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Expression of the Immune Regulator Tripartite-Motif 21 Is Controlled by IFN Regulatory Factors

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Tripartite-motif 21 (TRIM21) is an E3 ubiquitin ligase that regulates innate immune responses by ubiquitinating IFN regulatory factors (IRFs). TRIM21 is mainly found in hematopoietic cells in which its expression is induced by IFNs during viral infections and in systemic autoimmune diseases such as systemic lupus erythematosus and Sjögren’s syndrome. However, the exact molecular mechanism by which the expression of the Trim21 gene is regulated is unknown. In this study, we demonstrate that IFNs induce Trim21 expression in immune cells via IRFs and that IFN-α and IFN-β are the most potent inducers of Trim21. A functional IFN-γ-stimulated response element but no conserved IFN-γ-activated site was detected in the promoter of Trim21. IRF1 and IRF2 strongly induced Trim21 expression in an IFN-stimulated response element–dependent manner, whereas IRF4 and IRF8 strongly repressed the IRF1-mediated induction of Trim21. Consistent with this observation, baseline expression of Trim21 was elevated in Irf4−/− cells. Trim21, IRF1, and IRF2 expression was increased in PBMCs from patients with Sjögren’s syndrome compared with healthy controls. In contrast, IRF4 and IRF8 expression was not increased in PBMCs from patients. The IFN-γ–mediated induction of Trim21 was completely abolished by inhibiting protein synthesis with cycloheximide, and Trim21 expression could not be induced by IFN-γ in Irf1−/− cells, demonstrating that IFN-γ induces Trim21 indirectly via IRF1 and not directly via STAT1 activation. Our data demonstrate that multiple IRFs tightly regulate expression of Trim21 in immune cells, suggesting that a well-controlled expression of the E3 ligase TRIM21 is important for regulation of immune responses. The Journal of Immunology, 2013, 191: 000–000.

Tripartite-motif 21 (TRIM21) is a member of the tripartite-motif protein family characterized by three N-terminal domains: RING, B-box, and coiled-coil (1). The RING domain is critical for the E3 ubiquitin ligase activity of TRIMs (2, 3), whereas the B-box and coiled-coil domains are important for homo- or heteromultimerization (4, 5). Many TRIM proteins are functional IFN-stimulated response element but no conserved IFN-α/β responsive motif 21; TSS, transcription start site.

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The microarray data presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE48378.

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; CHIP-Seq, chromatin immunoprecipitation sequencing; CHX, cycloheximide; GAF, γ-activated factor; GAS, IFN-γ–activated site; IKNAR, IFN-α/β receptor; IFNGR, IFN-γ receptor; IRF, IFN regulatory factor; IRF8DBD, IFN regulatory factor 8 plasmid construct lacking the DNA-binding domain; IgG, IFN-stimulated gene; IgGf3, IFN-stimulated gene factor 3; ISRE, IFN-stimulated response element; p, phospho; qRT-PCR, quantitative RT-PCR; RLU, relative luminescence unit; Trim21, tripartite-motif 21; TSS, transcription start site.

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and STAT2, as well as other STATs (25, 26). Phosphorylated (p) STAT1 and pSTAT2 form a complex with IRF9 (IFN-stimulated gene factor 3 [ISGF3]) that subsequently activates the transcription of IFN-stimulated genes (ISGs) by binding to IFN-stimulated response elements (ISRE) (27–29). Importantly, type I IFN signaling additionally leads to formation of pSTAT1 homodimers, followed by a rapid induction of IRF1 (30, 31), which in turn binds to ISRE sites in the promoters of ISGs (32, 33). IFN-γ signaling also induces the phosphorylation of STAT1 and formation of pSTAT1 homodimers (IFN-γ–activated factor [GAF]) that bind to IFN-γ–activated sites (GAS) in promoters and transactivate IFNγ-dependent genes (34–36). Among these IFN-γ-induced genes is IRF1, which can in turn activate the transcription of ISRE-containing genes and thereby extend the range of genes induced by IFN-γ (32, 33).

To identify transcription factors involved in regulating the expression of Trim21, we used chromatin immunoprecipitation sequencing (ChIP-seq) data and phylogenetic footprinting and identified a conserved ISRE site just upstream of the transcription start site of Trim21. Using luciferase reporter assays, we found that IRF1 and IRF2 both strongly induced Trim21 expression and that IRF4 and IRF8 were potent repressors of Trim21 expression. We verified the physical binding of IRFs to the Trim21 ISRE site by DNA pulldown. The importance of IRFs in controlling Trim21 expression in vivo was confirmed using macrophages and T cells from Irf1+/−, Irf4fl/fl, and Cd4-Cre mice. Furthermore, IRF1 and IRF2, but not IRF4 and IRF8, had an increased expression together with Trim21 in patients with Sjögren’s syndrome and an IFN signature compared with controls. In all, our data provide evidence that the expression of the immune regulatory gene Trim21 is stringently regulated by IRFs.

