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Cooperation of TLR12 and TLR11 in the IRF8-Dependent IL-12 Response to Toxoplasma gondii Profilin

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TLRs play a central role in the innate recognition of pathogens and the activation of dendritic cells (DCs). In this study, we establish that, in addition to TLR11, TLR12 recognizes the profilin protein of the protozoan parasite Toxoplasma gondii and regulates IL-12 production by DCs in response to the parasite. Similar to TLR11, TLR12 is an endolysosomal innate immune receptor that colocalizes and interacts with UNC93B1. Biochemical experiments revealed that TLR11 and TLR12 directly bind to T. gondii profilin and are capable of forming a heterodimeric complex. We also establish that the transcription factor IFN regulatory factor 8, not NF-κB, plays a central role in the regulation of the TLR11- and TLR12-dependent IL-12 response of DCs. These results suggest a central role for IFN regulatory factor 8–expressing CD8+ DCs in governing the TLR11- and TLR12-mediated host defense against T. gondii. The Journal of Immunology, 2013, 191: 4818–4827.

Toxoplasma gondii, a protozoan parasite, infects all nucleated cells and regulates IL-12 production in response to T. gondii infection (9–11). Moreover, TLR11-dependent IL-12 production in response to T. gondii in vivo and in vitro is limited to the CD8+ DC population (7, 12), and the biochemical basis of this highly restricted IL-12 production in response to T. gondii profilin is unknown. In this study, we establish that TLR11 recognizes T. gondii profilin in a complex with another TLR11 family member, TLR12. Both TLR11 and TLR12 directly bind to T. gondii profilin, leading to initiation of the MyD88- and UNC93B1-dependent signaling cascade. Similar to TLR11, TLR12 appears to function as an intracellular TLR that directly interacts with UNC93B1. Furthermore, we reveal that the TLR11- and TLR12-mediated recognition of T. gondii profilin induces IFN regulatory factor (IRF)–8–dependent DC IL-12 production rather than the NF-κB signaling cascade. These results demonstrate that the selective TLR11- and TLR12-dependent activation of CD8+ DCs in response to T. gondii occurs because IRF8 expression is limited in this subset of DCs.

Materials and Methods

Animals
C57BL/6 (wild-type [WT]) mice were obtained from the University of Texas Southwestern Medical Center Mouse Breeding Core Facility. Tlr11−/−, Myd88−/−, NF-κB1−/−, NF-κB2−/−, and Ifn8−/− mice were described previously (7, 13–16). Irf4−/− (17) mice were obtained from The Jackson Laboratory. All animals were age- and sex-matched and maintained in the specific pathogen-free barrier facility at the University of Texas Southwestern Medical Center at Dallas. All experiments were performed using protocols approved by the Institutional Animal Care and Use Committees of the University of Texas Southwestern Medical Center.

Plasmid constructs and reagents
The pEGFPN1 and pmCherryN1 vectors were obtained from Clontech. The pcDNA3.1 vector was obtained from Invitrogen. Tlr12 was inserted between the Nhel and SacII sites of pEGFPN1 and pmCherryN1 using standard PCR techniques with the forward primer 5′-GCTAGC ATGCGGCCCAGATGGAGGCCACCCAGT-3′ and the reverse primer 5′-CGGCTGGTCCCGCCTCGGCCCACCTTG-3′. TLR12 was myc-tagged and inserted between the Nhel and XbaI sites of pcDNA3.1 using the forward primer 5′-TACCGAGCTCGGATCCACCATGCCCCGCATGGAGCG-3′ and the reverse primer 5′-GATATCTGAGATTTTTGTTCGTCCGCTCCTGGCC-3′.
GFP-tagged TLR11 cloned between the Nhel and SacII sites of pEGFPN1 and myc-tagged TLR12 cloned between the Nhel and XbaI sites of pcDNA3.1 have been described previously (18). Un9b1 was cloned into the XhoI and HindIII sites of pmCherryN1 and pEGFPN1 using the forward primer 5'-GGTTCCTCGAGATGAGGAGCTCCACACACG-3' and the reverse primer 5'-GGTTCTAAGCTTCTGCTCTAGGCCACATC-3'. The CD3 construct was a gift from Dr. Nicolai van Oers (University of Texas Southwestern Medical Center). All plasmids were prepared using the EndoFree Miniprep kit from Clontech.

