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Cooperation of TLR12 and TLR11 in the IRF8-dependent IL-12 response to *Toxoplasma gondii* profilin

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Abstract

Toll-like receptors play a central role in the innate recognition of pathogens and the activation of dendritic cells (DCs). Here, we established 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 heterodimer complex. We also established that the transcription factor IRF8, not NF-kB, 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 IRF8-expressing CD8+ DCs in governing the TLR11- and TLR12-mediated host defense against *T. gondii*.

Introduction

TLRs play a central role in detecting all types of pathogens, including viruses, bacteria, and parasites (1). The vertebrate TLR family consists of 13 members that can be grouped into six major families based on their sequence similarities (2). Closely related TLRs are involved in the recognition of similar microbial molecules, as illustrated by the analysis of TLR7 and TLR8, which are involved in the recognition of single-stranded RNA (3–5). In addition, TLRs of the same family are capable of forming heterodimers; for instance, TLR2 is known to function in TLR2-TLR1 or TLR2-TLR6 heterodimers (6). We have previously established that TLR11 is involved in the recognition of an unconventional profilin protein present in the protozoan parasite *Toxoplasma gondii* (7). Both *in vitro* and *in vivo* experiments revealed that the absence of TLR11 prevents the induction of IL-12 in response to the parasite or to purified *T. gondii* profilin (7). Furthermore, conditional deletion of the profilin gene in the parasite abolishes the production of pro-inflammatory cytokines by DCs,

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strongly suggesting that TLR11-mediated sensing of *T. gondii* profilin plays a major role in the initiation of IL-12 dependent immunity against the parasite (8). It is not known, however, if TLR11 directly senses its ligand or if other accessory proteins are involved in the recognition of *T. gondii* profilin. Because *T. gondii* can infect all nucleated cells and the cellular interactions between DCs and parasites are not completely understood, establishing the mechanism of immunity initiation is important (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 report, we established 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 revealed that the TLR11- and TLR12-mediated recognition of *T. gondii* profilin induces IRF8-dependent dendritic cell IL-12 production rather than the NF-kB 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 to this subset of DCs.

Material and Methods

Animals

C57BL/6 (WT) mice were obtained from the University of Texas Southwestern Medical Center Mouse Breeding Core Facility. *Tlr11*−/−, *Myd88*−/−, *NF-κB1*−/−, *NF-κB2*−/− and *Irf8*−/− 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 SPF 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 NheI and SacII sites of pEGFPN1 and pmCherryN1 using standard PCR techniques with the forward primer 5’-GCTAGC ATGCCCCGCATGGAGCGCCACCAGT-3’ and the reverse primer 5’-CCGCGGGTCGCGCTCCTGCCCGGCCTTG-3’. TLR12 was myc-tagged and inserted between the NheI and XbaI sites of pcDNA3.1 using the forward primer 5’-TACCGAGCTCGGATCCACCATGCCCCGCATGGAGCG-3’ and the reverse primer 5’-GATATCTGCAGAATTCTTACAGATCCTCTTCTGAGATGAGTTTTTGTTCGTCGCG CTCCTGCCCG-3’.

GFP-tagged TLR11 cloned between the NheI and SacII sites of pEGFPN1 and myc-tagged TLR11 cloned between the NheI and XbaI sites of pcDNA3.1 have been described previously (18). *Unc93b1* was cloned into the XhoI and HindIII sites of pmCherryN1 and pEGFPN1 using the forward primer 5’-GTTTCTCGAGATGAAGGAAGTCCCAACCAGC-3’ and the reverse primer 5’-GTTTCTAAGCTTCTGCTCCTCAGGCCCATC-3’. The CD3ζ construct was a gift from Dr. Nicolai van Oers (UT Southwestern). All plasmids were prepared using the Endofree Midiprep kit from Clontech.

Protein expression and purification

The extracellular portions of TLR11 and TLR12 were cloned into the pEGFP-N2 vector and were additionally tagged with the DED epitope (Patent RU2380373) using the forward primers 5'-AAGTCGACGCCACCATGGGCCGCTACTGGCT-3', 5'-AAGTCGACGCCACCATGCCCCGCATGGAGCG-3' and the reverse primers 5'-AAGGATCCTTTAAGTTCCAGAGTTTG-3', 5'-AAGGATCCCTCTGTTCCATGCGGACAATT-3', respectively.

