Title
Mechanistic characterization of the auto-inflammatory pathology caused by NFκB RelB deficiency

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Mechanistic characterization of the
auto-inflammatory pathology caused by NFκB RelB deficiency

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of
Philosophy in Molecular Biology

by

Héctor Ivan Navarro

2023
ABSTRACT OF THE DISSERTATION

Mechanistic characterization of the auto-inflammatory pathology caused by NFκB RelB deficiency

by

Héctor Ivan Navarro
Doctor of Philosophy in Molecular Biology
University of California, Los Angeles, 2023
Professor Alexander Hoffmann, Chair

Autoimmune and inflammatory diseases are leading causes of morbidity and death and arise from highly unique etiologies. Many autoimmune disorders involve dysregulation of the transcription factor NFκB/RelA, termed relopathies, and the interferon signaling pathway, termed interferonopathies. Previous reports have demonstrated that the loss of RelB results in autoimmunity and inflammatory pathology in both pediatric patients and mice. While recent studies reported immune sentinel cells to be critical mediators of the loss of RelB pathology, it remains unknown what dysregulated immune response pathways exist within these cells that drive the loss of RelB pathology.

In these studies, I took an unbiased approach to characterize innate responses of immune sentinel cells to determine both the functional role of dysregulated gene programs in the RelB-deficient pathology and the mechanistic regulation of these gene programs by RelB. I found that
loss of RelB in patient-derived fibroblasts and mouse myeloid cells results in elevated induction of hundreds of interferon-stimulated genes. To examine their functional role in RelB-deficient pathology, I generated compound mutant mice in which IFN signaling was genetically ablated. Removing hyper-expression of the interferon stimulated gene program did not ameliorate the autoimmune pathology of RelB knockout mice. Instead, I found that RelB suppresses a smaller set of pro-inflammatory response genes sharing a common NFκB binding motif in a manner that is independent of interferon signaling. Therefore, while transcriptomic profiling would describe the loss of RelB pathology as an interferonopathy, the functional genetic evidence indicates that the pathology in mice is interferon-independent.

To further determine the mechanism by which these pro-inflammatory genes were dysregulated by the loss of RelB, I performed biochemical and genome-wide analysis of RNA-Seq and ChIP-Seq data sets from innate immune cells derived from WT and RelB−/− mice. I found that the loss of RelB results in the hyper-binding by RelA to κB sites at or near the TSS of these IFN-independent pro-inflammatory genes, suggesting RelB may inhibit pro-inflammatory gene expression via competition with RelA for target gene promoters. To test this, I generated a novel RelB-DNA binding mutant mouse with three specific mutations in amino acids that contact the κB site. Indeed, while other cytoplasmic functions of RelB remained intact, the directed loss of RelB DNA binding function resulted in the hyper-expression of both IFN-dependent and independent gene expression and phenocopied the loss of RelB−/− inflammatory pathology. These results together suggest a key regulatory mechanism by the NFκB system in innate immune cells, in which RelB functions as a critical regulator of RelA pro-inflammatory gene expression and suppressor of autoimmune pathology.
The dissertation of Héctor Ivan Navarro is approved.

Jeffrey Aaron Long
Brigitte N. Gomperts
Genhong Cheng
William Edward Lowry
Alexander Hoffmann, Committee Chair

University of California, Los Angeles

2023
Dedication

I dedicate this thesis to my God, Savior, and Lord Jesus Christ.
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Philippians 3:13-14 “Brethren, I count not myself to have apprehended: but this one thing I do, forgetting those things which are behind, and reaching forth unto those things which are before, I press toward the mark for the prize of the high calling of God in Christ Jesus.”

1 Corinthians 2:1-2 “And I, brethren, when I came to you, came not with excellency of speech or of wisdom, declaring unto you the testimony of God. For I determined not to know anything among you, save Jesus Christ, and him crucified.”

תהלים 119:71-81

וְֹצִמֵהוּ לֵילְדָֽךְ לֵילְדִּי רָֽבְדִּלֵךְ אַֽלְּכִּלְּלֵךְ

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1. **Héctor I. Navarro**, Anna Fraser, Kim Ngo, Allison Daly, Yi Liu, Jennifer Chia, Alexander Hoffmann (exp. 2023) RelB suppresses autoimmunity and inflammatory gene expression by competing with RelA for binding to target gene promoters, in preparation.

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Chapter 1 - Introduction
Introduction of the Thesis

Autoimmune and Autoinflammatory Diseases

Autoimmune and inflammatory diseases are a major health concern with a growing list of approximately 150 life-long debilitating illnesses characterized by a dysregulation of the immune response resulting in aberrant damage to healthy host cells(1). These autoimmune and inflammatory diseases lead to incapacitating auto-toxicity in a variety of critical organs such as the brain, lung, pancreas, gastrointestinal tract, kidney, bone, and skin(2). While many autoimmune and inflammatory diseases present with common pathological characteristics, the etiological drivers of these pathologies are incredibly diverse(3) and are broadly characterized into two major categories, autoimmune diseases, characterized by a dysregulation of the adaptive immune system and more recently, autoinflammatory diseases (AIFs), characterized by a dysregulation of the innate immune system (4). Given this rapidly growing number of genetically described autoinflammatory diseases and the central role of chronic inflammation in other human pathologies, understanding mechanisms that lead to dysregulated chronic inflammation is critically important to wholistically address these pathologies. Recent efforts in characterizing AIFs have allowed for the classification of these diseases into the following five categories based on their underlying dysregulated mechanisms: inflammasomopathies, interferonopathies, unfolded protein responses/ER stress syndromes, relopathies, and uncategorized (5). However, diseases within these AIF categories arise from unique genetic mutations, therefore the pathological outcomes and symptoms of these diseases vary broadly within each AIF category and often have overlapping dysregulation of multiple immune response pathways (2,6–10). Therefore, understanding the dysregulated mechanisms driving
the etiology of these pathologies in a disease-specific manner is crucial to comprehensively tailor therapeutic approaches for patients with highly unique genetic lesions.

Loss of RelB Autoimmune and Inflammatory Pathology

A recent report of pediatric patients with a homozygous null mutation in the NFκB subunit, RelB, were characterized with autoimmune and inflammatory disease, combined immunodeficiency, failure to thrive, and significantly impaired ability to produce specific antibodies in vivo (11,12). The autoimmune pathology in these patients was further characterized and found to present with severe autoimmune skin diseases, rheumatoid arthritis involving altered aberrant thymic T-cell maturation, and reduced output of a skewed T-cell repertoire with expansion of clones (12). While dysregulation of the adaptive immune system in these patients was thoroughly characterized, a description of the dysregulation in the innate immune system was limited, but found normal levels of TNF secretion in patient-derived monocytes, and characterized elevated TNF-induced RelA binding in patient derived fibroblasts (12).

Characterization of the loss of RelB pathology in RelB−/− mouse models also reveals a stark autoimmune pathology with similarities to the human loss of RelB pathology: with muti-organ inflammation, thymic atrophy, reduction of thymocytes, impaired cell mediated immune response, but normal T and B cell development (13). These similarities suggest a conserved mechanism between the human pathology and mouse pathology and therefore these mice have been proven to be an invaluable tool for the characterization of some aspects of the loss of RelB pathology (13–16). However our knowledge remains limited and while innate immune dendritic cells have been shown to be key mediators of inflammation by the loss of RelB in mice, subsequent studies addressing the auto-inflammatory mechanisms that cause these immune sentinel cells to drive the pathology do not have a common consensus (17–19). This further calls
for a critical need for the characterization of dysregulated immune response pathways caused by the loss of RelB in inflammatory innate immune cells.

**NFκB signaling and regulation**

The NF-κB transcription factor signaling pathway is one of the most central immune response pathways that induces the expression of genes involved in cell survival, differentiation, and inflammation(20). NF-κB is made up of a family of subunits, RelA, cRel, RelB, p52, and p50, that dimerize to form multiple hetero- and homo dimeric transcription factors (21). These NF-κB transcription factors are tightly regulated from their transcriptional control in the nucleus via cytoplasmic sequestration by three isoforms of NF-κB inhibitor protein: IκBα, IκBβ, and IκBε (22), and by p105 and p100 which form the inhibitory high-MW kappaBsome (IκBδ) complex (23,24). Notably, IκBδ has been shown to directly interact with and sequester NF-κB dimers and release them upon stimulation (23).

**The role of RelB in NFκB regulation**

While NFκB is tightly regulated by NFκB inhibitory proteins and complexes, activity of certain NFκB subunits can be inhibited by other subunits. For example, from its discovery, RelB was uniquely found to act as both a transcriptional activator and a repressor of NFκB RelA transcriptional activation of metabolic and pro-inflammatory genes (25,26). A different report later found that RelB is required for the stabilization of p100, which enables the formation of the IκBδ complex, that inhibits RelA nuclear translocation and transcriptional activation of gene expression (23,27). RelB was also found to repress gene expression activity by facilitating repressive
Mechanistic Characterization of the Loss of RelB inflammatory pathology

In my studies, I sought to further understand the mechanistic dysregulation caused by the loss of RelB in immune sentinel cells by utilizing fibroblasts derived from a patient with a rare homozygous null mutation in the NFκB subunit, RelB. Given recent reports of elevated IFN signaling caused by the loss of RelB (31–33), I hypothesized that dysregulation of the IFN pathway may be present in sentinel cells derived from the loss of RelB patient. Further, I sought to understand if potential dysregulated autoinflammatory mechanisms are involved in driving the autoimmune inflammatory pathology. Therefore, I sought to utilize the loss of RelB mouse model to understand if these potentially dysregulated immune responses could be seen in the mouse autoimmune pathology, thus allowing the model to be used for further functional studies.

chromatin modifications through the formation of complexes with histone deacetylase and histone lysine acetyltransferase (28–30).
References:


Chapter 2

RelB-deficient autoinflammatory pathology presents as interferonopathy, but in mice is IFN-independent
RelB-deficient autoinflammatory pathology presents as interferonopathy, but in mice is IFN-independent

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**Conflict of Interest:**

The authors declare that research in this study was conducted in the absence of a conflict of interest.
ABSTRACT

**Background:** Autoimmune diseases are leading causes of ill health and morbidity and have diverse etiology. Two signaling pathways are key drivers of autoimmune pathology, interferon and NFκB/RelA, defining the two broad labels of interferonopathies and relopathies. Prior work established that genetic loss of function of the NFκB subunit RelB leads to autoimmune and inflammatory pathology in mice and humans.

**Objective:** We sought to characterize RelB-deficient autoimmunity by unbiased profiling of the stimulus-responses of immune sentinel cells, and to determine the functional role of dysregulated gene programs in the RelB-deficient pathology.

**Methods:** Transcriptomic profiling was performed on fibroblasts and dendritic cells derived from RelB-deficient patients and knockout mice, and transcriptomic responses and pathology were assessed in mice deficient in both RelB and the type I interferon receptor.

**Results:** We found that loss of RelB in patient-derived fibroblasts and mouse myeloid cells results in elevated induction of hundreds of interferon-stimulated genes. Removing hyper-expression of the interferon stimulated gene program did not ameliorate the autoimmune pathology of RelB knockout mice. Instead, we found that RelB suppresses a different set of inflammatory response genes in a manner that is independent of interferon signaling but associated with NFκB binding motifs.

**Conclusion:** While transcriptomic profiling would describe RelB-deficient autoimmune disease as an interferonopathy, the genetic evidence indicates that the pathology in mice is interferon-independent.

**Key Messages**
• Human and mouse NFκB RelB deficiency leads to multi-organ auto-immune pathology.

• Unbiased profiling of patient derived fibroblasts reveals a broad interferon gene signature which is also present in RelB knockout mouse dendritic cells.

• Compound deficiency of the interferon type I receptor completely ablates this gene program, but does not diminish RelB knockout autoimmune pathology, ruling out interferonopathy.

**KEYWORDS:** RelB, autoimmunity, inflammation, interferonopathy, relopathy, dendritic cells

**Capsule Summary:**
Loss of RelB reveals a broad interferon gene signature in patient-derived fibroblasts and mouse dendritic cells. While IFNAR deletion completely ablates this gene program, it does not diminish RelB knockout autoimmune pathology, ruling out interferonopathy.

**Abbreviations:**
AGS: Aicardi–Goutières syndrome
AI: autoimmune
AIF: autoinflammatory
CPM: counts per million
DC: dendritic cell
GO: gene ontology
HA20: A20 haplo-insufficiency
IFN: interferon
IFNAR: IFN-α/b receptor
ISG: interferon stimulated genes
ISRE: interferon sensitive response elements
MC: myeloid cell
PRR: pattern recognition receptor
SLE: systemic lupus erythematosus
TSS: transcription start site
INTRODUCTION

Autoimmune diseases (AI) and autoinflammatory (AIF) diseases are rapidly expanding categories of immune related disorders and a major health concern. Current studies estimate that there are around 150 life-long debilitating autoimmune diseases, characterized by a dysregulation of adaptive immune system, with no known cures (1) and a growing list of 40 genetically described autoinflammatory diseases, characterized by a dysregulation of the innate immune system (2). These diseases have chronic lifelong symptoms such as chronic fevers (3), arthritis (4), inflammatory bowel disease (5), hepatic and central nervous system inflammation (6,7), among other serious health issues. Our understanding of the role of chronic inflammation in other human disease such as cancer, heart disease, and psychiatric disorders is also growing rapidly (8). Therefore, understanding autoimmune and autoinflammatory diseases is critically important to therapeutically address the growing number of AI and AIF patients as well as other human diseases in which inflammation plays a key role. Autoinflammatory diseases have recently been categorized into the following five subsets based on underlying dysregulated mechanisms: inflammasomopathies, interferonopathies, unfolded protein responses/ER stress syndromes, relopathies, and uncategorized (9).

Of growing interest are interferonopathies, which are defined as diseases grouped by mendelian disorders associated with an upregulation of type I interferon (IFNs), first described in 2003 (10), and later officially categorized and termed (11). Type I IFNs are a class of antiviral, anti-inflammatory proteins first discovered in 1957 for their ability to induce influenza viral interference (12). Type I IFNs signal in a paracrine and autocrine manner via the IFN-α/b receptor (IFNAR) comprising of two subunits, IFNAR1 and IFNAR2. The IFN receptor has been shown to be expressed by virtually every nucleated cell, of hematopoietic and non-hematopoietic
origin, and while some cells are specialized producers of Type-I IFN, almost all cells are able to produce Type-I IFN (13). Upon Type I IFN binding to the IFNAR receptor, downstream transcription factor complex, ISGF3, is formed and induces the expression of interferon stimulated genes (ISGs) by binding to their promoter regions containing interferon-sensitive response elements (ISREs). Given the broad extent of the IFN-signaling network in human physiology, as one may expect, dysregulation of IFN signaling leads to a multitude of pathologies affecting various organs and organ systems. For example, USP18	extsuperscript{−/−} mice, lacking a negative regulator of IFN-signaling, UBP43, which leads to elevated levels of conjugates to ISG15, a potently induced ISGF3 target gene, develop brain injury, accompanied by hydrocephalus and early death with 50% of mice dying at 4 weeks of age (14,15). This pathology was also seen in human patients with ISG15 null mutations, causing death or seizures in 3 patients (16). Interferonopathies can cause other harmful effects on the CNS such as epilepsy, as well as psychomotor retardation in Aicardi–Goutières syndrome (AGS) (17) and have also been shown to lead to interstitial lung disease, arthritis, panniculitis lipodystrophy, necrotizing vasculitis, bone dysplasia, and early thrombotic events among other serious symptoms (18). Importantly, while we can broadly categorize AIFs into interferonopathies, the pathological outcomes and symptoms of diseases within this category vary broadly and often have overlapping dysregulation of various immune-related signaling pathways. Many AIF diseases also share AI disease characteristics involving dysregulation in both innate and adaptive immunity. Therefore, understanding the underlying mechanisms and etiology of these pathologies in a disease-specific manner is crucial to effectively tailor therapeutic approaches for patients with highly unique genetic lesions.
Recently, pediatric patients with a homozygous null mutation in the gene encoding NFκB subunit, RelB, were described (19–21). These patients presented a combined immunodeficiency phenotype with failure to thrive and a significantly impaired ability to produce specific antibodies \textit{in vivo} (20). Interestingly, these patients were also characterized with an autoimmune pathology presenting severe autoimmune skin disease and rheumatoid arthritis involving altered thymic T cell maturation with reduced output and production of a skewed T-cell repertoire with expansion of clones (21). While defects in the adaptive immune system were found and well described in these studies, the description of the patient innate immune function was more limited but appeared to behave normally relative to adaptive immune system, as measured by TNF secretion in patient derived monocytes. However, patient derived fibroblasts, key pathogen sensing sentinel cells, revealed to have elevated TNF-induced RelA DNA binding activity (21).