**Protein expression and purification**

The extracellular portions of TLR11 and TLR12 were cloned into the pEFGP-N2 vector and were additionally tagged with the DED epitope (patent RU2380373) using the forward primers 5'-AGCTCCGAGCAGCTCCCTCTGTC-3' and 5'-AATCTGAGCTCCCTCTGTC-3' and the reverse primers 5'-AAGATCTCTTTAAGTCCCCAAGTCTCTCTTGG-3' and 5'-AAAGATCTCCTGTCCTCACTGACAGTAATG-3', respectively.

To express the ectodomains of TLR11 and TLR12, Chinese hamster ovary (CHO)-S cells were stably transfected with the ectodomain constructs, and stable clones were selected with G418. The stably transfected cells were grown in CD CHO (Invitrogen) or DMEM/F12 media with 1% FBS. The TLR11 and TLR12 ectodomains were purified by affinity chromatography with a DED-specific mAb (clone 2E8; Proteinsynthesis, Moscow, Russia).

**Analysis of TLR12/profilin and TLR11/profilin interactions**

To analyze the interactions between T. gondii profilin and the ectodomains of TLR11 and TLR12, two ELISA-like assays were developed. In the first assay, ELISA plates were coated with T. gondii profilin (10 ng/ml) in 10 mM Tris (pH 6.0 or 8.0) and 150 mM NaCl. Free binding sites were blocked with 5% fat-free milk, and 1 µg/ml purified TLR11 or TLR12 ectodomain was added. After extensive washing steps, the presence of bound TLR11 and TLR12 was detected with a DED-specific mAb. In an alternative assay, the ELISA plates were initially coated with 10 µg/ml TLR11, TLR12, or TLR12 ectodomain in the same buffer. After blocking and washing steps, recombinant profilin (10 µg/ml) was added to the wells. Profilin was detected with a polyclonal rabbit anti–T. gondii profilin Ab developed in our laboratory.

To assess the formation of T. gondii profilin complexes with the TLR11 or TLR12 ectodomains, 2 µM T. gondii profilin was incubated with 0.2 µM purified TLR11 or TLR12 ectodomain for 2 h in 10 mM Tris (pH 6.0 or 8.0) and 150 mM NaCl. Biochemical fractionation of the protein complexes was performed by gel filtration chromatography with a Superdex 200 10/300 GL chromatographic separation column from GE Healthcare. The fast protein liquid chromatography fractions were then analyzed for the T. gondii profilin, TLR11, or TLR12 by ELISA.

**Cell culture, confocal microscopy, and immunoprecipitation**

The HEK293 cell line was obtained from the American Type Culture Collection and maintained in tissue culture flasks in RPMI 1640 medium containing 10% FBS (HyClone), 10 mM HEPES, 1 mM 2-ME, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.5 mg/ml l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. HEK293 cells were plated on glass cover slips and were transfected using Lipofectamine 2000 (Invitrogen). For immunofluorescence assays, HEK293 cells were grown on glass coverslips, transfected with the optimized ViraPower packaging mix (Invitrogen) and the mRNA-encoding pLenti6.4 CMV-EmGFP constructs.

**Results**

TLR12 colocalizes and interacts with TLR11 and UNC93B1

The cellular localization of TLRs plays a central role in determining access to ligands and downstream signaling molecules (20). Nucleic acid–sensing TLRs are localized intracellularly (21–25), and the chaperone-like protein UNC93B1 is indispensable for the proper localization and function of all RNA- and DNA-sensing TLRs (26–29). This is particularly evident from the phenotype of 3d mice, in which a point mutation in UNC93B1 results in the loss of function of TLR3, TLR7, TLR9, and TLR13 (26, 30). In contrast, the TLRs involved in sensing components of microbial cell walls or flagellin localize to the cell surface, and their signaling is impaired in the absence of functional UNC93B1 protein (20). We recently identified that, in addition to the nucleic acid–sensing TLRs, the protein-sensing receptor TLR11 directly interacts with UNC93B1 and localizes to the endolysosomal compartment (18). Furthermore, 3d mutants fail to respond to the TLR11 agonist T. gondii profilin in vitro and in vivo (18).