To express the ectodomains of TLR11 and TLR12, 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 monoclonal antibody (clone 2E8, Proteinsynthesis).

T. gondii profilin was expressed and purified as described previously (7). IL-12/23p40 and IL-12p70 ELISA kits were purchased from eBioscience.

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 μg/ml) in 10 mM Tris (pH 6.0 or pH 8.0) and 150 mM NaCl. Free binding sites were blocked with 5% fat free milk, and 1 μg/ml of the purified TLR11 or TLR12 ectodomain was added. After extensive washing steps, the presence of TLR11 and TLR12 was detected with a DED-specific monoclonal antibody. In an alternative assay, the ELISA plates were initially coated with 10 μg/ml of the TLR11, TLR12, or TLR13 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 antibody 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 hours in 10 mM Tris (pH 6.0 or pH 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 FPLC 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 ATCC and maintained in tissue culture flasks in RPMI 1640 medium containing 10% FBS (Hyclone), 10 mM HEPES, 1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.5 mg/ml L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK293 cells were plated on glass coverslips and were transfected using Lipofectamine 2000 (Invitrogen). For immunofluorescence assays, HEK293 cells were grown on glass slides, transfected with the TLR12 and TLR11 or TLR12 and UNC93B1 constructs for 36 h, and images were acquired with a Leica SPE microscope equipped with a 63× objective. Images were analyzed using Imaris software (Bitplane). Specifically, the merged image was analyzed using the "Co-localization" function using automatic thresholding and the resulting co-localization coefficients were copied into a text file and saved. We have analyzed >100 cells co-expressing TLR11 and TLR12 to calculate the average percent of colocalization. Statistical analyses were performed by importing the results from Imaris into GraphPad Prism software.

For immunoprecipitation experiments, HEK293 cells were cotransfected at 70% confluence with GFP- and myc-tagged constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. Thirty-six hours after transfection, the cells were lysed in lysis buffer (20 mM Tris-HCl (pH 8.0) 137 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 10% glycerol, Complete Mini Protease Inhibitors (Roche Applied Science) and 1 mM PMSF). The cell lysates were centrifuged, and the supernatants were used for immunoprecipitation with 2 μg of anti-GFP monoclonal antibody (anti-GFP-IP, clone 2G7, Proteinsynthesis, Russia) bound to protein A/G beads (Pierce). Equal quantities of eluted protein were subjected to SDS-PAGE, transferred to nitrocellulose, and blotted using anti-myc (Abcam) and anti-GFP (anti-GFP-BL, clone 3A9, Proteinsynthesis, Russia) antibodies.

siRNA, Lentiviral and Retroviral Transduction

Bone marrow-derived DCs were generated in the presence of Flt3L (100 ng/ml) purchased from eBioscience and were transfected with siRNA or infected with lentiviruses on day 3 of culture. The siRNA (FlexiTube siRNA) against TLR11 (SI01449497, SI01449504, SI01449483, SI01449490), TLR12 (SI01449469, SI01449476, SI01449455, SI01449462), TLR13 (SI01449511, SI01449518, SI01449525, SI01449532) and MyD88 (SI00196196, SI02713277, SI00196175, and SI00196182) were purchased from Qiagen. siRNA transfection was performed in a reverse manner, seeding the cells into a well already containing transfection mix according to the manufacturer's recommendations (Qiagen) but Lipofectamine 2000 (Invitrogen) was used instead of HiPerFect Transfection Reagent (Qiagen).