Materials and Methods

Animals

The generation of C57BL/6-Trim21fl/fl reporter mice has been described elsewhere (9). Irf1+/−, Irf4fl/fl, Irf6fl/fl, and Cd4-Cre mice were purchased from The Jackson Laboratory. The Irf4 and Irf8 alleles were inactivated in T cells by crossing Irf4fl/fl;Cd4-Cre and Irf8fl/fl;Cd4-Cre mice. Furthermore, Irf1 and Irf2, but not Irf4 and Irf8, had an increased expression together with Trim21 in patients with Sjögren’s syndrome and an IFN signature compared with controls. In all, our data provide evidence that the expression of the immune regulatory gene Trim21 is stringently regulated by IRFs.

Plasmids

Trim21 promoter fragments were amplified by PCR using the bacterial artificial chromosome RP23-311N6 as a template (BACPAC Resources Center, Oakland, CA). The PCR products were inserted into pCR-XL-TOP (Invitrogen), sequenced, and transferred to the pGla14 luciferase plasmid (Promega). The coding cDNA sequences of IRFs and PU.1 were amplified by RT-PCR from mouse splenocyte RNA and inserted into pCR-XL-TOP (Invitrogen), sequenced, and transferred to the FLG-CMV6c plasmid (Sigma-Aldrich). pTK- Renilla (Promega) was used as a normalization plasmid for reporter assays. ISRE-mutated luciferase reporter constructs were generated by PCR using long primers containing the mutations. pcDNA3-E47 was obtained from Addgene (plasmid 16059). Plasmid details and primer sequences are available upon request.

Cell culture and IFN stimulations

For analysis of GFP fluorescence in Trim21+/− reporter cells by flow cytometry, splenocytes were collected from C57BL/6-Trim21+/− reporter mice, perfused with 400 U/ml collagenase D (Roche) in HBSS, and incubated for 45 min at 37°C, followed by mechanical dissociation. Single-cell suspensions were obtained after RBC lysis by passing cells through a 70-μm cell strainer. Splenocytes were grown in 24-well plates (5 × 105 cells/well) in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS (Sigma-Aldrich), 2 mM l-glutamine, 100 U/ml penicillin–streptomycin, 20 mM HEPES, and 50 μM 2-ME. The cells were stimulated with 1000 U/ml murine IFN-α/γ (PBL IFN Source), IFN-β (PBL IFN Source), and IFN-γ (Millipore) for 24 h before flow cytometric analysis.

For experiments on the kinetics of Trim21 expression, spleens were harvested from C57BL/6 females after mechanical dissociation. After RBC lysis, single-cell suspensions were obtained by passing cells through a 40-μm cell strainer. Splenocytes were grown in 24-well plates (3 × 105 cells/well) and stimulated with murine IFN-α/γ, IFN-β, or IFN-γ at 500 U/ml for the indicated time points, followed by RNA extraction.

For analysis of dose-dependent effects on Trim21 expression, EL-4 cells were grown in 24-well plates (7.5 × 105 cells/well) and maintained in the same media as the splenocytes. The cells were stimulated with murine IFN-α/γ, IFN-β, and IFN-γ at indicated concentrations for 4 h. For the cycloheximide (CHX) experiments, EL-4 cells were grown in 6-well plates (3 × 105 cells/well) and were pretreated with CHX (Sigma-Aldrich) at 2.5 or 20 μg/ml for 30 min before adding IFNs at 500 U/ml. Cells were harvested after 4 h for RNA extraction.

Bone marrow–derived macrophages (BMDMs) were generated using L929-conditioned DMEM (Sigma-Aldrich) containing 20% 0.20-μm filtered medium from a confluent L929 culture, 10% FCS (Sigma-Aldrich), 2 mM l-glutamine, and 100 U/ml penicillin–streptomycin. After 7 d, the cells were plated in 6-well plates (5 × 105 cells/well) and stimulated the next day with 1000 U/ml IFN-α/γ, IFN-β, or IFN-γ for 6 h, followed by RNA extraction.

293T cells were maintained in DMEM (Sigma-Aldrich) supplemented with 10% FCS (Sigma-Aldrich), 2 mM l-glutamine, and 100 U/ml penicillin–streptomycin and grown in 24-well plates (1.5 × 105 cells/well).

