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Lymphotoxin β Receptor Activation on Macrophages Induces Cross-Tolerance to TLR4 and TLR9 Ligands

Nadin Wimmer,* Barbara Huber,* Nicola Barabas,* Johann Röhrl,* Klaus Pfeffer,† and Thomas Hehlgans*

Our previous studies indicated that lymphotoxin β receptor (LTβR) activation controls and downregulates inflammatory reactions. In this study, we report that LTβR activation on primary mouse macrophages results in induction of tripartite motif containing (TRIM) 30α, which negatively regulates NF-κB activation induced by TLR signaling. LTβR activation results in a downregulation of proinflammatory cytokine and mediator expression upon TLR restimulation, demonstrating that LTβR signaling is involved in the induction of TLR cross-tolerance. Specific knockdown experiments using TRIM30α-specific small interfering RNA abolished the LTβR-dependent induction of TRIM30α and LTβR-mediated TLR cross-tolerance. Concordantly, LTβR activation on bone marrow-derived macrophages induced cross-tolerance to TLR4 and TLR9 ligands in vitro. Furthermore, we have generated cell type-specific LTβR-deficient mice with ablation of LTβR expression on macrophages/neutrophils (LTβR<sup>F<sub>lox/lox</sub></sup> × LysM-Cre). In bone marrow-derived macrophages derived from these mice LTβR-induced cross-tolerance to TLR4 and TLR9 ligands was impaired. Additionally, mice with a conditional ablation of LTβR expression on macrophages (LTβR<sup>F<sub>lox/lox</sub></sup> × LysM-Cre) are resistant to LTβR-induced TLR4 tolerance in vivo. Collectively, our data indicate that LTβR activation on macrophages by T cell-derived lymphotoxin α<sub>1</sub>β<sub>2</sub> controls proinflammatory responses by activation of a TRIM30α-controlled, counterregulatory signaling pathway to protect against exacerbating inflammatory reactions. The Journal of Immunology, 2012, 188: 3426–3433.

Inflammation is a complex pathophysiological condition initially mediated primarily by innate immune cells in response to infection and/or tissue damage (1, 2). Innate immune cells detect and respond to danger signals such as pathogens and/or tissue damage by activating their TLRs on their cell surface. However, chronic and repeated stimulation through TLRs renders immune cells hyporesponsive to subsequent stimulation, a phenomenon known as TLR tolerance (3). The activation of innate immune cells triggers a robust but essential inflammatory response that needs to be tightly regulated (4, 5). Uncontrolled inflammatory reactions lead to extensive tissue damage and the manifestation of pathophysiologic conditions such as chronic inflammation, sepsis, and autoimmune disease (6). Membrane-anchored lymphotoxin (LT)α<sub>1</sub>β<sub>2</sub> and LIGHT, both members of the TNF superfamily, are functional ligands for the LTβ receptor (LTβR). Both ligands are expressed only on activated lymphocytes, NK cells, and a subset of follicular B cells, whereas the LTβR is primarily expressed on epithelial and stromal cells and cells of the myeloid lineage (7, 8). So far, most studies have focused on the critical role of LTβR signaling in the development and maintenance of secondary lymphoid organ integrity (9–11) and the control of dendritic cell-mediated immune homeostasis (12, 13). Furthermore, some reports have demonstrated a critical role for LTβR signaling for the protection against Citrobacter rodentium-induced colitis (14, 15) and Mycobacterium tuberculosis, Listeria monocytogenes (16), as well as cytomegalovirus (17) infections. Recent results have shown that ablation of LTβR signaling using either a functional inhibitor of LTβR activation (LTβR:lg) or LTβR-deficient mice or mice deficient for the T cell-derived ligand LTβ results in a significant aggravation of inflammation (18). Activation of the LTβR by its membrane-associated ligand LTαβ, but not LIGHT, seems to be crucial for the downregulation of the inflammatory response (19). However, the cellular and molecular mechanisms underlying this protective role of LTβR activation have so far not been elucidated.

In this study we have identified tripartite motif containing (TRIM) 30α, a negative regulator of TLR-induced NF-κB activation, as a target gene of LTβR signaling in bone marrow-derived macrophages (BMDM). LTβR-induced TRIM30α expression inhibits the production of proinflammatory cytokines and mediators upon TLR4 and TLR9 restimulation, demonstrating, to our knowledge, for the first time that LTβR activation induces cross-tolerance in TLR-induced cytokine and proinflammatory mediator production.

