


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

Maria Sjöstrand,^{1†} Berit Carow,²
William A. Nyberg,¹ Ruxandra
Covacu,³ Martin E. Rottenberg²
and Alexander Espinosa¹ 

¹Unit of Rheumatology, Center for Molecular Medicine L8:03, Department of Medicine, Karolinska Institutet, Karolinska University Hospital, Stockholm, ²Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, ³Unit of Neuroimmunology, Department of Clinical Neuroscience, Center for Molecular Medicine, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden

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[†]Center for Cell Engineering and Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA. Correspondence: Alexander Espinosa, Unit of Rheumatology, Department of Medicine, Center for Molecular Medicine, CMM L8:03, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden. Email: alexander.espinosa@ki.se Senior author: Alexander Espinosa

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.¹ A group of proteins involved in regulating innate immune responses is the Tripartite-motif (TRIM) family, comprising > 70 members.² Many TRIM proteins are E3 ligases that control pattern-recognition receptor responses via ubiquitination-dependent degradation or activation of signaling mediators.^{3,4} TRIM21 is an interferon

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.

(IFN) -stimulated E3 ligase that post-translationally modifies IFN regulatory factor 3 (IRF3), IRF5, IRF7, IRF8 and DDX41 with ubiquitin.^{5,6} *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.^{7,8} TRIM21 has also been shown to be important for IgG-mediated antiviral responses.⁹ Intriguingly, several studies on the role of TRIM21 in innate immune responses have yielded contradictory results.^{7–13} 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*^{-/-7} and wild-type mice on the C57BL/6J 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 lysed 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 iQ SYBR Green Supermix (Bio-Rad) or TaqMan assays (ThermoFisher, Waltham, MA). For SYBR based qRT-PCR, we used the following primers: *Nos2*-F CA GCTGGGCTGTACAAACCTT, *Nos2*-R CATTGGAAGTG AAGCGTTTCG, *Il12b*-F TCATCAGGGACATCATCAA ACC, *Il12b*-R GTGCTCCAGGAGTCAGGGTACT, *Il6*-F GAGGATACCACTCCCAACAGACC, *Il6*-R AAGTGCAT C-ATCGTTGTTTCATACA, and *Hprt*-F CTCATGGACTGA TTATGGACAGGAC, *Hprt*-R GCAGGTCAGCAAAGAAC 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° until total RNA isolation followed by qRT-PCR. To detect secreted cytokines, 1×10^5 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° 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° for 15 min, and were then stained with anti-CD11c-allophycocyanin (APC) (BD Biosciences, San Jose, CA) or anti-F4/80-APC (BD Biosciences, San Jose, CA) at 4° 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° for 15 min. Cells were then stained with the following panel for 30 min at 4° in PBS (1 mM EDTA, 2% FCS): TLR2-APC (Biolegend, San Diego, CA), CD206-phycoerythrin/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 *Cd34*, *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