Patients and gene expression analysis

PBMCs from 11 female RoSSA autoantibody-positive and untreated patients with primary Sjögren’s syndrome classified according to the revised European-American consensus criteria and 17 age- and gender-matched healthy controls without any immunological disease were collected. Total RNA was prepared using the Qiagen RNeasy kit. Using the Human exon 1.0 ST chip from Affymetrix, CEL files were preprocessed using the robust multi-array average algorithm as implemented in Affymetrix Power Tools-1.12.0. The IFN score was calculated as described previously (37). Microarray data are deposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) accession number GSE48378. Briefly, using the five top IFN-regulated genes with the lowest p value from the gene expression array FT3T, EPST1, OAS3, PEL1, and IFI6, a standardized expression level of each gene was summarized for each study subject, and a cutoff at 10 was used for defining presence or absence of an IFN signature.

The study was approved by the Regional Ethical Ethics Committee, Stockholm, Karolinska Institute, and all subjects gave informed written consent.

Quantitative RT-PCR

RNA from BMDMs and sorted naïve T cells was isolated using TRizol (Invitrogen) according to the manufacturer’s instructions. To enhance RNA precipitation, 10 μg glycerogen (Fermentas) was added. RNA from other cell types was purified using the RNaseasy Kit (Qiagen). The following primers were used for SYBR quantitative RT-PCR (qRT-PCR): 3′-CTCATGAGCT-ATTATAGCC-3′ (forward) and 5′-GCAATGGTGAGCTGATCTCAG-3′ (reverse); 3′-CTGACCACTGGACAGCAGAC-3′ (forward) and 5′-CCGATGTTCAACGAGGAACTAATAGCC-3′ (reverse); 3′-TGTCCTAGCATCTTCCTC-3′ (reverse) and 5′-CTGATGTCATACTTTGGGCG-3′ (forward) and 5′-TTGTCATACCTCTCAGTGG-3′ (reverse). The ΔΔCT method adjusted for amplification efficiency (Bio-Rad) was used to calculate expression relative to Hprt.

Reporter assays

293T cells were transfected with luciferase reporter plasmid, TK-Renilla normalization plasmid, and an IRF, PU.1, or E47 encoding plasmid using FugeneHD or X-treme Gene (Roche) and incubated for 24 h. The Dual-Luciferase Reporter Assay System (Promega) was used to quantify luminescence from transfected cells using a Modulus Single Tube Reader (Promega).

DNA pulldown

EL-4 cells were stimulated with 1000 U/ml IFN-γ for 6 h, and nuclear extracts were prepared with NE-PER reagent (Ferreme), according to the manufacturer’s instructions using protease and phosphatase inhibitors (Thermo Scientific, CA). Complementary oligonucleotides spanning the Trim21 ISRE site were synthesized by Eurofins MWG Operon. For each oligo pair, the sense oligo was biotinylated on the 5′-end. To generate dsDNA probes, oligos were annealed by incubating for 3 min at 95°C, followed by decreasing
temperature: 95°C → 15°C (ΔC = −1°C/min). The following sequences were as follows: ISRE-oligo sequence (sense), 5′-Biotin-TGCCCTGAGAGGAATTCCACAGCTGAGCAG-3′; mutated ISRE oligo sequence (sense), 5′-Biotin-TGCCCTGAGAGGAATTCCACAGCTGAGCAG-3′; and scrambled oligo sequence (sense), 5′-Biotin-ATAACCCGCAGATCGATACGGAATTTCTGAATTTGAGGAC-3′. For the binding reaction 3 μg biotinylated probe was mixed with 7 μg nuclear extract, 5 μg salmon sperm DNA (Sigma-Aldrich), and 3 μg BSA in a final volume of 15 μl in binding buffer (12% glycerol, 12 mM HEPES, 4 mM Tris pH 8, 60 mM KCl, 1 mM EDTA, and 1 mM DTT). After incubating at room temperature for 15 min, 30 μl streptavidin beads (1 mg/ml) resuspended in 5 μl binding buffer was added (Dynabeads; Invitrogen), and the reaction was further incubated for 10 min. After three washing steps with binding buffer, proteins bound to the probe were eluted by resuspending in 25 μl SDS-PAGE sample buffer containing 2-ME and incubating at 95°C for 5 min.