Furthermore, we have generated cell type-specific LTβR-deficient mice with ablation of LTβR expression on macrophages/neutrophils (LTβR<sup>F<sub>lox/lox</sub></sup> × LysM-Cre). BMDM derived from these mice are resistant to TLR4 and TLR9 tolerance in vitro. Additionally, no tolerance could be induced in the model of TLR4-induced cytokine production in vivo. Collectively, our data suggest that cell-type specific LTβR signaling is critically involved in the regulation of innate inflammatory immune reactions.

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Abbreviations used in this article: BMDM, bone marrow-derived macrophage; BMDN, bone marrow-derived neutrophil; ES, embryonic stem; LT, lymphotoxin; LTβR, lymphotoxin β receptor; RPEC, resident peritoneal exudate cell; siRNA, small interfering RNA; TRIM, tripartite motif containing.

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Materials and Methods

Generation of LTβR floxed mice

A genomic clone derived from a mouse 129sv/J genomic library encompassing the complete coding sequence for the LTβR locus has been described earlier (9). This clone was modified by introducing an FRT-flanked neomycin resistance cassette followed by a loxP signal at the 3' end into the EcoRI restriction site within the 5' untranslated region of the LTβR gene. Additionally, a second loxP site had been introduced into the second intron of the LTβR gene.

E14.1 embryonic stem (ES) cells were transfected with linearized LTβR targeting vector as described (20). G418-resistant ES cell colonies were picked. Homologous recombination was screened by PCR and subsequently confirmed by genomic Southern blotting after digest of the ES cell DNA with EcoRI and hybridization of the flanking probe, located 5' of the targeting vector in the genomic locus. Location and orientation of both loxP sites and the FRT flanked neomycin resistance cassette were verified by cloning of the corresponding PCR products and subsequent sequence analysis. Single integration of the targeting vector was verified by Southern blotting with the neomycin resistance cassette. Chimeric mice were generated as described (20). Deletion of the neomycin resistance cassette in vivo was achieved by breeding chimeric mice with FLPe deleter mice (21). Deletion of the neomycin resistance cassette was verified by Southern blotting. Mice were housed in an animal facility with barrier conditions. For genotyping by PCR the following primers were used: 5'-GAAGCATAGCATGTGTCACGG-3' and 5'-CTATTGAGGCAATGGG-GAAAGAGGAG-3'.

Mice

Female C57BL/6 mice (wild-type) were obtained from Janvier (Le Genest, France). LTβR-deficient mice (LTβR<sup>-/-</sup>), LTβRβ-deficient mice (LTβRβ<sup>-/-</sup>), and LIGHT-deficient mice (LIGHT<sup>-/-</sup>) have been described previously (9, 22-25). MyD88-deficient mice (MyD88<sup>-/-</sup>) and TNF-deficient mice (TNF<sup>-/-</sup>) were housed at the animal facility of the University of Regensburg and have been described previously (26, 27). Mice used for experiments were age, sex, and weight matched.

In vitro stimulation assays

The mouse macrophage cell line J774 (ATCC TIB-67) was maintained in humidified 5% CO<sub>2</sub> at 37°C in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 U/ml streptomycin. BMDM were generated as described previously (28). Stimulations were performed at day 7 in triplicates (1 x 10<sup>6</sup> cells/ml) using 10 μg/ml agonistic rat anti-mLTβR mAb (clone 5G11b, IgG2a; Hycult Biotech, Uden, The Netherlands), 10 μg/ml rat IgG (Sigma-Aldrich, Taufkirchen, Germany), or 100 ng/ml LPS from Salmonella enterica serotype abortus equi (Sigma-Aldrich, Hamburg, Germany) for the indicated times. Naive CD4<sup>+</sup> T cells were purified from single-cell suspensions of spleen using a CD4<sup>+</sup> T cell isolation kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany). T cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) for 14 h, additionally treated for 4 h with 10 μg/ml mLTβR-Ig or left unstimulated. T cells were washed three times with RPMI 1640 and cocomultured in increasing ratios (1:1, 1:5, and 1:10) with BMDM for 24 h in RPMI 1640 (Sigma-Aldrich) supplemented with 10% inactivated FCS, 100 U/ml penicillin, and 100 U/ml streptomycin.