The BLOCK-iT HiPerform Lentiviral Pol II miR RNAi Expression System (pLenti6.4 CMVp-EmGFP) was purchased from Invitrogen. The double-stranded oligo duplexes encoding miRNA against TLR12 (at position 858, 5’-TGCTGAGGAAATGCAAGTGGAGCAGTGTTTTGGCCACTGACTGACACTGCTCCT TGC ATTTCCT-3’ and 5’-CCTGAGGAAATGCAAGGAGCAGTGTCAGTCAGTGGCCAAAACACT GCTCCACTTGCATTTCCTC-3’; at position 1241, 5’-TGCTGTGTTATTGCCACTCAA CCAGAGTTTTGGCCACTGACTGACTCTGGTTGTGGCAATAACA-3’ and 5’-CCTGTG TTATTGCCACAACCAGAGTCAGTCAGTGGCCAAAACTCTGGTTGAGTGGCAATA ACAC-3’) were designed with Invitrogen’s RNAi Designer tool and ordered from Invitrogen, with pcDNA6.2-GW EmGFP LacZ supplied as a negative control. The lentiviral stocks were prepared by cotransfecting the 293FT producer cell line with the optimized ViraPower Packaging Mix (Invitrogen) and the miRNA-encoding pLenti6.4_CMVp_EmGFP constructs.

For IRF8 rescue experiments, Flt3L DCs were transduced with the retroviral supernatants by spinoculation (2400 rpm, 33°C, 1 hr) with 4 μg/ml polybrene on day 3 of the culture. Both pMSCV-IRF8 and the control vectors were described previously (19).

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). By contrast, the

TLRs involved in sensing components of microbial cell walls or flagellin localize to the cell surface, and their signaling is unimpaired 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 TLR12 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 co-expressed 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 co-localization (>80%), indicating that these innate receptors localize to similar intracellular compartments (Fig. 1A, Supplemental Fig. 1B). Additional confocal microscopy experiments revealed that TLR12 co-localizes with UNC93B1, further demonstrating that TLR12 is an intracellular 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 interact 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 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, lines 1 and 2). In addition, we observed strong interactions between TLR11 and TLR12, suggesting that these receptors can form a heterodimeric complex (Fig. 1C, line 3). This possibility 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 antibody. We established that TLR12 was capable of forming homodimers as well as heterodimers with TLR11 (Fig. 1C, lines 5 and 6). Surprisingly, TLR12 did not immunoprecipitate with MyD88 (Fig. 1C, line 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, since 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, line 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, lines 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, lines 10–12). Similarly, barely detectable interactions of TLR11 with TLR4 and TLR9 were seen under identical immunoprecipitaion 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 if 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 if 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 if 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 (PFTg). 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 antibody (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 if the ectodomains of TLR11 and TLR12 formed complexes with *T. gondii* profilin. We monitored the elution profiles of profilin (left) and the TLR ectodomains (right) alone, as shown in Fig. 2D–F. We next pre-incubated *T. gondii* profilin with the ectodomain of TLR11 or TLR12, allowed the complexes to form at pH 6.0, 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 co-elution 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 and 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 and Supplemental Fig. 2A–2C). 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 *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-kB transcriptional factors (31). We thus examined NF-kB activation triggered by TLR11 and TLR12 in response to *T. gondii* profilin. We observed that HEK 293 cells co-transfected with TLR11 and an NF-kB-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-kB in response to the agonist (Fig. 4A). Surprisingly, cells expressing both TLR11 and TLR12 did not trigger significant NF-kB 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 reveals that only CHO-K1 cells stimulated with *T. gondii* profilin triggered a very modest (2–3 fold) NF-kB 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-kBp65 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, and TLR9 (Supplemental Fig. 3A). We observed that cells overexpressing the examined TLRs activated NF-kB-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 if 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 were 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-kB inhibitor completely prevented TNF induction in response to CpG, but 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, while our experiments cannot rule out a possibility that NF-kB activity 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 TLR11 and TLR12 activation. The IL-12/23p40 promoter contains an IRF-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 the 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). By 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 and 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 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− DC produced IL-12/23p40 when pre-incubated 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 producing CD8+ DCs isolated from the spleen expressed increased levels of IRF8 compared to controls (Fig. 5E). In addition, 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 (Figure 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). While several innate immune cells, including DCs, macrophages, neutrophils, and pro-inflammatory 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 recombinant IL-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 report, we revealed that, in addition to TLR11, TLR12 plays a role in the regulation of IL-12 production by DCs in response to *T. 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 *T. gondii* profilin. These results biochemically define TLR11 and TLR12 as receptors for *T. gondii* profilin. Nevertheless, TLR11 but not TLR12 is involved in binding to MyD88. These results suggest that TLR11 and TLR12 binding to *T. gondii* profilin initiates the MyD88 signaling cascade via the TLR11 Toll/IL-1R (TIR) 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 *T. gondii* profilin, the TLR11 and TLR12 signaling cascade depends on the IRF8 transcription factor but does not induce significant activation of NF-κB. Furthermore, neither NF-κB1 nor NF-κB2 were required for DC IL-12 production in response to *T. gondii* profilin. This was in striking contrast with IRF8-deficient DCs that failed to induce detectable cytokine responses when stimulated with *T. 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 naïve CD8+ DCs to initiate IL-12 production in response to *T. 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 *T. gondii* profilin, but only after priming with IFN-γ is that dependent on IRF8 induction. The discovery of NF-κB independence for the TLR11 and TLR12 mediated induction of IL-12 in response to *T. gondii* profilin is in strong agreement with the phenotype of the *T. gondii* infected NF-κB1−/−, NF-κB2−/−, 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 *T. gondii* (50, 51), but in addition provide the first formal evidence describing direct *T. 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 *T. gondii*. In this regard our results differ from the report describing activation of NF-kB in response to TLR11 and TLR12 (50). It was

