed mice had a significant impact on the transcriptome. Transcriptional changes were not limited to gene expression; we showed that smoke induced signaling can modulate the splicing landscape of BMDMs and identified a prevalence of retained intron events in macrophages from smoked mice in addition to other events such as exon skipping (cassette exon). Interestingly, many of the proteins shown here to undergo alternative splicing are not themselves altered at the gene expression level in response to smoke

exposure, highlighting the importance of splicing analysis to uncover changes to the transcriptome that gene expression changes studies cannot detect.

Our differential gene expression analysis revealed disruption in many proteins with many of them being overexpressed following smoke exposure and regulated by transcription factors such as STAT1, STAT3 and STAT4, as well as CEBPB, CEBPD, and NFKB (Yang et al., 2006). Mechanistically, smoke induces oxidative stress in the lung which is thought to activate redox sensitive transcription factors (TFs), such as NFKB, leading to inflammatory pathway activation (Yang et al., 2006). Additionally, STAT3 and STAT4 are also reported to be activated by smoke exposure and STAT3 was shown to impact immune cell recruitment and inflammatory cytokine production following smoke exposure (Geraghty et al., 2013; Di Stefano et al., 2004). While it is not surprising that many of the protein coding genes activated by CS belong to pathways that are under the regulation of NFKB and STATs, our study is one of first to demonstrate this is occurring in the bone marrow compartment, a site distant from immediate smoke exposure. We confirmed this immune dysregulation at the protein level showing altered levels of IL9, IL16, GCSF, and VEGF, all of which have been reported to be disrupted in response to CS exposure (Tsantikos et al., 2018; Elisia et al., 2020; Andersson et al., 2004; Shiels et al., 2014; Volpi et al., 2011; Zou et al., 2018; Huang et al., 2017). Trends of dysregulation (up or downregulation) of these cytokines following CS exposure are complex and appear in some cases to be cell type specific as well as being dependent on the nature and duration of exposure.

A recent study has reported that lncRNAs can be activated or repressed post long-term smoke exposure (H. Zhang et al., 2019). Interestingly, we found an overall repression of macrophage lncRNAs and their associated protein coding genes in response to 8-week CS exposure, with *LincRNA-Cox2* being only one of three lncRNAs that were transcriptionally induced by CS. LncRNAs can serve as regulatory elements, modulating expression of either neighboring protein coding genes (*in cis*) or genes on distal chromosomes (*in trans*) (Robinson et al., 2020; Kopp and Mendell, 2018). Specifically, we show that induction of *lincRNA-Cox2* is accompanied by induction of associated protein coding gene *Ptgs2* (*Cox2*), which is consistent with our previous findings (Elling et al., 2018). Most importantly, several lncRNAs that were upregulated, or downregulated following CS exposure showed similar patterns of expression compared to their neighboring protein coding genes, suggesting that they are co-regulated. For this study we focused our efforts to understand how *lincRNA-Cox2* is functioning following CS exposure, but future work could focus on the other 11 lncRNAs, their neighboring protein coding genes and their regulatory roles following CS exposure.

Since *lincRNA-Cox2* is induced by CS exposure, we wanted to determine if it plays any mechanistic role in regulating immune gene expression following CS with a specific focus on the *trans* regulatory mechanisms of action of this lncRNA. In our previous work, we established that *lincRNA-Cox2* functions *in cis* to regulate its neighboring protein coding gene, *Ptgs2*. We illustrated through a knockout mouse model that *lincRNA-Cox2* promotes *Ptgs2* expression though an enhancer lncRNA

mechanism (Elling et al., 2018). To specifically investigate *lincRNA-Cox2* trans activity, we generated a "mutant" mouse model where splicing was impaired (Elling et al., 2018; Robinson et al., 2022) resulting in a knockdown of *lincRNA-Cox2* with no measurable impact on Ptgs2 expression (Elling et al., 2018). Utilizing this model allows us to investigate *lincRNA-Cox2 trans* regulatory activity independent of its function *in cis*. To this end, we exposed *lincRNA-Cox2* deficient mice and control mice to CS for 8 weeks and compared cytokine production across lung, BALF and serum. CS exposure led to a disruption in cytokine production where cytokines like IL16 and GCSF were dysregulated following CS exposure in the mutant mouse at a local and systemic level (**Fig.4.4**). Other cytokines like MIP2, Fractalkine and IL1A displayed differential expression in the *lincRNA-Cox2* mutant mouse following CS exposure suggesting that *lincRNA-Cox2* can impact some genes in a cell type specific manner. MIP2, MIP3B and VEGF were all found to be downregulated within the lungs of the *lincRNA-Cox2* deficient mice and interestingly they were all rescued back to WT levels in the *lincRNA-Cox2* MutxTg cross, indicating that *lincRNA-Cox2* can exert regulation on their production/secretion *in trans* (**Fig.3.4**).

Smoke exposure has been shown to lead to an exacerbated inflammatory response when a stimulus is introduced (Gaschler et al., 2009; Hardaker et al., 2010); where CS-exposed mice displayed increased pulmonary inflammation and lung damage as well as increased production of some key inflammatory cytokines. CS exposure has also been shown to alter the ability of macrophages to respond to inflammatory stimuli (Yang and Chen, 2018). We observed that following 4.5 weeks

exposure to CS the baseline levels of cytokines produced from BMDMs are similar between all genotypes and room air (RA) mice (**SFig.4.9**). Interestingly, 8 weeks of CS exposure impacted the baseline levels of cytokines in BMDMs compared to RA suggesting that prolonged exposure to CS over time can impact the baseline expression of genes emerging from the bone marrow niche (**Fig.4.1**). Genome wide RNA sequencing in combination with cytokine analysis revealed a skewed inflammatory response to LPS within smoke-exposed macrophages represented by dysregulation in coding genes expression and inflammatory cytokine secretion as well as an overall repression of lncRNA genes (**Fig.4.5**). This is especially interesting because it links CS exposure to a distorted immune response in which a macrophage has an altered response to a challenge, implicating CS in shaping the system's response to infections. This is supported by mounting evidence that implicates CS exposure in subverting immunity where CS results in an attenuated immune response in which the host fails to respond effectively to a bacterial or viral challenge (van Zyl-Smit et al., 2014; Phaybouth et al., 2006). Interestingly, loss of *lincRNA-Cox2* led to an exacerbated inflammatory response in LPS activated CS exposed macrophages, indicating that *lincRNA-Cox2* can shape the macrophage inflammatory response in CS exposed cells and alter their function. We do not yet know if it is only *lincRNA-Cox2's* expression in macrophages that drives the effects observed in the *in vivo* experiments in this study. Our recent work examining the role of *lincRNA-Cox2* in alveolar macrophages indicates that the gene functions in both resident and recruited macrophages and contributes to inflammation associated with acute lung injury (Robinson et al., 2022).

Further work is needed to determine the exact cell type contributing to the altered genes observed in this study in response to smoke exposure.

In conclusion our work sheds light on the broad impact of smoking on immune responses in the lung, serum and the bone marrow niche. We uncovered a novel impact of CS on gene expression and splicing in bone marrow derived macrophages. In addition, we uncovered a novel role for *lincRNA-Cox2* in regulating immune responses to smoke. It is important for us to broaden our understanding of the mechanism that underlies inflammation if we are to identify and develop effective areas for therapeutic intervention for smoke related inflammatory conditions such as COPD in the future.

4.5 Materials and Methods

4.5.1 Mice

Wild-type (WT) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred at the University of California, Santa Cruz (UCSC). All mouse strains were maintained under specific pathogen-free conditions in the animal facilities of UCSC and protocols performed in accordance with the guidelines set forth by UCSC and Weill Cornell Medical College Institutional Animal Care and Use Committees.

4.5.2 Smoke Exposure Protocol

Eight to twelve weeks old sex-matched mice were chosen at random and exposed to total body CS in a stainless-steel chamber using a whole-body smoke exposure device (Model TE-10 Teague Enterprises). CS is delivered via mainstream smoke via a TE-10 smoking machine in whole-body exposure chambers with 2-3hour sessions

daily from Monday through Friday. Each smoking session consists of 12 cycles, each lasting about 9 minutes and consuming 8 cigarettes per cycle to achieve 150 mg/m3 of CS particulate matter in the chamber. CS dose is calibrated using filter samples taken from each chamber and determining tobacco smoke particulate (TSP) numbers, using a timed sampling unit and gas volume meter. Once 150 mg/m3 of TSP is reached in the chamber it is maintained for 2 h, 5 days per week for 4.5 weeks or 8 weeks, as previously described (Cloonan et al., 2016; Mizumura et al., 2014). Age-matched male and female mice were used for all CS exposures. At the end of the exposure regimen, we euthanized mice by CO_2 narcosis.