Flow cytometry

Expression of LTβR was detected by FACS analysis (BD LSR II; BD Biosciences, San Jose, CA) using a specific rat anti-mLTβR biotin-conjugated Ab (clone 3CS, IgG1; eBioscience, San Diego, CA) or an irrelevant isotype-matched rat IgG as negative control followed by an allophycocyanin-conjugated streptavidin Ab.

Small interfering RNA experiments

J774 cells were transiently transfected with 1 μM TRIM30α-specific small interfering (siRNA) or scrambled siRNA using INTERFERin siRNA transfection reagent (Polyplus-Transfection, Illkirch, France) according to the manufacturer’s instructions. The siRNA constructs were designed as described (29).

TLR tolerance

The cells were not stimulated or were preincubated with 10 ng/ml rat IgG, 10 μg/ml agonistic anti-mLTβR mAb, or 100 ng/ml LPS (S. enterica serotype abortus equi; Sigma-Aldrich) for 30 h, washed twice with medium, and rechallenged with 100 ng/ml LPS or 1 μM CpG (Metabion, Martinsried, Germany) for 8 h or 200 ng/ml LPS and 20 ng/ml mouse IFN-γ (AbD Serotec, Kidlington, U.K.) for 24 h. TNF and IL-6 were measured in the supernatants by ELISA. Nitrite accumulation in the cell culture supernatants was measured by using the Griess assay.

On days 5 and 3 mice were i.p. pretreated with PBS, LPS (Escherichia coli, 0127:B8; Sigma-Aldrich) (50 μg/kg or 20 μg/kg), rat IgG (100 μg/mouse), or agonistic anti-mLTβR mAb (100 μg/mouse). On day 0 the mice were challenged with LPS at a dose of 10 μg/mouse. Serum was collected 1 h later and TNF was measured by ELISA. For analysis of the TRIM30α expression in vivo, mice were killed on day −2, resident peritoneal exudate cells (RPEC) were obtained by lavage of the peritoneum with 10 ml ice-cold RPMI 1640 containing 10% FCS.

RNA isolation and quantitative RT-PCR

Total RNA from cultured cells was extracted by using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. RNA was transcribed using the Promega (Mannheim, Germany) reverse transcription system following the manufacturer’s recommendations. Quantification of mouse TRIM30α mRNA was performed using an iQ multicolor real-time PCR detection system (Bio-Rad, München, Germany) following the manufacturer’s recommendations. For standardization, 18S RNA was amplified. Primers specific for TRIM30α were purchased from SABiosciences (Frederick, MD) following the manufacturer’s recommendations.

Western blotting

Analysis of TRIM30α protein expression was done using a polyclonal rabbit anti-TRIM30α antiserum generated by immunization of keyhole limpet hemocyanin-conjugated TRIM30α peptide (LHSQIKQNYLFLQ) synthesized by BioGenes (Berlin, Germany), as well as peroxidase-conjugated goat anti-rabbit IgG (whole molecule) Ab (Sigma-Aldrich). The detection of β-actin was performed with a peroxidase-conjugated chicken anti-mouse β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

ELISA

Mouse TNF, IL-6, and IFN-γ levels were measured with ELISA kits (R&D DuoSets; R&D Systems, Wiesbaden, Germany) according to the manufacturer’s protocol.

NO production

The NO production was determined using a Griess assay as described previously (30). The samples were analyzed in triplicates.

Statistics

Statistical analysis was performed using the Student t test. Data are expressed as mean ± SD, and p < 0.05 was considered statistically significant.

