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TRIF Signaling Drives Homeostatic Intestinal Epithelial Antimicrobial Peptide Expression

Silvia Stockinger,*,† Claudia U. Duerr,* Marcus Fulde,* Tamas Dolowschiak,*,‡ Johanna Pott,*, Ines Yang,*, Daniel Eibach,*, Fredrik Bäckhed,§ Shizuo Akira,*, Sebastian Suerbaum,*, Martijn Brugman,*, and Mathias W. Hornef*

Recent results indicate a significant contribution of innate immune signaling to maintain mucosal homeostasis, but the precise underlying signal transduction pathways are ill-defined. By comparative analysis of intestinal epithelial cells isolated from conventionally raised and germ-free mice, as well as animals deficient in the adaptor molecules MyD88 and TRIF, the TRL3 and TLR4, as well as the type I and III IFN receptors, we demonstrate significant TLR-mediated signaling under homeostatic conditions. Surprisingly, homeostatic expression of Reg3γ and Paneth cell enteric antimicrobial peptides critically relied on TRIF and, in part, TLR3 but was independent of IFN receptor signaling. Reduced antimicrobial peptide expression was associated with significantly lower numbers of Paneth cells and a reduced Paneth cell maturation and differentiation factor expression in TRIF mutant compared with wild-type epithelium. This phenotype was not transferred to TRIF-sufficient germ-free animals during cohousing. Low antimicrobial peptide expression in TRIF-deficient mice caused reduced immediate killing of orally administered bacteria but was not associated with significant alterations in the overall composition of the enteric microbiota. The phenotype was rapidly restored in a TRIF-independent fashion after transient epithelial damage. Our results identify TRIF signaling as a truly homeostatic pathway to maintain intestinal epithelial barrier function revealing fundamental differences in the innate immune signaling between mucosal homeostasis and tissue repair. The Journal of Immunology, 2014, 193: 4223–4234.

Innate immune receptors of the family of TLRs recognize conserved microbial structures and activate host defense mechanisms upon infection with pathogenic microorganisms. They are expressed by professional immune cells but also intestinal epithelial cells (IECs), although at a somewhat restricted spectrum (1, 2). Recent reports indicate that also in the absence of infectious challenge, innate immune stimulation of mucosal cells by ligands released from the gut microbiota occurs and contributes to maintenance of the intestinal epithelial barrier. A number of studies examined the role of epithelial innate immune signaling mediators and regulatory molecules in experimental models of intestinal inflammation and observed a significant influence on disease outcome (3–12). Only few studies, however, noted spontaneous mucosal dysfunction in gene-deficient animals, and these were restricted to central intermediates or regulators of the downstream TLR signal transduction pathway or induced effector molecules (6, 9, 12–18). Thus, the functional importance of TLR signaling under unchallenged, homeostatic conditions for the expression of epithelial effector functions of the mucosal barrier has remained largely undefined.

TLR recognition of the respective microbial ligand initiates signal transduction through two major pathways. Recruitment of MyD88 leads to NF-κB and MAPK activation. This pathway is activated by the IL-1R, as well as all TLRs, except the double-stranded RNA receptor TLR3. TRL3 and the LPS receptor TLR4 recruit the TIR domain–containing adaptor inducing IFN-β (TRIF, also called TIR domain–containing adaptor molecule 1 [TICAM1]) that activates NF-κB and MAPKs but additionally upregulates expression of type I IFN regulatory factor 3–dependent genes such as CCL5 (Rantes), IFN-β, or IFN-λ (19, 20). Subsequent binding of secreted IFN-α/β or -λ to the IFN receptor IFNAR or IL-28R, respectively, induces a variety of so-called IFN-stimulated genes (ISGs) (21). Thus, whereas impaired MyD88-dependent signaling blocks stimulation by most TLR ligands, as well as IL-1 and IL-18, signaling through TLR3 and, in part, TLR4 is affected in the absence of TRIF.

In this study, we performed a comparative expression analysis using primary small IECs isolated from mice under healthy...
conditions, as well as after transient mucosal damage, to characterize and define homeostatic innate immune signaling. IECs from germ-free (GF) and conventionally raised mice, as well as animals lacking the adaptor proteins TRIF and MyD88, the upstream receptors TLR3 and TLR4, as well as downstream signaling via the type I/III IFN loop, were included. The results revealed homeostatic innate immune receptor–dependent signaling of a large number of genes at the intestinal epithelium and identified important differences between homeostatic expression and signal transduction after mucosal challenge. Paneth cell antimicrobial peptide expression under homeostatic conditions required TRIF- and, in part, TLR3-mediated signaling but was independent of IFN signaling. Lack of TRIF-dependent signaling was compensated after mucosal damage with elevated production of antimicrobial peptides by alternative TRIF-independent signaling pathways. Thus, our results describe a distinct homeostatic epithelial cell signaling and identify TRIF-mediated signaling as innate immune pathway driving essential features of small-intestinal epithelial homeostasis.

Materials and Methods

Mice

C57BL/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). C57BL/6-Ticam1<−/−> mice were obtained from the Jackson Laboratory and bred locally. Trif<−/−>/Salmonella enterica

M. tuberculosis

Housing conditions were included in the analysis. GF mice were main-

In vitro mouse models

For bacterial challenge in vivo, 28- to 32-d-old mice were gavaged with 100 µl of a bacterial suspension in PBS using a 20-gauge feeding needle. After 2 h, mice were killed by cervical dislocation; the small intestine was isolated and divided into a proximal and a distal part. The luminal content was collected by flushing intestinal segments thoroughly with a defined volume of PBS (1 ml), and serial dilutions were plated onto tetracycline-containing brain heart infusion agar plates in case of L. monocytogenes infection or onto ampicillin-containing Luria-Bertani agar plates in case of S. Typhimurium infection. Mesenteric lymph nodes of 28- to 32-d-old wt, MyD88<−/−> and Trif<−/−>/PS2<−/−> animals were isolated, incubated shortly in 70% ethanol, and washed three times in PBS before homogenization, to determine the mucosal translocation of commensal bacteria. Bacterial numbers were determined by serial dilutions and plating on blood containing agar (Schaeder) medium followed by culture for 3–4 d at 37°C under anaerobic conditions. For transient epithelial damage, 28- to 32-d-old mice were i.p. injected with 100 µg human TNF-α kindly provided by Daniela Männel (University of Regensburg, Regensburg, Germany). Control mice were injected with 0.9% (v/v) NaCl solution. After 8 h, mice were killed by cervical dislocation, and the small intestine was isolated and either fixed in 4% formaldehyde solution or used for IEC isolation.