4.5.3 Cell culture

BMDMs were generated by culturing erythrocyte-depleted BM cells in DMEM supplemented with 10% FCS, 5 mL pen/strep (100×), 500 μL ciprofloxacin (10 mg/mL), and 10% L929 supernatant for 7 to 14 d, with the replacement of culture medium every 2 to 3 d. BMDMs were stimulated with 200ng/mL LPS.

4.5.4 PCR

RT-PCR validation was completed using three biological replicates. KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems).

Primers: Mse_Mib2_F1:AGGTGGACACCAAGAACCAG, Mse_Mib2_R1: GGATGCATGGTGTAGCAGTG. Band intensities were measured for each band in each condition and sample using ImageJ (Schneider et al., 2012). The relative abundance of each isoform was calculated using the equation to calculate percent

spliced in (PSI) PSI = inclusion counts/(inclusion counts + exclusion counts) in each condition and sample to validate the computationally derived delta PSI values.

4.5.5 Harvesting Bronchoalveolar Lavage Fluid (BALF)

Bronchoalveolar Lavage Fluid (BALF) was harvested as previously stated by Cloonan et al. (Cloonan et al., 2016). Briefly, 40 mice were euthanized via CO_2 asphyxiation, intubated with a 20G 3 1" catheter (SR-OX2025CA; Terumo), and the lungs were lavaged with ice cold PBS (10010-023; Life Technologies) supplemented with 0.5 mM EDTA (351-027-061; Quality Biological) in 0.5-ml increments for a total 4mLs. BAL was centrifuged at 1500 x rpm for 5 minutes. 1 ml red blood cell lysis buffer (Sigma Aldrich) was added to the cell pellet and left on ice for 15 minutes followed by centrifugation at 1500 x rpm for 5 minutes. The cell pellet was resuspended in 1 ml PBS and leukocytes were counted using a hemocytometer. Specifically, 20 µl was removed for cell counting performed in triplicate using a hemocytometer and 200 µl removed for cytocentrifuge preparations (Shandon Cytospin3, 350 rpm for 3 minutes) and stained using the Hema3 staining system (Fisher Scientific). The percentage of macrophages, lymphocytes and polymorphonuclear leukocytes (PMNs) were counted in a total of 300 cells, and absolute numbers of each leukocyte subset were calculated.

4.5.6 RNA sequencing libraries

Libraries were read on a High-SEq 4000 as paired-end 150-bp reads. Sequencing reads were aligned to the mouse genome (assembly GRCm38/mm10) using STAR. Differential gene-expression analyses were conducted using DESeq2. GO enrichment analysis was performed using PANTHER. Data was submitted to National Center for

Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database, accession GSE184571.

4.5.7 Alternative Splicing Analysis

Aligned reads (BAMs) were analyzed for alternative splicing events with rMATS v4.1.1 package (Shen et al., 2014) and JuncBASE (from Docker image mgmarin/juncbase:0.9). A custom GTF file combining long and short read sequencing data, and documented previously in Robinson *et al*. (Elektra K. Robinson et al., 2021), was used by the programs as the input annotation. Following statistical analysis, the results from both analyses were filtered based on FDR, percent splice in (PSI), and number of reads supporting the event.

In addition to filtering by FDR and PSI as described above, events were required to have total read support > 10.

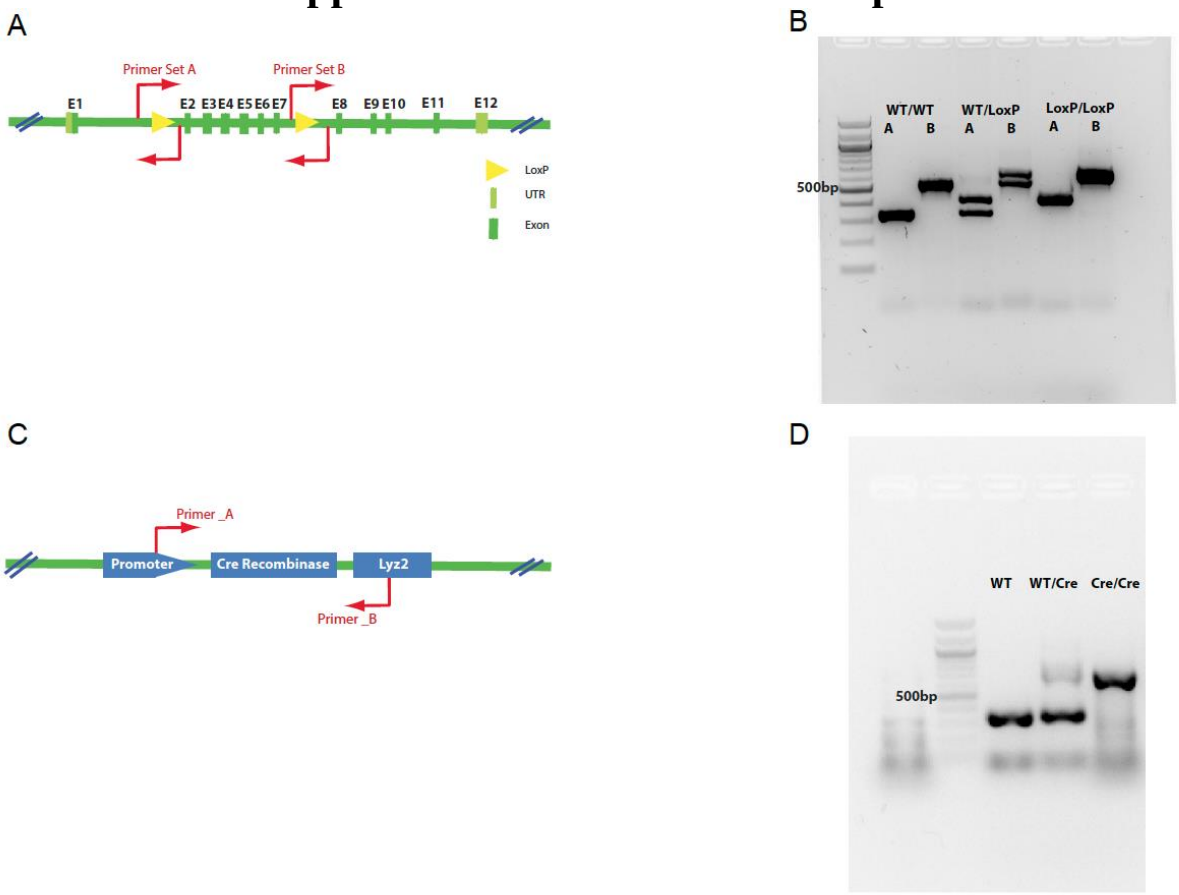
4.5.8 JuncBASE + DRIMSeq

In this analysis JuncBASE was used for generating event counts and DRIMSeq v1.20 was used for determining statistically significant events (Brooks et al., 2014; Nowicka and Robinson, 2016). Parameters used for JuncBASE were -j <intron coordinates from Gencode vM18> --jcn_seq_len 188. Parameters used for finding significantly differentially spliced events using DRIMSeq were: min_samps_gene_expr = 6, min_samps_feature_expr = 3, min_gene_expr = 10, min_feature_expr = 0.