Results

Generation of cell type-specific LTβR-deficient mice

To study the cellular and molecular mechanisms underlying the protective role of LTβR activation against an exacerbating inflammatory reaction, we generated macrophage/neutrophil-specific LTβR-deficient mice by crossing LTβR<sup>Δ/Δ</sup> mice with LysM-Cre mice (Fig. 1). To inactivate the LTβR cell type specifically we made use of both the Cre/loxP and FRT/FLPe technology (21, 31). In the LTβR targeting vector encompassing the entire genomic LTβR gene locus, a 5' loxP site was inserted into the second intron of the LTβR gene (Supplemental Fig. 1). Additionally, a neomycin marker cassette flanked by FRT sites and a 3' loxP site were inserted into the 3' untranslated region of the LTβR gene. The targeting vector was introduced into mouse ES cells and homologous recombination was verified by Southern blot and PCR analysis (Fig. 1A, 1B). After germline transmission, heterozygous LTβR<sup>Δ/Δ</sup> mice were crossed with FLPe deleter mice to delete the neomycin cassette in vivo. Deletion was verified by Southern blot analysis using a neomycin-specific probe (data not shown). LTβR<sup>Δ/Δ</sup> mice were born at expected Men-
mRNA expression could be detected (Fig. 2A). Examination of TRIM30 expression has only been shown to negatively regulate TLR-mediated NF-κB activation in BMDCs (29). Indeed, we found that in addition to LPS, stimulation of BMDM derived from wild-type mice with an agonistic anti-LTβR mAb induced TRIM30α mRNA expression (Fig. 2A). In contrast, in BMDM derived from LTβR-deficient mice no increase in TRIM30α mRNA expression could be detected (Fig. 2A). Examination of BMDM derived from LTβR<sup>−/−</sup> × LysM-Cre mice and control mice (LTβR<sup>−/−</sup>) revealed that cell type-specific ablation of LTβR expression on BMDM results in a strongly reduced ability to induce TRIM30α expression as a consequence of impaired LTβR signaling (Fig. 2B). A time course analysis of TRIM30α expression after LTβR activation on BMDM derived from wild-type mice revealed that TRIM30α mRNA expression is transiently induced as early as 8 h after LTβR stimulation, reaching a peak after 16 h stimulation (Fig. 2E). To evaluate TRIM30α protein expression in BMDM derived from wild-type, LTβR-deficient mice, LTβR<sup>−/−</sup> × LysM-Cre mice, and control mice (LTβR<sup>−/−</sup>/<sup>−/−</sup>) we used a TRIM30α-specific polyclonal antiserum and analyzed TRIM30α expression after LTβR activation by Western blot analysis. These results clearly demonstrate a transient LTβR-dependent induction of TRIM30α protein expression consistent with the pattern of its mRNA expression (Fig. 2C, 2D, 2F). Interestingly, no TRIM30α protein expression was detected in BMDM derived from LTβR<sup>−/−</sup> × LysM-Cre mice after LTβR stimulation, supporting the observation of a strongly reduced TRIM30α mRNA induction in these cells (Fig. 2A, 2B). To test for a possible role of neutrophils in our experimental model using LTβR<sup>−/−</sup> × LysM-Cre mice, we assessed LTβR expression on neutrophils (Gr1<sup>+</sup>, CD11b<sup>+</sup>, and F4/80<sup>+</sup>) isolated from bone marrow cells (BMDM) derived from both genotypes. Interestingly, only ∼6% of the Gr1<sup>+</sup>, CD11b<sup>+</sup>, and F4/80<sup>+</sup> cell population isolated from bone marrow cells (BMDM) derived from LTβR<sup>−/−</sup> × LysM-Cre mice expressed LTβR on the cell surface. LTβR expression was ablated on neutrophils isolated from LTβR<sup>−/−</sup> × LysM-Cre mice (Fig. 2G). Examination of BMDM derived from LTβR<sup>−/−</sup> × LysM-Cre mice and control mice (LTβR<sup>−/−</sup>/<sup>−/−</sup>) revealed no significant induction of TRIM30α expression in both genotypes (Fig. 2H). Collectively, these data indicate that LTβR expression was successfully inactivated on BMDM derived from LTβR<sup>−/−</sup> × LysM-Cre mice, but no significant TRIM30α induction could be detected after LTβR stimulation in BMDM derived from LTβR<sup>−/−</sup> × LysM-Cre mice.