Isolation of primary IECs and gene expression analysis

Epithelial cells were isolated from small-intestinal tissue by incubation in 30 mM EDTA in PBS as recently described (22, 23). RNA was isolated from epithelial cell preparations using the NucleoSpin RNA II kit from Machery-Nagel (Duren, Germany), according to the manufacturer’s instructions, and microarray analysis was performed using Whole Mouse Genome Oligo Microarray (4 × 44k; Agilent Technologies, Santa Clara, California). A total of 500 ng total RNA was used for reverse transcription and 1650 ng labeled cRNA for hybridization. GF or control animal IEC samples were labeled with either Cy3 or Cy5 following the manufacturer’s protocol, using four dye-swapped samples per group. mRNA samples from Trif<−/−>/PS2<−/−>, MyD88<−/−> and Trif<−/−>/PS2<−/−> animals were mixed and labeled with Cy3 using four samples per group. Arrays were scanned using an Agilent Technologies G2505C scanner and processed using Agilent’s Feature Extraction V10.7.3.1 software. Subsequently, the data were processed using R 2.12 and Bioconductor 2.7.7. Array data were normalized using VSN (24) and per probe, a linear model was fitted to the resulting expression values (25). Probes with p values < 0.05 after Benjamin–Hochberg multiple testing correction were considered significant. Hierarchical clustering was performed using Euclidean distance as a measure for dissimilarity (26). The array data are available in the Gene Expression Omnibus database under accession number GSE51260 (http://www.ncbi.nlm.nih.gov/geo/).

For real-time PCR analysis, RNA was isolated with TRIzol (Invitrogen) according to the manufacturer’s instruction. Reverse transcription was performed using 3 µg total RNA with RevertAid reverse transcriptase (Fermentas, St.-Leon-Rot, Germany). Real-time PCR was quantified using SYBR green (Invitrogen) and analyzed using the Pfaffl method to express relative expression of the target gene to the housekeeping gene Gapdh (27). The following forward and reverse primers were used in this study:

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Mmp7: 5'-TTTTGATGCAGCGCACTGCTA-3', 5'-ATGCTGCTGAGC-5' CAAACGAGATG-3', Reg3g: 5'-CCCTCTCTCTCTCAGACAT-5', 5'-ATATCTCCCTCCCAGGAAATCCT-3', Cryptd1: 5'-AAGAGATCACTAATGAGGAGAGAAGC-3', 5'-CGCAAGCAGGCTGTTGA-3', Isy15: 5'-AGCTGACAGGACGTCAGCAAT-3', 5'-TTGTGGCAATGCTTCTT-3', Oat2: 5'-GGATGCCTGCGGAGAATGCT-3', 5'-TGCTGGCTCTTGGAAACTG-3', Nio5: 5'-GGTGGGACCAAGGAGGAGA-3', 5'-GCTGCCCAGAGGTGTTGAC-3', and Gapdh (5'-GTCACACCAACTGTTAGGC-3', 5'-GCGATAGACTGTCGCTAG-3').
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Immunoblot analysis and ELISA

Isolated IECs were lysed in 500 µl lysis buffer (10 mM Tris-HCl pH 7.05, 50 mM NaCl, 30 mM NaPi, 50 mM Na2, 2 mM EDTA, 1% Triton X-100, protease inhibitor mixture (Roche)). Extracts were cleared by centrifugation, mixed with Laemmli buffer, and boiled for 10 min. Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Schwalbach, Germany), or in case of detection of cryptdins or α-defensin 2, polyvinylidenefluorid membranes (Invitrogen GmbH, Darmstadt, Germany) were used in combination with HRP-labeled secondary Abs from Jackson Immunoresearch (Seyburg, Germany), and Stat1 (BD Transduction Laboratories, Heidelberg, Germany) were used in combination with HRP-labeled secondary Abs against MMP7 (R&D Systems, Wiesbaden-Nordenstadt, Germany), β-actin (Sigma-Aldrich, Taun-kenkirchen, Germany), and Stat1 (BD Transduction Laboratories, Heidelberg, Germany) were used in combination with HRP-labeled secondary Abs from Jackson Immunoresearch (Suffolk, U.K.) and detected using the chemiluminescence detection system from Pierce (Rockford, IL). Abs against cryptdin 2 and Reg3g were used as described previously (28, 29).

Short sections of the distal small intestine were removed from healthy wt, MyD88<−/−>, and Trif<−/−>/PS2<−/−> animals, opened longitudinally, and incubated for 24 h at 37°C in cell culture medium supplemented with penicillin and streptomycin, to determine TNF released from intestinal tissue cells. TNF concentrations in serum or culture medium were analyzed by ELISA according to the manufacturer’s protocol (Nordic Biosite, Täby, Sweden).

