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Type II but not type I interferon signaling is indispensable for TLR7-promoted development of autoreactive B cells and systemic autoimmunity

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Abstract

TLR7 is associated with development of systemic lupus erythematosus (SLE), but the underlying mechanisms are incompletely understood. Although TLRs are known to activate type I interferon (T1IFN) signaling, the role of T1IFN and IFNγ signaling in differential regulation of TLR7-mediated antibody-forming cell (AFC) and germinal center (GC) responses, and SLE development has never been directly investigated. Using TLR7-induced and TLR7 overexpression models of SLE, we report here a previously unrecognized indispensable role of TLR7-induced IFNγ signaling in promoting AFC and GC responses, leading to autoreactive B cell and SLE development. T1IFN signaling on the other hand, only modestly contributed to autoimmune responses and the disease process in these mice. TLR7 ligand imiquimod (IMQ) treated IFNγ reporter mice show that $CD4^+$ effector T cells including follicular helper T (Tfh) cells are the major producers of TLR7-induced IFNγ. Transcriptomic analysis of splenic tissues from IMQ treated autoimmune-prone B6.Sle1b mice sufficient and deficient for IFNγR indicates that TLR7-induced IFNγ activates multiple signaling pathways to regulate TLR7-promoted SLE. Conditional deletion of *Ifngr1* gene in peripheral B cells further demonstrates that TLR7-driven autoimmune AFC, GC and Tfh responses and SLE development are dependent on IFNγ signaling in B cells. Finally, we show crucial B cell-intrinsic roles of STAT1 and T-bet in TLR7-driven GC, Tfh and

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plasma cell differentiation. Altogether, we uncover a non-redundant role for IFNγ and its downstream signaling molecules STAT1 and T-bet in B cells in promoting TLR7-driven AFC, GC and SLE development whereas T1IFN signaling moderately contributes to these processes.

Introduction

Systemic lupus erythematosus (SLE) is a debilitating autoimmune disease mediated by antibodies reactive to DNA- and RNA-associated self-antigens. Although extrafollicular antibody-forming cell (AFC) and follicular germinal center (GC) responses are traditionally thought to be involved mainly in anti-pathogen antibody responses, AFCs and GCs can also develop spontaneously in the absence of purposeful immunization or overt infection (1, 2). Dysregulation in spontaneous AFC (Spt-AFC) and Spt-GC responses in SLE patients and SLE-prone mice promotes autoreactive B cell survival and pathogenic autoantibody (autoAb) production (3–8). However, the mechanisms that drive Spt-AFC and Spt-GC responses which can lead to the development of autoreactive B cells, autoantibodies (autoAbs) and SLE are incompletely understood.

Genome-wide association studies (GWAS) revealed multiple risk alleles in the toll-like receptor (TLR) pathway that are strongly associated with SLE development (9, 10). Overexpression and overactivity of TLR7 are associated with development of SLE in humans and in mouse models (11–13). Recent collaborative works also have highlighted the significance of TLR7 in promoting extrafollicular double negative (DN) B cell populations involved in SLE pathogenesis (8). TLR7 gene escape from X chromosome inactivation enhances the B cell response to TLR7 stimulation (14). Studies in the MRL-lpr model highlight the opposing inflammatory and regulatory roles for TLR7 and TLR9, respectively, in extrafollicular autoreactive B cell responses and SLE development (15). Using a variety of SLE mouse models, we and others discovered that TLR7 signaling specifically in B cells promoted SLE development through formation of Spt-AFCs and Spt-GCs (11, 16, 17). These data highlight the importance of TLR7 signaling in promoting autoimmune AFC and GC responses, leading to the development of autoreactive B cells, autoAb production and SLE; however, mechanisms of TLR7-mediated autoimmunity and SLE development are not clearly defined.

Type I (T1IFN) and II (IFNγ) interferon signaling are both implicated in SLE (9, 18). An IFNα signature is associated with human SLE (19, 20). Lupus-prone mice deficient in IFNαR have reduced anti-nuclear antibodies (ANAs) and renal disease (21). Exogenous IFNα treatment accelerates the SLE phenotype in female (NZW x NZB) F1 mice with an early development of anti-dsDNA Abs and nephritis (22). The administration of IFNα in lupus-prone mice also leads to enhanced short-lived AFC and GC responses (23), and an altered B cell selection in the GCs (24). We and others recently showed a B cell-intrinsic role of T1IFN signaling in loss of tolerance and autoreactive B cell development in the AFC and GC pathways in lupus-prone mice (25, 26). SLE patients also showed an increased production of IFNγ (27, 28) and blockade of IFNγ normalizes IFN regulated gene expression in SLE patients (29). MRL/lpr and NZW/NZBF1 lupus-prone mice deficient in IFNγ have reduced autoAb production and renal disease (30–35). We and others discovered

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a B cell-intrinsic requirement of IFNγ signaling in Spt-AFC and Spt-GC responses, and SLE development (36, 37). These data indicate that both T1IFN and IFNγ signaling contribute to autoreactive B cell development in the AFC and GC pathways. However, the mechanisms and relative contribution of T1IFN and IFNγ signaling in TLR7-mediated AFC, GC and autoimmunity development have never been directly investigated using a TLR7-induced model, especially when T1IFN signaling is believed to be strongly associated with TLR signaling and SLE development.

T1IFN and IFNγ signaling mediate signals through signal transducer and activator of transcription 1 (STAT1). Interestingly, we previously reported a B cell-intrinsic requirement of STAT1 in the Spt-GC responses (36) which we also showed dependent on TLR7 signaling in B cells (16). However, how STAT1 downstream of T1IFN and IFNγ signaling in B cells control TLR7-mediated AFC and GC responses, promoting development of autoreactive B cells and SLE remain unanswered. Here we used TLR7-induced and -overexpression models of SLE to dissect the relative contribution of T1IFN and IFNγ signaling in driving autoimmune AFC and GC responses, promoting development of autoreactive B cells, autoAb production and SLE. We found that IFNγ and STAT1 signaling in B cells played an indispensable role in TLR7-promoted AFC and GC responses and SLE development, while global T1IFN signaling only moderately contributed to these processes. Using a TLR7-induced SLE model we show that TLR7 not only increased expression of T1IFN genes but also the IFNγ gene in splenic tissue compared to untreated mice. Our data from IFNγ reporter mice and RNA sequencing analysis indicate that TLR7-driven and T cell-produced IFNγ activates multiple signaling pathways to regulate TLR7-promoted SLE. Together, our data show that TLR7 drives autoreactive B cell development in the AFC and GC pathways and promotes SLE by activating T1IFN and IFNγ signaling in which IFNγ signaling plays an indispensable role which cannot be overcome by the activation of other downstream pathways of TLR7 signaling.

Materials and Methods

Mice

C57BL/6J (B6), B6.129S7-*Ifngr1*^{tm1Agt}/J (IFNγR1$^{-/-}$), B6(Cg)-*Ifnar1*$^{tm1.2Ees}$/J (IFNαR1$^{-/-}$), C.129S4(B6)-*Ifng*$^{tm3.1Lky}$/J mice (IFN-γ eYFP), B6.129S(Cg)-*Stat1*^{tm1Dlv}/J (STAT1$^{-/-}$), B6.129S-*Stat1*^{tm1Mam}/Mmjax (STAT1$^{fl/fl}$), C57BL/6N-*Ifngr1*$^{tm1.1Rds}$/J (IFNγR$^{fl/fl}$), B6.129-Tbx21^{tm2Srnr}/J (T-bet$^{fl/fl}$) and B6.SB-*Yaa*/J (B6.*yaa*) mice were originally purchased from The Jackson Laboratory and bred in house. The B6.Sle1b mice (congenic for the Sle1b sublocus) were described previously (38). B6.Sle1b^{Yaa} mice were generated by crossing B6.Yaa mice to B6.Sle1b mice. B6.CD23^{Cre} (B6.Cg-Tg(Fcer2a-cre)5Mbu, provided by Dr. Meinrad Busslinger were crossed to B6.Sle1b autoimmune mice in house. All animal studies were conducted at Penn State Hershey Medical Center in accordance with the guidelines approved by our Institutional Animal Care and Use Committee. Animals were housed in a specific pathogen free barrier facility.

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Imiquimod treatment

For epicutaneous imiquimod treatment, 5% imiquimod cream (Glenmark Pharmaceuticals) was applied on the ears of mice, 3 times weekly for 4–12 wks depending on the experiments as previously described (39, 40). For PCR array and RNAseq sample preparation, mice were treated for 4 wks. To study the systemic autoimmune responses, such as AFC, GC, Tfh and antibody responses, mice were treated for 8 wks. To evaluate immune complex deposition and kidney pathology, mice were treated for 12 wks (three times a wk for 8 wks followed by once a week for another 4 wks).