Furthermore, we investigated whether LTβR ligands (LTα/β, LIGHT) expressed on activated CD4<sup>+</sup> T cells are capable of inducing TRIM30α expression by activating the LTβR on BMDM. Such a mechanism would require cell–cell contact and imply the possibility of a crosstalk between activated lymphocytes and macrophages. Cocultivation of different ratios of activated CD4<sup>+</sup> T cells with BMDM derived from wild-type mice results in an increasing induction of TRIM30α mRNA expression (Fig. 3A). In

FIGURE 1. Conditional inactivation of the LTβR gene. (A and B) Generation of LTβR<sup>−/−</sup> mice. (A) Southern blot analysis of genomic DNA from E14.1 wild-type and targeted ES cells. Hybridization with the flanking probe yields a 17-kb fragment for the wild-type allele and a 4-kb fragment for the targeted allele. (B) PCR analysis of the integration of the 5′ loxP site after successful germline transmission. LTβR<sup><sub>−/−/−</sub></sup>, tail DNA from heterozygous mice; LTβR<sup><sub>−/−/wt</sub></sup>, tail DNA from homozygous mice; WT, tail DNA from control mouse.  

[C] Generation of macrophage/neutrophil-specific LTβR-deficient mice (LTβR<sup><sub>−/−</sub></sup> × LysM-Cre). (C) Southern blot analysis of LTβR gene deletion in BMDM. (D) Northern blot analysis of LTβR mRNA expression in BMDM derived from the indicated genotypes. (E) FACS analysis verifying the lack of LTβR protein expression on BMDM derived from LTβR<sup><sub>−/−</sub></sup> × LysM-Cre compared with the expression on LTβR<sup><sub>−/−</sub></sup> × LysM-Cre mice.
cells are currently under investigation.

To further confirm that LTβR activation on macrophages results in the induction of TRIM30α expression, we silenced TRIM30α expression by using siRNA in the mouse macrophage cell line J774, which we found positive for LTβR expression as revealed by RT-PCR and FACS analysis. Stimulation of J774 cells with agonistic anti-LTβR mAb (5G11, 10 μg/ml) at indicated time points revealed a polyclonal anti-TRIM30α or anti-β-actin (loading control) antiserum. More importantly, the induction of endogenous TRIM30α in J774 cells transfected with scrambled siRNA (Fig. 4B). We also noted that the expression of endogenous TRIM30α protein in nonstimulated J774 cells is inhibited using TRIM30α-specific siRNA (Fig. 4B). More importantly, the induction of TRIM30α protein expression after LTβR stimulation is efficiently inhibited using TRIM30α-specific siRNA compared with J774 cells transfected with scrambled siRNA (Fig. 4B).
Subsequently, we tested whether TRIM30α expression induced by LTβR activation regulates the production of proinflammatory mediators. Large amounts of TNF and IL-6 are detectable in the supernatant of J774 cells after stimulation with LPS (TLR4) and CpG (TLR9) whereas stimulation of these cells with agonistic anti-LTβR mAb could not induce proinflammatory cytokine expression (Fig. 4C, 4D and data not shown). As expected, pretreatment of J774 cells with LPS results in a significant suppression of TNF and IL-6 expression. Interestingly, pretreatment of J774 cells with anti-LTβR agonistic mAb resulted in a significant suppression of TNF and IL-6 expression compared with J774 cells not pretreated or cells pretreated with rat IgG (Fig. 4C, 4D). Additionally, in restimulation experiments using LPS and IFN-γ, a highly reduced production of NO, a key mediator of the antimicrobial immune response, was observed when J774 cells were prestimulated with agonistic anti-LTβR mAb (data not shown). Furthermore, we observed a higher cytokine production in nonstimulated J774 cells when expressing TRIM30α-specific siRNA compared with J774 cells expressing scrambled siRNA. Again, prestimulation of J774 cells with agonistic anti-LTβR mAb resulted in a significant suppression of TNF and IL-6 expression when transfected with scrambled siRNA upon restimulation with LPS. In contrast, no suppression of TNF and IL-6 production was detected in J774 cells prestimulated with agonistic anti-LTβR mAb when TRIM30α expression was silenced using specific TRIM30α siRNA (Fig. 4C, 4D). These results clearly demonstrate that LTβR prestimulation results in the suppression of proinflammatory mediators upon TLR restimulation in a TRIM30α-dependent manner.