observed that TLR11 or TLR12 can induce NF-kB response in the similarly transfected HEK 293 cells (50). Furthermore, it was suggested that similarly purified *Plasmodium falciparum* profilin can activate NF-kB 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 *T. gondii* instead of the endogenous protein (7, 8), we argue that the observed NF-kB activity triggered by *T. gondii* and malaria profilins likely stem from microbial contaminations present in the purified protein. This possibility is further supported by responsiveness of GM-CSF derived DCs to *T. gondii* profilin seen by Koblansky and colleagues (50). GM-CSF DCs, similar to CD8− splenic DCs lack IRF8 and do not produce IL-12 in response to purified *T. gondii* profilin unless primed with IFN-γ. Furthermore, the reported TLR11-independent but MyD88 dependent activity of *T. gondii* profilin is not supported by previous work from our, or other laboratories (7, 8, 52). While it is possible that experimental variations were responsible the observed differences in the TLR11 and TLR12 downstream signaling pathways activated by *T. gondii* profilin, we argue that in vivo and in vitro analysis of primary DCs revealed that *T. gondii* profilin induces IL-12 via a MyD88 and IRF8 dependent signaling pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011; 34:637–650. [PubMed: 21616434]
2. Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, Hood LE, Aderem A. The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci U S A. 2005; 102:9577–9582. [PubMed: 15976025]
3. Du X, Poltorak A, Wei Y, Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. European cytokine network. 2000; 11:362–371. [PubMed: 11022119]
4. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science (New York, N.Y.). 2004; 303:1529–1531.
5. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science (New York, N.Y.). 2004; 303:1526–1529.
6. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A. 2000; 97:13766–13771. [PubMed: 11095740]
7. Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS, Hieny S, Sutterwala FS, Flavell RA, Ghosh S, Sher A. TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science (New York, N.Y.). 2005; 308:1626–1629.
8. Plattner F, Yarovinsky F, Romero S, Didry D, Carlier MF, Sher A, Soldati-Favre D. Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response. Cell Host Microbe. 2008; 3:77–87. [PubMed: 18312842]
9. Denkers EY, Butcher BA, Del Rio L, Bennouna S. Neutrophils, dendritic cells and Toxoplasma. Int J Parasitol. 2004; 34:411–421. [PubMed: 15003500]