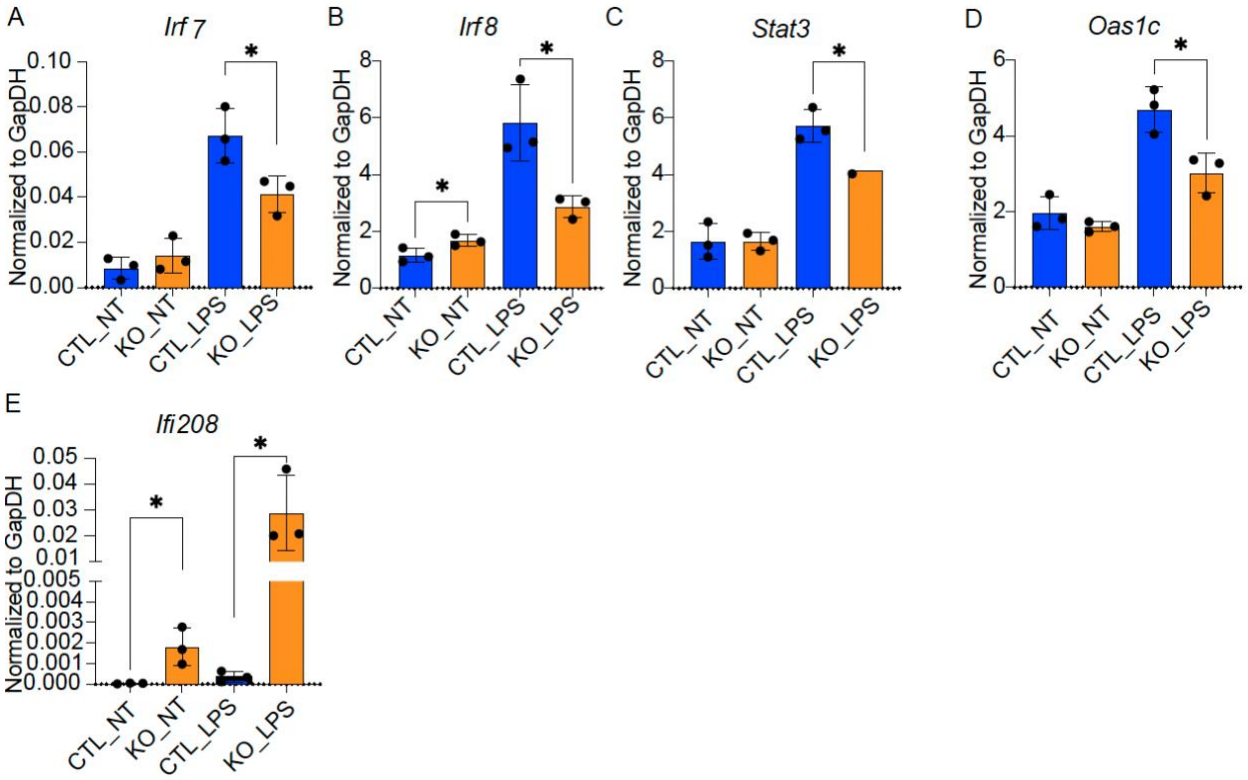
4.6 Acknowledgements

We thank Biorender for creating a platform to easily generate figures using “Biorender.com. This work was supported by the California Tobacco Related Disease Research Program grant 27IP-0017 to S.C and the Cornelius Hopper diversity award from the TRDRP to M.M.S.

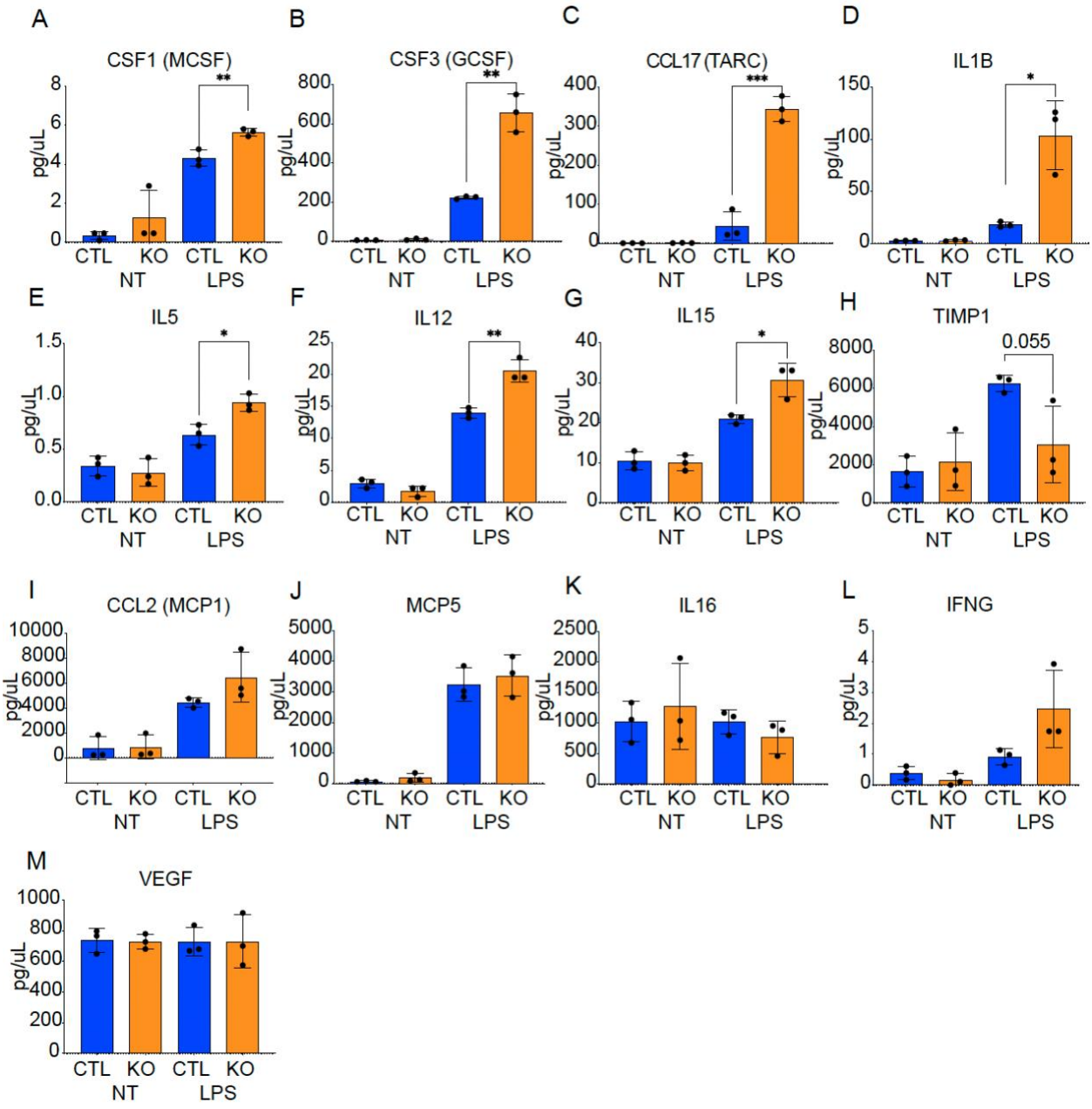
APPENDIX 1- Supplemental information to Chapter 2



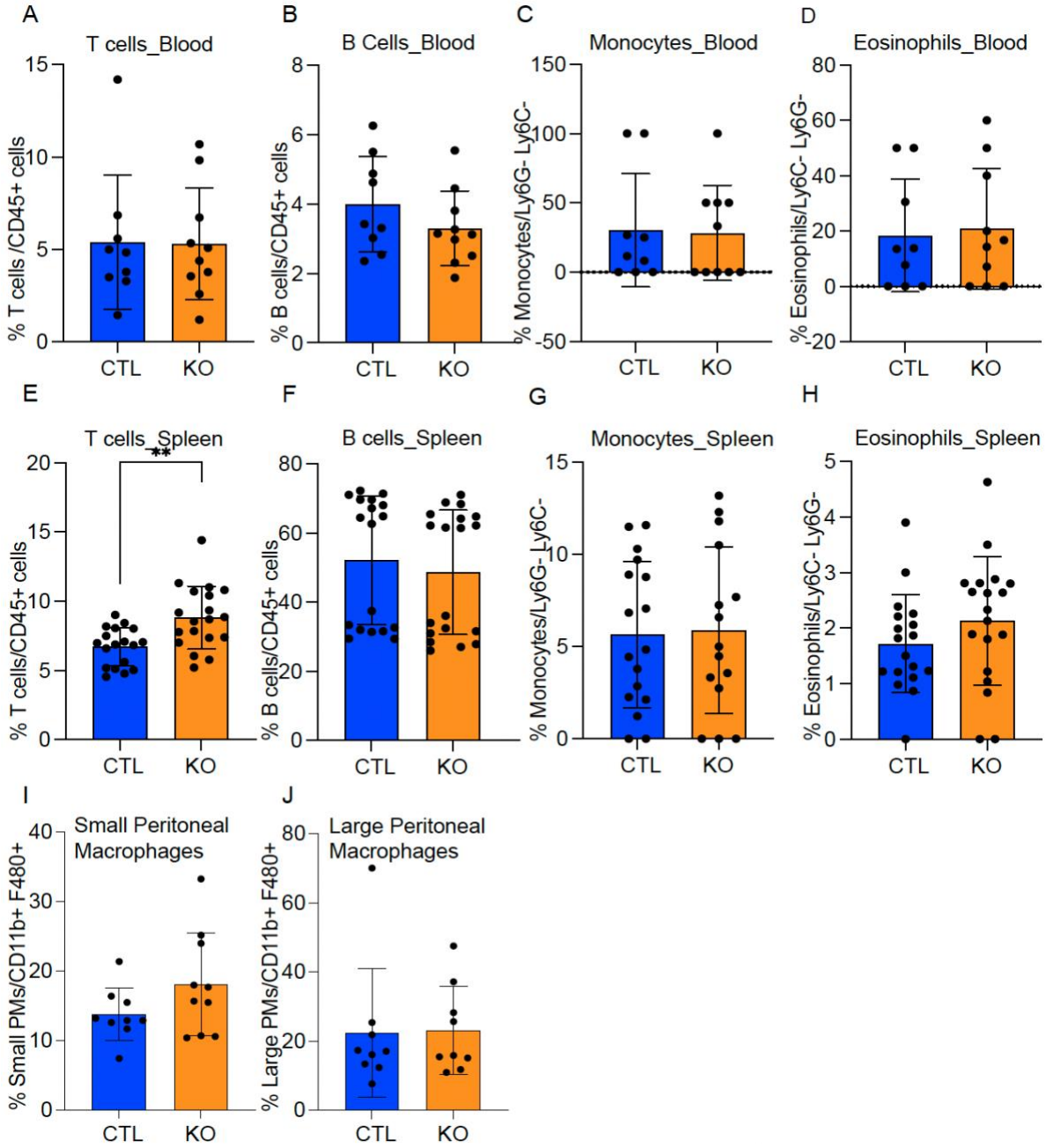
Supplementary figure 2.1 Genotyping HNRNPA2B1 KO mouse. A. Primer design for primer sets A and B which identify successful integration of the loxP sites flanking exons 2 and 7. **B.** Genotyping gel showing band sizes for HNRNPA2B1 locus as CTL, heterozygous for loxP and homozygous for loxP. **C.** Primer design to identify successful integration of cre recombinase downstream *Lyz2* promoter. **D.** Genotyping gel showing band sizes for CTL, Cre heterozygous and Cre homozygous locus.