Flow Cytometry

Flow cytometric analysis of total mouse splenocytes was performed using the following antibodies: B220-BV605 (RA3–6B2), CD4-AF700 (RMP4–5), CD44-APC (IM7), CD62L-PECy7 (MEK-14), PD1-PE (29F.1A12), NK1.1-biotin (PK136), Ly6G-PB (1A8), MHCII-PE-Cy7 (M5/114.15.2) (Biolegend, Inc.). GL7-FITC (GL-7), CD95- PeCy7, CXCR5-biotin (2G8), IFNγR-biotin (GR20), STAT1-PE (1/STAT1), CD11b-AF700 (M1/70), CD11c-FITC (HL3) (BD Biosciences). Ly6C- APC (HK1.4), PDC-PE (eBio927), CD8α (clone 53–6.7) (eBioscience). All cells were stained with fixable viability dye-eFluor780 (Invitrogen) prior to surface staining. Stained cells were analyzed using the BD LSR II flow cytometer (BD Biosciences). Data were acquired using FACSDiva software (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Immunofluorescence and ANA staining

Mouse spleens or kidneys were embedded in OCT compound and snap frozen over liquid nitrogen. 5μm thin sections were cut on a cryostat, mounted on ColorFrost plus microscope slides (Fisher Scientific, PA) and fixed in cold acetone for 20 min. The following antibodies and reagents were utilized for immunofluorescence staining of mouse spleen sections for germinal centers: PE-anti-CD4 (GK1.5, Biolegend); FITC-GL7 (RA3–6B2, BD Biosciences); APC-anti-IgD (11–26c2a, BD Biosciences). Kidney sections were stained for C3 using FITC-anti-C3 (ICL Laboratories) or Biotin-anti-IgG (Jackson Immunoresearch) followed by SA-PE. Anti-nuclear Ab (ANA) reactivity was detected by indirect immunofluorescence staining of HEp-2 cell slides using sera from indicated mice at a 1:50 dilution, and probed with FITC-rat anti-mouse Kappa (H139–52.1). The images of stained spleen and kidney sections were captured using the Leica DM4000 fluorescence microscope and analyzed using a Leica application suite-advanced fluorescence software (LAS-AF, Leica Microsystems). For the measurement of the GC area, a total of at least 10 randomly selected germinal centers ($GL\text{-}7^+$) per mouse spleen section from at least 5 mice of each genotype were measured for total area (μm^2) using the LAS-AF quantitation tool.

Kidney histopathology

Kidneys from 6 mo old mice were fixed in 10% neutral buffered formalin and embedded in paraffin. Kidney sections were cut at 3 μm and 6 μm thickness for periodic acid-Schiff (PAS) and H&E staining, respectively. All images were obtained with an Olympus BX51 microscope and DP71 digital camera using cellSens Standard 1.12 imaging software (Olympus America, Center Valley, PA). Two pathologists blinded to the genotype of the

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mice examined kidney sections. One kidney section per mouse was evaluated: each glomerulus was examined at 400x magnification and scored from 0 (normal) to 4 (severe) based on glomerular size and lobulation, presence of karyorrhectic nuclear debris, capillary basement membrane thickening, and the degree of mesangial matrix expansion and mesangial cell proliferation(41). H&E sections were scored for overall glomerular damage and interstitial inflammation as described (42).

ELISpot assay

ELISpot assays were performed as previously described (25, 43). Briefly, splenocytes in RPMI containing 10% fetal bovine serum were plated at a concentration of 1×10^6 cells/ well onto salmon sperm dsDNA- (Invitrogen), nucleosome- (histone from Sigma Aldrich plated on a layer of dsDNA coating)- or SmRNP- (Arotec Diagnostics) coated multiscreen 96-well filtration plates (Millipore, Bedford, MA). Serially diluted (1:2) cells were incubated for 12 h at 37°C. dsDNA-, nucleosome-, and smRNP-specific AFCs were detected by biotinylated anti-kappa Ab (Invitrogen) and streptavidin (SA)-alkaline phosphatase (Vector Laboratories) or alkaline phosphatase-conjugated anti-mouse IgG (Molecular Probes). Plates were developed using the Vector Blue Alkaline phosphatase Substrate Kit III (Vector Laboratories). ELISpots were enumerated and analyzed using a computerized ELISpot plate imaging/ analysis system (Cellular Technology).

ELISA

Serum autoAbs were measured using standard ELISA protocols as described (3). Briefly, total IgG autoAb titers were measured in ELISA plates coated with salmon sperm dsDNA, nucleosome or smRNP and detected with biotinylated secondary Ab followed by streptavidin (SA)-alkaline phosphatase (Vector Laboratories). The plates were developed using PNPP (*p*-Nitrophenyl Phosphate, Disodium Salt) (Thermo Fisher Scientific) substrates for alkaline phosphatase and read at λ405 nm on Synergy H1 (BioTek Instruments).

RNA Sequencing

RNA was extracted from spleen tissues by TRIzol as per the manufacturer's instructions (Ambion). Optical density values of extracted RNA were measured using NanoDrop to confirm an A260:A280 ratio above 1.9. RNA integrity number was measured using BioAnalyzer (Agilent Technologies). RNA 6000 Pico Kit to confirm RIN above 7. The cDNA libraries were prepared using the Ovation RNA-seq System V2 (NuGEN, San Carlos, CA) as per the manufacturer's instructions. The unique barcode sequences were incorporated in the adaptors for multiplexed high-throughput sequencing. The final product was assessed for its size distribution and concentration using BioAnalyzer High Sensitivity DNA Kit (Agilent Technologies) and then loaded onto TruSeq SR v3 flow cells on an Illumina HiSeq 2500 (Illumina) and run for 100 cycles using a paired-read recipe (TruSeq SBS Kit v3, Illumina) according to the manufacturer's instructions. Illumina CASAVA pipeline (released version 1.8, Illumina) was used to obtain de-multiplexed sequencing reads (fastq files) passed the default purify filter. Additional quality filtering used FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) to keep only reads that have at least 80% of bases with a quality score of 20 or more (conducted by fastq_quality_filter function) and reads left with 10 bases or longer after being end-trimmed with a base quality score of 20 (conducted

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by fastq_quality_trimmer function). A bowtie2 index was built for the mouse reference genome (GRCm38) using bowtie version 2.1.0. The RNA-seq reads were mapped using Tophat version 2.0.9(44) supplied by Ensembl annotation file; GRCm38.78.gtf. Gene expression values were computed using fragments per kilo base per million mapped reads (FPKM) values. Differential gene expression was determined using Cuffdiff tool which is available in Cufflinks version 2.2.1 supplied by GRCm38.78.gtf. Normalization was performed via the median of the geometric means of fragment counts across all libraries, as described (45). Statistical significance was assessed using a false discovery rate (FDR) threshold of 0.05 to determine the differentially abundant genes. Genes that were expressed at ≥ 1.5-fold differences between control and test samples were selected for further analysis. The heatmaps of selected genes were generated via the pheatmap package in R using Euclidean distance and Ward clustering method.

The accession number for the RNA-seq data reported in this paper is GEO: GSE141433 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE141433

Statistical analysis

P values were calculated using unpaired, nonparametric, Mann–Whitney, Student's *t*-test (for comparison of two groups) or one-way or two-way ANOVA, with a follow-up Tukey multiple-comparison test (for comparison of more than two groups) or two-way ANOVA, with a follow-up Sidak multiple-comparison test (for comparison of two groups). Ns = non-significant, * P < 0.05, ** P <0.01, *** P <0.001 and **** P <0.0001. GraphPad Prism 6 software was used.

Results

TLR7-driven AFC, GC and systemic autoimmunity model

To identify the mechanisms and determine the role of T1IFN and IFNγ signaling in differential regulation of TLR7-mediated autoimmunity, we have used a TLR7-induced SLE model in which we treated 8 wk old female mice with TLR7 agonist imiquimod (IMQ) as described (39). IMQ treated B6 (B6-IMQ) and autoimmune-prone B6.Sle1b mice (Sle1b-IMQ) displayed an increased number of splenocytes compared to untreated control counterparts (Fig. S1A). Overall percentage of $B220^{+}$ B cells and $CD4^{+}$ T cells was not significantly different in untreated and treated B6 and B6.Sle1b mice (Fig. S1B–C), but the numbers of B cells and $CD4^{+}$ T cells were higher in Sle1b-IMQ mice than three other groups (Fig. S1D–E). In addition, Sle1b-IMQ mice had much higher percentage of splenic $B220^{+}GL7^{hi}CD95^{hi}$ GC B cells and $CD4^{+}CD44^{hi}PD\text{-}1^{hi}CXCR5^{hi}$ Tfh cells than untreated B6.Sle1b mice (Fig. 1A–B). Flow cytometry gating strategy for GC B cells and Tfh cells is shown (Fig. S1F). Elevated GC and Tfh responses in Sle1b-IMQ mice were strongly associated with increased numbers of dsDNA- and nucleosome-specific splenic AFCs, serum ANA reactivity and autoantibody (autoAb) titers (Fig. 1 C–F). Treated B6 mice (B6-IMQ) had a two-fold higher GC and Tfh responses than untreated B6 mice but no autoAb production. We then treated 8 wk old mice with IMQ for 12 wks and found that Sle1b-IMQ mice had more severe nephritis than B6.Sle1b untreated mice and B6 treated mice (Fig. 1G–H). These elevated responses in Sle1b-IMQ mice were accompanied by an increased

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expression of interferon (IFN) gene signatures in the splenic tissue (Fig. 1I), and elevated IFNαR and IFNγR expression on B cells upon TLR7 stimulation *in vitro* (Fig. 1J). Thus Sle1b-IMQ mice represent a strong TLR7-driven systemic autoimmunity model with autoimmune phenotypes (elevated AFC, GC, Tfh, ANA and nephritis) closely resembling human SLE.