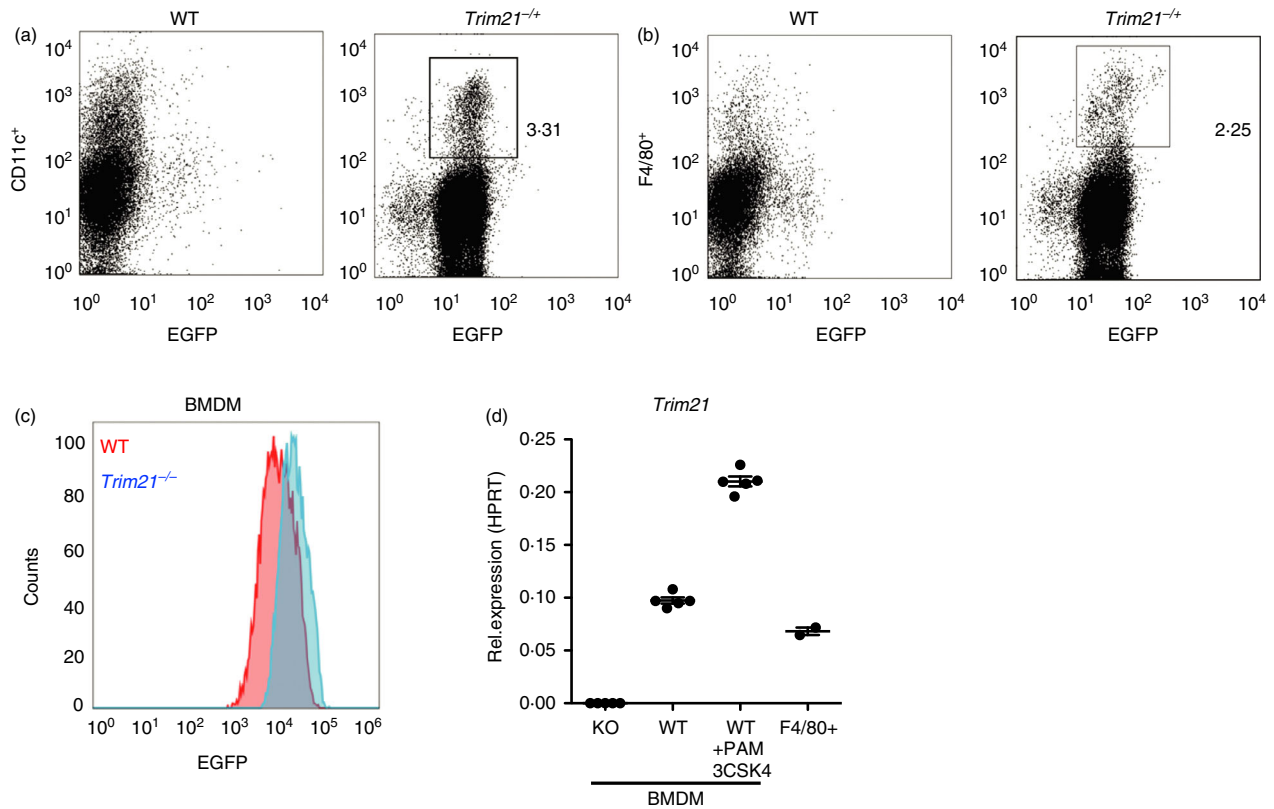
