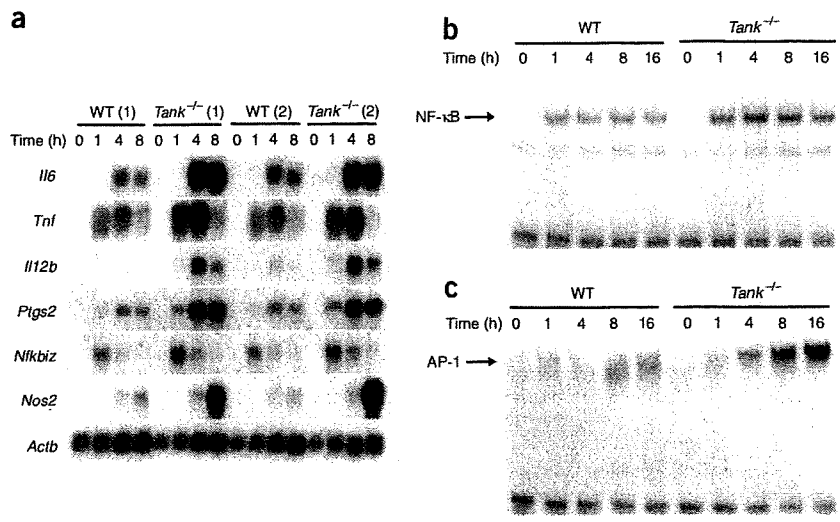

Figure 4 TANK negatively regulates the activation of NF-κB and AP-1 as well as gene expression in response to TLR7 stimulation in macrophages. (**a**) RNA blot analysis of the expression of *Il6*, *Tnf*, *Il12b*, *Ptgs2*, *Nfkbiz* and *Nos2* among total RNA extracted from wild-type and *Tank*$^{-/-}$ peritoneal macrophages stimulated for various times (above lanes) with 10 nM R-848. Bottom, rehybridization of the same membrane with an *Actb* probe (encoding β-actin). Data are from two independent experiments (1 and 2). (**b**,**c**) EMSA of the DNA-binding activity of NF-κB (**b**) and AP-1 (**c**) in nuclear extracts of wild-type and *Tank*$^{-/-}$ macrophages stimulated for various times (above lanes) with 10 μM R-848, assessed with NF-κB- and AP-1-specific probes. Arrows indicate induced NF-κB and AP-1 complexes. Data are representative of three independent experiments.

The results described above indicated that TANK negatively regulates the TLR-induced activation of NF-κB and AP-1. Activation of IRAK1 in response to R-848 was not enhanced in *Tank*$^{-/-}$ macrophages (**Fig. 5a**). Furthermore, IRAK1 was degraded after R-848 stimulation with similar kinetics in wild-type and *Tank*$^{-/-}$ macrophages (**Fig. 5b**), which indicated that TANK regulates signaling downstream of IRAKs. TANK has been reported to interact with the TRAF family members TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6. Among these, TRAF6 is needed for TLR signaling. As TRAF6 is ubiquitinated in response to TLR stimulation, we examined whether TANK modifies the ubiquitination of TRAF6. We found that induction of TRAF6 ubiquitination in response to R-848 stimulation was enhanced in *Tank*$^{-/-}$ macrophages compared with that in wild-type cells (**Fig. 5c**). Reciprocally, overexpression of TANK in human embryonic kidney (HEK293) cells inhibited the ubiquitination of TRAF6 (**Fig. 5d**). Together these results indicate that TANK inhibits TLR-induced activation of NF-κB and AP-1 by suppressing TRAF6 ubiquitination.

TANK is involved in BCR and CD40 signaling

Next we investigated the responses of *Tank*$^{-/-}$ B cells to mitogens such as TLR ligands and crosslinking of the BCR and CD40. After stimulation with R-848, CpG DNA, antibody to IgM (anti-IgM) or anti-CD40, *Tank*$^{-/-}$ B cells proliferated much more than did wild-type B cells (**Fig. 6a**). In contrast, splenic B cell death after culture without mitogen was similar in wild-type and *Tank*$^{-/-}$ mice (**Fig. 6b**), which indicates that TANK is not involved in the control of B cell apoptosis. In response to anti-CD40, B cells activate both canonical and noncanonical NF-κB. The noncanonical pathway is characterized by processing of the NF-κB2 precursor protein p100 to generate p52. We found that activation of noncanonical NF-κB in response to CD40 stimulation was similar in wild-type and *Tank*$^{-/-}$ B cells (**Fig. 6c**). In contrast, NF-κB DNA-binding activity was enhanced in *Tank*$^{-/-}$ B cells compared with that in wild-type B cells (**Fig. 6d**), and the band was supershifted by anti-p65 and anti-p50 (data not shown). Ubiquitination of TRAF6 after stimulation with anti-CD40 was also enhanced in *Tank*$^{-/-}$ B cells (**Fig. 6e**). Furthermore, BCR stimulation also induced enhanced activation of NF-κB and ubiquitination of TRAF6 in *Tank*$^{-/-}$ B cells (**Supplementary Fig. 3a,b**). Furthermore, expression of cyclin D2, an NF-κB-inducible protein, was higher in *Tank*$^{-/-}$ B cells than in wild-type B cells after stimulation with anti-CD40 or anti-IgM (**Supplementary Fig. 4**). These data suggest that TANK is involved in canonical but not noncanonical NF-κB-activation pathways in B cells.

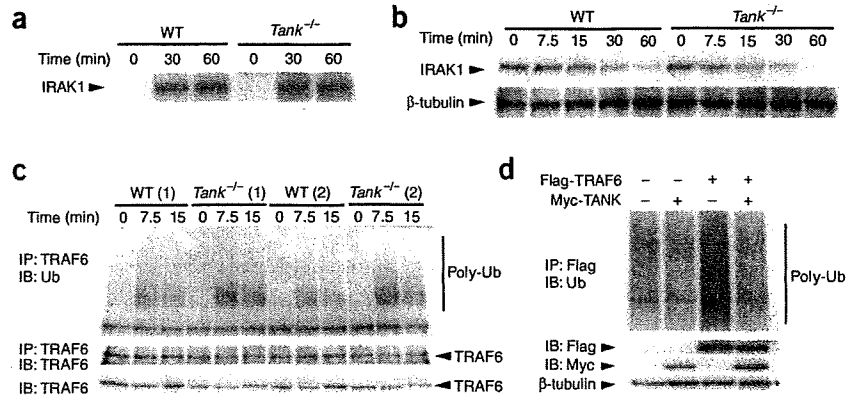

Figure 5 TANK controls TRAF6 ubiquitination in response to TLR7 stimulation in macrophages. (**a**) *In vitro* kinase assay of anti-IRAK1 immunoprecipitates from lysates of wild-type and *Tank*$^{-/-}$ peritoneal macrophages stimulated for various times (above lanes) with 10 μM R-848. Data are representative of two experiments. (**b**) Immunoblot analysis of whole-cell lysates of wild-type and *Tank*$^{-/-}$ macrophages stimulated for various times (above lanes) with 10 μM R-848, probed with anti-IRAK1. Below, immunoblot analysis of β-tubulin (loading control). Data are representative of two experiments. (**c**) Immunoblot (IB) analysis of anti-TRAF6 immunoprecipitates (IP) from lysates of macrophages treated for various times (above lanes) with R-848, probed with antibody to ubiquitin (Ub). Poly-Ub, polyubiquitin. Below, immunoblot analysis of TRAF6 (loading control), with (middle) and without (bottom) anti-TRAF6 immunoprecipitation. Data are from two independent experiments (1 and 2). (**d**) Immunoblot analysis of anti-Flag immunoprecipitates from lysates of HEK293 cells cotransfected with Flag-tagged TRAF6 and Myc-tagged TANK, probed with anti-ubiquitin. Below, immunoblot analysis of lysates without immunoprecipitation; β-tubulin, loading control. Data are representative of three independent experiments.

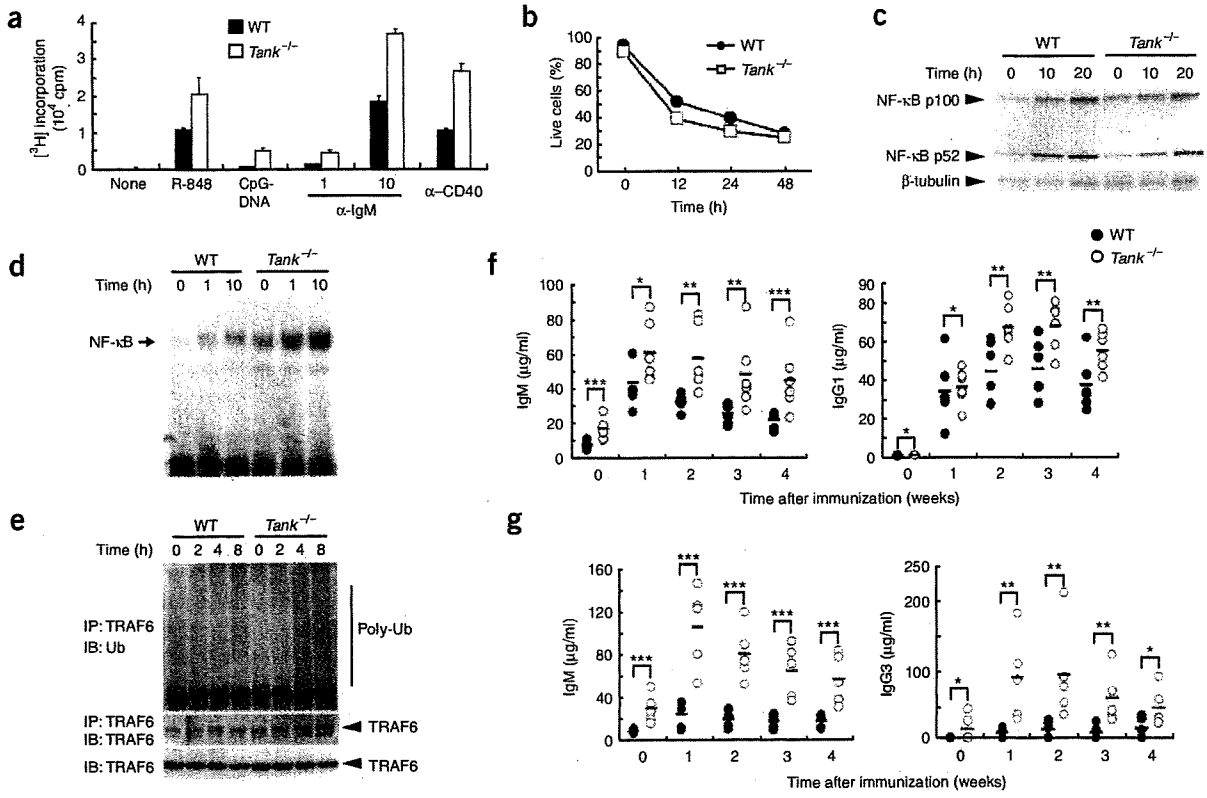

Figure 6 Enhanced activation of B cells in *Tank*$^{-/-}$ mice. (**a**) [^{3}H]thymidine incorporation by purified splenic B cells cultured for 48 h with R-848 (10 nM), CpG DNA (10 nM), anti-IgM (1 or 10 μg/ml) or anti-CD40 (1 μg/ml) and then pulsed with [^{3}H]thymidine (1 μCi) for the final 16 h, measured in a β-scintillation counter. (**b**) Viability of splenic B cells cultured for various times (horizontal axis) in the absence of cytokines, assessed by annexin V staining followed by flow cytometry. (**c**) Immunoblot analysis of the processing of p100 to p52 in whole-cell lysates of wild-type and *Tank*$^{-/-}$ B cells stimulated for various times (above lanes) with anti-CD40 (5 μg/ml). Bottom, immunoblot analysis of β-tubulin (loading control). (**d**) EMSA of NF-κB DNA-binding activity in nuclear extracts of wild-type and *Tank*$^{-/-}$ B cells stimulated for various times (above lanes) with anti-CD40 (5 μg/ml). Arrow indicates the induced NF-κB complex. (**e**) Immunoblot analysis of anti-TRAF6 immunoprecipitates from lysates of splenic B cells treated various times (above lanes) with anti-CD40 (5 μg/ml), probed with anti-ubiquitin. Bottom, immunoblot analysis of TRAF6 (loading control). (**f**) ELISA of the production of NP-specific IgM and IgG1 by mice immunized with NP-CGG, measured 1, 2, 3 and 4 weeks after immunization. (**g**) ELISA of the production of TNP-specific IgM and IgG3 by mice immunized with TNP-Ficoll, measured at 1, 2, 3 and 4 weeks after immunization. Each symbol represents an individual mouse; small horizontal lines indicate the mean (**f**,**g**). $^*P > 0.05$, $^{**}P < 0.05$ and $^{***}P < 0.01$, versus *Tank*$^{-/-}$ mice (two-tailed Student's *t*-test). Data are representative of three (**a** (error bars, s.d.) and **c**–**e**) or two (**b**) independent experiments, or single experiments with a total of five mice per genotype (**f**,**g**).