10. McKee AS, Dzierszinski F, Boes M, Roos DS, Pearce EJ. Functional inactivation of immature dendritic cells by the intracellular parasite Toxoplasma gondii. J Immunol. 2004; 173:2632–2640. [PubMed: 15294980]
11. Koshy AA, Dietrich HK, Christian DA, Melehani JH, Shastri AJ, Hunter CA, Boothroyd JC. Toxoplasma Co-opts Host Cells It Does Not Invade. PLoS Pathog. 2012; 8:26.
12. Reis e Sousa C, Hieny S, Scharton-Kersten T, Jankovic D, Charest H, Germain RN, Sher A. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. J Exp Med. 1997; 186:1819–1829. [PubMed: 9382881]
13. Scanga CA, Aliberti J, Jankovic D, Tilloy F, Bennouna S, Denkers EY, Medzhitov R, Sher A. Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. J Immunol. 2002; 168:5997–6001. [PubMed: 12055206]
14. Scharton-Kersten T, Contursi C, Masumi A, Sher A, Ozato K. Interferon consensus sequence binding protein-deficient mice display impaired resistance to intracellular infection due to a primary defect in interleukin 12 p40 induction. J Exp Med. 1997; 186:1523–1534. [PubMed: 9348310]
15. Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell. 1995; 80:321–330. [PubMed: 7834752]
16. Franzoso G, Carlson L, Poljak L, Shores EW, Epstein S, Leonardi A, Grinberg A, Tran T, Scharton-Kersten T, Anver M, Love P, Brown K, Siebenlist U. Mice deficient in nuclear factor (NF)-kappa B/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. J Exp Med. 1998; 187:147–159. [PubMed: 9432973]
17. Klein U, Casola S, Cattoretti G, Shen Q, Lia M, Mo T, Ludwig T, Rajewsky K, Dalla-Favera R. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. Nat Immunol. 2006; 7:773–782. [PubMed: 16767092]
18. Pifer R, Benson A, Sturge CR, Yarovinsky F. UNC93B1 is essential for TLR11 activation and IL-12-dependent host resistance to Toxoplasma gondii. J Biol Chem. 2011; 286:3307–3314. [PubMed: 21097503]
19. Tsujimura H, Tamura T, Gongora C, Aliberti J, Reis e Sousa C, Sher A, Ozato K. ICSBP/IRF-8 retrovirus transduction rescues dendritic cell development in vitro. Blood. 2003; 101:961–969. [PubMed: 12393459]
20. Barton GM, Kagan JC. A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat Rev Immunol. 2009; 9:535–542. [PubMed: 19556980]
21. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, Lien E, Nilsen NJ, Espevik T, Golenbock DT. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol. 2004; 5:190–198. [PubMed: 14716310]
22. Leifer CA, Kennedy MN, Mazzoni A, Lee C, Kruhlak MJ, Segal DM. TLR9 is localized in the endoplasmic reticulum prior to stimulation. J Immunol. 2004; 173:1179–1183. [PubMed: 15240708]
23. Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, Yamamoto A, Seya T. Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol. 2003; 171:3154–3162. [PubMed: 12960343]
24. Nishiya T, DeFranco AL. Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. J Biol Chem. 2004; 279:19008–19017. [PubMed: 14976215]
25. Barton GM, Kagan JC, Medzhitov R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat Immunol. 2006; 7:49–56. [PubMed: 16341217]
26. Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozat K, Mudd S, Mann N, Sovath S, Goode J, Shamel L, Herskovits AA, Portnoy DA, Cooke M, Tarantino LM, Wiltshire T, Steinberg BE, Grinstein S, Beutler B. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. Nat Immunol. 2006; 7:156–164. [PubMed: 16415873]