Findings in the RelB-null human pathology strikingly resembled previously reported phenotypes of \textit{RelB} \textsuperscript{−/−} mice (multi-organ inflammation, thymic atrophy, reduction of thymocytes, impaired cell mediated immune response, but normal T and B cell development (22), suggesting a conserved mechanisms between human and mouse pathology. In mice, functional adoptive transfer studies identified dendritic cells (DCs), key pathogen-sensing immune sentinel cells, to be key drivers of the lung inflammatory pathology seen in \textit{RelB} \textsuperscript{−/−} mice (23). Another study supported these findings by demonstrating a restoration of the thymic atrophy and the numbers of thymic Foxp3+ T-reg cells in \textit{RelB} \textsuperscript{−/−} mice upon adoptive transfer of \textit{RelB} \textsuperscript{+} DCs (24). However, subsequent studies that addressed the auto-inflammatory mechanisms that causes immune sentinel cells to drive the pathology have reported several different mechanisms and no consensus has emerged (25–27).
We aimed to further understand the mechanistic dysregulation of RelB-null immune sentinel cells as they contribute to $\text{RelB}^{-/-}$ auto-inflammatory disease. Given recent reports of elevated IFN signaling caused by RelB-deficiency (28–30), we hypothesized dysregulation of the IFN pathway may be present in RelB-null patient derived sentinel cells, potentially implying immediate clinically relevant characterization of the RelB null pathology as an interferonopathy. Indeed, we found that loss of RelB in patient-derived fibroblasts and mouse DCs, but not macrophages, results in elevated expression of interferon-stimulated genes (ISGs) that is dependent on elevated type I IFN signaling. However, to our surprise, while the hyper-expression of ISGs is a prominent aspect of the loss of RelB phenotype, complete ablation of type I IFN signaling in the mouse did not ameliorate the RelB pathology. We found instead that RelB directly suppresses a myriad of IFN-independent pro-inflammatory and immune response genes which promote a cell-intrinsic hyper-activated inflammatory state both in $\text{RelB}^{-/-}$ human fibroblasts and mouse DCs, which in turn are likely key contributors to the RelB-null autoimmune and autoinflammatory pathology.
Methods

Patient-Derived Fibroblasts

Total RNA was prepared as described (31). Strand-specific libraries were generated from 200 ng total RNA using the KAPA Stranded mRNA sequencing and Library Preparation Kit (Illumina). cDNA libraries were single-end sequenced (50bp) on an Illumina HiSeq 2000. RNA-seq reads were trimmed using cutadapt v1.12 (31) to remove low quality ends as well as removing remaining adapter sequence. Then reads were aligned on the human genome (hg38) using STAR software v2.5.2b (32). Aligned reads were filtered using samtools v1.3.1 (33) to keep only uniquely aligned reads. Gene expression quantification was done using featureCounts v1.5.1 (34) software and GENCODE v23 gene annotation (35). Differential expressed genes were selected using edgeR (36) with a 4 fold and 0.01 FDR threshold in either WT or patient samples. Clustering of differentially expressed genes was done using a kmean method to identify clusters of DEG with similar dynamic profiles. Gene ontology and motif analysis was done via homer suite considering regulatory regions within -4kb to 1kb from the transcription start site.

Mice and bone marrow-derived dendritic cells

Wild-type and transgenic mice were housed in pathogen-free conditions at University of California, Los Angeles. RelB+/ mice were generated by breeding RelB+/ mice and IFNAR+/RelB-/ mice were generated by mating IFNAR+/RelB+/ or IFNAR+/-RelB+/- mice to each other. All mice used for RNA-seq experiments were between 6 to 12 weeks at day of experiment, both male and female mice were used for experiments. Bone marrow cells were isolated from mouse femurs and cultured with M-CSF-containing L929-conditioned medium for BMDMs or with 20 ng/ml GM-CSF and 10 ng/ml IL-4 to produce BMDCs with half the media being replaced on day 3 and
6 as previously reported (37). Cells were stimulated with CpG (0.1µM) (Invivogen ODN 1668 Cat# tlrl-1668), or Poly(I:C) HMW (10µg/mL) (Invivogen Cat#tlrl-pic) and collected at specified timepoints in Invitrogen™ TRizol™ Reagent (Cat# 15-596-018), RNA was extracted using Qiagen RNeasy Mini Kit (Cat #74106) as described (39).

Transcriptome profiling

RNA was used for Illumina bead Arrays as described (31) and for RNA-seq as described (40). Briefly, libraries were prepped using KAPA Stranded mRNA-Seq Kit Illumina® platform KR0960 – v3.15 using 1µg of RNA per sample measured using Qubit 2.0 fluorometer. Final libraries were checked via agarose gel and multiplexed with a maximum of 24 samples per sequencing reaction. Libraries were sequenced using Illumina HiSeq 3000 with single end 50bp reads at the UCLA Technology Center for Genomics & Bioinformatics.

Bioinformatic Analysis

Reads were trimmed using cutadapt (31) (cutoff q=20) and mapped to the mm10 genome. Processed reads showed high quality reads and alignment scores. The October 2014 version of the Ensembl database was used to extract gene annotation information. CPM values were generated using edgeR (36) to normalize the raw counts data based on sequencing depth. To permit fold change calculations, a pseudocount of 1 CPM was added. Induced genes were selected using a log₂FC>1 cutoff for any stimulated timepoint relative to 0hr unstimulated control, transcripts with empty gene names were removed. Data was z-scored and plotted using the pheatmap R package. Fold differences of genes within heatmaps was calculated by first calculating the fold differences for all individual genes between genotype of interest and WT or
IFNAR−/− control CPM (Genotype X)/CPM (Genotype Y) for each individual time point. Average fold differences were then calculated by averaging the fold differences of all genes within each cluster for each individual time point. Gene ontology and motif analysis was done via homer suite considering regulatory regions within -1kb to 1kb from the transcription start site. Line graphs of individual genes were generated using GraphPad Prism.

*Tissue Isolation and Fixation*

Spleens were isolated from age matched mice immediately after euthanizing and subsequently rinsed with PBS. Excess PBS was removed from spleens and were subsequently weighed. Fixation was done in 10% Formaldehyde for 46-48hrs. Tissue was processed, sectioned, and hematoxylin and eosin stained by the UCLA Translational Pathology Core Laboratory (TPCL).

*REB approval*

All patient studies were approved by the SickKids Research Ethics Board (Protocol no. 100005598).
RESULTS

_Fibroblasts obtained from a human RelB-null donor show hyperexpression of type I interferon and interferon-stimulated genes._

To characterize the transcriptome-wide defects caused by the loss of RelB, we performed an unbiased differential gene expression analysis using fibroblasts obtained from a previously reported combined immunodeficiency patient (P1) with an AI disease pathology arising as a consequence from a rare homozygous mutation in the _RelB_ gene resulting in the complete loss of RelB protein, and their healthy close relative with homozygous copies of WT _RelB_ (Control) [20,21]. To experimentally model immune stimulation _via_ pattern recognition receptors (PRRs) that may occur in response to pathogen exposure or tissue injury, we stimulated fibroblasts with TLR3 agonist poly(I:C) (10µg/mL) in replicate. After collecting RNA at 5 time points (0, 2, 4, 8, 15 h) we performed whole transcriptome RNA-seq and bioinformatically identified 740 genes that were induced with a FC>4 and FDR <0.01 threshold in either WT or P1 replicate samples. Applying k-means clustering to the expression data of these genes, we identified 6 distinct clusters of differentially expressed genes that were hyper-expressed in P1 (_Fig. 1a_). A small proportion of genes clustered within cluster A (33 genes) and cluster B (63 genes) but resulted in weak or no motif enrichment results, however most genes clustered in hyper-expressed clusters C through F, which revealed to have average fold differences in counts per million (CPM) ranging from 1.6x and 14x between P1 and control fibroblasts at any of the measured timepoints (_Fig. 1a, Supplementary Table 1_). To identify potential regulatory features of these genes we performed motif enrichment analysis that considered -4kb to +1kB with respect to the transcription start site (TSS) of the regulatory region of each gene. Genes within hyper-expressed clusters C (102 genes) and D (175 genes) were statistically enriched for NFκB motifs in
regulatory regions, while hyper-expressed clusters E (210 genes) and F (157 genes) were statistically enriched for IRF and ISRE motifs (Fig. 1b). This analysis suggested two major categories of dysregulated genes: IFN-stimulated genes (ISGs) and NFκB-regulated genes. To identify potential biological functions, we performed gene ontology (GO) analysis. Clusters C and D were associated with terms invoking NFκB activating pathways such as “TNF” and “LPS signaling”. Clusters E and F were associated with terms invoking IFN-inducing pathways including Type I “interferon-α” and Type II “interferon-γ response” (Fig. 1c). Analysis of individual genes within clusters E and F revealed many hyper-expressed ISGs including Oasl, Oas2, Ifit1, Ifit2, Mx2, and Isg20 (Fig. 1d). Given that ISGs may be activated by Type I, II, and III IFNs (41), we evaluated the expression of IFN family members. We found Ifnb1 and Ifnl3 to be hyper-expressed in fibroblasts derived from P1 (Fig. 1d). Together, these data establish that loss of function of RelB results in hyper-induction of not only NFκB-associated genes but a large ISG expression program prompting the question of whether it drives the described autoimmune/autoinflammatory pathology.

Loss of RelB in mouse myeloid cells recapitulate hyperexpression of type I IFN and interferon stimulated gene programs observed in patient fibroblasts.

Adoptive transfer studies suggested that myeloid cells (MCs) play a key role in driving the lethal multi-organ inflammation in RelB−/− mice (23,24). We therefore undertook transcriptomic profiling of mouse MCs produced by differentiating wild-type and RelB−/− bone marrow cells with GMCSF + IL-4 or MCSF, which generate primarily dendritic cells or macrophages, respectively (38,42). We then stimulated these cells with TLR3 or TLR9 agonists poly(I:C)
(10µg/mL) - or CpG (0.1µM) over a 24-hour time course and performed unbiased differential gene expression analyses followed by k-means clustering. Our analysis of GMCSF MCs revealed 425 and 415 genes induced (log2FC>1) with poly(I:C) and CpG respectively. Differential gene expression analysis revealed clusters of genes which were hyper-expressed both in a basal state and upon poly(I:C)- or CPG- stimulation (Fig. 2a and 2b). Poly(I:C)-stimulated genes in hyper-expressed cluster A (72 genes) showed average fold differences in expression ranging from 1.5x to 3.9x between RelB⁻/⁻ and WT GMCSF MCs at any of the measured time points (Fig. 2a, Supplementary Table 2). Likewise, CpG stimulated genes in hyper-expressed cluster C (116 genes) showed average fold differences in expression ranging from 1.2x to 2.5x between RelB⁻/⁻ and WT GMCSF MCs at any of the measured time points (Fig. 2c, Supplementary Table 2), and shared 55 out of the 72 genes with poly(I:C) hyper-expressed cluster A. Motif enrichment analysis of hyper-expressed cluster A in poly(I:C)-stimulated GMCSF MCs revealed the ISRE as the top motif in a statistical enrichment analysis that considered -1kb to +1kB with respect to the TSS of the regulatory region of each gene (Fig. 2a, bottom). Similarly, in CpG-stimulated GMCSF MCs, hyper-expressed cluster C also yielded the ISRE as the top statistically enriched motif (Fig. 2c, bottom). Furthermore, GO analysis of these clusters revealed ISG-inducing pathways, “IFN-α response”, and “IFN-γ response” among the top terms for both clusters. These data suggested that hyper-expressed genes in these clusters are ISGs, perhaps elevated by hyper-induction of IFNs in RelB⁻/⁻ GMCSF MCs. We therefore analyzed a set of IFN genes and found Ifnb1 to be elevated, with concurrent hyper-expression of ISGs, Oasl, Oas1, Oas2, Ifit2, and Ifit3, in both poly(I:C)- and CPG-stimulated conditions (Fig. 2c and 2d). Like poly(I:C)-stimulated RelB-null patient-derived fibroblasts, RelB⁻/⁻ GMCSF MCs stimulated with poly(I:C) also revealed a gene cluster (cluster B) which was hyper-
expressed at the late 24h stimulated timepoint with an average expression fold difference of 1.45x between RelB<sup>−/−</sup> and WT GMCSF MCs. This cluster yielded NFκB as the top result in motif enrichment analysis. All other clusters in both poly (I:C) and CpG were unchanged, containing average expression fold differences ranging from .9x to 1.17x between RelB<sup>−/−</sup> and WT GMCSF MCs at all time points (Fig. 2a and 2c, Supplementary Table 2). Next, we asked whether myeloid cells differentiated with M-CSF (MCSF MCs) showed similar transcriptomic dysregulation as GMCSF MCs. We generated MCSF MCs from RelB<sup>−/−</sup> and WT control bone marrow and undertook an analogous analysis following stimulation. We identified 1,243 unique induced genes (log<sub>2</sub>FC>1) with LPS and performed differential gene expression analysis. In contrast to our findings with GMCSF MCs, our analysis of MCSF MCs revealed no differentially expressed gene clusters between WT and RelB<sup>−/−</sup> genotypes, with all clusters revealing average expression fold differences ranging from .9x to 1.1x between RelB<sup>−/−</sup> and WT MCSF MCs at all time points. (Fig. 2e, Supplementary Table 2). The lack of expression phenotype may be because MCSF MCs show lower levels of RelB expression compared to GMCSF MCs (39). Together, these results indicated that the loss of RelB results in a cell-specific transcriptome phenotype, with PAMP-stimulated GMCSF MCs showing broad and pronounced dysregulation of interferons and interferon-stimulated genes. Given the physiological importance of dendritic cells in both innate immunity as cytokine and chemokine producers (43), and adaptive immunity as antigen-presenting cells (44,45) and, in addition their demonstrated involvement in the RelB<sup>−/−</sup> autoinflammatory pathology (23,24), we proceeded with RelB<sup>−/−</sup> GMCSF MCs as a model system for further studies.
Compound deficiency of the type I interferon receptor ablates the hyperactivation of the interferon-stimulated gene program in RelB-deficient dendritic cells.