In contrast to B cells, wild-type and *Tank*$^{-/-}$ T cells proliferated to a similar degree after stimulation with anti-CD3 or anti-CD3 together with anti-CD28 (**Supplementary Fig. 2b**). When stimulated with phorbol 12-myristate 13-acetate and ionomycin *in vitro*, similar proportions of wild-type and *Tank*$^{-/-}$ CD4$^+$ T cells produced IFN-γ or IL-17 (**Supplementary Fig. 2c**). This suggests that TANK is not involved in the development of T helper type 1 or IL-17-producing T helper cells.

To explore the influence of TANK deficiency on antibody responses *in vivo*, we immunized wild-type and *Tank*$^{-/-}$ mice with the T cell–dependent antigen nitrophenol–chicken γ-globulin (NP-CGG) or the T cell–independent antigen trinitrophenyl-Ficoll (TNP-Ficoll). NP-specific IgG1 and IgM titers were higher in *Tank*$^{-/-}$ mice than in wild-type mice (**Fig. 6f**). TNP-specific IgG3 and IgM titers were also higher in *Tank*$^{-/-}$ mice than in wild-type mice (**Fig. 6g**). The difference between wild-type and *Tank*$^{-/-}$ mice was greater in response to immunization with TNP-Ficoll, which suggests that TANK may be more critical for T cell–independent immune responses than for T cell–dependent immune responses *in vivo*.

Intestinal microflora in the autoimmunity of *Tank*$^{-/-}$ mice

Proinflammatory cytokines are critical in the development of autoimmune disease. Overproduction of IL-6 and TNF in mice results in the development of mesangioproliferative glomerulonephritis and chronic polyarthritis, respectively. To investigate whether IL-6 or TNF is involved in disease pathogenesis in *Tank*$^{-/-}$ mice, we generated mice lacking IL-6 or TNF on the *Tank*$^{-/-}$ genetic background. The titers of anti-dsDNA antibodies were significantly lower in 5-month-old *Tank*$^{-/-}$*Il6*$^{-/-}$ mice than in 5-month-old *Tank*$^{-/-}$ mice (**Fig. 7a**). Moreover, IL-6 deficiency 'rescued' the glomerulonephritis that developed in *Tank*$^{-/-}$ mice (**Fig. 7b**). In contrast, TNF deficiency did not significantly alter the amount of anti-dsDNA antibody production in *Tank*$^{-/-}$ mice (**Fig. 7c**). To determine whether MyD88 deficiency protects against the disease progress, we crossed *Tank*$^{-/-}$ mice with *MyD88*$^{-/-}$ mice. Anti-dsDNA antibody titers were significantly lower in 5-month-old *Tank*$^{-/-}$*MyD88*$^{-/-}$ mice than in *Tank*$^{-/-}$ mice (**Fig. 7d**), which indicates that TLR and/or IL-1R family members are critical for the autoimmunity caused by TANK deficiency. The next question we addressed was how TLR and/or IL-1R signaling was activated to cause IL-6 production. Intestinal microflora has been shown to be involved

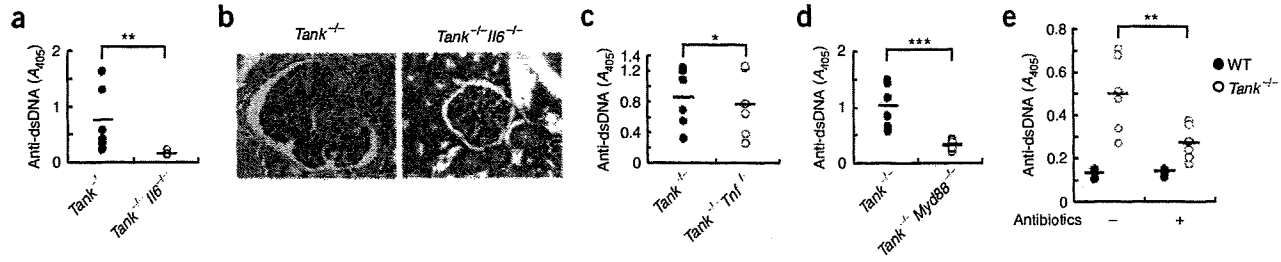

Figure 7 Antibiotic treatment, as well as deficiency of MyD88 or IL-6, ameliorates autoantibody production in $Tank^{-/-}$ mice. (**a**) Anti-dsDNA antibodies in serum from 5-month-old $Tank^{-/-}$and $Tank^{-/-}Il6^{-/-}$ mice. (**b**) Hematoxylin and eosin staining of kidney sections from $Tank^{-/-}$ mice and $Tank^{-/-}Il6^{-/-}$ mice. Original magnification, ×100. (**c**,**d**) ELISA of anti-dsDNA antibodies in $Tank^{-/-}$ and $Tank^{-/-}Tnf^{-/-}$ mice (**c**) or $Tank^{-/-}$ and $Tank^{-/-}Myd88^{-/-}$ mice (**d**). (**e**) ELISA of serum anti-dsDNA antibodies in 16-week-old wild-type and $Tank^{-/-}$ mice given drinking water (from birth onward) containing ampicillin (1 g/l), neomycin (1 g/l), vancomycin (0.5 g/l) and metronidazole (1 g/l); control wild-type and $Tank^{-/-}$ mice received untreated drinking water (–). Each symbol represents an individual mouse; small horizontal lines indicate the mean (**a**,**c**–**e**). $*P > 0.05$, $**P < 0.05$ and $***P < 0.01$, versus $Tank^{-/-}$ mice (two-tailed Student's *t*-test). Data are representative of single experiments with a total of six mice per genotype (**a**,**c**–**e**) or are representative of three experiments (**b**).

in the pathogenesis of autoimmune diseases, such as colitis in IL-10-deficient mice. Therefore, we treated $Tank^{-/-}$ mice orally with a combination of antibiotics to clear the intestinal microflora. The antibiotic treatment significantly ameliorated the production of anti-dsDNA antibodies (**Fig. 7e**), which suggests that continuous stimulation of TLRs by intestinal microflora contributes to the generation of autoantibodies in the absence of TANK.

DISCUSSION

Here we generated $Tank^{-/-}$ mice and have shown that TANK is essential for the negative regulation of canonical NF-κB signaling. $Tank^{-/-}$ mice had enhanced activation of macrophages and B cells in response to TLR ligands and antigens, which culminated in the development of fatal immune complex–mediated renal failure. Although TANK has been shown to positively regulate TBK1- and Ikki-mediated production of type I interferon by *in vitro* studies, analysis of $Tank^{-/-}$ mice showed that TANK was not needed for activation of the type I interferon pathway downstream of RLRs or TRIF. TANK forms a family with the adaptor proteins NAP1 and SINTBAD[38,39], which are composed of an amino-terminal coiled-coil domain and a TBK1-binding domain. NAP1 and SINTBAD have also been linked to the activation of TBK1 and Ikki downstream of virus sensors. Knockdown of NAP1, SINTBAD or TANK by small interfering RNA has been associated with impaired interferon responses. Hence, it is possible that these three proteins function redundantly in the activation of TBK1 and Ikki.

Although published studies have shown that TANK is a positive regulator of NF-κB, our results have shown that TANK is critical for the negative regulation of canonical NF-κB through suppression of TRAF6 ubiquitination. Lysine 63–type ubiquitination is important for the activation of TAK1 with the binding partners TAB2 and TAB3 in TLR signaling, and TANK may inhibit TRAF6 ubiquitination by directly binding to TRAF6 in response to TLR stimulation. Although A20 and CYLD have been identified as deubiquitinases[40–42], TANK does not contain a deubiquitination enzyme domain. Immunoprecipitation experiments showed that overexpressed A20 or CYLD failed to immunoprecipitate together with overexpressed TANK, which suggests that TANK may suppress ubiquitination of TRAF6 independently of A20 or CYLD (data not shown). Further studies are needed to assess the precise mechanism through which TANK modifies TRAF6. In addition, activation of canonical NF-κB in response to BCR and CD40 stimulation was augmented in $Tank^{-/-}$ B cells. Consistent with that, proliferation of B cells in response to TLR and BCR stimulation was much higher in $Tank^{-/-}$ mice. In TCR signaling, TRAF2 and TRAF6 are reported to participate in NF-κB activation downstream of the adaptors Bcl-10 and MALT1 (ref. 43). Given that TANK suppresses the polyubiquitination of TRAF6 in response to TLR stimulation in macrophages, it is possible that TANK suppresses BCR and CD40 signaling by regulating the activation of TRAF proteins in B cells. However, activation of non-canonical NF-κB was not enhanced in $Tank^{-/-}$ B cells, and it has been reported that TRAF3 controls mainly that activation in B cells[44]. Hence, these observations suggest that TANK is not involved in signaling downstream of TRAF3. Furthermore, TRAF2 can control noncanonical NF-κB as well as the development of marginal zone B cells. The relationship between TANK and TRAF2 needs to be explored further.

The disease caused by the absence of TANK was characterized by glomerulonephritis due to deposition of immune complexes in the glomeruli. In addition, anti-dsDNA antibodies and antinuclear antibodies were present in high concentrations in $Tank^{-/-}$ mice. These observations indicate that $Tank^{-/-}$ mice may represent a mouse model of lupus-like immune diseases. The phenotype of $Tank^{-/-}$ mice is reminiscent of that of mice that overexpress IL-6 in B cells[45], which is characterized by lymphadenopathy and plasmacytosis culminating in the development of severe glomerular nephritis. IL-6 is a pleiotropic cytokine responsible for fever, acute-phase protein expression, osteoclast activation and the development of IL-17-producing T helper cells and plasma cells. Indeed, $Tank^{-/-}$ macrophages showed enhanced production of proinflammatory cytokines, including IL-6 and TNF, in response to TLR stimulation. Furthermore, $Tank^{-/-}$ mice failed to produce autoantibodies and did not develop glomerulonephritis in the absence of IL-6. These results indicate that IL-6 is essential for the development of the $Tank^{-/-}$ B cells that are responsible for the production of autoantibodies. In contrast, $Tank^{-/-}$ T cells responded normally to TCR stimulation. Given that TANK is critical for inhibiting BCR-induced B cell activation, it is possible that the lack of TANK in B cells is important for the generation of autoimmune nephritis through aberrant activation of B cells in response to antigen stimulation.

The generation of anti-dsDNA antibodies in $Tank^{-/-}$ mice was significantly lower in response to oral treatment with antibiotics or in the absence of MyD88, which suggests that TLR signaling is critical for the development of autoimmune disease in $Tank^{-/-}$ mice. Although various proteins have been identified as negative regulators of TLR signaling, few mice lacking any a single one of these proteins

spontaneously develop autoimmune disease, with the exception of mice lacking A20. A20-deficient mice spontaneously develop multiorgan inflammation and premature death, which can be 'rescued' by MyD88 deficiency[46,47]. Unlike *Tank*$^{-/-}$ mice, A20-deficient mice do not develop immune complex–mediated glomerulonephritis. A20 controls TNF receptor signaling in addition to TLR signaling, yet the responses to TNF were not altered in *Tank*$^{-/-}$ cells. TNF is involved in the pathogenesis of organ-specific autoimmune diseases, such as rheumatoid arthritis and Crohn's disease[48]. Hence, the differences in the signaling pathways regulated by A20 and TANK may explain the differences in the types of autoimmune disease caused by A20 or TANK deficiency.

As oral treatment with antibiotics ameliorated autoantibody production in *Tank*$^{-/-}$ mice, constitutive stimulation of TLRs by intestinal microflora seems to be responsible for the generation of autoimmunity in the absence of TANK. Bone marrow–transfer experiments showed that hematopoietic cells were responsible for the death of *Tank*$^{-/-}$ mice (data not shown). Intestinal microflora contribute to the pathogenesis of inflammatory bowel disease[48,49], and the colitis observed in IL-10-deficient mice was 'rescued' by the absence of MyD88 (ref. 24), which suggests that TLR signaling is involved in the pathogenesis of inflammatory bowel disease. As TLRs are expressed on intestinal DCs and are responsible for sensing microbes in the intestine, it is possible that TANK controls the production of certain cytokines in intestinal tissues. Further studies are needed to understand why TANK deficiency causes autoimmune nephritis but not colitis.

In addition, the antigen-specific humoral immune responses to haptens were enhanced in *Tank*$^{-/-}$ mice. This may have been due to the enhanced DC and B cell activation in response to antigens and the adjuvant in *Tank*$^{-/-}$ mice. It will be useful to explore whether inhibition of TANK expression in certain cell types is beneficial for inducing antigen-specific immune responses *in vivo*. Modification of TANK may be helpful in vaccines administered together with an adjuvant. In summary, our results here have shown that TANK is a negative regulator of TLR and BCR responses. Future studies involving cell type–specific deletion of TANK will clarify the complex interaction between cells of the immune system needed to prevent the development of autoimmune disease.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession code. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A002312.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

T.K., O.T. and S.A. designed the research and analyzed data; T.K. generated *Tank*$^{-/-}$ mice and did most of the experiments; Y.T., Y.I. and T.T. did histological examination of kidneys; H.K. provided advice; and T.K., O.T. and S.A. prepared the manuscript.