IFNγ, but not type I IFN signaling is critical for TLR7-driven development of autoreactive B cells and SLE pathogenesis

Although the induction of T1IFNs by TLR signaling and their roles in SLE pathogenesis have been well described (46–48), we know little about how IFNγ signaling may contribute to TLR-mediated SLE autoimmunity. Therefore, using the TLR7-induced Sle1b-IMQ model, here we determined the relative contribution of T1IFN and IFNγ signaling in TLR7-mediated SLE autoimmune responses. Although IMQ-treated B6.Sle1b mice deficient in IFNαR (Sle1b.IFNαR$^{-/-}$-IMQ) had lower GC B cell and Tfh cell responses than Sle1b-IMQ mice, these responses were diminished in treated B6.Sle1b mice deficient in IFNγR (Sle1b.IFNγR$^{-/-}$-IMQ) (Fig. 2A, B). The frequency of GCs between Sle1b-IMQ and Sle1b.IFNαR$^{-/-}$-IMQ mice was similar, but GC sizes were smaller in Sle1b.IFNαR$^{-/-}$-IMQ mice than Sle1b-IMQ control mice whereas a negligible number of very small GCs was observed in Sle1b.IFNγR$^{-/-}$-IMQ mice (Fig. 2C, D). In addition, the number of dsDNA- and nucleosome-specific splenic AFCs (Fig. 2E), serum dsDNA-, nucleosome- and SmRNP-specific Abs (Fig. 2F) and serum ANA reactivity (Fig. 2G) were moderately reduced in Sle1b.IFNαR$^{-/-}$-IMQ mice whereas diminished in Sle1b.IFNγR$^{-/-}$-IMQ mice compared to Sle1b-IMQ control mice. Consistent with systemic autoimmune B and T cell responses, and serum ANA reactivity, immune complex (IC) deposition (IgG and C3) and severe glomerulonephritis (GN) in B6.Sle1b-IMQ mice were moderately reduced in Sle1b.IFNαR$^{-/-}$-IMQ mice whereas completely absent in Sle1b.IFNγR$^{-/-}$-IMQ mice (Fig. 2H–J).

Next, we determined the role of IFNγ signaling in SLE autoimmunity development in B6.Sle1b mice overexpressing TLR7 by breeding Sle1b.IFNγR$^{-/-}$ mice to B6.Yaa mice that carry an extra copy of TLR7 to generate Sle1b^{Yaa}IFNγR$^{-/-}$ mice. B6.Sle1b male mice expressing Yaa (Sle1b^{Yaa}) developed splenomegaly, which was reversed to a level observed in B6.Sle1b males in the absence of IFNγR (Fig. 3A). Elevated GC B cell (Fig. 3B, D, E) and Tfh cell (Fig. 3C) responses in Sle1b^{Yaa} mice were significantly reduced in Sle1b^{Yaa}IFNγR$^{-/-}$ mice. Similarly, the number of dsDNA- and nucleosome-specific splenic AFCs (Fig. 3F), serum dsDNA-, nucleosome- and SmRNP-specific Abs (Fig. 3G) and serum ANA reactivity (Fig. 3H) were significantly reduced in Sle1b^{Yaa}IFNγR$^{-/-}$ mice compared to Sle1b^{Yaa} control mice. Finally, IC deposition in the kidney and the developed GN in Sle1b^{Yaa} mice were also diminished in Sle1b^{Yaa}IFNγR$^{-/-}$ mice (Fig. 3I–K). Our data from TLR7-induced and -overexpression SLE models indicate that IFNγ signaling plays a non-redundant role in TLR7-promoted AFC and GC responses, and subsequent autoAb production and SLE pathogenesis, whereas T1IFN signaling only moderately contributes to these responses.

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IFNγ regulates gene expression of several cellular pathways to promote AFC and GC responses in TLR7-accelerated SLE

To investigate how IFNγ may promote autoreactive B cell development into the AFC and GC pathways in TLR7-accelerated SLE, we analyzed the gene transcripts in spleens of IMQ-treated B6.Sle1b and Sle1b.IFNγR$^{-/-}$ mice by RNA sequencing. Clustering of differentially expressed genes showed a distinct transcriptional profile in Sle1b.IFNγR$^{-/-}$-IMQ mice compared to B6.Sle1b-IMQ control mice. We found that 130 and 213 genes were up- and down -regulated, respectively in Sle1b.IFNγR$^{-/-}$-IMQ mice ($p < 0.05$; fold change ≥ 1.5; Fig. 4A). Functional classification of the differentially expressed genes with gene ontology analysis revealed many biological processes or the pathways that were downregulated (Fig. 4B) or upregulated (Fig. 4C) in the absence of IFNγR signaling. Genes differentially regulated between B6.Sle1b-IMQ and B6.Sle1b.IFNγR$^{-/-}$-IMQ splenic tissues were associated with gene transcription regulation (Fig. 4D), ubiquitination/proteasome complex (Fig. 4E), cell proliferation and migration (Fig. 4F), autoimmunity (Fig. 4G), inflammation (Fig. 4H), cellular and oxidative stress (Fig. 4I), cellular metabolism (Fig. 4J). These results indicate that IFNγ signaling promotes TLR7-mediated autoimmunity by regulating multiple biological processes.

CD4$^+$ T cells including Tfh produce TLR7-induced IFNγ

Considering the increased expression of the *Ifnγ* gene in splenic tissue (Fig. 1I) and regulation of multiple signaling pathways by IFNγ signaling (Fig. 4) in IMQ-treated mice, we next determined the cellular source of IFNγ in TLR7-mediated autoimmunity. We treated IFNγ-eYFP reporter mice with IMQ for 6 wks and found that CD4$^+$ T cells were the major producers of IFNγ although CD8$^+$ T cells and NK cells produced this cytokine to a certain extent (Fig. 5A). Of the CD4$^+$CD44^{hi} effector T cells, a higher frequency of pre-Tfh and Tfh cells produced IFNγ than non-Tfh cells upon IMQ treatment (Fig. 5B–D). DCs, macrophages, eosinophils, monocytes, and neutrophils did not produce IFNγ in these mice (Fig. 5E). These data together with increased IFNγR expression on B cells upon TLR7 stimulation *ex vivo* (Fig. 1J) point to an important role for TLR7 in regulating the IFNγ-IFNγR pathway in B cells by inducing T cell IFNγ production and upregulating IFNγR on B cells.

Absolute requirement of IFNγ signaling in B cells in regulating TLR7-mediated AFC, GC and lupus nephritis development

To determine how TLR7-induced and T cell-produced IFNγ may signal in B cells for regulating autoimmune AFC, GC and Tfh responses and promoting SLE development, we conditionally deleted the *Ifngr1* gene in peripheral B cells in B6.Sle1b mice (designated Sle1bIFNγR$^{fl/fl}$.CD23^{Cre+}). IMQ-treated Sle1bIFNγR$^{fl/fl}$.CD23^{Cre+} mice did not show splenomegaly that was observed in IMQ-treated Sle1bIFNγR$^{fl/fl}$ control mice (Fig. 6A, B). Sle1bIFNγR$^{fl/fl}$.CD23^{Cre+} mice also had markedly reduced B220$^+$GL-7^{hi}CD95^{hi} GC B cell and CD4$^+$CD44^{hi}CXCR5^{hi}PD-1^{hi} Tfh cell responses, including reduced frequency and size of GCs, compared to Sle1bIFNγR$^{fl/fl}$ mice (Fig. 6C–G). Sle1bIFNγR$^{fl/fl}$.CD23^{Cre+} mice also had diminished serum ANA reactivity (Fig. 6H), reduced serum autoAb titers (Fig. 6I) and splenic autoAb-producing AFCs (Fig. 6J). Dampened AFC, GC, and Tfh responses in

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Sle1bIFNγR$^{fl/fl}$.CD23^{Cre+} mice were strongly associated with diminished IC deposition in the kidney and with significantly improved kidney inflammation such as glomerulonephritis (GN), interstitial nephritis (IN) and vasculitis score (Fig. 6K–M). These data highlight the non-redundant role of IFNγ signaling in B cells in TLR7-mediated AFC, GC and Tfh responses, and the development of autoreactive B cells, autoAbs and lupus nephritis.

Crucial B cell-intrinsic roles of STAT1 and T-bet in TLR7-driven GC, Tfh and Ab responses which cannot be overcome by potential activation of the other TLR7 pathways

We next asked how much of a role STAT1 that functions downstream of IFNγ signaling played in TLR7-driven GC, Tfh and IgG Ab responses under a scenario in which several pathways (NF-kB, IRF5/7 and P38/MAPK) can be potentially activated by the strong *in vivo* TLR7 stimulation. We first treated B6 and B6.STAT1$^{-/-}$ mice with IMQ and found that STAT1 is absolutely required for the development of TLR7-driven GC B cell, Tfh cell and plasma cell (PC) responses (Fig. 7A–C). Next, by using conditional knockout mice with peripheral B cells deficient in STAT1 (STAT1$^{fl/fl}$CD23^{Cre+}), we found the requirement of B cell-intrinsic STAT1 in TLR7-mediated GC, Tfh, PC and IgG Ab responses (Fig. 7D–F). Consistent with these data B cells stimulated with TLR7 ligand had much higher STAT1 expression than unstimulated B cells (Fig. 7G). Similar to the increased STAT1 expression, T-bet downstream of STAT1 was also upregulated in B cells stimulated with TLR7 ligand (Fig. 7H). To solidify the importance of B cell intrinsic T-bet in TLR7 driven GC, Tfh and Ab responses, we treated T-bet$^{fl/fl}$CD23^{Cre+} mice where peripheral B cells are deficient in T-bet. We observed a crucial B cell-intrinsic role of T-bet in TLR7-mediated GC, Tfh and IgG Ab responses (Fig. 7I–K). These data indicate that STAT1 and T-bet downstream of TLR7 and IFNγ signaling play a significant role in TLR7-driven GC, Tfh and IgG Ab responses, which cannot be overcome by other pathways potentially activated by TLR7 stimulation.

