The receptor TREML4 amplifies TLR7-mediated signaling during antiviral responses and autoimmunity

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The molecules and pathways that fine-tune innate inflammatory responses mediated by Toll-like receptor 7 (TLR7) remain to be fully elucidated. Using an unbiased genome-scale screen with short hairpin RNA (shRNA), we identified the receptor TREML4 as an essential positive regulator of TLR7 signaling. Macrophages from Treml4<sup>−/−</sup> mice were hyporesponsive to TLR7 agonists and failed to produce type I interferons due to impaired phosphorylation of the transcription factor STAT1 by the mitogen-activated protein kinase p38 and decreased recruitment of the adaptor MyD88 to TLR7. TREML4 deficiency reduced the production of inflammatory cytokines and autoantibodies in MRL/lpr mice, which are prone to systemic lupus erythematosus (SLE), and inhibited the antiviral immune response to influenza virus. Our data identify TREML4 as a positive regulator of TLR7 signaling and provide insight into the molecular mechanisms that control antiviral immunity and the development of autoimmunity.

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pathogenesis of SLE in humans and mice. Male BXSB mice with a Y-linked autoimmune accelerator locus (Yaa) develop spontaneous SLE-like disease due to a duplication of a 4-megabase gene segment containing sequence encoding TLR7 transposed to the Y chromosome\textsuperscript{18,19}. This duplication is responsible for the autoimmune phenotype of Yaa male mice, because a reduction in the copy number of transcripts encoding TLR7 abrogates this disease\textsuperscript{20}. Furthermore, the progeny of mice of the MRL/lpr (MRL lymphoproliferation) strain, which spontaneously develop SLE, backcrossed to TLR7-deficient mice have much less renal disease and fewer autoantibodies to RNA-associated autoantigens\textsuperscript{21,22}. Finally, single-nucleotide polymorphisms in the gene encoding TLR7 have been identified that are associated with increased transcript expression and increased risk for SLE in humans\textsuperscript{23}. Together these observations indicate that TLR7 signaling is critical for SLE-specific autoimmunity.

To identify genes encoding molecules required for the TLR7-mediated activation of cells of the immune system, we performed a genome-scale RNA-mediated interference (RNAi)-based screen in which expression condition expression of mouse macrophages and identified TREML4 as a positive regulator of TLR7 signaling. TREML4 was required for TLR7-mediated responsiveness to TLR7 ligands and promoted TLR7-induced activation and phosphorylation of the MAPK p38 and the transcription factor STAT1 and the trafficking and localization of MyD88 and TLR7 to endosomes. We also found that TREML4 was critical for the antiviral immune response to the ssRNA influenza virus. Finally, TREML4 deficiency ameliorated inflammatory cytokine production and kidney disease in SLE-prone MRL/lpr mice. Thus, our findings demonstrate a critical role for TREML4 in promoting TLR7-mediated cytokine production during antiviral responses and the development of autoimmunity.

RESULTS
TREML4 positively regulates the TLR7 pathway
To identify genes and pathways required for TLR7-induced cell activation, we performed an unbiased genome-scale pooled lentivirus-based RNAi screen with a mouse short hairpin RNA (shRNA) library (with \(~4 \times 10^4\) members) developed by The RNAi Consortium\textsuperscript{24,25}. We used a construct in which expression of enhanced green fluorescent protein (GFP) is driven by the promoter of the gene encoding ELAM-1 ("endothelial leukocyte adhesion molecule 1"), which is responsive to the transcription factor NF-kB (NF-kB–GFP), and stably transduced RAW 264.7 mouse macrophages with this (to generate ‘RAW-GFP’ reporter cells). We transduced the RAW-GFP cells with the pool of \(~4 \times 10^4\) lentivirus-encoded mouse shRNAs. With this approach, \(~8 \times 10^3\) mouse genes were targeted by five or more independent shRNAs per gene, and cells received an average of \(~0.3\) shRNAs (multiplicity of infection, 0.3); each shRNA was introduced into \(~2 \times 10^4\) independent cells. We treated the shRNA-transduced cells with the synthetic TLR7-specific agonist gardiquimod (GRD) for 6 h and sorted the cells by flow cytometry into two populations, TNF\textsuperscript{lo}GFP\textsuperscript{lo} and TNF\textsuperscript{hi}GFP\textsuperscript{hi}, on the basis of their expression of tumor-necrosis factor (TNF) and NF-kB–GFP. We then amplified the shRNA sequences by PCR from genomic DNA isolated from the two cell populations and hybridized the products to complementary microarrays. To identify the shRNAs that inhibited GRD-induced expression of TNF and NF-kB (i.e., positive regulators of TLR7 signaling), we measured the overrepresentation of shRNAs that were more often present in the TNF\textsuperscript{lo}GFP\textsuperscript{lo} sorted population of RAW-GFP cells than in the TNF\textsuperscript{hi}GFP\textsuperscript{hi} subset. By this approach, we identified 257 such ‘enriched’ shRNAs with a difference in abundance of greater than twofold (Fig. 1a and Supplementary Table 1). In the TNF\textsuperscript{lo}GFP\textsuperscript{lo} subset, we identified enrichment for shRNAs that targeted genes encoding molecules with well-established roles in the TLR7 signaling pathway, such as TLR7, MyD88, IRAK2, IRAK4, Traf6 and Irf7 (Fig. 1a and Supplementary Table 1). In addition, we identified several genes encoding molecules that have not been previously described as positive regulators of TLR7 signaling (Fig. 1a and Supplementary Table 1). Among these, an shRNA targeting Treml4 had the eleventh greatest enrichment in our RNAi-based screen, among the \(~4 \times 10^4\) shRNAs tested (difference in abundance (log), 3.42-fold; Fig. 1a and Supplementary Table 1). To confirm the screen results, we independently transduced RAW-GFP reporter cells with lentivirus containing either of two different shRNAs that targeted two distinct regions of Treml4 mRNA and had different sequences in the seed region known to mediate off-target effects. Quantitative PCR and immunoblot analysis of the shRNA-transduced RAW-GFP cells showed that both effectively knocked down expression of Treml4 (Fig. 1b,c) and demonstrated a significant correlation between the reduced expression of Treml4 mRNA and TREML4 protein and diminished expression of TNF and NF-kB–GFP in response to the stimulation of TLR7 (Fig. 1d). These data demonstrated that TREML4 was a positive regulator of TLR7 signaling. To investigate whether TREML4 regulates the signaling pathways downstream of other TLRs, we transfected RAW-GFP cells with shRNA-encoding viruses to silence the expression of Myd88 or Treml4 and treated the cells with agonists for TLR2-TLR1 (the synthetic lipopeptide Pam\textsubscript{C3}CSK\textsubscript{4}), TLR2-TLR6 (zymosan), TLR3 (the synthetic RNA duplex poly[I:C]), TLR4 (lipopolysaccharide [LPS]), TLR9 (CpG DNA) or TLR13 (rRNA). Silencing of Treml4 expression significantly reduced the ability of RAW-GFP cells to induce expression of NF-kB–GFP and TNF in response to agonists of TLR7, TLR9 and TLR13 but not in response to other TLR ligands (Fig. 1e). These data demonstrated that TREML4 was a positive regulator for a limited sub-group of intra-cellular TLRs in RAW macrophages.

