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Reciprocal suppression between TGFβ signaling and TNF stimulation finetunes the macrophage inflammatory response

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Abstract

Inflammation plays a crucial role in the development of various disease conditions or is closely associated with them. Inflammatory cytokines like TNF often engage in interactions with other cytokines and growth factors, including TGFβ, to orchestrate inflammatory process. Basal/endogenous TGFβ signaling is a universal presence, yet the precise way TNF communicates with TGFβ signaling to regulate inflammation and influence inflammatory levels in macrophages has remained elusive. To address this question, this study utilized genetic approaches and a combination of molecular and cellular methods, including conditional TGFβ receptor knockout mice, human cells, RNAseq, ATACseq and Cut & Run-seq. The results reveal that the TGFβ signaling functions as a vital homeostatic pathway, curtailing uncontrolled inflammation in macrophages in response to TNF. Conversely, TNF employs two previously unrecognized mechanisms to suppress the TGFβ signaling. These mechanisms encompass epigenetic inhibition and RBP-J-mediated inhibition of the TGFβ signaling pathway by TNF. These mechanisms empower TNF to diminish the antagonistic influence exerted by the TGFβ signaling pathway, ultimately enhancing TNF's capacity to induce heightened levels of inflammation. This reciprocal suppression dynamic between TNF and the TGFβ signaling pathway holds unique physiopathological significance, as it serves as a crucial "braking" mechanism. The balance between TNF levels and the activity of the endogenous TGFβ signaling pathway plays a pivotal role in determining the overall extent of inflammation. The potential for therapeutically

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AUTHOR CONTRIBUTIONS

Yuhan Xia and Kazuki Inoue designed and performed the experiments, analyzed and curated raw data, prepared figures, and contributed to manuscript preparation. Ting Zheng and Yongli Qin performed all experiments and analyses, made figures and contributed to manuscript preparation for revision. Baohong Zhao conceived of and oversaw the project and wrote the manuscript. All authors reviewed and provided input on the manuscript for submission.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

augmenting the TGFβ signaling pathway presents an intriguing avenue for countering the impact of TNF and, consequently, developing innovative strategies for inflammation control.

1 | INTRODUCTION

TNF is a cytokine important for innate immunity, inflammation, and the regulation of host defense against microbial pathogens. It also plays a crucial pathogenic role in driving chronic inflammation and tissue damage in various inflammatory and autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and psoriasis arthritis (PsA).[1,2] Macrophages are major contributors to the induction and aggravation of inflammation. Stimulation of macrophages with TNF activates the canonical signaling pathways mediated by NF-κB and MAPKs, leading to the expression of well-known inflammatory genes encoding inflammatory cytokines, such as *Il6*, *Il1* and *Tnf*.[1] Moreover, TNF drives macrophages to produce a low amount of IFNβ, which in turn induces type-I IFN-stimulated genes (ISGs), such as *Mx1*, *Ifit1* and *Ifit2*, in macrophages.[3] The expression levels of many ISGs are elevated in RA synovial macrophages.[4] The inflammatory cytokines and ISGs triggered by TNF guide macrophages towards an intensified inflammatory state, thereby exacerbating inflammation.

Transforming growth factor β (TGFβ) is a pleiotropic cytokine that plays important roles in a variety of biological processes and functions, including morphogenesis, embryonic development, stem cell differentiation, and immune regulation. TGFβ binds to TGFβ receptor type II (*Tgfbr2*) that phosphorylates TGFβ receptor type I (*Tgfbr1*). Activation of TGFβ receptor type I further phosphorylates and activates the receptor-activated Smads (R-Smads), Smad2 and Smad3, which then translocate with the co-Smad, Smad4 into nuclei and coordinate with other transcription factors/co-activators to induce the expression of TGFβ responsive/target genes, such as *Id1*, *Id2*, *Id3*, *Cdkn1a and Cdkn2b*.[5–7] In addition to the canonical SMAD signaling, TGFβ can also regulate downstream cellular response via non-Smad pathways, such as the MAPK pathway.[6] The established feedback inhibitors of the TGFβ signaling pathway include inhibitory Smad (I-Smad) Smad7, Smad6, E3 ubiquitin protein ligases Smurf1/2, and other RING/U-box type E3 ubiquitin ligases that bind to Smad3 and Smad4 to induce their degradation.[5–9] The role of TGFβ is complicated and context dependent. For example, TGFβ is involved in both proinflammatory and suppressive immune responses, such as its distinct roles in different T cell subsets and differentiation stages, as well as in inflammatory reaction of myeloid cells in different stimulatory conditions.[10,11]

An essential factor contributing to the intricate regulation of diverse cellular responses by TGFβ is its extensive interactions with other cytokines and growth factors. The interactions between cytokines play a significant role in influencing their activities, especially in diverse pathological conditions, where distinct cytokine networks contribute to host responses and disease development. In many cases, the actions of TNF are observed in coordination with other cytokines, further emphasizing the complexity of cytokine-mediated responses and importance of crosstalk between different cytokines. The interaction between TNF and TGFβ has been reported. Some studies found antagonistic effects between these two

cytokines, whereas others observed synergistic effects, depending on cell types and various scenarios.[12–16] There is basal TGFβ signaling in most cells because of the presence of a low basal level of TGFβ in circulation and tissues. Despite the fact that macrophages at sites of inflammation, such as in RA, are continuously exposed to endogenous TGFβ,[17–22] little is known in terms of how the TGFβ signaling impacts TNF effects in inflammatory conditions, and vice versa.

In the present study, we discovered a mutually suppressive action between TGFβ signaling and TNF stimulation on the inflammatory response of macrophages. Importantly, we uncovered previously unrecognized molecular mechanisms by which TNF antagonizes the TGFβ signaling pathway through Recombinant recognition sequence binding protein at the Jκ Site (RBP-J) and epigenetic regulation. Furthermore, our research reveals the integration of signaling crosstalk between TNF and TGFβ at the chromatin level, enabling precise epigenetic regulation of inflammatory gene expression and responses in macrophages. Considering this newly identified molecular network, our findings have significant implications for the potential development of novel therapeutic strategies aimed at controlling inflammation in autoimmune and chronic inflammatory diseases.

2 | METHODS

2.1 | Mice

We generated mice with myeloid/macrophage-specific deletion of *Tgfbr2* or *Rbpj* by crossing the *Tgfbr2*$^{flox/flox}$ mice (The Jackson Laboratory, Stock No: 012603) or *Rbpj*$^{flox/flox}$ mice[23] with the mice with a lysozyme M promoter-driven Cre transgene on the C57BL/6 background (known as LysMcre; The Jackson Laboratory, Stock No: 004781). Gender- and age-matched *Tgfbr2*$^{flox/flox}$;LysMcre(+) mice (referred to as *Tgfbr2*$^{\Delta M}$) and their littermates with *Tgfbr2*$^{+/+}$;LysMcre(+) genotype as WT controls were used for experiments. Gender- and age-matched *Rbpj*$^{flox/flox}$;LysMcre(+) mice (referred to as *Rbpj*$^{\Delta M}$) and their littermates with *Rbpj*$^{+/+}$;LysMcre(+) genotype as WT controls were used for experiments. For TNF-induced inflammatory response in vivo, we used the established TNF-induced inflammatory mouse model with minor modifications as previously described.[24,25] Briefly, TNFα was administrated daily at the dose of 75 μg/kg to the calvarial periosteum of age- and gender-matched mice for five consecutive days. The blood serum was then collected to test cytokine induction by TNF stimulation. All animal procedures were approved by the Hospital for Special Surgery Institutional Animal Care and Use Committee (IACUC), and Weill Cornell Medical College IACUC.

2.2 | Reagents

Murine or human M-CSF, murine or human TNFα, and human TGFβ1 were purchased from PeproTech. Murine TGFβ1 was purchased from Themo Fisher Scientific.

2.3 | Cell culture

For human cell cultures, de-identified blood buffy coats (blood leukocyte preparations) were purchased from the New York Blood Center using a protocol approved by the Hospital for Special Surgery (HSS) Institutional Review Board. The blood buffy coats were anonymous

without any identifiable private information. As per Human Subjects Research in PHS SF424 (R&R) Application Guide, studies using purchased de-identified blood samples do not constitute human subject research; informed consent was not obtained at HSS. PBMCs from the buffy coats were isolated by density gradient centrifugation using Ficoll (Invitrogen Life Technologies, Carlsbad, CA). CD14(+) monocytes were purified from fresh PBMCs using anti-CD14 magnetic beads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. Human CD14(+) monocytes were cultured at a density of $15.6 \times 10^4/cm^2$ in α-MEM medium (Thermo Fisher Scientific) containing 10% FBS (Atlanta Biologicals), glutamine (2.4 mM, Thermo Fisher Scientific) and Penicillin–Streptomycin (Thermo Fisher Scientific) in the presence of human M-CSF (20 ng/mL; PeproTech, Rocky Hill, NJ) for 3 days to induce macrophages. The cells were then cultured with TNFα (40 ng/mL) and M-CSF (20 ng/mL) in the α-MEM medium for different times indicated in figure legends.

For mouse cell cultures, mouse bone marrow cells were harvested from the tibiae and femora of the age (8–16 week old)- and gender-matched mutant and control mice and cultured for 3 days in α-MEM medium containing 10% fetal bovine serum (FBS), glutamine (2.4 mM, Thermo Fisher Scientific), Penicillin–Streptomycin (Thermo Fisher Scientific) and CMG14–12 supernatant (the condition medium, which contained the equivalent of 20 ng/mL of rM-CSF and was used as a source of M-CSF). The attached bone marrow macrophages (BMMs) were scraped, seeded at a density of $4.5 \times 10^4/cm^2$, and cultured in α-MEM medium with 10% FBS, 1% glutamine and the condition medium for overnight. Except where stated, the cells were then treated without or with optimized concentrations of TNFα (40 ng/mL) in the presence of the condition medium for the times indicated in the figure legends.