Discussion

Cellular and molecular mechanisms by which toll-like receptor 7 (TLR7) overexpression or overactivity promotes autoreactive B cell development in the extrafollicular AFC and follicular GC pathways that are activated in SLE remain largely unknown. Here using the TLR7-induced and TLR7-overexpression models of SLE, as well as an IFNγ reporter mouse model, we demonstrate that TLR7 overactivation drives development of autoreactive B cells in both the AFC and GC pathways and promotes SLE by driving predominantly IFNγ production by T cells and mediating IFNγ signaling in B cells. TLR7-driven and T cell-produced IFNγ controls gene transcription in multiple cellular pathways important for immune cell survival, proliferation, metabolism and autoimmunity.

Several groups including ours, previously identified a B cell-intrinsic role of TLR7-MyD88 signaling in promoting autoimmune AFC and GC responses, leading to SLE pathogenesis in the mouse models (5, 11, 16, 17, 49). We and others also reported B cell-intrinsic roles of T1IFN and IFNγ signaling in regulating SLE-associated AFC, GC and autoAb responses in SLE-prone mice (25, 37, 43). Although TLR7 signaling is believed to drive T1IFN production and activation of T1IFN signaling in SLE (19, 47, 50, 51), how TLR7-driven IFNγ production and activation of IFNγ signaling in B cells may promote TLR7-mediated

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autoimmune AFC and GC responses and SLE development has previously not been addressed. Using the TLR7-induced SLE model and B cell-specific conditional deletion of the *Ifngr1* gene, we found that IFNγ signaling in B cells was essential for TLR7-mediated AFC, GC and Tfh responses, and glomerulonephritis (GN) development, whereas global T1IFN signaling deficiency moderately reduced these responses and was associated with less kidney pathology. Using IFNγ reporter mice we further established that TLR7 induced IFNγ production primarily by $CD4^+$ T cells, including pre-Tfh and Tfh. Together, our data demonstrate that TLR7-driven and T cell-produced IFNγ act non-redundantly to activate IFNγ signaling in B cells, promoting development of autoreactive B cells and SLE pathogenesis.

Although MRL/lpr and NZW/NZBF1 lupus-prone mice deficient in IFNγ or treated with anti- IFNγ blocking therapy had reduced autoAb production and renal disease (30–35), the role of IFNγ signaling in autoreactive B cell development in either the extrafollicular or the GC pathways in these mice was not tested. David Rawlings' and our groups previously discovered the role of IFNγ signaling in regulating SLE-associated GC responses; however, the link between TLR7 and IFNγ signaling in autoreactive B cell expansion in the GC pathway was not directly tested in these studies using a TLR7-induced SLE mouse model (36, 37). Here, we find a significantly reduced number of autoAb-producing AFCs and dampened GC responses in the spleens of TLR7-induced SLE-prone mice. Our current data highlight the importance of B cell-intrinsic IFNγ signaling downstream of TLR7 signaling in regulating autoreactive B cell development in both the extrafollicular and GC pathways, leading to TLR7-promoted autoantibody production and SLE development.

Synergy between TLR-MyD88 and IFNγ-STAT1 signaling in potentiating inflammatory responses in macrophages has previously been described (52). IFNγ-STAT1 signaling was found to aid TLR-mediated inflammatory cytokine production by regulating cellular metabolism (53) and gene transcription through chromatin remodeling of the inflammatory gene promoter and enhancer regions (54). MyD88 was also shown to directly interact with IFNγR to stabilize the IFNγ-induced cytokine and chemokine mRNA (55). In addition, STAT1 was shown to interact with cell cycle regulators cyclin D1 and Cdk4 (56). Consistent with these previous reports we found that global and B cell-specific deficiency of STAT1 resulted in reduced GC B cell and Tfh responses upon treatment with a TLR7 ligand, highlighting the importance of STAT1 in B cells in TLR7-mediated GC and Tfh responses. Published data and our current findings collectively suggest that TLR7-MyD88 and IFNγ-STAT1 signaling may synergize to activate multiple biological processes in B cells that are important for mediating SLE-associated AFC, GC and Tfh responses in TLR7-accelerated SLE autoimmunity.

Although T-bet has been associated with ABCs and class switching to the IgG2a/c isotype (57–59), we found significantly reduced TLR7-driven GC and Tfh responses in B cell conditional T-bet-deficient mice. Rubtsova et al. recently showed a B cell-intrinsic role of T-bet in B cell differentiation into ABCs, spontaneous GC B cells and plasma cells, and which subsequently increased kidney pathology (60). In addition, $CD11c^{hi}$T-bet^+ B cells have recently been described in SLE patients (61). An earlier report showed that TLR7 stimulation of B cells upregulated T-bet and that T-bet expression was elevated in 564Igi

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autoreactive B cells (62). Rubstova et al. similarly found that BCR and TLR7 signaling synergize with IFNγ signaling to drive T-bet expression in B cells (63). The Cancro group recently reported that T-bet expression is induced in B cells by TLR signaling, but not to BCR and/or anti-CD40 stimulation alone (64). Published data and our current observation together suggest that TLR and IFNγ-STAT1 signaling cooperate to drive T-bet expression in B cells which is important for GC, Tfh and Th1-biased IgG2c Ab responses. Consistent with this idea we found upregulation of STAT1 and T-bet in TLR7 stimulated B cells, implicating the involvement of both T-bet- and STAT1 in TLR7-mediated AFC, GC and SLE autoimmune responses.

TLR7 can potentially activate multiple signaling pathways (e.g., NF-κB, IRF5/7 and p38/MAPK) to promote autoimmunity (65). Both canonical and non-canonical NF-κB pathways have been shown to be important for foreign antigen-driven AFC and GC responses (66, 67), although the role of NF-κB in spontaneous or TLR7-mediated AFC and GC responses in SLE is unclear. Altered NF-κB activity was previously reported in B cells and T cells from SLE patients (68). IRF5-deficiency abrogates disease development in the FcγRIIB$^{-/-}$ Yaa model of SLE (69). Despite the potential activation of these pathways in TLR7-induced and TLR7-overexpression models of SLE, we found that IFNγR and STAT1 signaling in B cells played an important role in TLR7-mediated AFC and GC responses. Given the importance of both TLR7 and IFNγ-STAT1 signaling in autoimmune AFC and GC responses, we postulate that IFNγ-STAT1 signaling in B cells may cooperate with the NF-kB or IRF5/7 pathway to orchestrate a genetic program required for autoimmune AFC and GC responses in TLR7-accelerated SLE. Cooperation of NF-kB and STAT1 in the transcriptional control of anti-microbial genes in response to *in vitro* Listeria monocytogenes infection has previously been described (70).

Our RNA sequencing data derived from splenic tissues of IMQ treated Sle1b.IFNγR$^{-/-}$ and B6.Sle1b mice revealed differential expression of multiple genes and pathways involved in the autoimmune response. Specifically, multiple biological processes are disrupted in the absence of IFNγR signaling such as cell proliferation and migration, inflammation and cellular metabolism that can contribute to the development of autoimmunity. In support of the overall reduction in the number of autoantibody-producing AFCs in Sle1b.IFNγR$_1^{-/-}$ mice, the *xbp-1* gene that encodes for the transcription factor (XBP-1) and is required for plasma cell differentiation and autoimmune responses (71, 72), is downregulated in these mice. IL-6 signaling is involved in autoimmune germinal center formation and autoimmune disease progression likely by increasing the production of IFNγ (73, 74). Interestingly, *il6r* gene expression is downregulated in Sle1b.IFNγR$_1^{-/-}$ mice. In addition, the relationship between IL-6 and IFNγ signaling in autoimmunity is further supported by the upregulation of the *Smyd2_TSS3427* gene in Sle1b.IFNγR$^{-/-}$ mice, a gene that encodes a histone methyltransferase which negatively regulates IL-6 production (75). Finally, genes encoding chemokine receptor *Cxcr3* and its ligand *Cxcl9* that play an important role in autoimmunity development (76–78) are downregulated in the absence of IFNγR signaling. Overall, our data indicate that TLR7 stimulation induces expression of genes that are associated with autoimmune responses in an IFNγR signaling dependent manner.