Immunohistology, TUNEL staining, and fluorescence in situ hybridization

Ag retrieval in deparaffinized formaldehyde-fixed tissue sections was performed by boiling in 0.01 M sodium citrate buffer (pH 6.0). For MMP7 staining, slides were blocked with normal donkey serum (Jackson Immunoresearch, Suffolk, U.K.) and stained with 1:50 diluted goat polyclonal Abs against MMP7 (R&D Systems, Wiesbaden-Nordenstadt, Germany) in combination with a Cy3-conjugated donkey anti-goat secondary Ab (Jackson Immunoresearch). Reg3g (29) and cryptdin (28) staining was preceded by blocking of normal goat serum (Jackson Immunoresearch) followed by staining with rabbit anti-Reg3g or anti-cryptdin antisera in combination with a Cy3-conjugated goat anti-rabbit Ab. The cryptdin antisera was raised in rabbits using the mature processed form of cryptdin 2 (mouse α-defensin 2). Because of the high sequence identity of
most cryptdin family members and cross-reactivity of the antisera, the detected antimicrobial peptides are referred to as cryptdin throughout this article. Rabbit anti-lysozyme P antisera (1:250; Dako, Hamburg, Germany) or rabbit anti-Mucin2 antisera (1:500; provided by Gunnar C. Hansson, University of Göteborg, Göteborg, Sweden) was incubated for 1 h at room temperature and detected using a Texas Red–conjugated anti-rabbit secondary Ab (1:50; Jackson Immunoresearch). For TUNEL or alkaline phosphatase staining, deparaffinized formaldehyde-fixed tissue sections were analyzed with an in situ cell death detection kit (Roche, Mannheim, Germany) or Vector Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s recommendations. A mouse monoclonal anti–E-cadherin Ab (BD Biosciences, Heidelberg, Germany) followed by the appropriate AF488-conjugated secondary Ab (Molecular Probes, Jackson Immunoresearch, Suffolk, U.K.) or FITC-labeled wheat germ agglutinin (WGA) was used for visualization. For immunostaining, deparaffinized formaldehyde-fixed tissue sections were used. Sections were incubated with 50 ng of the unlabeled oligonucleotide probe BET42a in 10 ml hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 7.6, 0.01% NaDodSO4, 30% formamide) for 30 min at 37˚C to reduce nonspecific binding. Next, 5 ng of the specific Cy3-labeled labeled oligonucleotide probe (EUB338) together with 50 ng of the unlabeled oligonucleotide probe BET42a were added in 10 ml hybridization buffer for 90 min at 46˚C. Slides were washed in washing buffer (20 mM Tris-HCl pH 7.6, 0.01% NaDodSO4, 112 mM NaCl) for 15 min at 46˚C, rinsed with PBS, air-dried, and mounted in DAPI. For ALCAN blue staining, deparaffinized formaldehyde-fixed tissue sections were stained with Alcian Blue (Sigma-Aldrich, Taufenkirchen, Germany). Fluorescence in situ hybridization (FISH), paraffin-embedded terminal ileum tissue was sectioned, deparaffinized, and pretreated with 1 ml with lysozyme in 10 mM Tris-HCl pH 8.0 for 15 min and subsequently with 10 mg/ml lystostaphin in 10 mM Tris-HCl pH 8.0 for 15 min. After washing, sections were incubated with 50 ng of the unlabeled oligonucleotide probe BET42a in 10 ml hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 7.6, 0.01% NaDodSO4, 30% formamide) for 30 min at 37˚C to reduce nonspecific binding. Next, 5 ng of the specific Cy3-labeled labeled oligonucleotide probe (EUB338) together with 50 ng of the unlabeled oligonucleotide probe BET42a were added in 10 ml hybridization buffer for 90 min at 46˚C. Slides were washed in washing buffer (20 mM Tris-HCl pH 7.6, 0.01% NaDodSO4, 112 mM NaCl) for 15 min at 46˚C, rinsed with PBS, air-dried, and mounted in DAPI. Alcian blue staining, deparaffinized formaldehyde-fixed tissue sections were stained with Alcian Blue (Sigma-Aldrich, Taufenkirchen, Germany). Quantification of cell numbers was performed manually counting stained cells in a total of >100 crypts or villi in sections from at least 3 individual mice.

16S rDNA microbiota analysis
Healthy C57BL/6j wt, Trif-/-, or TriPLP mice were sacrificed and their intestine removed and divided into three parts (proximal small intestine, distal small intestine, and colon [including cecum]). The microbiota was analyzed as detailed by Yang et al. (30) with modifications. Bacterial DNA was isolated using the QIAamp DNA stool kit (Qiagen, Hilden) according to the manufacturer’s instructions. 16S rRNA gene fragments were amplified using 454 FLX titanium fusion primers with template-specific parts consisting of the universal bacterial primer 8F (5′-CTCAATCMTTTRAGAGTTT) and 541R (5′- CCTCGTCAATGATATGC) ampicons were sequenced from the 3′ end using Titanium chemistry on a 454 Genome Sequencer FLX (Roche), and raw data were processed with the 454 GS Run Processing Software (Roche). Sequences were further processed with the software package mothur, version 1.29.2 (31). Processing included removal of barcodes, primers, low-quality sequences, and low-quality sequence regions. PCR chimeras were identified usingUCHIME (32) with the mothur-provided “SILVA gold” reference set (31) and removed. Classification of sequences was based on Ribosomal Database Project classifier version 2.5 (33), with the number of bootstrap replicates increased to 1000, using a minimal bootstrap support of 70%. Chloroplast sequences and sequences that could not be identified to class level were culled. For identification to species level, sequences identified to a bacterial species were at least 97% well sequenced against a reference of Ribosomal Database Project database release 10 update 29, which had been modified with TaxCollector (34) for automated processing. Sequences were clustered into operational taxonomic units (OTUs) using ESPRIT-Tree (35) at an OTU similarity cutoff of 4%. This cutoff was chosen based on the normalized mutual information between species and OTU classification. For OTU classification, both 80% consensus identification and identification of species using silva gold were considered for each OTU, and the more detailed of the two classifications was chosen (see Supplemental Material). After subsampling to an equal number of sequences per sample, rarefaction curves were generated with mothur. The distribution of OTUs among the samples was visualized using the R script heat map (36) as described by Yang et al. (30). The R package vegan (37) was used for principal coordinates analyses (PCoA), using the “capscale” command in combination with the Bray-Curtis dissimilarity index, and for fitting the PCoA ordination. Canonical analysis of principal coordinates was performed using the function corPC from the R package vegan, to reduce nonspecific binding. Next, 5 ng of the specific Cy3-labeled labeled oligonucleotide probe (EUB338) together with 50 ng of the unlabeled oligonucleotide probe BET42a were added in 10 ml hybridization buffer for 90 min at 46˚C. Slides were washed in washing buffer (20 mM Tris-HCl pH 7.6, 0.01% NaDodSO4, 112 mM NaCl) for 15 min at 46˚C, rinsed with PBS, air-dried, and mounted in DAPI. Alcian blue staining, deparaffinized formaldehyde-fixed tissue sections were stained with Alcian Blue (Sigma-Aldrich, Taufenkirchen, Germany). Quantification of cell numbers was performed manually counting stained cells in a total of >100 crypts or villi in sections from at least 3 individual mice.