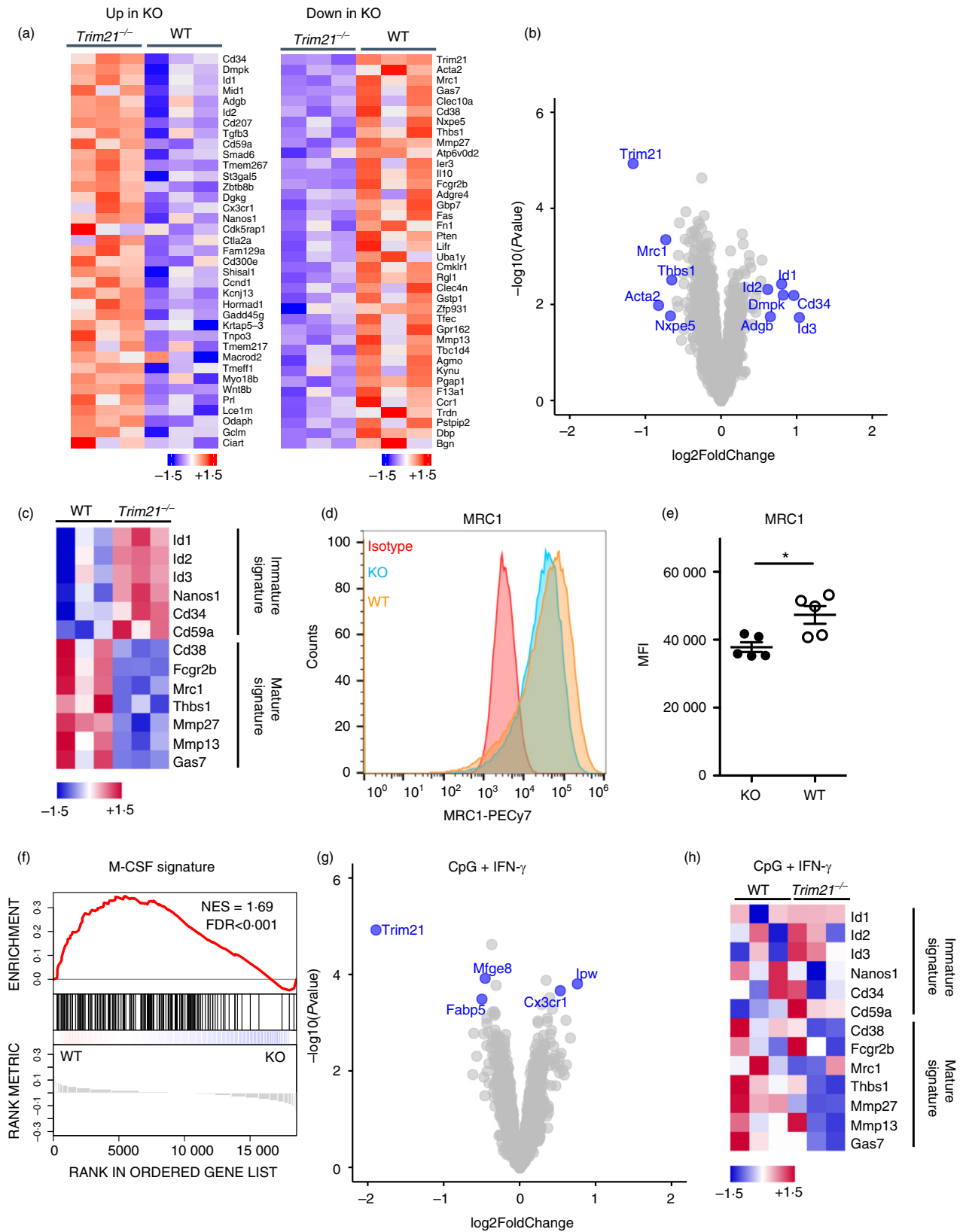