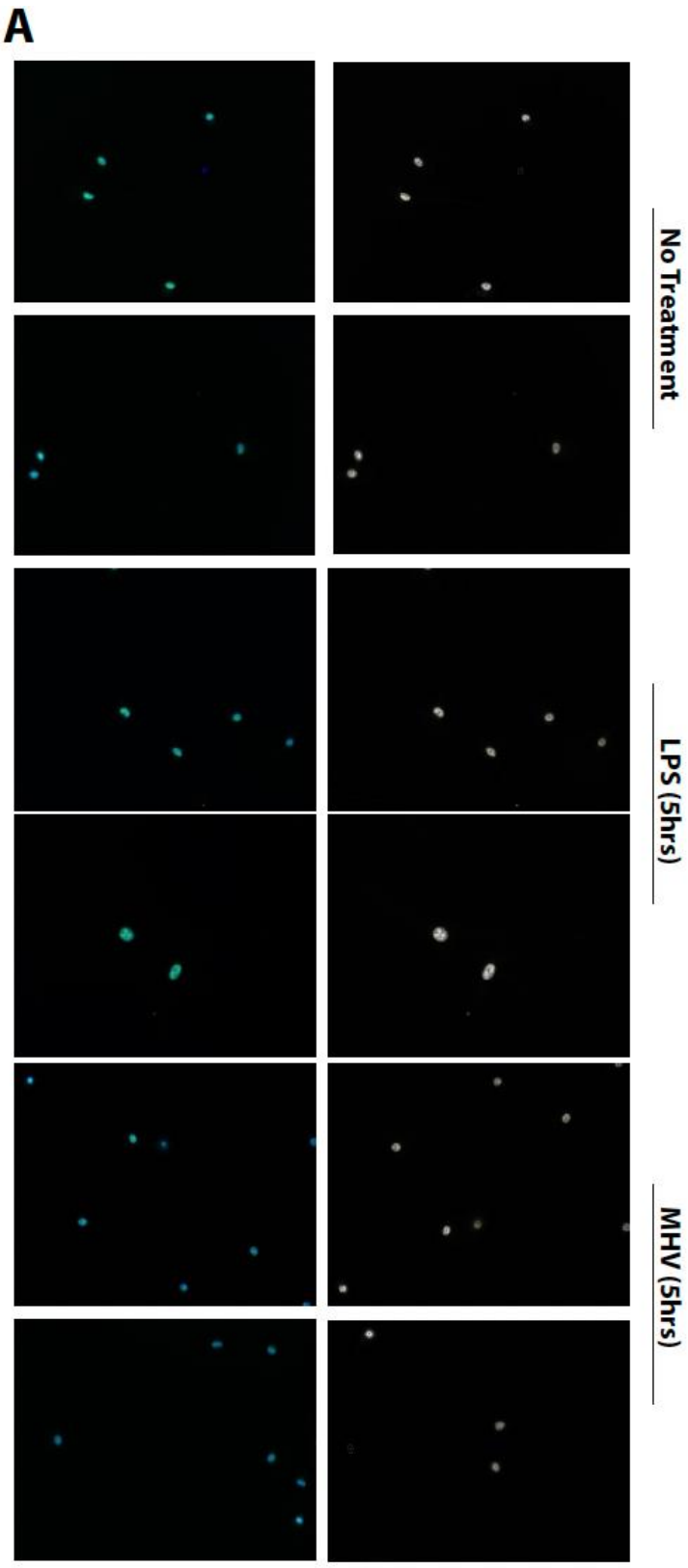
Supplementary figure 2.2 HNRNPA2B1 regulates IFN response genes transcriptionally. A-E. Normalized qRTPCR results for **A.** *Irf7***, B.** *Irf8***, C.** *Stat3***, D.** *Oas1c* and **E.** *Ifi208* Showing changes in CTL and KO BMDMs at baseline and after LPS stimulation. Student's t-tests were performed using GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines (*$P \leq 0.05$).



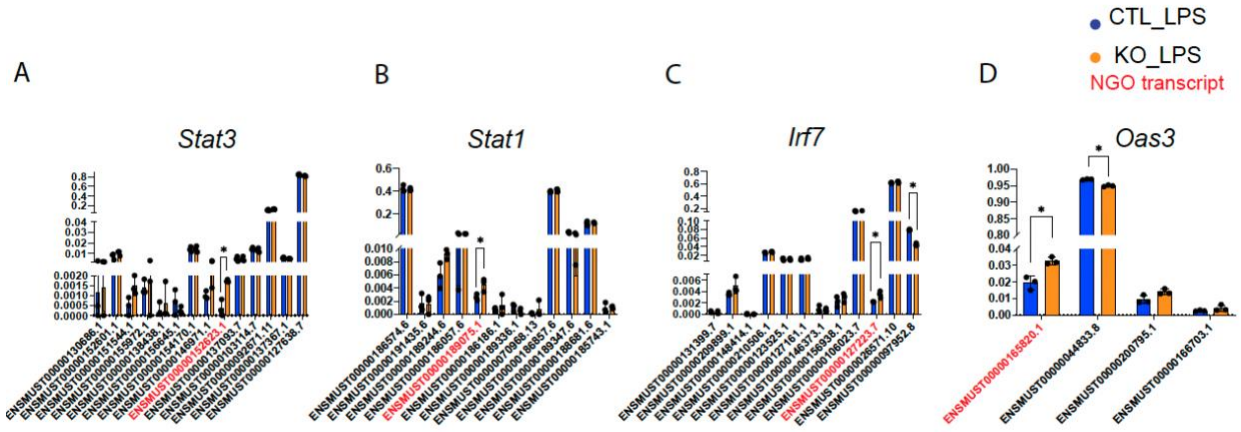
Supplementary figure 2.3 HNRNPA2B1 regulates inflammatory cytokine production under stimulus. A-M. Multiplex ELISA results of inflammatory cytokine changes in CTL and KO BMDM supernatant after treatment with LPS for 18 hrs. **A**. MCSF, **B.** GCSF, **C.** TARC, **D.** IL1B, **E.** IL5, **F.** IL12, **G.** Il15, **H.** TIMP1, **I.** MCP-1, **J.** MCP-5, **K.** Il16, **L.** IFNG, **M.** VEGF. Student's t-tests were performed using GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines (*P ≤ 0.05, **P ≤ 0.01).



Supplementary figure 2.4 Immune cell profiling in blood and spleen of CTL and KO mice. A-D. Immune cell levels in CTL and KO mice blood analyzed using flow cytometry **A.** T cells**, B.** B cells**, C.** Monocytes**, D.** Eosinophils**. E-H.** Immune cell levels in CTL and KO mice spleen analyzed using flow cytometry **E.** T cells**, F.** B cells**, G.** Monocytes**, H.** Eosinophils**. I.** Levels of small peritoneal macrophages**, J.** Levels of large peritoneal macrophages.


A
No Treatment
LPS (5hrs)
MHV (5hrs)

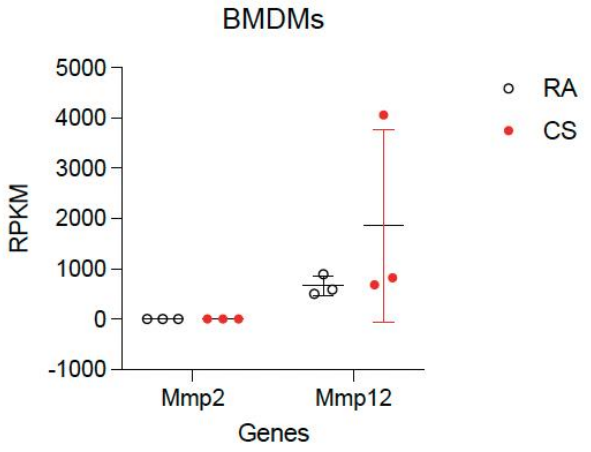
Supplementary figure 2.5 HNRNPA2B1 remains in the nucleus at baseline and after exposure to stimulus. A. Immunofluorescence Images of primary BMDMs. Nuclei stained with DAPI, HNRNPA2B1 stained with an anti-mouse secondary antibody conjugated to Alexa 488. Image processing using Fiji, nuclei boundaries were drawn using masks on DAPI channel and overlaid on to green channel to show that HNRNPA2B1 exists exclusively in the nucleus.