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ONLINE METHODS

Generation of *Tank*$^{-/-}$ mice. *Tank* was isolated from genomic DNA extracted from embryonic stem cells (GSI-I) by PCR. The targeting vector was constructed by replacement of a 2.0-kilobase fragment encoding the *Tank* open reading frame with a neomycin-resistance gene cassette; the gene encoding herpes simplex virus thymidine kinase driven by the promoter of the gene encoding phosphoglycerate kinase was inserted into the genomic fragment to facilitate negative selection. After transfection of the targeting vector into embryonic stem cells, colonies doubly resistant to the aminoglycoside G418 and gancyclovir were selected, screened by PCR and further confirmed by Southern blot analysis. Homologous recombinants were microinjected into blastocysts from C57BL/6 female mice, and heterozygous F_1 progenies were intercrossed to obtain *Tank*$^{-/-}$ mice. *Tank*$^{-/-}$ mice on the 129Sv × C57BL/6 background and their littermate controls were used.

Mice and cells. *MyD88*$^{-/-}$ and *Tnf*$^{-/-}$ mice have been described[4,23]. *Il6*$^{-/-}$ mice were provided by T. Yasui. All animal experiments were carried out with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University). At 3 d after injection of 2 ml of 4.0% (wt/vol) thioglycollate medium (Sigma), peritoneal exudate cells were isolated from the peritoneal cavities of mice by washing with ice-cold Hank's buffered-salt solution (Invitrogen). Resting B cells were isolated from splenocyte single-cell suspensions by positive selection with anti-B220 magnetic beads (Miltenyi Biotec). T cells were isolated from splenocyte single-cell suspensions by positive selection with anti-Thy-1.2 magnetic beads (Miltenyi Biotec). Cell purity was confirmed to be above 90% by flow cytometry.

Reagents. MALP-2 was provided as described[7]. LPS from *Salmonella minnesota* strain Re-595 was from Sigma-Aldrich. Poly(I:C) was from Amersham Biosciences. R-848 was provided by the Pharmaceuticals and Biotechnology Laboratory of the Japan Energy Corporation. The CpG oligonucleotide was synthesized as described[7]. Polyclonal anti-IRAK1 has been described[7].

Measurement of cytokines and autoantibodies. Concentrations of cytokines in culture supernatants and serum were measured by ELISA. ELISA kits for mouse TNF and IL-6 were from R&D Systems. The ELISA kit for mouse IFN-α was from PBL Biomedical Laboratories. ELISA kits for mouse anti-dsDNA antibodies and antinuclear antibody were from Alpha Diagnostic International. Serum immunoglobulin concentrations were measured as described[50].

Histological analysis. Formalin-fixed tissues were stained with hematoxylin and eosin or periodic acid–Schiff reagent. For detection of renal IgG deposits, kidneys were rapidly frozen in liquid nitrogen and cryostat sections 2 μm in thickness were fixed for 15 min in 100% (vol/vol) acetone. Sections were incubated overnight at 4 °C with FITC-conjugated goat anti–mouse IgG (67228; ICN Biomedicals), FITC-conjugated donkey anti–mouse IgM (715-095-050; Jackson ImmunoResearch), FITC-conjugated sheep anti–human C3c complement (433004; Thermo Electron) or FITC-conjugated anti–mouse C1q (RmC7H8; Cedarlane Laboratories), each at a concentration of 10 μg/ml.

RNA hybridization. Peritoneal macrophages were treated for 0, 1, 4 and 8 h with 10 nM R-848, and total RNA was extracted with the TRIzol reagent (Invitrogen). The extracted RNA was separated by electrophoresis, transferred to nylon membranes and hybridized with various cDNA probes. For detection of the expression of *Tank* mRNA, a 319–base pair fragment (nucleotides 350–669) of Tank cDNA was used as a probe. The same membranes were rehybridized with an *Actb* probe.

***In vitro* kinase assay.** Peritoneal macrophages stimulated with 10 nM R-848 were lysed and immunoprecipitated with anti-IRAK1. Then, IRAK1 activity was measured by *in vitro* kinase assay as described[7].

Immunoblot analysis. Peritoneal macrophages were treated for various times with 10 nM R-848, then were lysed in a lysis buffer composed of 1.0% (vol/vol) Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and a protease inhibitor 'cocktail' (Roche). Lysates were separated by SDS-PAGE and analyzed by immunoblot. Polyclonal anti-TANK (2141) was from Cell Signaling. Polyclonal anti-TRAF6 (sc-7221), monoclonal anti-Ub (F-7), monoclonal anti-β-tubulin (D-10) and anti-cyclin D2 (34B1-3) were from Santa Cruz Biotechnology.

EMSA. Nuclear extracts were prepared from peritoneal macrophages (4×10^6) stimulated with 10 nM R-848 as described[7], then were incubated with or without antibodies to NF-κB p65 (C-20) or p50 (D-17; Santa Cruz) and were further incubated with a probe specific for NF-κB DNA-binding sites, before being separated by electrophoresis and visualized by autoradiography.

Immunoblot, immunoprecipitation and *in vivo* ubiquitination assays. Peritoneal macrophages (4×10^6) were stimulated for various times with 10 nM R-848. Immunoblot analysis and immunoprecipitation were done as described[7]. For detection of *in vivo* ubiquitination of TRAF6, cell lysates were boiled for 10 min at 90 °C in 1% (wt/vol) SDS for removal of noncovalently attached proteins, followed by immunoprecipitation with anti-TRAF6 in 0.1% (wt/vol) SDS lysis buffer in the presence of protease inhibitors. Ubiquitin was detected by immunoblot analysis.

B cell and T cell proliferation assays. Purified splenic B cells (5×10^4) were cultured for 48 h in 96-well plates with various concentrations of R-848, CpG DNA, anti-IgM (Jackson ImmunoResearch) or anti-CD40 (HM40-3, PharMingen). Purified splenic T cells were stimulated for 48 h with plate-bound anti-CD3 alone (1 or 5 μg/ml; 2C11; Pharmingen) or with anti-CD3 (1 μg/ml) plus anti-CD28 (1 μg/ml; 37.51; Pharmingen). Samples were pulsed with 1 μCi [^{3}H]thymidine for the final 16 h and ^{3}H uptake was measured with a β-scintillation counter (Packard).

***In vivo* immunization and ELISA.** Mice were immunized intraperitoneally with 50 μg NP-CGG (Biosearch Technologies) precipitated with Imject alum (Pierce) or with 25 μg of TNP-Ficoll (Biosearch Technologies). Antigen- and isotype-specific antibodies in serum collected from peripheral blood at various time points were measured by ELISA on plates coated with NP-BSA or TNP-BSA (Biosearch Technologies). Alkaline phosphatase–conjugated antibodies to mouse IgM (1020-04), IgG1 (1070-04) and IgG3 (1100-04) were from Southern Biotechnology.

Cell viability. Purified splenic B cells (1×10^6) were cultured for various periods in RPMI medium containing 10% (vol/vol) FCS. Cell viability was assessed with annexin V–indocarbocyanine (BioVision) and a FACSCalibur (Becton Dickinson).

Construction of TANK expression plasmids. Full-length mouse TANK cDNA was obtained by PCR from a mouse cDNA library and was cloned into the Myc-pcDNA3 vector.

Statistical analysis. Statistical significance was calculated with the two-tailed Student's *t*-test. *P* values of less than 0.05 were considered significant.

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REVIEW

Immune responses of TLR5$^+$ lamina propria dendritic cells in enterobacterial infection

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Abstract Toll-like receptors (TLRs) recognize distinct microbial components and induce innate immune responses. TLR5 has been shown to recognize bacterial flagellin. Unlike other TLRs, TLR5 is not expressed on conventional dendritic cells or macrophages. By contrast, TLR5 is mainly expressed on intestinal CD11c$^+$ lamina propria cells (LPCs), which do not express TLR4. These cells detect pathogenic bacteria and secreted proinflammatory cytokines, mainly in a TLR5-dependent manner. Notably, transport of pathogenic *Salmonella typhimurium* from the intestinal tract to mesenteric lymph nodes was impaired in *Tlr5*$^{-/-}$ mice, suggesting that CD11c$^+$ LPCs expressing TLR5 are used by *S. typhimurium* for systemic infection. CD11c$^+$ LPCs consist of four subsets distinguished by differential expression patterns of CD11c and CD11b. CD11c^{hi}CD11b^{hi} LPDCs have been identified as TLR5-expressing cells. In response to flagellin, these LPDCs induce the differentiation of naive B cells into IgA$^+$ plasma cells via a mechanism independent of gut-associated lymphoid tissue (GALT), and trigger the differentiation of antigen-specific Th17 and Th1 cells. These LPDCs have unique properties in that they can induce acquired immunity as well as innate immunity via TLR5 in the intestine.

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Keywords Lamina propria · TLR5 · IgA · Th17 · Autophagy

Introduction

Toll-like receptors (TLRs) recognize a variety of microbial components and induce innate immune responses [1]. They are abundantly expressed on 'professional' antigen-presenting cells (APCs) such as macrophages and conventional dendritic cells (DCs) and serve as an important link between the innate and adaptive immune responses. To date, 13 TLRs have been identified in mammals. Bacterial flagellin is a structural protein that forms the main portion of flagella and promotes bacterial chemotaxis and bacterial adhesion to and invasion of host tissues [2]. TLR5 recognizes the conserved domain in flagellin monomers and triggers proinflammatory as well as adaptive immune responses [3, 4]. TLR5 is expressed on the basolateral surfaces of intestinal epithelial cells and is thought to be key in the recognition of invasive flagellated bacteria at the mucosal surface [5]. Human intestinal epithelial cell lines produce chemokines in response to flagellin, leading to subsequent migration of immature DCs [6]. Furthermore, TLR5 is highly expressed in the human lung and a common TLR5 polymorphism in humans causes susceptibility to legionellosis [7, 8]. Although accumulating evidence suggests that TLR5 is critical for host defense against enterobacterial infection, the in vivo function of TLR5 has not been fully elucidated.

Identification of TLR5-expressing cells in the intestine

Unlike other TLR family members, TLR5 is not expressed on macrophages and conventional DCs in mice. Instead,

TLR5 is highly expressed in the small intestine [9]. In contrast to the findings of a previous report, TLR5 expression was not high in intestinal epithelial cells (IECs). IECs could not produce any inflammatory cytokines in response to flagellin, although they did produce defencin-β3 and CCL27. On the other hand, TLR5 was highly expressed in lamina propria cells (LPCs). TLR family members are preferentially expressed on APCs. In the LP of the mouse small bowel, DCs have been thought to be the dominant APCs. Interestingly, a considerable amount of TLR5 mRNA was detected in $CD11c^+$ LPCs, but none was detected in $CD11c^-$ LPCs. Thus, $CD11c^+$ LPCs were identified as TLR5-expressing cells in the intestine (Fig. 1) [9].

Innate immune responses by $CD11c^+$ LPCs

Because the intestine is constantly exposed to food antigens (Ags) and commensal bacteria, it is the consensus that most mucosal DCs induce tolerance, but not inflammation. In the Peyer's patches and mesenteric lymph nodes, conventional DCs consist of $CD11c^{hi}CD11b^+CD8a^-$, $CD11c^{hi}CD11b^-CD8a^+$ and $CD11c^{hi}CD11b^-CD8a^-$ subsets. In addition, $CD11c^{int}$ plasmacytoid DCs exist in these sites [10]. DCs in the Peyer's patch produce interleukin 10 (IL-10) rather than IL-12, polarize naive T cells toward T helper type 2 (Th2) or regulatory T (Treg) cells, and induce the differentiation of immunoglobulin A $(IgA)^+$ plasma cells [11]. Flagellin-stimulated $CD11c^+$ LPCs do not produce IL-10 and tumor-necrosis factor (TNF)-α, but instead produce IL-6 and IL-12 [9]. Furthermore, $CD11c^+$ LPCs express chemokines, prostaglandins, antimicrobial peptides and molecules involved in cellular adhesion, cytoskeletal organization and intracellular transport in response to flagellin [9]. Accordingly, $CD11c^+$ LPCs have a tendency to induce inflammatory responses rather than tolerance when exposed to flagellin.