Figure 1. *Trim21* expression in macrophages and dendritic cells. *Trim21* expression was determined by EGFP fluorescence in splenocytes from wild-type (WT) and *Trim21*^{-/-} mice stained for CD11c (a) and F4/80 (b). *Trim21* expression was determined by EGFP fluorescence in WT and *Trim21*^{-/-} bone-marrow-derived 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).

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 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 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.



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.

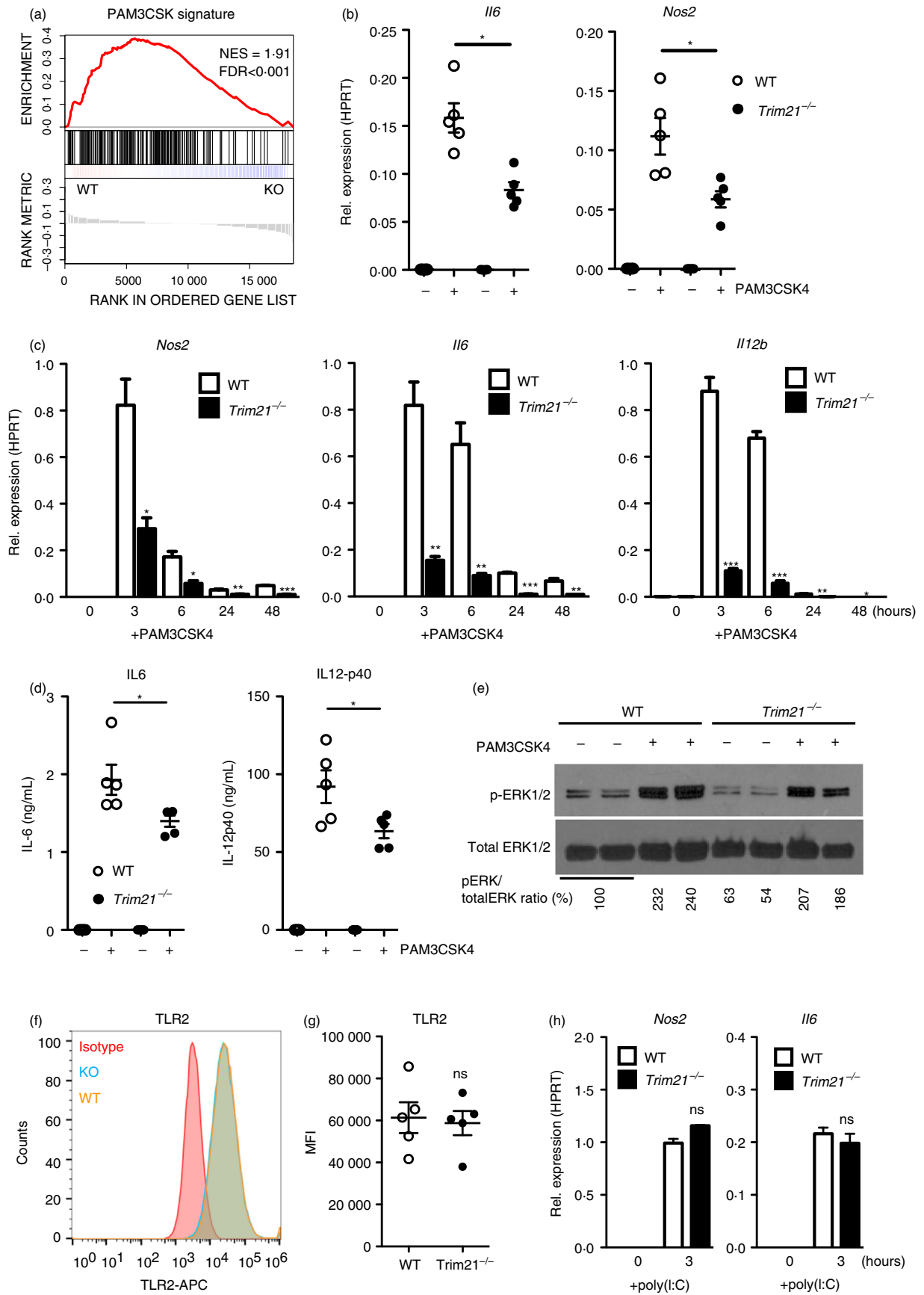


Figure 3. *Trim21*^{-/-} bone-marrow-derived macrophages (BMDMs) are hyporesponsive to stimulation with PAM3CSK4. Gene-set enrichment analysis of Toll-like receptor 2 (TLR2) -activation (PAM3CSK4) signature genes in *Trim21*^{-/-} (knockout; KO) and wild-type (WT) BMDMs at baseline (a). *Trim21*^{-/-} and WT BMDMs were stimulated with 0.1 µg/ml PAM3CSK4 for 6 hr followed by quantitative RT-PCR against *Nos2* (iNOS) and *Il6* (IL-6) (*n* = 5) (b). *Trim21*^{-/-} and WT BMDMs were stimulated with 0.1 µg/ml PAM3CSK4 for 3–48 hr followed by quantitative RT-PCR against *Nos2* (iNOS), *Il6* (IL-6) and *Il12b* (IL-12-p40) (*n* = 3) (c). *Trim21*^{-/-} and WT BMDMs were stimulated with 0.1 µg/ml PAM3CSK4 for 24 hr, followed by ELISA to measure secreted IL-6 and IL-12-p40 protein (*n* = 5) (d). Immunoblotting against phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 in BMDMs stimulated with 0.1 µg/ml PAM3CSK4 for 6 hr (e). *Trim21*^{-/-} and WT BMDMs were stimulated with 1.0 µg/ml poly(I:C) for 3 hr followed by quantitative RT-PCR against *Nos2* and *Il6* (*n* = 3) (f). Surface expression of TLR2 on BMDMs was determined by flow cytometry (*n* = 5) (g, h). Statistics for gene expression data were calculated using the Student's *t*-test, and statistical testing for flow cytometry data was performed using two-sided Mann–Whitney test (**P* < 0.05, ***P* < 0.01, ****P* < 0.005). The experiment was performed three times with similar results. Gene-set enrichment was calculated as a normalized enrichment score. Multiple testing was corrected using false-discovery rate calculated based on gene-set permutations.