We next investigated whether overexpression of TREML4 could amplify TLR signaling. We transfected human embryonic kidney (HEK) cells stably expressing TREML4 with plasmids containing CDNA encoding TLR2, TLR3, TLR4, TLR7, TLR9 or TLR13 and stimulated the cells with the cognate agonists of these TLRs. Overexpression of TREML4 significantly amplified TLR7-, TLR9- and TLR13-mediated induction of IL8 mRNA in HEK cells transfected to express TLR7, TLR9 or TLR13, respectively (Fig. 1f and Supplementary Fig. 1a). HEK cells overexpressing TREML4 in the absence of TLR7 overexpression did not produce IL8 mRNA in response to TLR7 agonists (Fig. 1f), which indicated that TREML4 was not activated directly by TLR7 ligands.

To investigate whether TREML4 regulates TLR7 signaling through its association with intracellular adaptors, we transfected TLR7-expressing HEK cells with plasmid encoding wild-type TREML4, TREML4 lacking the cytoplasmic domain or TREML4 containing a point substitution in the transmembrane domain (with replacement of leucine for the lysine at position 210) and measured IL8 mRNA following stimulation with the TLR7 ligand GRD. We found only a modest requirement for the cytosolic domain of TREML4 for TLR7 signaling (Fig. 1g). In contrast, HEK cells expressing TREML4 lacking the cytoplasmic domain had significant impairment in TLR7-induced expression of IL8 mRNA compared with that of HEK cells expressing wild-type TREML4 (Fig. 1g), which indicated that the charged lysine residue in the transmembrane domain was required for the TREML4-mediated amplification of TLR7 signaling.

Human and mouse TREML4 share about 40% amino acid identity; however, human TREML4 is truncated compared with its mouse...
TREML4 positively regulates TLR7 signaling. (a) Expression analysis of shRNAs present in the TNFαGFPφ sorted population of RAW-GFP macrophages treated for 6 h with GRD; results are presented relative to those of the TNFαGFPφ subset. Area outlined in green indicates 257 ‘enriched’ shRNAs with a difference in expression (log2 values) of over twofold (right margin, mRNAs targeted); P = 0.05 for those shRNAs, and P = 0.012 for shRNA targeting Treml4 mRNA (Mann-Whitney test). (b) Quantitative PCR analysis of Treml4 mRNA in RAW-GFP cells transduced with lentivirus containing either of two different shRNAs targeting Treml4 (shTREML4) or control shRNA scrambled sequence (shCtrl). (c) Immunoblot analysis of TREML4 and GAPDH (loading control throughout) in RAW-GFP cells transduced with shRNA-containing lentivirus as in (d) Flow cytometry of RAW-GFP cells transduced with control shRNA or shRNA targeting Treml4 (hairpin 2 in b) and left untreated (UT) or treated for 6 h with GRD (10 µg/ml). Numbers in top right quadrants indicate percent TNF-α-FITC+ cells. P < 0.01 shCtrl versus shTREML4, GRD treated (Mann-Whitney test). (e) Induction of TNF-α and NF-κB in RAW-GFP cells transduced with lentivirus containing shRNA targeting MyD88 (shMyD88) or TREML4 (horizontal axis) and left untreated or treated for 4 h with specified TLR agonists (key), analyzed by flow cytometry and presented relative to induction after transduction of control shRNA, set as 100%. (f) Quantitative PCR analysis of IL8 mRNA in HEK cells left untransfected (HEK) or transfected to overexpress TREML4 alone (HEK-TREML4) or express TREML4 alone (HEK-TLR7) or both in combination (HEK-TLR7-TREML4), and treated for 3 h with various concentrations of GRD (horizontal axis); results are presented relative to those of untreated HEK cells (0). (g) Quantitative PCR analysis of IL8 mRNA in HEK cells left untransfected or transfected to express TLR7 (key) plus wild-type TREML4 (TREML4(WT)), TREML4 with a point substitution in the transmembrane domain (TREML4(K210L) or TREML4 lacking the cytoplasmic domain (TREML4(Δcyto)), or no TREML4 (Mock), and treated for 3 h with GRD (10 µg/ml); results are presented relative to those of mock-transfected HEK cells. (h) Quantitative PCR analysis of IL8 mRNA in THP-1 cells transfected with lentivirus encoding control shRNA or five different shRNAs targeting various sequences in human TREML4 (1–5, horizontal axis), and treated for 3 h with GRD (10 µg/ml); results are presented relative to those of untreated cells transfected with shCtrl. *P < 0.05 and **P < 0.01 (Mann-Whitney test).

Data are from one experiment representative of two experiments (a,c) or one experiment representative of three experiments (b,d–h; median and s.d.).

counterpart due to insertion of a stop codon just downstream of sequence encoding the charged lysine residue in the transmembrane domain. To determine whether human TREML4 regulates TLR7 signaling, we silenced TREML4 expression in the human monocyte cell line THP-1 by lentiviral transduction of TREML4-specific shRNA. THP-1 cells in which TREML4 was silenced had lower expression of IL8 mRNA following stimulation with the TLR7 ligand GRD than did THP-1 cells transfected with nontargeting control shRNA (Fig. 1h), which indicated an evolutionarily conserved function for TREML4 in the regulation of TLR7 signaling.

Because TREML4 binds to late apoptotic and necrotic cells and because nucleic acids can accumulate in apoptotic blebs or leak from necrotic cells, we investigated whether nucleic-acid ligands for TLR7, TLR9 and TLR13 could bind to TREML4 to induce TREML4 signaling. Because little is known about the signaling pathways and targets downstream of TREM receptors, we constructed a chimeric protein containing the extracellular domain of TREML4 fused to the intracellular domain of the TNF receptor TNFR1 and overexpressed it in HEK cells. This TREML4-TNFR1 chimeric receptor allowed us to quantify the binding of ligand to TREML4 through induction of the TNFR1-dependent signaling pathway that leads to the activation of NF-κB and expression of IL8 mRNA in HEK cells. The addition of late apoptotic cells to the culture medium induced robust induction of IL8 mRNA in cells expressing TREML4-TNFR1 but not those expressing a control construct of the C-type lectin receptor dectin-1 fused to TNFR1 (Supplementary Fig. 1b); this indicated a direct interaction between TREML4 and apoptotic cells. In contrast, following stimulation with GRD, CpG DNA or rRNA, we observed only weak induction of IL8 mRNA (less than twofold) in cells transfected to express TREML4-TNFR1 compared with its expression in mock-transfected cells (Supplementary Fig. 1b); this suggested that nucleic acids were not cognate ligands for TREML4. Together these results identified mouse and human TREML4 as positive regulators of signaling via TLR7, TLR9 and TLR13.

TREML4 positively regulates signaling via TLR7, TLR9 and TLR13

We performed quantitative PCR analysis to assess the expression of Treml4 mRNA in various organs of wild-type C57BL/6 mice. Treml4 was expressed mainly in the spleen (Supplementary Fig. 2a). Treml4 expression was low or absent on endothelial cells, B cells, T cells and natural killer cells but was detectable on macrophages, dendritic cells (DCs), neutrophils, bone marrow–derived DCs (BMDCs) and bone marrow–derived macrophages (Supplementary Fig. 2b), consistent with published reports suggesting restricted TREML4 expression on splenic macrophages and DCs. In addition, in vitro stimulation with the TLR7 ligand GRD induced a rapid increase in Treml4 mRNA expression in cultured splenic macrophages (Fig. 2a).

To further investigate the role of TREML4 in regulating TLR7 responses, we generated TREML4-deficient (Treml4−/−) mice in which TREML4 expression was ablated by replacement of exons 2–4, which encode most of the extracellular domain, with a neomycin-resistance cassette (Fig. 2b,c and Supplementary Fig. 2c). To determine whether TREML4 was required for TLR7–induced gene expression in splenic macrophages, we measured the induction of
mRNA encoding various cytokines, chemokines and transcription factors in GRD-stimulated splenic macrophages from Treml4−/− mice and their wild-type (Treml4+/+) littersates. Compared with the gene induction in Treml4+/+ cells, Treml4−/− splenic macrophages had significant impairment in the induction of 21 genes known to be activated by the TLR7 pathway, such as those encoding pro-inflammatory cytokines and chemokines and type I interferons, as well as interferon-induced genes, such as Cxcl10, Cxcl9 and Stat1 (Fig. 2d). We also stimulated Treml4+/+ and Treml4−/− splenocytes with agonists for TLR2, TLR3, TLR4, TLR7, TLR9 or TLR13. Treml4−/− splenocytes had significant impairment in GRD-, CpG DNA- and RNA-induced expression of Tnf mRNA compared with that of Treml4+/+ macrophages (Fig. 2e), which confirmed the results obtained by shRNA-mediated knockdown of TREML4 in RAW cells (Fig. 1e). Enzyme-linked immunosorbent assay (ELISA) of the supernatants of GRD-, CpG DNA- or LPS-stimulated splenocytes showed that in response to stimulation with GRD, secretion of TNF, IL-12 p40 (IL-12p40), interferon-β (IFN-β) and the interferon-inducible chemokine CXCL10 from Treml4−/− macrophages was significantly impaired compared with that of Treml4+/+ macrophages (Fig. 2f), while in response to stimulation with CpG DNA, the secretion of IFN-β and CXCL10 from Treml4−/− and Treml4+/+ macrophages was similar, but the secretion of TNF and IL-12p40 from Treml4−/− macrophages was significantly impaired compared with that of Treml4+/+ macrophages (Fig. 2f). These results suggested that TLR7-mediated activation of the interferon pathway was TREML4 dependent, while TLR9-mediated activation of the interferon pathway was TREML4 independent. We found no difference between Treml4+/+ and Treml4−/− macrophages in their cytokine production in response to stimulation with LPS (Fig. 2f), which indicated that TREML4 was not involved in regulating the TLR4 pathway.

We also investigated whether TREML4 modified TLR-induced cytokine production in neutrophils. We isolated CD11b+Ly6c− cells (neutrophils) from the bone marrow of Treml4−/− mice by magnetic bead-based negative selection and found that these cells had significant impairment in GRD- and RNA-induced expression of Cxcl2 mRNA compared with that of wild-type neutrophils (P<0.01 Mann-Whitney test; Supplementary Fig. 2d), which suggested that the TLR7- or TLR13-mediated induction of Cxcl2 in neutrophils was TREML4 dependent. In contrast, Treml4−/− neutrophils migrated normally in response to the peptide chemoattractant FMLP (formyl-methionyl-leucyl-phenylalanine) in a Transwell chemotaxis assay and had normal ability to ingest opsonized latex beads by phagocytosis compared with that of wild-type neutrophils (Supplementary Fig. 2e,f); this suggested that the migration and phagocytic pathways in neutrophils were TREML4 independent. Collectively, these data indicated that TREML4 was a nonredundant positive regulator of signaling via TLR7, TLR9 and TLR13 in splenic macrophages and neutrophils.

**TREML4 is critical for responses to ssRNA and influenza virus**

To investigate whether TREML4 also regulated TLR7 responses to other synthetic and natural ligands, such as the synthetic imidazoquinoline compound R-848 and the ssRNA influenza virus strain A/Puerto Rico/8/34 (PR8) intranasally into Treml4+/+ and Treml4−/− mice and used quantitative PCR analysis, flow cytometry and ELISA to assess induction of the expression of TNF, IL-12p40 (encoded by Il12b), IFN-β and CXCL10 (at the level of mRNA and protein) in the spleen and serum at 8 h after injection. Splenocytes from Treml4+/+ mice given injection of GRD, R-848 or influenza virus had high expression of Tnf, Il12b, Ifnb and Cxcl10 mRNA (Fig. 3a). In contrast, splenocytes isolated from Treml4−/− mice given injection of GRD, R-848 or influenza virus showed marked impairment in the expression of these

Figure 2 Regulation of TLR7 signaling by TREML4. (a) Quantitative PCR analysis of Trem4 mRNA in F4/80+ splenic macrophages isolated from Treml4−/− mice treated for 1, 3 or 8 h with GRD (10 µg/ml) or CpG DNA; results are presented relative to those of Gapdh mRNA (control gene). (b) Quantitative PCR analysis of Trem4 mRNA in the blood of Trem4−/−, Trem4+/− and Trem4−/− mice; results presented as in a. (c) Immunoblot analysis of TREML4 and GAPDH in spleen extracts isolated from Trem4−/−, Trem4+/− and Trem4−/− mice. (d) Quantitative PCR analysis of genes encoding inflammatory molecules (horizontal axis) in Trem4−/− and Trem4+/− F4/80+ splenic macrophages treated for 4 h with GRD (10 µg/ml); results are presented relative to those of untreated cells. (e) Quantitative PCR analysis of Tnf mRNA in Trem4−/− and Trem4+/− F4/80+ splenic macrophages treated for 4 h with various TLR ligands (horizontal axis); results are presented relative to those of untreated cells. (f) ELISA of TNF, IL-12p40, IFN-β and CXCL10 in supernatants of Trem4−/− and Trem4+/− F4/80+ splenic macrophages treated for 18 h with CpG DNA; results are presented relative to those of untreated cells.
inflammatory cytokine-encoding mRNAs (Fig. 3a). Intracellular flow cytometry showed that F4/80+CD11b+CD11c+ splenic macrophages from Treml4−/− mice given injection of GRD, R-848, influenza virus or CpG DNA produced significantly less TNF protein than did their Treml4+/+ counterparts (Fig. 3b). The amount of TNF or IL-12p40 protein in the serum was significantly lower in Treml4−/− mice than in Treml4+/+ mice following injection of GRD, R-848, CpG DNA, LPS or influenza virus (Fig. 3c). The serum concentration of IFN-β and CXCL10 was significantly lower in Treml4−/− mice given injection of GRD, R-848 or influenza virus than in their Treml4+/+ counterparts, but expression of these was induced by CpG DNA or LPS at similar level in Treml4−/− cells and Treml4+/+ cells (Fig. 3c), which indicated that the TLR7-mediated activation of the interferon pathway in vivo was TREML4 independent, while the TLR9- or TLR4-mediated activation of the interferon pathway in vivo was TREML4 dependent.

To further investigate the role of TREML4 in the antiviral host defense against influenza virus, we monitored weight loss and viral load in Treml4+/+ and Treml4−/− mice infected intranasally with a sublethal dose of influenza virus. Treml4−/− mice lost weight with kinetics similar to the weight loss of their Treml4+/+ littermates during the first week of infection (Fig. 3d). However, Treml4−/− mice continued to lose weight by day 9 after infection, while the weight of the Treml4+/+ mice plateaued and then began to gain steadily (Fig. 3d). Wild-type mice cleared the influenza virus between 10 d and 14 d after primary infection, and most Treml4+/+ mice survived, while the viral burden in the lungs of Treml4−/− mice was significantly higher than that of Treml4+/+ mice at day 10, and a majority of the Treml4−/− mice succumbed to infection (Fig. 3e, f). Together these data demonstrated that TREML4 was needed to control the early antiviral response to intranasal infection with influenza virus and to restrict the viral load in the lungs of infected mice.

To determine whether TREML4 was required for the immune response to other pathogenic viruses, we measured the expression of Tnf mRNA by quantitative PCR in splenic macrophages isolated from wild-type and Treml4−/− mice and then cultured with vesicular stomatitis virus (a ssRNA virus) or cytomegalovirus (a dsDNA virus). Treml4−/− macrophages had significant impairment in Tnf mRNA expression induced by vesicular stomatitis virus or cytomegalovirus compared with that of wild-type macrophages (* P < 0.01 (Mann-Whitney test); Supplementary Fig. 3a). This suggested that TREML4...
might have an important role in the antiviral immune response to a wide range of viruses that are pathogens in humans.

To determine whether the TREML4 deficiency in Treml4−/− mice had affected the cellular expression of TLR7 and/or members of the TLR7 signaling pathway, we assessed the expression of TLR7 in splenic macrophages and DCs from Treml4−/− mice. As shown by intracellular flow cytometry, TLR7 expression in Treml4−/− cells was similar to that in wild-type cells (Supplementary Fig. 3b). In addition, we found that untreated splenic macrophages from wild-type and Treml4−/− mice expressed similar amounts of genes encoding molecules critical for TLR7 signaling, such as Ifnar1, Ifnar2, Tlr7, Myd88, Irak1, Irak3, Irak4, Traf6, Tbk1, Syk, Dap12, Irf5, Irf7, Stat1 and Stat2 (Supplementary Fig. 3c), which suggested that decreased expression of TLR7 or other components of the TLR7 pathway did not account for the observed hyporesponsiveness of Treml4−/− mice to challenge with GRD, R-848 or influenza virus. Collectively, these data demonstrated an essential role for TREML4 in the antiviral host response to infection with influenza virus.

**TREML4 controls the activation of MAPKs and STAT1**

We next assessed the molecular mechanism of the hyporesponsiveness of Treml4−/− macrophages to TLR7 agonists. To determine whether TREML4 regulates TLR ligand–induced activation of MAPKs, we prepared BMDCs from Treml4+/+ and Treml4−/− mice and stimulated the cells with LPS, GRD or CpG DNA, then assessed, by intracellular flow cytometry, the accumulation of phosphorylated kinases Erk1 and Erk2 (collectively called ‘p44p42’ here) and phosphorylated p38. Stimulation with GRD caused a rapid increase (within 15 min) in phosphorylated p44p42 and phosphorylated p38 in Treml4+/+ BMDCs but not in Treml4−/− BMDCs (Fig. 4a,b). Following stimulation with CpG DNA, the phosphorylation of p44p42 was impaired in Treml4−/− BMDCs compared with that in their Treml4+/+ counterparts, but the phosphorylation of p38 was not (Fig. 4a,b); this suggested that TREML4 regulated the TLR7 and TLR9 signaling pathways differently. We observed no difference in the ability of LPS to activate MAPKs in Treml4+/+ BMDCs versus Treml4−/− BMDCs (Fig. 4a,b).

Activation of the transcription factor STAT1 is required for the production of interferons and interferon–regulated genes. Full activation of STAT1 occurs by phosphorylation of its Ser727 and Tyr701. Phosphorylation of STAT1 at Ser727 is mediated by TLR-induced p38, while phosphorylation of STAT1 at Tyr701 is TLR independent and autocrine signaling via type I interferons. Thus, phosphorylation of STAT1 at Ser727 is mediated by TLR–interferon–STAT1 signaling pathways.

The activation of transcription factor STAT1 is required for the production of interferons and interferon–regulated genes. Full activation of STAT1 occurs by phosphorylation of its Ser727. Phosphorylation of STAT1 at Ser727 is mediated by TLR-induced p38, while phosphorylation of STAT1 at Tyr701 is TLR independent on autocrine signaling via type I interferons. Thus, phosphorylation of STAT1 at Ser727 is TLR dependent and interferon independent, while phosphorylation of STAT1 at Tyr701 is TLR independent and interferon dependent. To determine whether TREML4 regulates the TLR7–induced phosphorylation of STAT1 at Ser727, we stimulated Treml4+/+ and Treml4−/− BMDCs with LPS, GRD or CpG DNA and assessed by intracellular flow cytometry the induction of such phosphorylation. We observed this phosphorylation in Treml4+/+ BMDCs stimulated with LPS, GRD or CpG DNA and in Treml4−/− BMDCs stimulated with LPS or CpG DNA but not in Treml4−/− BMDCs stimulated with GRD (Fig. 4c). These data indicated that the TLR7–mediated activation of p38 and phosphorylation of STAT1 at Ser727 were TREML4 dependent, while the TLR4- or TLR9–mediated activation of p38 and STAT1 was TREML4 independent. In addition, we found that the phosphorylation of STAT1 at Tyr701 was significantly impaired in Treml4−/− BMDCs treated with GRD compared with that in their Treml4+/+ counterparts (Fig. 4d), which suggested that TREML4 also amplified signaling via the IFNAR1 receptor for interferons. Because expression of the genes encoding IFN-α, IFN-β and CXCL10 is induced by STAT1, these observations were consistent with the reduced induction of these genes in response to stimulation of TLR7 but not in response to stimulation of TLR4 or TLR9 and suggested that TREML4 might mediate the cross-talk among the TLR, interferon and STAT1 signaling pathways.

**TREML4 regulates trafficking of MyD88 and TLR7**

To determine whether TREML4 regulates the intracellular trafficking of MyD88 to TLR7, we investigated whether TLR7 and MyD88 interacted following stimulation with GRD, TLR7 and MyD88 immunoprecipitated together in GRD–stimulated Treml4+/+ BMDCs, while we did not observe this association in Treml4−/− BMDCs (Fig. 5a). The binding and recruitment of MyD88 to TLR7 was TREML4 dependent (Fig. 5a). We also assessed by confocal microscopy the recruitment of MyD88 to TLR7 in BMDCs stimulated for 5 or 15 min with GRD and stained with fluorescence–labeled antibody to TLR7 (anti-TLR7) and anti–MyD88. We observed significant impairment in the trafficking and co–localization of MyD88 and TLR7 in Treml4−/− BMDCs compared with that in Treml4+/+ BMDCs (Fig. 5b), which suggested that
TREML4 regulated TLR7 signaling by controlling the recruitment of MyD88 to TLR7. To investigate whether TREML4 regulates the trafficking of TLR7 from the endoplasmic reticulum to endosomal and lysosomal compartments after stimulation, we stimulated Treml4+/+ and Treml4−/− BMDCs left unstimulated (−) or stimulated (+) for 15 min with GRD, followed by immunoprecipitation (IP) with anti-TLR7 (α-TLR7) or isotype-matched control antibody (isotype). (b,c) Double immunofluorescence of TLR7 plus MyD88, EEA1 or LAMP-1 on Treml4+/+ and Treml4−/− BMDCs left untreated or stimulated for 15 min with GRD (left), and quantification of such results for cells treated for 5 min or 15 min with GRD (right). Original magnification, ×400. *P < 0.01 (Student’s t-test). Data are from one experiment representative of at least three independent experiments (mean and s.d. in b,c).

TREML4-deficiency reduces autoimmunity in mice

To assess the effect of TREML4 deficiency on autoimmunity, we backcrossed Treml4−/− mice to MRL/lpr mice (which spontaneously develop an autoimmune disease with clinical features similar to human SLE, including autoantibody generation, activation of cells of the immune system and lethal glomerulonephritis) for eight generations. We used a HEp-2 antinuclear antibody (ANA) indirect fluorescent antibody assay for detecting antibodies to both RNA- and DNA-containing autoantigens in the serum of mice deficient in TREML4 (Fig. 6a). In both male Treml4−/− MRL/lpr mice and female Treml4−/− MRL/lpr mice, ELISA to quantify autoantibodies to dsDNA and the RNA-containing autoantigen Sm-RNP showed that the generation of autoantibodies to DNA and Sm-RNP was significantly lower in Treml4−/− MRL/lpr mice than in Treml4+/+ MRL/lpr mice (Fig. 6d). In addition, the highly specific Crithidia luciliae immunofluorescence assay showed substantially less binding of autoantibodies to the dsDNA of the C. luciliae kinetoplast in serum from Treml4+/+ MRL/lpr mice than in that from Treml4−/− MRL/lpr mice (data not shown). These results were consistent with published reports demonstrating impairment in the generation of autoantibodies to RNA- and DNA-containing autoantigens in MRL/lpr mice deficient in TLR7 signaling and TLR9 signaling.

We observed significantly fewer CD4+ T cells, CD8+ T cells and plasmacytoid DCs in the spleen and lymph nodes of Treml4−/− MRL/lpr mice than in those of Treml4+/+ MRL/lpr mice (Fig. 6e), as well as fewer activated CD44+ CD4+ T cells in the spleen of Treml4−/− MRL/lpr mice than in that of Treml4+/+ MRL/lpr mice (Fig. 6f). Splenocytes from Treml4−/− MRL/lpr mice had significantly lower expression of Cxcl10, Il1a, Il1b, Tnf, Il12b, Ifnb and Ifna mRNAs than did those from Treml4+/+ MRL/lpr mice (Fig. 6g), and the concentration of IFN-γ in the serum was significantly lower in Treml4−/− MRL/lpr mice than in Treml4+/+ MRL/lpr mice (Fig. 6h). Glomerular size, cellularity and deposition of protein in periodic acid Schiff-stained kidney sections were diminished in Treml4−/− MRL/lpr mice compared with that in Treml4+/− MRL/lpr mice (Fig. 6i). Furthermore, the composite renal disease score, which includes several parameters of nephritis (such as glomerular deposition of immunoglobulin G, proteinuria and blood urea nitrogen concentration), was significantly lower in Treml4−/− MRL/lpr mice than in Treml4+/− MRL/lpr mice (Fig. 6j). Consistent with their diminished kidney disease, Treml4−/− MRL/lpr mice showed significantly better survival (~88% survival at 26 weeks) than did Treml4+/− MRL/lpr mice (~50% survival at 26 weeks) (Fig. 6k). Finally, both male Treml4−− MRL/lpr mice and female Treml4−/− MRL/lpr mice were equally protected against SLE, including autoantibody generation, activation of cells of the immune system, inflammatory cytokine production and nephritis (data not shown). Collectively, these data demonstrated an essential role for TREML4 in the regulation of autoimmunity.
The ligands for receptors of the TREM family have largely remained elusive. Our data obtained with HEK cells transfected to express a chimeric TREML4-TNFR1 reporter suggested that none of the TLr ligands tested bound to or activated TREML4 directly. While the TREML4 ligand remains unknown, we did demonstrate that amplification of TLr7 signaling by TREML4 was dependent on the charged nature immunology association with the adaptor DAP12 (‘DNAX-activation protein, 12 kDa’), which bears an immunoreceptor tyrosine-based activation motif. TREML4 also associates with DAP12 but not with DAP10 or Fc receptors. DAP12-deficient macrophages have been shown to secrete increased amounts of cytokines afterTLr stimulation, which indicates that receptors that pair with DAP12 negatively regulate signaling through TLrs. Those data are in agreement with studies showing that the receptor TREM2, which pairs with DAP12, negatively regulates TLr signaling. Furthermore, those patients with Nasu-Hakola disease who develop bone cysts and presenile dementia have been found to have loss-of-function mutations in either the gene encoding DAP12 or that encoding TREM2, which indicates that this receptor–signaling adaptor pair is crucial for bone remodeling and brain function. In addition, variants in TREM2 have been associated with an increased risk of Alzheimer’s disease. Thus, DAP12 and TREM2 negatively regulate inflammatory pathways; however, what remains unclear is how DAP12 signaling downstream of TREM1 and TREML4 is involved in the positive regulation of TLr signaling.
pathways. One possibility is that TREM receptors may regulate TLR signaling in a DAP12-independent manner. Indeed, soluble forms of TREM1 and TREM2 are released from cells activated by inflammatory stimulation, and a soluble form of TREM4 has been shown to bind to dead cells. These soluble TREM receptors may interact with as-yet-unidentified ligands on cells to amplify or suppress inflammatory pathways. Additional studies are needed to address whether different signaling pathways are activated via soluble forms of TREM receptors versus the membrane-bound forms.

While the overexpression and genetic loss-of-function experiments indicated that TREM4 was essential in promoting TLR7 signaling, because the ligand for TREM4 is unknown, we were unable to induce TREM4 signaling directly and identify its function(s) in the absence of TLR7 signaling. The development of specific agonists of TREM4 will be very useful as a tool with which to delineate the key downstream signaling pathways responsible for TREM4-mediated amplification of signaling via TLRs and interferons, while antagonists of TREM4 may have therapeutic applications in dampening inflammatory autoimmune diseases. It is also possible that no endogenous ligands exist for TREM4 and perhaps, like other receptors such as TREM2, its expression and signaling through adaptors controls cellular functions such as transport to the cell surface, shedding of soluble receptors from the ectodomain, phagocytosis and the expression of co-stimulatory molecules. We found that similar to mouse TREM4, human TREM4 also positively regulated TLR7 signaling. Patients with acute coronary syndrome and coronary artery calcification have high expression of human TREM4 (refs. 39,40). Such results are consistent with the proposal of a role for TREM4 as an activating receptor and indicate it might have a role in cardiovascular disease. Together these data suggest that increased TREM4 expression could be a useful biomarker for the early detection of acute and chronic inflammatory disease in patients.

The ‘rescue’ of the autoimmune phenotype in MRL/lpr mice by deletion of TREM4 is similar to results obtained with MRL/lpr mice lacking MyD88, TLR7, or both TLR7 and TLR9, or MRL/lpr mice given injection of dual antagonists of TLR7-TLR9, which have much lower autoantibody titers to the autoantigens DNA and RNA and also have ameliorated disease (Ref. 41). We propose that in the absence of TREM4, MyD88 fails to traffic to TLR7, which results in disruption of the activation of p38 and the phosphorylation of STAT1. Similar to STAT1-deficient mice, mice lacking TREM4 are unable to produce interferon or express interferon-inducible genes in response to TLR7 stimulation and are protected from the development of lupus disease. Together these data demonstrate critical role for TREM4 in regulating TLR7 signaling and that therapeutic blockade of TREM4 holds considerable promise for the prevention of autoimmunity.

Although it is possible that therapy with antagonistic anti-TREM4 could be used to prevent and/or dampen lupus disease, caution may be warranted, as chronic therapeutic inhibition of signaling via TLR7 and TLR9 could potentially lead to increased susceptibility to infectious complications with microbial pathogens. While mice with mutations in genes encoding specific TLRs or key TLR signaling molecules are more susceptible to infection, their clinical disease signs are often mild and occur mainly in experimental settings with high doses of pathogens. Furthermore, these mice are healthy in standard husbandry conditions, which suggests that other pathways can overcome these defects. This is true for infection with influenza virus, which is controlled by at least three different classes of pattern-recognition receptors, including TLR7, TLR8 and NLRP3 (a member of the RIG-1 (retinoic acid–inducible gene I) and Nod (nucleotide-binding oligomerization domain)-like receptor family). The recognition of ssRNA from influenza virus by TLR7 mediates signaling that leads to the production of antiviral cytokines, such as type I interferons, but TLR7 signaling is not needed for an intact CD8+ T cell response. Instead, signaling via NLPR3 and the cytokine receptor IL-1R in DCs generates protective adaptive immunity by promoting CD8+ T cell responses to influenza virus. Finally, increased susceptibility to viral infections has not been noted in patients with SLE who have been treated with hydroxychloroquine, a molecule that raises lysosomal pH and inhibits TLR signaling, or in patients with SLE enrolled in clinical trials who have received inhibitors of TLR7 and TLR9. Thus, while mice deficient in TREM4 are more susceptible to infection with influenza virus, additional studies are needed for full analysis of the role of TREM4 in antiviral host defense to determine whether it may be possible to therapeutically inhibit TREM4-mediated TLR7 signaling in the long term for the treatment of autoimmune diseases without the potentially harmful complications that could arise from viral infections.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Z.G.R.-O., A.P., W.F.P., N.H. and T.K.M. planned the research, analyzed and interpreted data and wrote the manuscript; Z.G.R.-O., A.P. and T.K.M. did most of the experiments; A.P., J.W.G. and W.F.P. performed and analyzed ELISA, PCR and mouse pathology studies; G.S.C., D.E.R., A.D.L. and J.E.K. analyzed and interpreted data; M.T. helped with mouse breeding, genotyping and the production of lentiviruses encoding shRNA; M.T., E.K. and T.K.M. contributed to the generation of TREM4-deficient mice; and all authors participated in editing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Reagents. Reagents were from Sigma-Aldrich unless stated otherwise. DMEM and RPMI-1640 medium were from Invitrogen. Complete medium consisted of RPMI-1640 medium or DMEM supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine and 10% FCS. The following antibodies were used in this study: anti-TREM1 (16E5; BioLegend), anti-GADPH (7.11; Sigma-Aldrich), anti-TNF (MPF-XT22; R&D Systems), antibody to STAT1 phosphorylated at Tyr701 (58D6; Cell Signaling), antibody to STAT1 phosphorylated at Ser727 (D3B7; Cell Signaling), antibody to phosphorylated p44p42 (D13.14.4E; Cell Signaling), antibody to phosphorylated p38 (2B10; Cell Signaling), antibody-MyD88 (2E9C2; Thermo Scientific), anti-TLR7 (PA5-23489; Thermo Scientific), anti-TLR7 (PA5-23126), anti-CD11b (M1/70; ebioscience), anti-CD11c (N418; ebioscience), anti-F4/80 (BM8, ebioscience), anti-EEA1 (1G11; ebioscience) and anti-LAMP-1 (1D4B; ebioscience). All TLR agonists were from Invivogen. The HEK293T cell line (HCL41517) was from Fisher Scientific, and human THP-1 cells (TIB-202) were from American Type Culture Collection. All cell lines were determined negative for mycoplasma by analysis with a mycoplasma-detection PCR Kit (Sigma). Influenza virus strain A/Puerto Rico/8/34 (PR8; VR-1469; American Type Culture Collection) was grown in Madin Darby canine kidney cells in the Means laboratory.

Plasmids. The CDNA encoding mouse TREM1 was provided by H. Hemmi and R. Steinman. Plasmids encoding mouse TLR proteins were obtained from D. Golenbock or were from Invivogen.

Mice. All mice were maintained under micro-isolation in specific pathogen-free conditions at the Massachusetts General Hospital animal facility under a protocol approved by the Institutional Animal Care and Use Committee. For the generation of TREML4-deficient mice, a targeting vector containing 6,239 base pairs (bp) of 5′-flanking genomic DNA and 4,366 bp of 3′-flanking genomic DNA (HCL4517) was from Fisher Scientific, and human THP-1 cells (TIB-202) were from American Type Culture Collection. All cell lines were determined negative for mycoplasma by analysis with a mycoplasma-detection PCR Kit (Sigma). Influenza virus strain A/Puerto Rico/8/34 (PR8; VR-1469; American Type Culture Collection) was grown in Madin Darby canine kidney cells in the Means laboratory.

Isolation of BMDCs. For the enrichment of BMDCs, mice were killed and bone marrow cells were collected from the femurs and tibiae. Following treatment with red blood cell lysis buffer (00-4333-57; ebioscience), the remaining cells were suspended at a final concentration of 1 × 10^6 cells/ml in complete RPMI medium supplemented with 10% supernatant of J558L mouse myeloma cells as a source of the cytokine GM-CSF. Cells were seeded in non–tissue culture–treated Petri dishes and incubated at 37 °C with 5% CO2, and cells were fed every 3 d. At day 8, cells were harvested and seeded according the experimental approach.

Purification of bone marrow–derived neutrophils. Marrow cavities of the femurs of 8-week-old mice were flushed with DMEM. After hypotonic lysis of red blood cells, mature neutrophils were isolated by centrifugation for 30 min at 28 °C and 500g over discontinuous Percoll gradients consisting of 55%, 65% and 75% (vol/vol) Perc in PBS. Mature neutrophils were recovered at the interface of the 65% and 75% fractions. Neutrophil purity was >90% as assessed by flow cytometry with staining with anti-Ly6G (RB6-8C5; ebioscience) and anti-CD11b (M1/70; ebioscience).

Pooled shRNA screen. The genome-scale pooled shRNA assays were performed with 2.5 × 10^5 RAW-GFP reporter cells for each replicate placed in culture medium containing 4 µg/ml polybrene and a pool of ~4 × 10^6 shRNA-containing lentiviruses to achieve a multiplicity of infection of 0.3. After 24 h, the cells infected with that library were split into T175 flasks and were selected in culture medium containing puromycin. Five days later, 1 × 10^6 infected cells were treated for 6 h with GRD, then were harvested, stained with antibodies to detect intracellular TNF (MP6-XT22; R&D Systems) and sorted into two populations by flow cytometry on the basis of low and high dual expression of TNF and NF-κB-GFP. The shRNA sequences were amplified by PCR from the genomic DNA isolated from the sorted cells and were hybridized to complementary microarrays for analysis of the abundance of each shRNA with Dchip software.

Real-time quantitative PCR. Total RNA was extracted with an RNaseasy kit and was treated with DNase according to the manufacturer's protocol (Qiagen), and each sample was reverse-transcribed with MultiScribe reverse transcriptase (Applied Biosystems). Each 25-µl sample for quantitative PCR contained 2 µl of cDNA, 12.5 µl of 2× SYBR Green Master Mix (Applied Biosystems) and 500 nM of sense and antisense primers. The sequence of oligonucleotide primers (designed on the PrimerBank or Primer3 websites and obtained from Integrated DNA Technologies) were as follows: human GAPDH, 5′-AGTTAGAGTGCCGAGTC-3′ and 5′-GAAGATGTGGATGATGATT-3′; human IL-8, 5′-CTGGCGCGTGGCTCTTGTG-3′ and 5′-CCTTGGCAAACTCGACCT-3′; mouse TREM1, 5′-GGGTCGTTCGAGTCATGTT-3′ and 5′-GGTATCAAAACAACCGCCAG-3′; and 5′-GGGTCGTTCGAGTCATGTT-3′ and 5′-GGTATCAAAACAACCGCCAG-3′; mouse IL-1α, 5′-GGCACCCTTACACCTCAGACT-3′ and 5′-TGGCAGTCCATATTTAACAAAGTGTT-3′; mouse TNF, 5′-CCCCCTACACTCGATCATCTTTCT-3′ and 5′-GTGACCTGATCTCTTTTCTTACGACCTGATCATCTTTCT-3′; mouse CCL2, 5′-CAGAGTTCTGGCTTGGGATCCA-3′ and 5′-ATGGTTCTCCGTCGAAGGACT-3′; and mouse CCR7, 5′-GAGGATTTTCTGGGTATTGCTTGGGATCCA-3′ and 5′-ATGGTTCTCCGTCGAAGGACT-3′. PCR amplification conditions were as follows: 94 °C for 3 min; 30 cycles of 30 s each at 94 °C, 58 °C and 72 °C; followed by 72 °C for 10 min of incubation at 72 °C.
mouse IFN-α/β, 5′-TGTAGCTACTACTGGTCAGC-3′ and 5′-GATC TCTTATGCAAGATTGCG-3′; mouse IL-23α, 5′-AATAATTGCGGC CGTATCCAGT-3′ and 5′-GCTCCCTTTTGAAGATGTCG-3′; mouse MyD88, 5′-CTATGTTCTCATAACCTTGGT-3′ and 5′-AAATGCGGATG TGGGTCAGC-3′; mouse IRAK1, 5′-CCCACTGTTGATATGTGCG-3′ and 5′-GAGATTGTCAGGACCTGAGC-3′; mouse IRAK4, 5′-CATACC ACCTTAAATGGG-3′ and 5′-GGAACATTTGTATGTCGTCAGC-3′; mouse IRAK5, 5′-CTGCTGTTGATGCCTTTAATT-3′ and 5′-GGAGA AACCTCTAAGGTGCG-3′; mouse TRAF6, 5′-AAACGCAGAAATGTT CTCCCT-3′ and 5′-ACTTGGACCTAATCCATTAG-3′; mouse TBK1, 5′-ACTCTGTATCTCTAGTCTGA-3′ and 5′-TCTCTGGAGA CTACAGCCATT-3′; mouse SYK, 5′-CTACCTGGCAGGACAGC-3′ and 5′-GCCATTGATGCTCTCTCGATG-3′; mouse DAP12, 5′-GAGTAGA CTCTTTGCAAGATGC-3′ and 5′-CTCTGATCTGGAGGAACCA-3′; mouse IKKγ, 5′-AGAGCAGGGAATCACTGAG-3′ and 5′-TGGAT GTCACCGCTTTTTATAG-3′ and mouse STAT2, 5′-CTGAAGGAGG AACAGAGATGTC-3′ and 5′-CAGGGTTGTAAATCGGGCAA-3′. Emitted fluorescence for each reaction was measured three times during the annealing-extension phase, and amplification plots were analyzed with MX4000 software, version 3.0 (Stratagene). The quantity of gene expression was generated by comparison of the fluorescence generated by each sample with standard curves of known quantities, and the calculated number of copies was divided by the number of copies of the housekeeping gene encoding GAPDH.

**Histological assessment.** Histopathologic examination of kidney samples was done after routine fixation and embedding of the tissues in paraffin. Tissue sections from the skin were cut and stained with periodic acid–Schiff stain. All slides were coded and evaluated in a blinded manner in terms of the identity of the sample. Pathological changes in the kidney were assigned grades according to the presence of glomerular, interstitial, perivascular inflammation and deposition of immunocomplexes. Scores ranging from 0 (normal) to 4 (most severely inflamed) were assigned for each of the four features. A minimum of 50 glomeruli were assessed to determine the glomerular index in each mouse. Sections were visualized with a Nikon Eclipse ME600 fluorescent microscope equipped with a high-resolution DXM1200C Nikon digital camera. Data were analyzed with NIS-Elements software (Nikon) and Adobe Photoshop.

**Autoantibody profiles.** ANA and *Crithidia lucilae* immunofluorescence assays (Bio-Rad) were performed according to the manufacturer’s instructions, with serum at a dilution of 1:160–1:5120 or 1:80 for human shTREML4 (5), CCTCCATCAATGCTCTGAGA. Plasmids were purified with a QIAprep Miniprep kit (Qiagen). Plasmids were then transfected into HEK293T cells along with the plasmids pCMV-DR8.2 dpvr and pCMV-BSVG for the production of lentivirus. RAW-GFP or THP-1 cells were placed in 24-well tissue culture dishes (2 × 10⁵ cells per well) and were infected, followed by incubation for 2 d. Infected cells were selected in complete RPMI medium containing 10% (vol/vol) FBS and puromycin (5 µg/ml) and were tested 1 week after infection. The knockdown efficiency of shRNA was determined by quantitative PCR.

**Phosphorylation-specific flow cytometry.** Samples underwent enriched for BMDCs as described above. BMDCs (2 × 10⁶ cells per ml) were seeded in 5-ml polypropylene tubes containing complete RPMI medium. Cells were treated for 25 min at 37 °C with 5% CO₂ with 10 µg/ml of gadolinium (TLR7), 100 ng/ml LPS (TLR4) or 10 µU recombinant IFN-γ (Prepotech). Treated cells were fixed for 10 min at 4 °C with cold 2% paraformaldehyde. Cells were washed three times with cold flow cytometry buffer (PBS supplemented with 2% FCS) and were permeabilized for 20 min on ice with cold 100% methanol. Cells were washed twice for removal of methanol and were stained with the following fluorescence-labeled antibodies: allopurinocyanine–anti-CD11c (N418; BioScience), Alexa Fluor 488–conjugated antibody to p44/42 phosphorylated at Tyr202 and Tyr204 (D13.14.4E; Cell Signaling), phycoerythrin–conjugated antibody to pT38 phosphorylated at Thr180 and Tyr182 (28B10; Cell Signaling), antibody to STAT1 phosphorylated at Ser727 (D3B7; Cell Signaling) and antibody to STAT1 phosphorylated at Thr701 (58D6; Cell Signaling). Secondary staining for phosphorylated STAT1 was performed with Alexa Fluor 488–conjugated goat antibody to rabbit IgG (A-11080; Thermo Scientific). Samples were analyzed with a Becton Dickinson FACScalibur, and data were collected with CellQuest software and analyzed with FlowJo software (X version for Mac).

**Immunoblot analysis and co-immunoprecipitation.** Co-immunoprecipitation experiments were performed using BMDCs seeded in six-well plates at a final concentration of 1 × 10⁶ cells per ml in complete RPMI medium. Cells were incubated for 15 min at 37 °C with 5% CO₂ with 10 µg/ml gadolinium. Cells were harvested and lysed in 1× lysis buffer (Cell Signaling). Lysates were incubated for 24 h at 4 °C with anti–mouse TLR7 (PA-5-23489; Thermo Scientific) or the isotype-matched control antibody–mouse IgG (PA-5-23090; Thermo Scientific). Supernatants were incubated overnight at 4 °C with protein G agarose beads. Precipitated proteins were eluted, separated by SDS-PAGE and analyzed by immunoblot with anti–mouse MyD88 (2E9C2; Thermo Scientific).

**Confocal microscopy.** BMDCs were seeded in confocal dishes (Mattek) at a final concentration of 1 × 10⁶ cells per ml in complete RPMI medium. The following day, cells were treated for 5 min or 15 min at 37 °C with 10 µg/ml GRD. Cells were carefully washed with PBS and were fixed for 10 min at room temperature in the dark with 2% PFA. Fixed cells were permeabilized for 1 h at 37 °C with SAP buffer (2% saponin in PBS). Following permeabilization, BMDCs were stained intracellularly with anti–mouse TLR7 (PA-5-23489; Thermo Scientific), anti–mouse MyD88 (2E9C2; Thermo Scientific), anti–mouse EE1A (1G11; eBioscience) or anti–mouse LAMP-1 (1D4B; eBioscience), followed by staining with the secondary antibody Alexa Fluor 488–conjugated goat anti-rabbit (35552; Invitrogen) or Alex Fluor 550–conjugated goat anti-mouse antibody (84540; Invitrogen). Cells were washed three times with PBS. Finally, DNA was stained for 10 min at room temperature with 1:1,000 dilution of DAPI (4,6-diamidino-2-phenylindole; Invitrogen). Cells were visualized with a Nikon Eclipse ME600 fluorescence microscope equipped with a high-resolution DXM1200C Nikon digital camera. Data were analyzed with NIS-Elements software (Nikon) and Adobe Photoshop.
Transwell chemotaxis assay. Chemotaxis was assayed in 96-well chemotaxis chambers with polycarbonate membranes (pore size, 5 µm; Neuroprobe). CyQUANT dye mix was used for quantification of migrating cells. After 2 h of incubation, fluorescence was measured in a FluoroSkan Ascent fluorescent plate reader. fMLP was placed in the bottom well of a Neuroprobe chemotaxis chamber. Cells were spun (100 µl, containing on average 4 × 10⁴ cells), then were resuspended in 50 µl DMEM and placed on top of the filter in the chemotaxis chamber. After 3 h of incubation, the number of cells migrating to the lower chamber was determined with CyQUANT. The chemotactic index was determined by division of the number of migrating cells (as determined by CyQUANT fluorescence) by the number of input cells, and the results were normalized to 1 on the basis of the migration of untreated cells.

Infection with influenza virus. Mice were infected intranasally with a live influenza virus strain PR8 at a dose of 30–50 EID per gram body weight in 50 µl PBS. Some mice were killed on day 10 after infection, the lungs were collected and the viral load was determined by quantitative PCR with the following primers: forward primer, 5′-CGGTCCA AATTCCTGCTGA-3′; and reverse primer, 5′-CATTGCTTCCA ATCCA-3′. Other mice were monitored daily for 14–18 d for weight loss and survival.

Statistical analysis. Statistical calculations were performed with a statistical software package (GraphPad Prism 5.0d). For comparisons of two groups, mean ± s.e.m. values were analyzed by the two-tailed unpaired Student t test with the Bonferroni correction applied for multiple comparisons. For comparisons of greater than two groups, significance was determined by one- or two-way analysis of variance (ANOVA) with correction or Mann-Whitney Test. For survival studies, significance was determined with the log rank test.