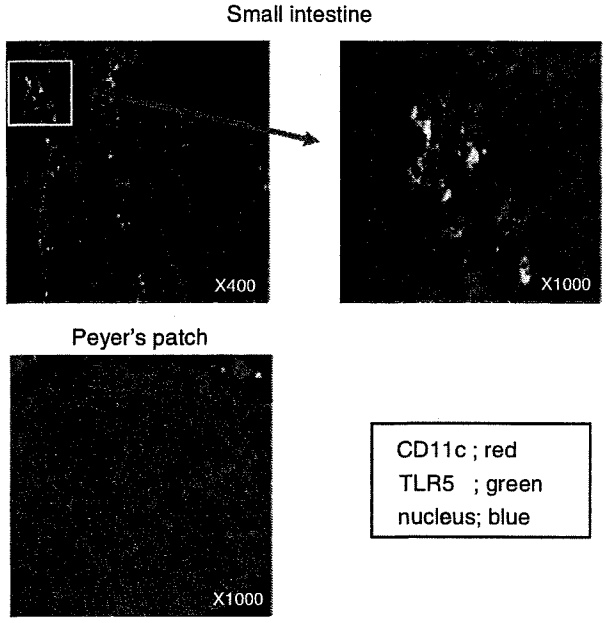

Fig. 1 TLR5 is highly expressed on $CD11c^+$ LPCs. Confocal microscopy of frozen tissue sections of the small intestine and Peyer's patch of C57BL/6 mice, fixed and stained with antibodies specific for CD11c (*red*) and TLR5 (*green*) [9]

Recognition of bacteria by $CD11c^+$ LPCs

TLR4, which is abundantly expressed on innate immune cells such as conventional DCs and macrophages, recognizes lipopolysaccharide (LPS) of Gram-negative bacteria [1]. However, $CD11c^+$ LPCs do not produce any inflammatory cytokines in response to LPS owing to their low expression of TLR [49]. Conventional DCs, which express TLR4 but not TLR5, recognize Gram-negative flagellated bacteria, mainly via TLR4, while $TLR4^-TLR5^+CD11c^+$ LPCs produce inflammatory cytokines after exposure to such bacteria [9]. The unique profile of TLR expression in $CD11c^+$ LPCs seems to be closely related to the specific environment in the intestine. Most commensal bacteria in the intestine are Gram-negative anaerobic rod bacteria, which contain LPS in their cell walls. The low expression of TLR4 may allow $CD11c^+$ LPCs to avoid inducing excessive immune responses to commensal bacteria. Instead, $CD11c^+$ LPCs may induce inflammatory responses to pathogenic flagellated bacteria, which are able to invade the LP, via TLR5. However, some commensal bacteria also have flagella. Recently, it was reported that α- and ε-proteobacteria can change the TLR5 recognition site in flagellin without losing flagellar motility [12]. Furthermore, some commensal bacteria in the intestine suppress flagellin expression [13]. Unlike pathogenic bacteria, commensal bacteria may have mechanisms to escape TLR5-mediated host detection in the intestine.

Role of TLR5 in *Salmonella typhimurium* infection

S. typhimurium is a facultative intracellular pathogenic bacterium that causes typhoid-like disease in mice. In oral infection, *S. typhimurium* are captured by sub-epithelial DCs after transport through M cells in Peyer's patches (PPs) or intra-epithelial DCs in LPs [14, 15]. After internalization, *S. typhimurium* inject effector proteins into the cytoplasm via a type III secretion system and inhibit phagosome-lysosome fusion. *S. typhimurium* can safely replicate in *Salmonella*-containing vacuoles (Fig. 2) [16]. On the other hand, bacteria-laden DCs undergo maturation and migrate to the T-cell zones of PPs or draining

mesenteric lymph nodes (MLNs). These mature DCs are also thought to be responsible for the dissemination of *S. typhimurium* via the blood stream to the liver and spleen [13, 17]. Although CD11c$^+$ LPCs express inflammatory cytokines after exposure to *S. typhimurium* in vitro, via a TLR5-dependent mechanism, *Tlr5*$^{-/-}$ mice showed resistance to oral *S. typhimurium* infection. The transport of *S. typhimurium* from the LP to MLNs was impaired in *Tlr5*$^{-/-}$ mice. As *S. typhimurium* could not fully activate and induce maturation of *Tlr5*$^{-/-}$ CD11c$^+$ LPCs, migration of bacteria–laden CD11c$^+$ LPCs may be inefficient in *Tlr5*$^{-/-}$ mice. *S. typhimurium* may use CD11c$^+$ LPCs as carriers during systemic infection by inversely utilizing the host defense activity of TLR5 (Fig. 3) [9].

Four subsets of CD11c$^+$ LPCs in the small intestine

CD11c$^+$ LPCs in murine small intestine consist of four subsets distinguished by differential expression patterns of CD11c and CD11b: two subsets of DCs (CD11c^{hi}CD11b^{low} and CD11c^{hi}CD11b^{hi}), macrophages (CD11c^{int}CD11b^{int}) and eosinophils (CD11c^{int}CD11b^{mid}) (Fig. 4a). The CD11c^{hi}CD11b^{low} and CD11c^{hi}CD11b^{hi} subsets have a DEC-205$^+$ major histocompatibility complex (MHC) class II-high CD80$^+$CD86$^+$CD103$^+$ surface phenotype. In addition, the CD11c^{hi}CD11b^{hi} subset is moderately F4/80 positive, suggesting that this subset expresses both DC (DEC-205) and macrophage (F4/80) markers. The CD11c^{int}CD11b^{int} subset consists of F4/80$^+$DEC-205$^-$ MHC class II$^+$ phagocytic macrophages. The CD11c^{int} CD11b^{mid} subset consists of eosinophils with uniquely shaped nuclei and eosinophilic granules, which express CD80 but not MHC class II (Fig. 4b) [18]. Among the four subsets of CD11c$^+$ LPCs in the mouse small intestine, CD11c^{hi}CD11b^{hi} lamina propria dendritic cells (LPDCs) specifically express TLR5 (Fig. 4c). This subset produces IL-6 and IL-12 in response to flagellin in a TLR5-dependent manner. However, they did not produce IL-10, TNF-α or IL-23. CD11c^{hi}CD11b^{hi} LPDCs also express TLR9, and produce IL-6 and IL-12 in response to CpG DNA. In contrast to CD11c^{hi}CD11b^{hi} LPDCs, other CD11c$^+$

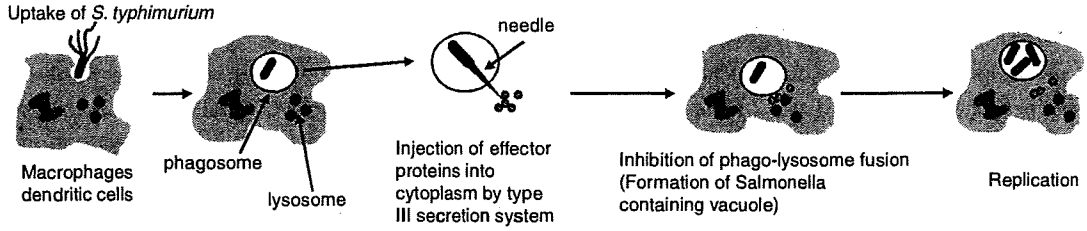

Fig. 2 Type III secretion system of *S. typhimurium*. *S. typhimurium* inject effector proteins into the cytoplasm via a type III secretion system and inhibit phagosome-lysosome fusion. *S. typhimurium* can safely replicate in *Salmonella*-containing vacuoles

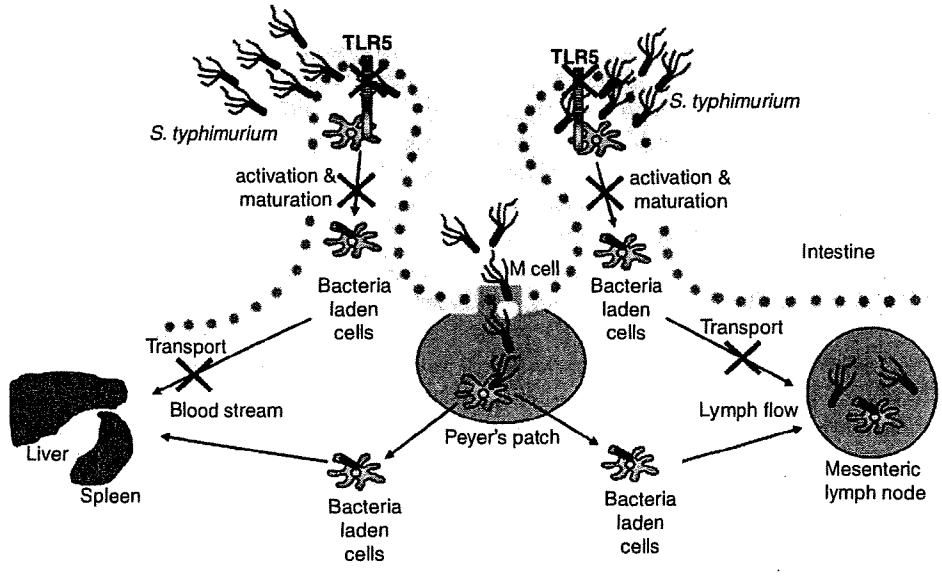

Fig. 3 Impaired transport of *S. typhimurium* in *Tlr5*$^{-/-}$ mice. *S. typhimurium* are captured by sub-epithelial DCs after transport through M cells in PPs or intra-epithelial DCs in LPs. Bacteria-laden DCs undergo maturation and migrate to the T-cell zones of PPs or draining MLNs. These maturate DCs are also responsible for the dissemination of *S. typhimurium* via the blood stream to the liver and spleen. The transport of *S. typhimurium* from the LP to MLNs was impaired in *Tlr5*$^{-/-}$ mice. As *S. typhimurium* could not fully activate and induce maturation of *Tlr5*$^{-/-}$ CD11c$^+$ LPCs, migration of bacteria–laden CD11c$^+$ LPCs may be inefficient in *Tlr5*$^{-/-}$mice

subsets in the LP do not produce such cytokines in response to flagellin. Thus, $CD11c^{hi}CD11b^{hi}$ LPDCs are responsible for TLR5-mediated innate immune responses in the intestinal LP [18].

TLR5 and intestinal IgA production

The intestine is known as an organ that produces large amounts of secretory IgA [19]. Intestinal gut-associated lymphoid tissues (GALTs) such as PPs, ILFs and MLNs are major sites for generation of IgA^+ plasma cells in the intestine. IgA^+ plasma cells are induced there via a mechanism dependent on Ag, $CD4^+$ follicular B helper T cells and the formation of germinal centers (GCs) [20–22]. Suppressive $Foxp3^+CD4^+$ T cells in PPs can differentiate efficiently into cells with characteristics of follicular B helper T cells, which then participate in the induction of GCs and IgA production in the gut[23]. $CD103^+$ GALT DCs produce retinoic acid (RA), which induces the selective expression of gut homing receptors, such as integrin $\alpha4\beta7$ and CCR9, on differentiated IgA^+ plasma cells for gut homing [24, 25]. However, differentiation of IgA^+ cells does not necessarily require T cell help and GC formation. GALT DC-derived RA and cytokines synergistically act on naïve B cells, leading to the generation of T cell–independent IgA^+ cells [25, 26]. Furthermore, some IgM^+ B cells, especially peritoneal B1 cells, directly migrate to the gut LP in a sphingosine 1-phosphate (S1P)-dependent manner and differentiate into IgA^+ plasma cells in the LP with the help of stroma cells [27]. Commensal bacteria induce natural secretory IgA, and this process is mediated by commensal bacteria-laden DCs [28]. Furthermore, DCs in the small intestinal LP send protrusions into the lumen through an intercellular gap between epithelial cells via a mechanism dependent on the chemokine receptor CX3CR1, and actively sample luminal bacteria [29]. Although accumulating evidence suggests the involvement of DCs in the generation of IgA^+ plasma cells in the LP, it is not known what subset of DCs is responsible for this event. Interestingly, $CD11c^{hi}CD11b^{hi}$ LPDCs are responsible for the generation of IgA^+ plasma cells in the LP. Flagellin-stimulated $CD11c^{hi}CD11b^{hi}$ LPDCs efficiently induced the differentiation of $B220^-$ IgA^+ plasma cells in the absence of T cells in a TLR5-dependent manner. Similar to $CD103^+$ DCs in GALTs, $CD11c^{hi}CD11b^{hi}$ LPDCs specifically express *Aldh1a2* mRNA, which encodes retinal dehydrogenase 2 (RALDH2). $CD11c^{hi}$ $CD11b^{hi}$ LPDC-derived RA synergistically acts on naïve B cells with TLR5-mediated inflammatory cytokines, leading to the induction of IgA^+ plasma cells. Furthermore, TLR5 is critical for IgA synthesis in vivo. Mice lacking the transcription factor Id2 do not develop GALTs, yet they retain intestinal IgA production. In wild-type mice, around 20% of IgA^+ plasma cells exist in the small intestinal LP. Interestingly, about 10% of IgA^+ cells are found in the LP of $Id2^{-/-}$ mice, which confirms that gut IgA can be generated without GALTs. Although $Tlr5^{-/-}$ mice did not have fewer IgA^+ B cells, $Id2^{-/-}$ $Tlr5^{-/-}$ mice had far fewer IgA^+ cells in the LP [18]. Thus, TLR5 signaling in $CD11c^{hi}CD11b^{hi}$ LPDCs is essential for GALT-independent IgA synthesis.