We next tested whether TRIM30α expression induced by LTβR activation regulates the production of proinflammatory cytokines and mediators in primary macrophages. Large amounts of TNF

**FIGURE 3.** LTβR-mediated TRIM30α expression induced by activated T cells is independent of TNF expression and the MyD88 signaling pathway. (A) Quantitative RT-PCR analysis of TRIM30α induction after coculturing of BMDM derived from wild-type mice or LTβR-deficient mice with activated CD4+ T cells isolated from wild-type or LTβRIg/LIGHT double-deficient mice for 24 h. Additionally activated and LTβR:Ig-pretreated CD4+ T cells were incubated with BMDM derived from wild-type mice. Nonactivated CD4+ T cells were used as negative control. Data are expressed as means ± SD. Representative data from one out of two independent experiments are shown. (B and C) Quantitative RT-PCR analysis of TRIM30α induction in BMDM derived from wild-type mice and MyD88-deficient mice (B) or derived from wild-type mice and TNF-deficient mice (C) after stimulation with LPS (100 ng/ml), polynosinic-polycytidylic acid (25 µg/ml) agonistic anti-LTβR mAb (5G11, 10 µg/ml), or rat IgG (10 µg/ml) as control. Data are expressed as means ± SD. Representative data from one out of three independent experiments are shown.

**FIGURE 4.** LTβR signaling inhibits the production of TNF and IL-6 by inducing TRIM30α expression. (A and B) Analysis of TRIM30α mRNA expression by quantitative RT-PCR (A) and TRIM30α protein expression by Western blot (B) in J774 cells after transfection (24 h) with TRIM30α-specific siRNA or scrambled siRNA followed by stimulation with agonistic anti-LTβR mAb (5G11, 10 µg/ml). (C and D) ELISA of TNF (C) or IL-6 (D) using supernatant of J774 cells transfected (24 h) with specific TRIM30α siRNA or scrambled siRNA before stimulated with agonistic anti-LTβR mAb (5G11, 10 µg/ml), rat IgG (10 µg/ml), or LPS (100 ng/ml) for 30 h followed by restimulation with LPS (100 ng/ml) for 8 h. Differences were considered significant with a p value of <0.05. **p < 0.001, ***p < 0.0001.
and IL-6 are detectable in the supernatant of BMDM derived from wild-type mice, LTBR<sup>Box/Box</sup> × LysM-Cre mice, or LTBR<sup>Box/Box</sup> mice after stimulation with TLR4 ligand (LPS) and TLR9 ligand (CpG), whereas stimulation with agonistic anti-LTBR mAb alone could not induce proinflammatory cytokine expression (Fig. 5A–D and data not shown). No difference in the induction of TLR tolerance was observed in BMDM derived from LTBR<sup>Box/Box</sup> mice or LTBR<sup>Box/Box</sup> mice using TLR4 and TLR9 agonists (data not shown). As expected, prestimulation of BMDM derived from LTBR<sup>Box/Box</sup> mice but not from LTBR<sup>Box/Box</sup> mice with agonistic anti-LTBR mAb resulted in hyporesponsiveness for the induction of TNF and IL-6 after restimulation with the TLR ligands LPS or CpG (Fig. 5A–D). These results were further verified by using BMDM derived from LTBR-deficient mice and wild-type mice (Supplemental Fig. 2A–D). Additionally, restimulation with LPS and IFN-γ resulted in a highly reduced production of NO when using BMDM derived from LTBR<sup>Box/Box</sup> mice cells prestimulated with agonistic anti-LTBR mAb in contrast to BMDM derived from LTBR<sup>Box/Box</sup> × LysM-Cre mice (Fig. 5E). Collectively, these data support the conclusion that LTBR activation on macrophages induces TRIM30α expression, which renders these cells hyporesponsive with respect to proinflammatory cytokine and mediator expression.