ChIP-seq

Previously published ChIP-Seq reads for CTCF and RAD21 (38) and H3K4me3, H3K4me2, and H3K4me1 (39) were displayed in the Integrated Genome Browser (40). The median values are shown, and error bars represent range (min-max). The graph is representative of two independent experiments. (Fig. 1A). To verify that the induction of Trim21 expression is indeed induced by IFNs in a dose-dependent manner and that type I IFNs (especially IFN-β) are more potent at inducing Trim21 transcription than IFN-γ (Fig. 1A). To verify that the induction of Trim21 expression by IFNs is not an artifact because of the use of tumor cell lines or extensive culture of primary cells in vitro, we treated freshly isolated mouse splenocytes with IFNs and analyzed Trim21 mRNA levels over time. Both type I and type II IFNs induced Trim21 expression in splenocytes. However, compared with IFN-γ,

Immunoblotting

Proteins were separated on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membranes were blocked in 5% nonfat milk in 0.05% TBS-Tween before incubating with a 1:200 dilution of anti-IRF1 (Santa Cruz Biotechnology) or anti-STAT1 (Santa Cruz Biotechnology) Ab, followed by an anti-rabbit HRP-conjugated Ab (DakoCytomation). For anti-FLAG immunoblotting membranes were blocked in 5% nonfat milk in 0.05% TBS-Tween and incubated with anti-FLAG (Sigma-Aldrich) diluted 1:3000. This was followed by incubation with anti-rabbit HRP-conjugated Ab (DakoCytomation) diluted 1:2,000 and with anti-β-actin-HRP (Sigma Aldrich) diluted 1:50,000.

Flow cytometry and cell sorting

Splenocytes were incubated with mouse Fc block (BD Biosciences) for 5 min at 4°C and then stained for 20 min at 4°C with the following fluorescent Ab combinations: CD8-PE (BD Biosciences), CD4-PerCP, NK.1.1-allophycocyanin (BioLegend), and CD3-allophycocyanin-Cy7; GR1-PE (BD Biosciences), CD11b-PerCP, and F4/80-allophycocyanin (BioLegend); or CD19-PE, B220-PerCP, and CD11c-allophycocyanin (BD Biosciences). The different cell populations were defined as follows: T, CD3+; B, B220+CD19+; NK, CD3−NK1.1−; dendritic cell, CD11c+; M, CD11b+F4/80+; and GR, GR-1+. Ifn1−/−, Ifn4−/−, Ifn8−/−, and wild-type CD4+ T cells were purified from pooled spleen and lymph node cell suspensions using CD4-positive MACS selection (Miltenyi Biotec), and naive CD4+CD62LhighCD44low T cells were subsequently sorted by FACS.

**FIGURE 1.** Trim21 expression is induced by type I and type II IFNs. (A) EL-4 cells were treated with 100, 500, and 1000 U/ml IFNs for 4 h and Trim21 mRNA levels were determined by qRT-PCR and normalized to Hprt. The graph is representative of two independent experiments. (B) Freshly isolated mouse splenocytes from two mice were stimulated with 500 U/ml IFN in triplicates for indicated time points. Trim21 mRNA levels were determined by qRT-PCR and normalized to Hprt. The median values are shown, and error bars represent range (min-max). The graph is representative of two independent experiments. (C) Splenocytes from C57BL/6-Trim21−/− reporter mice were stimulated with 1000 U/ml IFN for 24 h, and the GFP fluorescence was determined by flow cytometry. Graph shows mean fluorescent intensity (MFI) values for GFP from three C57BL/6-Trim21−/− mice.
stimulation with type I IFNs led to a more rapid and higher expression of \( \text{Trim21} \), with the strongest effect observed after IFN-\( \beta \) stimulation (Fig. 1B). The IFNAR has been reported to form a more stable complex with IFN-\( \beta \) compared with IFN-\( \alpha \), which may explain the stronger effect of IFN-\( \beta \) on inducing and maintaining the expression of \( \text{Trim21} \) (43, 44).

IFNs clearly induced the expression of \( \text{Trim21} \) in splenocytes, but it is possible that \( \text{Trim21} \) expression differs between different splenic immune cell populations. To address this issue, we cultured splenocytes from \( \text{Trim21}^{+/GFP} \) reporter mice, and measured the basal expression level and induction of \( \text{Trim21}-\text{GFP} \) expression in response to IFNs by flow cytometry (Fig. 1C). \( \text{Trim21} \) had a low basal expression in B cells and granulocytes compared with T cells, NK cells, macrophages, and dendritic cells. Interestingly, the induction of \( \text{Trim21} \) by IFNs also differed between these cell types, being lowest in granulocytes and B cells.