The high sequence similarity of TLR11 and TLR12 (2) prompted us to examine TLR12 localization and interaction with UNC93B1. First, we expressed GFP-tagged TLR11 in the HEK293 cells and observed that TLR12 is an intracellular innate immune receptor (Supplemental Fig. 1A). We further confirmed an intracellular localization of TLR12 in mouse macrophages transfected with TLR12-GFP (Supplemental Fig. 1A). Finally, we coexpressed mCherry-tagged TLR12 with GFP-tagged TLR11 and observed that TLR12, similar to TLR11, is an intracellular innate immune receptor (Fig. 1A, Supplemental Fig. 1B). Moreover, TLR12 and TLR11 exhibited a high degree of colocalization (>80%), indicating that these innate receptors localize to similar intracellular compartments (Fig. 1A, Supplemental Fig. 1B). Additional confocal microscopy experiments revealed that TLR12 colocalizes with TLR11 in >80% of cells.
with UNC93B1, further demonstrating that TLR12 is an intra-
cellular innate immune receptor (Fig. 1B).

Formation of TLR homodimers or heterodimers is an essential
step to trigger the signaling cascade initiated by the recruitment
of TLR adaptor proteins. Because TLRs in the same family can in-
teract with each other, TLR11 and TLR12 colocalized and have
a similar expression pattern (Supplemental Fig. 1C), we examined
the interactions of these proteins with each other and with proteins

![FIGURE 1. TLR12 localizes intracellularly and associates with TLR11 and UNC93B1. (A) HEK293 cells were plated on glass coverslips and
cotransfected with TLR11-GFP and TLR12-mCherry. After 24 h, the cells were imaged with a Leica SPE microscope. Original magnification ×63. (B) HEK293 cells were plated on glass coverslips and cotransfected with TLR12-GFP and UNC93B1-mCherry. After 24 h, the cells were imaged with a Leica SPE microscope. Original magnification ×63. (C) HEK293 cells were cotransfected with 1) TLR11-GFP and myc-MyD88, 2) TLR11-GFP and myc-TLR11, 3) TLR11-GFP and myc-TLR12, 4) TLR12-GFP and myc-MyD88, 5) TLR12-GFP and myc-TLR11, 6) TLR12-GFP and myc-TLR12, 7) UNC93B1-GFP and myc-MyD88, 8) UNC93B1-GFP and myc-TLR11, 9) UNC93B1-GFP and myc-TLR12, 10) CD3ζ-GFP and myc-MyD88, 11) CD3ζ-GFP and myc-TLR11, or 12) CD3ζ-GFP and myc-TLR12. Thirty-six hours after transfection, the cell lysates were immunoprecipitated (IP) with anti-GFP Ab, and the immunoprecipitated proteins were analyzed by immunoblotting (IB) with anti-myc or anti-GFP Abs to detect myc- or GFP-tagged proteins, respectively. A representative result from five experiments is shown.

essential to the TLR11 signaling pathway, MyD88 and UNC93B1.
We observed that TLR11 strongly interacted with MyD88 and was
also capable of forming a homodimer, as evident from interactions
between GFP- and myc-tagged TLR11 expressed in the same cells
(Fig. 1C, lanes 1 and 2). Additionally, we observed strong inter-
actions between TLR11 and TLR12, suggesting that these receptors
can form a heterodimeric complex (Fig. 1C, lane 3). This possi-
bility was further explored in reciprocal experiments in which
GFP-tagged TLR12 was immunoprecipitated and the presence of TLR11 and MyD88 in a complex with TLR12 was analyzed with the anti-myc Ab. We established that TLR12 was capable of forming homodimers as well as heterodimers with TLR11 (Fig. 1C, lanes 5 and 6). Surprisingly, TLR12 did not immunoprecipitate with MyD88 (Fig. 1C, lane 4), indicating that these proteins do not directly interact. Our experiments revealed a formation of TLR11/MyD88 complex in the absence of profilin or TLR12, because we expressed TLR11 and MyD88 in human 293 cells that lack TLR12. At the same time, TLR12 does not interact with MyD88 under any examined conditions (Fig. 1C and data not shown).