**LTBR activation on macrophages induces TLR4 tolerance in vivo**

To further characterize the function of LTBR-induced TRIM30α expression in vivo we made use of the LPS-induced tolerance model. Pretreatment of wild-type mice with low doses of LPS on day 5 and day 3 before LPS rechallenge resulted in strongly reduced TNF serum levels compared with wild-type mice pretreated with PBS or rat IgG. Interestingly, pretreatment with agonistic anti-LTBR mAb also resulted in strongly reduced TNF serum levels after LPS rechallenge comparable to the levels observed after the pretreatment with low doses of LPS. This effect was abolished when using inactivated agonistic anti-LTBR mAb, thus excluding a possible LPS contamination of the Ab preparation (Supplemental Fig. 2E). Furthermore, LTBR-deficient mice were no longer able to mount a LPS tolerance induction by the treatment with anti-LTBR mAb, although reduced TNF serum levels were detected by pretreatment of these mice with low doses of LPS before LPS rechallenge (Supplemental Fig. 2F). The analysis of LTBR<sup>Box/Box</sup> × LysM-Cre mice in our experimental model demonstrated that pretreatment with agonistic anti-LTBR mAb does not result in reduced TNF serum levels, suggesting that LTBR signaling in macrophages seems to be involved in TLR tolerance in our experimental model (Fig 6B). As anticipated, pretreatment of the LTBR in LTBR<sup>Box/Box</sup> mice resulted in strongly reduced TNF serum levels, comparable to levels detected after pretreatment with low doses of LPS (Fig. 6A) or observed after LPS pretreatment of wild-type mice (Supplemental Fig. 2E). Whereas in both LTBR<sup>Box/Box</sup> × LysM-Cre mice and LTBR<sup>Box/Box</sup> mice LPS induces TRIM30α expression, the stimulation of LTBR<sup>Box/Box</sup> mice with agonistic anti-LTBR mAb results in the upregulation of TRIM30α mRNA expression, and no induction of TRIM30α mRNA was detected in LTBR<sup>Box/Box</sup> × LysM-Cre mice (Fig. 6C). This observation matches our additional findings of TRIM30α induction after stimulation with agonistic anti-LTBR mAb in LTBR-deficient mice (Supplemental Fig. 2G). These results clearly demonstrate that LTBR signaling in macrophages...
induces TRIM30α expression, which subsequently results in tolerance to the TLR4 ligand in vivo.

**Discussion**

Most studies on LTβR signaling have focused on the organization, development, and maintenance of lymphoid tissues and their role in adaptive immune responses (7). Our data demonstrate that LTβR signaling is very important for the control of innate immune reactions by inducing a negative feedback mechanism in myeloid cells while mounting the early innate immune response. LTβR-mediated induction of TRIM30α seems to be tightly controlled on mRNA as well as on the protein level, resulting in the suppression of proinflammatory cytokine and mediator expression in vitro and in vivo.

In addition to LTβR activation using an agonistic anti-LTβR mAb or activated CD4+ T cells expressing LTβR ligands, we also found that recombinant mouse LIGHT was capable of inducing TRIM30α expression in BMDM in vitro (data not shown). So far, LIGHT has been described as a costimulatory cytokine in T cell activation by interacting mainly with HVEM expressed on T cells (23, 35), but it is not yet known whether the interaction with the LTβR contributes to TRIM30α induction in vivo.

Although activation of TLR-mediated signaling pathways initiating an early inflammatory response are indispensable for protecting the host against pathogenic organisms, an excessive and/or prolonged activation may lead to both acute and chronic inflammatory diseases. Therefore, the intensity and duration of TLR responses must be tightly regulated. Downregulation of TLR signaling, called TLR tolerance, as well as cross-tolerance among various TLR ligands might have been developed to prevent excessive inflammatory damage to the host. Most likely TLR tolerance is achieved through multiple mechanisms involving the induction of negative regulators such as A20, IRAK-M, MyD88, as well as SOCS-1 (36–38). However, these negative regulators mediating self and cross-tolerance are induced by TLR signaling pathways. Based on our observation that activated lymphocytes expressing LTβR ligands induce TRIM30α expression in myeloid cells, it is tempting to speculate that activated lymphocytes as part of the adaptive immune response interact and communicate with myeloid cells to induce a signaling pathway that negatively regulates the early innate inflammatory response.

TRIM30α is a member of the TRIM superfamily, many of which are expressed upon TLR activation and in an IFN-dependent manner (39, 40). It has been shown that members of the TRIM superfamily are involved in a broad range of biological processes that are associated with innate immune reactions (41). A recent study showed that TRIM30α interacts with TAK1 and promotes the degradation of TAB2 and TAB3, resulting in an inhibition of TRAF6 auto-ubiquitylation and consequently the inhibition of NF-κB activation, which ultimately reduced proinflammatory cytokine expression (29). Because TAK1 plays a crucial role in NF-κB activation through other signaling pathways such as those initiated by TNF and IL-1 (42), TRIM30α might also play a role in regulating inflammatory responses in addition to those initiated by TLR activation.

