TRIM21 controls Toll-like receptor 2 responses in bone-marrow-derived macrophages

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Summary
TRIM21 is an interferon-stimulated E3 ligase that controls the activity of pattern-recognition signaling via ubiquitination of interferon regulatory factors and DDX41. Previous studies on the role of TRIM21 in innate immune responses have yielded contradictory results, suggesting that the role of TRIM21 is cell specific. Here, we report that bone-marrow-derived macrophages (BMDMs) generated from Trim21−/− mice have reduced expression of mature macrophage markers. Reflecting their reduced differentiation in response to macrophage colony-stimulating factor (M-CSF), Trim21−/− BMDMs had decreased expression of M-CSF signature genes. Although Trim21−/− BMDMs responded normally to Toll-like receptor 9 (TLR9) activation, they produced lower levels of pro-inflammatory cytokines in response to the TLR2 agonist PAM3CSK4. In line with this, the response to infection with the Bacillus Calmette–Guérin strain of Mycobacterium bovis was also diminished in Trim21−/− BMDMs. Our results indicate that TRIM21 controls responses to TLR2 agonists.

Keywords: macrophage; Toll-like receptor 2; TRIM21.

Introduction

Innate immune responses are under firm control by a large number of negative and positive regulators, enabling strong responses against pathogens while at the same time minimizing tissue damage and promoting timely resolution. A group of proteins involved in regulating innate immune responses is the Tripartite-motif (TRIM) family, comprising > 70 members. Many TRIM proteins are E3 ligases that control pattern-recognition receptor responses via ubiquitination-dependent degradation or activation of signaling mediators. TRIM21 is an interferon (IFN) -stimulated E3 ligase that post-translationally modifies IFN regulatory factor 3 (IRF3), IRF5, IRF7, IRF8 and DDX41 with ubiquitin. Trim21−/− mice develop strong T helper type 17-mediated contact hypersensitivity responses and have enhanced type I IFN responses against DNA viruses resulting in reduced viral titers. TRIM21 has also been shown to be important for IgG-mediated antiviral responses. Intriguingly, several studies on the role of TRIM21 in innate immune responses have yielded contradictory results. This may be due to the use of different cell types, different protocols for triggering of innate immune signaling, or to differences between the

Abbreviations: APC, allophycocyanin; BCG, Bacillus Calmette–Guérin; BMDM, bone marrow-derived macrophage; ERK1/2, extracellular signal-regulated kinase 1/2; FCS, fetal calf serum; HRP, horseradish peroxidase; IFN, interferon; IL-6, interleukin-6; IRF3, interferon regulatory factor 3; M-CSF, macrophage colony-stimulating factor; PBS, phosphate-buffered saline; TLR, Toll-like receptor; TRIM, Tripartite-motif
two commonly used Trim21−/− strains.11 Trim21−/− mouse embryonic fibroblasts have strongly increased responses to poly(I:C) and lipopolysaccharide, whereas Trim21−/− bone-marrow-derived macrophages (BMDMs) do not demonstrate enhanced reactivity to the same Toll-like receptor (TLR) ligands.11 This suggests that the phenotype of Trim21−/− BMDMs is masked by redundancy or compensatory mechanisms, or that TRIM21 is necessary for proper differentiation of BMDMs.11 To better understand the role of TRIM21 in BMDMs, we have compared gene expression profiles and TLR responses between Trim21−/− and wild-type BMDMs.

Material and methods

Cell culture

We isolated bone marrow cells from Trim21−/−− and wild-type mice on the C57BL/6 background, followed by erythrocyte lysis and differentiation into macrophages. For time–course experiments with PAM3CSK4 and Mycobacterium bovis Bacillus Calmette–Guérin (BCG), we used complete Dulbecco’s modified Eagle’s medium supplemented with supernatant from L929 cell cultures as previously described.14 In other quantitative RT-PCR (qRT-PCR) experiments, and in ELISA and immunoblotting experiments, BMDMs were instead generated using 20 ng/ml recombinant mouse macrophage colony-stimulating factor (M-CSF) (R&D Systems, Minneapolis, MN) in complete Dulbecco’s modified Eagle’s medium. The study was approved by the Ethical Review Committee North, Stockholm County.