In additional immunoprecipitation experiments, we also revealed that UNC93B1 interacted with TLR12 (Fig. 1C, lane 8). These results confirm the results of the imaging experiment, which demonstrated colocalization of these molecules (Fig. 1B). In the same experiments, and as we have previously reported, UNC93B1 interacted with TLR11 but not with MyD88 (Fig. 1C, lanes 7 and 8). As a negative control in all experiments, we expressed an irrelevant transmembrane protein, CD3ζ, which was similarly fused to GFP. Under identical immunoprecipitation conditions, CD3ζ did not interact with MyD88, TLR11, or TLR12 (Fig. 1C, lanes 10–12).

Similarly, barely detectable interactions of TLR11 with TLR4 and TLR9 were seen under identical immunoprecipitation conditions (Supplemental Fig. 1D), proving the specificity of TLR11/TLR11 and TLR11/TLR12 interactions. Taken together, our results suggest that TLR11 and TLR12 are capable of forming homo- and heterodimers, and, although both proteins interact with UNC93B1, only TLR11 is capable of recruiting MyD88 to the receptor complex.

**TLR11 and TLR12 directly bind to T. gondii profilin**

The interaction of TLR12 with TLR11 and UNC93B1 prompted us to investigate whether TLR12 cooperates with TLR11 in the recognition of *T. gondii* profilin. We previously isolated T. gondii profilin as an IL-12–inducing protein whose activity was dependent on TLR11, UNC93B1, and MyD88, but we did not investigate a role for TLR12 in those experiments (7, 8, 18). Furthermore, it is unknown whether TLR11 directly interacts with *T. gondii* profilin. To formally address these questions, we expressed and purified recombinant extracellular portions (ectodomains) of TLR11 and TLR12 (Supplemental Fig. 1E) and examined whether the ectodomains of TLR11 and TLR12 interacted with *T. gondii* profilin.

We first developed an ELISA-like assay in which plates were coated with recombinant *T. gondii* profilin (or BSA and ectodomain of TLR13 as control proteins) and investigated the binding of TLR11 and TLR12 to *T. gondii* profilin. We observed that both TLR11 and TLR12 strongly interacted with *T. gondii* profilin (Fig. 2A). As expected from the endolysosomal localization of TLR11 and TLR12, binding to the ligand was pH-dependent, and although both receptors strongly interacted with profilin at pH 6.0 (Fig. 2A), the binding was diminished at higher pH (Fig. 2B and data not shown). We also performed the reciprocal experiment in which highly purified TLR11 and TLR12 ectodomains (Supplemental Fig. 1E) were immobilized and soluble *T. gondii* profilin was added. The binding of *T. gondii* profilin to the TLR11 and TLR12 ectodomains was detected with a profilin-specific Ab (Fig. 2C). These experiments also revealed strong, pH-dependent interactions between TLR11 and TLR12 and *T. gondii* profilin (Fig. 2A–C). Both control proteins, BSA and TLR13, failed to demonstrate interactions with *T. gondii* profilin above the background levels (Fig. 2A–C).

To further examine the formation of the TLR11/profilin and TLR12/profilin complexes, we used size-exclusion chromatography to determine whether the ectodomains of TLR11 and TLR12 formed complexes with *T. gondii* profilin. As shown in Fig. 2D–F, we monitored the elution profiles of profilin (left) and the TLR ectodomains (right) alone. We next preincubated *T. gondii* profilin with the ectodomain of TLR11 or TLR12, allowed the complexes to form at pH 6.0, and then performed gel filtration chromatography and tested each fraction for the presence of profilin and the TLR11 or TLR12 ectodomains. We observed that both TLR11 and TLR12 formed complexes with *T. gondii* profilin, as demonstrated by the coelution of *T. gondii* profilin and TLR11 (Fig. 2D) or *T. gondii* profilin and TLR12 (Fig. 2E) in the same fractions. As expected from the ELISA data (Fig. 2A, 2C), TLR13 and BSA failed to form a complex with *T. gondii* profilin (Fig. 2F and data not shown). These data formally establish that both TLR11 and TLR12 can directly interact with *T. gondii* profilin and form a ligand/receptor complex.