TRIF-dependent antimicrobial peptide expression
Antimicrobial peptides are critical components of the enteric mucosal barrier. In the small intestine, the largest group comprises the α-defensins (in mice also called cryptidins) produced by Paneth cells located at the lower end of intestinal crypts (38, 39). The proform of cryptidins is cleaved by MMP7. Processing is required to obtain antimicrobial activity, and secretion into the gut lumen is considered the main regulatory mechanism (40). In contrast, Reg3γ is an antimicrobial protein that is regulated at the transcriptional level and exhibits a less restricted expression pattern with synthesis by epithelial cells in the crypt and lower villous region (41). Fig. 2 confirms the critical role of TRIF for the homeostatic expression of MMP7, Reg3γ, and cryptdin in healthy, unchallenged mice by quantitative RT-PCR (Fig. 2A), immunoblotting (Fig. 2B, 2C), and immunofluorescent staining (Fig. 2D–F). A less pronounced role of MyD88 was found for homeostatic Reg3γ expression (Fig. 2A, 2B) in accordance with published results (42, 43). Although the absence of TRLR4 and TLR3 moderately influenced the expression of Reg3γ on the mRNA level, protein expression in TLR4-/- and TLR3-deficient IECs was comparable with wt levels (Fig. 2A, 2B). In accordance with previous work, MMP7 and Reg3γ expression were reduced in the absence of the gut microbiota (44, 45) (Fig. 2A, 2B). In contrast, the levels of Paneth cell–derived cryptidins on the mRNA level were largely maintained in the absence of the microflora as previously reported (46) (Fig. 2A). Lower protein levels of cleaved cryptdin were in accordance with reduced MMP7 expression (Fig. 2B, 2C). Although in part significant, differences in MMP7, Reg3γ, and cryptdin expression on the mRNA and protein level found in mice deficient in type I or III IFN signaling were rather variable.

Results

Comparative intestinal epithelial gene expression analysis
IECs were isolated from healthy 28-d-old wt, as well as Trif-/-, Trif-/-, MyD88-/-, and TriPLP mice (31) animals; total mRNA was prepared, and the global mRNA expression profile was determined. Subsequently, the lack of expression of the knocked-out genes was confirmed and global background, signal, and hybridization were verified to confirm that RNA hybridization was satisfactory. We then analyzed significantly differentially expressed genes between wt and the respective knockouts. Fig. 1A indicates the number of genes differentially regulated in IECs of the respective gene-deficient animals in comparison with wt animals (Fig. 1A, labeling next to the boxes) and illustrates the genes simultaneously significantly altered in the absence of two functionally dependent signaling molecules (Fig. 1A, labeling next to the arrows). Surprisingly, the number of TRIF-dependent genes by far exceeded the number of genes regulated by the adaptor protein MyD88. TLR3 played a more dominant role for maintenance of homeostatic gene expression than TLR4. The cluster dendrogram confirmed the more pronounced effect in IECs derived from animals devoid of TLR3- and TRIF-mediated innate immune signaling (Fig. 1B). Subsequently, genes with well-established function in IECs such as antimicrobial peptide production were analyzed, and marked differences were noted between the groups examined (Fig. 1C). Again, these differences were particularly evident in the absence of TRIF signaling and mostly dependent on TLR3. In contrast, MyD88 was critical to maintain expression of the majority of ISGs.
Lack of antimicrobial peptide synthesis enhances the survival of orally administered bacterial pathogens

To test the functional consequences of reduced antimicrobial peptide production in the absence of the adaptor protein TRIF, we removed and lumina!y exposed intestinal tissue of wt and Trif\textsuperscript{LPS2/LPS2} mice to enteropathogenic \textit{L. monocytogenes} (Fig. 3A). The number of viable bacteria was determined 2 \textit{h} after administration to address the presence of antibacterial activity. In accordance with studies showing an important role of antimicrobial peptides in intestinal immunity against \textit{L. monocytogenes}, a significantly enhanced number of viable bacteria were isolated from the intestinal lumen of Trif\textsuperscript{LPS2/LPS2} animals as compared with wt mice. Similar results were additionally obtained in vivo after oral administration of a high (10\textsuperscript{10} CFU; Fig. 3B) or a low (10\textsuperscript{6} CFU; Supplemental Fig. 3A) dose of \textit{L. monocytogenes}. In both cases, significantly higher numbers of bacteria were grown from intestinal tissue of Trif\textsuperscript{LPS2/LPS2} mice as compared with wt animals 2 \textit{h} after administration. This effect appeared to be specific for the reduced antimicrobial peptide-derived antibacterial activity because no significant effect was noted after administration of the enteropathogenic bacterium \textit{S. Typhimurium}, known to exhibit a reduced susceptibility to killing by Reg3\textgamma and Paneth cell–derived cryptdins (41, 47) (Supplemental Fig. 3B, 3C). Also, the translocation of commensal bacteria into the mesenteric lymph nodes was not significantly enhanced in the absence of TRIF and MyD88, respectively (Supplemental Fig. 3D).