Microarray

BMDMs were harvested in cold phosphate-buffered saline (PBS) and lyzed in RLT buffer (Qiagen, Hilden, Germany). RNA isolation and quality control were performed at the Bioinformatics and Expression Analysis (BEA) facility at Karolinska Institutet, followed by standard protocol for hybridization to Mouse Gene Chip 1.0 ST (Affymetrix, Santa Clara, CA). CEL files from microarrays were preprocessed and normalized with robust multi-array average using the R package ‘oligo’,15,16 Expression data were filtered using the R package ‘genefilter’, significance analysis of microarrays was performed using the R package ‘siggenes’, and volcano plots were generated using the R packages ‘ggplot2’, ‘ggrepel’ and ‘gghighlight’. Heat maps for microarray data were generated using the Multi Experiment Viewer (MeV) software.17

Quantitative RT-PCR

Total RNA was isolated using TRIzol followed by reversed transcription using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). The qRT-PCR was performed using the qSYBR Green Supermix (Bio-Rad) or TaqMan assays (ThermoFisher, Waltham, MA). For SYBR based qRT-PCR, we used the following primers: Nos2-F CA GCTGGGCTGTACAACTT, Nos2-R CATIGGAAATG AACCGGTTCG, Il12b-F TCACTAGGGACATCTCACC, Il12b-R GTGCTCCAGGATCGCTACT, Il6-F GAGGATACCCTCCCCAGACCC, Il6-R AAGTGGCATTAC- TCTGTTGTCTACATA, and Hprt-F CTCACTGGACTGA TTAGGCACAGGC, Hprt-R GAGGTCTGCAAGAAGAC TTATAGCC. For quantification of Trim21, we used a TaqMan assay (Mm00447364_m1) targeting Trim21 exons that are deleted in the Trim21−/− mice (exons 5 and 6), and normalized the expression to Hprt (Mm01545399_m1) (ThermoFisher Scientific).

TLR stimulation experiments

To determine the expression genes by qRT-PCR, 2 × 10^6 BMDMs per well were seeded in triplicates for each time-point. Cells were either infected with M. bovis BCG at a multiplicity of infection of 5, or stimulated with 0–1 µg/ml PAM3CSK4 (Invivogen, San Diego, CA), 1 µg/ml poly(I:C) (Invivogen) or 1 µg/ml CpG-ODN M362 (Alexis Biochemicals, San Diego, CA) with 100 U/ml IFN-γ (R&D Systems). Cells were lysed in TRIZol after 3, 6, 24 and 48 hr, and kept at −80°C until total RNA isolation followed by qRT-PCR. To detect secreted cytokines, 1 × 10^6 BMDMs were seeded in 48-well plates and stimulated with 0–1 µg/ml PAM3CSK4 (Invivogen, San Diego, CA) for 24 hr. Supernatants were collected and assayed for interleukin–6 (IL–6) and IL–12–p40 using the Mouse IL–12 p40 NonAllele-specific Quantikine ELISA or Mouse IL–6 NonAllele-specific Quantikine ELISA kits (R&D Systems, Minneapolis, MN).

Gene-set enrichment analysis

Gene-set enrichment analysis was performed using the GenePattern module (Broad Institute, Cambridge, MA) and visualized using the replotGSEA script in R.18 Gene sets were downloaded from the Molecular Signature Database v5.2 (Broad Institute, Cambridge, MA). We used the following gene signatures for gene-set enrichment analysis: GSE5099_UNSTIM_VS_MCSF_TREATED_MONOCYTE_DAY7_UP (M-CSF signature), GSE17721_CTRL_VS_PAM3CSK4_6H_BMDC_UP (PAM3CSK signature) and GSE22935_UNSTIM_VS_12H_MBOVIS_BCG_STIM_MACROPHAGE_UP (BCG signature).