While the expression of ISGs is primarily driven by the transcription factor complex ISGF3 or STAT1 homodimers (GAF) which are downstream of IFN signaling (46), many ISGs also contain NFκB motifs and have been shown to be regulated by NFκB (47–49). However, the potential role of NFκB RelB in regulating ISGs or other immune response genes remains largely unknown. Given the hyper-expression of IFNb, it remained unclear whether hyperexpression of ISGs is caused by elevated IFNb signaling and secondary ISGF3 activation, or by IFN-independent regulatory mechanisms more directly caused by the loss of RelB. To distinguish between these two mechanisms, we generated an IFNAR−/−RelB−/− double mutant mouse which results in complete ablation of IFNb signaling and thus a loss of secondary IFN-dependent gene expression programs, while IFN-independent gene expression remains. Using bone marrow from double IFNAR−/−RelB−/−, single IFNAR−/−, single RelB−/−, and WT mice, we produced GMCSF MCs and performed RNA-seq followed by differential gene expression analysis and k-means clustering.

To distinguish between dysregulated responses that were interferon-dependent or independent, we aimed to identify clusters with hyper-expressed genes shared between RelB−/− and IFNAR−/− RelB+− GMCSF MCs and that were not hyper-expressed in single IFNAR−/− cells. We therefore first identified 1,123 induced (Log2FC>1) genes upon CpG (0.1µM) stimulation in WT or RelB−/− MCs, and further filtered for genes that were hyper-expressed (FC>1.5) in RelB+− MCs relative to WT MCs. We identified 334 genes which met these criteria and then examined their expression patterns in IFNAR−/− and IFNAR−/−RelB−/− MCs. Using k-means clustering on these genes of interest, our analysis identified hyper-expressed cluster A (215) which had average fold
differences ranging from 1.8x to 2.1x between IFNAR−/−RelB−/− MCs relative to WT MCs and 1.4x to 1.6x relative to single IFNAR−/−MCs at all observed timepoints (Fig. 3a, Supplementary Table 3), suggesting these may be genes directly affected by the loss of RelB, and independently of IFN signaling. On the other hand, the induction of genes in hyper-expressed cluster B (119 genes) was lost in both IFNAR−/−RelB−/− and single IFNAR−/−MCs, having average CPM fold changes differences of .6x in both IFNAR−/−RelB−/− and IFNAR−/− MCs relative to WT MCs at the late 8h stimulated timepoint. These data suggested that genes in this cluster are ISGs and are hyper-expressed in RelB−/− due to elevated IFNb signaling. Motif enrichment analysis (-1kb to +1kB from the TSS) of cluster A genes hyper-expressed specifically in RelB−/− and IFNAR−/− RelB−/− mutants revealed NFκB as the top statistically enriched motif and GO analysis revealed NFκB-inducing pathways “CD40”, “TNF”, and “LPS-signaling” as the top terms (Fig. 3b). As expected, motif enrichment analysis of IFN-dependent cluster B revealed ISRE as the top statistically enriched motif and GO analysis resulted in ISG-inducing pathways, “IFN-λ” and “IFN-b”, as the top terms (Fig. 3b). Our analysis of poly(I:C) (10ug/mL) stimulated MCs also yielded an IFN-dependent and independent cluster (Supplementary Fig. S1), with the IFN-dependent cluster sharing 44 out of 82 genes with the CpG IFN-dependent cluster, and the and the IFN-independent clusters sharing 58 out of 130 genes. (Supplementary Table 3, Supplementary Table 4).

These data suggested that while there are ISGs that are indirectly hyper-expressed by the loss of RelB, genes directly dysregulated by the loss of RelB are primarily NFκB-regulated immune response genes. Analysis of individual genes within the IFN-independent cluster A and genes with similar IFN-independent expression patterns revealed a myriad of NFκB-regulated pro-inflammatory genes including inflammatory chemokines, Ccl5 and Ccl22, co-stimulatory
molecules Cd80 and Cd86, canonical and non-canonical NFκB stimulating receptor Cd40. As well as IFN signaling activators and p-IRF3 kinases, Map3k14 (NIK), and Ikbke (IKKε) (50,51) (Fig. 3c). We found these genes were also hyper-expressed in IFNAR<sup>−/−</sup>RelB<sup>−/−</sup> MCs upon poly (I:C) stimulation (Supplementary Fig. S2). We then asked if these genes may also be hyper-expressed in RelB-null patient samples and we found 51 out of 334 of these genes were hyper-expressed in human RelB-null fibroblast from P1 (Fig. 3d). Together, these data suggest that while the ISG transcriptome signature is a substantial portion of the hyperexpression in RelB-null immune sentinel cells, RelB-loss also results in hyperexpression of many immune response genes via IFN-independent mechanisms.

Ablation of the elevated type I IFN stimulated gene program does not rescue RelB-null pathology.

Having established that a significant proportion of the hyper-expressed genes associated with RelB-loss are due to elevated IFN signaling, we asked whether these genes cause or contribute to the inflammatory pathology described for the RelB<sup>−/−</sup> mouse. To answer this, we examined the health state of RelB<sup>−/−</sup>, and IFNAR<sup>−/−</sup>RelB<sup>−/−</sup> double mutant mice, as well as controls. Upon phenotypical analysis, the 4-week old IFNAR<sup>−/−</sup>RelB<sup>−/−</sup> mouse appeared runted, much smaller than its healthy heterozygous littermate, and similar to RelB<sup>−/−</sup> mice (22) (Fig. 4a). IFNAR<sup>−/−</sup>RelB<sup>−/−</sup> mice were found to have marked splenomegaly similar to RelB<sup>−/−</sup> mice (22) (Fig. 4b). The spleen from IFNAR<sup>−/−</sup>RelB<sup>−/−</sup> weighed 2.9x and 2.5x more than spleens from WT and single IFNAR<sup>−/−</sup> mice respectively (Fig. 4c). Additionally, histological analysis of IFNAR<sup>−/−</sup>RelB<sup>−/−</sup>-spleens revealed marked red pulp expansion and a reduction in white pulp, similar to both our observations and prior reported findings in the RelB<sup>−/−</sup> mice (22) (Fig. 4d). Upon assessment of
serum cytokine levels, $RelB^{-/-}$ mice revealed elevated levels of CXCL10 and IL-6 ($p=.051$), consistent with previous reports in the skin and lungs of $RelB^{-/-}$ mice, respectively (23,52). However, the levels of these elevated cytokines in the serum were not diminished by $IFNAR$ deletion (Fig. S3). These data together identify two unique classes of genes regulated by NFκB subunit RelB in immune sentinel cells relevant to human pathology, IFN-dependent ISGs, and IFN-independent inflammatory genes. Most importantly these data determine clinically relevant findings in understanding which genes play critical roles in loss of RelB autoinflammatory pathology. While we have shown that the loss of RelB indirectly regulates ISGs via Type I IFN signaling, these genes seem to play a superfluous role in critical aspects of the multi-organ inflammation seen in $RelB^{-/-}$ mice, instead RelB directly suppresses IFN-independent genes that are likely the critical drivers of inflammation by immune sentinel cells. These data inform future studies for clinical targets in treating loss of RelB and other autoimmune and autoinflammatory disorders.
DISCUSSION

Here we report a molecular characterization of auto-inflammatory disease caused by RelB-deficiency. Taking an unbiased approach via transcriptome analysis, we found fibroblasts derived from RelB-deficient patients showed hyper-expression of IFN-b and interferon-stimulated genes (ISGs) when exposed to the PRR agonist poly(I:C). This was also seen in mouse DCs (Fig. 2a) and found to be dependent on signaling via the type I interferon receptor, IFNAR (Fig. 1a, 3a). Initially, we expected to provide evidence to categorize the RelB<sup>-/-</sup> null pathology as an interferonopathy-driven AIF disease, thereby providing a clinically relevant therapeutic target to ameliorate the auto-inflammatory pathology. However, we found that compound knockout IFNAR<sup>-/-</sup>RelB<sup>-/-</sup> mice showed no amelioration of critical aspects of the auto-inflammatory disease characteristic of RelB-deficient mice. Compound knockouts remained drastically runted, presented hunched backs, and had enlarged abdomens, like RelB<sup>-/-</sup> mice (22). Additionally, no improvement in the organ inflammation was seen as measured by splenomegaly or histological analysis of red and white pulp.

Given the pronounced ISG expression signature in RelB-deficient human fibroblasts and murine dendritic cells, this is a surprising result. We wondered whether the compound mutant might have residual ISG expression via STAT1 or IRF3-dependent compensatory mechanisms (53,54), but detailed analysis confirmed that ISG expression was diminished to baseline or below by the IFNAR knockout mutation (Fig. 4e). Thus, the pathology in C57/Bl6 mice resulting from RelB-deficiency, despite being associated with a pronounced interferon signature, is not an interferonopathy. However, given that our studies were done with a specific congenic mouse strain, under pathogen-free conditions, we cannot rule out type I IFN involvement in RelB knockout pathologies in other genetic backgrounds or in the context of diverse microbial
exposure, which may be relevant to human pathology (55). Additionally, we cannot rule out the potential role of other classes of interferons given that our approach was restricted to deletion of type I interferon signaling.

What may be causing or contributing to the pathology if elevated ISGs are not? We focused our attention to IFN-independent genes that remain elevated when IFNAR is also knocked out. We identified a group of pro-inflammatory genes that were elevated in RelB-null patient-derived fibroblasts, as well as in murine RelB−/− DCs. Importantly, this gene cluster remained elevated in DC derived from IFNAR−/−RelB−/− mice. Our analysis of this cluster of dysregulated genes revealed the NFκB motif as the top motif enriched near the regulatory regions of these genes. Interestingly, in fibroblasts from patients in a different family in which RelB expression is reduced but not absent, a cluster of hyper-induced genes also showed enrichment of the NFκB motif (19). This supports the notion that RelB-deficiency leads to a pathology that can be categorized as a relopathy.

Among these dysregulated genes associated with the RelB relopathy are potent pro-inflammatory chemokines, Ccl5 and Ccl22. Ccl5 is a potent regulator of inflammation and chemotaxis and is of great therapeutic interest in diseases involving immune dysregulation such as inflammatory bowel disease, atherosclerosis, hepatic inflammation, and many cancers (56), importantly Ccl5 is a potent recruiter of T cells into sites of inflammation and can also recruit macrophages, eosinophils, and basophils (57). Given that MCs reside in all peripheral tissues and fibroblasts are found in most tissues of the body (59), Ccl5 hyper-expressing fibroblasts and GMCSF MCs in RelB−/− mice and human patients may explain the initial recruitment of lymphocytes into inflamed organ tissues. MCs are also professional antigen presenting cells responsible for initiating antigen-specific T-cell immunity. T-cells require a secondary co-
stimulation signal after TCR binding to become active, of which CD80, CD86, and CD40 are key co-stimulatory signaling molecules (60,61). The hyper-expression of CD80, CD86, and CD40 in IFNAR<sup>−/−</sup>RelB<sup>−/−</sup> GMCSF MCs, suggests that these cells may exist in an intrinsic auto-inflammatory state that may hyper-activate T-cell mediated immune response at sites of inflammation in RelB<sup>−/−</sup> mice. This is consistent with findings in RelB-null human patients of high peripheral T cell numbers with clonally expanded populations as shown by TCR-V<sub>b</sub> analysis (20,21), along with previous reports demonstrating that the multiorgan inflammation, myeloid hyperplasia, and inflammatory skin lesions are T-cell dependent mechanisms in RelB<sup>−/−</sup> mice (52,62). In addition, the cluster of IFN-independent RelB<sup>−/−</sup> dysregulated genes included Ikbke, as well as non-canonical NFκB activator Map3k14, which are both established Type I IFN inducers (51,63), prompting the question of whether these may contribute to the onset of IFN dysregulation. However, since our data indicate that the ISG expression program is not contributing to the auto-inflammatory pathology, this question was not further pursued.

In clinical settings, hyper-expression of ISGs, a hallmark of interferonopathies, has been associated with hepatosplenomegaly, meningoencephalitis, interstitial lung disease, recurrent unexplained fever, inflammatory organ damage, high mortality, and autoimmune characteristics (18,64,65), some of which are symptoms seen in the RelB<sup>−/−</sup> pathology. Clinical diagnosis of interferonopathies relies on an “interferon score” by measuring the expression of a panel of interferon-stimulated genes (66), a type-I IFN-response-gene score (IRG-S), cytokine profiling, clinical phenotyping, or next-generation sequencing (NGS) (18). Testing therapeutic targets along the IFN-axis in many human trials showed moderate success with monoclonal antibodies against IFNAR in treating systemic lupus erythematosus (SLE) (67), a well characterized interferonopathy (68). However, patient response rates remained below 50%, and many other
clinical anti-IFN trials have produced mixed results (64). In the mouse, lupus models have a higher level of success with anti-IFNAR antibody therapy extending survival from ~20% in controls to ~70% with treatment (69), raising the question of what may account for the large variation in response to anti-interferon therapy.

Interestingly, in multiple studies SLE patients have also been characterized with A20 haplo-insufficiency (HA20), an autoinflammatory relopathy, presenting with systemic inflammation and increased NFκB-mediated pro-inflammatory cytokines (70,71), however these studies did not test for an IFN-signature. A separate study characterizing 30 patients with mutations in the, TNFAIP3 gene (encoding A20), and 8 other clinically diagnosed HA20 patients showed that many of these patients were previously diagnosed with diseases associated with interferonopathies and other inflammatory mechanisms such as SLE, autoimmune hepatitis, and juvenile idiopathic arthritis (72–74).

Our studies presented here and by Roifman et al. (19) indicate that in RelB-deficient autoimmunity a presentation of interferonopathy is secondary to relopathy-type auto-inflammatory mechanisms, establishing a hierarchical relationship. While prior molecular characterization of human monogenic or polygenic pathologies suggests similarities to RelB-deficiency in the presentation of the pathology, it remains unclear whether the described molecular mechanisms and hierarchy apply. Yet, these findings may provide a potential explanation for the lack of efficacy in interferonopathy patients to therapeutics that target the IFN-axis (75). In sum, our findings emphasize that a presentation of interferonopathy does not necessarily render the interferon pathway an effective drug target and underscores the need for continued characterization of NFκB-driven auto-inflammatory mechanisms to develop effective therapies for relopathies.
Acknowledgments

Chapter 2 is a modified presentation of material that has been published as “RelB-deficient autoinflammatory pathology presents as interferonopathy, but in mice is IFN-independent” by Hector I. Navarro, Yi Liu, Anna Fraser, Diane Lefaudeux, Jennifer J. Chia, Linda Vong, Chaim M. Roifman, and Alexander Hoffmann. The dissertation author was the primary investigator and author of this material. Linda Vong prepared and generated human patient samples for Figure 1A. Diane Lefaudeux processed, analyzed, and generated the figure for Figure 1A. Anna Fraser analyzed the data and generated the figures for Figures 2a, 2c, and 2e. Yi Liu generated samples and provided RNA from CpG and poly(I:C) stimulated BMDCs for RNA-Seq analysis for Figures 3a, 3b, 3c, Figure 4e, Figure S1, and Figure S2. Jennifer J. Chia analyzed the histological sections from Figure 4d.

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Author Contributions

A.H. and H.I.N. designed and conceived the research; H.I.N., Y.L. performed experimental work; H.I.N., A.F., D.L., J.C. analyzed the data; H.I.N., C.R., A.H. discussed and interpreted results; L.V., C.R., A.H. procured samples and funding; H.I.N., A.H. wrote and Y.L., D.L., C.R. edited the manuscript.
Data Availability Statement

The experimental data is available on the Gene Expression Omnibus: GSE224515 and is available in the supplemental data.

Conflict of Interest

The authors declare that research in this study was conducted in the absence of a conflict of interest.