**TLR12 is involved in the regulation of the IL-12 response in response to *T. gondii* profilin**

To examine the role of TLR12 in the regulation of DC IL-12 responses triggered by *T. gondii* profilin, we knocked down TLR12 or MyD88 in Flt3L-derived DCs by lentivirus-mediated delivery of miRNA targeting TLR12 (Fig. 3A, Supplemental Fig. 2A–C). We observed that the transduction of DCs with the TLR12- or MyD88-targeted miRNA dramatically reduced the IL-12p40 level secreted by DCs in response to *T. gondii* profilin (Fig. 3A). Importantly, the production of IL-12/23p40 was unimpaired in DCs similarly infected with control lentiviruses. These results strongly suggest that TLR12 plays a role in the response to *T. gondii* profilin (Fig. 3A). At the same time, miRNA against TLR12 had no effects on the IL-12 responses triggered by other tested TLR ligands (Supplemental Fig. 2C), indicating a selective role for TLR12 in the *T. gondii* profilin recognition. Similarly, we observed that all tested siRNA sequences against TLR12 (Supplemental Fig. 2D) dramatically reduced IL-12p40 secretion by DCs stimulated with *T. gondii* profilin (Fig. 3B). In additional control experiments and in agreement with the previous studies (7, 13) we observed a similar suppression of IL-12p40 production using siRNA against TLR11 and MyD88 (Supplemental Fig. 2E) but not against TLR13 (Supplemental Fig. 2E). Thus, these experiments revealed that, in addition to TLR11 and MyD88, TLR12 is required for the induction of IL-12 by DCs in response to *T. gondii* profilin.

**IRF8 regulates TLR11- and TLR12-dependent IL-12 responses**

Activated TLR homodimers or heterodimers trigger cytokine production via the activation of NF-κB transcripational factors (31). We thus examined NF-κB activation triggered by TLR11 and TLR12 in response to *T. gondii* profilin. We observed that HEK 293 cells cotransfected with TLR11 and an NF-κB–driven luciferase reporter did not induce significant levels of luciferase activity in response to *T. gondii* profilin (Fig. 4A). Similarly, we established that TLR12-expressing cells did not activate NF-κB in response to the agonist (Fig. 4A). Surprisingly, cells expressing both TLR11 and TLR12 did not trigger significant NF-κB activation in response to any tested concentration of *T. gondii* profilin (Fig. 4A and data not shown). The inability of TLR11 and TLR12 to trigger NF-κB–driven luciferase activity was also seen in RAW264.7 cells (data not shown), and an additional screening revealed that only CHO-K1 cells stimulated with *T. gondii* profilin triggered a very modest (2- to 3-fold) NF-κB activity in response to *T. gondii* profilin (data not shown). Most importantly, purified CD8⁺ splenic DCs, which produce high levels of IL-12/23p40 in response to *T. gondii* profilin (data not shown), failed to demonstrate translocation of NF-κBp65 from the cytosolic compartment to the nucleus (Fig. 4B). These results are in contrast to those for other tested TLRs, including TLR2, TLR3, TLR4, TLR5, TLR7,
FIGURE 2. *T. gondii* profilin (PFTg) directly interacts with TLR11 and TLR12. (A) ELISA plates were coated with *T. gondii* profilin (10 μg/ml) or BSA (10 μg/ml) in 10 mM Tris (pH 6.0) and 150 mM NaCl. After blocking free binding sites with 5% fat-free milk, plates were incubated with 1 μg/ml purified TLR11 (green), TLR12 (red), TLR13 (black), or BSA (blue). Binding of the TLR ectodomains was detected with a DED-specific mAb. All ELISA steps were performed at pH 6.0. (B) ELISA plates were coated with *T. gondii* profilin (10 μg/ml) or BSA (10 μg/ml) in 10 mM Tris (pH 8.0) and 150 mM NaCl. After blocking free binding sites with 5% fat-free milk, plates were incubated with 1 μg/ml purified TLR11 (green), TLR12 (red), TLR13 (black), or BSA (blue). Binding of the TLR ectodomains was detected with a DED-specific mAb. All ELISA steps were performed at pH 8.0. (C) ELISA plates were coated with 10 μg/ml TLR11, TLR12, TLR13, or BSA in 10 mM Tris (pH 6.0) and 150 mM NaCl. After blocking and washing steps, recombinant profilin (10 μg/ml) was added to the wells. Profilin was detected with a *T. gondii* profilin–specific rabbit polyclonal Ab. All ELISA steps were performed at pH 6.0. A representative result from at least 10 experiments is shown. (D) *T. gondii* profilin (2 μM) alone (blue) or preincubated with 0.2 μM purified TLR11 ectodomain (red). Biochemical fractionation of *T. gondii* profilin or *T. gondii* profilin and TLR11 (red) was performed by gel filtration chromatography, and each fraction was tested for *T. gondii* profilin by ELISA (left). In the right panel, the *T. gondii* profilin plus TLR11 fractions (red) were additionally tested for TLR11 compared with TLR11 alone (green) and analyzed according to the same gel filtration chromatography procedure with a Superdex 200 10/300 GL chromatographic separation column. (E) *T. gondii* profilin alone (blue), *T. gondii* profilin and TLR12 (red), or TLR12 alone (Figure legend continues)
and TLR9 (Supplemental Fig. 3A). We observed that cells over-expressing the examined TLRs activated NF-κB–driven luciferase expression in response to the corresponding ligands, although to a different degree for each of the tested TLRs (Supplemental Fig. 3A).