Flow cytometry

For isolation of splenic dendritic cells and macrophages, mouse spleens were perfused with 400 U/ml of collagenase D (Roche, Basel, Switzerland) in Hanks’ balanced salt solution and incubated for 45 min at 37°C followed by
mechanical dissociation. Splenocytes were first incubated with anti-CD16/32 (Fc-block) (Biolegend, San Diego, CA) in PBS (1 mM EDTA, 2% fetal calf serum (FCS)) at 4°C for 15 min, and were then stained with anti-CD11c-allophycoycyanin (APC) (BD Biosciences, San Jose, CA) or anti-F4/80-APC (BD Biosciences, San Jose, CA) at 4°C in PBS with 1 mM EDTA, 2% FCS. The BMDMs were first incubated with anti-CD16/32 (Fc-block) (Biolegend, San Diego, CA) in PBS (1 mM EDTA, 2% FCS) at 4°C for 15 min, and were then stained with anti-CD11c-allophycoycyanin (APC) (BD Biosciences, San Jose, CA) or anti-F4/80-APC (BD Biosciences, San Jose, CA) at 4°C in PBS with 1 mM EDTA, 2% FCS. The BMDMs were first incubated with anti-CD16/32 (Fc-block) (Biolegend, San Diego, CA) in PBS (1 mM EDTA, 2% FCS) at 4°C for 15 min. Cells were then stained with the following panel for 30 min at 4°C in PBS (1 mM EDTA, 2% FCS): TLR2-APC (Biolegend, San Diego, CA), CD206-phycocerythrin/Cy7 (Biolegend, San Diego, CA), CD38-BV510 (BD Biosciences, San Jose, CA) and F4/80-APC/Cy7 (Biolegend, San Diego, CA). After washing twice, the cells were acquired using a Gallios flow cytometer (Beckman Coulter, Brea, CA) followed by data analysis using FLOWJO v10 (FlowJo, Ashland, OR).

Immunoblotting

Cell lysates for immunoblotting were prepared using CellLytic M (Sigma Aldrich, St Louis, MO) supplemented with the Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific). Proteins were separated using 4%–20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad). This was followed by the transfer of proteins to Amersham Hybond polyvinylidene fluoride membranes (GE Healthcare, Chalfont St Giles, UK), and blocking of membranes in 5% non-fat milk in 0.1% Tween–TBS for 1 hr. For immunoblotting, we used the following antibodies: anti-extracellular signal-regulated kinase 1/2 (anti-ERK1/2; #9102; Cell Signaling Technologies, Danvers, MA), anti-phospho-ERK1/2 (#9106; Cell Signaling Technologies). The following secondary antibodies were used: anti-mouse IgG-horseradish peroxidase (HRP) (#7076; Cell Signaling Technologies), and anti-rabbit IgG-HRP (#7074S; Cell Signaling Technologies). The binding of HRP-conjugated antibodies was visualized using Clarity Western ECL Substrate (Bio-Rad). All antibodies were used at concentrations recommended by the manufacturers. Quantification of bands was performed using ImageJ (National Institutes of Health, Bethesda, MA).

Results

Expression of Trim21 in macrophages and dendritic cells

To verify that Trim21 is expressed in macrophages and dendritic cells, we used the EGFP reporter inserted into the Trim21 locus. We used heterozygous Trim21+/− mice in flow cytometry and could demonstrate that splenic dendritic cells (CD11c+) and splenic macrophages (F4/80+) both express Trim21 (Fig. 1a,b). To verify that BMDMs generated in vitro also express Trim21, we analyzed EGFP fluorescence by flow cytometry and found that Trim21+/− BMDMs were EGFP positive (Fig. 1c). To validate the expression of Trim21 with an additional method, we used qRT-PCR to quantify Trim21 expression in BMDMs and in splenic macrophages (Fig. 1d). By analyzing the expression of Trim21 in mononuclear myeloid cells using a public RNA-seq data set (GSE122108), we found that the highest expression of Trim21 is in yolk sac macrophages (data not shown).