Figure Legends
**Human Fibroblasts**

### Motifs

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### Gene Ontology Terms

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<td>Regulation of Cardioblast Diff.</td>
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### Gene Expression

#### poly(I:C) stimulation (Hrs.)

- **Ifnb1**: Exp. (Control R1/R2, Patient 1 R1/R2)
- **Ihhi3**: Exp. (Control R1/R2, Patient 1 R1/R2)
- **Oas1**: Exp. (Control R1/R2, Patient 1 R1/R2)
- **Oas2**: Exp. (Control R1/R2, Patient 1 R1/R2)
- **Ifit1**: Exp. (Control R1/R2, Patient 1 R1/R2)
- **Ifit2**: Exp. (Control R1/R2, Patient 1 R1/R2)
- **Mx2**: Exp. (Control R1/R2, Patient 1 R1/R2)
- **Ifnl3**: Exp. (Control R1/R2, Patient 1 R1/R2)
- **Ifnb**: Exp. (Control R1/R2, Patient 1 R1/R2)
Fig. 1 Fibroblasts obtained from a human *RelB*-null donor show hyperexpression of type I interferon and interferon-stimulated genes.  

a Heatmap of z-scored log₂CPM of all poly(I:C)(10µg/ml)-induced (FC>4 and FDR <0.01) genes in Control or P1 (RelB-null) derived fibroblasts (740 genes). Each row represents individual genes, and each column is from an individual time point post stimulation. R1 and R2 are experimental replicates. Red and blue colors represent distance from mean log₂CPM value for each gene. 

b Top two results of known motif analysis results for gene clusters from Fig. 1a. Motif analysis considered -4kb to +1kB with respect to the transcription start site (TSS). Cluster B did not generate motifs with given parameters. (N.A.=No motif result) 

c Gene ontology results for gene clusters from Fig. 1a. 

d Line graphs of gene expression (log₂CPM) for *Ifn*-b, and *Ifn*- λ3, and several ISGs during poly(I:C)-stimulation (0,2,4,8 and 15hr) (blue line represents control patient derived fibroblasts, Rep. 1= large circle, Rep.2= small circle, red line represents P1 patient derived fibroblasts, Rep. 1= large square, Rep.2= small square).
Mouse GMCS MCs

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Mouse MCSF MCs

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**Cluster B**
- **GO Terms**
  - IFN-β Targets
  - Interferon Gamma Response
  - Interferon Alpha Response

**Cluster C**
- **GO Terms**
  - Inflammatory Response
  - Immune System Process
  - TNF

**Cluster D**
- **GO Terms**
  - Interferon Gamma Response
  - Innate Immune Response
  - Interferon Alpha Response

**Cluster B**
- **GO Terms**
  - LPS Response
  - TNF-α Signaling via NF-κB
  - TNF Targets

**Cluster C**
- **GO Terms**
  - Interferon Alpha Response
  - Interferon Gamma Response
  - Interferon Alpha Response

**Cluster D**
- **GO Terms**
  - Interferon Gamma Response
Fig. 2 Loss of RelB in mouse myeloid cells recapitulate hyperexpression of type I IFN and interferon stimulated gene programs. a Heatmap of z-scored microarray mRNA expression of all poly(I:C)-induced (10µg/ml) (Log₂FC >1) genes in WT GMCSF MCs (425 genes) (upper panel). Motif analysis and GO results for gene clusters from Fig. 2a (lower panel) b Line graphs of mRNA expression during poly(I:C)-stimulation (0,1,3,8 and 24hr), blue line (circle) represents WT GMCSF MCs, red line (square) represents RelB⁻/⁻ GMCSF MCs c (Top) Heatmap of z-scored mRNA expression of all CPG-induced (0.1µM) (Log₂FC >1) genes in WT MCs (415 genes). c (Bottom) Motif analysis and GO results for gene clusters from Fig. 2c d Line graphs of mRNA expression during CpG-stimulation (0,1,3,8 and 24hr), same color key as Fig 2b. e (Left) Heatmap of z-scored mRNA expression of all LPS-induced (Log₂FC >1) genes in WT or RelB⁻/⁻ MCSF MCs (1,243 unique genes). e (Right) Motif analysis and gene ontology results from gene cluster B of Fig. 2e. Each row in heatmaps represents individual genes, each column is an individual time point during stimulation. Motif analysis showing top statistically significant motif analysis result (known or de-novo) considering -1kb to +1kB with respect to the TSS.
Cluster A
(\(n=215\))

Cluster B
(\(n=119\))

Z-score of \(\log_2(\text{CPM})\)

Mouse BMDCs

Human Fibroblast IFN Independent Genes
Fig. 3 Type I IFN receptor compound deficiency ablates the elevated IFN-stimulated gene program but reveals other immune response genes suppressed by RelB independent of type I IFN signaling.

a Heatmap of z-scored CPM of all $RelB^{+/-}$ hyper-expressed genes in WT, $RelB^{-/-}$, $IFNAR^{-/-}$, and $IFNAR^{-/-}RelB^{-/-}$ MCs. Genes selected for CpG-induced (0.1µM) ($\log_2$FC >1) in WT or $RelB^{-/-}$ MCs & hyper-expressed (FC >1.5) in $RelB^{+/-}$ MCs relative to WT MCs at any time point (334 genes). Each row represents individual genes, each column is from an individual time point during stimulation. b Top known motif analysis and gene ontology results for gene clusters from Fig. 3a. c Line graphs of CPM for IFN-independent hyper-expressed genes from cluster A from Fig.3a and genes with similar functions during CpG-stimulation (0,1,3, and 8hr), dark blue line (closed circle) represents WT GMCSF MCs, dark red line (closed square) represents $RelB^{+/-}$ GMCSF MCs, light blue line (open circle) represents $IFNAR^{-/-}$ GMCSF MCs, light red line (open square) represents $IFNAR^{-/-}RelB^{-/-}$ GMCSF MCs. d Line graphs of log$_2$CPM for genes from cluster A in Fig.3a and genes with similar functions in patient derived fibroblasts from Fig.1a. Poly(I:C)-stimulation (0,2,4,8 and 15hr), (blue line represents control patient derived fibroblasts, Rep. 1= large circle, Rep.2= small circle, red line represents P1 patient derived fibroblasts, Rep. 1= large square, Rep.2= small square). Motif analysis considered -1kb to +1kB with respect to the TSS.
Double Heterozygous Litter-Mate

Mouse BMDCs

CpG stimulation (Hrs.)

Spleen Weights (g)

P=.38

P=2x10^-4

P=.010

P=.55

P=.27
Fig. 4 Ablation of the elevated type I IFN-stimulated gene program does not rescue RelB-null pathology.

a Representative image of $\text{IFNAR}^{-/-};\text{RelB}^{-/-}$ (Right) and healthy litter mate (Left) at 4 weeks of age, ruler for scale. b Representative image of spleens from $\text{IFNAR}^{-/-};\text{RelB}^{-/-}$ mice (Right) and healthy litter mate (Left) c Spleen weights from age-matched WT (solid dark blue), $\text{RelB}^{-/-}$ (solid dark red), $\text{IFNAR}^{-/-}$ (checkered light blue), $\text{IFNAR}^{-/-}\text{RelB}^{-/-}$ (checkered light red), and $\text{IFNAR}^{+/-}\text{RelB}^{+/-}$ (solid light blue) mice. Error bars indicate S.D. Statistical analysis was done using unpaired 2-tailed students t-test d Representative images from histology of WT, $\text{RelB}^{-/-}$, $\text{IFNAR}^{-/-}$, and $\text{IFNAR}^{-/-}\text{RelB}^{-/-}$ spleens, demonstrating loss of white pulp and expansion of red pulp in $\text{IFNAR}^{-/-}\text{RelB}^{-/-}$ spleens. White bar for scale (bottom right, 50µm) e Line graphs of gene expression (CPM) for ISGs showing loss of induction in $\text{IFNAR}^{-/-}$ (light blue, open circle) and $\text{IFNAR}^{-/-}\text{RelB}^{-/-}$ (light red, open square) mice during CpG-stimulation (0,1,3, and 8hr). Dark blue line (closed circle) represents WT GMCSF MCs, dark red line (closed square) represents $\text{RelB}^{-/-}$ GMCSF MCs, light blue line (open circle) represents $\text{IFNAR}^{-/-}$ GMCSF MCs, light red line (open square) represents $\text{IFNAR}^{-/-}\text{RelB}^{-/-}$ GMCSF MCs.
<table>
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<th>IFNAR&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>IFNAR&lt;sup&gt;−/−&lt;/sup&gt; RelB&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<td>B</td>
<td>(n=82)</td>
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<td>C</td>
<td>(n=50)</td>
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Z-score of log2(CTP)
Fig. S1 RelB suppresses some immune response genes independently of type I IFN signaling with poly(I:C) stimulation. Heatmap of z-scored CPM of all RelB\(^{-/-}\) hyper-expressed genes in WT, RelB\(^{-/-}\), IFNAR\(^{-/-}\), and IFNAR\(^{-/-}\)RelB\(^{-/-}\) MCs. Genes selected for poly(I:C) (10\(\mu\)g/mL) induced (Log\(2\)FC >1) in WT or RelB MCs at any time point & hyper-expressed (FC>1.5) in RelB\(^{-/-}\) MCs relative to WT MCs at any time point (262 genes). Each row represents individual genes, and each column is from an individual time point post stimulation.
Poly IC Stimulated Mouse BMDCs - Interferon Independent

Poly IC Stimulated Mouse BMDCs - Interferon Dependent

poly(I:C) stimulation (Hrs.)
Fig. S2 Individual IFN independent hyper expressed immune response genes in IFNAR<sup>−/−</sup> RelB<sup>−/−</sup> MCs upon poly(I:C) stimulation. Line graphs of gene expression (CPM) for IFN-independent genes hyper-expressed genes from cluster A from Fig.S1 and genes with similar functions during poly(I:C) stimulation (0,1,3, and 8hr) (dark blue line (circle) represents WT GMCSF MCs, dark red line (square) represents RelB<sup>−/−</sup> GMCSF MCs, light blue line (triangle) represents IFNAR<sup>−/−</sup> GMCSF MCs, light red line (triangle) represents IFNAR<sup>−/−</sup>RelB<sup>−/−</sup> GMCSF MCs.
Serum Cytokine Levels

**CXCL10**

- WT
- RelB<sup>-/-</sup>
- IFNAR<sup>-/-</sup>
- IFNAR<sup>-/-</sup>RelB<sup>-/-</sup>

P = .006

**IL-6**

- WT
- RelB<sup>-/-</sup>
- IFNAR<sup>-/-</sup>
- IFNAR<sup>-/-</sup>RelB<sup>-/-</sup>

P = .051
Fig. S3 Serum cytokine levels derived from WT, RelB<sup>−/−</sup>, IFNAR<sup>−/−</sup>, and IFNAR<sup>−/−</sup>RelB<sup>−/−</sup> mice.

Bar graphs of cytokine concentrations (pg/mL) for CXCL10 and IL-6. Solid blue bar represents serum from WT mice (n=3), solid red bar represents serum from RelB<sup>−/−</sup> mice (n=2), checkered blue bar represents serum from IFNAR<sup>−/−</sup> mice (n=2), checkered red bar represents serum from IFNAR<sup>−/−</sup>RelB<sup>−/−</sup> mice (n=1).
References:


74. Gedik KC, Lamot L, Romano M, Demirkaya E, Piskin D, Torreggiani S, et al. The 2021 European Alliance of Associations for Rheumatology/American College of Rheumatology points to consider for diagnosis and management of autoinflammatory type I

Chapter 3

NF-κB RelB suppresses autoimmunity and inflammatory gene expression by competing with RelA for binding to target gene promoters
Title: NF-κB RelB suppresses autoimmunity and inflammatory gene expression by competing with RelA for binding to target gene promoters

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ABSTRACT

Many auto-inflammatory disorders arise as a consequence of NF-κB dysregulation, termed relopathies. Genetic loss of the NF-κB subunit, RelB, in humans and mice leads to autoimmunity and lethal multi-organ inflammatory pathology; our recent study showed that the pathology is independent of type I interferon signaling and revealed dysregulation of a myriad of pro-inflammatory genes which contain NF-κB RelA motifs at their regulatory regions. It remains unknown how the loss of RelB leads to the dysregulation of these RelA motif containing pro-inflammatory genes. Here, we report biochemical and genome-wide analyses of dendritic cells derived from WT and RelB mutant mice. We found that the loss of RelB results in elevated binding by RelA to κB sites at or near the TSS of a subset of hyper-expressed genes, including previously characterized IFN-independent NF-κB pro-inflammatory genes. To test whether RelB may inhibit RelA binding to target gene promoters via competition for κB sites, we generated a new mouse strain that harbors targeted point mutations in the RelB-DNA binding domain guided by X-ray crystallographic structures to eliminate high affinity DNA binding. We found that the DNA binding function of RelB is required to suppress both IFN-dependent and IFN-independent inflammatory gene expression, and the targeted loss of the RelB-DNA binding function is alone sufficient to phenocopy the RelB⁻/⁻ inflammatory pathology. These results provide insight into the biological mechanism of RelB as a regulator of pro-inflammatory gene expression and a suppressor of auto-immune pathology.

Introduction:

Current studies estimate that there are around 150 life-long autoimmune diseases, that are characterized by dysregulation of the adaptive immune system, with no known cures (1), affecting
approximately 5-8% of the world wide population (2). There is also a growing list of 40 genetically described autoinflammatory diseases, characterized by dysregulation of innate immune responses (3). One of the most common immune response pathways associated with autoimmune and autoinflammatory disease is NF-κB (4–7). NF-κB plays a central role in inducing the expression of genes involved in cell survival, differentiation, and inflammation (8–11), and is comprised of a family of five individual subunits RelA, cRel, RelB, p52, and p50, that dimerize to form multiple hetero- and homo dimeric transcription factors (12,13). Genetic loss of the RelB subunit in mice results in multiorgan inflammatory pathology characterized by several phenotypic lesions such as mixed inflammatory cell infiltration in several organs, including lungs and liver, as well as myeloid splenomegaly due to extramedullary hematopoiesis, and thymic atrophy (14). Transcriptomic studies using fibroblasts obtained from a previously reported patient with a combined immunodeficiency (CID) and autoimmune pathology resulting from a rare homozygous RelB mutation leading to the complete loss of RelB revealed a pronounced dysregulation of interferon-stimulated gene expression (15–17). This dysregulated gene program was also seen in dendritic cells derived from RelB−/− mice, cells that have been shown to be key mediators of autoimmunity in the loss of RelB pathology (17). These data suggested the categorization of the loss of RelB autoimmune pathology as an interferonopathy characterized as a group of inherited autoinflammatory diseases with a dysregulation of the interferon pathway (18–21). However, genetic ablation of IFN-signaling with IFNAR−/− RelB−/− mice did not improve critical aspects of the RelB−/− pathology (17). Further analysis of dendritic cells derived from IFNAR−/− RelB−/− mice revealed a subset of dysregulated NF-κB RelA motif-containing pro-inflammatory genes that are the likely drivers of the pathology, suggesting its classification as a relopathy (an NF-κB-related autoinflammatory disease) (22–24). Given that these NF-κB proinflammatory genes were found
to be hyper-expressed in the loss of RelB human patient derived fibroblasts, and NF-κB signaling was also found to be dysregulated in a separate study with patients containing a different mutation leading to reduced levels of RelB (25), this calls for critical attention in understanding how the loss of RelB leads to the dysregulation of these NF-κB motif-containing pro-inflammatory genes.

Inflammatory gene expression by NFκB is tightly regulated by NFκB inhibitor proteins, IκBs. Three inhibitors, IκBα, IκBβ, and IκBɛ, allow for NFκB activation via the canonical pathway as stimulus-induced phosphorylation by the NEMO-containing IκB kinase targets them to degradation (26). (27,28). A fourth IκB activity is mediated by a multi-meric complex (IκBsome) consisting of IκB domain-containing p105 and p100, encoded by nfkbi and nfkb2 genes, respectively. The IκBsome controls the pool of NFκB that is available to bind to IκBα, IκBβ, and IκBɛ, and hence activatable via the canonical NFκB pathway (29,30). Once NFκB dimers are activated, they bind the same DNA sequence motif, with preferences between homo- and heterodimers, but remarkably little difference between the heterodimeric RelA:p50, cRel:p50 and RelB:p50 dimers (31).