2.4 | qPCR

For quantification of mRNA, reverse transcription and real-time PCR were performed as previously described.[26] mRNA amounts were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The primers for real-time PCR were as follows:

Tnf: 5′-CCCTCACACTCAGATCATCTTCT-3′ and 5′-G CTACGACGTGGGCTACAG-3′; *Il6*: 5′-TACCACTTCAC AAGTCGGAGGC-3′ and 5′-CTGCAAGTGCATCATCGT TGTTC-3′; *Ifnb1*: 5′-TTACACTGCCTTTGCCATCC-3′ and 5′-AGAAACACTGTCTGCTGGTG-3′; *Mx1*: 5′-GG CAGACACCACATACAACC-3′ and 5′-CCTCAGGCTAG ATGGCAAG-3′; *Ifit1*: 5′-CTCCACTTTCAGAGCCTTCG-3′ and 5′-TGCTGAGATGGACTGTGAGG-3′; *Ifit2*: 5′-AA ATGTCATGGGTACTGGAGTT-3′ and 5′-ATGGCAATTA TCAAGTTTGTGG-3′; *Id1*: 5′-AGGTGAACGTCCTGCT CTACGA-3′ and 5′-CAGGATCTCCACCTTGCTCACT-3′; *Id3*: 5′-CACTTACCCTGAACTCAACGCC-3′ and 5′-CC CATTCTCGGAAAAGCCAG-3′; *Cdkn1a*: 5′-GCAGATCC ACAGCGATATCC-3′ and 5′-CAACTGCTCACTGTCCAC GG-3′; *Cdkn2b*: 5′-AGCTGGATCTGGTCCTTGAG-3′ and 5′-GATCCAAGAATTTCCCTTGC-3′; *Smad7*: 5′-TT CGGACAACAAGAGTCAGCTGGT-3′ and 5′-AGCCTTG

ATGGAGAAACCAGGGAA-3′; *Tgfbr1*: 5′-TCTGGATCAG GTTTACCACTGC-3′and 5′-AAACCGACCTTTGCCAATG C-3′; *Tgfbr2*: 5′-ATGAGCAACTGCAGCATCAC-3′ and 5′-G CAAACCGTCTCCAGAGTAATG-3′; *Gapdh*: 5′-ATCAAG AAGGTGGTGAAGCA-3′ and 5′-AGACAACCTGGTCCTC AGTGT-3′.

2.5 | ELISA

Mouse serum IFN-β was measured by using VeriKine-HS Mouse Interferon Beta Serum ELISA Kit (PBL Assay Science) according to the manufacturer's instruction. Mouse serum IL-1β was measured by IL-1β Mouse Uncoated ELISA Kit (Thermo Fisher Scientific) and IL-6 by using IL-6 Mouse Uncoated ELISA Kit (Thermo Fisher Scientific) according to the manufacturer's instruction.

2.6 | RNA-seq and analysis

For RNAseq using human macrophages, total RNA was extracted from cultured primary human macrophages using RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB) was used to purify poly-A+ transcripts and generate libraries with multiplexed barcode adaptors following the manufacturer's instructions. All samples passed quality control analysis using a Bioanalyzer 2100 (Agilent). High-throughput sequencing (50 bp, single-end) was performed using the Illumina Hiseq 4000 in the Weill Cornell Medicine Genomics Resources Core Facility with a sequencing depth between 30 to 50 million reads per sample. RNA-seq reads were aligned to the human genome (GRCh38) using HISAT2 with default parameters. Reads were counted by HTseq-count and edgeR was used to estimate the transcript abundances as counts per million (CPM) values and calculate adjusted *p*-value (adj.*p*-value) and log2 fold-change (Log_2FC). Genes with low expression levels (<1 CPM) in all conditions were filtered from downstream analyses. Benjamini–Hochberg false discovery rate (FDR) procedure was used to correct for multiple testing. Genes with adjusted *p*-value < .05 and fold-change of at least 1.5 were identified as differentially expressed genes (DEG) between conditions using the edgeR analysis of three RNA-seq biological replicates from different donors.

For RNAseq using murine macrophages from WT and $Rbpj^{\Delta M}$, total RNA extraction from cultured primary murine macrophages, poly-A+ selection and generation of libraries were performed as mentioned above. All samples passed quality control analysis using a Bioanalyzer 2100 (Agilent). High-throughput sequencing (50 bp, single-end) was performed using the Illumina Hiseq 2500 or 4000 in the Weill Cornell Medicine Genomics Resources Core Facility with a sequencing depth between 30 to 50 million reads per sample. RNA-seq reads were aligned to the mouse genome (mm10) using HISAT2 with default parameters. Reads were counted by HTseq-count. Differential expression analyses and batch correction were performed using DESeq2 to calculate adjusted *p*-value (adj.*p*-value) and log2 fold-change (Log_2FC). Benjamini–Hochberg FDR procedure was used to correct for multiple testing. Genes with adjusted *p*-value < .05 and fold-change of at least 1.5 were identified as differentially expressed genes (DEG) between conditions of two RNA-seq biological replicates.

2.7 | ATAC-seq and analysis

ATAC-seq was performed according to the Omni-ATAC protocol as previously described.[27] Briefly, 50 000 cells were collected and washed with cold ATAC-Resuspension Buffer (RSB) containing 0.1% NP40, 0.1% Tween-20, and 0.01% Digitonin. Cells were lysed with cold ATAC-RSB containing 0.1% Tween-20. Pelleted nuclei were incubated with transposition mix (25 μL 2× TD buffer (Illumina), 2.5 μL transposase (Illumina), 16.5 μL PBS, 0.5 μL 1% digitonin, 0.5 μL 10% Tween-20, 5 μL nuclease-free water) for 30 min at 37°C in a thermomixer at 1000 rpm. Transposed DNA was purified using DNA Clean & Concentrator (Zymo Research). We amplified library fragments using previously published barcoded primers,[28] with the following PCR conditions: 72°C for 5 min; 98°C for 30 s; and thermocycling at 98°C for 10 s, 63°C for 30 s and 72°C for 1 min for a total of 10–13 cycles. The libraries were purified using DNA Clean & Concentrator. Library quality and quantification were assessed with an Agilent Bioanalyzer at the Weill Cornell Medicine Genomics Resources Core Facility. Barcoded sample libraries were pooled for a final concentration of 4 nM. Sequencing was performed on Illumina Hiseq4000 (50 bp, single-end) at the Weill Cornell Genomics Resources Core Facility. Sequenced reads were aligned to reference human genome (GRCh38) using Bowtie2 with default parameter. The read depth was 80 to 90 million reads for each sample. The total number of mapped reads in each sample was normalized to one million mapped reads. Peak calling was performed using MACS2[29] with a *q*-value cutoff of 0.01. Differential accessibility analysis of peaks was performed with Diffbind (Bioconductor. http://bioconductor.org/packages/release/bioc/vignettes/DiffBind/inst/doc/DiffBind.pdf). Differentially accessible peaks were defined as FDR < 0.01 and fold-change of at least 2. The peaks were assigned to each gene locus, including 20 kb upstream of transcription start site, gene body and 5 kb downstream of transcription termination site. For visualizing the ATAC-seq data, bigwig files were created from bam files with deeptools,[30] normalized using the Counts Per Million mapped reads (CPM) method, and then the peaks were visualized in Integrative Genomics Viewer (IGV).[31]

2.8 | Cut & Run-seq and analysis

Cut & Run was performed as previously described.[32] Totally 500 000 cells were used per condition. Briefly, cells were washed by wash buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM Spermidine, 1× Complete™ Protease Inhibitor Cocktail (Roche)), then mixed with Concanavalin A beads and permeabilized with Cell Permeabilization Buffer (wash buffer containing 0.01% Digitonin). The cells were then incubated with the primary antibodies (H3K4me3: 07–473, Millipore, 1:100; H3K27me3: 07–449, Millipore, 1:100) for 16 h at 4°C, followed by incubation with protein A-MNase (Cell Signaling Technology) for 1 h at 4°C. MNase was activated with $CaCl_2$ for 2 h at 4°C. After adding stop buffer (340 mM NaCl, 20 mM EDTA, 4 mM EGTA, 50 μg/mL RNase A, 50 μg/mL Glycogen), samples were incubated for 30 min at 37°C to release chromatin from cells. Fragmented chromatins were collected and purified with DNA Clean & Concentrator (Zymo Research). Library preparation was performed using NEBNext® Ultra™ II DNA Library Prep Kit according to manufacturer's instruction. Sequencing was performed on the Illumina NextSeq 500 (50 bp, paired-end). The read depth was 8 to 10 million reads for each condition. Then, sequenced reads were aligned to reference human genome (GRCh38) using Bowtie2 with the parameter, *--end-to-end --very-sensitive --no-mixed --dovetail --no-discordant --phred33*

-I 10 -X 700. Peak calling was performed using MACS2[29] with a *q*-value cutoff of 0.01. For visualizing the peaks of histone modifications, bigwig files were created from bam files with deeptools, normalized using the CPM method, and then the peaks were visualized in IGV.[31]

2.9 | Immunoblot analysis

Total cellular extracts were obtained using lysis buffer containing 150 mM Tris–HCl (pH 6.8), 6% SDS, 30% glycerol, and 0.03% Bromophenol Blue; 10% 2-ME was added immediately before harvesting cells. Cell lysates were fractionated on 7.5% SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and incubated with specific antibodies. Western Lightning plus-ECL (PerkinElmer) was used for detection. TGFBR1 antibody (PA5–86551, 1:1000) was from Thermo Fisher Scientific; TGFBR2 (sc-17792, 1:500), and p38α (sc-535, 1:3000) antibodies were from Santa Cruz Biotechnology; p-Smad2/3 (D27F4, 1:1000) antibody was obtained from Cell Signaling Technology.