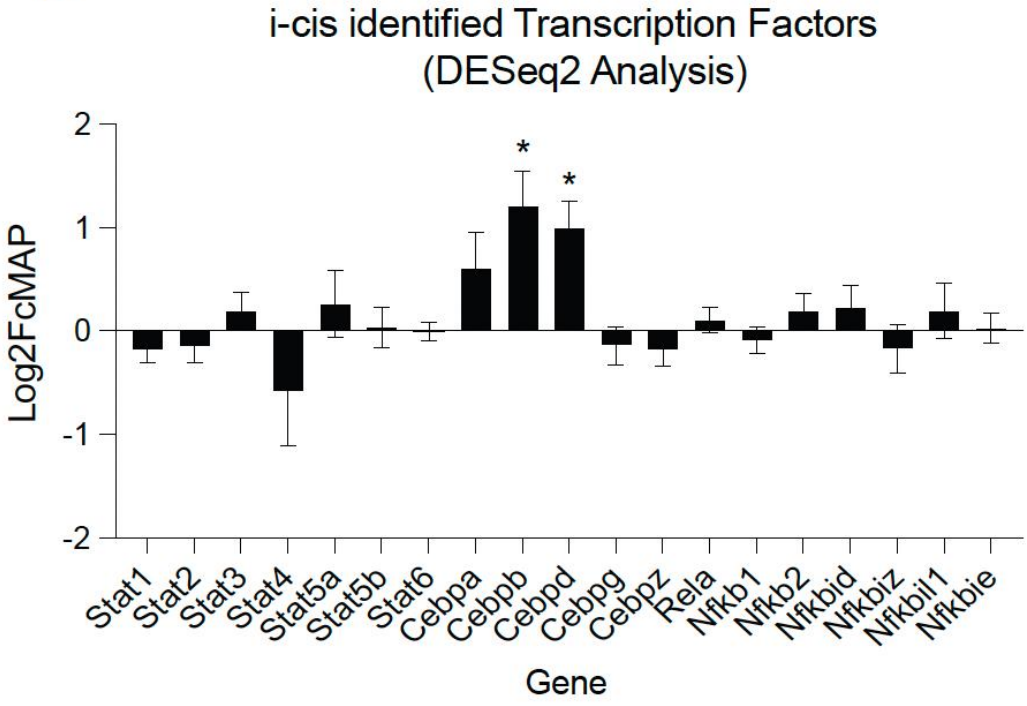
Supplementary figure 2.6 HNRNPA2B1 regulates alternative splicing of interferon response genes. A-D. Isoform usage levels analyzed by IUTA showing switching in isoform usage between CTL and KO BMDMs in **A.** *Stat3***, B.** *Stat1***, C.** *Irf7* **and D.** *Oas3***.**

APENDIX 2- Supplemental information to Chapter 4

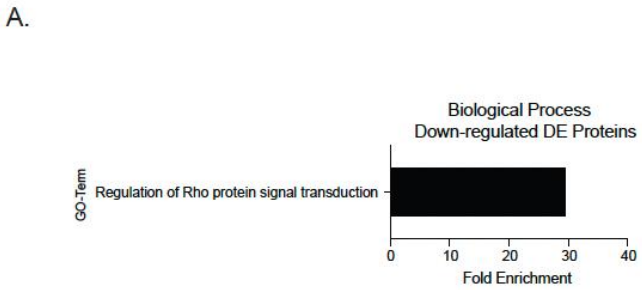
A.


BMDMs
5000
4000
3000
2000
1000
0
-1000
RPKM
RA
CS
Mmp2
Mmp12
Genes

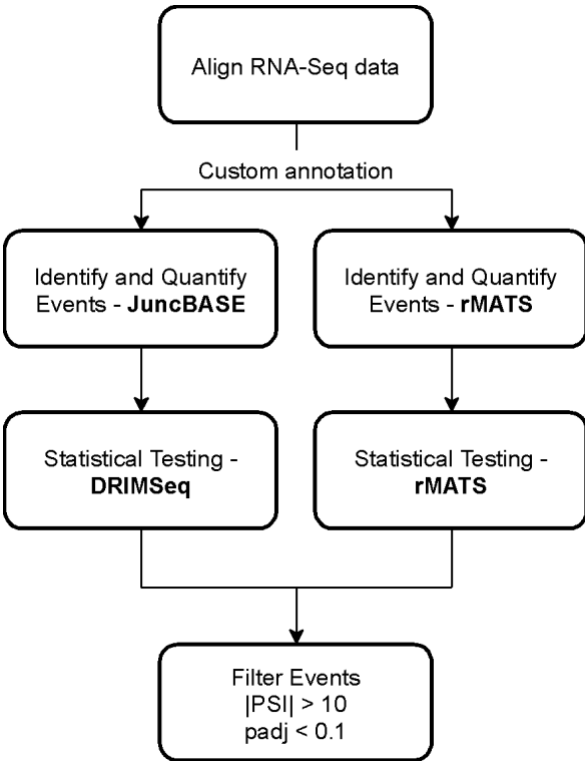
B.


i-cis identified Transcription Factors
(DESeq2 Analysis)
2
1
0
-1
-2
Log2FcMAP
*
*
Stat1
Stat2
Stat3
Stat4
Stat5a
Stat5b
Stat6
Cebpa
Cebpb
Cebpd
Cebpg
Cebpz
Rela
Nfkb1
Nfkb2
Nfkbid
Nfkbiz
Nfkbil1
Nfkbie
Gene

Supplementary Figure 4.1 Cigarette smoke COPD does not globally regulate the transcriptional expression of inflammatory transcription factors or MMPs. (A) Reads per kilobase of exon per million reads mapped (RPKM) of MMP2 and 12 as determined by DESeq2 analysis of BMDMs from room air *vs.* cigarette smoked mice (B) Bar plot of Log2FcMAP determined by DESeq2 analysis of BMDMs from room air *vs.* cigarette smoked mice. Asterisks indicate statistically significant differences between mouse lines using DESeq2 (*p < 0.05).

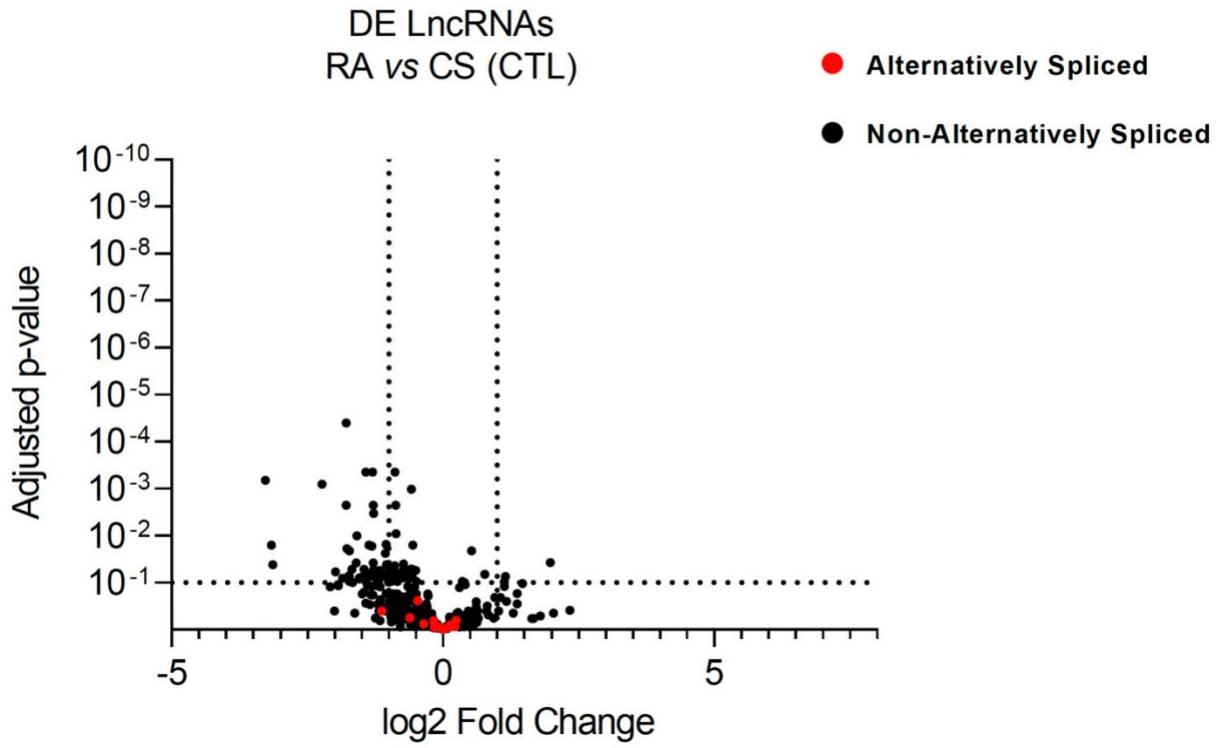


Supplementary Figure 4.2 Gene ontology analysis of CS down-regulated protein-coding genes in BMDMs. (A) The associated biological process of down-regulated differentially expressed protein-coding genes from CS exposure using DAVID tools.