IgA class-switch recombination (CSR) is severely impaired in inducible nitric oxide synthase (iNOS)-deficient mice [30]. iNOS regulates the expression of transforming growth factor-β receptor (TGF-βR) II and plays a critical role in T-cell-dependent IgA CSR. iNOS is also involved in T-cell-independent IgA CSR through the production of a proliferation-inducing ligand (APRIL, also called Tnfsf13) and a B-cell-activating factor of the TNF family (BAFF, also called Tnfsf13b). iNOS is preferentially expressed in DCs in both GALTs and LPs. The number of $iNOS^+$ DCs was reduced in the mucosa-associated lymphoid tissues (MALTs) of germ-free mice, $Myd88^{-/-}$ mice and $Tlr2^{-/-}4^{-/-}9^{-/-}$ mice, suggesting that this DC subset is induced via a TLR-dependent mechanism. These $iNOS^+$ DCs have a $TNF\text{-}\alpha^+CD11b^+$MHC class $II^+CD80^+CD86^{lo}Ly6C^+Ly6G^-Gr\text{-}1^+Mac3^+$ surface phenotype, revealing that they are presumably a TNF-α/iNOS-producing DC (TipDC) subset, originally identified in mice infected with *Listeria monocytogenes* [31, 32]. Although both $iNOS^+$ DCs and $CD11c^{hi}CD11b^{hi}$ LPDCs are important for IgA synthesis in the intestine, their phenotypes and functions are completely different (Fig. 5a). Based on the expression of surface molecules, $iNOS^+$ DCs are similar to $CD11c^{int}CD11b^{int}$ macrophages in the LP, which were recently shown to induce $Foxp3^+$ regulatory T cells via IL-10 and TGF-β production [33]. Whereas $iNOS^+$ DCs produce TNF-α, $CD11c^{hi}CD11b^{hi}$ LPDCs produce IL-6 and IL-12, but not TNF-α. Although $iNOS^+$ DCs supply BAFF and APRIL, $CD11c^{hi}CD11b^{hi}$ LPDCs can not. By contrast, the expression of RALDH isoforms were ambiguous in $iNOS^+$ DCs. Thus, $CD11c^{hi}CD11b^{hi}$ LPDCs and $iNOS^+$ DCs have separate roles in IgA CSR and may work cooperatively with each other in the generation of IgA^+ plasma cells (Fig. 5b) [18, 30].

$CD11c^{hi}CD11b^{hi}$ LPDC-mediated $CD4^+$ helper T cell (Th) response

$CD11c^{hi}CD11b^{hi}$ LPDCs are critical for the induction of Th responses as well as IgA production in the intestine [18, 33]. Th17 cells have recently emerged as a third T cell subset that produces IL-17, plays an essential role in protection against certain extracellular pathogens and induces

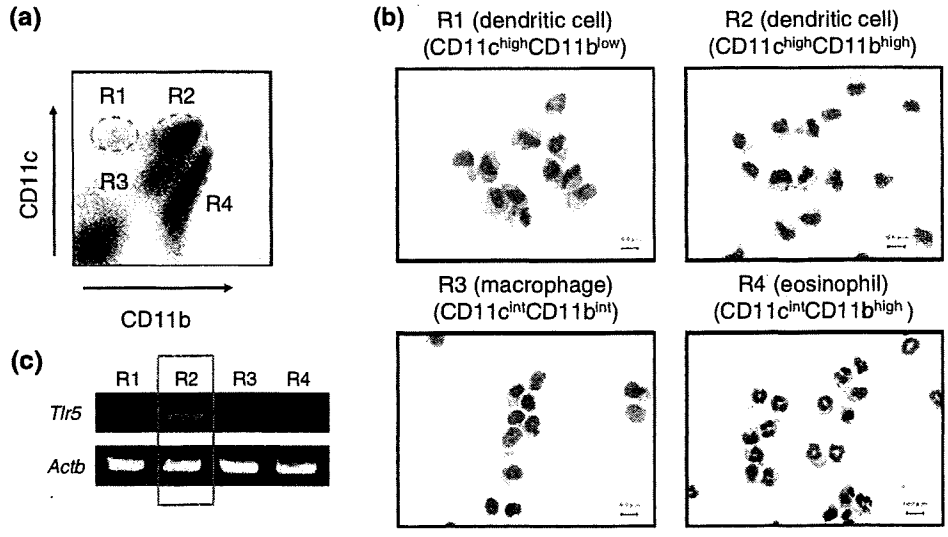

Fig. 4 Four subsets of $CD11c^+$ LPCs. **a** Flow cytometry of intestinal low-density LPCs stained for CD11b and CD11c, before and after sorting. **b** May-Grunwald-Giemsa staining of four leukocyte subsets (gated in a) from the lamina propria. *Scale bars* 10 μm. **c** RT-PCR of *Tlr5* expression in the four leukocyte lamina propria subsets. *Actb* encodes β-actin [18]

(a)

	iNOS+ DC	$CD11c^{hi}CD11b^{hi}$ LPDC
Surface molecules	$CD11b^+$, $CD11c^{low}$, MHC classII+, $CD80^+$, $CD86^{low}$, $Ly6c^+$, Ly6G-, $Gr\text{-}1^+$, $Mac3^+$	$CD11b^{high}$, $CD11c^{high}$, MHC $classII^{high}$, $CD80^+$, $CD86^+$, $Dec205^+$, $F4/80^+$, $CD103^+$
TLR expression	?	TLR5, TLR9
iNOS expression	+	-
cytokines	TNF-α	IL-6, IL-12
Induction of BAFF and APRIL	+	-
Expression of RALDH isoforms	Partially	RALDH2

(b)

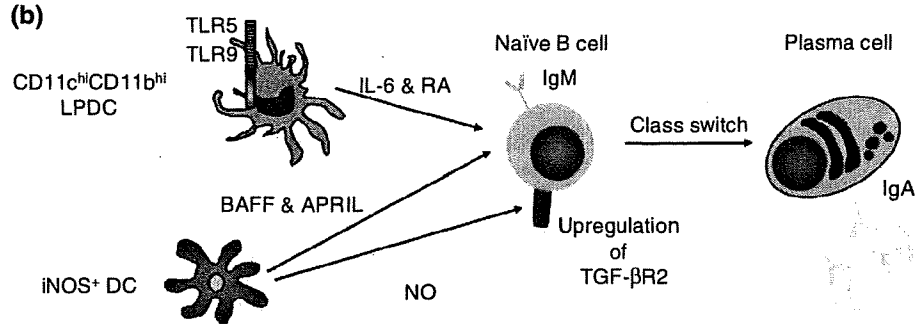

Fig. 5 $iNOS^+$ DCs and $CD11c^{hi}CD11b^{hi}$ LPDCs. **a** Expression of surface molecules and phenotypes in $iNOS^+$ DCs. **b** $CD11c^{hi}CD11b^{hi}$ LPDCs and $iNOS^+$ DCs cooperatively act on naïve B cells to induce IgA^+ plasma cells [30]

inflammation and severe autoimmunity [34]. Differentiation of Th17 cells is initiated by TGF-β and IL-6 (Fig. 6) [35–37]. IL-6 signaling activates STAT3 and the lineage-determining transcription factor RORγt [38]. RORα, another member of the ROR family, is induced in a STAT3-dependent manner and may act synergistically with RORγt to induce Th17 cells [39]. IL-21 expression is induced in developing Th17 cells by IL-6. IL-21 acts autocrinely on Th17 cells to amplify this population [40, 41]. IL-23 serves to expand the previously differentiated Th17 cell population [35]. In humans, IL-1 has been reported to be involved in the generation of Th17 cells [42]. In addition, recent reports have shown that interferon regulatory factor (IRF) 4, Runt-related transcription factor 1 (Runx1) and aryl hydrocarbon receptor (AHR) are essential for Th17 cell differentiation [43–46]. Interestingly, Th17 cells preferentially exist in the intestinal LP of healthy mice [38]. Whereas conventional DCs induce Th1 cells in

response to TLR ligands, CD11c^{hi}CD11b^{hi} LPDCs induce RORγt^{+} functional Th17 cells as well as Th1 cells from naïve CD4^{+} T cells in response to flagellin in vitro. In addition, these DCs induce the generation of both Th17 cells and Th1 cells in an antigen (Ag)-specific manner in vivo. CD11c^{hi}CD11b^{hi} LPDCs produce IL-6 in response to flagellin, and this process is essential for the induction of Th17 cell differentiation [18]. However, it remains unclear why CD11c^{hi}CD11b^{hi} LPDCs, but not conventional DCs, have the ability to induce Th17 cells.

A series of recent studies showed that RA negatively regulates Th17 cell differentiation [47–49]. In every study, RA effectively inhibited in vitro differentiation of Th17 cells induced by IL-6 plus TGF-β in a dose-dependent manner. Although CD11c^{hi}CD11b^{hi} LPDCs express RALDH2, they specifically induce Th17 cells [18]. Interestingly, the effect of RA on DC-mediated Th17 cell differentiation differs according to its concentration. RA at high concentration (10 μM) inhibits the differentiation of Th17 cells as well as Th1 cells, suggesting that high concentrations of RA inhibit both Th17 and Th1 cell differentiation induced by LPDCs. On the other hand, the RAR inhibitor LE540 inhibits the differentiation of Th17 cells, but not Th1 cells, suggesting that RA from LPDCs is necessary for Th17 cell differentiation. Interestingly, LPS-stimulated SPDCs can induce Th17 cells to the same extent as flagellin-stimulated LPDCs (R2) following the addition of RA at low concentration (1 nM). Furthermore, 10 μM RA abolished Th1 cell differentiation induced by LPS-stimulated SPDCs [18]. Thus, RA at low concentrations (1 nM) may have a positive effect on DC-mediated Th17 cell differentiation. According to a previous report, the plasma RA level is usually on the order of 10 nM [24]. It is well known that GALT DCs, but not DCs from other central lymphoid tissues, such as the spleen or peripheral lymph nodes (PLNs), produce RA, which upregulates expression of the chemokine receptor CCR9 and the integrin $\alpha_4\beta_7$ on CD8^{+} precursors, Ag-specific CTLs, CD4^{+} T cells (Th1 and Th 2) and B cells [24, 25, 50–52]. Iwata et al [24] clearly showed that RA effectively enhanced $\alpha 4\beta 7$ expression on CD8^{+} T cells even at concentrations in the range 0.1–1 nM. Because CD11c^{hi}CD11b^{hi} LPDCs could induce B220^{-}IgA^{+} plasma cells from naïve B cells and CCR9 expression on differentiated plasma cells effectively in vitro, the RA released from LPDCs is functional [18]. Although it is difficult to determine the

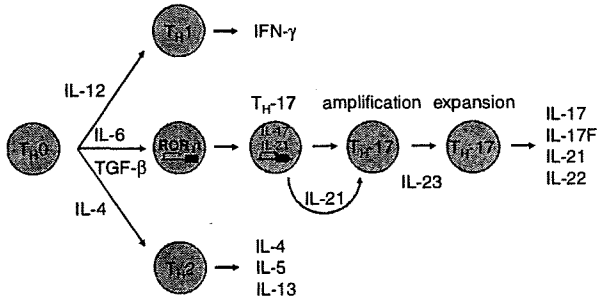

Fig. 6 Differentiation of Th17 cells. Th17 cell differentiation is initiated by TGF-β and IL-6. IL-6 signaling activates Stat3 and the lineage-determining transcription factor RORγT. IL-21 is induced by developing Th17 cells in response to IL-6 and autocrinely acts on Th17 cells to amplify this population. IL-23 serves to expand the previously differentiated Th17 cell population

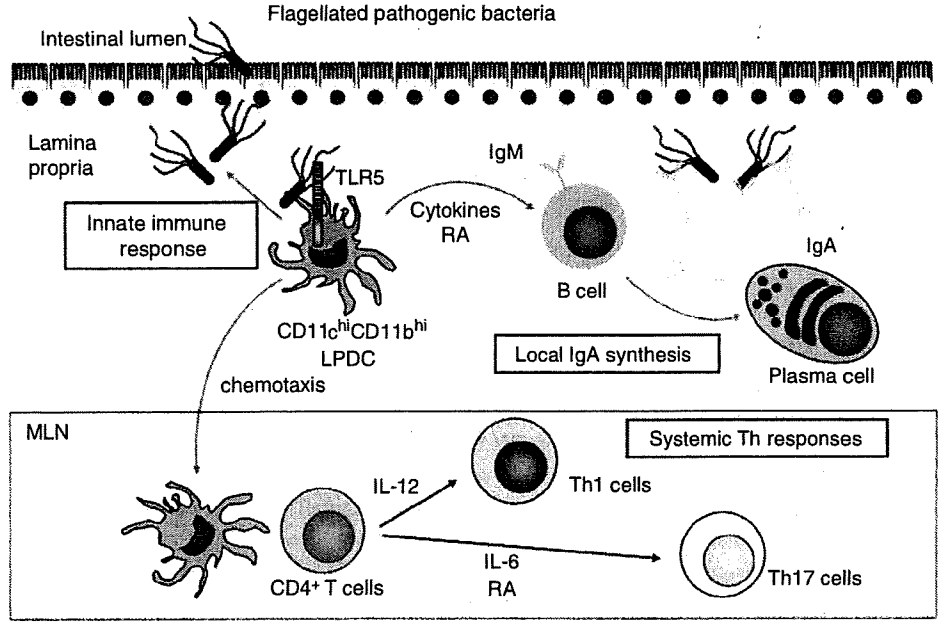

Fig. 7 Roles of CD11c^{hi}CD11b^{hi} LPDCs in the small intestine. CD11c^{hi}CD11b^{hi} LPDCs recognize invasive flagellated bacteria via a TLR5-dependent mechanism and induce innate immune responses. They also work against bacterial infection by inducing 'local' IgA secretion and 'systemic' T helper responses through TLR5 stimulation

local concentrations of retinoic acid secreted by $CD11c^{hi}CD11b^{hi}$ LPDCs, LPDC-derived RA acts as a positive regulator of Th17 cell differentiation. Thus, we have to reconsider the effects of RA on Th17 responses more cautiously.