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In summary, our data define a previously unrecognized indispensable role for IFNγ signaling and its downstream transcription factors STAT1 and T-bet in B cells in promoting TLR7-accelerated AFC and GC responses and SLE autoimmunity. In contrast, we find moderate effects of T1IFN signaling on these processes. Our current T1IFN signaling data reflect the moderate efficacy of the clinical trials for anti-IFNα or anti-IFNαR therapy in SLE patients. Future treatment approaches could aim to reduce TLR7 signaling in combination with T1IFN or IFNγ signaling to a threshold that can prevent or treat SLE patients and maintain protection against pathogenic infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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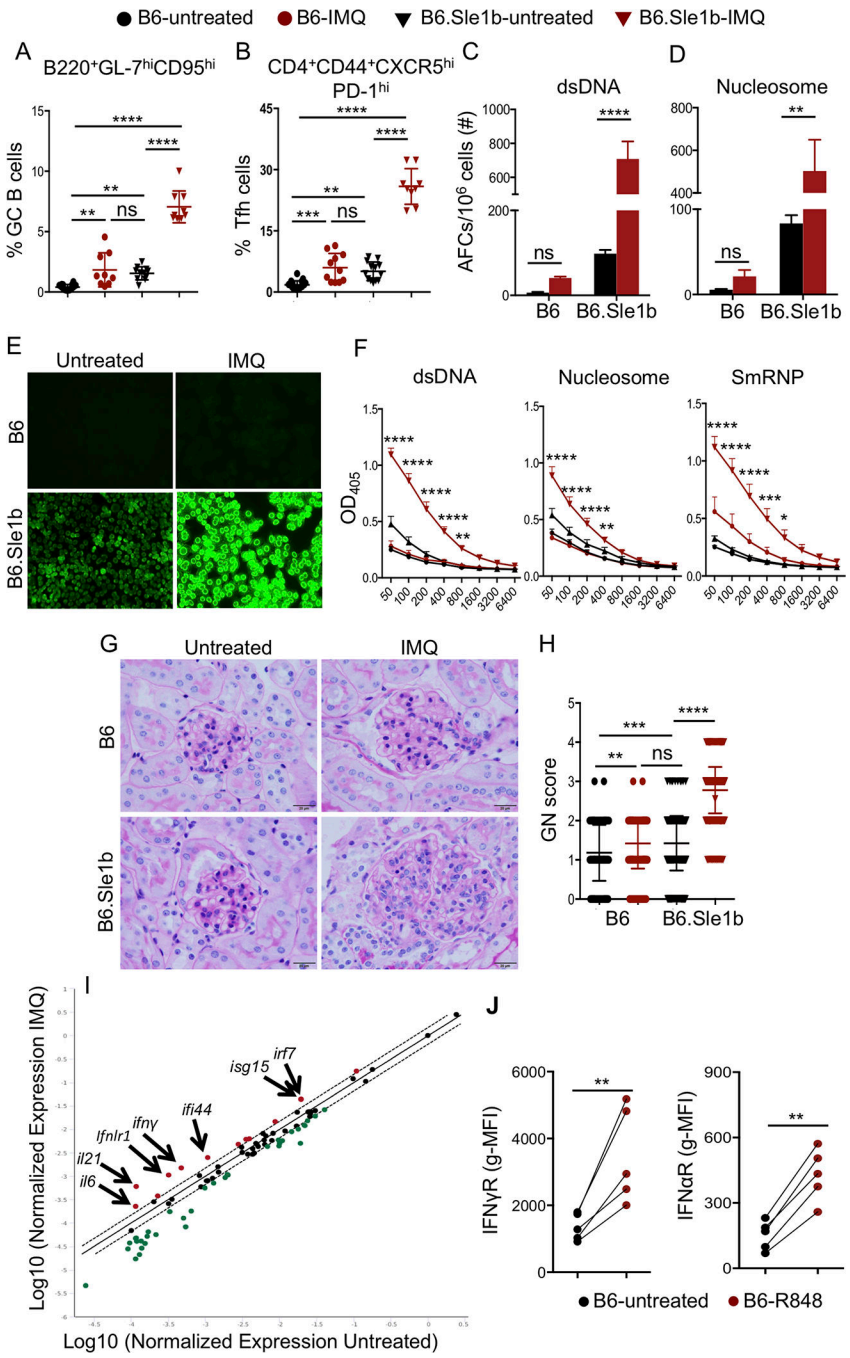

Figure 1. Imiquimod (IMQ) induced mouse model of SLE.

Eight-ten wk old C57BL/6 (B6) and B6.Sle1b mice were epicutaneously treated on the ears with TLR7 ligand imiquimod (IMQ) for 8 wks. The percentages of splenic $B220^{+}GL7^{hi}CD95^{hi}$ GC B cells (A) and $CD4^{+}CD44^{hi}PD\text{-}1^{hi}CXCR5^{hi}$ Tfh cells (B) from these mice were obtained through flow cytometric analysis. (C-D) The number of dsDNA- and nucleosome-specific AFCs in the spleens was quantified by ELISPOT assay. These data represent 4 mice of each group. ANA reactivity (E) and serum titers of dsDNA-, nucleosome- and SmRNP-specific Abs (F) in these mice (6 mice of each group) are shown.

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Representative images of kidney sections stained with PAS (G) and glomerulonephritis (GN) score (H) are shown. (I) B6.Sle1b mice were untreated or treated with IMQ for 4 wks and the spleen tissues were processed for interferons and interferon receptors PCR array. Arrows in the scatter plots represent upregulated genes upon IMQ treatment. (J) Expression of IFNγR and IFNαR on B cells before and after stimulation with R848 for 48 hr. These data represent 2–3 experiments and each symbol indicates an individual mouse (A, B, J) or a glomerulus (H). Statistical analysis was performed by one-way (A-B, H) or two-way (C-D, F) ANOVA, with a follow-up Tukey multiple-comparison test or unpaired, nonparametric Mann-Whitney Student's t-test (J). Ns, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Error bars in A, B and H represent mean ± SD, and in F, C and D represent mean ± SEM.

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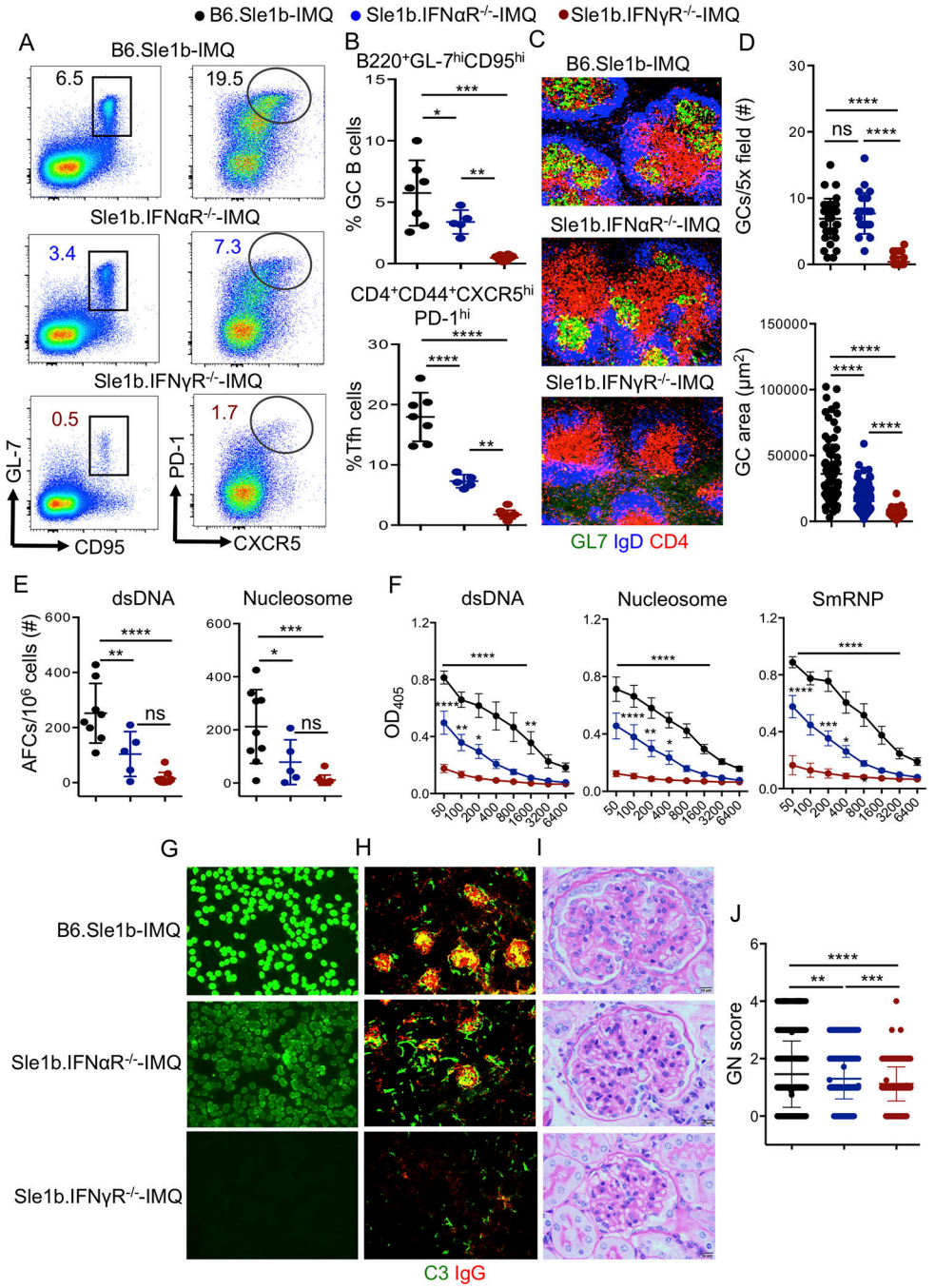

Figure 2. Differential roles of type I and II IFNs in TLR7-mediated AFC, GC, and Tfh responses and SLE development.