2.10 | ChIP-qPCR

Cells (10×10^6 cells per condition) were crosslinked for 10 min at room temperature with 0.8% formaldehyde solution followed by 5 min quenching with 125 mM glycine. Cells were pelleted at 4°C and washed with ice-cold PBS twice. The crosslinked cells were lysed with buffer LB1 with protease inhibitors on ice for 10 min. The nuclei were pelleted, resuspended in buffer LB2 and incubated for 10 min on ice. The lysis samples were resuspended and sonicated in buffer LB3 using a Bioruptor Pico device (Diagenode) for 6 cycles of 30 s on/30 s off. After sonication, samples were centrifuged at 12 000 rpm for 10 min at 4°C and 10% of sonicated cell lysates was taken as input. The chromatin lysates were incubated with Protein A/G magnetic beads (Themo Fisher) with 5 μg of the appropriate antibody overnight at 4°C. RBP-J antibody (#5313) was from Cell Signaling Technology. After overnight incubation, antibody-bound magnetic beads were washed twice with Low salt buffer, twice with High salt buffer, once with LiCl wash buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 250 mM LiCl, 1% NP-40), and once with TE with 50 mM NaCl. Cross-links were reversed by overnight incubation at 65°C. Input and ChIP DNA were treated with RNase A and Proteinase K to remove RNAs and proteins. DNA was purified with MinElute PCR Purification Kit (Qiagen). DNA was analyzed by qPCR and normalized relative to total input. The qPCR primers used in the ChIP assay: *Tgfbr1* promoter locus (2 kb upstream of TSS): 5′-AAGGTTTCCCATGTGAGGCTAG-3′ and 5′-GCATGCAGCAAAGACTGAAG-3′; *Tgfbr2* promoter locus (2 kb upstream of TSS): 5′-TACGCTGAAATCGC TTGCTC-3′ and 5′-AAAGTCCCAGTCAGTCCAAGAC-3′.

2.11 | Statistical analyses

Statistical analyses were performed using Graphpad Prism® software. Two-tailed Student's *t*-test was applied when there were only two groups of samples. In the case of more than two groups of samples, one-way analysis of variance (ANOVA) was used with one condition, and two-way ANOVA was used with more than two conditions. ANOVA was followed by post-hoc Turkey's multiple comparisons. $p < .05$ was considered statistically significant; *p value < .05 and **p value < .01. The data displayed normal distribution. The estimated variance was similar between experimental groups. Data are presented as the mean ± SD or ± SEM as indicated in the figure legends.

3 | RESULTS

3.1 | TGFβ signaling suppresses TNF-induced inflammatory response in macrophages

To examine the effects of TGFβ signaling on TNF action, we generated *Tgfbr2* conditional knock out (cKO) mice, in which *Tgfbr2* is specifically deleted in myeloid lineage macrophages by crossing *Tgfbr2*$^{flox/flox}$ mice with *LysMcre* mice (*Tgfbr2*$^{f/f}$;*LysMCre*; *hereafter referred to as Tgfbr2*$^{\Delta M}$). *LysMcre*$^{+}$ littermates served as wild type controls (here-after referred to as WT). In these TGFβ receptor type II cKO mice, the TGFβ signaling in macrophages is absent. TNF induces the expression of inflammatory cytokines and ISGs in bone marrow derived macrophages (BMMs), as expected (Figure 1A). Strikingly, deletion of *Tgfbr2* enhanced the expression of inflammatory cytokines, such as *Tnf*, *Il6* and *Ifnb*, and ISGs, such as *Ifit1*, *Ifit2* and *Mx1*, in BMMs stimulated with TNF. We further investigated whether this change would occur in vivo. We employed a well-established TNF induced mouse chronic inflammatory response model[33] in *Tgfbr2*$^{\Delta M}$ and the WT control mice. We found that *Tgfbr2* deficiency markedly increased the serum levels of inflammatory cytokines, including IL-1β, IL-6 and IFN-β in response to TNF treatment (Figure 1B). These results collectively indicate that the TGFβ signaling plays an inhibitory role in TNF-induced inflammatory gene expression.

3.2 | TNF regulates histone modification to suppress the TGFβ signaling pathway in macrophages

We next explored the impact of TNF stimulation on the TGFβ signaling in macrophages. To address this, we performed gene expression profiling using high-throughput sequencing of RNAs (RNA-seq) from the human macrophages derived from CD14(+) peripheral blood monocytes (PBMCs) to identify transcriptomic changes of the endogenous TGFβ signaling pathway with or without TNF treatment. We found that TNF induces the expression of the negative regulators of TGFβ signaling, such as *SMURF1*, *SMURF2*, *SMAD6* and *SMAD7*, in macrophages (Figure 2A). Conversely, TNF stimulation led to the suppression of key elements in the TGFβ signaling pathway responsible for signal transduction, including the receptors *TGFBR1* and *TGFBR2*, the signaling transducers *SMAD2/3/4*, and co-regulators (Figure 2A). Additionally, TNF inhibited the expression of endogenous *TGFB1* (Figure 2A). Consequently, the expression of TGFβ signaling target genes, such as *ID1/2/3*, *CDKN1A*, *and CDKN2B*, was significantly suppressed by TNF (Figure 2A). These findings clearly indicate that TNF stimulation profoundly dampens TGFβ signaling.

Since gene expression is often regulated at the chromatin level, we then asked whether TNF stimulation influences the chromatin accessibility and histone modification at the loci of genes involved in the TGFβ signaling pathway in macrophages. To address this, we first performed ATAC-seq to investigate the regulation of chromatin accessibility by TNF. We observed that TNF nearly does not affect the chromatin accessibility at the loci of *TGFBR1*, *TGFBR2*, CREBBP, *SMURF1*, *SMURF2*, *SMAD6*, and *ID1/2/3*. We then turned to look into the histone modification at these loci. To this end, we performed Cut&Run-seq for the H3K4me3 histone mark, associated with transcriptional activation, and the H3K27me3 histone mark, associated with transcriptional inhibition. The presence of H3K4me3 signals is particularly notable in the vicinity of the transcriptional start sites (TSS). We found that

H3K4me3 signal levels at the loci of genes responsible for TGFβ signaling transduction, such as *TGFBR1*, *TGFBR2*, and *CREBBP* (Figure 2B), as well as TGFβ target genes like *ID1/2/3* (Figure 2D), were dampened by TNF. Conversely, gene loci associated with negative regulation of the TGFβ pathway, such as *SMURF1*, *SMURF2*, *and SMAD6* (Figure 2C), displayed heightened H3K4me3 signal levels following TNF stimulation. In contrast, the presence of H3K27me3 signals was less frequent at gene loci. Strikingly, alterations induced by TNF in H3K27me3 levels at the gene loci of *TGFBR1*, *CREBBP*, *SMAD6*, *ID1*, *and ID3* exhibited an opposing pattern to that of H3K4me3 signals (Figure 2B–D). Importantly, these shifts in histone marks align consistently with the transcriptomic changes observed in these genes. This collective evidence underscores a robust epigenetic foundation underlying the mechanisms through which TNF effectively suppresses the endogenous TGFβ signaling pathway in macrophages.

3.3 | RBP-J antagonizes the TNF effects on the TGFβ signaling pathway

RBP-J was initially identified as a key regulator in the canonical Notch signaling pathway. More recent studies have unveiled its important role as a significant player in macrophage polarization, TLR and TNF signaling pathways.[26,34–37] Since TNF activates RBP-J,[38] we then asked whether RBP-J regulates the TNF effects on the TGFβ signaling pathway. We generated *Rbpj* conditional knock out (KO) mice, in which *Rbpj* is specifically deleted in myeloid lineage macrophages by crossing *Rbpj* $^{flox/flox}$ mice with *LysMcre* mice (*Rbpj* $^{f/f}$;*LysMCre*; *hereafter referred to as Rbpj*$^{\Delta M}$). *LysMcre*$^{+}$ littermates served as wild type controls (hereafter referred to as Ctrl). We performed RNAseq to investigate how RBP-J influences the transcriptomic changes in macrophages stimulated with TNF. We found that RBP-J deficiency drastically impacted the TNF-induced gene expression (Figure 3A) and pathways (Figure 3B) in macrophages. Specifically, TNF signaling, cytokine and chemokine pathways are enriched in WT macrophages in response to TNF. In contrast, TGFβ signaling, metabolic and osteoclast signaling pathways are highly enriched in *Rbpj*$^{\Delta M}$ cells by TNF stimulation (Figure 3B). The expression levels of *Tgfbr1*, *Tgfbr2*, and TGFβ signaling target genes *Id1*, *Id2*, *Id3*, *Cdkn1a* and *Cdkn2b* were higher in *Rbpj*$^{\Delta M}$ BMMs than those in the control cells both before and after TNF treatment (Figure 3C). However, the expression levels of *Smad6*, *Smurf1* and *Smuf2* were decreased by RBP-J deficiency before and after TNF treatment (Figure 3C). The elevated TGFβ signaling effect by RBP-J deficiency appears to be long-term, as indicated by the TGFβ signaling target *Id1 and Id3* expression (Figure 3D). We then examined whether RBP-J influences TGFβ signaling transduction. We observed that RBP-J deletion resulted in increased phosphorylated Smad2/3 levels induced by TGFβ (Figure 3E). These data indicate that RBP-J plays an inhibitory role in TNF-mediated inhibition of TGFβ signaling.