$CD11c^{hi}CD11b^{hi}$ LPDCs and regulatory T cells

Recent reports show that $CD103^+$ DCs in MLNs and LPs have a role in regulating immunity [53, 54]. $CD103^+$ DCs migrate from the LP to MLNs in a CCR7-dependent manner and induce $FoxP3^+$ regulatory T (Treg) cells via their derived RA [55, 56]. $CD103^+$ DCs seem to be the same population as $CD11c^{hi}CD11b^{hi}$ LPDCs, because $CD11c^{hi}CD11b^{hi}$ LPDCs specifically express CD103 and RALDH2 [18]. $CD11c^{hi}CD11b^{hi}$ LPDCs may have opposite functions, inducing both immunological tolerance and protective immune responses. When $CD11c^{hi}CD11b^{hi}$ LPDCs take up food antigens under physiological conditions, they may induce immunological tolerance by promoting the development of T reg cells. When $CD11c^{hi}CD11b^{hi}$ DCs take up pathogenic bacteria during infection, they are activated and induced to maturate via innate immune receptors, leading to the activation of acquired immunity through the induction of proinflammatory cytokines and costimulatory molecules. Thus, $CD11c^{hi}CD11b^{hi}$ DCs may play critical roles in the decision to mount tolerant or protective immune responses in intestine by using innate immune receptors such as TLR5 as switches.

Conclusion

In this review, we have summarized the unique characteristics of TLR5-expressing LPDCs. These DCs induce innate immune responses by recognizing flagellated pathogenic bacteria via TLR5. They also work against bacterial infection by inducing 'local' IgA secretion and 'systemic' T helper responses through TLR5 stimulation (Fig. 7). Because IL-17 influences various kinds of cells to produce proinflammatory cytokines and induces the activation and migration of neutrophils, $CD11c^{hi}CD11b^{hi}$ LPDCs and Th17 cells may be involved in the pathogenesis of intestinal bowel diseases, such as Crohn's disease. In addition, $CD11c^{hi}CD11b^{hi}$ LPDCs might be useful targets of mucosal vaccination based on their ability to induce Th1 responses and IgA synthesis. Future studies on $CD11c^{hi}CD11b^{hi}$ LPDCs will lead to new medical treatments.

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Direct Stimulation of *tlr5*$^{+/+}$ CD11c^{+} Cells Is Necessary for the Adjuvant Activity of Flagellin[1]

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Flagellin is a highly effective adjuvant, but the cellular mechanism underlying this activity remains uncertain. More specifically, no consensus exists as to whether flagellin activates dendritic cells (DC) directly or indirectly. Intramuscular immunization with flagellin-OVA fusion protein resulted in enhanced in vivo T cell clustering in draining lymph nodes and IL-2 production by OVA-specific CD4^{+} T cells. Immunization with flagellin-OVA also triggered greater levels of Ag-specific CD4^{+} T cell proliferation than immunization with flagellin and OVA as separate proteins. To determine whether flagellin, in the context of a fusion protein with OVA, was acting directly on DC, we used a combination of CD4^{+} T cell adoptive transfers and bone marrow chimera mice in which the presence or absence of potential *tlr5*$^{+/+}$ CD11c^{+} cells was controlled by injection of diphtheria toxin. The Ag-specific CD4^{+} T cell response in mice with CD11c^{+} cells from a *tlr5*$^{-/-}$ background and mixed populations of all other hematopoietic cells was dramatically reduced in comparison to mice that had DC from *tlr5*$^{-/-}$ and wild-type backgrounds. Immunization of *MyD88*$^{-/-}$*tlr5*$^{+/+}$ mice revealed that the enhanced response following immunization with flagellin-OVA is dependent on signaling via the TLR5-MyD88 pathway as well as enhanced Ag uptake and processing resulting from Ag targeting via TLR5. In summary, our data are consistent with the conclusion that direct stimulation of *tlr5*$^{+/+}$ CD11c^{+} cells is necessary for the adjuvant activity of a flagellin fusion protein and that this adjuvant effect requires signaling through TLR5. *The Journal of Immunology*, 2009, 182: 7539–7547.

Flagellin, the major structural protein of bacterial flagella (1) and the ligand for TLR5 (2–4), is a potent systemic and mucosal adjuvant (5–13). Although the adjuvant activity of flagellin is widely accepted to stem from its ability to induce dendritic cell (DC)[3] maturation (10, 14–16), the mechanism, either direct or indirect, has not been demonstrated. Indeed, some researchers have concluded that flagellin does not exert a direct effect on DC (15, 17).

Numerous studies have examined the effect of treatment with flagellin on DC. Didierlaurent et al. (14) demonstrated that incubation of splenic and bone marrow-derived murine DC (BMDC) with flagellin results in modest up-regulation of MHC class II, CD86, CD80, and CD40 and that immunization of mice adoptively transferred with OVA-specific CD4^{+} T cells with flagellin and OVA promoted proliferation of OVA-specific cells. Ablation of CD80 or CD86 expression reduced the ability of murine DC to promote Ag-specific CD4^{+} T cell proliferation and Ab production following immunization with flagellin (10). These findings support the hypothesis that flagellin activates DC and that DC are a crucial cell type in the cellular mechanism of flagellin adjuvant activity, but these findings do not directly address the route of activation.

Several groups have reported that flagellin can promote the activation of myeloid-derived human DC in vitro (15, 18–20). Although Means et al. (15) found that flagellin could promote the in vitro activation of human DC, they also reported that incubation of murine BMDC with flagellin did not trigger up-regulation of CD80, CD86, or CCR7 and concluded that flagellin does not mature murine DC. In vivo experiments examining the effect of i.v. injection of flagellin revealed slight up-regulation of CD80 and CD40 expression by CD11b^{+} and CD8α^{+} splenic DC as well as substantial up-regulation of CD86 expression by CD8α^{+} DC (17). On the basis of this finding and the observation that splenic DC from mice that were irradiated and reconstituted with *MyD88*$^{-/-}$ bone marrow showed reduced modulation of CD80, CD86, and CD40 expression following i.v. injection of flagellin, Salazar-Gonzalez et al. (17) concluded that the in vivo effect of flagellin on DC occurs through an indirect mechanism.

To date, no published study has demonstrated an ability of flagellin to directly stimulate DC in vivo. Experiments on in vitro-generated, BMDC are potentially a direct test, but the weak response generated in these cells raises the possibility that BMDC are not sufficiently differentiated to have acquired flagellin responsiveness. Additionally, some studies have used concentrations of flagellin that are far in excess of what other have found to be required for a maximal response (10^{-10} M). Consequently, the responses in these studies may result from stimulation with contaminating endotoxins or nucleic acids. In view of these shortcomings, we developed an experimental model to determine the requirement for direct stimulation of *tlr5*$^{+/+}$ DC in the in vivo immune response.

Materials and Methods

Mice

C57BL/6, CD11c-diptheria toxin receptor (DTR)/GFP (21), and B6.PL-Thy1a (CD90.1^{+}) mice were obtained from The Jackson Laboratory. TCR transgenic OT-II mice (22), which recognize residues 323–339 of chicken OVA in the context of I-A^{b}, were provided by Dr. E. Hiltbold (Wake Forest

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[3] Abbreviations used in this paper: DC, dendritic cell; BMDC, bone marrow-derived murine DC; DTR, diptheria toxin receptor.

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University School of Medicine). $MyD88^{-/-}$ (23) and $tlr5^{-/-}$ (24) mice have been described previously. All mice were housed in the Wake Forest University School of Medicine animal facility in accordance with institutional and U.S. Department of Agriculture guidelines. All mouse experiments were approved by the Institutional Animal Care and Use Committee.

Immunogens

Recombinant his-tagged *Salmonella* FliC (flagellin) and the nonsignaling flagellin truncation 229 were produced as described previously (9, 25). Recombinant his-tagged flagellin-OVA fusion protein was produced by replacing the hypervariable region of flagellin with OVA and expressing the fusion protein in *E. coli* using a pET29a expression vector. All recombinant proteins were purified using a metal-affinity resin and Acrodisc Mustang Q and E membranes (Pall), which remove nucleic acids and endotoxins. Contaminating endotoxin levels were verified to be <30 pg LPS/μg protein by *Limulus* amebocyte lysate assay (Associates of Cape Cod). Activity of flagellin and flagellin-OVA were verified by measuring TNF-α production in cultures of RAW424 cells stably transfected with mouse TLR5 (26). OVA was purchased from Sigma-Aldrich. Contaminating nucleic acids and endotoxins were removed from the OVA by treatment with Acrodisc Mustang Q and E membranes.

BMDC cultures

Bone marrow was harvested from the femurs and tibias of C57BL/6 mice. RBC were lysed with ammonium chloride lysing buffer (BioWhittaker), and the bone marrow cells were plated in a 24-well plates at a density of 5×10^5 cells/well. Cells were cultured in recombinant mouse GM-CSF for 6 days with media changes on days 2, 4, and 5. On day 6, cells were harvested, counted, and replated in a 48-well plate at 5×10^5 cells/well. Cells were stimulated with flagellin, flagellin 229, flagellin-OVA, *E. coli* LPS (Sigma-Aldrich), or poly(I:C) (InvivoGen). Twenty-four hours later, supernatant was harvested for analysis by ELISA using the OptEIA kit IL-6 (BD Biosciences). Cells were harvested for flow cytometric analysis of CD80 (16-10A1) and CD86 (GL1) expression using Abs from BD Biosciences.

Generation of bone marrow chimeras and OT-II adoptive transfers

Six- to 8-wk-old, wild-type, female C57BL/6 mice received whole-body irradiation of 900 rad from a ^{127}Cs irradiator and then were injected with 1×10^6 bone marrow cells from CD11c-DTR/GFP mice (21) and 1×10^6 bone marrow cells from wild-type or $tlr5^{-/-}$ mice. All donor and recipient mice were sex-matched. Bone marrow chimera mice were maintained on acid water (pH 2.7) for 4 wk following irradiation. Twelve weeks following bone marrow cell transfer, chimera mice were injected via the tail vein with 3×10^6 CFSE-labeled OT-II cells. Twenty-four hours following OT-II cell transfer, chimera mice were injected with 10 ng of diphtheria toxin (DT) per gram of body weight. This dose of toxin resulted in elimination of 87% of splenic CD11c$^+$ cells in CD11c-DTR/GFP mice (data not shown). Eighteen hours after injection of DT, adoptive transfer recipient mice were immunized by i.m. route with 1×10^{-11} mol (0.8 μg) of flagellin-OVA. CD4$^+$ T cells were enriched by negative selection before adoptive transfer in all experiments using bone marrow chimera mice, $MyD88^{-/-}$, and $tlr5^{-/-}$ mice. Immunizations were performed 1 day following cell transfer for all experiments except ones in which mice were treated with DT.

Immunofluoresence

Tissue samples were prepared as described previously (11). OT-II cells were identified on the basis of CD90.1 expression using the anti-CD90.1^{FITC} (OX-7 from BD Biosciences) and rabbit anti-FITC^{AF594} (Invitrogen) Abs. DC were revealed by staining with the CD11c-specific Ab N418 directly conjugated to AF647 (Biolegend), and CD4$^+$ cells were identified by staining with RMN-4 directly conjugated to AF488 (BD Biosciences). CD19$^+$ cells were identified with the mAb 1D3, and CD3$^+$ cells were identified with 145-2C11 (from BD Biosciences). Slides were imaged using a Nikon Eclipse TE300 microscope and a Retiga EX camera. Overlays were composed using Adobe PhotoShop 7.0, and cell counts were performed using ImageJ.

Flow cytometric analysis

CFSE labeling was performed by incubating 2.5×10^6 cells/ml serum-free PBS containing 2 μM CFSE (Invitrogen) for 10 min at room temperature. Adoptively transferred OT-II cells were discriminated on the basis of CD90.1 (OX-7) and CD4 (RM4-5) expression. For restimulation experiments, lymph node cell suspensions generated from the draining lymph nodes were restimulated in vitro with 30 μg/ml $OVA_{323-339}$ in RPMI 1640 with 10% FBS for 5 h. Brefeldin A (BD Biosciences) was added for the last 2.5 h of culture. Staining for IL-2 was performed using cytofix/cytoperm solution (BD Biosciences) following by staining with mouse IL-2-specific Ab (JES6-5H4). Data were analyzed using FloJo 7.2.5 (Tree Star). Absolute cell numbers were determined by flow cytometric counting (27).