A functional ortholog to TRIM30α in humans has not yet been identified based on sequence similarity; however, a similar pathway seems to operate in human cells. Using the human monocyte cell line THP-1 we were able to induce LTβR-mediated TLR tolerance (data not shown), suggesting that some proteins seem to regulate proinflammatory cytokine and mediator expression in an LTβR-dependent manner in human cells of myeloid origin.

The exact molecular mechanisms by which LTβR induces TRIM30α expression in myeloid cells are currently under investigation; however, within this study, using a novel conditional LTβR mouse line, important information on the role of LTβR/
TRIM30a actions could be provided that is essential for protection against an exacerbating inflammatory immune response.

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Disclosures

The authors have no financial conflicts of interest.
Supplemental Figure 1

LTjIR genomic locus

Targeting vector

Targeted allele

LTjIR (floxed) allele

Supplemental Figure 1
Supplemental Figure 2

A

B

C

D

E

F

G

Pretreatment: * rat IgG umLTjR mAb

Challenge: LPS LPS LPS

BMDM C57BL/6

BMDM LTjR −

TNF (pg/ml)

IL-4 (pg/ml)

TNF (pg/ml)

NO (μM)

Pretreatment: * rat IgG umLTjR mAb

Challenge: LPS LPS LPS

Pretreatment: * rat IgG umLTjR mAb

Challenge: CpG CpG CpG

Pretreatment: * rat IgG umLTjR mAb

Challenge: LPS+IFN LPS+IFN LPS+IFN

Pretreatment: * rat IgG umLTjR mAb

Challenge: LPS LPS LPS

C57BL/6

LTjR −

TNF (ng/ml)

TNF (pg/ml)

TNF (pg/ml)

TNF (pg/ml)

Treatment:

C57BL/6

LTjR −

C57BL/6

LTjR −
**Supplemental Fig. 1:** Conditional inactivation of the LTβR gene.

Targeting strategy used for conditional inactivation of the LTβR gene (E: EcoR1 restriction-site). Numbers above gene structure indicate fragment size; numbers below gene structure correspond to the exon structure of the LTβR gene. Primers and the size of PCR products are indicated.

**Supplemental Fig. 2:** LTβR signalling in BMDM inhibits the production of pro-inflammatory cytokine and mediator expression *in vitro* and *in vivo*.

A-D, TNF (*A* and *C*), IL-6 (*B*) or NO production (*D*) in the supernatant of BMDM derived from wild-type mice and LTβR-deficient mice stimulated with agonistic anti-LTβR monoclonal antibody (5G11, 10 μg/ml) or rat IgG (10 μg/ml) for 30 h before re-stimulation with LPS (100 ng/ml) for 8 h (*A* and *B*), CpG (1 μM) for 8 h (*C*) or LPS (200 ng/ml) and IFN-γ (20 ng/ml) for 24 h (*D*). Data are expressed as mean ± SD. Statistical significance was determined using Student’s *t*-test. Differences were considered significant with a p-value of p< 0.05. *, p< 0.05 ; **, p< 0.001 ; ***, p< 0.0001. Representative data from one out of three independent experiments are shown.

*E* and *F*, ELISA of TNF serum levels in wild-type mice (*n*=5) (*E*) and LTβR-deficient mice (*n*=5) (*F*) after pre-treatment with agonistic anti-LTβR monoclonal antibody (5G11, 100 μg/mouse) or rat IgG (100 μg/mouse) or LPS (50 μg/kg and 20 μg/kg) following challenge with LPS (10 μg/mouse) for 1 h. Data are expressed as mean ± SD. Statistical significance was determined using Student’s *t*-test. Differences were considered significant with a p-value of p< 0.05. *, p< 0.05 ; **, p< 0.001. *G*, Quantitative RT-PCR analysis of TRIM30α expression in RPC derived from wild-type mice (*n*=3) and LTβR-deficient mice (*n*=3) after pre-treatment with agonistic anti-LTβR monoclonal antibody (5G11, 100 μg/mouse) or rat IgG (100 μg/mouse) or LPS
(50 μg/kg and 20 μg/kg) in vivo. Representative data from one out of two independent experiments are shown.