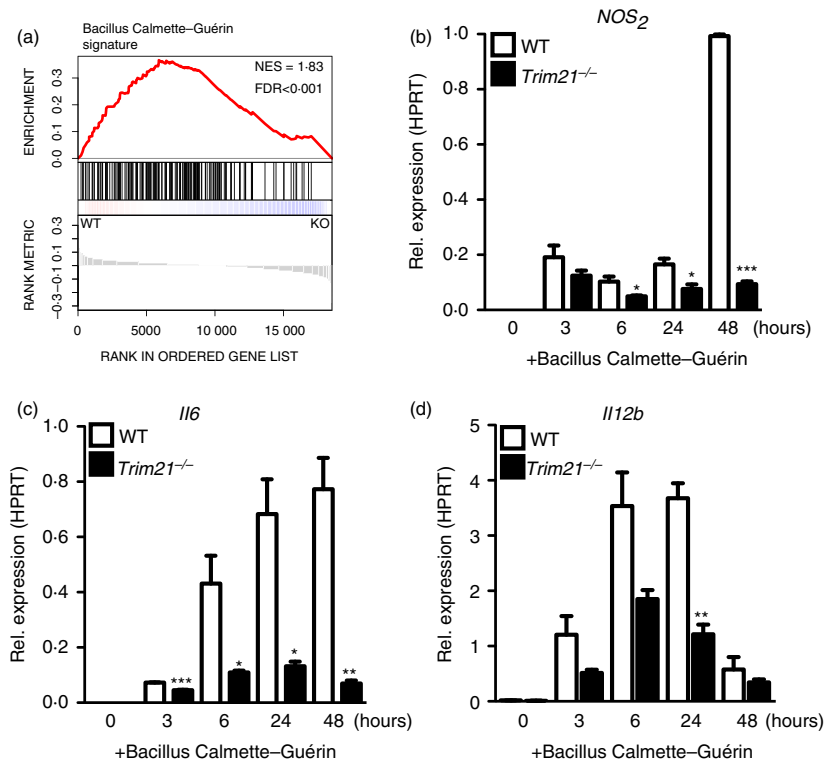
Trim21^{-/-} BMDMs are hyporesponsive to stimulation with BCG

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

Figure 4. *Trim21*^{-/-} bone-marrow-derived macrophages (BMDMs) are hyporesponsive to *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG). Gene-set enrichment analysis of BCG-activation signature genes in *Trim21*^{-/-} and wild-type (WT) BMDMs at baseline (a). *Trim21*^{-/-} and WT BMDMs were stimulated with *M. bovis* BCG (multiplicity of infection = 5) for 3–48 hr followed by quantitative RT-PCR against *Nos2* (iNOS), *Il6* (IL-6) and *Il12b* (IL-12-p40) (b–d). Statistics were calculated using the Student's *t*-test. The experiment was performed twice with similar results (*n* = 3) (**P* < 0.05, ***P* < 0.01, ****P* < 0.005). Gene-set enrichment was calculated as a normalized enrichment score. Multiple testing was corrected using false-discovery rate calculated based on gene-set permutations.



with data from Foltz *et al.*,²⁸ 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,²⁹ 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 TLR2-specific E3 ubiquitin ligase (PPP1R11) has recently been described, demonstrating that TLR2 signaling can directly be controlled by ubiquitination.³⁰ The change in cytokine production after TLR2 activation of *Trim21*^{-/-} BMDMs is of a similar moderate magnitude to changes reported after knockdown of other E3 ligases, including TRIM13²⁶ and CHIP.²⁷ This is expected, because E3 ligases are modulators of signaling rather than necessary components of the signaling pathways. Importantly, previous reports have also found that TRIM21 activates TAK1,^{9,31} demonstrating that TRIM21 can directly control TLR signaling. There are no added antibodies in our experimental set up, 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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