Representative flow cytometry plots (A) and percentage (B) of $B220^{+}GL7^{hi}CD95^{hi}$ GC B cells and $CD4^{+}CD44^{hi}PD\text{-}1^{hi}CXCR5^{hi}$ Tfh cells in imiquimod (IMQ) treated B6.Sle1b, Sle1b.IFNαR$^{-/-}$ and Sle1b.IFNγR$^{-/-}$ female mice. (C) Representative spleen sections stained with GC B cell marker GL7 (green), naïve B cell marker IgD (blue) and T cell marker CD4 (red). (D) Number of GCs per 5x Field (top panel) and GC areas (bottom) were measured in 10 mice of each genotype. (E) Numbers of dsDNA- and nucleosome-specific splenic AFCs in these mice were quantified by ELISPOT assay. dsDNA-, nucleosome- and

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SmRNP-specific serum IgG titers (F) and serum ANA reactivity (G) were measured by ELISA and HEp-2 assays, respectively. Data in F and G represent 6 mice of each genotype. (H) Representative kidney sections from these mice were stained with anti-C3 (green) and anti-mouse IgG (red). Representative periodic acid-Schiff (PAS) stained kidney sections (I) and GN score (J) from all three genotypes are shown. These data represent three experiments and each symbol indicates an individual mouse (B, E) or a GC (D) or a glomerulus (J). Statistical analysis was performed by one-way (B, D, E, J) or two-way (F) ANOVA, with a follow-up Tukey multiple-comparison test. ns, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Error bars in B, D, E and J represent mean ± SD, and in F represent mean ± SEM.

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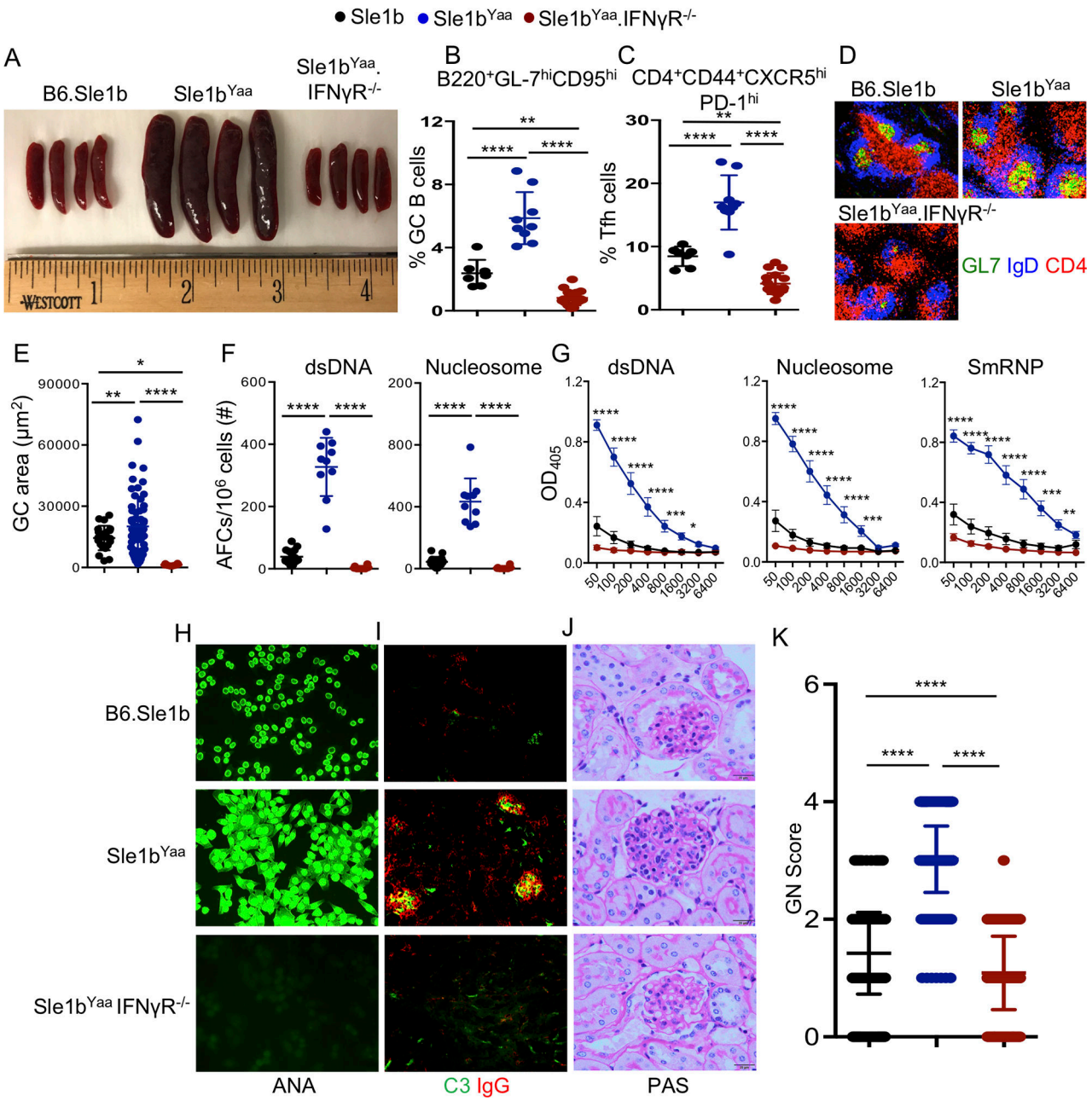

Figure 3. Essential role of IFNγ in the Yaa mouse model of SLE.

Representative images of spleen size (A), the percentages of $B220^{+}GL7^{hi}CD95^{hi}$ GC B cells (B) and $CD4^{+}CD44^{hi}PD\text{-}1^{hi}CXCR5^{hi}$ Tfh cells (C) are shown. Histological images of splenic GCs (D) stained for GC B cell marker, GL7 (green), mature B cell marker, IgD (blue), and T cell marker, CD4 (red) and GC areas (E) are shown. Number of splenic dsDNA- and nucleosome-specific AFCs (F), serum Ab titers against dsDNA, nucleosome and SmRNP (G), and serum ANA reactivity (H) are illustrated. Data in G and H represent 7–10 mice of each group. Representative images of kidney sections stained with anti-C3 (green) and anti-IgG (red) (I). Representative images of PAS stained kidney sections (J) and GN score (K). These data were derived from 6–8 mo old B6.Sle1b, $Sle1b^{Yaa}$ and

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Sleb^{Yaa}IFNγR$^{-/-}$ mice and represent 2–3 experiments. Each symbol represents a mouse (B-F) or a glomerulus (H). Statistical analysis was performed by one-way (B-C, E-F, K) or two-way (G) ANOVA, with a follow-up Tukey multiple-comparison test. ns, non-significant; *, P <0.05; **, P <0.01; ***, P <0.001; ****, P <0.0001. Error bars in B, C, E, F and K represent mean ± SD, and in G represent mean ± SEM.

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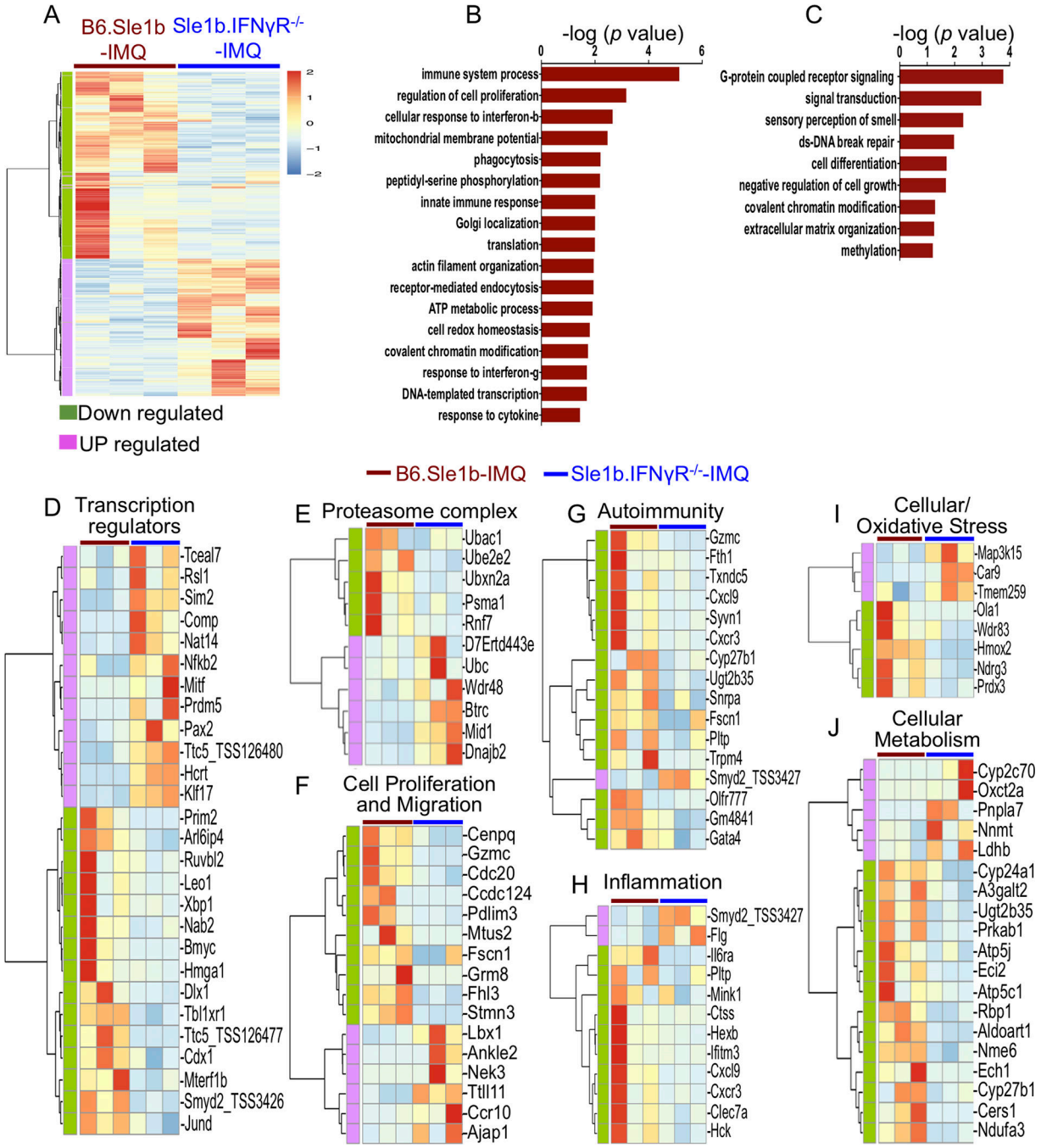

Figure 4. Transcriptome and gene ontology analysis of IMQ-treated Sle1b.IFNγR$^{-/-}$ and B6.Sle1b splenic tissue RNA.