Statistics

Statistical analysis of data was performed with SigmaStat 3.10 (Systat Software) or GraphPad Prism 5 for Windows (GraphPad Software). For normally distributed data sets, significance was determined using the Student's *t* test. The significance of data sets, which were not normally distributed or of unequal variance, was determined using the Mann-Whitney rank-sum test. Values of $p < 0.05$ were considered significant. Error bars represent the SEM.

Results

In vitro stimulation of BMDC

Since conflicting evidence regarding the effect of flagellin on BMDC might be due to contaminants in the flagellin preparations used in some of these studies, we first determined that our flagellin and flagellin-OVA did not signal in a TLR5-independent fashion. Incubation of TLR5-negative RAW 264.7 cells with flagellin or flagellin-OVA over a broad concentration range did not result in TNF-α production (data not shown; see also Refs. 13 and 26). Having established that our proteins did not contain stimulatory factors other than flagellin, we proceeded with experiments on the effect of flagellin on BMDC stimulated in vitro. This approach has the benefit of eliminating the effect of non-DC cell types on DC activation. Bone marrow cells were cultured for 6 days with murine GM-CSF before stimulation with flagellin (10^{-9} M), flagellin-OVA (10^{-9} M), inactive flagellin 229 (10^{-9} M), LPS (250 ng/ml), or poly(I:C) (25 μg/ml). BMDC stimulated with flagellin or flagellin-OVA produced equivalent and extremely low levels of IL-6 compared with BMDC stimulated with the other TLR agonists but slightly higher than with media or flagellin 229 ($p < 0.001$) (Fig. 1*A*). Stimulation with flagellin or flagellin-OVA resulted in slight up-regulation of CD80 but not CD86. As with cytokine production, the effect on expression of CD80 and CD86 was less than that seen following stimulation with other TLR agonists, although stimulation with poly(I:C) did not effect expression of CD80 (Fig. 1*B*). These results are consistent with either of two hypotheses. Flagellin activates DC in vivo by an indirect mechanism, or alternatively, flagellin can directly activate DC in vivo but that in vitro-generated DC have not reached the point at which they have acquired flagellin responsiveness—either for lack of TLR5 expression or required intracellular signaling components. Since low levels of IL-6 production and slight up-regulation of CD80 and CD86 were observed (Fig. 1), it is likely that the cultures do indeed contain flagellin-responsive cells, but they represent only a small fraction of the total cell population. In either case, BMDC are clearly a poor model for studying the effect of flagellin on DC.

Immunization with flagellin-OVA promotes clustering and IL-2 production by Ag-specific CD4$^+$ T cells

Ingulli et al. (28) showed that 24 h following immunization, Ag-specific CD4$^+$ T cells cluster around Ag-loaded DC. Subsequent intravital microscopy studies have revealed that these clusters represent the second of three distinct phases of DC-mediated, CD4$^+$ T cell priming during which phase-stable T cell-APC synapses form (29, 30). We hypothesized that immunization with flagellin-OVA would result in maturation of Ag-loaded DC and that these mature DC would be more effective at promoting stable T cell-DC interactions than DC in mice, which were immunized with an equal dose of OVA. C57BL/6 mice received 3×10^6 OT-II CD4$^+$

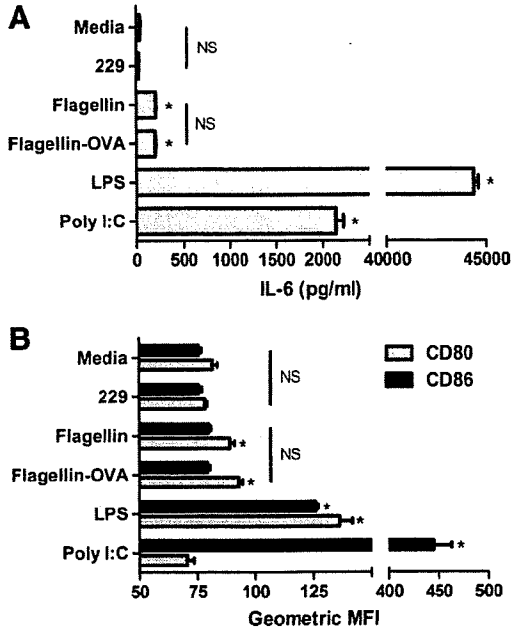

FIGURE 1. Flagellin triggers low-level IL-6 production and slight up-regulation of CD80 by BMDC. Bone marrow cells were cultured for 6 days in GM-CSF, before replating and stimulation with flagellin 229 (10^{-9} M), flagellin (10^{-9} M), flagellin-OVA (10^{-9} M), LPS (250 ng/ml), or poly(I:C) (25 μg/ml). *A*, IL-6 production following in vitro stimulation of BMDC. *B*, Modulation of CD80 and CD86 following in vitro stimulation of BMDC. *, $p < 0.05$ compared with flagellin 229 control. NS indicates no statistically significant difference between conditions.

T cells and 1 day later were immunized with 1×10^{-11} mol of flagellin-OVA or OVA only. Twenty-four hours after immunization, the draining popliteal lymph nodes were harvested and frozen in OCT. Frozen tissue sections were cut and stained for CD4, CD90.1, and CD11c. The minimum requirement for a cluster was three contiguous CD90.1$^+$ cells in the CD4$^+$ region of the lymph node cortex. Representative images for OVA and flagellin-OVA immunized mice are shown in Fig. 2, *A* and *B*. At least 200 CD90.1$^+$ cells were counted from each lymph node. In mice that were immunized with flagellin-OVA, 24% of the CD90.1$^+$ cells were in clusters. By contrast only, 4% of the OVA-specific T cells in the draining lymph nodes of mice immunized with OVA alone were in clusters (Fig. 2*C*).

On the basis of the enhanced clustering of OVA-specific cells in mice immunized with flagellin-OVA, we hypothesized that immunization with the fusion protein would also enhance IL-2 production and, consequently, clonal expansion by the responding CD4$^+$ T cell population. OT-II cells were transferred into C57BL/6 mice as described above and immunized with OVA, flagellin-OVA, or PBS (as a control). Mice were sacrificed 3 days after immunization, and cell suspensions generated from the draining popliteal lymph nodes were restimulated in vitro with 30 μg/ml $OVA_{323-339}$ peptide for 5 h. IL-2 production was measured by intracellular cytokine staining, and the total numbers of lymphocytes, CD4$^+$ T cells, and CD90.1$^+$ cells were also determined using flow cytometry-based measurement in conjunction with fluorescent microspheres (27). Immunization with flagellin-OVA stimulated an increase in the total number of lymphocytes and CD4$^+$ cells recovered from the draining node (Fig. 3*C*). This finding is consistent with the results of a prior study (11) in which we demonstrated that flagellin is a potent inducer of T and B lymphocyte recruitment to draining lymph nodes following immunization.

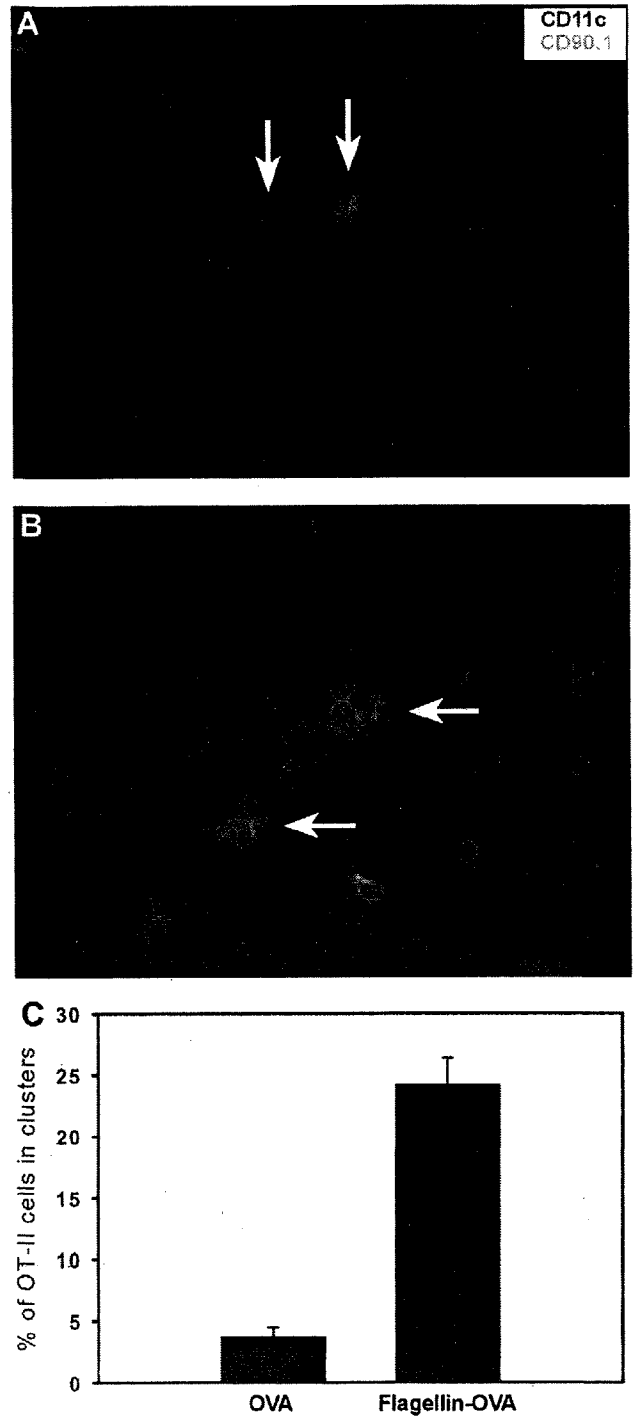

FIGURE 2. Immunization with flagellin-OVA fusion protein promotes clustering of OVA-specific CD4$^+$ T cells. Draining popliteal lymph nodes were harvested from adoptive transfer recipient mice 24 h following immunization. Adoptively transferred CD4$^+$ OVA-specific cells were identified by staining lymph node tissue sections with CD90.1-specific Ab (green). CD11c$^+$ cells were identified by staining with mouse CD11c-specific Ab (red). Arrows indicate examples of single CD90.1$^+$ OVA-specific T cells from OVA-immunized mice (*A*) and clusters of CD90.1$^+$ OVA-specific cells from flagellin-OVA-immunized mice (*B*). *C*, Clustering of Ag-specific cells was quantified by counting at least 200 cells from each lymph node. Immunization with flagellin-OVA promoted significantly more clustering than immunization with OVA alone ($p < 0.001$). The data in this experiment were obtained from three mice per group.

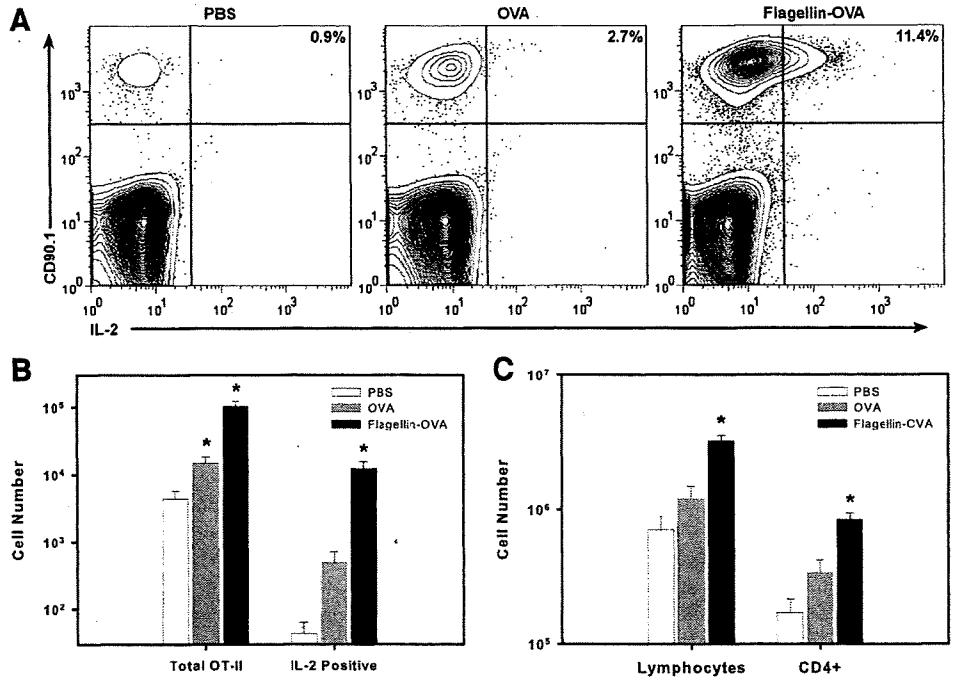

FIGURE 3. Immunization with flagellin-OVA fusion protein enhances IL-2 production by OVA-specific CD4^{+} T cells. C57BL/6 mice were i.v. injected with 3×10^{6} CD4^{+}CD90.1^{+} OT-II T cells. Twenty-four hours later, mice were immunized i.m. with 1×10^{-11} mol of flagellin-OVA fusion protein. Mice were sacrificed 3 days following immunization, and cells recovered from the draining popliteal lymph nodes were restimulated in vitro with 30 μg/ml $OVA_{323-339}$ for 5 h. Brefeldin A was added for the final 2.5 h of culture. *A*, IL-2 production was determined by intracellular cytokine staining. Plots are gated on CD4^{+} cells. Total numbers of CD90.1^{+}CD4^{+} and CD90.1^{+}CD4^{+}IL-2^{+} cells (*B*) and lymphocytes (*C*) and polyclonal CD4^{+} cells were determined by flow cytometric quantification. The data in this experiment were obtained from four mice per group. The experiment was repeated twice with similar results.