To directly examine whether NF-κB plays a role in regulation of IL-12 production by DCs triggered by T. gondii profilin, DCs were next isolated from NF-κB1−/− and NF-κB2−/− mice. These DCs are deficient in classical and alternative NF-κB pathways, respectively. Our experiments revealed that neither NF-κB1 nor NF-κB2 was required for IL-12 production by DCs triggered by T. gondii profilin (Fig. 4C). Furthermore, we also observed that TLR9 can also engage the NF-κB–independent IL-12 production by DCs (Fig. 4C). At the same time, TLR9-induced TNF production was NF-κB–dependent (Fig. 4C). Furthermore, a pan-NF-κB inhibitor completely prevented TNF induction in response to CpG, but it had no effect on IL-12 production by DCs stimulated with T. gondii profilin and only partially reduced TLR9-mediated induction of IL-12. Overall, although our experiments cannot rule out a possibility that NF-κB may be triggered by TLR11 and TLR12 in other cell types besides DCs, this signaling pathway is not required for IL-12 production in response to T. gondii profilin.

The inability of T. gondii profilin to trigger significant levels of NF-κB activation in transfected cells or primary DCs prompted us to examine transcription factors regulating IL-12 responses after T. gondii profilin. The IL-12/23p40 promoter contains an NF-κB-binding site (32), and CD8+ DCs are enriched for the IRF8 transcription factor (19, 33–37). Therefore, we examined the role of IRF transcription factors in the TLR11- and TLR12-dependent cytokine responses triggered by T. gondii profilin. We first purified splenic CD11c+ DCs from WT, IRF4−/−, and IRF8−/− mice and stimulated these cells with T. gondii profilin or CpG, a TLR9 agonist. We observed that, in the absence of IRF8 but not IRF4, splenic DCs failed to secrete IL-12p40 in response to TLR11/TLR12 agonist (Fig. 5A). These results indicate that IRF8 is essential for IL-12/23p40 production in response to T. gondii profilin. Control experiments with CpG demonstrated that IRF8 also plays a role in the regulation of IL-12/23p40 but not in TNF production in response to TLR9 activation (Fig. 5A).

IRF8, an important regulator of IL-12 production, is also indispensable for the development of CD8+ DCs (19, 33, 36). Others and we have previously established that CD8+ DCs are the major source of IL-12 in response to TLR11 activation (7, 12, 38). Thus, the lack of detectable IL-12/23p40 and IL-12p70 (not shown) production in the absence of IRF8 has two potential explanations.