B6.Sle1b and Sle1b.IFNγR$^{-/-}$ mice were treated with imiquimod (IMQ) for 4 weeks and the splenic tissues were processed for RNA sequencing. (A) Heat map showing the relative expression pattern of the top 343 differentially expressed genes between the groups. Fold-change values were visualized as $\log_{10}$ (FC). Gene ontology and pathway analysis for the genes that were down regulated (B) or upregulated (C) in Sle1b.IFNγR$^{-/-}$-IMQ mice (FC > 2 and P value < 0.05). (D-J) Heat maps display differentially expressed genes that were categorized based on their functions in particular biological processes (FC >1.5 and P value

< 0.05). Hierarchical-clustering analysis was conducted for the transcript levels of the differentially expressed genes (Ward's clustering algorithm and Euclidean distance measure; n = 3, each sample was pooled from four mice of each genotype).

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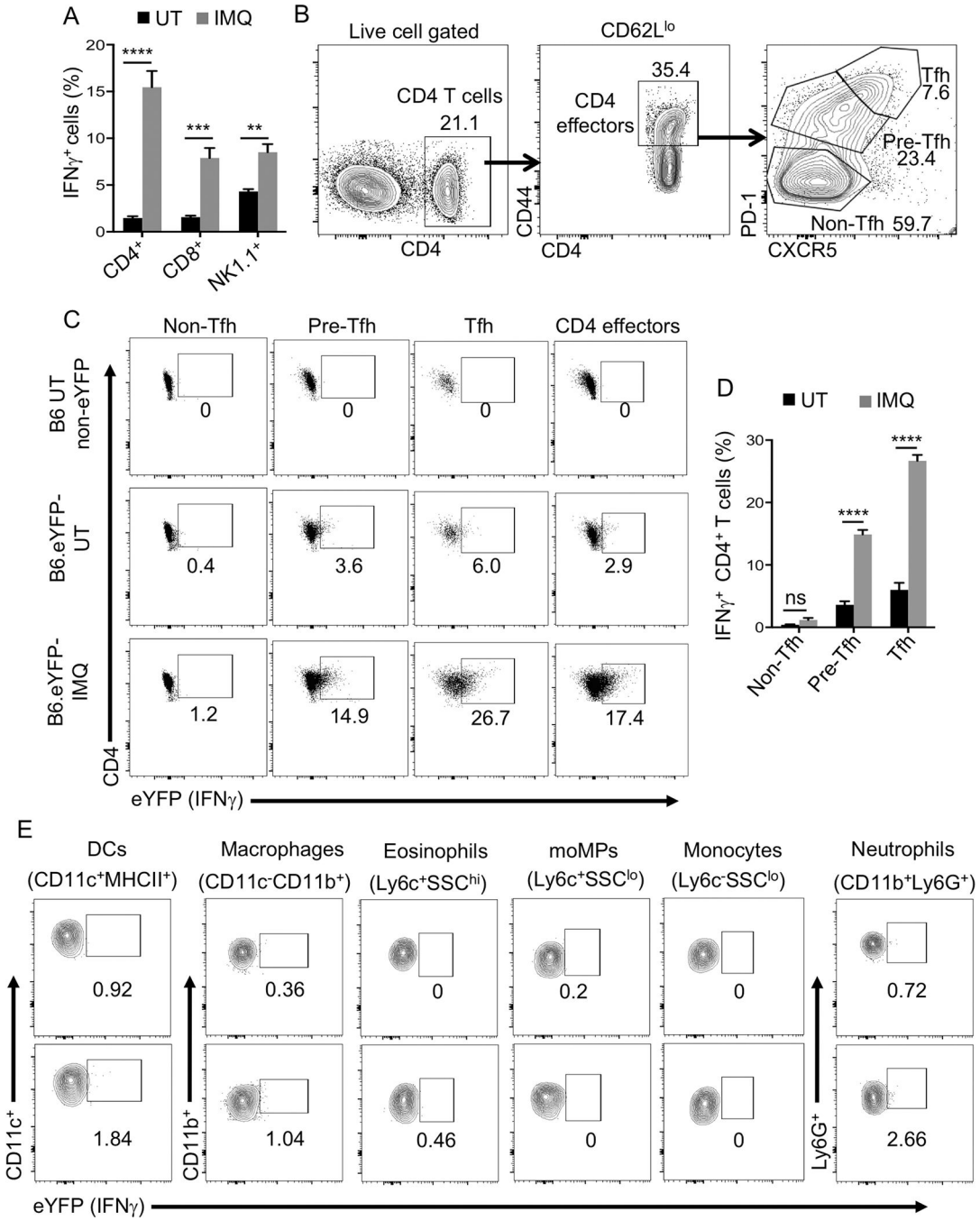

Figure 5. CD4$^+$ T cells are the major IFNγ producers upon TLR7 stimulation.

Three to four mo old B6 (non-eYFP) and eYFP-IFNγ reporter (B6.eYFP) mice were left untreated (UT) or treated with imiquimod (IMQ) for 4 wks and splenocytes were analyzed for eYFP expressing cells by flow cytometry. (A) Bar diagrams show the percentage of eYFP$^+$ (IFNγ$^+$) total CD4$^+$, CD8$^+$ and NK1.1$^+$ cells. These data represent 6 mice per group. (B) Flow cytometry gating strategy for CD4$^+$CD44^{hi} effector T cells that are subdivided into CD4$^+$PD-1^{lo}CXCR5^{lo} non-Tfh, CD4$^+$PD-1^{int}CXCR5^{int} pre-Tfh and CD4$^+$PD-1^{hi}CXCR5^{hi} Tfh. (C) Representative flow cytometry dot plots from indicated mouse strains show

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frequency of eYFP-expressing (IFNγ^+) cells in non-Tfh, pre-Tfh, Tfh and CD4 effector cells. (D) Bar diagrams show the percentage of IFNγ^+ cells in non-Tfh, pre-Tfh and Tfh cells in untreated (UT) and IMQ treated B6.eYFP mice. These data represent 5 mice of each group. (E) Flow cytometry contour plots represent eYFP expression in the indicated myeloid cell populations. moMPs, monocyte-derived macrophages. Statistical analysis was performed by unpaired, nonparametric Mann-Whitney Student's *t*-test or two-way ANOVA, with a follow-up Sidak multiple-comparison test. ns, non-significant; **, P <0.01; ***, P <0.001; ****, P <0.0001. Error bars in A and D represent mean ± SEM.