While RelA is known as a potent transcriptional activator that controls inflammatory gene expression, RelB was characterized as both a transcriptional activator and a repressor of metabolic and inflammatory genes (32–34). RelB which is typically constitutively nuclear, was found to repress gene expression activity by facilitating repressive chromatin modifications through the formation of complexes with histone deacetylase and histone methyltransferase (35,36), but was also found to stabilize p100 and sequester RelA in a non-productive complex (29,37,38). Thus it remains unclear what mechanism lead to dysregulated pro-inflammatory gene expression and autoimmunity in RelB-deficient patients and mice (15,16). Given the role of dendritic cells in the loss of RelB pathology and other autoimmune diseases (39–42) and the high expression of RelB
in mouse and human dendritic cells (43,44), we sought here to characterize the mechanism by which RelB represses inflammatory gene expression in dendritic cells from RelB$^{-/-}$ mice. We found that RelB$^{-/-}$ dendritic cells showed elevated RelA binding to chromatin, and addressed the hypothesis that the two family members compete for binding the same κB sites in vivo. To test this, we generated a RelB DNA binding mutant mouse strain in which RelB lacks 3 amino acid side chains that make specific contacts with κB site nucleotides and performed biochemical and genome-wide analysis of RNA-Seq and ChIP-Seq data. Our results support a model in which simple competition for κB sites endows RelB with the important function to broadly dampen immune response gene expression and thereby reduce the risk for auto-inflammatory and auto-immune disease.

Materials and Methods

*Design of RelB DNA binding mutants*

The pBABE puro vector containing RelB were constructed for RelB$^{DB/DB}$ mice by first amplifying the coding region fragment of murine RelB corresponding to the desired amino acids 1 to 558 by the polymerase chain reaction (PCR), inserted into the *EcoRI* and *BamHI* sites of retroviral vector pBABE-puro. Mutagenesis using Agilent QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) were performed for RelB point mutations. DNA binding mutant within amino-terminal of RelB were mutated by a single or triple substitution at residues Arg117, Tyr120, and Glu123 to Ala (R117A, Y120A, R123A). RelB-Y120A was obtained by PCR using a pair of oligonucleotide (5’-tgccatgctcggcGCggtgaggcgcgctccgccg and 5’-gcggcctcgacctcgGCggggactgcc). RelB-R117A/Y120A/E123A mutant was obtained using
a pair of oligonucleotide (5’-cagcgtgcatgGCcttccgcGCcgagtgcGCgggccgctcggcc and 5’-ggccgacgcgggccGCgcactcgGCgcggaagGCcatgccacgctg).

*Generation of cell line harboring RelB DNA binding mutants*

Immortalized *relb*/*nfkb2*−/− fibroblast cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% bovine calf serum (BCS), 1% penicillin-streptomycin and 1% L-Glutamine. Plat-E cells were cultured in 10% fetal calf serum, 1% penicillin-streptomycin, 1% L-Glutamine, and supplemented with 1µg/ml Puromycin, and 10µg/ml of Blasticidin.

For transfection, Platinum-E (Plat-E) retroviral packaging cell line (45), a modified cell line derived from HEK-293T, were plated on 10-cm plates 16-hr prior at 50% confluent in DMEM supplemented with 10% fetal calf serum, 1% penicillin-streptomycin and 1% L-Glutamine. Cells were transfected using 300µl of Opti-MEM medium (ThermoFisher Scientific) and polyethylenimine (PEI; 1µg/µl in 1xPBS pH4.5, Polysciences #23966-2) with 7µg of retroviral construct DNA, pBABE puro empty vector (EV control) or RelB expressing constructs (4:1 ratio of PEI (µl):plasmid DNA (µg)). Transfection complex (Opti-MEM medium, PEI reagent, plasmid DNA) were incubated for 15minutes at room temperature then added drop-wise to Plat-E cells for 6 hours. Transfected media was replaced with fresh DMEM media containing 10% fetal calf serum, 1% penicillin-streptomycin and 1% L-Glutamine. Cells were further incubated for a total of 48-hr post-transfection and prior to collecting viruses. Viruses-containing supernatant were filtered through a 0.45 µm filter and used to infect *relb*/*nfkb2*−/− MEFs with the addition of 4µg/ml Polybrene. 48-hr post-infection of MEFs with viruses the stably transduced cells were selected with 2.5µg/ml puromycin for a total of 72 hours. Post selection with puromycin, puromycin-containing medium is removed and cells are passaged twice for recovery prior to being expanded.
in culture for experiments. Empty vector (EV), pBABE puro vector without the RelB gene fragment is used as a negative control, to maintain a stable retrovirally transduced RelB knockout cell line, meanwhile pBABE puro containing full-length RelB (RelB-WT) is used as a positive RelB expressing cell line.

**Biochemical characterization of RelB DNA binding mutants**

Western blotting and electrophoretic mobility shift assay (EMSA) were performed with standard methods as described previously (Shih et al., 2012; Werner *et al*., 2005; Hoffmann *et al*., 2002). For co-immunoprecipitation assay of RelB, whole cell lysates (WCL) from 3 x 10^6 cells were lysed with RIPA buffer [50mM Tris-Cl (pH 7.5), 150mM NaCl, 1mM EDTA, 0.10% SDS, 1% TritonX-100] containing 1 mM PMSF and 1 mM DTT. For co-immunoprecipitation assay, unstimulated WCLs were incubated overnight at 4°C with 5µg anti-RelB antibody conjugated to prewashed protein-G beads (Dynabeads Protein-G magnetic beads, Life Technologies). The beads were then washed thoroughly in 1X TBS-T buffer pH 7.4, and eluted with 100µL 1xSDS sample buffer containing 2.5% β-mercaptoethanol and analyzed by western blotting. Western blotting were probed with antibodies specific for RelB (Santa Cruz Biotechnology, sc-226), RelA (Santa Cruz Biotechnology, sc-372), IκBα (Santa Cruz Biotechnology, sc-371), and p105/p50 (BioBharati Lifescience, BB-AB0080). For EMSA, MEF cells were stimulated with 1 ng/ml TNF and nuclear extracts were harvested at indicated times (h). 2.5 µL total normalized nuclear extracts were incubated for 15 minutes with 0.01 pmol of P^32-labeled 38bp double-stranded oligonucleotide containing two consensus κB sites (5’-GCTACAAAGGACTTTCCGCTGGGACTTTCCAGGGAGG-3’ ) in binding buffer [10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10% glycerol, 1% NP-40, 1mM EDTA, 0.1 mg ml⁻¹
Poly(deoxyinosinic-deoxycytidylic)], in a final reaction of 6 µL. The reaction mixtures were run on a non-denaturing 5% acrylamide (30:0.8) gel containing 5% glycerol and 1X TGE buffer [24.8 mM Tris, 190 mM glycine, 1 mM EDTA] at 200 volts for 2 hours. The gel was visualized by Typhoon Scanner (GE Healthcare Life Sciences). BMDC whole cell lysates were collected by scraping cells on ice and transferring to Eppendorf tube with subsequent PBS washes and resuspension in 100µl of 1x Lamelli Sample Buffer (Bio-rad #1610737) and then heated to 95 degrees for 5 minutes and stored at 80 °C before use. Nuclear cell lysates for BMDCs were collected after stimulation by scraping cells on ice and transferring to Eppendorf tube with subsequent PBS washes, cells were then resuspended in 200µl of Cytoplasmic Extract Buffer (10 mM HEPES-KOH pH 7.9, 10 mM KCl, .1 mM EDTA, 1 mM DTT, 1 mM PMSF, 4µg/ml Leupeptin, 0.001mM Pepstatin A, 0.01TIU/ml Aprotinin, and Phosphatase Inhibitor Cocktail Set I and II, 1 /100µL (EMD Millipore, 524624-1SET and 524625-1SET )) and incubated for 10 minutes. After incubation, 2 µl of 10% NP-40 was added and incubated for 30 seconds followed by vortexing for 30 seconds. Cells were spun down three times at 12000xg for 40 seconds at room temperature. Nuclei were then collected and resuspended in Nuclear Extract Buffer (20nM HEPES pH7.9, 420 mM NaCl, 1.5 mM KCl, .2 mM EDTA, 1 mM DTT, 1 mM PMSF, 4µg/ml Leupeptin, 0.001mM Pepstatin A, 0.01TIU/ml Aprotinin, and Phosphatase Inhibitor Cocktail Set I and II, 1 /100µL (EMD Millipore, 524624-1SET and 524625-1SET )). Western blot quantifications were done via previously established methods (46).

**Generation of the RelB DNA binding mutant mouse strain**

The RelB<sup>DB<sub>DB</sub></sup> mutant mouse line was generated by Ingenious Targeting Laboratory. A donor sequence encoding the mutant RelB protein was used to generate, via homologous recombination,
a tagged embryonic stem cell line, that was implanted to yield heterozygous mice. These mice were then bred with a mouse line constitutively expressing the Flp recombinase to remove the Neo resistance marker included in the homologous donor sequence. We then back-crossed the resultant mice with wild-type C57BL/6J mice to remove the Flp background and generate homozygous knocking $RelB^{DB/DB}$ mice.

**Mouse husbandry**

Wild-type, knockout and knockin mice were housed in pathogen-free conditions at University of California, Los Angeles. $RelB^{-/-}$ mice were generated by breeding $RelB^{+/-}$ mice and $IFNAR^{-/-}RelB^{-/-}$ mice were generated by mating $IFNAR^{+/-}RelB^{-/-}$ to each other. All mice used for experiments were between 4 to 12 weeks at day of experiment, both male and female mice were used for experiments.

**Bone Marrow-Derived Dendritic Cells**

Bone marrow cells were isolated from mouse femurs and cultured with 20 ng/ml GM-CSF and 10 ng/ml IL-4 to produce BMDCs with half the media being replaced on day 3 and 6 as previously reported [35]. Cells were stimulated with CpG (0.1μM) (Invivogen ODN 1668 Cat# tlrl-1668), or Poly(I:C) HMW (10μg/mL) (Invivogen Cat#tlrl-pic) and collected at specified timepoints in Invitrogen™ TRIzol™ Reagent (Cat# 15-596-018) for RNA and was extracted using Qiagen RNeasy Mini Kit (Cat #74106) as described (Shih et al 2012).

**Transcriptome profiling**

RNA was used for Illumina bead Arrays as described (Cheng et al 2017) and for RNA-seq as described (Sen et al 2020). Briefly, libraries were prepped using KAPA Stranded mRNA-Seq Kit
Illumina® platform KR0960 – v3.15 using 1µg of RNA per sample measured using Qubit 2.0 fluorometer. Final libraries were checked via agarose gel and multiplexed with a maximum of 24 samples per sequencing reaction. Libraries were sequenced using Illumina HiSeq 3000 with single end 50bp reads at the UCLA Technology Center for Genomics & Bioinformatics.

**Bioinformatic Analysis**

Reads were trimmed using cutadapt (1) (cutoff q=20) and mapped to the mm10 genome. Processed reads showed high quality reads and alignment scores. The October 2014 version of the Ensembl database was used to extract gene annotation information. CPM values were generated using edgeR (5) to normalize the raw counts data based on sequencing depth. To permit fold change calculations, a pseudocount of 1 CPM was added. Induced genes were selected using a log$_2$FC>1 cutoff for any stimulated timepoint relative to 0hr unstimulated control, transcripts with empty gene names were removed. Data was z-scored and plotted using the pheatmap R package. Fold differences of genes within heatmaps was calculated by first calculating the fold differences for all individual genes between genotype of interest and WT or $IFNAR^+$ control CPM (Genotype X)/CPM (Genotype Y) for each individual time point. Average fold differences were then calculated by averaging the fold differences of all genes within each cluster for each individual time point. Gene ontology and motif analysis was done via homer suite considering regulatory regions within -1kb to 1kb from the transcription start site. Line graphs of individual genes were generated using GraphPad Prism.

**ChIP Sample Generation and Data Preprocessing**
ChIP-seq was performed as previously described (Barish et al., 2010; Lee et al., 2006) with anti-RelA antibody (Cell Signaling, 8242). Approximately 10 million BMDCs were used per sample. Two chemical crosslinkers, DSG and PFA, were used during sample preparation at concentrations of 1.0mM and 1.0%, respectively. Sonication was performed on a Covaris M220 focused ultrasonicator. ChIP-seq libraries were prepared using KAPA HyperPrep Kits (Roche). Reads were aligned using Hisat2 to the mouse genome (NCBI37/mm9). Peaks were called if they were enriched compared to input samples and had a false discovery rate of < 0.01 using HOMER software (Heinz et al., 2010). To compare peaks across samples, a master probe which contained all peaks from every sample was generated using BEDTools (Quinlan et al., 2010). Then SeqMonk was used to find raw read counts for each peak in the master probe. RPKMs were generated using the length of each peak and the depth of sequencing for each sample.

*Tissue Isolation and Fixation*

Spleens were isolated from age matched mice immediately after euthanizing and subsequently rinsed with PBS. Excess PBS was removed from spleens and were subsequently weighed. Fixation was done in 10% Formaldehyde for 46-48hrs. Tissue was processed, sectioned, and hematoxylin and eosin stained by the UCLA Translational Pathology Core Laboratory (TPCL).

*Results*

**Characterization of IFN-independent driven multi-organ inflammation in RelB−/− mice.**
Loss of RelB in mice results in a multiorgan inflammatory pathology characterized by various phenotypic lesions such as mixed inflammatory cell infiltration in several organs, including lungs and liver, as well as myeloid splenomegaly due to extramedullary hematopoiesis, and thymic atrophy, characterized by an average of 30% decrease in thymic weights (14). Transcriptomic studies using fibroblasts obtained from a previously reported patient (P1) with a combined immunodeficiency (CID) and autoimmune pathology resulting from a rare homozygous RelB mutation leading to the complete loss of RelB revealed a pronounced dysregulation of interferon-stimulated gene expression which was also seen in dendritic cells derived from RelB−/− mice (17). Initially, these data suggested the categorization of the loss of RelB pathology as an interferonopathy, however, genetic ablation of IFN-signaling in a compound mutant IFNAR−/−RelB−/− mouse strain did not improve critical aspects of the loss of RelB pathology such as splenomegaly, runted growth, and elevated serum cytokine levels (17). Here we examined other aspects of the loss of RelB pathology that have been previously reported (14,39,40). We therefore produced compound IFNAR−/−RelB−/−mice and compared them to WT, single RelB−/−, and single IFNAR−/− littermates. As previously reported, IFNAR−/−RelB−/−mice were runted compared to littermate controls (Fig. 1a), and displayed splenomegaly. Thymic atrophy was assessed by weight, and thymi were found to be significantly smaller in IFNAR−/−RelB−/−mice (Fig. 1b) with weights being substantially lower than controls, analogous to the previously reported defect in single RelB−/− mice versus controls (14) (Fig. 1c). Histologic sections of lung and liver appeared unremarkable in WT and IFNAR−/− mice; in contrast, there was marked lymphocytic and granulocytic inflammatory infiltrates in diffuse and perivascular distributions in IFNAR−/−RelB−/− mice (Fig. 1d and e), similar to the previously described findings for RelB−/− mice (14). Common among previous reports characterizing the loss of RelB pathology, dendritic cells are key regulators of the loss of RelB
inflammatory pathology in in various tissues (39,40) and while IFN signaling was shown to be dysregulated in dendritic cells derived from RelB\(^{-/-}\) mice (17), the present phenotypic and pathological characterization provides further evidence that the loss of RelB pathology is largely driven independently of IFN signaling.