3.4 | RBP-J inhibits the expression of TGFβ receptors in macrophages

We next asked how RBP-J suppresses the TGFβ signaling pathway. Since we have found that RBP-J restrains the TGFβ signaling transduction and target gene expression, we wondered whether RBP-J affects the expression of TGFβ receptors, which are the most upstream of TGFβ signaling. As shown in Figure 4A, we discovered that RBP-J deficiency significantly elevated the expression of *Tgfbr1* and *Tgbfr2*. Furthermore, the protein levels of TGFβRI and TGFβRII were enhanced in *Rbpj*$^{\Delta M}$ BMMs (Figure 4B). We then performed

ChIP assay using the WT control and *Rbpj*$^{\Delta M}$ BMMs, and identified RBP-J occupancy at the promoter loci of *Tgfbr1* and *Tgfbr2* (Figure 4C). These results collectively demonstrate that RBP-J binds to the promoters of *Tgfbr1* and *Tgfbr2*, and suppresses the expression of TGFβ receptors in macrophages.

3.5 | RBP-J aggravates TNF-induced inflammatory response in macrophages

Next, we wondered what the role of RBP-J is in TNF-induced inflammatory response. TNF induces IFNβ production and ISG expression in macrophages. From our RNAseq data, we found that the TNF-induced gene expression of inflammatory cytokines, such as *Tnf* and *Il6*, and ISGs, such as *Mx1*, *Ifit1*, *Ifit2*, was markedly decreased by RBP-J deficiency (Figure 5A). We further confirmed the expression changes of these genes using qPCR (Figure 5B). We then asked whether RBP-J promotes inflammatory cytokine production in vivo. We developed TNF induced mouse chronic inflammatory response model[33] in the WT control and *Rbpj*$^{\Delta M}$ mice, and we found that the serum levels of inflammatory cytokines, including IL-1β, IL-6 and IFN-β induced by TNF stimulation were significantly dampened in the *Rbpj*$^{\Delta M}$ mice compared to the control mice (Figure 5C). These results demonstrate that RBP-J plays an important role in the promotion of macrophage inflammation induced by TNF.

4 | DISCUSSION

Many disease conditions are either caused by inflammation or closely associated with it. TNF serves as a pivotal inflammatory cytokine, often acting as a catalyst and exacerbating inflammatory states across a range of disease contexts. Consequently, there exists paramount importance in unraveling the mechanisms that govern TNF-mediated inflammation. In the present study, we have discerned a critical inhibitory role played by the TGFβ signaling in the production of inflammatory cytokines and ISG expression induced by TNF in macrophages (Figure 6). The basal/endogenous TGFβ signaling, functioning as a homeostatic mechanism, adeptly fine-tunes TNF-triggered inflammation to prevent unbridled inflammatory conditions. Intriguingly, TNF stimulation exerts a potent suppressive effect on the basal/endogenous TGFβ signaling pathway, as illustrated in Figure 6. This mechanism empowers TNF to attenuate the intrinsic antagonistic impact of the TGFβ signaling pathway, thereby facilitating the induction of heightened inflammatory responses. This reciprocal suppression dynamic between TNF and the TGFβ signaling pathway carries unique physiopathological significance, as it represents a pivotal “braking” mechanism. The equilibrium between the intensity of TNF and the activity of endogenous TGFβ signaling contributes significantly to the overall level of inflammation. Appropriately augmenting the TGFβ signaling pathway holds the potential to counteract the influence of TNF, offering a promising avenue for the development of novel therapeutic strategies aimed at inflammation control.

This study has unveiled two previously unrecognized molecular mechanisms through which TNF exerts suppression upon the TGFβ signaling pathway. These mechanisms involve epigenetic inhibition and RBP-J-mediated interference of the TGFβ signaling pathway by TNF. Epigenetic modifications are instrumental in shaping the functionality of the genome.

The findings from the epigenetic studies conducted within this research underscore this concept, elucidating the integration of signaling crosstalk between TNF and the TGFβ pathway at the chromatin level. This integration enables precise epigenetic regulation of inflammatory gene expression and responses within macrophages. Furthermore, RBP-J has been observed to bind to the promoters of TGFβ receptors, leading to the suppression of TGFβ signaling by inhibiting their expression. It is worth exploring possible molecular connections between these mechanisms. For instance, investigating whether RBP-J plays a role in the epigenetic regulation of TGFβ signaling. Previous literature has indicated dynamic binding of RBP-J, both dependent and independent of Notch signaling, to its target genes in various scenarios.[39–41] RBP-J also extensively interacts with chromatin-modifying complexes and enzymes, such as PRC, LSD1, and KDM5, to modulate active or repressive histone marks that, in turn, regulate gene expression.[42] Therefore, it is highly possible that RBP-J may also suppress TGFβ signaling through epigenetic modifications of gene loci. This discovery could potentially unveil new therapeutic targets for mitigating TNF-mediated inflammation in various diseases.

TNF suppresses the expression of Tgfbr2 in the WT cells. Interestingly, Tgfbr2 expression also temporarily decreases on day1, then increases on day 2 and 3 after TNF treatment in the *Rbpj* deficient cells. The reason for this temporal decrease is unclear, presumably due to RBP-J independent mechanisms that decrease Tgfbr2 in the *Rbpj* deficient cells. Nonetheless, the expression levels of Tgfbr2 in the *Rbpj* deficient cells are still significantly higher than those in the WT control cells on day1, which is similar to other time points. These results collectively indicate that RBP-J plays an important inhibitory role in Tgfbr2 expression.

In addition to its role in inhibiting TGFβ signaling by targeting TGFβ receptors, RBP-J is presumed to have the capacity to directly regulate the expression of inflammatory genes induced by TNF. Existing literature provides evidence that RBP-J deletion results in reduced production of inflammatory cytokines, such as TNF, IL-6, and IL-12, in response to Toll-like receptor (TLR) activation, ultimately leading to a decrease in endotoxin-induced lethality in mice.[37] Notably, RBP-J has been observed to bind to the promoter region of *Il6* and inhibit its transcriptional activity.[37,43] While it remains uncertain whether RBP-J similarly binds to the regulatory loci of other genes responsible for inflammatory cytokines and interferon-stimulated genes (ISGs), particularly in response to inflammatory stimuli like TNF or LPS, the possibility certainly exists. Moreover, RBP-J has the capability to form complexes with various molecular partners to jointly regulate the expression of these genes, thereby establishing a regulatory network that collaborates with the TGFβ signaling pathway to modulate the inflammatory response in macrophages.

TGFβ needs to be activated from the latent form to exert its biological activity. TGFβ is produced by a variety of cells and tissues. In healthy individuals, a serum level of TGFβ, generally less than 1.5 ng/mL, is sustained to fulfill its homeostatic function.[20,44,45] Similarly, culture media typically contain a low level of TGFβ, within the 1–2 ng/mL range, reflecting physiological/basal concentrations. The results from our RNAseq data show a basal level of TGFβ target gene expression in macrophages, indicating that the TGFβ in the culture medium is biologically active.

Our research has uncovered that the TGFβ signaling plays a pivotal role in dampening TNF-induced inflammatory gene expression and cytokine production in macrophages. However, the precise mechanisms through which the TGFβ signaling accomplishes this function remain enigmatic. In a prior study, we observed that prolonged exogenous TGFβ treatment of macrophages led to a reduction in chromatin accessibility and a decrease in the active histone mark H3K4me3 level, while simultaneously increasing the suppressive histone mark H3K27me3 level at ISG loci. These findings illustrated the epigenetic suppression of ISG loci by TGFβ signaling.[33] It is conceivable that the basal TGFβ signaling, such as that in our culture experiments, may similarly employ epigenetic mechanisms to inhibit TNF function, even though the basal/physiological TGFβ levels are typically lower than those achieved with exogenous treatment. It would be also interesting to explore dose effects of TGFβ on TNF-induced inflammation and associated mechanisms in future studies.

As macrophages are different in many aspects from monocytes,[46,47] we tested how TNF stimulation affects TGFβ signaling pathway in freshly isolated human monocytes. As shown in Figure S1, the response of monocytes to TNF is different from macrophages. In contrast to macrophages, TNF treatment moderately increases the expression of TGFβ receptors, as well as the target genes of the TGFβ signaling pathway, such as RBL1 and RBL2. The expression of the negative regulators of the TGFβ signaling pathway, however, appears in different patterns between genes. For example, TNF reduces the expression of SMAD7, but enhances SMURF2. These results indicate that the response of freshly isolated monocytes to TNF is different from macrophages in terms of the expression of genes involved in the TGFβ signaling pathway.

In the present study, we showed that TNF regulates histone modification in different TGFβ related genes, at least, for 1 day. These epigenetic modifications are in line with the kinetics of the gene expression changes in response to TNF. To test how long this regulation would last, we examined the expression of several typical TGFβ related genes in a longer time course (Figure S2). We found that the suppression of TGFβ receptors can last for, at least, 6 days. The expression of the negative regulators of the TGFβ signaling pathway, such as SMAD7 and SMURF2, was increased by TNF at day1, but suppressed at later time points. The target genes of the TGFβ signaling pathway responded variously to TNF. For example, RBL1 was suppressed by day 1, and then its expression returned to the basal levels. RBL2 expression was continuously inhibited for a long time by TNF treatment, at least for 6 days. These results suggest more complex changes in the transcriptome and likely epigenome that occur in macrophages after a prolonged TNF exposure.