The \( \text{Trim21} \) promoter contains an ISRE

To identify the molecular mechanisms involved in the regulation of \( \text{Trim21} \) expression, we looked for histone modifications and genomic regions important for regulating the expression of \( \text{Trim21} \). We used ChIP-seq data from mouse pro-B cells to investigate genomic regions important for regulating the expression of \( \text{Trim21} \). The core promoter is just upstream of the predicted TSS (Fig. 2A). This experiment clearly showed that IRF1, IRF2, and IRF8 are able to initiate transcription via binding to the ISRE site in the \( \text{Trim21} \) promoter thus indicating that the induction of \( \text{Trim21} \) expression following IFN stimulation is most likely mediated by IRFs.

\( \text{Trim21} \) expression is induced by IRF1 and IRF2 and repressed by IRF4 and IRF8

To test whether IRFs can initiate \( \text{Trim21} \) transcription via binding to the ISRE site in the \( \text{Trim21} \) promoter, we cotransfected an \( \text{IRF1} \) encoding plasmid with each of the five promoter constructs described in Fig. 3A. This experiment clearly showed that \( \text{IRF1} \) could induce expression only of promoter fragments containing the ISRE (Fig. 3B). Because all members of the IRF family can potentially bind to ISRE sites, we investigated the ability of other IRFs to induce or repress \( \text{Trim21} \) expression using luciferase reporter assays (Fig. 3C). In addition to IRF1, we found that IRF2, but no other IRFs, could transactivate the \( \text{Trim21}-\text{Luc} \) reporter.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** The \( \text{Trim21} \) promoter contains an ISRE. (A) ChIP-seq data from primary naive mouse B cells revealed genomic regions regulating \( \text{Trim21} \) expression. H3K4me1 and H3K4me2 and H3K4me3 histone marks typical of transcriptionally active regions were identified immediately upstream of exon 1 indicating an active promoter. (B and C) The region −3 kb to +0.5 kb (TSS = +1) was analyzed by phylogenetic footprinting and a highly conserved ISRE site was identified in the promoter region of \( \text{Trim21} \). No putative GAS site was identified.
under these conditions. Depending on the cell type, IRF2 can either act as an antagonist of IRF1 or synergize with IRF1 in activating expression of ISRE-dependent promoters (45, 46). In this study, we clearly demonstrate that IRF1 and IRF2 do not counteract each other in transactivating Trim21 expression (Fig. 3D). Notably, the Trim21 promoter contains three repeats of the

![Figure 3](image-url)
GAAA element, whereas previous studies analyzing the simultaneous effect of IRF1 and IRF2 used reporter constructs containing only one or two GAAA elements, suggesting that IRF1 and IRF2 can simultaneously bind separate single GAAA elements of the Trim21 ISRE to facilitate the formation of active transcriptional complexes. By contrast, we observed that IRF4 and IRF8 significantly repressed the basal expression of Trim21 (Fig. 3C). This basal expression of Trim21 is likely due to binding of the ISRE site by endogenously expressed IRF1 and IRF2. IRF4 and IRF8 may thus repress basal expression from the Trim21 promoter by competing with endogenous IRF1 and IRF2 for binding to the ISRE site. To confirm that the different IRF constructs were expressed at a similar level after transfection, we performed immunoblotting (Fig. 3E).

IRF1 and IRF2 activate Trim21 expression via the triple GAAA element in the ISRE site of Trim21

To prove that the identified ISRE in the Trim21 promoter is a bona fide ISRE, we generated Trim21 reporter constructs with mutations in the ISRE sequence (Fig. 4A). Mutations of single GAAA elements did not affect the ability of IRF1 to induce expression (Fig. 4B), suggesting that the different GAAA elements are redundant and that IRF1 has the ability to bind to each of the individual elements. By contrast, mutation of all three GAAA elements of the ISRE abolished IRF1-induced expression (Fig. 4B). All three combinations of two simultaneously mutated GAAA elements still allowed for IRF1-dependent expression from the Trim21 ISRE (data not shown). Similar results were observed for IRF2, demonstrating that the ISRE is a bona fide binding site for IRF1 and IRF2 (Fig. 4C).

To confirm the physical binding of IRF1 to the Trim21 ISRE site, we performed DNA pull-down experiments. We treated EL-4 cells with IFN-γ for 6 h and prepared nuclear extract to enrich for active nuclear IRF1. Equal fractions of the nuclear extract were then incubated with biotinylated probes, containing an intact Trim21 ISRE site, a mutated ISRE site, or a scrambled ISRE site. IRF1 was only pulled down by the intact ISRE probe and not by the mutated or scrambled probes, demonstrating that IRF1 can physically bind the ISRE in the Trim21 promoter (Fig. 4D).