One explanation is that IRF8 is essential for the TLR11 and TLR12 signaling pathway. Alternatively, the lack of IL-12 in the supernatants of T. gondii profilin–activated IRF8−/− CD11c+ cells could be explained by a deficiency in the CD8+ DC subset. To test these hypotheses, we generated DCs from IRF8-deficient cells and reconstituted IRF8 expression using retroviral transduction. As a negative control, IRF8−/− DCs were infected with an empty vector (Fig. 5B). In addition to IRF8−/− cells, WT, TLR11−/−, and MyD88−/− cells were transduced with IRF8 or control vectors. We observed that the expression of exogenous IRF8 in IRF8−/− DCs was sufficient for the reconstitution of TLR11- and TLR12-dependent sensitivity to T. gondii profilin, as measured by the secretion of IL-12p40 by these cells (Fig. 5B, left). In contrast, IRF8−/− cells infected with the control vector failed to secrete IL-12p40 in response to any tested concentration of T. gondii profilin (Fig. 5B, right). As expected, IRF8 expression in TLR11- or MyD88-deficient cells did not rescue the responsiveness of DCs to T. gondii profilin (Fig. 5B). Notably, IRF8 regulated IL-12/23p40 secretion but not TNF production by DCs in response to the TLR9 agonist (Fig. 5B, Supplemental Fig. 3B). These results suggest that the IL-12 response is initiated by activation of TLR11 and TLR12 by T. gondii profilin and that the responses initiated by the activation of TLR9 by CpG are regulated by IRF8.

To formally exclude a possibility that IRF8 expression resulted in induction of TLR11-, TLR12-, and MyD88-dependent T. gondii profilin sensitivity by rescuing development of CD8+ like DCs, we next generated fully differentiated bone marrow–derived macrophages and induced IRF8 expression by recombinant IFN-γ (Supplemental Fig. 3C). As a negative control, macrophages were also generated from IRF8−/− cells. We observed that IRF8 induction by IFN-γ was sufficient for acquisition of T. gondii profilin responsiveness as measured by IL-12p40 production. In contrast, IRF8−/− macrophages failed to do so (Fig. 5C). Similarly, sort-purified fully differentiated WT but not IRF8−/− splenic CD8+ DCs produced IL-12/23p40 when preincubated with IFN-γ prior to stimulation with T. gondii profilin (Fig. 5D). At the same time IFN-γ had a minor effect on TLR11 and TLR12 expression (Supplemental Fig. 3D, 3E). These data argue against a possibility that IFN-γ regulates responsiveness to T. gondii profilin via regulation of TLR11 and TLR12 expression.

To expand on our in vitro experiments, we next examined the expression of IRF8 and IL-12/23p40 in DCs after injection of profilin in WT and IRF8−/− mice. We observed that all IL-12/23p40–pro-
ducing CD8⁺ DCs isolated from the spleen expressed increased levels of IRF8 compared with controls (Fig. 5E). Additionally, IRF8 expression was essential for the IL-12 response triggered by *T. gondii* profilin in vivo (Fig. 5E). Induction of IRF8 in CD8⁺ DCs resulted in appearance of IL-12/23p40–producing cells in response to *T. gondii* profilin, which was increased in conditions where mice were primed with rIFN-γ (Fig. 5F).

Taken together, these experiments formally established that IRF8, but not NF-κB, regulates DC IL-12 production in response to the TLR11/TLR12 agonist *T. gondii* profilin.