**Loss of RelB leads to elevated RelA binding to a subset of genomic κB sites.**

Considering these data, we focused our attention to the previously identified NF-κB driven pro-inflammatory gene expression that remain dysregulated in bone marrow dendritic cells (BMDCs) derived from *IFNAR\(^{-/-}\)/RelB\(^{-/-}\)* mice (17). Given that these NF-κB pro-inflammatory genes were also found to be dysregulated in patient-derived fibroblasts from RelB-null human patients (17), we aimed at a further mechanistic characterization of the suppression of NF-κB driven pro-inflammatory gene expression by RelB.

While previous studies have reported some differences in binding specifies to NF-κB motifs by certain NF-κB homodimers, all NF-κB hetero-dimers bind the classical 10 base pair palindromic κB motif GGRNNNNNYCC. In particular, RelB-containing heterodimers have shown DNA binding specificities similar to cRel and RelA heterodimers (31). We therefore asked if RelB and RelA may compete for binding to the same κB sites. We approached this question by first examining whether the loss of RelB may result in altered RelA binding at NF-κB motifs that would normally be bound by RelB in WT conditions. To answer this, we generated dendritic cells from HSCs derived from both *RelB\(^{-/-}\)* and *WT* mice and performed ChIP-seq for NF-κB subunits, RelA and RelB. We first analyzed the distribution of the relative binding strength of all RelA binding sites between *RelB\(^{-/-}\)* and *WT* BMDCs. Differences in binding strength were normally distributed and centered at a 1:1 ratio. Binding sites at the 90\(^{th}\) percentile displayed a log\(_2\)(.41) fold change.
in binding between \( \text{RelB}^- \) BMDCs and \( \text{WT} \) BMDCs. Whereas binding events in the bottom 10% displayed a \( \log_2(-.59) \) fold change (Fig. 2a). To assess whether sequences bound by RelB in \( \text{WT} \) BMDCs display altered RelA binding in \( \text{RelB}^- \) BMDCs, we selected for RelB binding events induced > 2-fold by LPS stimulation (10ng/mL) (Log\(_2\)FC>1) in \( \text{WT} \) BMDCs and subsequently analyzed the changes in RelA binding at these locations in \( \text{RelB}^- \) BMDCs. Using k-means clustering we found 3 predominant RelA binding patterns at these sites. Cluster A (n=1,580) contained binding events which revealed elevated binding by RelA in \( \text{RelB}^- \) BMDCs, with an average of \( \log_2\)FC\((.56) \) between \( \text{RelB}^- \) and \( \text{WT} \) BMDCs (Fig. 2b & 2c). Cluster B (n=2,135) contained RelA binding events that were unchanged in \( \text{RelB}^- \) BMDCs with an average of \( \log_2\)FC\((-0.08) \) between \( \text{RelB}^- \) and \( \text{WT} \) BMDCs (Fig. 2b & 2c), and binding events in cluster C (n=719) displayed a reduction in RelA binding with an average of \( \log_2\)FC\((-1.09) \) between \( \text{RelB}^- \) and \( \text{WT} \) BMDCs (Fig. 2b & 2c). Furthermore, motif analysis of sequences within these clusters confirmed the specificity of these binding events to RelA and RelB antibodies with appx. 60%-80% of peaks within each cluster containing NF-κB binding elements (Fig. 2e). These data reveal that while the majority of RelA-DNA binding events are unchanged in the absence of RelB, the loss of RelB affects binding of RelA to a portion of NF-κB binding elements which are also bound by RelB in a \( \text{WT} \) setting. This suggests that RelA and RelB may compete for binding to NF-κB binding elements genome-wide.

**Elevated RelA binding to promoter regions is correlated with elevated gene expression.**

We next sought to understand if sites with elevated RelA binding in the absence of RelB are enriched at or near genes that are hyper-expressed in \( \text{RelB}^- \) BMDCs. To answer this, we first
annotated all RelA binding event to the nearest gene, resulting in the annotation of several RelA binding events to some genes. Since we were specifically interested in RelA binding events that showed elevated binding as a consequence of the loss of RelB, we filtered our data to assign each gene with only the one RelA binding event displaying the highest fold change between RelB\(^{-/-}\) and WT BMDCs. After our filtering we found the ratio between RelA binding events in RelB\(^{-/-}\) vs. WT BMDCs was normally distributed and centered around 1.1. RelA binding events in the top 90\(^{th}\) percentile displayed log\(_2\)FC(.56) and the bottom 10\(^{th}\) percentile had a log\(_2\)FC(-.28) (Fig. 3a). Using our previously published RNA-seq data sets from WT and RelB\(^{-/-}\) BMDCs, we then asked if the pro-inflammatory genes previously reported to be dysregulated in RelB\(^{-/-}\) BMDCs contained NF-κB regulatory elements that showed elevated binding by RelA. To answer this, for every gene induced > 2-fold upon CpG stimulation (0.1µM) in either WT or RelB\(^{-/-}\) BMDCs, we plotted the RelB\(^{-/-}\) hyper-expression phenotype against the elevated RelA binding phenotype and highlighted the previously reported IFN-independent NF-κB pro-inflammatory genes that were hyper-expressed in RelB\(^{-/-}\) BMDCs. We found that 98 of 117 (84%) of these genes fell within the elevated binding and hyper-expressed quadrant (Fig. 3b). Further, loss of RelB resulted in a statistically significant increase in RelA binding at enhancer regions of these NF-κB pro-inflammatory genes relative to genes with unchanged gene expression between RelB\(^{-/-}\) and WT BMDCs (Fig. 3c). We then compared the genome browser tracks of RelA binding events at or near the promoter regions of previously reported IFN-independent NF-κB pro-inflammatory genes that were hyper-expressed in RelB\(^{-/-}\) BMDCs. While many RelA binding events were directly on the promoter regions, the average distance between RelA binding events and their respective TSS was -1,352 bp (Supplementary Table 1). We observed that the loss of RelB resulted in elevated binding by RelA at or near the TSS of NF-κB pro-inflammatory genes
including *Cd40, Ikbke, Map3k14, Ccl5, Ccl22*, and *Cd80* (**Fig. 3d**). These data provide evidence that in a RelB deficient context, hyper-expressed pro-inflammatory genes seem to show elevated binding by RelA at or near their respective TSS.

**RelB requires its DNA binding function to suppress autoimmune pathology.**

These data suggests that RelB may suppress the expression of NF-κB pro-inflammatory genes and potentially the inflammatory pathology via competition with RelA for binding to sites at or near the TSS regulatory promoter regions of these genes. On the other hand, RelB has other functions that do not require DNA binding. For example, it has also been reported that the loss of RelB results in reduced stability of the *nfkb2 p100* protein, a component of the IκBsome which sequesters RelA in the nucleus thereby limiting the pool of transcriptionally active NF-κB (29,37,47). RelB has also been reported to dimerize with RelA into a complex that cannot bind DNA (48), potentially inhibiting the availability of transcriptionally active RelA:RelA and RelA:p50 dimers. We therefore aimed to determine whether RelB suppresses the autoinflammatory pathology seen in *RelB<sup>−/−</sup>* mice via its DNA binding function or other functions mediated by protein-protein interactions. To distinguish between these two possibilities, we aimed to generate a novel RelB mutant mouse ([RelB<sup>DB/DB</sup>]) with a directed loss of DNA binding function. Using the previously reported crystal structure of RelB showing the protein–DNA binding interface (49), we first designed and characterized RelB variants with a single amino acid substitution (RelB<sup>V120A</sup>) or a triple substitution within the amino-terminal DNA binding domain of RelB, converting Arg117, Tyr120, and Glu123, to Alanine (R117A, Y120A, R123A) (**Fig. 4a**). Having confirmed the ectopic expression of these RelB protein variants to be at similar levels to
wild type RelB protein in RelB−/− 3T3 cells (Fig. S1), we measured their ability to bind DNA via electrophoretic mobility shift assay. We found that while wild type RelB protein was able to bind NF-κB binding elements, single RelBY120A and triple RelBR117A, Y120A, R123A mutant proteins were both unable to bind to NF-κB binding elements, confirming the directed loss of DNA binding function (Fig. 4b). To ensure maximal reduction in DNA binding affinity we decided to use the triple mutant design for generating the RelB DNA mutant mouse (RelBDB/DB). As expected, analysis of BMDCs produced from bone marrow cells derived from these RelBDB/DB mice revealed similar baseline levels of p100 protein relative to BMDCs generated from WT mice (Fig. 4c and Fig. S2) and did not show elevated levels of RelA nuclear translocation upon stimulation with CpG (10nM) (Fig. 4d).

Upon phenotypic assessment, RelBDB/DB mutant mice appeared runted and much smaller than WT littermates, were hunched, had scaly skin, and enlarged abdomens similar to RelB−/− mice at 8 weeks of age (14) (Fig. 4e). RelBDB/DB mice were found to have marked splenomegaly as reported in RelB−/− mice, with spleens from RelBDB/DB mice weighing on average 1.7x more than spleens from WT mice (Fig. 4f). To determine whether this pathology was type I interferon dependent or independent we bred the new RelBDB/DB mutant strain into the IFNAR−/− mouse strain to produce a compound mutant. Similar to our previous findings, splenomegaly was found to be independent of IFN signaling, with spleens from IFNAR−/−RelBDB/DB mice weighing on average 2.1x and 1.9x more than spleens from WT and single IFNAR−/− mice, respectively (Fig. 4f). Histologic sections of spleens showed a reduction in white pulp and expansion of red pulp in RelBDB/DB and IFNAR−/−RelBDB/DB spleens when compared with WT and IFNAR−/− controls (Fig. S3). In the lung, histologic sections from both RelBDB/DB and IFNAR−/−RelBDB/DB mice showed a moderate lymphocytic perivascular infiltrate (Fig. 4g). Histologic sections of livers from
RelB<sup>DB/DB</sup> mice showed moderate periportal and centrilobular lymphocytic infiltrates; IFNAR<sup>−/−</sup> RelB<sup>DB/DB</sup> livers also showed periportal and centrilobular lymphocytic infiltrates but appeared less extensive (Fig. 4h). RelB<sup>DB/DB</sup> mice also revealed thymic atrophy that was not rescued by IFNAR<sup>−/−</sup>-RelB<sup>DB/DB</sup> compound mutant mice similar to RelB<sup>−/−</sup> mice (Fig S4).

**RelB suppresses type I IFN signaling and pro-inflammatory genes via DNA binding function.**

Given that phenotypic and histological analysis of the RelB<sup>DB/DB</sup> mutant mice revealed a similar autoinflammatory pathology as previously characterized in RelB<sup>−/−</sup> mice, we wondered whether the DNA binding function or other functions mediated by protein-protein interactions of RelB are required for suppressing the IFN-dependent and/or IFN-independent pro-inflammatory genes that are hyper-expressed in RelB<sup>−/−</sup> mice. To distinguish between these two mechanisms, we isolated BMDCs derived from WT, RelB<sup>−/−</sup>, RelB<sup>DB/DB</sup>, or single IFNAR<sup>−/−</sup>, IFNAR<sup>−/−</sup>-RelB<sup>−/−</sup>, and IFNAR<sup>−/−</sup>-RelB<sup>DB/DB</sup> compound mutant mice which produce mice with complete ablation of type I IFN signaling and thus a loss of secondary IFN-dependent gene expression programs, while IFN-independent gene expression remains intact. We then stimulated and collected cells at various timepoints (0, 1, 3, and 8hr.) and performed RNA-seq followed by differential gene expression analysis.

To identify genes that were hyper-expressed by complete loss of RelB, we first selected for genes that were induced by CpG (0.1µM) (log<sub>2</sub>FC>1) in either WT or RelB<sup>−/−</sup> BMDCs and hyper-expressed (FC>1.5) in RelB<sup>−/−</sup> BMDCs relative to WT BMDCs and subsequently plotted the expression of these genes across BMDCs derived from WT, RelB<sup>−/−</sup>, RelB<sup>DB/DB</sup>, single IFNAR<sup>−/−</sup>,
IFNAR\textsuperscript{+}/RelB\textsuperscript{−}, and IFNAR\textsuperscript{−}/RelB\textsuperscript{DB/DB} mice. Using k-means clustering, we identified an IFN-dependent hyper-expressed cluster B (93 genes) containing genes hyper expressed in RelB\textsuperscript{−/−} BMDCs which had average fold differences ranging from 1.25x to 1.5x compared to WT BMDCs at all observed time points. The expression of genes in this cluster was absent in all IFNAR\textsuperscript{−/−} containing BMDCs with average fold differences of .5x-.6x in all IFNAR\textsuperscript{−/−} containing BMDCs relative to WT BMDCs at the late 8h stimulated timepoint, suggesting they were IFN-dependent ISGs (Fig. 5a, Supplementary Table 2). Notably, we found this cluster of genes was also hyper-expressed in BMDCs derived from RelB\textsuperscript{DB/DB} mice, with fold differences ranging from 1.35x to 1.5x between RelB\textsuperscript{DB/DB} and WT BMDCs at all observed time points. As expected, motif analysis of cluster B produced the interferon sensitive response element (ISRE) as the top statistically enriched motif and gene ontology analysis revealed interferon signaling stimulating pathways “interferon beta” and “interferon alpha” among the top terms (Fig. 5b).

Analysis of cluster A (178 genes) revealed genes that were hyper-expressed in all RelB\textsuperscript{−/−} containing conditions but were not hyper-expressed in BMDCs derived from single IFNAR\textsuperscript{−/−} mutant mice, suggesting these were previously established IFN-independent NF-κB pro-inflammatory genes. Again, we found these genes were also hyper-expressed in BMDCs derived from the RelB\textsuperscript{DB/DB} and IFNAR\textsuperscript{−/−}/RelB\textsuperscript{DB/DB} compound mutant mice. These genes revealed average fold differences ranging from 1.3x to 1.5x between RelB\textsuperscript{DB/DB} BMDCs relative to WT BMDCs, 1.6x to 2.0x between IFNAR\textsuperscript{−/−}/RelB\textsuperscript{DB/DB} BMDCs relative to WT BMDCs, and 1.2x to 1.3x between IFNAR\textsuperscript{−/−}/RelB\textsuperscript{DB/DB} BMDCs relative to single IFNAR\textsuperscript{−/−} BMDCs at all observed timepoints (Fig. 5a, Supplementary Table 4). Motif analysis of cluster A revealed NF-κB as the top statistically enriched motif for these genes and gene ontology analysis revealed NF-κB activating pathways, “CD40”, “LPS” and “TNF-a signaling”, among the top GO terms (Fig. 5b). Analysis of individual
genes in the IFN-dependent hyper-expressed cluster B were previously established ISGs including Mx1, Isg20, Oasl2, Ifi35, Ifit1, Isg15, Oaslb, as well as Ifn-b, which were also hyper-expressed in BMDCs derived from RelBDB/DB mice (Fig. 5c). Notably, the expression of previously established hyper-expressed NF-κB pro-inflammatory genes such as Map3k14, Ikbke, Ccl22, Ccl5, Cd40, Cd80, and Cd86 were also hyper-expressed in RelBDB/DB and IFNAR−/−RelBDB/DB BMDCs relative to WT and single IFNAR−/− BMDCs. (Fig. 5c). These data together provide strong evidence that RelB suppresses both IFN-independent and dependent pro-inflammatory gene expression via direct binding to DNA.