IFN-γ induces Trim21 expression indirectly via IRF1, not directly via STAT1

IFN-γ is a proinflammatory cytokine that induces a large set of genes involved in immune responses, including many TRIM genes (20). IFN-γ signals through the IFN-γ receptor (IFNGR) and induces the formation of pSTAT1 homodimers that enter the nucleus and activate transcription of target genes by binding to GAS sites. Although Trim21 expression was induced by IFN-γ, we could not find any putative GAS site in the Trim21 promoter. We hypothesized that IFN-γ indirectly stimulates Trim21 expression via induction of IRF1 and subsequent binding of IRF1 to the Trim21 ISRE site and not via direct binding of pSTAT1 homodimers to the Trim21 promoter. To test this, we pretreated EL-4 cells with CHX to block protein synthesis, stimulated the cells with type I and type II IFNs, and subsequently analyzed IRF1, STAT1, and Trim21 expression. CHX treatment completely blocked the induction of nuclear IRF1 by type I and type II IFNs but had only a minor effect on the levels of nuclear STAT1 (Fig. 5A). Importantly, addition of CHX dramatically reduced the induction of Trim21 by IFNs, suggesting that optimal induction of Trim21 is
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FIGURE 5. IRF1 is necessary for optimal Trim21 expression. (A) EL-4 cells were pretreated with CHX for 30 min and stimulated by IFNs for 4 h. Nuclear extracts were prepared, and the IRF1 and STAT1 molecules were detected by Western blot analysis. Both the STAT1a (91 kDa) and STAT1b (84 kDa) isoforms were detected. (Two nonspecific bands between 120 and 150 kDa were also detected.) (B) EL-4 cells were pretreated with CHX for 30 min and stimulated by IFNs for 4 h before RNA extraction. Trim21 mRNA levels were determined by qRT-PCR and normalized to Hprt (FC, fold change over non–IFN-stimulated cells). The graph is representative of two independent experiments. (C) Wild-type and Irf3/− cells (CD4+CD62LhighCD44low) T cells were isolated, and Trim21 mRNA levels were determined by qRT-PCR and normalized to Hprt (n = 5). (D) BMDCs from Irf1+/+ and Irf1−/− mice were stimulated with IFNs for 6 h, and Trim21 expression levels were determined by qRT-PCR. One representative experiment of two is shown, n = technical triplicates of individual wells. (E) BMDCs from wild-type mice were stimulated with IFNs for 6 h, and Ifn2 expression levels were determined by qRT-PCR. One representative experiment of two is shown, n = technical triplicates. All statistical calculations were performed with Student t test, and all error bars represent SEM.

Independent of Stat1 but requires the presence of IRF1 (Fig. 5B). To first confirm the critical role of IRF1 in Trim21 basal expression, we sorted naive CD4+ T cells from wild-type and IRF1-deficient mice. Expression of Trim21 was significantly lower in Irf1−/− cells compared with wild-type cells, demonstrating the necessary role for IRF1 in maintaining the basal expression level of Trim21 (Fig. 5C). To subsequently verify that IRF1 is necessary for IFN-γ-induced Trim21 expression, we treated IRF1-deficient BMDCs with IFNs and observed that IFN-γ could not induce Trim21 expression in the absence of IRF1, whereas the induction of Trim21 by IFN-α and IFN-β was almost unchanged (Fig. 5D). Interestingly, IFN-γ, although a potent inducer of IRF1 expression, was unable to induce IRF2 expression, whereas IFN-α and IFN-β strongly induced both IRF1 and IRF2 (Fig. 5A, 5E). In all, these data suggest that Trim21 induction by type II IFN relies critically on IRF1, whereas Trim21 induction by type I IFN is mediated by both IRF1 and IRF2.