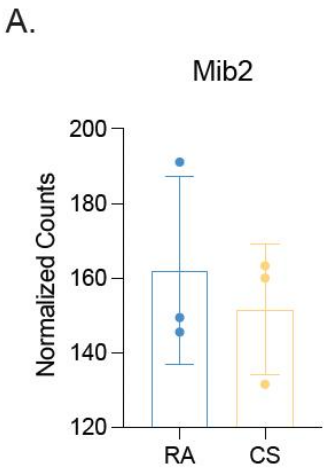


Supplementary Figure 4.3 Computational pipeline and comparison of t-test and DRIMSeq alternative splicing events.

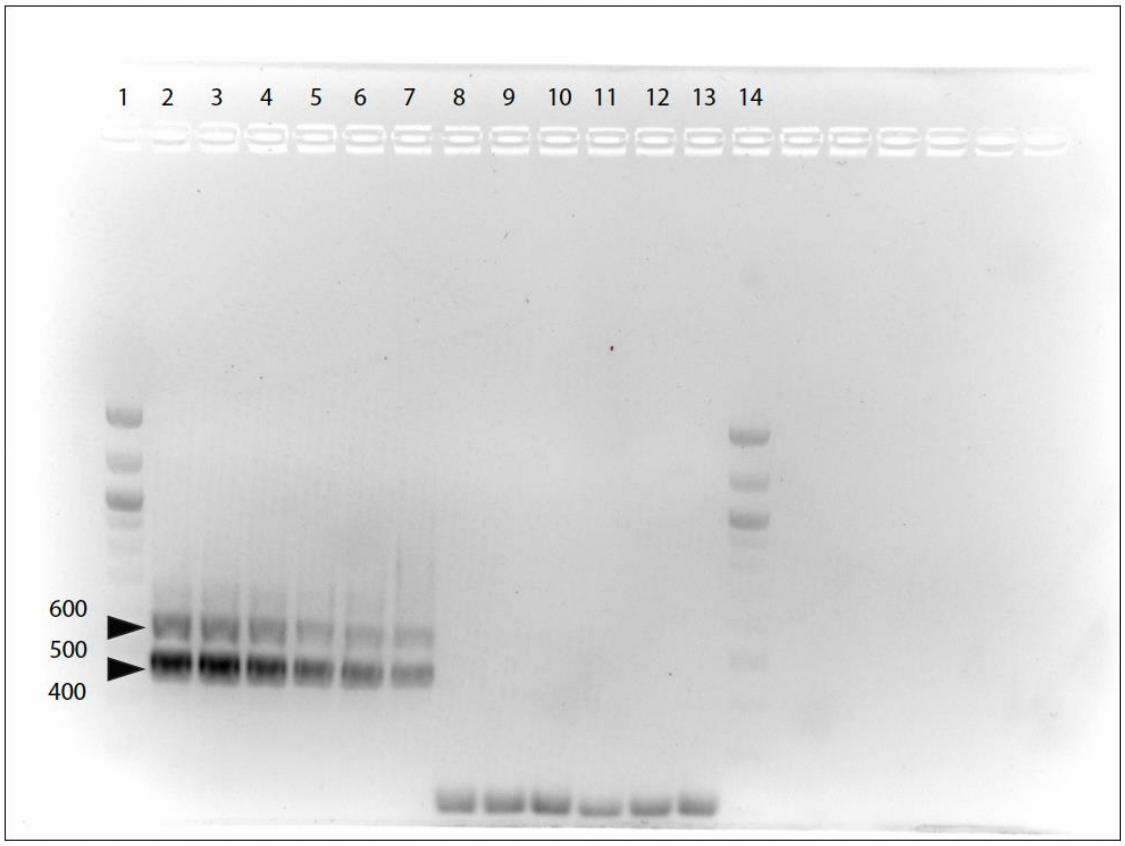
(A) Bioinformatic pipeline for human and mouse RNA-seq data. Alternative splicing event-type classification of significant differential splicing events (|ΔPSI| > 10 and adjusted p-value < 0.1) in mouse macrophages ± cigarette smoke (CS) exposure.



Supplementary Figure 4.4 Cigarette Smoke induces alternative splicing in long non-coding RNA bone marrow-derived macrophages. (A) Volcano plot of all differentially expressed long non-coding RNA when comparing BMDMs from room air (RA) to cigarette smoke (CS) mice with significantly differentially spliced genes (FDR < 0.1, |ΔPSI| > 10) across at least one event type marked.



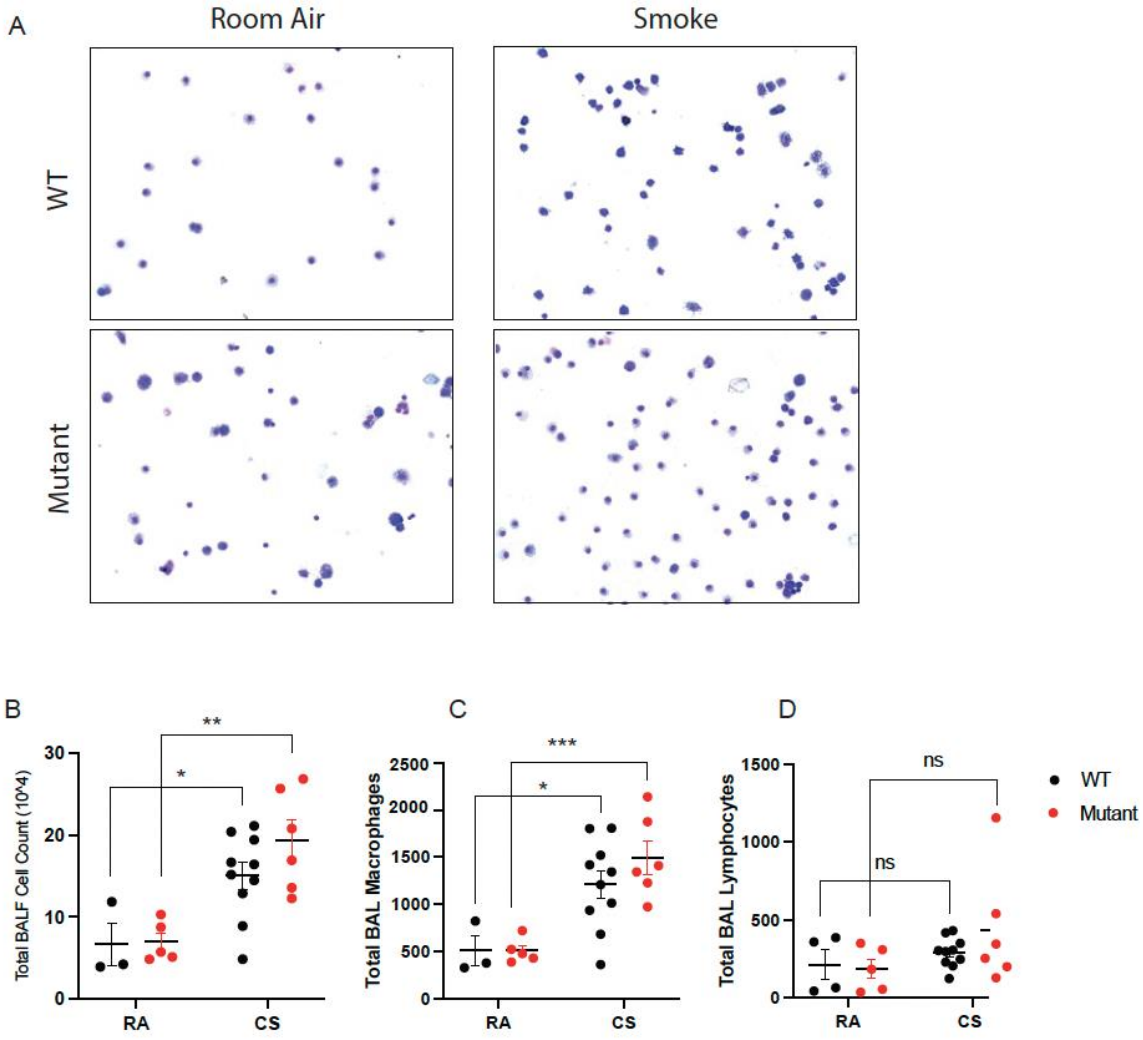
Supplementary Figure 4.5 Mib2 is not differentially expressed in bone marrow-derived macrophages from cigarette smoke exposed mice. (A) The normalized counts and DESeq2 analysis of *Mib2*.