Interestingly, TNF has been shown to induce the expression of TGFβ in lung fibroblasts.[15,48] In contrast to the response observed in macrophages in this study, TNF was reported to elevate TGFβ receptor expression and enhance the TGFβ signaling pathway in fibroblasts.[49] Consequently, the interplay between TNF and TGFβ signaling pathways is notably intricate, contingent upon cell types and the specific stimulation context. The reciprocal repression observed between TNF and TGFβ signaling within macrophages, along with the underlying mechanisms unveiled in this study, contribute significantly to our understanding of the inhibition of inflammation mediated by TGFβ in macrophages. For example, in chronic inflammatory conditions such as rheumatoid arthritis, macrophages are a key player in

the inflammation and pathogenesis of this disease. TNF drives inflammation and tissue destruction, and TGFβ signaling is highly enriched in RA.[33] However, it is unclear how TNF and TGFβ signaling crosstalk in macrophages in this disease setting. The discoveries in this study provide a platform of molecular mechanisms and offer promising potential for novel therapeutic strategies aimed at controlling inflammation, such as in RA, based on these mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The datasets that support the findings of this study and were generated by the authors as part of this study have been deposited in the Gene Expression Omnibus database with the accession code: GSE171843 and GSE242269.

Abbreviations:

TGFβ	Transforming growth factor beta
TNF	Tumor necrosis factor

REFERENCES

1. Kalliolias GD, Ivashkiv LB. TNF biology, pathogenic mechanisms and emerging therapeutic strategies. Nat Rev Rheumatol. 2016;12:49–62. [PubMed: 26656660]
2. Brenner D, Blaser H, Mak TW. Regulation of tumour necrosis factor signalling: live or let die. Nat Rev Immunol. 2015;15:362–374. [PubMed: 26008591]
3. Yarilina A, Park-Min KH, Antoniv T, Hu X, Ivashkiv LB. TNF activates an IRF1-dependent autocrine loop leading to sustained expression of chemokines and STAT1-dependent type I interferon-response genes. Nat Immunol. 2008;9:378–387. [PubMed: 18345002]
4. Yarilina A, Ivashkiv LB. Type I interferon: a new player in TNF signaling. Curr Dir Autoimmun. 2010;11:94–104. [PubMed: 20173389]
5. Morikawa M, Derynck R, Miyazono K. TGF-beta and the TGF-beta family: context-dependent roles in cell and tissue physiology. Cold Spring Harb Perspect Biol. 2016;8:a021873. [PubMed: 27141051]
6. Tzavlaki K, Moustakas A. TGF-beta signaling. Biomolecules. 2020;10:487. [PubMed: 32210029]

7. Massague J TGFbeta signalling in context. Nat Rev Mol Cell Biol. 2012;13:616–630. [PubMed: 22992590]
8. Imamura T, Oshima Y, Hikita A. Regulation of TGF-beta family signalling by ubiquitination and deubiquitination. J Biochem. 2013;154:481–489. [PubMed: 24165200]
9. Luyckx I, Verstraeten A, Goumans MJ, Loeys B. SMAD6-deficiency in human genetic disorders. NPJ Genom Med. 2022;7:68. [PubMed: 36414630]
10. Sanjabi S, Oh SA, Li MO. Regulation of the immune response by TGF-beta: from conception to autoimmunity and infection. Cold Spring Harb Perspect Biol. 2017;9:a022236. [PubMed: 28108486]
11. Batlle E, Massague J. Transforming growth factor-beta signaling in immunity and cancer. Immunity. 2019;50:924–940. [PubMed: 30995507]
12. Yoshimatsu Y, Kimuro S, Pauty J, et al. TGF-beta and TNF-alpha cooperatively induce mesenchymal transition of lymphatic endothelial cells via activation of activin signals. PLoS One. 2020;15:e0232356. [PubMed: 32357159]
13. Liu ZW, Zhang YM, Zhang LY, et al. Duality of interactions between TGF-beta and TNF-alpha during tumor formation. Front Immunol. 2021;12:810286. [PubMed: 35069596]
14. Verrecchia F, Mauviel A. TGF-beta and TNF-alpha: antagonistic cytokines controlling type I collagen gene expression. Cell Signal. 2004;16:873–880. [PubMed: 15157666]
15. Sullivan DE, Ferris M, Pociask D, Brody AR. Tumor necrosis factor-alpha induces transforming growth factor-beta1 expression in lung fibroblasts through the extracellular signal-regulated kinase pathway. Am J Respir Cell Mol Biol. 2005;32:342–349. [PubMed: 15653932]
16. Yamane K, Ihn H, Asano Y, Jinnin M, Tamaki K. Antagonistic effects of TNF-alpha on TGF-beta signaling through downregulation of TGF-beta receptor type II in human dermal fibroblasts. J Immunol. 2003;171:3855–3862. [PubMed: 14500687]
17. Schlaak JF, Pfers I, Meyer Zum Buschenfelde KH, Marker-Hermann E. Different cytokine profiles in the synovial fluid of patients with osteoarthritis, rheumatoid arthritis and seronegative spondylarthropathies. Clin Exp Rheumatol. 1996;14:155–162. [PubMed: 8737721]
18. Lettesjo H, Nordstrom E, Strom H, et al. Synovial fluid cytokines in patients with rheumatoid arthritis or other arthritic lesions. Scand J Immunol. 1998;48:286–292. [PubMed: 9743215]
19. Canete JD, Llena J, Collado A, et al. Comparative cytokine gene expression in synovial tissue of early rheumatoid arthritis and seronegative spondyloarthropathies. Br J Rheumatol. 1997;36:38–42. [PubMed: 9117172]
20. Miossec P, Naviliat M, Dupuy d'Angeac A, Sany J, Banchereau J. Low levels of interleukin-4 and high levels of transforming growth factor beta in rheumatoid synovitis. Arthritis Rheum. 1990;33:1180–1187. [PubMed: 2390123]
21. Mieliauskaite D, Venalis P, Dumalakiene I, Venalis A, Distler J. Relationship between serum levels of TGF-beta1 and clinical parameters in patients with rheumatoid arthritis and Sjogren's syndrome secondary to rheumatoid arthritis. Autoimmunity. 2009;42:356–358. [PubMed: 19811300]
22. Fava R, Olsen N, Keski-Oja J, Moses H, Pincus T. Active and latent forms of transforming growth factor beta activity in synovial effusions. J Exp Med. 1989;169:291–296. [PubMed: 2909658]
23. Li S, Miller CH, Giannopoulou E, Hu X, Ivashkiv LB, Zhao B. RBP-J imposes a requirement for ITAM-mediated costimulation of osteoclastogenesis. J Clin Invest. 2014;124:5057–5073. [PubMed: 25329696]
24. Takayanagi H, Kim S, Matsuo K, et al. RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. Nature. 2002;416:744–749. [PubMed: 11961557]
25. Kitaura H, Zhou P, Kim HJ, Novack DV, Ross FP, Teitelbaum SL. M-CSF mediates TNF-induced inflammatory osteolysis. J Clin Invest. 2005;115:3418–3427. [PubMed: 16294221]
26. Xu H, Zhu J, Smith S, et al. Notch-RBP-J signaling regulates the transcription factor IRF8 to promote inflammatory macrophage polarization. Nat Immunol. 2012;13:642–650. [PubMed: 22610140]
27. Corces MR, Trevino AE, Hamilton EG, et al. An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. Nat Methods. 2017;14:959–962. [PubMed: 28846090]

28. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods. 2013;10:1213–1218. [PubMed: 24097267]
29. Feng J, Liu T, Qin B, Zhang Y, Liu XS. Identifying ChIP-seq enrichment using MACS. Nat Protoc. 2012;7:1728–1740. [PubMed: 22936215]
30. Ramirez F, Ryan DP, Gruning B, et al. deepTools2: a next generation web server for deep-sequencing data analysis. Nucleic Acids Res. 2016;44:W160–W165. [PubMed: 27079975]
31. Robinson JT, Thorvaldsdottir H, Winckler W, et al. Integrative genomics viewer. Nat Biotechnol. 2011;29:24–26. [PubMed: 21221095]
32. Skene PJ, Henikoff S. An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. elife. 2017;6:e21856. [PubMed: 28079019]
33. Xia Y, Inoue K, Du Y, et al. TGFbeta reprograms TNF stimulation of macrophages towards a non-canonical pathway driving inflammatory osteoclastogenesis. Nat Commun. 2022;13:3920. [PubMed: 35798734]
34. Kopan R, Ilagan MX. The canonical notch signaling pathway: unfolding the activation mechanism. Cell. 2009;137:216–233. [PubMed: 19379690]
35. Zhao B TNF and Bone Remodeling. Curr Osteoporos Rep. 2017;15:126–134. [PubMed: 28477234]
36. Zhao B Intrinsic restriction of TNF-mediated inflammatory Osteoclastogenesis and bone resorption. Front Endocrinol. 2020;11:583561.
37. Hu X, Chung AY, Wu I, et al. Integrated regulation of toll-like receptor responses by notch and interferon-gamma pathways. Immunity. 2008;29:691–703. [PubMed: 18976936]
38. Ng C, Qin Y, Xia Y, Hu X, Zhao B. Jagged1 acts as an RBP-J target and feedback suppresses TNF-mediated inflammatory osteoclastogenesis. J Immunol. 2023;211:1340–1347. [PubMed: 37756541]
39. Hamidi H, Gustafason D, Pellegrini M, Gasson J. Identification of novel targets of CSL-dependent notch signaling in hematopoiesis. PLoS One. 2011;6:e20022. [PubMed: 21637838]
40. Castel D, Mourikis P, Bartels SJ, Brinkman AB, Tajbakhsh S, Stunnenberg HG. Dynamic binding of RBPJ is determined by notch signaling status. Genes Dev. 2013;27:1059–1071. [PubMed: 23651858]
41. Wang H, Zou J, Zhao B, et al. Genome-wide analysis reveals conserved and divergent features of Notch1/RBPJ binding in human and murine T-lymphoblastic leukemia cells. Proc Natl Acad Sci USA. 2011;108:14908–14913. [PubMed: 21737748]
42. Schwanbeck R The role of epigenetic mechanisms in notch signaling during development. J Cell Physiol. 2015;230:969–981. [PubMed: 25336183]
43. Plaisance S, Vanden Berghe W, Boone E, Fiers W, Haegeman G. Recombination signal sequence binding protein Jkappa is constitutively bound to the NF-kappaB site of the interleukin-6 promoter and acts as a negative regulatory factor. Mol Cell Biol. 1997;17:3733–3743. [PubMed: 9199307]
44. Becker-Merok A, Eilertsen GO, Nossent JC. Levels of transforming growth factor-beta are low in systemic lupus erythematosus patients with active disease. J Rheumatol. 2010;37:2039–2045. [PubMed: 20682675]
45. Manolova I, Gerenova J, Ivanova M. Serum levels of transforming growth factor-beta1 (TGF-beta1) in patients with systemic lupus erythematosus and Hashimoto's thyroiditis. Eur Cytokine Netw. 2013;24:69–74. [PubMed: 23614931]
46. Netea MG, Nold-Petry CA, Nold MF, et al. Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Blood. 2009;113:2324–2335. [PubMed: 19104081]
47. Seow V, Lim J, Iyer A, et al. Inflammatory responses induced by lipopolysaccharide are amplified in primary human monocytes but suppressed in macrophages by complement protein C5a. J Immunol. 2013;191:4308–4316. [PubMed: 24043889]
48. Sullivan DE, Ferris M, Nguyen H, Abboud E, Brody AR. TNF-alpha induces TGF-beta1 expression in lung fibroblasts at the transcriptional level via AP-1 activation. J Cell Mol Med. 2009;13:1866–1876. [PubMed: 20141610]

49. Voloshenyuk TG, Hart AD, Khoutorova E, Gardner JD. TNF-alpha increases cardiac fibroblast lysyl oxidase expression through TGF-beta and PI3Kinase signaling pathways. Biochem Biophys Res Commun. 2011;413:370–375. [PubMed: 21893029]

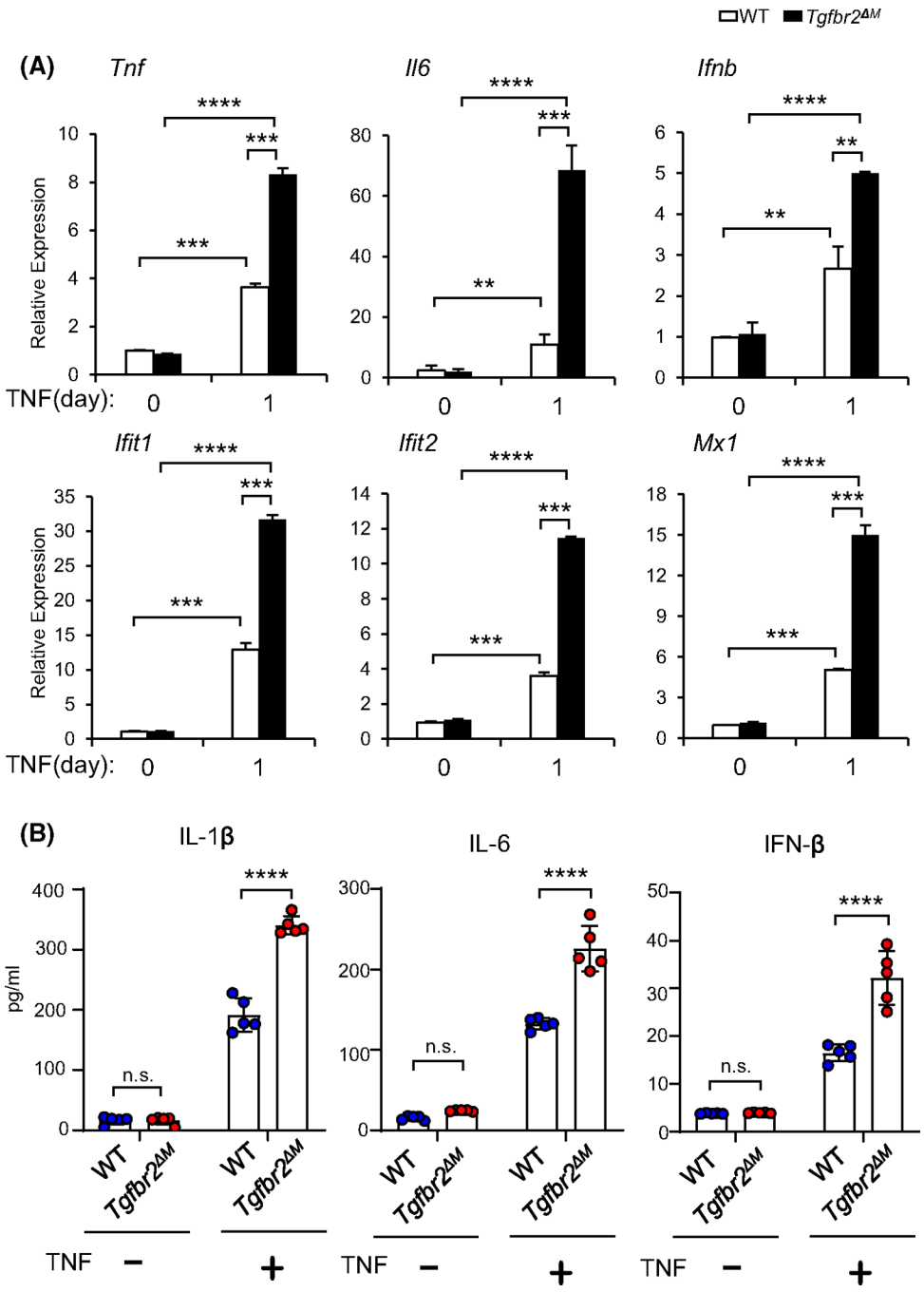

FIGURE 1.

Tgfbr2 deficiency enhances TNF-induced inflammatory response in macrophages. (A) qPCR analysis of mRNA expression of *Tnf*, *Il6*, *Ifnb1*, *Ifit1*, *Ifit2* and *Mx1* in the bone marrow macrophages of WT and $Tgfbr2^{\Delta M}$ mice stimulated with TNF (40 ng/mL) for the indicated times. (B) ELISA analysis of IL-1β, IL-6 and IFN-β levels in the serum from the 12-week-old male WT and $Tgfbr2^{\Delta M}$ mice after TNF-induced supra-calvarial inflammation (n = 5/group). (A, B) $^{**}p < .01$; $^{***}p < .001$; $^{****}p < .0001$; ns: not statistically significant by two-way ANOVA. Error bars: (A) Data are mean ± SD; (B) Data are mean ± SE.