Consistent with the enhanced clustering of cells in mice immunized with fusion protein, we observed a 4- to 5-fold increase in the percentage of IL-2-producing cells within the OT-II population recovered from mice immunized with flagellin-OVA compared with OVA alone (Fig. 3*A*). The percent increase, when compounded with the increase in the total number of OVA-specific cells, resulted in a 25-fold increase in the absolute number of IL-2^{+} cells recovered from mice immunized with flagellin-OVA compared with mice immunized with OVA alone (Fig. 3*B*). IL-2 production was restricted to cells that had divided (data not shown). The enhanced T cell clustering, IL-2 production, and proliferation seen in mice immunized with flagellin-OVA is consistent with but not formal proof of the hypothesis that flagellin-OVA activates DC by a direct mechanism that involves TLR5-dependent uptake of the fusion protein and thus increased levels of processed Ag for presentation to OVA-specific T cells.

Immunization with a single flagellin-OVA fusion protein is superior to immunization with flagellin plus OVA as separate proteins

If flagellin directly stimulates DC in vivo, then flagellin-OVA should be significantly more potent than flagellin and OVA as separate proteins in the induction of OVA-specific CD4^{+} T cell proliferation since the DC that bind flagellin will also receive OVA. Thus, Ag uptake should be far more efficient than obtained with just OVA. However, if the mechanism of DC activation by flagellin is indirect, flagellin-OVA and flagellin plus OVA should exhibit equal potency. To test these possibilities, C57BL/6 mice were injected via the tail vein with 3×10^{6} CFSE-labeled, OVA-specific cells and i.m. immunized the following day with 1×10^{-12}, 1×10^{-11}, or 1×10^{-10} mol of OVA, flagellin plus OVA, or flagellin-OVA. Three days later, the mice were sacrificed, and OVA-specific cell proliferation was measured in cells recovered from the draining popliteal lymph nodes.

Immunization with flagellin-OVA fusion protein results in enhanced Ag-specific cell division compared with immunization with flagellin plus OVA or OVA alone (Fig. 4). Immunization with 1×10^{-10} mol of OVA alone stimulated an average of 0.43 divisions/cell in the original starting population. However, a similar level of cell division, an average of 0.37 divisions/cell, was achieved following immunization with one-tenth as much (1×10^{-11} mol) flagellin plus OVA. In contrast immunization with 1×10^{-11} mol of flagellin-OVA resulted in an average of 0.93 cell

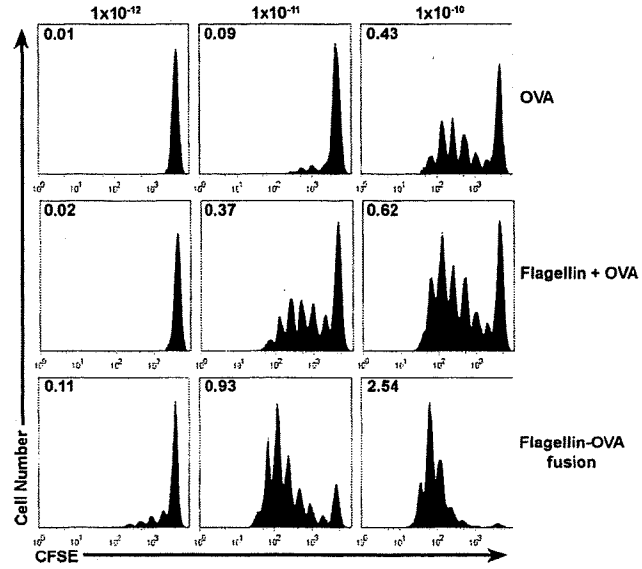

FIGURE 4. In vivo T cell proliferation in response to immunization with OVA, flagellin plus OVA, or flagellin-OVA fusion protein. C57BL/6 mice were i.v. injected with 3×10^{6} CFSE-labeled, CD4^{+}CD90.1^{+} OT-II T cells. Twenty-four hours later, mice were immunized i.m. with 1×10^{-12}, 1×10^{-11}, or 1×10^{-10} mol of OVA, flagellin and OVA, or flagellin-OVA fusion protein. Mice were sacrificed 3 days following immunization, and proliferation by the OT-II population in the draining, popliteal lymph node was compared based on CFSE dilution. Numbers indicate the division index, which is the average number of divisions that a cell present in the starting population has undergone. Histograms are gated on CD4^{+}CD90.1^{+} cells. The data in this experiment were obtained from four mice per group. The experiment was repeated twice with similar results.

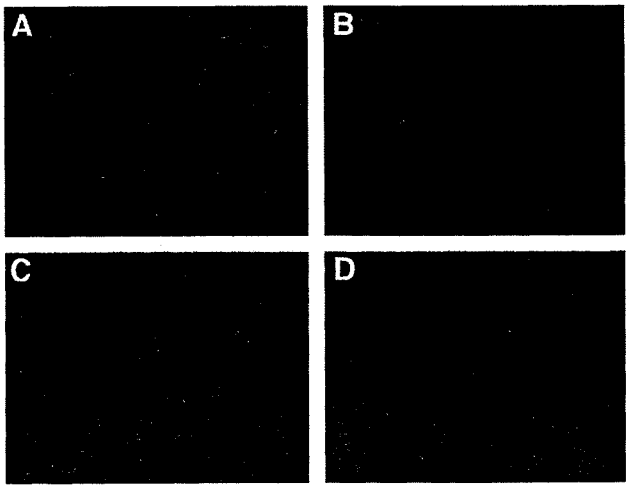

FIGURE 5. Treatment of CD11c-DTR mice with DT causes depletion of GFP$^+$ cells in lymph nodes but does not affect normal lymph node architecture 18 h after treatment. CD11c$^+$ cells are revealed by GFP expression in CD11-DTR mice treated with PBS (*A*). The level of GFP detected was greatly reduced in mice treated with DT (*B*). Sections stained with CD19-specific (red) and CD3-specific (blue) Abs revealed no difference in normal lymph node architecture in T and B lymphocyte zones between PBS control mice (*C*) and DT-treated mice (*D*). The data in this experiment were obtained from four mice per group.

divisions by the original starting population. Although the addition of flagellin as a separate protein has a clear adjuvant effect, the effect is not as great as immunization with flagellin fusion protein. Immunization with 1×10^{-12} mol of the fusion protein resulted in detectable levels of proliferation by OVA-specific cells, but at this low dose, immunization with flagellin plus OVA or OVA alone did not stimulate OVA-specific cell proliferation. Notably, after immunization with the middle and high doses, the fraction of cells in the undivided population is much smaller in mice immunized with fusion protein than in mice immunized with flagellin plus OVA or OVA alone. These results clearly demonstrate that immunization with flagellin-OVA fusion protein promotes a stronger response than immunization with equal doses of flagellin plus OVA as separate proteins. This finding is consistent with, but not conclusive evidence for, a direct effect of flagellin-OVA on DC.

tlr5$^{+/+}$ DC are necessary for the in vivo adjuvant effect of flagellin

To determine whether the in vivo adjuvant effect of flagellin-OVA involves TLR5-positive DC and thus a direct effect of flagellin-OVA, we developed a bone marrow chimera system in which irradiated mice were reconstituted with a combination of CD11c-DTR/GFP and wild-type bone marrow or CD11c-DTR/GFP and *tlr5*$^{-/-}$ bone marrow. Although wild-type murine cells are insensitive to treatment with DT (31), CD11c-DTR/GFP mice express the primate DTR under the control of the CD11c promoter (21), and thus, CD11c$^+$ cells in these mice are fully sensitive to the toxic effect of DT. The histological effects of DT treatment of these mice has been thoroughly examined (32) and confirmed the original report that injection of these mice with DT results in the transient depletion of CD11c$^+$ cells. In confirmation of prior published studies, we found that DT treatment resulted in 85–90% depletion of CD11c$^+$ cells in CD11c-DTR/GFP mice as measured by flow cytometry (data not shown). Depletion of GFP$^+$ cells was also confirmed by histological analysis (Fig. 5, *A* and *B*) as well as preservation of normal T and B lymphocyte microenvironments in lymph nodes of CD11c-DTR/GFP mice treated with PBS (Fig. 5*C*) or DT (Fig. 5*D*). Bone marrow from the CD11c-DTR/GFP mice used in combination with bone marrow from *tlr5*$^{-/-}$ mice afford the unique opportunity to create a chimera mouse in which, following treatment with DT, the vast majority of the CD11c$^+$ cells are from a *tlr5*$^{-/-}$ background, whereas CD11c$^-$ hemopoietic cell populations in these mice should be 50% *tlr5*$^{-/-}$ and 50% *tlr5*$^{+/+}$. For these studies, we have assumed equal engraftment of the different types of bone marrow used to reconstitute irradiated mice. Due to the very low levels of TLR5 surface expression by flagellin responsive cells (our unpublished observations), we were unable to detect TLR5 expression by cells from normal C57BL/6 or bone marrow chimera mice. However, if TLR5-expressing DC are present in draining lymph nodes, then such cells and the adjuvant effect of flagellin would be lost in mice reconstituted with CD11c-DTR/GFP plus *tlr5*$^{-/-}$ bone marrow and treated with DT. If lymph node DC do not normally express TLR5, then DT treatment should have no significant effect on the adjuvant activity of flagellin in these chimera mice.

Twelve weeks following irradiation and reconstitution, chimera mice received 3×10^6 CD4$^+$ enriched, CFSE-labeled OT-II cells and the following day were injected with DT or PBS (as a control). This combination of reconstitution and toxin treatment resulted in four groups of mice, three of which had populations of DC from *tlr5*$^{+/+}$ mice (C57BL/6 and/or CD11c-DTR/GFP) and one of which that had DC from a *tlr5*$^{-/-}$ background. Each group contained four mice. Eighteen hours after injection with DT or PBS, mice were immunized with 1×10^{-11} mol of flagellin-OVA. Three days after immunization, CFSE dilution by OVA-specific OT-II cells recovered from the draining lymph node was measured by flow cytometry. OVA-specific cells recovered from the group of mice that were reconstituted with CD11c-DTR/GFP plus wild-type bone marrow and treated with PBS (Fig. 6*A*) or DT (Fig. 6*B*) exhibited substantial levels of OVA-specific T cell proliferation. A similar pattern of OVA-specific T cell proliferation was also observed with mice reconstituted with CD11c-DTR/GFP and *tlr5*$^{-/-}$ bone marrow and treated with PBS (Fig. 6*C*). In striking contrast, OVA-specific T cell proliferation was dramatically reduced in mice reconstituted with CD11c-DTR/GFP and *tlr5*$^{-/-}$ bone marrow and treated with DT (Fig. 6*D*). The small extent of proliferation is probably due to the incomplete (85–90%) deletion of the CD11c-DTR/GFP DC in the presence of DT. The dramatic difference in proliferation by OVA-specific cells in mice containing *tlr5*$^{+/+}$ DC and mice with DC from a *tlr5*$^{-/-}$ background clearly demonstrates that the adjuvant effect of flagellin-OVA requires *tlr5*$^{+/+}$ DC. Similar results were obtained in a separate experiment following intranasal immunization with flagellin-OVA (three mice per group).

Enhanced efficacy of flagellin-OVA fusion protein requires expression of TLR5 and MyD88

The requirement for a TLR5-expressing DC might simply be due to enhanced uptake of flagellin-OVA by a TLR5-dependent endocytic mechanism and not because of any significant contribution of TLR5 signaling. Indeed, in other systems, targeting Ags to specific populations of DC has been shown to significantly enhance the in vivo CD4$^+$ T cell response (33, 34), albeit at higher Ag doses and in combination with adjuvants. To address these possibilities, we compared the response to immunization with flagellin-OVA fusion protein in *tlr5*$^{-/-}$ and *MyD88*$^{-/-}$ mice since flagellin signaling via TLR5 is MyD88 dependent (35, 36).

In the first set of experiments, CFSE-labeled OT-II cells were transferred into C57BL/6, *tlr5*$^{-/-}$, or *MyD88*$^{-/-}$ mice. One day following transfer, mice were immunized i.m. with 1×10^{-11} mol