Reduced maturation of Trim21+/− BMDMs in response to M-CSF

Members of the IRF transcription factor family are important for proper differentiation and polarization of macrophages.19-22 As TRIM21 is an E3 ubiquitin ligase for several IRFs, we hypothesized that the maturation of Trim21+/− BMDMs might be affected. We performed microarray experiments (GeneChip Mouse Gene 1·0 ST Array) to identify changes in gene expression between wild-type and Trim21+/− BMDMs. The top up-regulated and down-regulated genes are displayed in heat maps (Fig. 2a), and as a volcano plot (Fig. 2b). Analyzing the gene expression data, we found that Trim21+/− BMDMs had an altered expression of genes typical of mature and undifferentiated (immature) macrophages (Fig. 2c). This included reduced expression of Cd38 (cyclic ADP ribose hydrolase) and the mannose receptor Mrc1 (MRC1/Cd206), and increased expression of immature genes such as Cds34, Nanos1 and Id1. However, we did not observe any differences in morphology and cell numbers when comparing the differentiation of wild-type and Trim21+/− BMDMs (data not shown). Since minor changes in mRNA levels do not always translate into changes in protein levels, we tried to verify these observations on the protein level using flow cytometry. We could only verify a reduced surface expression of MRC1 in Trim21+/− BMDMs (Fig. 2d,e), and not for, for example, Cd38 (data not shown). We found a reduced M-CSF signature in Trim21+/− BMDMs (Fig. 2f), suggesting that Trim21+/− bone marrow myeloid progenitors have a reduced response to M-CSF and therefore do not differentiate efficiently to BMDMs in vitro. To test whether Trim21+/− BMDMs retained the ability to mature, we treated BMDMs with CpG-ODN and IFN-γ for 24 hr. The expression of macrophage maturation markers was similar between wild-type and Trim21+/− BMDMs after TLR9 activation, suggesting that Trim21+/− BMDMs matured normally in response to CpG-ODN (Fig. 2g). Also, treating Trim21+/− BMDMs with CpG-ODN and IFN-γ led to reduced expression of immature markers (Fig. 2h). In all, these results indicate that Trim21+/− BMDMs have a slightly reduced response to M-CSF.

Trim21+/− BMDMs are hypo-responsive to stimulation with the TLR2 ligand PAM3CSK4

To identify altered TLR signaling pathways in Trim21+/− BMDMs, we compared tonic TLR signaling between Trim21+/− and wild-type BMDMs
Trim21−/− and wild-type BMDMs. Tonic TLR signaling occurs as the result of bacterial contaminants in serum, as previously reported.23–25 By comparing the expression of TLR-induced genes at baseline, we discovered a reduced TLR2-activation (PAM3CSK4) gene signature in Trim21−/− BMDMs (Fig. 3a). This suggested that TRIM21 is necessary for optimal responses to TLR2 agonists. To verify this, we generated Trim21−/− and wild-type BMDMs and stimulated them with the synthetic TLR2 agonist PAM3CSK4 for 6 hr. Indeed, the induction of Il6 (IL6) and Nos2 (iNOS) was reduced in Trim21−/− BMDMs after stimulation with PAM3CSK4 (Fig. 3b). We then generated new Trim21−/− and wild-type BMDMs and stimulated them with PAM3CSK4 for between 3 and 48 hr and found a clearly reduced response in Trim21−/− BMDMs at all time-points (Fig. 3c). The secretion of IL-6 and IL-12-p40 was also diminished in stimulated Trim21−/− BMDMs as determined by ELISA (Fig. 3d). Furthermore, the ratio of phosphorylated ERK1/2 (p-ERK1/2) to total ERK1/2 was reduced in stimulated Trim21−/− BMDMs (Fig. 3e). The reduced response to TLR2 activation was not to the result of lower expression of TLR2 at the cell surface (Fig. 3f,g). In contrast, the TLR3 agonist poly(I:C) induced similar expression of

Figure 1. Trim21 expression in macrophages and dendritic cells. Trim21 expression was determined by EGFP fluorescence in splenocytes from wild-type (WT) and Trim21−/− mice stained for CD11c (a) and F4/80 (b). Trim21 expression was determined by EGFP fluorescence in WT and Trim21−/− bone-marrow-derived macrophages (BMDMs) (c). The expression of Trim21 in BMDMs from Trim21−−/− (knockout, KO) and WT mice, and in WT splenic macrophages (F4/80+), was determined by quantitative RT-PCR (d).