\textit{The Trif}\textsuperscript{LPS2/LPS2} microbiota is able to drive epithelial antimicrobial peptide expression

The observed phenotype in Trif\textsuperscript{LPS2/LPS2} mice could result from a direct TRIF-mediated effect on epithelial cell differentiation and antimicrobial peptide expression. Alternatively, TRIF via an unknown mechanism might exert a significant influence on the composition of the enteric microbiota, and the altered microbiota might, in turn, be responsible for the detected difference. To examine the potential of the enteric microbiota of Trif\textsuperscript{LPS2/LPS2} mice to promote enteric antimicrobial peptide expression, we cohoused 7- to 8-wk-old GF wt mice with conventional Trif\textsuperscript{LPS2/LPS2}, specific pathogen-free mice for 3 wk, and the enteric antimicrobial peptide expression was comparatively analyzed in GF wt and freshly conventionalized wt animals. Immunostaining, Western blot, and quantitative RT-PCR illustrated the stimulatory potential of the Trif\textsuperscript{LPS2/LPS2} microbiota to induce epithelial Reg3\textgamma expression in GF animals, although a direct comparison of the potential of wt versus Trif\textsuperscript{LPS2/LPS2} microbiota was not performed (Fig. 4A–C). In addition, no downregulation of MMP7 and

\textbf{FIGURE 1.} Comparative analysis of intestinal epithelial gene expression in wt, Tlr3\textsuperscript{−/−}, Tlr4\textsuperscript{−/−}, MyD88\textsuperscript{−/−}, and Trif\textsuperscript{LPS2/LPS2} mice. (A) Overall gene expression profile in isolated IECs of 28-d-old healthy mice according to the established signal transduction pathways. The number of genes differentially expressed in IECs between knockout and wt (9900 for Tlr3\textsuperscript{−/−}, 3533 for Tlr4\textsuperscript{−/−}, 323 for MyD88\textsuperscript{−/−}, and 10036 for Trif\textsuperscript{LPS2/LPS2}) are displayed next to the boxes representing the respective knockouts. The overlaps in significantly differentially regulated genes between the knockouts are indicated with numbers next to the arrows connecting the knockouts. (B) Cluster dendrogram indicating the segregation of the expression profile of individual mice according to their genotype. (C) Comparative expression analysis in respect to genes known to critically contribute to epithelial barrier formation and ISGs in wt, Tlr3\textsuperscript{−/−}, Tlr4\textsuperscript{−/−}, MyD88\textsuperscript{−/−}, and Trif\textsuperscript{LPS2/LPS2} mice. The expression values are normalized by calculating Z-scores for the expression values for each gene.
FIGURE 2. TRIF critically determines intestinal epithelial antimicrobial peptide expression and drives antibacterial activity under homeostatic conditions. 

(A) Quantitative RT-PCR for Mmp7, Reg3γ, and Cryptdin1 in IECs isolated from 28-d-old C57BL/6 wt, TLR3/−/−, TLR4/−/−, MyD88/−/−, and TRIF/LPS2/LPS2 mice, as well as from GF mice (n = 4/genotype or condition). (B and C) Immunoblot for MMP7 and Reg3γ (B), as well as cryptdin (C), in total lysate of primary IECs. Each lane represents an individual mouse. Recombinant mature cryptdin 2 was loaded as a positive control for the anti-cryptdin Ab. Quantitative analysis of protein expression was performed using ImageJ software. All protein levels were normalized against the corresponding actin levels. (D–F) Immunofluorescence staining for Reg3γ (D), cryptdin 2 (E), and MMP7 (F) in small-intestinal tissue of wt and TRIF/LPS2/LPS2 mice. Counterstaining was performed with DAPI (blue) (D–F), WGA (D), or E-cadherin (green) (E and F). Scale bars, 50 µm (D); 25 µm (E and F). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
cryptdin 2 expression in Paneth cells was noted in previously GF animals after colonization with the microbiota from Trif<sup>LPS2/LPS2</sup> mice (Fig. 4B, 4D).

In accordance, no significant difference was noted in the composition, anatomical distribution, and density of the enteric microbiota in wt (C57BL/6), Trif<sup>+/+LPS2</sup>, and Trif<sup>LPS2/LPS2</sup> animals. Cohoused wt (Trif<sup>+/+</sup>), Trif<sup>+/LPS2</sup>, and Trif<sup>LPS2/LPS2</sup> siblings from six litters born after crossing heterozygous parent animals were examined by high-throughput 16S rDNA sequencing. The data set used for OTU clustering contained 543,669 sequences with an average length of 284 bp (range 100–509 bp). After subsampling to 969 sequences per sample, individual samples contained 16 to 140 OTUs, with 710 OTUs overall. AMOVA analysis with both Bray-Curtis and mother-based Jaccard indices did not reveal significant differences between genotypes, neither for combined analysis of samples from all intestinal sites nor for separate comparisons of proximal small intestine, distal small intestine, and colon samples. PCoA analysis based on Bray-Curtis distances, in combination with factor fitting, did not detect significant effects of genotype or sex for any of the intestinal sections (Fig. 5A, 5B). It did, however, reveal significant differences between litters (Supplemental Material). According to AMOVA analysis, colon samples were significantly different between litters A and D, A and E, B and D, B and F, and C and F; distal small-intestine samples were significantly different between litters A and D, A and E, B and F, as well as E and F; and proximal small-intestine

**FIGURE 3.** Lack of enteric antimicrobial peptide synthesis enhances the survival of bacterial pathogens within the gut lumen. (A) Segments obtained from proximal (left panel) or distal (right panel) small intestine of wt and Trif<sup>LPS2/LPS2</sup> mice were filled with PBS containing 10<sup>5</sup> CFU <i>L. monocytogenes</i> and ligated. After 2 h, luminal washings were performed, and the number of viable intraluminal bacteria was determined by serial plating. Data represent a summary of 3 individual experiments using 17 wt and 14 Trif<sup>LPS2/LPS2</sup> mice in total; whiskers on box plots show minimum and maximum. Statistical analysis was performed using the Mann–Whitney <i>U</i> test (*<i>p</i> = 0.0131, **<i>p</i> = 0.0083). (B) wt and Trif<sup>LPS2/LPS2</sup> mice were orally infected with 10<sup>10</sup> CFU <i>L. monocytogenes</i> by gavage. The number of viable bacteria (CFU) in the proximal (left panel) and distal (right panel) part of the small-intestinal lumen were determined 2 h postinfection. Data represent a summary of two individual experiments using 13 wt and 12 Trif<sup>LPS2/LPS2</sup> mice in total; whiskers on box plots show minimum and maximum. Statistical analysis was performed using the Mann–Whitney <i>U</i> test (**<i>p</i> = 0.0043, ***<i>p</i> = 0.0003). See also Supplemental Fig. 3.