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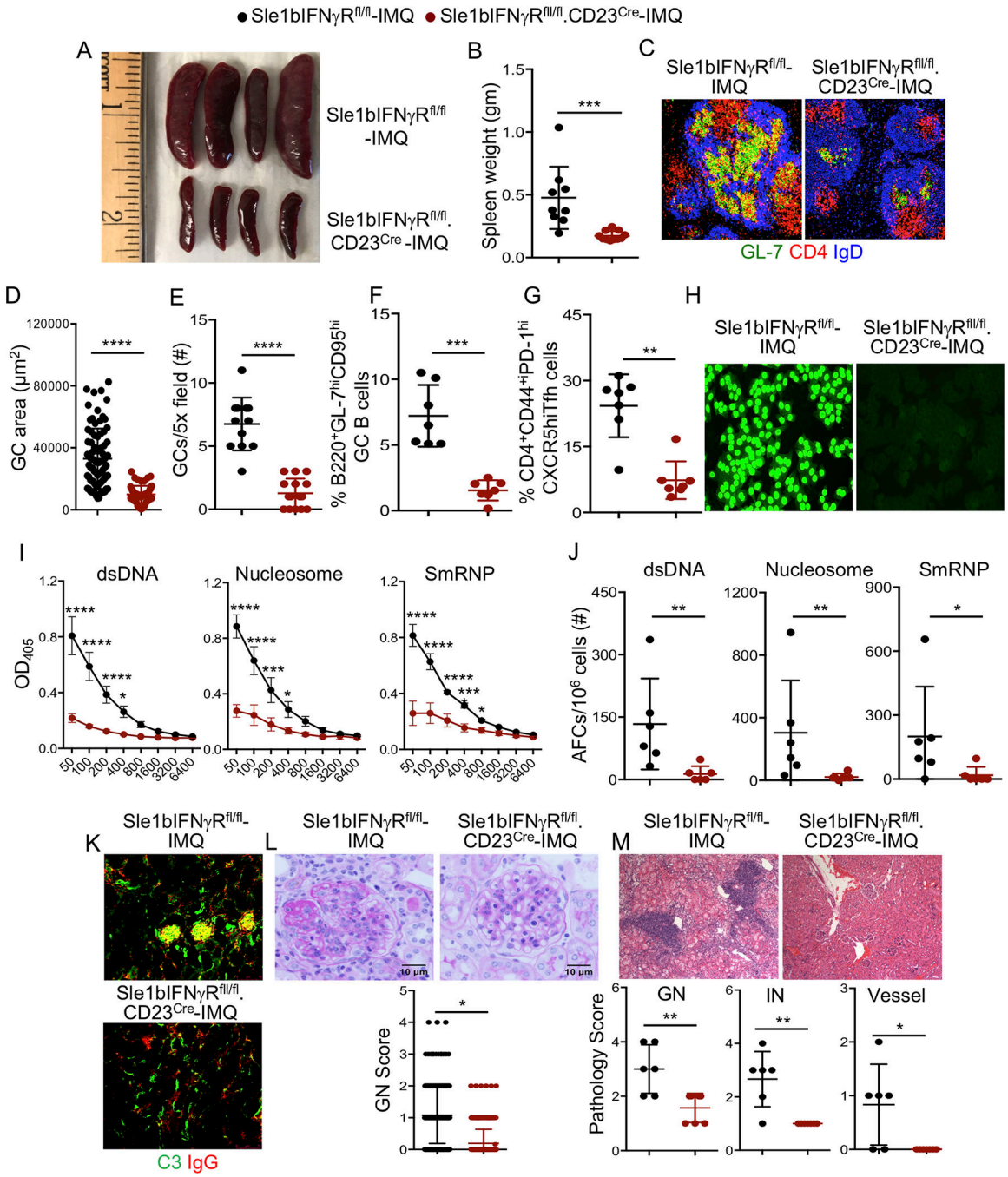

Figure 6. B cell-intrinsic IFNγ signaling is necessary for TLR7-mediated autoimmune responses and lupus nephritis development.

(A-M) Sle1bIFNγR$^{fl/fl}$ and Sle1bIFNγR$^{fl/fl}$.CD23^{Cre+} mice were treated with IMQ for 12 wks. Spleen pictures (A) and their weight (B) are shown. Representative spleen sections were stained for GCs (C) with GL7 (green), CD4 (red), and IgD (blue); and the areas (D) and the frequency of GCs (E) were measured. The percentage of B220^{+}GL7^{hi}CD95^{hi} GC B cells (F) and CD4^{+}CD44^{hi}PD-1^{hi}CXCR5^{hi} Tfh cells (G) were measured by flow cytometry. Serum ANA reactivity (H) and autoAb titers (I) against dsDNA, nucleosome and SmRNP are shown. Data in (H) and (I) represent 6 mice of each genotype. Numbers of splenic

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dsDNA-, nucleosome-, and SmRNP specific AFCs were quantified by ELISPOT assay (J). (K) Representative kidney sections were stained with anti-C3 (green) and anti-IgG (red). Representative kidney sections were stained with PAS (L) or H&E (M) and scored for glomerulonephritis (GN), interstitial nephritis (IN), or inflammation in the vessels. These data represent 2–3 experiments and each symbol indicates an individual mouse (B, F-G, J, M) or a GC (D-E) or a glomerulus (L). Statistical analysis was performed by unpaired, nonparametric Mann-Whitney Student's *t*-test or two-way ANOVA, with a follow-up Sidak multiple-comparison test (I). ns, non-significant; *, P <0.05; **, P <0.01; ***, P <0.001; ****, P <0.0001. Error bars in B, D, E, F, G, J, L and M represent mean ± SD, and in I represent mean ± SEM.

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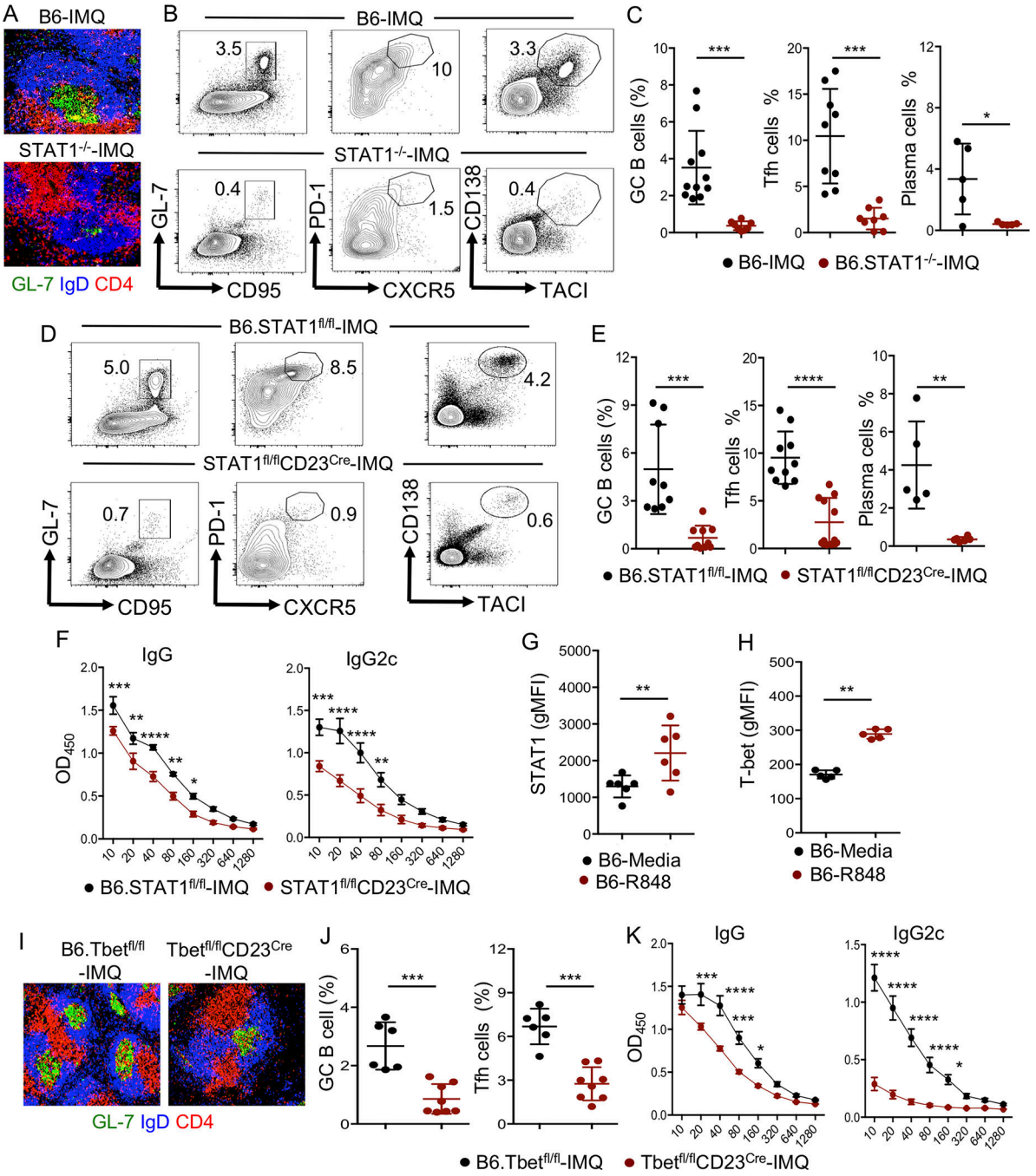

Figure 7. B cell-intrinsic roles of STAT1 and T-bet in TLR7-mediated GC, Tfh and Ab responses.

(A) Representative histological images of splenic GCs in B6 and B6.STAT1$^{-/-}$ mice treated with imiquimod (IMQ) for 8 weeks. Spleen sections were stained for GC B cell marker, GL7 (green), mature B cell marker, IgD (blue), and T cell marker, CD4 (red). (B) Flow cytometry gating strategy and (C) the percentage of GC B cells, Tfh and plasma cells in B6 and B6.STAT1$^{-/-}$ mice are shown. (D, E) B6.STAT1$^{fl/fl}$ control mice and STAT1$^{fl/fl}$CD23^{Cre+} conditional knockout mice were treated with IMQ for 8 weeks before performing similar analyses as in (B, C). (F) Serum IgG and IgG2c Abs from mice described in (D, E) were

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measured by ELISA. STAT1 (G) and T-bet (H) expression in unstimulated (media) and TLR7 stimulated B cells were measured by intracellular staining (ICS) and flow cytometry analysis. (I) Representative histological images of splenic GCs, (J) percentage of GC B cells and Tfh, and (K) total serum IgG and IgG2c Abs in IMQ treated B6.STAT1$^{fl/fl}$ and STAT1$^{fl/fl}$CD23^{Cre+} mice were analyzed as in (A, B, C and F). Statistical analysis was performed by unpaired, nonparametric Mann-Whitney Student's *t*-test (C, E, G-H, J) or two-way ANOVA, with a follow-up Sidak multiple-comparison test (F, K). ns, non-significant; *, P <0.05; **, P <0.01; ***, P <0.001; ****, P <0.0001. Error bars in C, E, G, H and J represent mean ± SD, and in F and K represent mean ± SEM.