Figure 2. Reduced expression of macrophage maturation genes in Trim21−/− bone-marrow-derived macrophages (BMDMs). Heat map of top up-regulated and down-regulated genes in Trim21−/− (knockout; KO) BMDMs (a). Volcano plot of gene expression in Trim21−/− BMDMs versus wild-type (WT) BMDMs (b). Expression of macrophage progenitor and maturation genes in unstimulated WT and Trim21−/− BMDMs (c). Surface expression of MRC1 (CD206) on BMDMs was determined by flow cytometry (n = 5) (d, e). Gene-set enrichment analysis of macrophage colony-stimulating factor (M-CSF) signature genes in unstimulated WT and Trim21−/− BMDMs (f). Volcano plot of gene expression in stimulated Trim21−/− BMDMs versus stimulated WT BMDMs [CpG + interferon-γ (IFN-γ), 24 hr] (g). Expression of macrophage progenitor and maturation genes in WT and Trim21−/− BMDMs stimulated with CpG + IFN-γ for 24 hr (h). Heat maps display individual expression values as z-scores. Gene-set enrichment was calculated as a normalized enrichment score. Statistical testing for flow cytometry data was performed using two-sided Mann–Whitney test (*P < 0.05). Multiple testing was corrected using false-discovery rate calculated based on gene-set permutations. © 2019 The Authors. Immunology published by John Wiley & Sons Ltd., Immunology, 159, 335–343
Nos2 and Il6 in wild-type and Trim21−/− BMDMs (Fig. 3h). The decreased response of Trim21−/− BMDMs in response to PAM3CSK4 was of a similar magnitude to what have been reported after knockdown of other E3 ligases.26,27 In all, these data indicate that Trim21−/− BMDMs have a deficient response to TLR2 agonists.
Trim21−/− BMDMs are hyporesponsive to stimulation with BCG

PAM3CSK4 is a synthetic triacylated lipopeptide that mainly activates TLR2; therefore, we asked whether TRIM21 was also necessary for full responses to more complex stimuli. To this end, we compared the responses of Trim21−/− and wild-type BMDMs infected with M. bovis BCG. By comparing the expression of BCG-induced genes at baseline, we discovered a reduced BCG-activation gene signature in Trim21−/− BMDMs (Fig. 4a). Trim21−/− BMDMs infected with BCG showed reduced expression of Il12b, Il6 and Nos2 transcripts compared with wild-type BMDMs (Fig. 4b-d).

Discussion

The role of TRIM21 in innate immune responses is complex and depends on cell type and the nature of the stimulatory signal. Several studies on the cellular function of TRIM21 have been performed in immortalized cell lines, warranting further studies using primary immune cells. Here we investigated the role of TRIM21 in mouse BMDMs and found that Trim21−/− BMDMs have a reduced response to M-CSF. Furthermore, Trim21−/− BMDMs were hyporesponsive to TLR2 stimulation by both the synthetic ligand PAM3CSK4 and M. bovis BCG, indicating that TRIM21 also plays an important role in response to more complex stimuli. Our results fit well
with data from Foltz et al., who identified TRIM21 as a critical factor in the defense against the obligate intracellular parasite Toxoplasma gondii. Since both M. bovis BCG and T. gondii activate TLR2 signaling, it is possible that TRIM21 is an important factor for the defense against intracellular parasites that trigger TLR2 signaling.

The exact mechanism by which TRIM21 controls TLR2 signaling remains unclear. The E3 ligase activity of TRIM21 might enhance TLR2 signaling by promoting the activity of TLR2 signaling mediators, or by degrading negative regulators of TLR2 signaling. Interestingly, a recent study identified TRIM21 as a negative regulator of TLR2 signaling. This is expected, because E3 ubiquitin ligases are modulators of signaling rather than necessary components of signaling pathways. Importantly, previous reports have also found that TRIM21 activates TAK1, demonstrating that TRIM21 can directly control TLR signaling. There are no added antibodies in our experimental setup, demonstrating that the observed effect of TRIM21 deficiency is antibody independent.

In all, our results indicate that TRIM21 is involved in regulating innate immune responses against TLR2 agonist including lipoproteins, peptidoglycans and lipoteichoic acids. The role of TRIM21 in TLR2 responses in vivo, and the exact molecular mechanism, remain to be investigated.

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Author contributions
MS designed experiments and performed cell stimulations and gene expression analysis with Affymetrix microarray and quantitative RT-PCR; BC designed experiments, and performed cell stimulations and infections with BCG; WN performed quantitative RT-PCR analysis and immunoblotting on BMDMs. RC performed analysis of gene expression data; MR designed experiments; AE designed experiments and wrote the manuscript.

Disclosure
The authors have no conflicts of interest.

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