**FIGURE 4.** The Trif<sup>LPS2/LPS2</sup> microbiota is sufficient to drive Reg3<sup>γ</sup> expression and does not suppress Paneth cell antimicrobial peptide expression in GF animals. (A) Immunofluorescence staining for Reg3<sup>γ</sup> (red) in small-intestinal tissue of Trif<sup>LPS2/LPS2</sup>, wt C57BL/6 GF, and wt C57BL/6 GF mice conventionalized through cohousing with Trif<sup>LPS2/LPS2</sup> mice for 3 wk (ex GF). Counterstaining was performed with DAPI (blue) and WGA (green). Scale bar, 50 μm. (B) Immunoblot for Reg3<sup>γ</sup> and MMP7 and (C) quantitative RT-PCR for Reg3<sup>γ</sup> in total primary IECs isolated from wt C57BL/6 GF and wt C57BL/6 GF mice conventionalized through cohousing with Trif<sup>LPS2/LPS2</sup> mice for 3 wk (ex GF). Each lane in (B) represents an individual mouse. Quantitative analysis of protein expression was performed using ImageJ software. All protein levels were normalized against the corresponding actin levels. (D) Immunofluorescence staining for MMP7 and cryptdin in small-intestinal tissue of Trif<sup>LPS2/LPS2</sup>, wt C57BL/6 GF, and wt C57BL/6 GF mice conventionalized through cohousing with Trif<sup>LPS2/LPS2</sup> mice for 2 wk (ex GF). Counterstaining was performed with DAPI (blue) and phalloidin (green). Scale bar, 25 μm. **<i>p</i> < 0.01.
samples were significantly different between litters A and D, B and F, C and D, as well as C and E (experiment-wise error rate 0.05 for each intestinal section, Bonferroni correction). Also, the anatomical distribution of bacteria in the terminal small intestine visualized using a eubacterial FISH probe did not reveal any detectable difference between wt (C57BL/6) and \( \text{Trif}^{LPS2/LPS2} \) mice (Fig. 5C). Finally, the bacterial genome copy number, and thus the number of bacteria in the proximal and distal small intestine, as well as colon, was similar in wt (C57BL/6), \( \text{Trif}^{+/LPS2} \), and \( \text{Trif}^{LPS2/LPS2} \) mice (Fig. 5D). These results suggest that the bacterial composition, distribution, and density of the enteric microbiota are independent of TRIF and able to support antimicrobial peptide expression.

**TRIF-independent upregulation of antimicrobial peptides in response to epithelial damage**

We next investigated the expression of antimicrobial peptides in wt (C57BL/6) and \( \text{Trif}^{LPS2/LPS2} \) mice after epithelial damage. Only a few models of small-intestinal epithelial damage, however, have been described. The widely used model of i.p. administration of TNF was chosen because it induces rapid (i.e., within hours) damage of the small-intestinal epithelium in a TRIF-independent
fashion (48–50). Of note, TRIF mutant mice exhibited normal serum TNF levels and mucosal TNF production, suggesting that reduced TNF production was not involved in the observed phenotype of TRIF mutant mice (data not shown and Fig. 6A). Expression of MMP7 and Reg3γ in wt mice was moderately enhanced 8 h after TNF treatment (Fig. 6B, left panel). Surprisingly, a markedly enhanced epithelial Reg3γ expression, as well as upregulation of MMP7, was also found in Trif<sup>LPS2/LPS2</sup> mice after TNF-induced damage (Fig. 6B, right panel). Immunofluorescent staining confirmed strong TRIF-independent upregulation of Reg3γ in response to TNF treatment and revealed a markedly enhanced synthesis of Paneth cell–derived cryptdin and MMP7 (Fig. 6C–E). These results indicate that distinct regulatory pathways govern antimicrobial peptide expression under homeostatic as compared with challenged conditions, and that the loss of antimicrobial peptide expression in Trif<sup>LPS2/LPS2</sup> mice is rapidly compensated in a TRIF-independent fashion upon mucosal challenge.

Mechanism of reduced intestinal Paneth cell numbers in the absence of TRIF signaling

The number of lysozyme-positive Paneth cells was significantly reduced in the absence of TRIF (Supplemental Fig. 2D, 2E). Therefore, we next investigated possible mechanisms of reduced Paneth cell numbers and antimicrobial peptide production. No differences in the expression of atonal homolog 1 (Atoh1) and the SAM pointed domain containing ETS transcription factor (Spdef), transcription factors involved in the differentiation of the secretory cell types, were detected in wt versus Tlr3<sup>−/−</sup> or Trif<sup>LPS2/LPS2</sup> mice. This is in line with our findings on normal numbers of Muc2<sup>+</sup> goblet cells (Supplemental Fig. 2A, 2B, 2E), because Atoh1 also drives the differentiation of all secretory epithelial cell lineages and Spdef is involved in the generation of both goblet cells and Paneth cells (51). In contrast, expression of the HMG-box transcription factor Sox9 and the ephrin B1 receptor Ephb3 was reduced in both Tlr3<sup>−/−</sup> and Trif<sup>LPS2/LPS2</sup> mice (Fig. 7A). Sox9 critically contributes to Paneth cell differentiation, whereas Ephb3 restricts the localization of Paneth cells to the crypt region (52–54). Both transcription factors thus are highly specific for Paneth cell differentiation and function. In addition, we examined loss of Paneth cell viability using TUNEL staining. Whereas TUNEL<sup>+</sup> cells were detected at the villous tip of both wt and Trif<sup>LPS2/LPS2</sup> mice, no TUNEL staining was detected within the crypt region in lysosome-positive Paneth cells of both mouse strains (Fig. 7B). Together, these results point toward a role of TRIF signaling in the differentiation of Paneth cells and their ability to produce antimicrobial peptides.