Discussion:

Our study aimed to elucidate the mechanism by which RelB acts to suppresses the hyper-expression of pro-inflammatory genes and inflammatory pathology seen in RelB−/− mice (14) (17). We found that the loss of RelB pathology is driven interferon-independently in all aspects of the pathology including in the lung, liver, and thymus (Fig. 1a-c). Our RNA-Seq and ChIP-Seq analysis of dendritic cells from RelB−/− mice revealed elevated binding by RelA to a subset of genomic κB sites (Fig. 2a-c) and elevated binding by RelA to the promoter regions of the previously identified IFN-independent pro-inflammatory genes was correlated with their hyper-expression caused by the loss RelB (Fig. 3b-d). These data suggested that RelB may inhibit the expression of these genes via competing with RelA for binding to their promoter regions on the DNA. On the other hand, previous reports have found that RelB is required for the stabilization of p100, a core component of the IkBsome complex such that loss of RelB resulted in elevated RelA nuclear translocation (29,30,37). We also identified this effect in BMDCs derived from RelB−/− mice (Fig. 4c). Therefore, we aimed to identify whether the nuclear DNA binding function or other
protein-protein interaction function of RelB was required for suppressing pro-inflammatory gene expression and the inflammatory pathology seen in RelB\(^{-/-}\) mice. To answer this, we generated a novel RelB\(^{DB/DB}\) mouse with a directed loss of the DNA binding function (Fig. 4a & 4b) that maintained cytoplasmic p100 stabilization function and did not result in elevated RelA nuclear translocation (Fig. 4c & 4d). We found that while the cytoplasmic functions of RelB remained intact, the directed loss of RelB DNA binding function alone resulted in the hyper-expression of both type I IFN-dependent and independent pro-inflammatory gene expression similar to the pathology seen by complete loss of RelB (Fig. 5a-d) (17). Further, upon phenotypic analysis, RelB\(^{DB/DB}\) mutant mice appeared runted and much smaller than WT littermates at 8 weeks of age, similar to the RelB\(^{-/-}\) mice (Fig. 4e) (14). RelB\(^{DB/DB}\) mice presented with hunched backs, scaly skin, enlarged abdomens, thymic atrophy, splenomegaly, and moderate lymphocytic perivascular infiltrate in the lungs, and less extensive periportal and centrilobular lymphocytic infiltrates in the liver. Like RelB\(^{-/-}\) mice, these critical aspects of the inflammatory pathology were not rescued by the generation of IFNAR\(^{-/-}\)/RelB\(^{DB/DB}\) compound mutant mice (Fig. 4e-h, Fig. S3-S4).

While we identified that the RelB DNA binding function is required to suppress both the pro-inflammatory gene expression and inflammatory pathology seen in RelB\(^{-/-}\) mice, further studies are warranted to understand whether the loss of other specific functions of RelB may also produce the inflammatory pathology. Additionally, while BMDCs in our cell culture system demonstrate competition between RelA and RelB for binding to promoter regions of target genes, further experiments are required to understand whether other members of the NF-κB family may also be involved. Our findings also do not determine the exact function of RelB and RelA upon binding to DNA, so it remains to be determined whether RelB may be suppressing gene expression upon DNA binding through previously reported epigenetic mechanisms (35,36,50) or may simply
be inhibiting RelA transcriptional activity by occupying the promoter regions of these genes. Interestingly though, it has been shown that RelB and H3 lysine methyltransferase G9a generate epigenetic silencing at the interleukin-1β promoter, and the loss of RelB similarly results in increased binding by RelA to the IL-1β promoter (36).

Upon activation of the non-canonical NF-κB pathway, RelB most commonly binds to the processed form of p100, p52, the NF-κB subunit encoded by nfkb2. This leads to the formation of the RelB/p52 hetero-dimer which functions as the pre-dominant dimer downstream of the non-canonical NF-κB signaling pathway (51,52). Interestingly nfkb2 has also been established as an inhibitor of dendritic cell activation, with nfkb2−/− DCs revealing elevated MHC class II and costimulatory molecule expression and an enhanced ability to induce CD4+ T cell responses (53). Mice deficient in nfkb2 with a complete loss of p100 protein also demonstrate defects in humoral responses, germinal center reactions, and secondary lymphoid development, but develop only a mild autoimmune pathology in a subpopulation of mice, with the great majority having unaffected lifespans (54–57). These data support our findings that the stabilization of p100 function by RelB is not the key mechanism leading to lethal mutli-organ pathology in RelB−/− mice. A hypothesized mechanism for the milder nfkb2−/− pathology has been put forth in which the absence of p100 may facilitate the nuclear translocation of RelB/p50 dimers and activate non-canonical signaling in this fashion and therefore compensate for loss of RelB/p52 dimers (58,59).

In other studies NF-κB p50 has also been shown to have antagonistic effects on RelA. Given that p50 homo-dimers lack a transactivation domain (60), but are able to bind DNA and compete with RelA for binding to κB sites at regulatory regions, p50 homodimers are thought to acts as repressors of NF-κB gene expression. Indeed overexpression of p50 was shown to suppress
RelA activated expression of several immune response genes such as *H-2K1*, *ICAM1*, and *IL-2* (61–63). Interestingly B-cell lymphoma factor 3 (Bcl-3) is another IκB-like regulator of NF-κB signaling (64), and has been shown to predominantly interact with repressive p50 and p52 homodimers. Being located primarily in the nucleus (64–66), its main function towards NF-κB lies in altering the binding of NF-κB factors to DNA (66). Bcl-3 can also bind directly to p50 and form stable complexes to maintain the nuclear localization of p50 homo-dimers (66), and upon the phosphorylation of Bcl-3, it has been shown to increase the binding of p50 homodimers to DNA in thymocytes (65), and inhibit the binding of RelA hetero-dimers to κB sites (67).

Interestingly, while single *bcl3*⁻/⁻ mice demonstrate minor defects in T cell-mediated immunity, germinal center reactions, and secondary lymphoid organ development, *bcl3*⁻/⁻ mice display an even milder pathology than *nfkb2*⁻/⁻ mice (59,68,69). However, compound *nfkb2*⁻/⁻*bcl3*⁻/⁻ mutant mice reveal severe lethal multi-organ pathology that was remarkably similar to *RelB*DB/DB and *RelB*⁻/⁻ mice (Fig 4A) (59). *Nfkb2*⁻/⁻*bcl3*⁻/⁻ mice were runted in size, hunched, had scaly skin, and marked immune infiltration in the liver, lungs, and skin that was mediated by T-cells and also revealed severely disrupted thymic architecture (59), but the molecular characterization of this pathology remains to be understood. These data support our proposed model in which elevated levels of nuclear RelA translocation caused by the loss of p100 protein alone is not sufficient for severe pathology. Further, the loss of DNA binding competition between repressive p50 homodimers and RelA at specific κB sites caused by deletion of *bcl-3* alone is also insufficient to cause severe pathology. Instead, the *nfkb2*⁻/⁻*bcl3*⁻/⁻ combination leading to elevated nuclear RelA levels and elevated κB site availability from loss of repressive nuclear p50 homodimers may lead to elevated RelA binding to κB sites that reach the threshold needed to result in severe lethal inflammatory pathology. Given the high levels of nuclear RelB in dendritic cells (43,44) and the
established genome-wide competition between RelB and RelA for binding at κB sites (Fig. 4a-c), our model proposes that loss of RelB DNA binding function alone results in elevated RelA DNA binding to a broad range of NF-κB pro-inflammatory genes. Therefore, RelB is unable to dampen RelA DNA binding activity at these pro-inflammatory genes, ultimately leading to severe inflammatory pathology similar to nfkб2−/−bcl3−/− mice. This is further underscored by the fact that the drastic autoimmune pathology and pro-inflammatory gene expression changes we observe are associated with only slight 1.5-4x fold differences in RelA binding to promoter regions of these genes.

In summary, while RelB has been previously been described as a transcriptional activator (33,71,72), our findings of RelB in suppressing pro-inflammatory gene expression are reminiscent of the earliest reports of RelB as a transcriptional repressor (32). While in other contexts RelB functions as a transcriptional activator, in BMDCs it functions as a repressor of transcriptional activity. These data together highlight the fine tuning of the NF-κB signaling system, and notably in the BMDC context, RelB seems to play a key role in regulating RelA activity. These data provide critical mechanistic insights into the autoinflammatory mechanisms leading to the loss of RelB pathology and other NF-κB mediated pathologies. While substantial inflammatory consequences are seen at the phenotypic level, our findings highlight that the loss of RelB pathology, although caused by a minor lesion in one subunit of NF-κB, ultimately leads to broad reaching deleterious effects in other arms of the NF-κB family. Finally, these data demonstrate that loss of RelB pathology and others like it may likely require therapeutic approaches focused on fine tuning these slight changes in dysregulated gene regulatory mechanisms, these approaches will likely lead to the most promising clinical interventions. Given the extensive list of pathologies linking NF-κB to autoimmunity and the emerging human pathologies caused by mutations in other
NF-κB family members also leading to similar dysregulation of IFN-dependent and IFN-independent pro-inflammatory gene expression (6,7,73), these data will likely provide broad and immediate insights for these autoimmune and inflammatory diseases.

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Chapter 3 is a modified presentation of material that is in preparation for publication as “NF-κB RelB suppresses autoimmunity and inflammatory gene expression by competing with RelA for binding to target gene promoters” by Hector I. Navarro, Allison Daly, Kim Ngo, Anna Fraser, Jennifer J. Chia, Yi Liu, and Alexander Hoffmann. The dissertation author was the primary investigator and author of this material. Jennifer J. Chia analyzed the histological sections from Figures 1d and 1e, Figures 4g and 4h, and Figure S3. Yi Liu generated samples and provided LPS stimulated BMDCs for ChIP-seq analysis in Figures 2a-d and Figures 3a-d. Yi Liu generated samples and provided RNA from CpG stimulated BMDCs for RNA-seq analysis for Figures 5a-d. Allison Daly generated and processed ChIP-seq data for Figures 2a-d and Figures 3a-d. Anna Fraser analyzed data for Figure 2B and Figure 3B. Kim Ngo designed, generated, and characterized the RelB DNA binding mutant proteins in Figures 4A, 4B, S1 and generated RelB<sup>DB/DB</sup> mutant mice for Figures 4d-h, Figures 5a-d, and Figures S2-S4.

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Author Contributions
A.H. and H.I.N. designed and conceived the research; H.I.N., Y.L., K.N., A.D. performed experimental work; H.I.N., A.F., A.D., J.C. analyzed the data; H.I.N., A.H. discussed and interpreted results; A.H. procured samples and funding; H.I.N., A.H. wrote and Y.L. and A.D. edited the manuscript.

**Conflict of Interest:**

The authors declare that research in this study was conducted in the absence of a conflict of interest.

**Figure Legends:**
Figure 1: Characterization of IFN-independent driven multi-organ inflammation in RelB<sup>−/−</sup>mice.

A) IFNAR<sup>−/−</sup> IFNAR<sup>−/−</sup> littermate RelB<sup>−/−</sup>

B) IFNAR<sup>−/−</sup> IFNAR<sup>−/−</sup> RelB<sup>−/−</sup>

C) Age-Matched Thymic Weights

D) Lung Histology

E) Liver Histology

WT  RelB<sup>−/−</sup>  IFNAR<sup>−/−</sup>  IFNAR<sup>−/−</sup> RelB<sup>−/−</sup>  IFNAR<sup>−/−</sup> HGB<sup>−/−</sup>  IFNAR<sup>−/−</sup> HGB<sup>−/−</sup> RelB<sup>−/−</sup>
Figure 1: Characterization of IFN-independent driven multi-organ inflammation in RelB-/- mice. a Representative image of IFNAR-/-RelB-/- (Right) and IFNAR-/- litter mate (Left) at 4 weeks of age, ruler for scale. b Representative image of thymi from IFNAR-/-RelB-/- mice (Right) and IFNAR-/- litter mate (Left) c Thymic weights from age-matched WT (dark blue), RelB-/- (dark red), IFNAR-/- (light blue, checkered), IFNAR-/-RelB-/- (light red, checkered) mice. (***=p<.001, **=p<.01, *=p<.05); error bars indicate S.D. Statistical analysis was done using unpaired 2-tailed students t-test d Representative images of H&E stained sections of WT, RelB-/-, IFNAR-/-, and IFNAR-/-RelB-/- mouse lungs (d) and liver (e). Scale bar indicates 50µm; n=2-3.
Figure 2: Loss of RelB leads to elevated RelA binding to a subset of genomic \( \kappa \)B sites.

A. All RelA ChIP-seq Peaks in WT vs. RelB\(^{-/-}\) 

- Mean: 0.06
- 90th %: 0.41
- 10th %: -0.59

B. \( \alpha \)-RelA CHIP

<table>
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<th>WT</th>
<th>RelB(^{-/-})</th>
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<td>LPS</td>
<td>+</td>
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Cluster analysis:

- Cluster A: 1e-353, 933/1396
- Cluster B: 1e-446, 1086/1891
- Cluster C: 1e-144, 476/591

C. RelA binding in RelB\(^{-/-}\) vs. WT

D. de novo Motif Analysis

Cluster A: NF-\( \kappa \)B

Cluster B: NF-\( \kappa \)B

Cluster C: NF-\( \kappa \)B
**Figure 2:** Loss of RelB leads to elevated RelA binding to a subset of genomic κB sites.  

a. Histogram of log$_2$FC ($RelB^{-/-}$/$WT$) RelA Binding (RPKM) for all ChIP peaks with RPKM>10, descriptive statistics= mean: log$_2$(−0.06), 90$^{th}$ percentile: log$_2$(0.41), 10$^{th}$ percentile log$_2$(−0.59).  

b. Heatmap of z-scored RelA and RelB-ChiP peaks (RPKM) in all RelB-bound peaks in $WT$ and $RelB^{-/-}$ BMDCs. Peaks selected for all LPS (10ng/mL) induced RelB-ChiP binding events (log$_2$FC>1) at 1hr LPS timepoint in $WT$ BMDCs, min RPKM>10 in any condition. Heatmaps for RelA and RelB-ChiP are ordered by RelA k-means clustering and separately z-scored.  

c. Scatter plot showing the means of log$_2$FC ($RelB^{-/-}$/WT) RelA Binding for clusters A-C from Fig. 2b.  

e. Top de novo motif analysis results for genomic regions from clusters A-C from Fig 2C. Motif analysis considered motif sizes up to 200bp.
Figure 3: Elevated RelA binding to promoter regions is correlated with elevated gene expression.

A) ReLA peaks associated with LPS-induced genes

B) ReLA binding vs. Gene Expression

C) ReLA binding in RelB-/- vs. WT

D) Heatmaps of gene expression for various genes under LPS treatment for 1 hour with and without RelB.
Figure 3: Elevated RelA binding to promoter regions is correlated with elevated gene expression. 
a Histogram of filtered highest fold change RelA-ChIP peaks for all genes. Peaks were annotated to the nearest gene promoter region and filtered for the highest fold change \( \log_{2} \text{FC} (\text{RelB}^{−/−}/\text{WT}) \) RelA binding (RPKM) for each individual gene, all peaks are RPKM>10, descriptive statistics= mean: \( \log_{2}(1.14) \), 90\(^{\text{th}}\) percentile: \( \log_{2}(0.55) \), 10\(^{\text{th}}\) percentile \( \log_{2}(-0.28) \).  
b Scatter plot showing the \( \log_{2} \text{FC} (\text{RelB}^{−/−}/\text{WT}) \) in RelA binding (RPKM) at 1hr LPS stimulation compared to all CPG induced genes (Log\(_{2}\text{FC}>1\)) in WT or RelB\(^{−/−}\) BMDCs, 8hr peak gene expression timepoint shown. Previously characterized IFN-independent RelB\(^{−/−}\) hyper expressed genes are highlighted in red, all other induced genes are colored grey.  
b Scatter plot comparing the \( \log_{2} \text{FC} (\text{RelB}^{−/−}/\text{WT}) \) RelA binding (RPKM) of IFN-independent RelB\(^{−/−}\) hyper expressed genes and genes with unchanged expression (.93<FC<1.07 relative to WT) at 8hr. CpG stimulation timepoint.  
d Representative genome browser tracks for ChIP peaks near promoter regions (<4kb) of TSS for IFN-independent RelB\(^{−/−}\) hyper expressed genes. IGV tracks from WT BMDCs are represented in blue, IGV tracks from RelB\(^{−/−}\) BMDCs are represented in red. RelB\(^{−/−}\) (red) and WT (blue) RelA-ChIP tracks (top row) are overlayed for visual purposes.
Figure 4: RelB requires its DNA binding function to suppress autoimmune pathology.