IRF4 and IRF8 block the IRF1-mediated induction of Trim21 expression

Because IRF4 and IRF8 could inhibit the basal activity of the Trim21 promoter (Fig. 3C), we asked whether they could also block the IRF1-mediated induction of Trim21. To answer this, we cotransfected 293T cells with IRF1 and IRF4 or IRF8 and measured the induction of the Trim21 luciferase reporter. Strikingly, both IRF4 and IRF8 completely abolished the IRF1-mediated induction of Trim21 expression (Fig. 6A). Because IRF8 can form complexes with IRF1 and PU.1, the possibility remained that IRF8 would be able to induce Trim21 expression when combined with IRF1 in the presence of PU.1. However, cotransfection of IRF8 with Irf1 and Pu.1 could not rescue the IRF8-mediated inhibition of IRF1-induced Trim21 expression (Fig. 6A). Because both IRF4 and IRF8 can form activating complexes with PU.1 and E47 (47), we also evaluated the transcriptional activating capacity of IRF4 and IRF8 in the presence of these two factors. Neither Pu.1 nor E47 could rescue the IRF4- or IRF8-mediated inhibition of Trim21 expression (data not shown), suggesting that IRF4 and IRF8 are bona fide inhibitors of the Trim21 gene. To test whether the repressive effect of IRF8 was due to competitive binding to the ISRE, we generated a mutated IRF8 plasmid construct lacking the DNA-binding domain (IRF8ΔDBD). IRF8ΔDBD was unable to fully antagonize IRF1-mediated induction of Trim21 expression when transfected in a 1:1 ratio with IRF1, strongly suggesting that IRF8 blocks Trim21 expression by binding to the ISRE in the Trim21 promoter (Fig. 6B). To verify the effect of IRF4 and IRF8 on Trim21 expression in primary cells, we quantified Trim21 expression in naive Irf4−/− or Irf8−/− CD4+ T cells by qRT-PCR. Trim21 expression was strongly increased in Irf4−/− T cells compared with wild-type T cells but was unaffected in Irf8−/− T cells (Fig. 6C), suggesting that the low levels of IRF4, but not of IRF8, in naive T cells are sufficient to repress Trim21 expression.

IRF and Trim21 expression in Sjögren’s syndrome

Sjögren’s syndrome is an autoimmune disease with increased levels of type I IFN and overexpression of type I IFN–stimulated
genes, the so-called IFN signature (48–50). Using PBMCs from patients with a verified IFN signature, we found that TRIM21 is indeed expressed at higher levels compared with healthy controls (Fig. 6D). Consistent with the increased TRIM21 expression, both IRF1 and IRF2 were also expressed at significantly higher levels in patients with Sjögren’s syndrome when compared with the matched controls. In contrast, IRF4 and IRF8 expression did not differ between patients and healthy controls. Taken together with our mechanistic data, the data suggest that the increased levels of IRF1 and IRF2 in patients with Sjögren’s syndrome may lead to overexpression of TRIM21 and confirm the relevance of our observations also in the context of a human autoimmune disease.

Discussion
IFNs are critical for an effective immune defense against infections and cancer. However, an uncontrolled production of IFNs can lead to autoimmune disease; therefore, IFN signaling and IFN production is tightly controlled by several factors including suppressor of cytokine signaling 1–3, PIAS1–4, and Src homology region 2 domain-containing phosphatase 2 (PTPN11) reviewed in Ref. 51. TRIM21 is a novel regulator of innate immune responses, acting by ubiquitinating IRF3, IRF5, IRF7, and IRF8, thus controlling the production of, for example, IFN-β and the p40 subunit of IL-12/IL-23 (8, 9, 16). Lack of TRIM21 leads to enhanced production of type I IFN and inflammatory cytokines after TLR stimuli (8, 9). The expression of Trim21 itself is strongly induced by IFNs as a negative feedback loop, but the molecular details of this have so far not been described previously. In this study, we characterize the promoter of Trim21, and identify IRFs as transcription factors that both induce and repress the expression of Trim21. More precisely, we show that a functional ISRE is present in the Trim21 promoter and that IRF1 and IRF2 activate Trim21 transcription, whereas IRF4 and IRF8 repress it.

We initially observed that Trim21 was induced by both type I and type II IFNs, which suggested that Trim21 transcription may be induced not only by IRFs, but also directly by pSTAT1 homodimers that form downstream of both IFNAR and IFNGR signaling (32, 33). However, we observed that Trim21 was not induced by IFN-γ in Ifn1−/− cells, demonstrating that IFN-γ-mediated induction of Trim21 is strictly IRF1 dependent. A molecular basis for this result comes from the observation that there is no GAS element in the Trim21 gene. Furthermore, ectopic expression of either IRF1 or IRF2 strongly induced Trim21 expression, indicating that these factors are individually sufficient to induce Trim21. Interestingly, endogenous IRF2 was induced by type I IFNs but not by IFN-γ, explaining why Trim21 induction by
IFN-γ was completely abolished in Irrf1−/− cells compared with wild-type cells, whereas Trim21 induction by type I IFN was only marginally affected. Furthermore, these data might also explain why type I IFNs induce significantly more expression of Trim21 than IFN-γ does.