Discussion

The intestinal mucosa represents both a tight barrier against commensal and pathogenic microorganisms, as well as a highly efficient absorptive surface for nutrients and water. Both functions are the result of a long coevolution between microbial organisms and the mammalian host, and are absolutely required to maintain the host’s life. Until recently, the intestinal epithelial barrier was considered to represent a largely static, developmentally deter-

![FIGURE 6. TRIF-independent antimicrobial peptide expression after transient mucosal damage. (A) TNF secretion by small-intestinal tissue explants from 11–12 individual C57BL/6, MyD88<sup>−/−</sup>, and Trif<sup>LPS2/LPS2</sup> mice incubated in cell culture medium for 24 h at 37°C. TNF concentrations were measured by ELISA. (B) C57BL/6 wt and Trif<sup>LPS2/LPS2</sup> mice were injected i.p. with 0.9% (w/v) NaCl or 100 μg human TNF. Eight hours postinfection, IECs were isolated and protein lysates were subjected to immunoblotting for MMP7 and Reg3γ. Each lane represents an individual mouse. Quantitative analysis of protein expression was performed using ImageJ software. All protein levels were normalized against the corresponding actin levels. (C-E) Immunofluorescent staining for Reg3γ in duodenum (C) and cryptdin (D) as well as MMP7 (E) in terminal ileum of Trif<sup>LPS2/LPS2</sup> mice 8 h after i.p. injection of 0.9% (w/v) NaCl (− TNF) or 100 μg huTNF (+ TNF). Counterstaining was performed with DAPI (blue) and E-cadherin (green). Scale bars, 50 μm (C); 25 μm (D and E). *p < 0.05, **p < 0.01.
from four individual 28-d-old C57BL/6 wt, mice. Expression levels of Paneth cell differentiation factors Atoh1, Spdef, Sox9, and Ephb3 in IECs derived from LPS2/LPS2 mice. Counterstaining was performed with DAPI (blue) and TRIF mice. Reduced expression of Paneth cell differentiation factors in the absence of TRIF. (A) Gene expression levels of Paneth cell differentiation factors Atoh1, Spdef, Sox9, and Ephb3 in IECs derived from four individual 28-d-old C57BL/6 wt, Trif<sup>LPS2;LPS2</sup>, and Tlr3<sup>LPS2;LPS2</sup> mice. (B) TUNEL staining in small-intestinal tissue of C57BL/6 wt and Trif<sup>LPS2;LPS2</sup> mice. Counterstaining was performed with DAPI (blue) and lysozyme (green). Tissue sections of three mice were independently examined. Original magnification ×100. *p < 0.05, **p < 0.01.

FIGURE 7. Reduced expression of Paneth cell differentiation factors in the absence of TRIF. (A) Gene expression levels of Paneth cell differentiation factors Atoh1, Spdef, Sox9, and Ephb3 in IECs derived from four individual 28-d-old C57BL/6 wt, Trif<sup>LPS2;LPS2</sup>, and Tlr3<sup>LPS2;LPS2</sup> mice. (B) TUNEL staining in small-intestinal tissue of C57BL/6 wt and Trif<sup>LPS2;LPS2</sup> mice. Counterstaining was performed with DAPI (blue) and lysozyme (green). Tissue sections of three mice were independently examined. Original magnification ×100. *p < 0.05, **p < 0.01.

The enteric microbiota provides a large variety of immunostimulatory ligands. Its influence under physiological conditions is illustrated in GF mice that exhibit diminished epithelial proliferation and a less developed mucosal immune system (44, 65, 66). TLR4 and TLR3 are well-known to initiate TRIF-dependent signaling. Endotoxin levels derived from food and the gut microbiota reach significant concentrations within the intestinal lumen (22). Although the local concentration of TLR3 ligands under healthy conditions is unknown, dsRNA species released from both exfoliated host cells and commensal bacteria were recently reported to drive TLR3/TRIF-dependent protective gene expression at the intestinal mucosa (67–69). This could also explain the sustained expression of Paneth cell antimicrobial peptides under GF conditions in accordance with a previous report (46). The detectable but less pronounced phenotype in single TLR3- or TLR4-deficient mice suggests a redundant function of these receptors. In addition, TLR2 and TLR5, known to be present at the intestinal epithelium, were recently shown to recruit TRIF and might therefore contribute to epithelial TRIF-dependent signaling (62, 70–72).

The analysis of specific epithelial transcription factors indicated unaltered expression of more broadly involved transcription factors such as Atoh1 or Spdef that both contribute to secretory cell lineage development (51). This finding is in line with the preserved goblet cell numbers in Trif<sup>LPS2;LPS2</sup> mice. In contrast, Sox9 and Ephb3 more specifically act on Paneth cells. Epithelium-specific, Sox9-deficient mice completely lack Paneth cells (52, 54). The reduced levels of Sox9 expression might therefore explain the lower numbers of Paneth cells and antimicrobial peptide expression found in TRIF mutant mice. The Ephb3 receptor is stimulated by Ephrin B1 and B2 ligands, and is driven by the β-catenin/TCF signaling pathway. Receptor stimulation affects cell modeling, migration, and cell matrix adhesion, and restricts the localization of Paneth cells to the lower crypt region (53). This localization is critical for the maintenance of the crypt-villus structure because Paneth cells via Notch signaling repress premature differentiation of neighboring Lgr5<sup>+</sup> stem cells into the secretory cell lineage (39).