Supplementary Figure 4.6 Full PCR gel image of *Mib2* retained intron event across samples

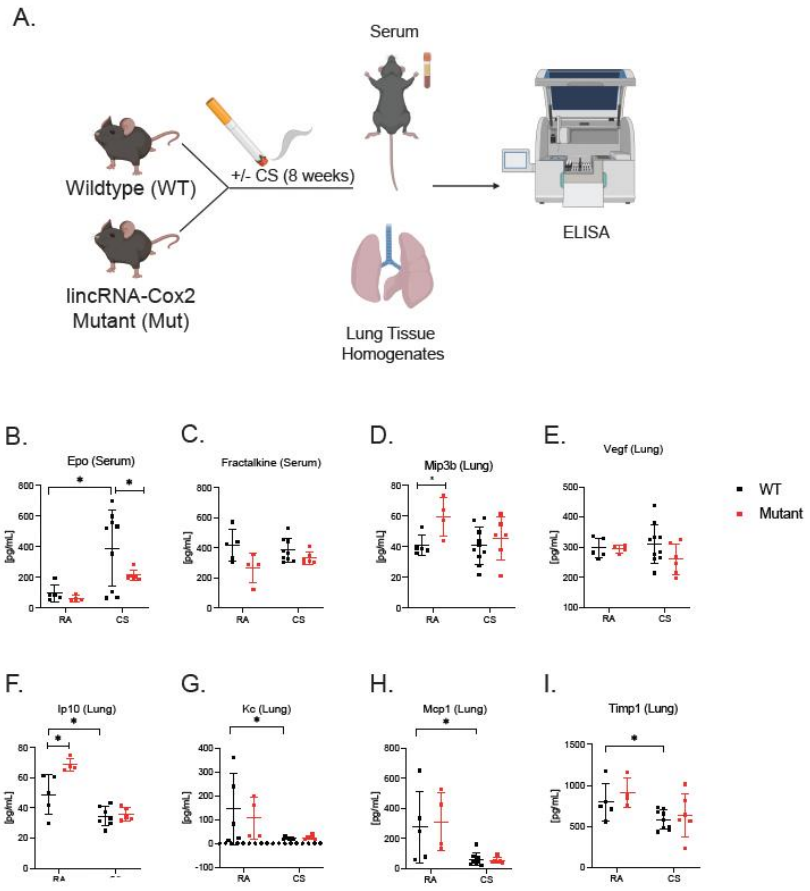
(A) PCR gel results of Mib2 at the RI event site and loading control HPRT in biological triplicates. Lanes 1 and 14 are 100 bp ladder. Lanes 2 through 4 are RA samples 1,2,3 and lanes 5 through 7 are CS samples 1,2,3 showing PCR amplification of the Mib2 RI

event. When the intron is retained a band of size 548 bps is expected, otherwise a band of size 452 bps. Lanes 8 through 13 are RA samples 1,2,3 followed by CS samples 1,2,3 showing amplification of the housekeeping gene Hprt.

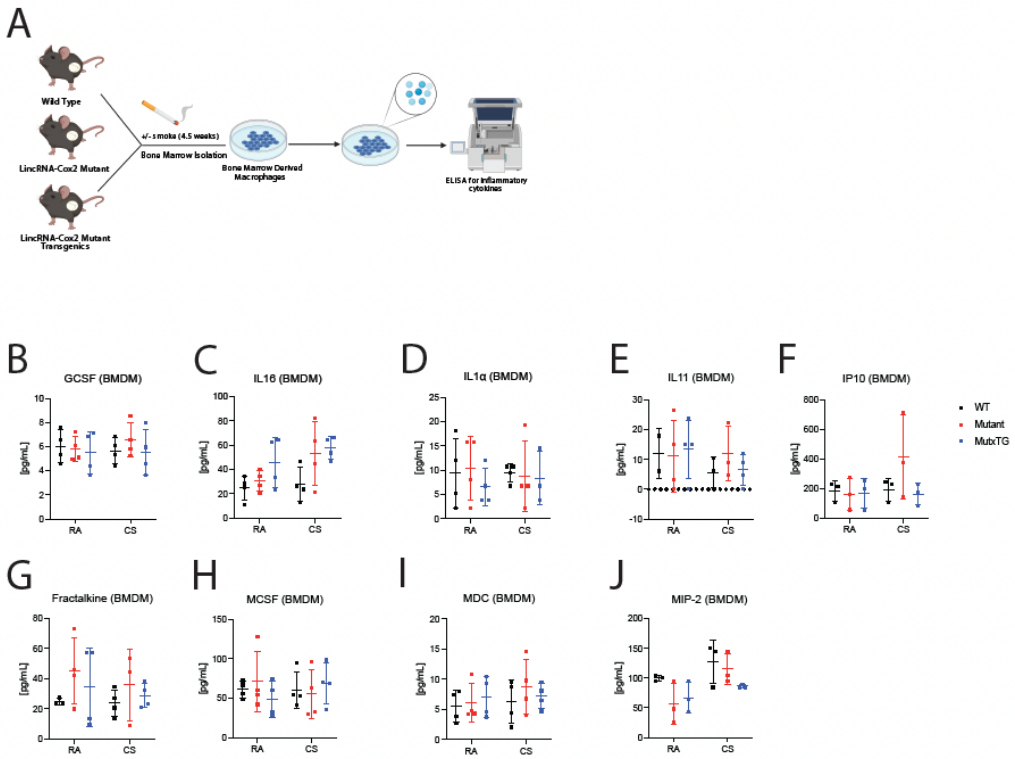


Supplementary Figure 4.7 Cigarette smoke increases total cell and macrophage numbers in BALF from CS exposed mice. (A) Cytospin images of total cells and macrophages in BALF from Wild type (WT) and *lincRNA-Cox2* mutant mice. Number of (B) total cells, (C) macrophages and (D) lymphocytes from BALF of CS and RA treated WT and *lincRNA-Cox2* mutant mice. Each dot represents an individual animal. Error bars represent standard deviation of biological replicates. Asterisks indicate statistically significant differences between mouse lines using Student's t-tests

(*p < 0.05, **p < 0.01, ***p < 0.001). Student's t tests were performed using GraphPad Prism to obtain p values. NS indicates not significant.



Supplementary Figure 4.8 Changes in inflammatory cytokine levels between WT and *lincRNA-Cox2* mutant mice under RA and CS treatments for 8 weeks. ELISAs were performed on serum for (B) Epo and (C) Fractalkine. ELISAs were performed on normalized lung tissue homogenates on (D) MIP3B, (E) VEGF, (F) IP10, (G) KC, (H) MCP1 and (I) TIMP1. Each dot represents an individual animal. Error bars represent standard deviation of biological replicates. Asterisks indicate statistically significant differences between mouse lines using Student's t-tests (*p < 0.05, **p < 0.01, ***p < 0.001). Student's t tests were performed using GraphPad Prism to obtain p values.



Supplementary Figure 4.9 *lincRNA-Cox2* does not regulate cytokines in BMDMs from cigarette smoked mice.

(A) Experimental schematic for BMDM harvested from WT, lincRNA-Cox2 mutant and *lincRNA-Cox2* MutxTg mice exposed to room air or cigarette-smoked. Supernatants were harvested and ELISAs were performed to measure (B) GCSF, (C) IL16, (D) IL1A, (E) IL11, (F) IP10, (G) Fractalkine, (H) MCSF, (I) MDC and (J) MIP2. Each dot represents an individual animal. Error bars represent standard deviation of biological triplicates. Asterisks indicate statistically significant differences between mouse lines using Student's t-tests (*$p < 0.05$, **$p < 0.01$). Student's t tests were performed using GraphPad Prism to obtain p values.

CHAPTER 5: Conclusion and future directions

This thesis is focused on expanding our traditional view of gene regulation which is typically restricted to transcriptional changes. In this thesis, I have presented evidence of alternative splicing as well as non-coding RNAs, specifically lncRNAs, playing a vital role in modulating gene expression under inflammatory and environmental stimuli. Majority of this work is myeloid centric; advocating for the essential role played by macrophages in the initial stages of infection where they regulate inflammatory activation intensity and as a result change the outcome of infection. Here, I will describe major conclusions from the information presented in every chapter as well as major future studies that can further advance our understanding of inflammatory signaling modes of regulation.

5.1 Sequencing technologies advancements are required to unravel the complexity of the transcriptome

Advances in sequencing technologies have been tremendously valuable in studying immune regulation through providing a wealth of information that can be analyzed alongside differential expression. Information about splicing, RNA modifications and isoform usage can be gleaned from NGS and long read sequencing, providing a great resource for transcriptional and post transcriptional changes in each cell type leading to different inflammatory outcomes. While functional assays are the bread and butter for biological studies, sequencing has been widely utilized as a

starting point to kick off investigation into gene involvement in a variety of cellular functions.