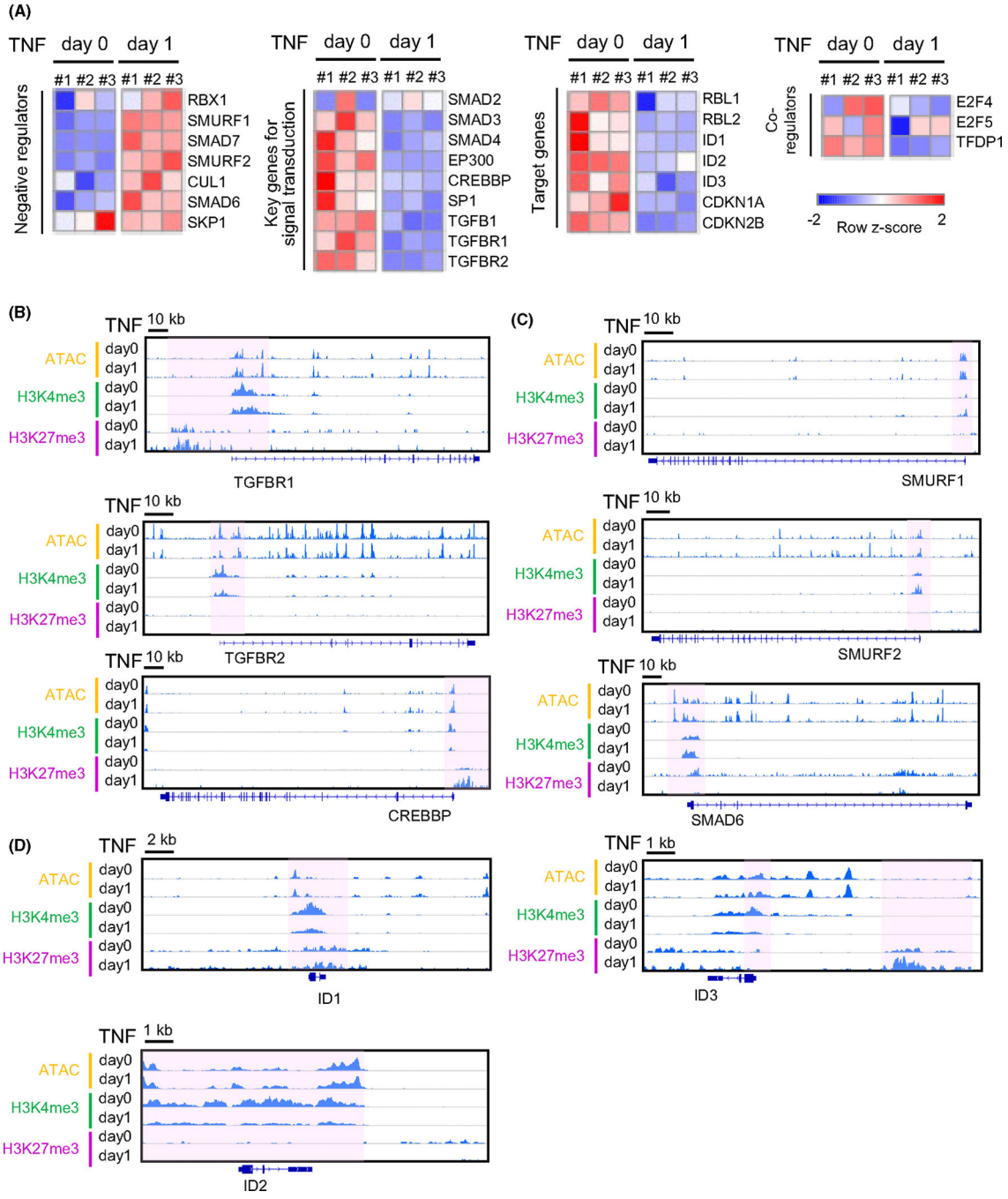

FIGURE 2.

TNF suppresses basal TGFβ signaling in macrophages. Human macrophages derived from CD14(+) monocytes were stimulated by TNF (40 ng/mL) for 0 or 1 day. (A) Heatmaps of genes of negative regulators, key genes for signal transduction, targets and co-regulators of the TGFβ signaling pathway regulated by TNF stimulation. Row z-scores of CPMs were shown in the heatmaps. (B–D) Representative Integrative Genome Browser tracks displaying normalized tag density profiles for ATAC-seq, H3K4me3 and H3K27me3 Cut&Run-seq

signals at *TGFBR1*, *TGFBR2* and *CREBBP* (B); *SMURF1*, *SMURF2* and *SMAD6* (C); *ID1*, *ID2* and *ID3* (D). Data are representative of 2 biological replicates.

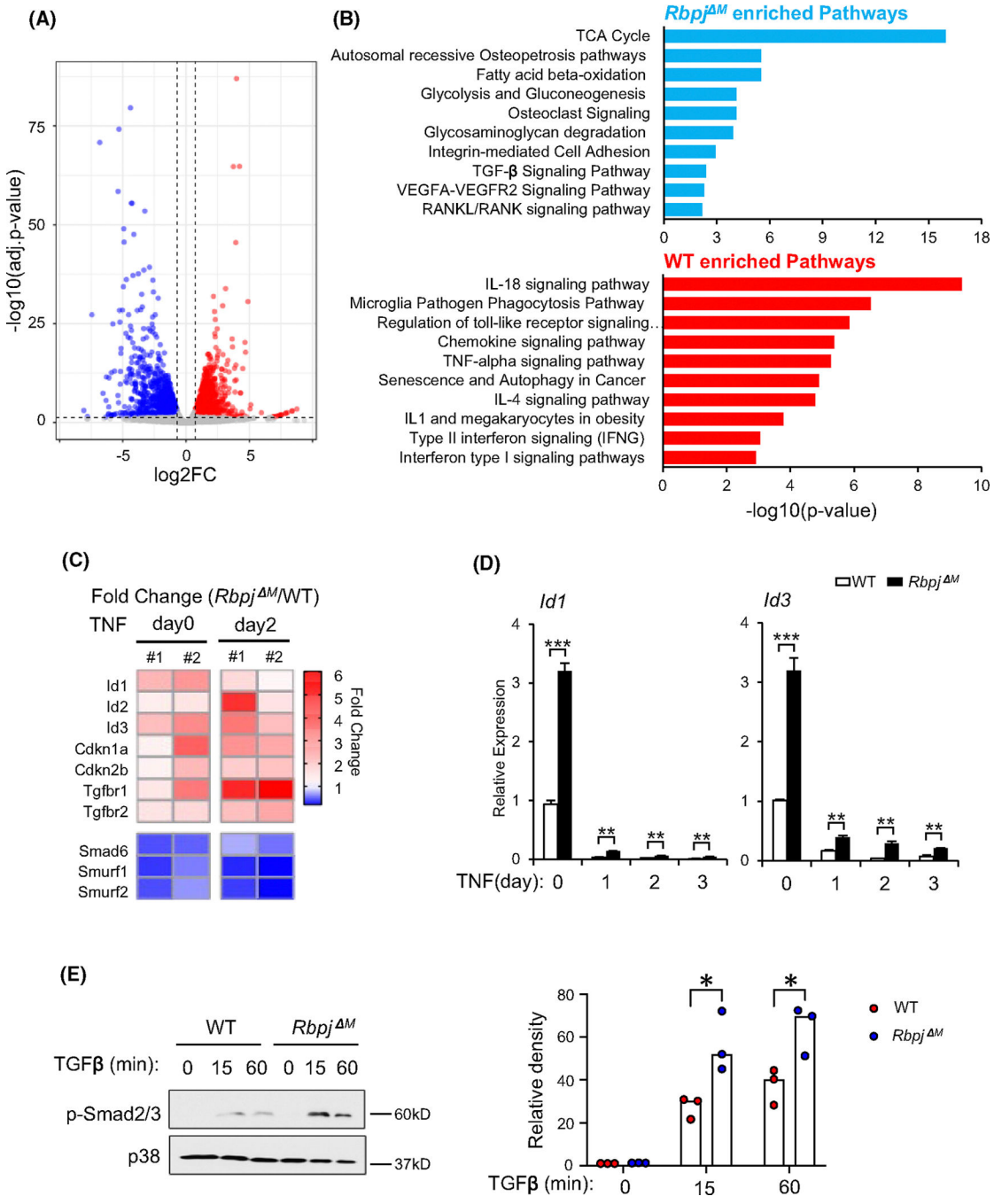

FIGURE 3.

Rbpj deficiency enhances TGFβ signaling in *Rbpj*$^{\Delta M}$ macrophages. (A) Volcano plot of RNA-seq analysis of TNF-induced differentially expressed genes at 48 h after TNF stimulation with significant (adj.*p*-value < .01) and greater than 2-fold changes between WT and *Rbpj*$^{\Delta M}$ BMMs. (B) Pathway analysis of the differentially expressed genes. Enriched pathways in WT (upper panel) and *Rbpj*$^{\Delta M}$ BMMs (lower panel) were shown. (C) RNAseq–based heatmaps showing the fold changes (*Rbpj*$^{\Delta M}$/WT) of the expression of genes involved in the TGFβ signaling pathway at basal and TNF stimulation of WT and *Rbpj*$^{\Delta M}$ BMMs for

2 days. Fold changes ($Rbpj^{\Delta M}$/WT) of gene expression (CPMs) were shown in the heatmap. Fold changes with >1 indicate higher gene expression levels in $Rbpj^{\Delta M}$ BMMs than in WT cells, and vice versa. #1, biological replicate 1. #2, biological replicate 2. (D) qPCR analysis of mRNA expression of *Id1 and Id3* in WT and $Rbpj^{\Delta M}$ BMMs stimulated with TNF for the indicated times. (E) Immunoblot analysis of phospho-Smad2/3 in WT and $Rbpj^{\Delta M}$ BMMs stimulated with TGFβ (1 ng/mL) for the indicated times. p38 was measured as a loading control. Right panel: quantification of the relative band density using Image J software. n = 3 independent experiments. (D, E) *$p < .05$; **$p < .01$; ***$p < .001$; by two-way ANOVA. Error bars: Data are mean ± SD.