Although we did not detect increased numbers of TUNEL<sup>+</sup> Paneth cells in TRIF mutant mice, we cannot formally exclude enhanced loss of Paneth cells in the absence of functional TRIF signaling. TRIF together with RIP1, Fas-associated death domain, procaspase 8, and cFLIP forms the atypical death complex leading to caspase 3-mediated, TNF-independent noninflammatory apoptosis (73, 74). This mechanism helps to preserve the epithelial integrity and provides protection from viral infection in the small intestine (75, 76). Simultaneously, the atypical death complex inhibits the induction of RIP3-dependent necroptosis, an inflammatory form of cell death. Epithelial deletion of Fas-associated death domain and caspase 8 leads to massive necrotic cell death associated with spontaneous enteric and colonic inflammation and, of note, Paneth cell loss (18, 77). Interestingly, at least two different RIP3-dependent necroptosis-inducing pathways exist. Whereas colonic inflammation is diminished in the absence of the microbiota, MyD88, or TNF, Paneth cell deletion is still observed (77, 78). We therefore hypothesize that the absence of also a third complex member, TRIF, might induce a milder form of epithelial necroptosis leading to reduced Paneth cell antimicrobial peptide synthesis in TRIF mutant mice. A recent report on continuous TLR3-mediated IFN stimulation by commensal enteric bacteria suggests that also the atypical death complex might be formed under homeostatic conditions (69).

Paneth cell loss in TRIF-deficient mice was independent of TNF in accordance with a recent report (77); in fact, TNF by itself has crucial significance. The restoring signaling pathway is currently unknown. It might be rather unspecific because cohabing of TRIF mutant mice with conventional wt animals also enhanced Paneth cell antimicrobial peptide expression (data not shown). However, reduced Paneth cell antimicrobial peptide
expression in TRIF mutant mice might also result from a direct influence of TRIF signaling on Paneth cell susceptibility to endoplasmic reticulum stress and autophagosome formation, previously associated with reduced Paneth cell numbers (79–81). Finally, TRIF-induced IFN regulatory factor 3–dependent IFN induction could directly or indirectly be involved in the observed phenotype. TRIF-mediated IFN synthesis was shown to protect from infection with the enteropathogen Yersinia enterocolitica (82). In accordance, epithelial expression of the ISGs inducible NO synthase and Stat1 were affected in the absence of TRIF. Both inducible NO synthase and Stat1, however, are known to underlie more complex regulatory circuits, and the expression of classic ISGs like Oasl and ISG15 relied on MyD88 rather than TRIF (Fig. 1C).

An important role of antimicrobial peptides both in the regulation of the microbial flora and in the host defense against pathogenic bacteria has been demonstrated in mice and humans (47, 83, 84). Enteric antimicrobial peptides in the adult small intestine belong to the family of cryptidins (α-defensins), cryptdin-related sequence peptides, or C-type lectins. Paneth cell–derived cryptidins and cryptdin-related sequence peptides are constitutively expressed, and enzymatic processing and peptide secretion regulate their release (46, 85). Expression of the processing enzyme MMP7, but not of cryptidins, was diminished in the absence of intestinal bacteria in accordance with previous results (46, 85). Also, homeostatic expression of the transcriptionally regulated bactericidal C-type lectin Reg3γ was found to depend on TRIF-mediated signaling, particularly evident in the duodenal and jejunal epithelium. In accordance, commensal bacteria had previously been shown to induce expression of Reg3γ (8, 41–43). The reduced antimicrobial peptide production in TRIF-deficient mice was functionally relevant, causing significantly enhanced survival of commensal bacteria in mesenteric lymph node tissue in the bactericidal C-type lectin Reg3γ. Also, homeostatic expression of the transcriptionally regulated bactericidal C-type lectin Reg3γ was found to depend on TRIF-mediated signaling, particularly evident in the duodenal and jejunal epithelium. In accordance, commensal bacteria had previously been shown to induce expression of Reg3γ (8, 41–43). The reduced antimicrobial peptide production in TRIF-deficient mice was functionally relevant, causing significantly enhanced survival of the enteropathogenic bacterium L. monocytogenes within the intestinal lumen. Reduced enteric antibacterial activity might lower the efficacy of the mucosal barrier and allow tissue penetration of commensal bacteria as recently described (86). Our comparative analysis of wt, MyD88−/−, and TRIF−/− mice, however, did reveal only a nonsignificant trend toward higher numbers of commensal bacteria in mesenteric lymph node tissue in the absence of MyD88- or TRIF-dependent signaling. Also, the overall microbiota composition, number, and anatomical distribution were unaffected in TRIF−/− mice. Although this finding was unexpected, similar findings have been described in MyD88 and single TLR-deficient mice (87). Cohousing experiments of TRIF-deficient conventional mice with wt GF mice illustrated the ability of the TRIF microbiota to induce epithelial Reg3γ expression. In the absence of a direct comparison of conventional TRIF mutant with conventional wt animals, however, we cannot formally exclude a diminished stimulatory activity of the TRIF microbiota on Reg3γ expression. In conclusion, our results indicate a significant contribution of TRIF-mediated innate immune signaling for homeostasis of the small-intestinal mucosa. Although the analysis of isolated epithelial cells suggests the detection of epithelial-intrinsic signaling effects, indirect immune cell-mediated mechanisms cannot be excluded. Antimicrobial peptide production was significantly impaired in the absence of TRIF, leading to an enhanced survival of bacterial pathogens. Importantly, TRIF-independent compensatory pathways were induced after mucosal damage and are able to rapidly reconstitute host defense activation in accordance with previous reports (3, 4). Thus, despite the involvement of innate immune signaling during both homeostasis and mucosal damage, the underlying signaling pathways appear to differ significantly. Multiple signaling pathways may converge to upregulate mucosal host defense and repair under stress conditions, whereas maintenance of the epithelial barrier function under homeostatic conditions significantly relies on TRIF.

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Disclosures

The authors have no financial conflicts of interest.

References