In the first chapter, I discuss LncRNAs, which are emerging as valuable targets to study in the scope of immune regulation as many have been identified as immune modifying through functioning in *cis* or *trans* to regulate immune gene expression. They represent attractive biomarkers or druggable targets for inflammatory and autoimmune diseases which requires highly developed tools to accurately quantify changes in lncRNA expression and isoform dominance in variable disease models.

Alternative splicing is the process by which a gene is able to code for multiple isoforms thus maximizing the complexity of gene function. Alternative splicing was found to be responsible for differences in gene isoforms under inflammatory conditions and to provide a new layer of regulation that's separate from gene expression changes. While short read NGS is able to capture some low resolution alternative splicing information, long read sequencing presents a valuable tool that's able to accurately call isoform expression and detect novel isoforms in highly repetitive regions of the genome.

Advances in long read sequencing enable an accurate and complete re-construction of the genome with better visibility of repetitive regions and structural isoforms in addition to splicing and modification data. At the same time, advances in single cells RNA sequencing (scRNA-seq) enable a better understanding of differences in the genome on a cell-cell basis. Collectively, new technologies are

being developed with the aims of parsing the complexity of the genome regulatory mechanisms, especially immune regulatory mechanisms and push our current understanding of what we now know as modes of regulation.

5.2 Novel role of HNRNPA2B1 in regulating IFNG signaling through alternative splicing.

Heterogeneous nuclear ribonucleoproteins family (HNRNPs) are highly involved in regulation of RNA metabolism and maturation, specifically they are highly tied to alternative splicing of transcripts, which allows them to function in gene regulation across many diverse biological processes. In the second chapter, I discuss the novel role of HNRNPA2B1 in regulating immune activation of macrophages under stimulus, specifically promoting IFNG signaling leading to macrophage activation which in turn drives inflammatory cytokine production and pathogen clearance. We used a conditional knockout mouse model using a cre-lox system which depletes HNRNPA2B1 in myeloid cells only and showed that mice that lack HNRNPA2B1 do not respond effectively to LPS in an endotoxic shock model where mice failed to promote pre-inflammatory cytokine activation. Similarly, knockout mice were found to be more sensitive to Salmonella infection as they died earlier and displayed higher bacterial burden in addition to lower systemic pro-inflammatory cytokine levels.

Mechanistically, HNRNPA2B1 was able to control IFNG signaling pathway including major transcription factors (such as JAKs and STATs) as well as major signaling molecules through alternative splicing of the IFNG receptor. In KO

macrophage, HNRNPA2B1 depletion results in favoring the noncoding IFNGR transcript which desensitizes the macrophage to IFNG and dampens macrophage activation signals such as MHCII and CD86. This in turn, attenuates T cell activation and adaptive immunity and sensitized the KO mouse to pathogens. These findings stress the complex role of alternative splicing in proper macrophage function which in turn impacts the organism's ability to combat infection and highlights the need for a careful and accurate assessment of alternative splicing role in immune activation regulation.

In order to unravel the complex splicing changes in HNRNPA2B1 KO macrophages we performed Nanopore long read sequencing on KO macrophages to discover any new functional isoforms that are formed due to absence of HNRNPA2B1, or any known isoforms that could be functional in regulating IFNG signaling cascade through differential isoform production. Functional validation of such isoforms can be challenging due to their tendency to undergo NMD, making these transcripts harder to quantify on a gel. Thus, high precision long read sequencing presents the most direct approach to investigate isoform changes and to identify alternative splicing events. We were able to identify a large number of genes that undergo alternative splicing when HNRNPA2B1 is depleted, a large portion of which were involved in inflammatory signaling, more specifically in IFN response.

While these findings are helpful in bringing us closer to understanding the mechanism of HNRNPA2B1 regulation of IFNG signaling, we still need to better

distinguish transcripts that directly bind to HNRNPA2B. Enhanced crosslinking and immunoprecipitation (eCLIP) can help us identify transcripts bound directly to HNRNPA2B1, by cross examining the list of resulting transcripts with alternatively spliced genes list we can confirm transcripts regulated by HNRNPA2B1 through alternative splicing.

5.3 HNRNPA2B1 role in regulating inflammatory activation in RA FLS

HNRNPA2B1's newly discovered role in regulating IFN signaling, in addition to its implication in RA pathogenesis through abundance of autoantibodies against it in patient's sera, makes it an attractive target to study in relation to RA. We were able to show that HNRNPA2B1 role in regulating IFN signaling is conserved in FLS cells from human RA patients, where silencing HNRNPA2B1 weakened inflammatory overactivation and lowered IFN gene expression in FLS cells. This data is especially remarkable given the role IFNG plays in promoting FLS immune overactivation in RA, through silencing HNRNPA2B1 we were able to weekend IFN activation without completely abrogating it, thus potentially alleviating patient's symptoms. Whether this approach is truly beneficial, it needs to be further tested in a T cell specific RA model in mice, such as Collagen Induced Arthritis (CIA). Inducing CIA in conditional HNRNPA2B1 KO mice could help us test whether dampening IFNG signaling in macrophages could lower T cell activation and thus prevent FLS overactivation in the RA model.

5.4 CS exposure impacts the macrophage inflammatory signaling.

Cigarette smoke (CS) is recognized as a driver of inflammation through alterations in macrophage function , leading to disruption of homeostasis and subsequent pathogenesis such as inflammation and COPD. In chapter 3, we investigate the mechanisms by which CS alters macrophage inflammatory gene expression through a chronic smoke exposure model. We found that macrophages from smoke-exposed mice upregulate expression of inflammatory genes, especially ones involved in the NF-kB and JAK-STAT pathways. In this chapter, through the use of RNA sequencing and bioinformatic techniques we were able to identify a few mechanisms by which this differential gene expression could be regulated.

5.5 *LincRNACox2* regulates inflammatory gene expression in *trans* post smoke exposure.

In chapter three, we found that CS exposure resulted in differential expression of several lncRNAs, most importantly, *lincRNA-Cox2* which was identified as a significantly induced lncRNA following smoke exposure, similar to its neighboring protein-coding gene Ptgs2 (Cox2). Previous results from the lab identified *lincRNA-Cox2* as a *cis* acting regulator of its neighboring protein-coding gene Ptgs2 using a KO mouse model. Additionally, *lincRNACox2* deficient mutant (Mut) and *lincRNACox2* transgenic (Tg) mouse models enabled us to determine that *lincRNACox2* transcriptionally regulates several immune genes in trans within the spleen and lung in an LPS induced endotoxic shock model. In chapter 3, we report on

lincRNACox2 novel function in regulating macrophage's inflammatory response to smoke exposure. Through our mouse models, we were able to show that *lincRNACox2* functions in *trans* to regulate immune genes within the lung following CS exposure. Most importantly, we found that some cytokines such as Il16, GCSF and others were dysregulated in the *lincRNACox2* Mut mouse post CS exposure, additionally, we were able to rescue the dysregulation in some cytokines and chemokines when *lincRNACox2* was reintroduced in the MutxTg mouse. Thus, we were able to show that *lincRNACox2* contributes to the coordination of immune genes in macrophages post CS exposure.

Collectively, these results point to the involvement of a lincRNA in regulating the inflammatory response machinery and alter macrophage response to an environmental stimulus (CS) through gene expression regulation. Despite the wealth of information we generated to investigate the *lincRNACox2* function, there is still much to be learned regarding the mechanism of regulation. An exciting future direction is to perform pull down experiments on *lincRNACox2* in macrophages to discover any binding partners that facilitate its regulatory function under inflammatory or CS exposed conditions. In addition, it is not known yet how the altered macrophage response post CS exposure affects the mouse overall response to infection, infecting CS exposed mice with a bacterial or viral infection could help us better understand the impact of long-term CS exposure on the organism's sensitivity to infections. These experiments will advance our understanding of *lincRNACox2*

involvement in immune modulation of macrophages and the subsequent impact on the organism's response to infections.

5.6 CS induced alternative splicing in macrophages

Alternative splicing is a key regulator of inflammation and a potential mechanism that links CS exposure to abnormal inflammatory responses in macrophages in COPD. In chapter three, we used two different bioinformatics tool to interrogate splicing changes in macrophages exposed to smoke. We found a variety of alternative splicing events induced in macrophages after CS, and we were able to validate one interesting event occurring in *Mib2*. These findings showcased alternative splicing as an additional mechanism by which CS exposure is able to coordinate changes in the macrophage transcriptome and induce disruption in normal macrophage function. Long read RNA sequencing presents an attractive next step to accurately study the changes in the transcriptome of CS exposed macrophages and will provide a wealth of information to push our understanding of how the macrophage responds to smoke.

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