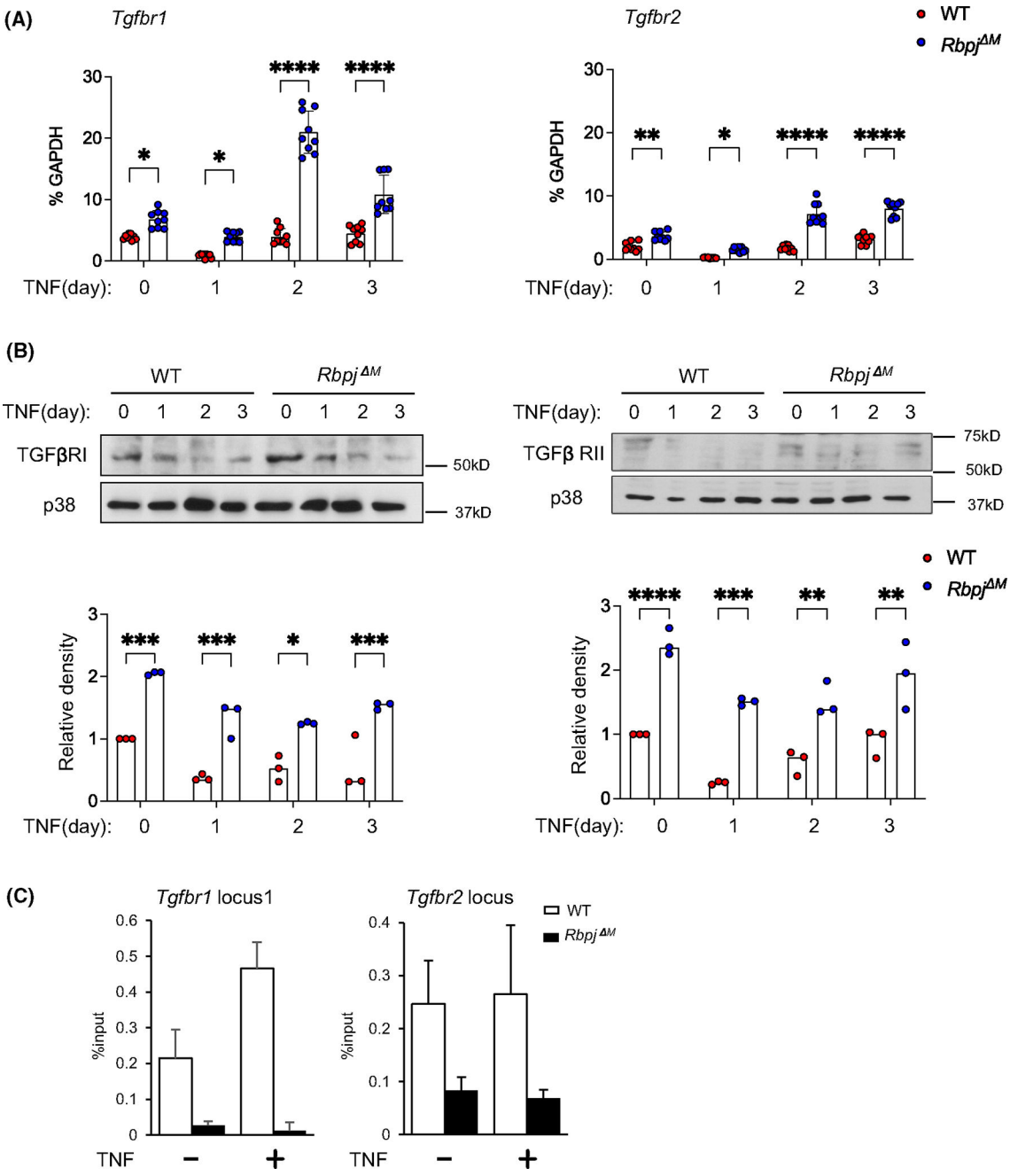

FIGURE 4.

RBP-J inhibits the expression of TGFβ receptors in macrophages. (A) qPCR analysis of mRNA expression of *Tgfbr1* and *Tgfbr2* in WT and *Rbpj*$^{\Delta M}$ BMMs stimulated with TNF for the indicated times. Data are from 9 replicates from 3 independent experiments. (B) Immunoblot analysis of *Tgfbr1* and *Tgfbr2* in WT and *Rbpj*$^{\Delta M}$ BMMs stimulated with TNF for the indicated times. p38 was measured as a loading control. Lower panel: quantification of the relative band density using Image J software. $n = 3$ independent experiments. (C) ChIP analysis of RBP-J occupancy at the *Tgfbr1* and *Tgfbr2* promoter loci in the WT or

Rbpj$^{\Delta M}$ BMMs stimulated with or without TNF (40 ng/mL) for 48 h. (A, C) *$p < .05$; **$p < .01$; ****$p < .0001$ by two-way ANOVA. Error bars: (A, C) Data are mean ± SD.

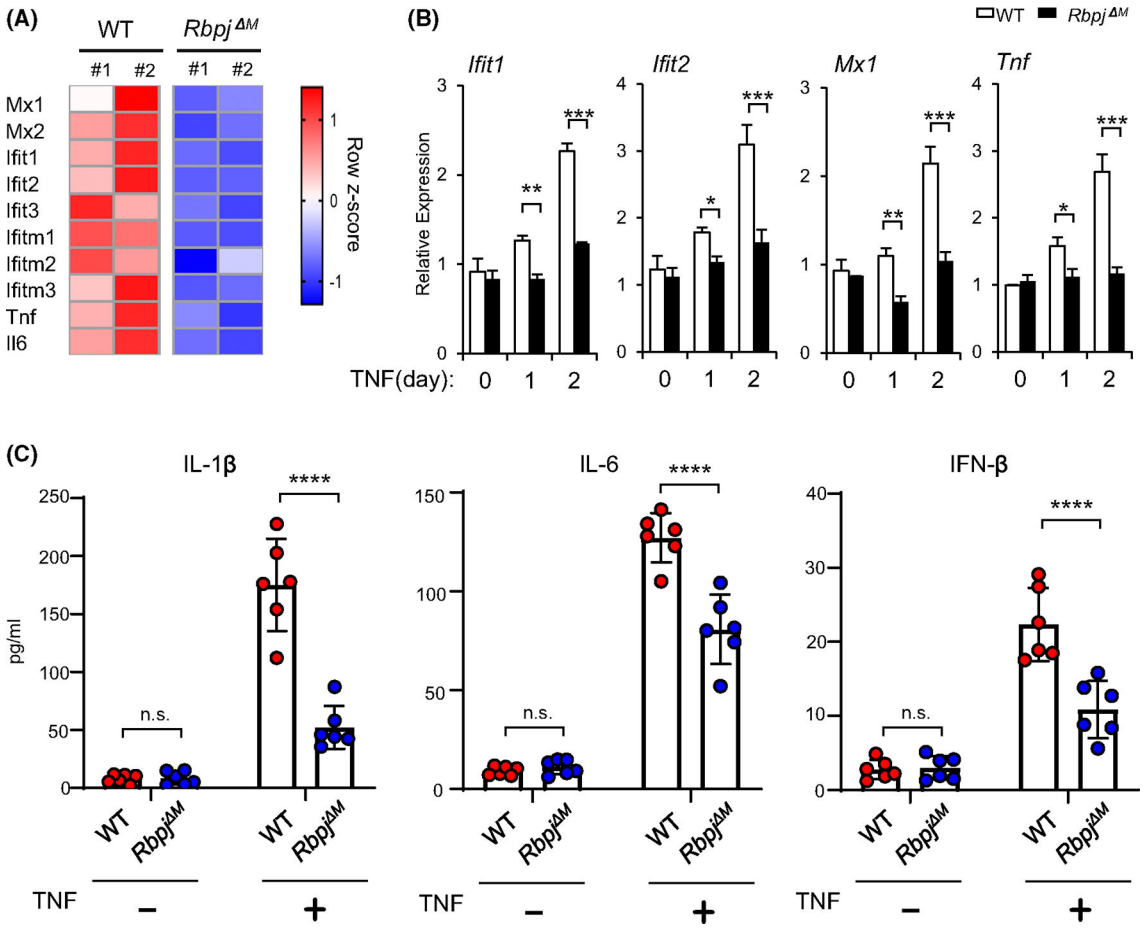

FIGURE 5.

RBP-J deletion attenuates TNF-induced inflammatory response in macrophages. (A) RNAseq–based expression heatmaps of the ISGs, *Tnf* and *Il6* in WT and $Rbpj^{\Delta M}$ BMMs stimulated by TNF for 2 days. Row *z*-scores of CPMs of genes were shown in the heatmap. #1, biological replicate 1. #2, biological replicate 2. (B) qPCR analysis of mRNA expression of *Ifit1*, *Ifit2*, *Mx1* and *Tnf* in the WT and $Rbpj^{\Delta M}$ BMMs stimulated with TNF (40 ng/mL) for the indicated times. (C) ELISA analysis of IL-1β, IL-6 and IFN-β levels in the serum from the 12-week-old male WT and $Rbpj^{\Delta M}$ mice developed TNF-induced inflammatory model ($n = 6$/group). (B, C) $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; $^{****}p < .0001$; ns: not statistically significant by two-way ANOVA. Error bars: (B) Data are mean ± SD; (C) Data are mean ± SE.

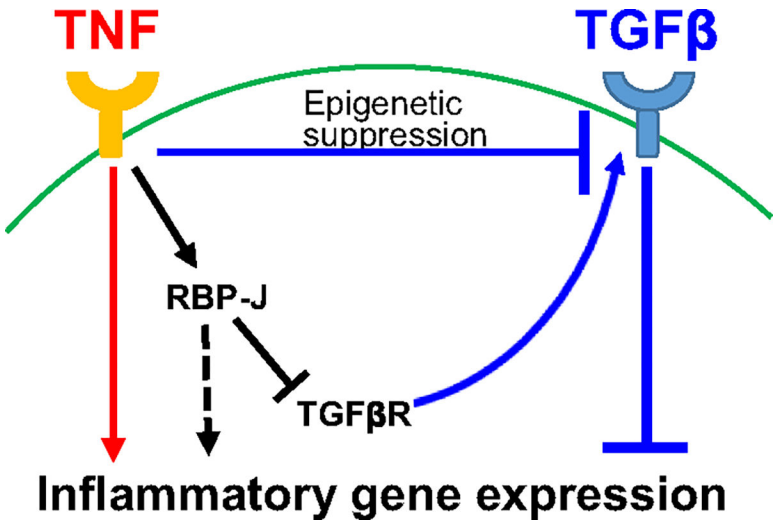

FIGURE 6.

A model showing the reciprocal suppression between TGFβ signaling and TNF stimulation during macrophage inflammatory response. In this newly identified molecular network, the basal/endogenous TGFβ signaling functions as a homeostatic “braking” system to suppress TNF-induced inflammation. Conversely, TNF takes advantage of mechanisms mediated by epigenetics and RBP-J to counteract the action of TGFβ signaling to heighten inflammation in macrophages. The overall balance between the strengths of the TNF and TGFβ signaling pathways contributes to the inflammatory state in macrophages.