A) Designing RelB DNA Binding mutants

B) relb<sup>−/−</sup> ifnb2<sup>−/−</sup> 3T3 Cells

C) WT | RelB<sup>−/−</sup> | RelB<sup>−/−</sup>  p100  R1  R2  R3

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D) CPG (10nM)                   0  15  30  60  120  240  0  15  30  60  120  240 min

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E) WT | RelB<sup>−/−</sup>

F) Age-Matched Spleen Weights

G) Lung Histology

H) Liver Histology
Figure 4: RelB requires its DNA binding function to suppress autoimmune pathology. a Crystal structure of novel RelB DNA binding domain mutant with alanine substitutions at R117, Y120, E123. b EMSA supershift assay with pBABE puro (EV), RelB\textsuperscript{WT}, Rel\textsuperscript{Y120A}, and RelB\textsuperscript{DB/DB} reconstitution in rebl\textsuperscript{-/-}p52\textsuperscript{-/-}p50\textsuperscript{-/-} 3KO 3T3 cells stimulated 30 min with TNF-\alpha (1 ng/mL). c Western Blot for p100 and B-tubulin from whole cell lysates of unstimulated BMDCs derived from WT, RelB\textsuperscript{-/-}, and RelB\textsuperscript{DB/DB} mice., n=3. d Western Blot for RelA and p84 on nuclear fractions of time course CpG (10nM) stimulated BMDCs derived from WT and RelB\textsuperscript{DB/DB} mice., n=2. e Representative images of RelB\textsuperscript{DB/DB} (left) and WT litter mate (right) at 8 weeks of age, ruler for scale. f Spleen weights from age-matched WT (dark blue), RelB\textsuperscript{DB/DB} (dark green), IFNAR\textsuperscript{-/-} (light blue, checkered), IFNAR\textsuperscript{-/-}RelB\textsuperscript{DB/DB} (light green, checkered) mice. (**=p<.01, *=p<.05, n.s.= not significant); error bars indicate S.D. Statistical analysis was done using unpaired 2-tailed students t-test. g Representative images from H&E stained sections of WT, RelB\textsuperscript{DB/DB}, IFNAR\textsuperscript{-/-}, and IFNAR\textsuperscript{-/-}RelB\textsuperscript{DB/DB} lungs (g), and livers (h). Scale bar indicates 50\mu m; n=2-3.
Figure 5: RelB suppresses type I IFN signaling and pro-inflammatory genes via DNA binding function.

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Z-score of CPM

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92
Fig. 5: RelB suppresses type I IFN signaling and pro-inflammatory genes via DNA binding function. 

a Heatmap of z-scored CPM of all RelB\(^{-/-}\) hyper-expressed genes in WT, RelB\(^{-/-}\), RelB\(^{DB/DB}\), IFNAR\(^{-/-}\), and IFNAR\(^{-/-}\)RelB\(^{-/-}\), IFNAR\(^{-/-}\)RelB\(^{DB/DB}\) BMDCs. Genes selected for CpG (0.1\(\mu\)M)-induced (log\(_2\)FC>1) in WT or RelB BMDCs at any time point & hyper-expressed (FC>1.5) in RelB\(^{-/-}\) BMDCs relative to WT BMDCs at any time point (271 genes). Each row represents individual genes, and each column is from an individual time point post stimulation.

b Top result of known motif analysis for gene clusters from Fig. 5a. Motif analysis considered -1kb to +1kB with respect to the transcription start site (TSS), Gene ontology (GO) results for gene clusters from Fig. 5a.

c Line graphs of gene expression (CPM) for IFN-b and interferon stimulated genes upon CpG-stimulation (0,1,3, and 8hr) dark blue line (circle) represents WT BMDCs, dark red line (square) represents RelB\(^{-/-}\) BMDCs, dark green line (triangle) represents RelB\(^{DB/DB}\) BMDCs, light blue line (inverted triangle) represents IFNAR\(^{-/-}\) BMDCs, light red line (diamond) represents IFNAR\(^{-/-}\)RelB\(^{-/-}\) BMDCs, light green line (circle) represents IFNAR\(^{-/-}\) RelB\(^{DB/DB}\) BMDCs.

d Line graphs of gene expression (CPM) for IFN-independent hyper-expressed genes from cluster A from Fig. 5a and genes with similar functions during CpG-stimulation (0,1,3, and 8hr) dark blue line (circle) represents WT BMDCs, dark red line (square) represents RelB\(^{-/-}\) BMDCs, dark green line (triangle) represents RelB\(^{DB/DB}\) BMDCs, light blue line (inverted triangle) represents IFNAR\(^{-/-}\) BMDCs, light red line (diamond) represents IFNAR\(^{-/-}\) RelB\(^{DB/DB}\) BMDCs, light green line (circle) represents IFNAR\(^{-/-}\) RelB\(^{DB/DB}\) BMDCs.
S1

α-RelB IP

RelB (75-63-48-35)
Fig S1: Western Blot for RelB of RelB-IP samples from WT 3T3 cells and relb<sup>−/−</sup>/p52<sup>−/−</sup>/p50<sup>−/−</sup>
3KO 3T3 cells reconstituted with RelB<sup>WT</sup>, RelB<sup>Y120A</sup>, and RelB<sup>R117A, Y120A, R123A</sup>.
p100 Protein Level

WT
RelB
RelB

n.s.

**

p100 Protein Levels
(Normalized to WT Max)
Fig S2: Bar graphs of quantifications for p100 and B-tubulin from western blot of whole cell lysates of unstimulated BMDCs derived from WT, RelB−/−, and RelBDB/DB mice. Blue bar represents WT BMDCs, red bar represents RelB−/− BMDCs, green line represents RelBDB/DB BMDCs. (**=p<.01, n.s.=not significant) n=3, Error bars indicate S.D. Statistical analysis was done using unpaired 2-tailed students t-test.
Spleen Histology

WT

RelB$^{DB/DB}$

IFNAR$^{-/-}$

IFNAR$^{-/-}$ RelB$^{DB/DB}$
Fig S3: Representative images from H&E stained sections of WT, RelB\(^{DB/DB}\), IFNAR\(^{-/-}\), and IFNAR\(^{-/-}\) RelB\(^{DB/DB}\) spleens. Scale bar indicates 50µm; n=2-3
Age-Matched Thymic Weights

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** n.s.
Fig S4: Thymic weights from age-matched WT (dark blue), \( RelB^{DB/DB} \) (dark green), \( IFNAR^{-/-} \) (light blue, checkered), \( IFNAR^{-/-}RelB^{DB/DB} \) (light red, checkered) mice. (**=p<.01, n.s.= not significant); error bars indicate S.D. Statistical analysis was done using unpaired 2-tailed students t-test.
References:


46. Davarinejad H. Quantifications of Western Blots with ImageJ.


Chapter 6

Conclusion and future perspectives
**Conclusion and future perspectives**

Together these studies provide a comprehensive molecular characterization of the autoinflammatory pathology caused by the loss of RelB. First, I undertook an unbiased transcriptomic analysis of fibroblasts derived from patients with a rare RelB-null mutation (1,2) and discovered a prominent dysregulated Type I IFN gene signature (3). My analysis revealed the Type I IFN, *Ifnb* to be hyper-expressed in these patient-derived fibroblasts, resulting in the hyper-expression of *Ifnb* responsive ISGs, a phenotype that was conserved in dendritic cells derived from *RelB*−/− mice (3). Given the potential clinical implications of these findings, I sought to understand whether this elevated IFN signaling played a critical role in driving the inflammatory pathology, and further, if attenuating the IFN pathway would remedy the pathology. To test this, I generated an *IFNAR*−/−*RelB*−/− compound mutant mouse, and while I was able to achieve complete ablation of dysregulated ISG expression, I surprisingly found that the compound mutant *IFNAR*−/−*RelB*−/− mice showed no amelioration of critical aspects of the inflammatory pathology. These *IFNAR*−/−*RelB*−/− mice remained runted, presented with hunched backs, enlarged abdomens, thymic atrophy, splenomegaly, and revealed marked lymphocytic and granulocytic inflammatory infiltrates in diffuse and perivascular distributions of the lung and liver. These data provided compelling evidence that the while the inflammatory pathology seen in *RelB*−/− mice presents as an interferonopathy(4–7), it is however interferon independent. If this remarkable dysregulation in interferon signaling seems to play a superfluous role, I wondered what dysregulation may remain to drive the disease.

I therefore continued my analysis focusing on IFN-independent genes that remain dysregulated in *IFNAR*−/−*RelB*−/− mice. I identified an IFN-independent cluster containing pro-inflammatory genes commonly sharing an NFkB binding motif at or near their transcriptional
start sites that remained dysregulated in BMDCs derived from IFNAR\(^{\pm}\)/RelB\(^{\pm}\) mice. My analysis of individual genes within this cluster identified non-canonical NFκB activator Map3k14 (NIK) and Ikbke (IKKε), which have both been shown to be potent activators of the type I IFN transcription factor, IRF3 (8–11). I wondered whether the hyper-expression of these genes may in fact play a role in hyper-activating type I IFN signaling but given that IFN signaling did not seem to play a role in driving the pathology, I did not pursue this further. Among these IFN-independent pro-inflammatory genes, I also found co-stimulatory surface proteins Cd40, Cd80, and Cd86 which are involved in T cell activation of the adaptive immune response (12,13), as well as potent T-cell recruiting chemokines Ccl5 and Ccl22 (14–16). Notably, a recent report of distinct patients with a different mutation resulting in reduced RelB expression also revealed a dysregulation of NFκB motif containing pro-inflammatory genes (17). These data together indicate that the hyper-expression of NFκB regulated pro-inflammatory genes are likely the drivers of the loss of RelB inflammatory pathology and calls critical attention to the need for mechanistic characterization of how RelB acts to suppress these pro-inflammatory genes.

In my follow up study, I aimed to mechanistically describe how RelB acts to suppresses the hyper-expression of these IFN-independent pro-inflammatory genes and the inflammatory pathology seen in RelB\(^{\pm}\) mice (18). Previous reports have found that RelB is required for the stabilization of p100, a core component of the RelA inhibiting complex, IκBδ, and that loss of RelB resulted in reduced levels of cytoplasmic IκBδ and elevated RelA nuclear translation (19–21). I also identified this effect in BMDCs derived from RelB\(^{\pm}\) mice. In my study, I found that the loss of RelB resulted in elevated RelA binding to a subset of genomic κB sites and revealed elevated binding to the promoter regions of the previously identified IFN-independent pro-inflammatory genes that are hyper-expressed as a result of the loss RelB. These data suggested
that RelB may inhibit the expression of these genes via competition with RelA for DNA binding to their promoter regions. Therefore, I aimed to identify whether the cytoplasmic p100 stabilization or the nuclear DNA binding function of RelB was required for suppressing pro-inflammatory gene expression and the inflammatory pathology seen in RelB<sup>−/−</sup> mice. To answer this, using previously reported structures of RelB (22), I generated a novel RelB<sup>DB/DB</sup> mouse with a directed loss of the RelB DNA binding function that maintained cytoplasmic p100 stabilization function and did not result in elevated RelA nuclear translocation. I found that while the cytoplasmic function of RelB remained intact, the directed loss of its DNA binding function resulted in the hyper-expression of both Type I IFN-dependent and independent pro-inflammatory gene expression. Further, upon phenotypic analysis, RelB<sup>DB/DB</sup> mutant mice appeared runted and much smaller than WT littermates, similar to the RelB<sup>−/−</sup> mice at 8 weeks of age (18). These mice presented with hunched backs, enlarged abdomens, thymic atrophy, splenomegaly, and moderate lymphocytic perivascular infiltrate in the lungs, and less extensive periportal and centrilobular lymphocytic infiltrates in the liver. Like RelB<sup>−/−</sup> mice, these critical aspects of the inflammatory pathology were not rescued by the generation of IFNAR<sup>−/−</sup>RelB<sup>DB/DB</sup> compound mutant mice.

While I found the RelB DNA binding function is required to suppress pro-inflammatory gene expression and the inflammatory pathology seen in RelB<sup>−/−</sup> mice, I cannot determine whether the loss of other functions of RelB may also produce the inflammatory pathology. These findings also do not determine the exact function of RelB and RelA upon DNA binding, so it remains unclear whether RelB may be suppressing gene expression upon DNA binding through previously reported epigenetic mechanisms (23–25), or may simply be inhibiting RelA transcriptional activity by occupying the promoter regions of these genes. Interestingly though, it
has been shown that RelB and H3 lysine methyltransferase G9a generate epigenetic silencing at the interleukin-1β promoter, and the loss of RelB similarly results in increased binding by RelA to the IL-1β promoter (26). Additionally, nfkβ2 has also been established as an inhibitor of dendritic cell activation, with nfkβ2−/− DCs demonstrating elevated levels of activation and ability to stimulate T-cell responses (27). Mice with a loss of nfkβ2, and therefore a complete loss of p100 protein, reveal only a mild autoimmune pathology in a subpopulation of mice, with the great majority having unaffected lifespans (28–31), supporting our model in which RelB does not suppress autoimmune pathology primarily through the stabilization of p100 protein. However, Nfkβ2−/−bcl3−/− mice that lack both p100 and bcl3, a primarily nuclear protein (32–34) which stabilizes nuclear levels of the p50 homodimer, a competitor to RelA DNA binding (35–37), revealed a remarkably similar pathology to RelBDB/DB and RelB−/− mice (38). Supporting our model that elevated levels of nuclear RelA translocation caused by the loss of p100 protein alone is not sufficient for severe pathology, nor the removal of an inhibitor of RelA binding to a limited number of κB sites (38–40). Instead RelB acts a genome wide competitor to RelA in binding κB sites at a many inflammatory genes genome wide, and loss of RelB DNA binding function alone leads to elevated binding by RelA to many pro-inflammatory genes and therefore severe autoimmune pathology.

While RelB has been previously been described as a transcriptional activator(23,41), my findings of RelB in suppressing pro-inflammatory gene expression are reminiscent of the earliest reports of RelB as a transcriptional repressor (42). While in other contexts RelB functions as a transcriptional activator, in BMDCs it functions as a transcriptional repressor. These data together highlight the fine tuning of the NFκB signaling system, and notably in the BMDC context, RelB seems to play a unique role in regulating RelA activity. This is further underscored
by the fact that the drastic autoimmune pathology and pro-inflammatory gene expression changes I observe are associated with only slight 1.5-4x fold differences in RelA binding to their promoter regions. In summary these data provide critical mechanistic insights into the autoinflammatory mechanisms leading to the loss of RelB pathology. While drastic inflammatory consequences are seen at the phenotypic level, my findings highlight that the loss of RelB pathology may likely require therapeutic approaches focused on fine tuning these slight changes in dysregulated gene regulatory mechanisms. These approaches will likely lead to the most promising clinical interventions.

References:


