Release from UNC93B1 reinforces the compartmentalized activation of select TLRs

Nucleic acid-sensing Toll-like receptors (TLRs) are subject to complex regulation to facilitate the recognition of microbial DNA and RNA while limiting the recognition of an organism’s own nucleic acids. Failure to properly regulate these TLRs can lead to autoimmune and autoinflammatory diseases. Intracellular localization of these receptors is thought to be crucial for the discrimination between self and non-self, but the molecular mechanisms that reinforce compartmentalized activation of intracellular TLRs remain poorly understood. Here we describe a mechanism that prevents the activation of TLR9 from locations other than endosomes. This control is achieved through the regulated release of the receptor from its trafficking chaperone UNC93B1, which occurs only within endosomes and is required for ligand binding and signal transduction. Preventing release of TLR9 from UNC93B1, either by mutations in UNC93B1 that increase affinity for TLR9 or through an artificial tether that impairs release, results in defective signalling. Whereas TLR9 and TLR3 are released from UNC93B1, TLR7 does not dissociate from UNC93B1 in endosomes and is regulated by distinct mechanisms. This work defines a checkpoint that reinforces the compartmentalized activation of TLR9, and provides a mechanism by which activation of individual endosomal TLRs may be distinctly regulated.

Disruption of TLR9 signalling but not trafficking

To dissect the mechanisms by which UNC93B1 regulates TLR signalling, we performed a scanning-alanine mutagenesis screen of UNC93B1 in RAW264.7 macrophages (see Methods). The screen identified an UNC93B1 mutation (SKN to AAA at residues 282–284 in loop 5, hereafter referred to as UNC93B1SKN) that abolished signalling of TLR9, but did not affect TLR7 or TLR3 (Fig. 1a, b). Notably, TLR9 trafficking appeared unaltered, as receptor cleavage, which occurs within endosomes, was unaffected (Fig. 1c). Also, UNC93B1SKN itself trafficked normally to LAMP1+ endosomes (Extended Data Fig. 1a). Disruption of TLR9 signalling without abrogating trafficking to endosomes was unexpected and suggested a new trafficking-independent function for UNC93B1.

We individually mutated each amino acid within the SKN motif to alanine and determined that the S282A mutation was sufficient to abolish TLR9 signalling, whereas K283A and N284A were not (Extended Data Fig. 1b). Similar to the UNC93B1SKN mutant, UNC93B1(S282A)-expressing macrophages failed to respond to TLR9, whereas TLR3, TLR7, TLR5 and TLR13 responses were unaffected (Fig. 1d and Extended Data Fig. 1c–f). Induction of IFNβ after stimulation with CpG-A conjugated to the cationic lipid Dotap (DOTAP–CpG-A) was also abrogated (Fig. 1e). Again, levels of cleaved TLR9 in phagosomes were similar compared to wild-type cells, indicating normal trafficking (Fig. 1f).

UNC93B1(S282A) inhibits DNA binding to TLR9

On the basis of the lumenal position of the UNC93B1(S282A) mutation, we reasoned that ligand binding might be affected. Indeed, CpG-B binding to TLR9 was reduced in mutant cells (Fig. 2a). Similar to the UNC93B1SKN mutant, UNC93B1(S282A)-expressing macrophages failed to respond to TLR9, whereas TLR3, TLR7, TLR5 and TLR13 responses were unaffected (Fig. 1d and Extended Data Fig. 1c–f). Induction of IFNβ after stimulation with CpG-A conjugated to the cationic lipid Dotap (DOTAP–CpG-A) was also abrogated (Fig. 1e). Again, levels of cleaved TLR9 in phagosomes were similar compared to wild-type cells, indicating normal trafficking (Fig. 1f).

We considered the possibility that a loss of interaction between UNC93B1(S282A) and TLR9 during trafficking might impair ligand binding to TLR9. Indeed, CpG-B binding to TLR9 was reduced in mutant cells (Fig. 2a). To rule out differences in DNA uptake or sampling, we verified that all cell lines were equally capable of endocytosing CpG-B (Extended Data Fig. 2a) and that the ligands were effectively delivered to LAMP1+ endolysosomes containing TLR9 (Extended Data Fig. 2b, c). We also ruled out defects in TLR9 dimerization and defects in the association between N-terminal and C-terminal fragments after receptor cleavage (Extended Data Fig. 3a, b). Altogether, these results suggested a reduced ability of TLR9 to bind ligand in UNC93B1(S282A)-expressing cells unrelated to known mechanisms of receptor regulation.

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binding. By contrast, TLR9 showed greater interaction with the UNC93B1 SKN and S282A mutants (Fig. 2b and Extended Data Fig. 4a). Interaction with TLR7 was unaffected (Extended Data Fig. 4b). Furthermore, the interaction with TLR9 was not observed when the neighbouring UNC93B1 residues K283 or N284 were mutated (Extended Data Fig. 4c). We confirmed the increased interaction between TLR9 and UNC93B1(S282A) by proximity ligation assay (Fig. 2c) and in purified phagosomes (Fig. 2d).

The binding interfaces between UNC93B1 and TLR9

Examination of additional UNC93B1 mutants within loop 5 for their effect on TLR9 signalling and binding (Extended Data Fig. 5a) identified a larger region within UNC93B1 (spanning amino acids 270–284 and containing Ser282) in which mutations either abolished or increased the interaction with TLR9 (Extended Data Fig. 5b). Irrespective of the direction, all mutations consistently reduced TLR9 signalling (Extended Data Fig. 5c), which highlights the importance of an optimal binding affinity between UNC93B1 and TLR9 for proper TLR9 function. Most of the mutations had little or no effect on TLR7 function (Extended Data Fig. 5c). To identify the reciprocal binding region in TLR9 that engages with the loop 5 region of UNC93B1, we used a set of TLR9 chimeric variants in which varying segments of the juxtamembrane and transmembrane domains had been replaced with the corresponding sequences of TLR3. As TLR3 is unaffected by UNC93B1(S282A) (Extended Data Fig. 1c), we reasoned that this strategy could identify crucial residues within TLR9 that mediate interaction with UNC93B1. The analysis identified a short motif of five amino acids (LSWDC) in the juxtamembrane region of TLR9 that when reverted to the corresponding TLR3 sequence (PFEIL) restored function and normal interaction in the presence of UNC93B1(S282A) (Extended Data Fig. 5d, e). We conclude that the LSWDC motif within TLR9 engages with loop 5 of UNC93B1 and that mutations within this interaction surface can alter TLR9–UNC93B1 association and affect TLR9 function.

We next explored the importance of loop 5 for function of human UNC93B1. Two out of three reported single nucleotide polymorphisms (SNPs) within the respective UNC93B1 region (from the NCBI dbSNP database, with minor allele frequencies between 8.5 × 10⁻² and 9.1 × 10⁻⁴) reduced TLR9 signalling (Extended Data Fig. 5f). The reduction in
explain the attenuated ligand binding of TLR9 when forcefully bound to receptor in an unfavourable conformation for ligand binding, which would signalling. UNC93B1 might interfere with DNA recognition by keeping the ‘sticky’ effect that prevents efficient release of TLR9 in endosomes.

The altered chemistry of the UNC93B1 mutant might have an overall in endosomes (Fig. 3b and Extended Data Fig. 7b), which suggests that TLR9 can be trafficked to endosomes before ligand binding and activation. We investigated this possibility by using cellular fractionation to separate ER and endosomes and measured the extent of the TLR9–UNC93B1 interaction in each organelle preparation (Fig. 3a and Extended Data Fig. 9a). The selected endosomal fractions contained high levels of CpG-B–biotin ligand (when fed to cells), indicating that we had enriched the relevant signal of TLR7 and UNC93B1 in endosomes is crucial for a distinct regulatory mechanism, which accompanies a manuscript, we describe how the continued association of UNC93B1 and TLR9 is mediated by a distinct interaction surface. Notably, the sensitivity of TLR3 to UNC93B1(S282A) suggests that interaction with UNC93B1 in endosomes (Extended Data Fig. 9b); however, the insensitivity of TLR7 to UNC93B1 is mediated by a distinct interaction surface. Notably, the sensitivity of TLR3 to UNC93B1(S282A) suggests that interaction with UNC93B1(S282A) might have an overall ‘sticky’ effect that prevents efficient release of TLR9 in endosomes.

On the basis of these results, we considered a release model, in which TLR9 must dissociate from UNC93B1 for efficient ligand binding and signalling. UNC93B1 might interfere with DNA recognition by keeping the receptor in an unfavourable conformation for ligand binding, which would explain the attenuated ligand binding of TLR9 when forcefully bound to UNC93B1(S282A) (Extended Data Fig. 7c). To test this model independently and more directly, we engineered a cysteine bridge between TLR9 and UNC93B1 to tether the two proteins together and prevent release. We thereby focused on the previously identified loop 5 binding region of UNC93B1 and the juxtamembrane region of TLR9 (Fig. 3c). We identified a pair of UNC93B1 and TLR9 cysteine mutants (UNC93B1(281C) and TLR9(812C)) that trafficked to endosomes (Extended Data Fig. 8a) yet remained attached through an intramolecular disulfide bond (Fig. 3c, top left, and Extended Data Fig. 8b). Preventing release from UNC93B1 through this tether completely abrogated TLR9 signalling (Fig. 3c, bottom left). Notably, UNC93B1(281C) and TLR9(812C) were functional when expressed alone, ruling out the possibility that the cysteine mutations simply created non-functional proteins (Fig. 3c, bottom left, and Extended Data Fig. 8c).

Next, we asked whether other endosomal TLRs also require release from UNC93B1 for signalling. Similar to TLR9, TLR3 released from UNC93B1 in endosomes (Extended Data Fig. 9b); however, the insensitivity of TLR3 to UNC93B1(S282A) suggests that interaction with UNC93B1 is mediated by a distinct interaction surface. Notably, the interaction between TLR7 and UNC93B1 did not decrease in endosomes (Fig. 3d and Extended Data Fig. 9c, d), which suggests that TLR7 can bind ligand and signal while associated with UNC93B1. In fact, in an accompanying manuscript, we describe how the continued association of TLR7 and UNC93B1 in endosomes is crucial for a distinct regulatory mechanism that prevents TLR7-dependent autoimmunity. Thus, UNC93B1 uses different mechanisms to regulate activation of nucleic acid-sensing TLRs in endosomes.

**TLR9 must be released from UNC93B1 to function**

Because an enhanced association with UNC93B1 is detrimental to TLR9 function, we reasoned that TLR9 might require release from UNC93B1 in endosomes before ligand binding and activation. We investigated this possibility by using cellular fractionation to separate ER and endosomes and measured the extent of the TLR9–UNC93B1 interaction in each organelle preparation (Fig. 3a and Extended Data Fig. 9a). The selected endosomal fractions contained high levels of CpG-B–biotin ligand (when fed to cells), indicating that we had enriched the relevant signalling endosomes for TLR9 (Extended Data Fig. 6). As predicted, the association of UNC93B1 and TLR9 was weaker in endosomes than in the ER (Fig. 3b and Extended Data Fig. 7a). Furthermore, UNC93B1(S282A) showed an overall stronger association with TLR9, both in the ER and in endosomes (Fig. 3b and Extended Data Fig. 7b), which suggests that the altered chemistry of the UNC93B1 mutant might have an overall ‘sticky’ effect that prevents efficient release of TLR9 in endosomes.

On the basis of these results, we considered a release model, in which TLR9 must dissociate from UNC93B1 for efficient ligand binding and signalling. UNC93B1 might interfere with DNA recognition by keeping the receptor in an unfavourable conformation for ligand binding, which would explain the attenuated ligand binding of TLR9 when forcefully bound to UNC93B1(S282A) (Extended Data Fig. 7c). To test this model independently and more directly, we engineered a cysteine bridge between TLR9 and UNC93B1 to tether the two proteins together and prevent release. We thereby focused on the previously identified loop 5 binding region of UNC93B1 and the juxtamembrane region of TLR9 (Fig. 3c). We identified a pair of UNC93B1 and TLR9 cysteine mutants (UNC93B1(281C) and TLR9(812C)) that trafficked to endosomes (Extended Data Fig. 8a) yet remained attached through an intramolecular disulfide bond (Fig. 3c, top left, and Extended Data Fig. 8b). Preventing release from UNC93B1 through this tether completely abrogated TLR9 signalling (Fig. 3c, bottom left). Notably, UNC93B1(281C) and TLR9(812C) were functional when expressed alone, ruling out the possibility that the cysteine mutations simply created non-functional proteins (Fig. 3c, bottom left, and Extended Data Fig. 8c).

Next, we asked whether other endosomal TLRs also require release from UNC93B1 for signalling. Similar to TLR9, TLR3 released from UNC93B1 in endosomes (Extended Data Fig. 9b); however, the insensitivity of TLR3 to UNC93B1(S282A) suggests that interaction with UNC93B1 is mediated by a distinct interaction surface. Notably, the interaction between TLR7 and UNC93B1 did not decrease in endosomes (Fig. 3d and Extended Data Fig. 9c, d), which suggests that TLR7 can bind ligand and signal while associated with UNC93B1. In fact, in an accompanying manuscript, we describe how the continued association of TLR7 and UNC93B1 in endosomes is crucial for a distinct regulatory mechanism that prevents TLR7-dependent autoimmunity. Thus, UNC93B1 uses different mechanisms to regulate activation of nucleic acid-sensing TLRs in endosomes.
UNC93B1 release required for TLR9 responses in vivo

Finally, we tested the importance of TLR9 release from UNC93B1 in vivo. We introduced the UNC93B1(S282A) mutation into the germine of mice using Cas9 genome editing (Extended Data Fig. 10a). Bone marrow-derived macrophages (BMMs) from these mice showed a selective loss of TLR9 responses, whereas TLR7, TLR3 and TLR4 responses were normal (Fig. 4a). Type I IFN production by plasmacytoid dendritic cells in response to TLR9 stimulation was similarly ablated (Fig. 4b). Lastly, B cells from UNC93B1(S282A) mice did not proliferate in response to CpG-B, but showed normal proliferation in response to resiquimod (R848) and lipopolysaccharide (LPS) (Fig. 4c and Extended Data Fig. 10b). These data support, across several cell types and with endogenous protein levels, a model in which TLR9 must be released from UNC93B1 in endosomes to function.

The differential regulation of TLR9 and TLR7 that we describe may explain the enigmatic observation of TLR9 and TLR7 contributing distinctly to the pathology of certain autoimmune diseases8,9,10. TLR9 function is strictly linked to proper trafficking and localization, both through UNC93B1 association, as we describe here, and through the requirement for ectodomain proteolysis and ligand processing in endosomes9,20. Accordingly, overexpression of TLR9 does not induce disease18. By contrast, TLR7 appears subject to more 'tunable' regulation that dampens but does not eliminate signalling (see our accompanying paper45), and overexpression of TLR7 is sufficient to break tolerance and drive autoimmunity20,29. Why distinct mechanisms of regulation have evolved for such functionally similar innate receptors remains unclear. One possibility is that differences in the trafficking of TLR7 and TLR9 influence the likelihood that self RNA or DNA will be encountered; indeed, TLR9 traffics to endosomes via the plasma membrane whereas TLR7 is thought to bypass the plasma membrane10. Alternatively, the nature of the ligands recognized by each receptor may require differing degrees of tunability. Recent work has revealed that TLR7 and TLR8 ligands are quite simple (for example, TLR7 recognizes the purine nucleoside guanosine together with a 3′-mer uridine-containing single-stranded RNA20–24). In this case, avoiding self recognition may require more subtle modulation of signalling than is necessary for TLR9. Regardless of any teleological rationale, dissecting the mechanisms that underlie differential regulation of these TLRs should reveal new opportunities for therapeutic manipulation of TLR activation.

Online content

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1. Majer, O., Liu, B. & Barton, G. M. Nuclear acid-sensing TLRs: trafficking and regulation.Curr. Opin. Immunol. 44, 26–33 (2017).
2. Deane, J. A. et al. Control of toll-like receptor 7 expression is essential to restrict autoimmunity and dendirctic cell proliferation. Immunity 27, 801–810 (2007).
3. Fukushima, R. et al. UNC93B1 restricts systemic lethal inflammation by orchestrating Toll-like receptor 7 and 9 trafficking. Immunity 35, 69–81 (2011).
4. Mouchess, M. L. et al. Transmembrane mutations in Toll-like receptor 9 bypass the requirement for ectodomain proteolysis and induce fatal inflammation. Immunity 35, 721–732 (2011).
5. Pitsikas, P. et al. Autoimmune B cell responses to RNA-related antigens due to TLR7 gene duplication. Science 312, 1668–1672 (2006).
6. Subramanian, S. et al. A TLR7 translocation accelerates systemic autoimmunity in murine lupus. Proc. Natl Acad. Sci. USA 103, 9970–9975 (2006).
7. Barton, G. M., Kagan, J. C. & Medzhitov, R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat. Immunol. 7, 49–56 (2006).
8. Kim, Y. M., Brinkmann, M. P., Paquet, M. E. & Ploegh, H. L. UNC93B1 delivers nucleotide-sensing toll-like receptors to endosomes. Nature 452, 234–238 (2008).
9. Fukushima, R. et al. UNC93B1 biases Toll-like receptor responses to nucleic acid in dendritic cells toward DNA: but against RNA-sensing. J. Exp. Med. 206, 1355–1359 (2009).
10. Lee, B. L. et al. UNC93B1 mediates differential trafficking of endosomal TLRs. eLife 2, e00931 (2013).
11. Latz, E. et al. Ligand-induced conformational changes allosterically activate Toll-like receptor 9. Nat. Immunol. 8, 772–779 (2007).
12. Ohto, U. et al. Structural basis of CpG and inhibitory DNA recognition by Toll-like receptor 9. Nature 520, 702–705 (2015).
13. Onji, M. et al. An essential role for the N-terminal fragment of Toll-like receptor 9 in DNA sensing. Nat. Commun. 4, 1949 (2013).
14. Majer, O., Liu, B., Kreuk, L. S. M., Krogan, N. & Barton, G. M. UNC93B1 recruits syntenin-1 to dampen TLR7 signalling and prevent autoimmunity. Nature 10.1038/s41586-019-1612-6 (2019).
15. Christensen, S. R. et al. Toll-like receptor 7 and 9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. Immunity 25, 477–482 (2006).
16. Nickerson, K. M. et al. TLR9 regulates TLR7- and MyD88-dependent autoantibody production and disease in a murine model of lupus. J. Immunol. 184, 1840–1848 (2010).
17. Chan, M. P. et al. DNase II-dependent DNA digestion is required for Toll-like receptor 9. Nat. Immunol. 6, 585–583 (2005).
18. Ewald, S. E. et al. The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. Nature 456, 658–662 (2008).
19. Park, B. et al. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. J. Exp. Med. 206, 1339–1350 (2009).
20. Roberts, A. W. et al. Tissue-resident macrophages are locally programmed for silent clearance of apoptotic cells. Nature 435, 913–927 (2005).
21. Shibata, T. et al. Guanosine and its modified derivatives are endogenous ligands for TLR7. Int. Immunol. 28, 211–222 (2016).
22. Tan, H. et al. Toll-like receptor 8 senses degradation products of single-stranded RNA. Nat. Struct. Mol. Biol. 22, 109–115 (2015).
23. Zhang, Z. et al. Structural analysis reveals that Toll-like receptor 7 is a dual receptor for guanosine and single-stranded RNA. Immunity 45, 737–748 (2016).

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Methods and reagents

The following antibodies were used for immunoblot, immunoprecipitations, or flow cytometry: rat anti-HA as purified antibody or matrix (3F10, Roche), mouse anti-Flag as purified antibody or matrix (M2, Sigma-Aldrich), anti-V5 (rabbit polyclonal, AB3792, Millipore), anti-mouse LAMP1 (goat polyclonal, AF4320, R&D Systems), anti-calnexin (rabbit polyclonal, ADI-SPA 860-F, Enzo Life Sciences), rabbit anti-Rab5 (CSB1, Cell Signaling), anti-EEA1 (rabbit polyclonal, 2411S, Cell Signaling), mouse anti-GM130 (610822, BD), anti-TLR9 (B33A4, gift from K. Miyake), anti-GAPDH (GT239, GeneTex), anti-TNF-APC (MP6-XT22, ebioscience), CD16 (65B, Biologend), purified anti-CD16/32Fc Block (2.4G2, BD), goat anti-mouse IgG-AlexaFluor680 (Invitrogen), goat anti-rat IgG-AlexaFluor680 (Invitrogen), rabbit anti-goat IgG-AlexaFluor680 (Invitrogen), goat anti-mouse IRDye 800CW (Licor), donkey anti-rabbit IRDye 680RD (Licor), goat anti-rat IRDye 800CW (Licor). For immunofluorescence: rat anti-HA (3F10, Roche), rabbit anti-LAMP1 (ab24170, Abcam), donkey anti-rat AlexaFluor488 (Jackson Immunoresearch), donkey anti-rabbit AlexaFluor647 (Jackson Immunoresearch). Cells were mounted in Vectashield Hard Set Mounting Medium for Fluorescence (Vector Laboratories). For PLA: rabbit anti-HA (ab9110, Abcam), mouse anti-Flag (M2, Sigma-Aldrich). For ELISA: anti-mouse TNF purified (IF3D34, ebioscience), anti-mouse TNF Biotin (XT3/XT22, ebioscience), streptavidin HRP (BD Pharmingen).

The following TLR ligands were used: CpG-B (ODN1168: TTCACTGACGTTCCTGATGC, all phosphorothioate linkages) and CpG-A (ODN1185S: GCTGGTACAAGTTGACGCTTTGACG, all phosphorothioate linkages), asterisk indicate phosphorothioate linkages were synthesized by Integrated DNA Technologies (Cy3 or biotin was attached to the 5′ end for imaging or biochemistry experiments), R848 (Invivogen), PolyIC HMW (Invivogen), ultrapure Flagellin-ST (Invivogen), ORN Sa19 (Invivogen), and LPS (Invivogen). Human IL-1β was purchased from Acros. Lipofectamine-LTX reagent (Invitrogen) was used following the QuickChange II Site-directed Mutagenesis protocol from Agilent Technologies. The following MSCV-based retroviral vectors were used to express TLR7 and TLR9 in cell lines: MSCV2.2 (IRES-GFP), MSCV-Thy1.1 (IRES-Thy1.1), or MIGR2 (IRES-CD2). TLR7 and TLR9 were fused to haemagglutinin (YPYDVPDYA) at the C-terminal end. TLR7 sequence was synthesized after codon optimization by Invitrogen’s GeneArt Gene Synthesis service, as previously described16.

Plasmid constructs

For additional site-directed mutagenesis, AccuPrime Pfx DNA polymerase (Invitrogen) was used following the QuickChange II Site-directed Mutagenesis protocol from Agilent Technologies. The following MSCV-based retroviral vectors were used to express TLR7 and TLR9 in cell lines: MSCV2.2 (IRES-GFP), MSCV-Thy1.1 (IRES-Thy1.1), or MIGR2 (IRES-CD2). TLR7 and TLR9 were fused to haemagglutinin (YPYDVPDYA) at the C-terminal end. TLR7 sequence was synthesized after codon optimization by Invitrogen’s GeneArt Gene Synthesis service, as previously described16.

Mice

Mice were housed under specific-pathogen-free conditions at the University of California, Berkeley. All mouse experiments were performed in accordance with the guidelines of the Animal Care and Use Committee at UC Berkeley. C57BL/6j mice were from the Jackson Laboratory. UNC93B1(S282A) mice were generated using Cas9 genome editing. The guide RNA used was: TGCTGCGCGGCAGCGTCCGAAGG. The single-stranded oligonucleotide template contained a 91-bp homology arm on the PAM proximal site and a 39-bp homology arm on the PAM distal site. Four phosphothioate linkages were incorporated into the ends of the oligo template (one at the 5′ and three at the 3′ end of the oligo). In brief, female C57BL/6j mice at 4 weeks of age were superovulated and mated overnight with C57BL/6j male mice (~8 weeks old). Zygotes were collected from superovulated females and were placed in KSO medium (MR106D, Millipore) before use. Embryos were transferred to 10 µl Opti-MEM reduced serum medium (31985062, Thermo Fisher Scientific) and then mixed with 10 µl CRISPR reagent (that is, Cas9, gRNA, single-stranded oligonucleotide). This 20 µl mixture (embryos in RNP solution) was undergone electroporation in a 1 mm electroporation cuvette using a Gene Pulser XCell electroporator (1652660, Bio-Rad). After electroporation, 96 embryos were transferred to four CD1 recipients via oviduct transfer. Offspring were genotyped by sequencing for the correct targeted allele and further bred to ensure germline transmission. For experiments we used Unc93b1+/- and Unc93b1-/- mice as littermate controls. Unless noted, mice were analysed at 5–8 weeks of age. In total, a minimum of 8 mice per group were analysed, divided between three experiments.

Type I interferon production by plasmacytoid dendritic cells

Bone marrow was flushed and red blood cells were lysed using ammonium-chloride-potassium (ACK) buffer (Gibco). Then, 10^5 cells per well were seeded into 96-well flat-bottom plates. Cells were stimulated with various concentrations of CpG-A for 16 h. The next day, supernatants were transferred onto L-292 ISRE-luciferase reporter cells to determine the amount of released type I IFN. Recombinant mouse IFN-β (PBL InterferonSource) was used for the standard curve. Reporter cells were incubated in bone marrow supernatants for 8 h, lysed by passive lysis (Promega) and luciferase activity was measured on a LMaxII-384 luminometer (Molecular Devices).

B cell proliferation assay

Splenocytes were digested with collagenase 8 (Sigma) and DNase-1 for 45 min and red blood cells were lysed using ACK buffer (Gibco). Splenocytes were labelled with 12.5 µg ml^(-1) CFSE (Invitrogen) for 10 min at 37 °C and immediately underlaid with 3 ml fetal calf serum (FCS) to spin out carboxyfluorescein succinimidyl ester (CSFE). Cells were taken up in RPMI medium (containing 10% FCS, 1-glutamine, penicillin–streptomycin,
cells were sorted on a BD FACSAria Fusion Sorter to match expression overnight at 32°C and protein expression was checked 48 h later. Target supernatant (with polybrene at 5 µg ml⁻¹) was used to infect target cells were incubated at 32°C. Forty-eight hours after transfection, viralfectamine LTX reagent. Twenty-four hours after transfection, cells

to TOPO cloning.

response to TLR7 stimulation and sequencing of the targeted region on Cas9-GFP two days later. Correct targeting was verified by loss of

Macrophages were transfected with Cas9(D10A) and all four guide TAGTAGCGG; (2) CGGAGTGGTCAAGAACGTGCTGG; (3) TTCGGAAT-

(Extended Data Fig. 10c). For the quantification, a proliferation index was determined by dividing the gMFI of CFSE of the unstimulated control by the gMFI CFSE of the stimulated sample (CFSEstimulated:CFSEsample) and plotted along the ligand titration.

Plasmid constructs
AccuPrime PfX DNA polymerase (Invitrogen) was used for site-directed mutagenesis using the QuikChange II Site-directed Mutagenesis protocol from Agilent Technologies. The following murine stem-cell virus (MSCV)-based retroviral vectors were used to express UNC93B1, TLR9, TLR7 and TLR3 in cell lines: MSCV-PuromCherry (IRES-PuromycinR-T2A-
mCherry), MSCV2.2 (IRES-GFP), MSCV-Thy1.1 (IRES-Thy1.1) and MIGR2 (IRES-hCD2). 3× Flag (DYKDHDGDYKDHDIDYKDDDDK) was fused to the C terminus of UNC93B1. TLR9, TLR7 and TLR3 were fused to haemagglutinin (YPYDVPDYA) at the C-terminal end. The TLR7 sequence was synthesized after codon optimization by Invitrogen’s GeneArt Gene Synthesis service as previously described10. TLR9 chimeras of the juxtamembrane and transmembrane regions were previously described1.

Cells and tissue culture conditions
All cell lines used in this study have been tested negative for mycoplasma and positively authenticated by 16-Marker STR DNA Profiling (IDEXX). HEK293T cells were obtained from American Type Culture Collection (ATCC). GP2-293 packaging cell lines were obtained from Clontech. The above cell lines were cultured in DMEM complete medium supplemented with 10% (v/v) FCS, l-glutamine, penicillin–streptomycin, sodium pyruvate and HEPES (pH 7.2; Invitrogen). RAW264.7 macrophage cell lines (ATCC) were cultured in RPMI 1640 (same supplements as above).

BMMs were differentiated for seven days in RPMI complete medium (same supplements as above plus 0.00034% (v/v) β-mercaptoethanol) and supplemented with M-CSF containing supernatant from 3T3-CSF cells.

To generate HEK293T UNC93B1−/− cells, guide RNAs were designed and synthesized as gBlocks as previously described16 and subcloned into pUC19 (guide RNA: CTCACCTAGGCGTCTACC). Humanized Cas9-2xNLS-GFP was a gift from the Doudna laboratory, University of California, Berkeley. HEK293T cells were transfected using Lipofectamine LTX with equal amounts of the guide RNA plasmid and Cas9 plasmid. Seven days post transfection cells were plated in a limiting-dilution to obtain single cells. Correct targeting was verified by PCR analysis and loss of response to TLR9 and TLR7 stimulation in an NF-κB dilution to obtain single cells. Correct targeting was verified by PCR

dilution factor (based on the volume loaded and the pooled fraction volume). The relative proportion of UNC93B1-bound TLR was calculated as the ratio of the quantified UNC93B1-bound TLR over the total TLR amount (converted to percentage for Extended Data Fig. 9d, the total amount of each TLR in the pooled endosomal fractions was calculated by multiplying the band intensities by the dilution factor (based on the volume loaded and the pooled fraction volume). The relative proportion of UNC93B1-bound TLR was calculated as the ratio of the quantified UNC93B1-bound TLR over the total TLR amount (converted to percentage for Extended Data Fig. 9d). To normalize across different experiments, equal amounts of protein standards were loaded in each gel and band intensities were used as a normalization reference.

Streptavidin pull-down assays were performed on cells fed CpG-B-biotin for 4 h, lysed in NP-40 buffer (as above) and cleared of insoluble debris. Lysates were incubated for 2 h with streptavidin magnetic beads (pre-blocked with 1% BSA-PBS), rotated at 4°C, and washed four times in lysis buffer. Precipitates were boiled in SDS–PAGE buffer at room temperature for 1h. Proteins were separated by SDS–PAGE (Bio-Rad TGX precast gels) and transferred to Immobilon PVDF membranes (Millipore) in a Trans-Blot Turbo transfer system (Bio-Rad). Membranes were probed with the indicated antibodies and developed using the Licor Odyssey Blot Imager. Relative band intensities were quantified using Fiji (ImageJ)26. For co-immunoprecipitation efficiencies in Extended Data Fig. 9d, the total amount of each TLR in the pooled endosomal fractions was calculated by multiplying the band intensities by the dilution factor (based on the volume loaded and the pooled fraction volume). The relative proportion of UNC93B1-bound TLR was calculated as the ratio of the quantified UNC93B1-bound TLR over the total TLR amount (converted to percentage for Extended Data Fig. 9d).

Retroviral transduction
For retroviral transduction of RAW macrophages, VSV-G-pseudotyped retrovirus was made in GP-293 packaging cells (Clontech). GP-293 cells were transfected with retroviral vectors and pVSV-G using Lipofectamine LTX reagent. Twenty-four hours after transfection, cells were incubated at 32°C. Forty-eight hours after transfection, viral supernatant (with polybrene at 5 µg ml⁻¹) was used to infect target cells overnight at 32°C and protein expression was checked 48 h later. Target cells were sorted on a BD FACSAria Fusion Sorter to match expression or drug-selected with Puromycin, starting 48 h after transduction. Efficiency of drug selection was verified by equal mCherry expression of target cells.

For retroviral transduction of BMMs, bone marrow was collected and cultured in RPMI containing M-CSF for two days. Progenitor cells were transduced with viral supernatant (produced as above) on two successive days by spinfection for 90 min at 32°C. Forty-eight hours after the second transduction cells were put on puromycin selection and cultured in RPMI containing M-CSF until collection on day 8.

Luciferase assays
Activation of NF-kB in HEK293T cells was performed as previously described48. In brief, transfections were performed in OptiMEM-I (Invitrogen) with LTX transfection reagent (Invitrogen) according to manufacturer’s guidelines. Cells were stimulated with CpG-B (200 nM–1µM), R848 (100–200 ng ml⁻¹) or human IL-1β (20 ng ml⁻¹) after 24 h, and lysed by passive lysis after an additional 12–16 h. Luciferase activity was measured on an LMaxII-384 luminometer (Molecular Devices).

Immunoprecipitation and western blot analysis
Cells or purified phagosomes were lysed in NP-40 buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 5mM EDTA and supplemented with EDTA-free complete protease inhibitor cocktail (Roche) and 1 mM PMSF). After incubation at 4°C on a rotator, lysates were cleared of insoluble material by centrifugation. For immunoprecipitations, lysates were incubated with anti-HA matrix or anti-Flag matrix (pre-blocked with 1% BSA-PBS) overnight, and washed four times in lysis buffer the next day. For immunoprecipitations with anti-TLR9 (B33A4), cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM CaCl₂, 10% glycerol, 1 mM dithiothreitol (DTT), 0.5% NP-40, supplemented with EDTA-free complete protease inhibitor cocktail (Roche) and 1 mM PMSF. Cleared lysates were incubated with 8 µg ml⁻¹ anti-TLR9 (B33A4) overnight. The next day, 50 µl Protein Gagarose (pre-blocked with 1% BSA-PBS) was added to lysates for 1–2 h and washed four times in lysis buffer. Precipitated proteins were denatured in SDS–PAGE buffer at room temperature for 1h. Proteins were separated by SDS–PAGE (Bio-Rad TGX precast gels) and transferred to Immobilon PVDF membranes (Millipore) in a Trans-Blot Turbo transfer system (Bio-Rad). Membranes were probed with the indicated antibodies and developed using the Licor Odyssey Blot Imager. Relative band intensities were quantified using Fiji (ImageJ)26. For co-immunoprecipitation efficiencies in Extended Data Fig. 9d, the total amount of each TLR in the pooled endosomal fractions was calculated by multiplying the band intensities by the dilution factor (based on the volume loaded and the pooled fraction volume). The relative proportion of UNC93B1-bound TLR was calculated as the ratio of the quantified UNC93B1-bound TLR over the total TLR amount (converted to percentage for Extended Data Fig. 9d).

To visualize disulfide bond formation in RAW macrophages, cells were lysed in buffer containing 1% dodecyl-β-D-maltoside (DDM) and 0.2% choleryl hemisuccinate (CHS) detergents for 2 h at 4°C. After removing insoluble material, lysates were incubated with haemagglutinin matrix for 2–4 h and washed four times in buffer containing 0.25% DDM and CHS. Protein was eluted in lysis buffer containing 10% DDM and 300 µg ml⁻¹ haemagglutinin peptide for 1 h at room temperature. Eluates were divided in half and denatured in either reducing (+DTT) or non-reducing (–DTT) SDS buffer for 1h at room temperature.
Flow cytometry
Cells were seeded into non-treated tissue culture 24-well plates or round-bottom 96-well plates. The next day, cells were stimulated with the indicated TLR ligands. To measure TNF production, brefeldinA (BD GolgiPlug) was added to cells 30 min after stimulation, and cells were collected after an additional 5.5 h. Cells were stained for intracellular TNF with a Fixation & Permeabilization kit, according to manufacturer’s instructions (eBioscience).

To measure DNA uptake, cells were fed Cy3-fluorescent-tagged CpG-B for the indicated amounts of time (control cells for no uptake were pre-chilled and stimulated on ice). Cells were washed three times with ice-cold PBS, fixed in 1% paraformaldehyde (PFA), 2% FCS in PBS, and pre-chilled and stimulated on ice (also see ‘Microscopy’ section). The post-processed super-resolution Z-series was analysed in Imaris x64 9.2.1. In brief, surface volumes for each cell were generated using the dispersion of the PLA signal as a proxy for the cell boundary. Surface volumes were masked and the numbers of PLA spots within the masked areas were quantified using the spot finder tool. Spot size was set to 0.25 µm and threshold to 2100.

Proximity ligation assay
Coverslips (high-performance, 18×18 mm, thickness no. L.5 Zeiss) were acid-washed in 3 M HCl, washed extensively in water, dipped in 70% ethanol and allowed to air-dry. Cells were plated onto coverslips and allowed to settle overnight. Coverslips were washed with PBS, fixed with 4% PFA in PBS for 15 min, and permeabilized with 0.5% saponin in PBS for 5 min. To quench PFA autofluorescence, coverslips were treated with sodium borohydride, 0.1% saponin in PBS for 10 min. After three washes in PBS, cells were subjected to the PLA staining protocol. All steps, including blocking, incubations with primary antibodies (rabbit anti-HA (ab9110) and mouse anti-Flag (M2)) and Duolink PLA probes, ligase and polymerase were followed as per the manufacturer’s instructions. Cells were mounted in Duolink In situ mounting medium plus DAPI and imaged on a Zeiss Elyra PS.1 with a 63×/1.46 oil immersion objective (also see ‘Microscopy’ section). The post-processed super-resolution Z-series were analysed in Imaris x64 9.2.1. In brief, surface volumes for each cell were generated using the dispersion of the PLA signal as a proxy for the cell boundary. Surface volumes were masked and the numbers of PLA spots within the masked areas were quantified using the spot finder tool. Spot size was set to 0.25 µm and threshold to 2100.

Phagosome isolation
Cells in a confluent 15-cm dish were incubated with approximately 10⁶ magnetic beads (1-µm size, Polysciences) for 4 h. After rigorous washing in PBS, cells were scraped into 10 ml sucrose homogenization buffer (SHB; 250 µM sucrose, 3 mM imidazole, pH 7.4) and pelleted by centrifugation. Cells were resuspended in 2 ml SHB plus protease inhibitor cocktail with EDTA (Roche) and 1 mM PMSF and disrupted by 25 strokes in a steel dounce homogenizer. The disrupted cells were gently rocked for 10 min on ice to free endosomes. Beads were collected with a magnet (Dynal) and washed four times with SHB plus protease inhibitor. After the final wash, phagosome preparations were denatured in 2× SDS buffer at room temperature for 1 h and analysed by western blot.

Cell fractionation by sucrose density-centrifugation
Cells in four confluent 15-cm dishes were washed in ice-cold PBS, scraped in 10 ml SHB and pelleted by centrifugation. Cells were resuspended in 2 ml SHB plus protease inhibitor cocktail with EDTA (Roche) and 1 mM PMSF and disrupted by 25 strokes in a steel dounce homogenizer. The disrupted cells were centrifuged for 10 min at 1,000g to remove nuclei. Supernatants were loaded onto continuous sucrose gradients (percentage iodixanol: 0, 10, 20, 30) and ultracentrifuged in an SW41 rotor at 113,832g for 2 h (Optima L-90K Ultracentrifuge, Beckman Coulter). Twenty-two fractions of 420 µl were collected from top to bottom, and 100 µl of each fraction were denatured in SDS buffer for western blot analysis. For immunoprecipitations, three fractions corresponding to ER or endosomes were combined and lysed for 1 h after addition of protease inhibitor cocktail and NP-40 to a final concentration of 1%. Co-immunoprecipitation with anti-HA or anti-Flag matrix was performed as described above.

CpG-B–biotin dot blot
Cells in four confluent 15-cm dishes were fed 1 µM CpG-B–biotin in 4 h and cell fractionation was performed as described in the ‘Cell fractionation by sucrose density-centrifugation’ section. The individual fractions were lysed in 0.5% NP-40 and 3 µl lysate per fraction was spotted onto an Ambion BrightStar Plus Nylon membrane. The membrane was air-dried and DNA crosslinked in a Stratalinker on the auto-crosslink setting (L,200 (>100) m cm²). The membrane was washed in TBS plus...
0.1% Tween 20 (TTBS) containing 0.1% SDS for 1 h at room temperature to reduce background, followed by blocking in Odyssey blocking buffer for 1 h at room temperature. The membrane was incubated with Streptavidin-IR 680 (L5,000) for 30 min at room temperature, washed three times in TTBS and imaged on a Licor Odyssey scanner.

β-Hexosaminidase activity assay
Ten microlitres of each gradient fraction was mixed with 50 µl of substrate solution (6 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminidine (Sigma) dissolved in assay buffer (50 mM citrate-phosphate buffer, pH 4.5, containing 0.1% Triton X-100) and incubated for 20 min at 37 °C. Enzymatic reaction was stopped by adding 100 µl Stop buffer (20 mM Na2CO3, 110 mM glycine) and fluorescence was read at excitation 365, emission 450 nm in black assay plates.

Quantification and statistical analysis
Statistical parameters, including the number of independent repeats, the number of biological replicates per representative experiment, and statistical significance, are reported in the figures and legends. Representative images have been repeated at least three times, unless otherwise stated. Data are judged to be statistically significant when \( P < 0.05 \) by two-tailed Student’s \( t \)-test. To compare the means of different groups, a one-way ANOVA followed by a Tukey’s post-test was used. To compare means of different groups across a dose response, a two-way ANOVA followed by a Tukey’s or Sidak’s post-test was used. *\( P < 0.05 \), **\( P < 0.01 \) and ***\( P < 0.001 \). Statistical analysis was performed in GraphPad PRISM 7 (GraphPad Software). No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
The datasets generated during and/or analysed during the current study are either included within the manuscript or are available from the corresponding author on reasonable request. Source Data for Fig. 4 and Extended Data Fig. 10 are included in the online version of the paper. Gel source data can be found in Supplementary Fig. 1.

25. Mali, P. et al. RNA-guided human genome engineering via Cas9. Science 339, 823–826 (2013).
26. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).
27. Carpenter, A. E. et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol. 7, R100 (2006).

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Author contributions
O.M. and G.M.B designed experiments. O.M., B.L., B.J.W., L.S.M.K. and E.V.D. performed experiments and analysed the data. O.M. performed the initial alanine mutagenesis screen. O.M. wrote the manuscript. G.M.B., O.M. and B.L. revised and edited the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to G.M.B.
Reprints and permissions information is available at http://www.nature.com/reprints.
Extended Data Fig. 1 | A luminal UNC93B1 mutation results in defective TLR9 signalling despite normal trafficking. **a**, Colocalization of UNC93B1 (red) and LAMP1 (green) in macrophages expressing the indicated UNC93B1 alleles using super-resolution structured illumination microscopy. Representative images are shown. Boxed areas are magnified. The plot shows quantification of the percentage of total UNC93B1 within LAMP1+ endosomes. Each dot represents an individual cell. P values determined by unpaired two-tailed Student’s t-test. Data are from a single experiment. Scale bars, 10 µm. **b**, UNC93B1(S282A) is sufficient for the TLR9 signalling defect. NF-κB luciferase assay in HEK293T cells stimulated with CpG-B (1 µM) for 16 h. Data are normalized to UNC93B1-independent human IL-1β responses and expressed as luciferase fold change over unstimulated controls. n = 3 biological replicates. **c**, Intracellular cytokine staining of TNF in macrophage lines expressing the indicated UNC93B1 alleles after stimulation with CpG-B (1 µM), R848 (100 ng ml⁻¹), poly(I:C) (100 ng ml⁻¹), Sa19 (a TLR13-specific RNA agonist; 200 ng ml⁻¹), flagellin (100 ng ml⁻¹) or LPS (10 ng ml⁻¹). Grey histograms show unstimulated controls. d, e, TNF production of the indicated macrophage lines after 8 h stimulation with increasing concentrations of CpG-A (d), or LPS (50 ng ml⁻¹) (e). n = 2 biological replicates. ***P < 0.0001, determined by unpaired two-tailed Student’s t-test. Blot below shows UNC93B1 expression levels. f, qRT–PCR analysis of Tnfa expression in the indicated macrophage lines 8 h after stimulation with DOTAP–CpG-A (1 µM) or LPS (10 ng ml⁻¹). n = 3 biological replicates. ***P = 0.0003 (S282A versus WT), ***P = 0.0002 (HR versus WT), unpaired two-tailed Student’s t-test. Data are mean ± s.d. and representative of two independent repeats unless noted otherwise.
Extended Data Fig. 2 | UNC93B1(S282A) does not affect DNA delivery to TLR9-containing endosomes. a, Uptake of Cy3-labelled CpG-B (1 µM) of macrophage lines expressing the indicated alleles of UNC93B1–Flag. Data are mean ± s.d. relative uptake compared to wild-type at 60 min. n = 3 biological replicates. P values determined by two-way ANOVA followed by a Tukey’s post-test (WT versus SKN). b, Colocalization of CpG-A (red), TLR9–HA (green) and LAMP1 (magenta) in macrophage lines shown in a after incubation with Cy3-labelled CpG-A (1 µM) for 2 h using super-resolution structured illumination microscopy. Representative images are shown. Boxed areas and areas containing white lines are magnified. The histograms display fluorescent intensity plots of pixels along the white lines. Shaded areas highlight regions of colocalization of CpG-A, TLR9 and LAMP1. c, Quantification of the percentage of CpG-A colocalized with TLR9. Each dot represents an individual cell, n = 4 (WT), n = 5 (SKN) and n = 5 (HR). Data are mean ± s.d. ***P < 0.0001, unpaired two-tailed Student’s t-test. Scale bars, 5 µm. Data are from a single experiment.
Extended Data Fig. 3  UNC93B1(S282A) does not affect TLR9 dimerization or the association between N-terminal and C-terminal cleavage products of TLR9.  a, Macrophage lines co-expressing TLR9–HA and TLR9–V5 together with the indicated UNC93B1–Flag alleles were subjected to haemagglutinin immunoprecipitation followed by V5 immunoblot. TLR9 levels in whole-cell lysates are also shown.  b, Macrophage lines expressing TLR9–HA and the indicated UNC93B1–Flag alleles were subjected to immunoprecipitation with an anti-TLR9 antibody specific to the N-terminal cleavage fragment (B33A4), followed by immunoblot of the C-terminal TLR9 fragment with an anti-HA antibody. Data are representative of at least two independent experiments.
Extended Data Fig. 4 | UNC93B1(S282A) shows a stronger interaction with TLR9, but not TLR7. a, The UNC93B1 mutants SKN and S282A display a stronger association with TLR9. Immunoprecipitation of TLR9–HA from macrophage lines expressing the indicated UNC93B1 alleles, followed by immunoblot of UNC93B1–Flag. b, The UNC93B1 mutants SKN and S282A do not affect the interaction with TLR7. Immunoprecipitation of UNC93B1–Flag from macrophage lines expressing TLR7–HA and the indicated UNC93B1 alleles, followed by immunoblot of TLR7–HA. c, Immunoprecipitation of UNC93B1–Flag from macrophage lines expressing the indicated UNC93B1 alleles, followed by immunoblot of TLR9–HA. All blots are representative of at least two independent experiments. KA, K283A; NA, N284A; SA, S282A.
Extended Data Fig. 5 | Identification of residues within loop 5 of UNC93B1 that mediate interaction with TLR9. 

a, Schematic of the tested loop 5 mutants of UNC93B1 and the relative TLR9 responses indicated in shades of grey; white indicates a response equivalent to wild type, and black indicates no response. Asterisks show human UNC93B1 SNPs that have been tested in f. 

b, A larger region in loop 5 of UNC93B1 mediates binding to TLR9. Immunoprecipitation of UNC93B1–Flag from macrophage lines expressing the indicated UNC93B1 mutants (spanning amino acids 267–284, and non-functional HR) followed by immunoblot of TLR9–HA. Data are representative of two independent experiments.

c, Intracellular cytokine staining of TNF in macrophage lines shown in b after stimulation with CpG-B (25 nM), R848 (100 ng ml⁻¹) or LPS (10 ng ml⁻¹). Shaded histograms show unstimulated controls. Data are representative of three independent experiments.

d, Schematics showing relative positions and sequence alignment (bottom) of swapped regions within the TLR9/3 chimaeras. Coloured regions indicate TLR3 sequences. NF-κB luciferase assay in HEK293T cells transiently transfected with the indicated TLR9 and UNC93B1 mutants and stimulated with CpG-B (200 nM) for 16 h. Data are normalized to UNC93B1-independent human IL-1β responses and expressed as luciferase fold change over unstimulated controls. Data are mean ± s.d., n=3 biological replicates. P values determined by one-way ANOVA followed by a Tukey’s post-test. For CpG-B stimulations: P=0.0048 (WT versus G270S), P=0.0113 (WT versus R277Q), P=0.9994 (WT versus G283R). Data are representative of four independent experiments. For R848 stimulations: P=0.2001 (WT versus G270S), P=0.0002 (WT versus R277Q), P=0.9933 (WT versus G283R). Data are representative of three independent repeats experiment. 

e, TLR9 mutants that rescue signalling in the presence of UNC93B1(S282A) also show normal binding to UNC93B1(S282A). Haemagglutinin immunoprecipitation of the indicated TLR9 mutants transiently expressed in HEK293T cells stably expressing the indicated UNC93B1–Flag alleles, followed by immunoblot of UNC93B1–Flag. Data are representative of three independent experiments.

f, Human UNC93B1 variants with SNPs in loop 5 show decreased TLR9 signalling. NF-κB luciferase assay in HEK293T cells expressing TLR9 or TLR7 and the indicated human UNC93B1–Flag variants and stimulated with CpG-B (250 nM) or R848 (250 ng ml⁻¹) for 16 h, respectively. Data are normalized to Renilla expression and expressed as RLU. Data are mean ± s.d., n=3 biological replicates. P values are determined by one-way ANOVA followed by a Tukey’s post-test. For CpG-B stimulations: P=0.0001 (WT versus G270S), P=0.9999 (WT versus R277Q), P=0.0001, TLR9Mut1; P<0.0001, TLR9Mut2; P<0.0001, TLR9Mut3; P=0.0020, TLR9Mut4; P=0.0171). Data are representative experiment of two independent repeats. 

g, Expression levels of the UNC93B1 mutants used in f. *P<0.05, **P<0.01, ***P<0.001.
Extended Data Fig. 6 | Cellular fractionation showing the distribution profiles for CpG-B–biotin ligand and β-hexosaminidase. Macrophages were stimulated for 4 h with biotinylated CpG-B (1 µM) and subjected to subcellular fractionation by density-gradient centrifugation. The distributions of TLR9–HA, CpG-B, LAMP1 and β-hexosaminidase activity are shown. Data are representative of two independent experiments.
Extended Data Fig. 7 | The TLR9–UNC93B1 association is reduced in endosomes compared to the ER. a, Input controls of TLR9 and UNC93B1 (relates to Fig. 3b). TLR9 and UNC93B1 levels in pooled ER or endosome fractions from macrophage lines expressing TLR9–HA and the indicated UNC93B1 alleles. Data are representative of three independent experiments. b, Increased interaction between TLR9 and UNC93B1(S282A) in endosomes. Immunoprecipitation of UNC93B1–Flag from pooled endosome fractions followed by immunoblot for TLR9–HA. Input controls are also shown. Data are representative of three independent experiments. Bar graph shows the quantification of TLR9 bound to UNC93B1 in pooled endosome fractions, normalized by UNC93B1–Flag levels in endosome fractions. Data are mean ± s.d., each dot represents data from an independent experiment (n = 3). *P = 0.0413, unpaired two-tailed Student’s t-test. c, Release model of TLR9.
Extended Data Fig. 8 | Tethering of TLR9 and UNC93B1. a, Cysteine mutants of TLR9 and UNC93B1 do not affect trafficking of TLR9 to endosomes. Immunoblot of TLR9–HA from macrophage lines expressing the indicated TLR9–HA and UNC93B1–Flag cysteine mutants. Data are representative of two independent experiments. b, TLR9–HA immunoblot under non-reducing conditions after immunoprecipitation of TLR9–HA from macrophage lines shown in a. The high molecular mass band indicates disulfide bond formation between UNC93B1 and TLR9. Data are representative of two independent experiments. c, UNC93B1-tethered TLR9 is unable to signal. NF-κB luciferase assay in HEK293T cells expressing the indicated cysteine mutant combinations and stimulated with CpG-B (1 µM) for 16 h. Data are normalized to Renilla expression and expressed as luciferase fold change over unstimulated controls. Data are mean ± s.d., n = 3 biological replicates. P values determined by unpaired two-tailed Student’s t-test. Data are representative of three independent experiments.
Extended Data Fig. 9 | TLR3 but not TLR7 releases from UNC93B1 in endosomes. 

**a**, Subcellular fractionation of macrophages lines showing the distributions of TLR3, TLR7 and TLR9 across fractions. The pooled endosome and ER fractions for subsequent co-immunoprecipitations are highlighted. 

**b**, Immunoprecipitation of TLR9–HA and TLR3–HA from pooled ER or endosome fractions of macrophage lines expressing wild-type UNC93B1. Immunoprecipitated TLR–HA levels were normalized across fractions and probed for levels of UNC93B1–Flag. Bar graph shows the quantification of UNC93B1 bound to TLR3 between ER and endosome fractions. Data are mean ± s.d., each dot represents data from an independent experiment (n = 3). **P = 0.0039, paired two-tailed Student’s t-test.** 

**c**, Input controls of TLR9, TLR7 and UNC93B1 in pooled ER and endosome fractions (relates to Fig. 3d). 

**d**, Less TLR3 or TLR9 is associated with wild-type UNC93B1 in pooled endosome fractions compared to TLR7. Immunoprecipitation of UNC93B1–Flag from pooled endosome fractions (as shown in **a**) followed by immunoblot for TLR3–HA, TLR7–HA or TLR9–HA. Bar graph shows the calculated relative proportion of UNC93B1-bound TLR compared to total amount of the same TLR in the pooled endosome fractions. Data are mean ± s.d., each dot represents data from an independent experiment (n = 3). **P = 0.0032 (TLR3 versus TLR7) and P = 0.0036 (TLR7 versus TLR9), determined by unpaired two-tailed Student’s t-test.** All immunoblots are representative of three independent experiments.
Extended Data Fig. 10 | Generation of UNC93B1(S282A) knock-in mice and B-cell stimulation with LPS. a, CRISPR-Cas-9 strategy to generate UNC93B1(S282A) knock-in mice. Blue line indicates the guide sequence. Red bases indicated the edited codon. A representative sequencing trace of genomic DNA from an edited founder mouse is shown. b, B cell proliferation in CSFE-labelled splenocyte cultures of the indicated mouse genotypes after stimulation for 3 days with increasing doses of LPS. The proliferation index is defined as the gMFI of CSFEunstim/CFSEsample as in Fig. 4c. Each curve shows the dose response of cells from three mice. Data are mean ± s.d. P values determined by two-way ANOVA followed by a Sidak’s post-test. c, Gating strategy for B cell stimulation assay.
Reporting Summary

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When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- [ ] n/a
- [x] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- [ ] Only common tests should be described solely by name; describe more complex techniques in the Methods section.
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  - Give P values as exact values whenever suitable.
- [ ] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- [ ] For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- [ ] Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated
- [ ] Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

**Data collection**
- Immunoblot images: Odyssey Infrared Imaging System (version 2.1), LI-COR Biosciences
- Immunofluorescence staining data: ZEN (version 1.1.2.0), Carl Zeiss
- ELISA data: SoftMax Pro (version 5.0.1), Molecular Devices Corp.
- Flow cytometric data: FACSDiva (version 6.1.3), BD Biosciences
- Luciferase assay data: SoftMax Pro (version 5.4), MDS Analytical Tech.

**Data analysis**
- Flow cytometry data was analyzed with FlowJo 10.1r7.
- Microscopy images were processed in ZEN 2012 1.1.2.0 (Carl Zeiss).
- Colocalization analysis was performed with CellProfiler 2.2.0.
- Immunoblot quantification was performed in Fiji 1.0 (ImageJ).
- All other data/graphs were plotted and analyzed using Prism 5.0c or 6.0 (GraphPad).
- Statistical analysis was performed with Prism 5.0c, 6.0 or 7.0.

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Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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- A list of figures that have associated raw data
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The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical methods were used to predetermine sample sizes. Sample sizes for studies with mice were determined by availability of animals of the correct genotypes or were based on the numbers used in previous publications, in which comparable sample sizes resulted in statistically significant results. Statistical tests (as described in the Methods) were used to determine statistical significance. |
| Data exclusions | No data were excluded from analyses. |
| Replication | Every experiment was repeated (with similar results) at least twice. In most cases, experiments were repeated greater than 3 times with similar results. |
| Randomization | Randomization was not appropriate for this study. Littermate mice placed into groups based on their genotypes to enable meaningful comparisons. |
| Blinding | Investigators were blinded to the genotype of samples for analysis on mouse immune cells. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
| --- | --- |
| ☒ ☒ | Unique biological materials |
| ☒ ☒ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☒ ☒ | Palaeontology |
| ☒ ☒ | Animals and other organisms |
| ☒ | Human research participants |

Methods

| n/a | Involved in the study |
| --- | --- |
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials
Any unique biological resources will be made readily available to any investigators at non-profit institutions after publication and upon request. Requests from for-profit corporations will be handled and negotiated by our university’s technology transfer office. Unique mouse strains will also be made available, given that the investigators provide written assurance that the animals will be used in accordance with their institution’s IACUC guidelines.

Obtaining unique materials
Antibodies

The following antibodies were used for immunoblots and immunoprecipitations: anti-HA as purified antibody or matrix (clone 3F10; Ab: 11867423001, 1:1000; matrix: 1185016001, Roche), anti-FLAG as purified antibody or matrix (M2; Ab: F1804, 1:2000; matrix: M8823; Sigma-Aldrich), anti-V5 (AB3792, 1:2500, Millipore), anti-mLamp-1 (AF4320, 1:1000, R&D Systems), anti-Calnexin (ADI-SPA-860, 1:1000, Enzo Life Sciences), anti-Gapdh (GT239, 1:5000, GeneTex), anti-TLR9 (rabbit polyclonal, B33A4, kind gift from Kensuke Miyake, 0.075 µg/ml), anti-Rab5 (3547, 1:1000, Cell Signaling), anti-EEA1 (2411S, 1:1000, Cell Signaling), anti-GM130 (610822, 1:300, BD), goat anti-mouse IgG-AlexaFluor680 (A21058, 1:10000, Invitrogen), rabbit anti-goat IgG-AlexaFluor680 (A21088, 1:10000, Invitrogen), goat anti-mouse IRDye 800CW (926-32210, 1:1000, Licor), donkey anti-rabbit IRDye 680RD (926-68073, 1:10000, Licor), goat anti-mouse IRDye 800 (926-68073, 1:10000, Licor), goat anti-rabbit IRDye 800 (926-68073, 1:10000, Licor), goat anti-rabbit IgG-AlexaFluor680 (A21096, 1:10000, Invitrogen).

Antibodies for immunofluorescence were: rat anti-HA (3F10, 11867423001, 1:200, Roche), rabbit anti-Lamp1 (ab24170, 1:500, Abcam), donkey anti-rat IgG-AlexaFluor488 (712-545-150, 1:500, Jackson Immunoresearch), donkey anti-rabbit IgG-AlexaFluor647 (711-606-152, 1:500, Jackson Immunoresearch). Cells were mounted in Vectashield Hard Set Mounting Medium for Fluorescence (H-1400, Vector Laboratories).

For ELISA: anti-mouse TNFα purified (1F3F3D4, 14-7325-85, 1.5µg/ml, eBioscience), anti-mouse TNFα-biotin (XT3/XT22, 13-7326-85, 1ug/ml, eBioscience), Streptavidin-HRP (554066, 1:8000, BD Pharmingen).

For PLA: anti-HA (ab24170, 1:200, Abcam), anti-FLAG (M2, F1804, 1:200, Sigma-Aldrich). Antibodies and reagents used for flow cytometry were: anti-TNFα (MP6-XT22, 17-7321-82, 1:500, eBioscience), purified anti-CD16/32 Fc Block (2.4G2, 553141, 1:500, BD), CD19 (eBio1D3, 25-0193-82, 1:200, eBioscience).

Validation

The custom-designed rabbit polyclonal antibody against TLR9 for IB studies was validated previously and published in DOI: 10.1038/ncomms2949. All other commercial antibodies were validated by manufacturers for indicated species and application. Antibodies used for flow cytometry and immunofluorescence microscopy were further validated by staining known populations of cells. When possible, genetic controls were used to confirm specificity of any staining. Antibodies used for biochemistry were further validated by performing pilot experiments using samples with known parameters.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) RAW264 macrophage cell line was obtained from ATCC. HEK293 cells were obtained from ATCC.

Authentication Cells lines were authenticated via morphology, flow cytometry (measuring surface markers), and functional assays. Individual lines were not kept in culture for longer than 2 months.

Mycoplasma contamination Cell lines were screened for mycoplasma. Also, individual lines were not kept in culture for longer than 2 months. New lines were thawed from cryopreserved stocks previously confirmed as mycoplasma-negative.

Commonly misidentified lines (See ICLAC register) None of the cell lines used are listed in the ICLAC database.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals Mouse, C57BL/6, males and females, ages ranging from 3 weeks to 4 months. Mouse, Unc93b1(S282A) knockin mouse, males and females, ages ranging from 3 weeks to 4 months.

Wild animals The study did not involve wild animals.

Field-collected samples The study did not involve field-collected samples.

Flow Cytometry

Plots

Confirm that:

☑ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☑ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
☑ All plots are contour plots with outliers or pseudocolor plots.
☑ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation Toll-like receptor signaling in RAW macrophages, BMMs, and BM-DCs: Cells were seeded into non-treated tissue culture 24-well plates or round-bottom 96-well plates. The next day cells were stimulated with the indicated TLR ligands. To measure TNFα production, BrefeldinA (BD GolgiPlug, BD Biosciences) was added to cells 30 min after stimulation, and cells were collected after
an additional 5.5 h. Dead cells were excluded using a fixable live/dead stain (Violet fluorescent reactive dye, Invitrogen). Cells were stained for intracellular TNFα with a Fixation & Permeabilization kit according to manufacturer’s instructions (eBioscience). For B cell proliferation assays, spleens were digested with collagenase 8 (Sigma) and DNase-I for 45 min and red blood cells were lysed using ACK buffer (Gibco). Splenocytes were labeled with 12.5ug/mL CFSE (Invitrogen) for 10min at 37°C and immediately underlayed with 3ml FCS to spin out CSFE. Cells were taken up in media (RPMI/10%FCS/L-glutamine/Pen-Strep/HEPES/Sodium pyruvate/beta-mercaptoethanol), counted, and seeded at 200,000 cells per well in round-bottom 96-well plates. Cells were incubated in media with various concentrations of CpG-B, R848, or LPS for 72 h. Flow cytometry was used to analyze stimulated cells. Live, singlet cells were pre-gated on CD19+ and cell proliferation was determined by the geometric mean fluorescence intensity (gMFI) of CFSE. For the quantification, the gMFI CSFE of the unstimulated control was divided by the gMFI CSFE of the stimulated sample (CSFEUnstim:CSFESample) and plotted along the ligand titration.

**Instrument**
BD Fortessa or X20 flow cytometer (BD Biosciences)

**Software**
FlowJo 10.1r7.

**Cell population abundance**
Unc93b1-/- Raw macrophages retrovirally transduced with Unc93b1 mutants were sorted on mCherry to 95-99% purity.

**Gating strategy**
RAW macrophages, BMMs, bone marrow cells, and B cells were gated in the following order to determine the frequencies of live cells: 1) FSC-A/SSC-A, 2) Live/dump, 3) Singlets (FSC-H/FSC-A). The precise gating strategy for B cells is outlined in Ext. Data Fig. 10c. For intracellular TNFα staining, the frequencies of TNFα positive cells of live cells are displayed.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.