**Discussion**

Induction of IL-12 is the crucial step for the activation of the immune responses required for host protection from *T. gondii* (39). This cytokine is indispensable for triggering IFN-γ production by NK and T cells (40), and the failure to initiate the IL-12– and IFN-γ–mediated host responses results in acute susceptibility to the parasite (41–43). Whereas several innate immune cells, including DCs, macrophages, neutrophils, and proinflammatory monocytes, are capable of producing IL-12 in response to *T. gondii* (9, 44), the experimental infection of mice lacking CD8⁺ DCs has established...
that these immune cells are indispensable for initial IL-12 production during parasitic infection (38). Furthermore, the acute susceptibility to *T. gondii* in the absence of CD8+ DCs can be rescued by treatment with rIL-12, indicating that the major function of these DCs is TLR-dependent recognition of the parasite and production of IL-12 (38, 45). These in vivo results are in strong agreement with in vitro experiments that have demonstrated that CD8+ DCs are the major IL-12-producing cells among splenocytes stimulated with *T. gondii* (7, 12). TLR11, MyD88, and IRF8 have also been shown to be indispensable for DC IL-12 responses in vivo and in vitro (7, 13, 14, 46). The absence of either of these molecules greatly diminishes IL-12 production triggered by purified *T. gondii* profilin, but the precise connections between TLR11, the TLR adaptor protein MyD88, and the transcriptional factor IRF8 were not well understood. Furthermore, it was not clear why TLR11-dependent induction of IL-12 is limited to CD8+ DCs and how this receptor recognizes *T. gondii* profilin.
In this study, we reveal that, in addition to TLR11, TLR12 plays a role in the regulation of IL-12 production by DCs in response to To. gondii profilin. TLR12 is homologous to TLR11, is localized intracellularly, and similar to TLR11, and directly interacts with UNC93B1, an endoplasmic reticulum–resident protein. Additionally, both TLR11 and TLR12 are not only capable of forming a heterodimeric complex but also directly interacts with To. gondii profilin. These results biochemically define TLR11 and TLR12 as receptors for To. gondii profilin. Nevertheless, TLR11 but not TLR12 is involved in binding to MyD88. These results suggest that TLR11 and TLR12 binding to To. gondii profilin initiates the MyD88 signaling cascade via the TLR11 Toll/IL-1R domain. Analysis of the downstream events of the TLR11 and TLR12 signaling pathways revealed that although both receptors are important for the regulation of IL-12 production by DCs in response to To. gondii profilin, the TLR11 and TLR12 signaling cascades depend on the IRF8 transcription factor but do not induce significant activation of NF-κB. Furthermore, neither NF-κB1 nor NF-κB2 were required for DC IL-12 production in response to To. gondii profilin. This was in striking contrast with IRF8-deficient DCs that failed to induce detectable cytokine responses when stimulated with To. gondii profilin. Moreover, IRF8 overexpression or induction by IFN-γ was sufficient for induction of responsiveness to the TLR11/TLR12 agonists. These results demonstrate that the restricted ability of naive CD8+ DCs to initiate IL-12 production in response to To. gondii profilin occurs because IRF8 expression is limited to this DC subset. Moreover, other DC subsets and macrophages can produce IL-12 when stimulated with To. gondii profilin, but only after priming with IFN-γ is this dependent on IRF8 induction. The discovery of NF-κB independence for the TLR11- and TLR12-mediated induction of IL-12 in response to To. gondii profilin is in strong agreement with the phenotype of the To. gondii–infected NF-κB1−/−, NF-κB2−/−, and c-Rel−/− mice producing unimpaired IL-12 in response to the parasitic infection (47–49). Our study is in agreement with the recent reports describing a requirement for TLR12 in induction of IL-12 response to To. gondii (50, 51), but, additionally, to our knowledge it provides the first formal evidence describing direct To. gondii profilin interaction with highly purified TLR11 and TLR12. Most importantly, our study uncovered a MyD88- and IRF8-dependent but NF-κB–independent pathway in DCs for the induction of IL-12 in response to To. gondii. In this regard our results differ from the report describing activation of NF-κB in response to TLR11 and TLR12 (50). It was observed that TLR11 or TLR12 can induce NF-κB response in the similarly transfected HEK 293 cells (50). Furthermore, it was suggested that similarly purified Plasmodium falciparum profilin can activate NF-κB in GM-CSF–derived DCs (50). Knowing that P. falciparum and other tested malaria profilins do not trigger TLR11 activation, as is evident from both the biochemical and genetic experiments in which P. falciparum profilin was expressed in To. gondii instead of the endogenous protein (7, 8), we argue that the observed NF-κB activity triggered by To. gondii and malaria profilins likely stems from microbial contaminations present in the purified protein. This possibility is further supported by responsiveness of GM-CSF–derived DCs to To. gondii profilin seen by Koblansky et al. (50). GM-CSF DCs, similar to CD8+ splenic DCs, lack IRF8 and do not produce IL-12 in response to purified To. gondii profilin unless primed with IFN-γ. Furthermore, the reported TLR11–dependent but MyD88–dependent activity of To. gondii profilin is not supported by previous work from our laboratory or other laboratories (7, 8, 52). Although it is possible that experimental variations were responsible for the observed differences in the TLR11 and TLR12 downstream signaling pathways activated by To. gondii profilin, we argue that in vivo and in vitro analysis of primary DCs revealed that To. gondii profilin induces IL-12 via a MyD88- and IRF8-dependent signaling pathway.

**Disclosures**

The authors have no financial interests of conflict.

**References**