Although ectopic expression of IRF1 or IRF2 was sufficient to induce Trim21 expression, we could not detect induction of Trim21 expression after transfection with IRF3, IRF5, IRF7, and IRF9 plasmids. This could be because these factors are dependent on posttranslational modifications or complex formation for their transcriptional activity (52, 53). By contrast, ectopic expression of IRF1 and IRF2 is sufficient for induction of Trim21 because functional binding to ISRE sites by these factors does not require posttranslational modifications (54–56). It remains possible that IRF3, IRF5, IRF7, and IRF9 can activate Trim21 expression after proper posttranslational modifications, for example, phosphorylation of IRF3 by TANK-binding kinase 1 after activation of TLR3 or formation of IISG3 complexes after type I IFN stimulation. Pretreatment with CHX dramatically reduced the induction of Trim21 by IFNs, even though the levels of nuclear STAT1 were almost unchanged, indicating that Trim21 induction is independent of pSTAT1 homodimers and IISG3. In contrast, because of its short half-life, nuclear IRF1 levels were completely ablated by pretreatment with CHX. The very short half-life of IRF1 (30–60 min) compared with IRF2 (~8 h) (57, 58), and the dramatic effect of CHX on Trim21 expression, indicates that IRF1 is the most important factor for Trim21 induction after stimulation with IFNs.

IRF4 is a transcription factor important mainly in the adaptive immune system where it regulates the activation and differentiation of B and T cells (59). IRF4 is induced by T cell activation via TCR signaling, and the dramatic effects of IRF4 on Trim21 expression suggests that Trim21 plays a role in T cell biology as previously suggested by Ishii et al. (60). The higher expression level of IRF4 in B cells compared with T cells (http://www.immmgen.org/data-browse/index.html) could explain the lower basal expression and weaker induction of Trim21 in B cells following IFN stimulation, because IRF4 can repress both the basal and IRF1-induced expression of Trim21. It is also possible that the low induction of Trim21 in B cells and granulocytes could be due to cell-specific epigenetic modifications (e.g., H3K9me2 histone modifications) in the Trim21 locus in these two cell types (61).

Expression analysis in patients with Sjögren’s syndrome and an IFN signature verified increased expression of Trim21, IRF1, and IRF2. In contrast to IRF1 and IRF2, the expression of IRF4 and IRF8 was not increased in PBMCs of patients with Sjögren’s syndrome. IRF1 and 2 are induced by type I IFNs but IRF4 and 8 are not, and it is therefore possible that IRF1 and 2 increase TRIM21 expression in response to type I IFN in systemic autoimmune disease, whereas IRF4 and 8, which are induced by other factors, repress TRIM21 expression in other situations, for example in cells stimulated with Ag or pathogen-associated-molecular patterns. Our data also confirm Trim21 as part of the IFN-signature, which relates to disease activity in systemic autoimmune.

It has previously been suggested that ISGs with ISRE sites containing multiple GAAA elements are regulated differently than ISGs with ISRE sites containing only one or two GAAA elements (62). Indeed, as shown by our mutational analysis of the Trim21 ISRE site that contains three GAAA elements, the destruction of only one or two GAAA elements still leaves at least one GAAA element available for IRF binding and allows for induction of Trim21 expression. Cotransfection with IRF1 and IRF4 in a 1:1 ratio resulted in a complete loss of IRF1-mediated Trim21 induction. This suggests that IRF4 has a stronger affinity than IRF1 for the GAAA elements and that IRF4 therefore can outcompete IRF1 in binding to the ISRE site of the Trim21 promoter when present at a 1:1 ratio. However, IFN stimulation with increasing levels of IRF1 leads to release of the repression and induction of Trim21 expression. Genes containing ISRE sites with triple GAAA elements, or alternatively tandem ISRE sites with double GAAA elements (e.g., Isg15, Oasl, Mx1, Ifi1, and Trim21) are more strongly induced by IFNs than genes containing ISRE sites with only one or two GAAA elements (63). We suggest that for strongly induced ISGs with multiple GAAA elements the negative regulation by IRF4 and IRF8 may play an important role in controlling their induction and the subsequent IFN response. This might be achieved for example by a higher affinity of IRF4 and IRF8 for ISRE sites in such ISGs, leading to an efficient block of ISG expression. Thus, multiple GAAA elements may not be an important feature for strong induction during immune responses, but also for tight regulation of IFN dependent genes by IRFs.

In all, the data presented in this study show for the first time, to our knowledge, that Trim21, an E3 ubiquitin ligase involved in the control of IRF-mediated immune responses, is itself regulated by IRFs at the transcriptional level. The induction of Trim21 expression observed in type I IFN responses, for example, during antiviral defense and systemic autoimmune diseases, is mainly mediated by IRF1 and IRF2. In contrast, induction of IRF4 and IRF8 is a mechanism for turning Trim21 expression off such that effector and memory B and T cells are generated effectively. Taken together, these data help us mechanistically connect the elevated expression of Trim21 via IRF1 and IRF2 with the IFN signature observed in systemic autoimmune diseases.

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