27. Kim YM, Brinkmann MM, Paquet ME, Ploegh HL. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. Nature. 2008; 452:234–238. [PubMed: 18305481]
28. Brinkmann MM, Spooner E, Hoebe K, Beutler B, Ploegh HL, Kim YM. The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. J Cell Biol. 2007; 177:265–275. [PubMed: 17452530]
29. Lee BL, Moon JE, Shu JH, Yuan L, Newman ZR, Schekman R, Barton GM. UNC93B1 mediates differential trafficking of endosomal TLRs. Elife. 2013; 2:19.
30. Oldenburg M, Kruger A, Ferstl R, Kaufmann A, Nees G, Sigmund A, Bathke B, Lauterbach H, Suter M, Dreher S, Koedel U, Akira S, Kawai T, Buer J, Wagner H, Bauer S, Hochrein H, Kirschning CJ. TLR13 Recognizes Bacterial 23S rRNA Devoid of Erythromycin Resistance-Forming Modification. Science (New York, N.Y.). 2012; 20:20.
31. Dunne A, O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. Sci STKE. 2003; 25
32. Ma X, Chow JM, Gri G, Carra G, Gerosa F, Wolf SF, Dzialo R, Trinchieri G. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. J Exp Med. 1996; 183:147–157. [PubMed: 8551218]
33. Aliberti J, Schulz O, Pennington DJ, Tsujimura H, Reis e Sousa C, Ozato K, Sher A. Essential role for ICSBP in the in vivo development of murine CD8alpha + dendritic cells. Blood. 2003; 101:305–310. [PubMed: 12393690]
34. la Sala A, He J, Laricchia-Robbio L, Gorini S, Iwasaki A, Braun M, Yap GS, Sher A, Ozato K, Kelsall B. Cholera toxin inhibits IL-12 production and CD8alpha+ dendritic cell differentiation by cAMP-mediated inhibition of IRF8 function. J Exp Med. 2009; 206:1227–1235. [PubMed: 19487420]
35. Qi CF, Li Z, Raffeld M, Wang H, Kovalchuk AL, Morse HC 3rd. Differential expression of IRF8 in subsets of macrophages and dendritic cells and effects of IRF8 deficiency on splenic B cell and macrophage compartments. Immunol Res. 2009; 45:62–74. [PubMed: 18663414]
36. Schiavoni G, Mattei F, Sestili P, Borghi P, Venditti M, Morse HC 3rd, Belardelli F, Gabriele L. ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. J Exp Med. 2002; 196:1415–1425. [PubMed: 12461077]
37. Tailor P, Tamura T, Morse HC 3rd, Ozato K. The BXH2 mutation in IRF8 differentially impairs dendritic cell subset development in the mouse. Blood. 2008; 111:1942–1945. [PubMed: 18055870]
38. Mashayekhi M, Sandau MM, Dunay IR, Frickel EM, Khan A, Goldszmid RS, Sher A, Ploegh HL, Murphy TL, Sibley LD, Murphy KM. CD8alpha(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites. Immunity. 2011; 35:249–259. [PubMed: 21867928]
39. Pifer R, Yarovinsky F. Innate responses to Toxoplasma gondii in mice and humans. Trends Parasitol. 2011; 27:388–393. [PubMed: 21550851]
40. Trinchieri G. Interleukin-12: a cytokine at the interface of inflammation and immunity. Adv Immunol. 1998; 70:83–243. [PubMed: 9755338]
41. Gazzinelli RT, Hieny S, Wynn TA, Wolf S, Sher A. Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. Proc Natl Acad Sci U S A. 1993; 90:6115–6119. [PubMed: 8100999]
42. Hunter CA, Subauste CS, Van Cleave VH, Remington JS. Production of gamma interferon by natural killer cells from Toxoplasma gondii-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha. Infect Immun. 1994; 62:2818–2824. [PubMed: 7911785]
43. Khan IA, Matsuura T, Kasper LH. Interleukin-12 enhances murine survival against acute toxoplasmosis. Infect Immun. 1994; 62:1639–1642. [PubMed: 7909536]
44. Dunay IR, Sibley LD. Monocytes mediate mucosal immunity to Toxoplasma gondii. Curr Opin Immunol. 2010; 22:461–466. [PubMed: 20537517]

45. Hou B, Benson A, Kuzmich L, DeFranco AL, Yarovinsky F. Critical coordination of innate immune defense against Toxoplasma gondii by dendritic cells responding via their Toll-like receptors. Proc Natl Acad Sci U S A. 2011; 108:278–283. [PubMed: 21173242]

46. Kucera K, Koblansky AA, Saunders LP, Frederick KB, De La Cruz EM, Ghosh S, Modis Y. Structure-based analysis of Toxoplasma gondii profilin: a parasite-specific motif is required for recognition by Toll-like receptor 11. J Mol Biol. 2010; 403:616–629. [PubMed: 20851125]

47. Caamano J, Tato C, Cai G, Villegas EN, Speirs K, Craig L, Alexander J, Hunter CA. Identification of a role for NF-kappa B2 in the regulation of apoptosis and in maintenance of T cell-mediated immunity to Toxoplasma gondii. J Immunol. 2000; 165:5720–5728. [PubMed: 11067930]

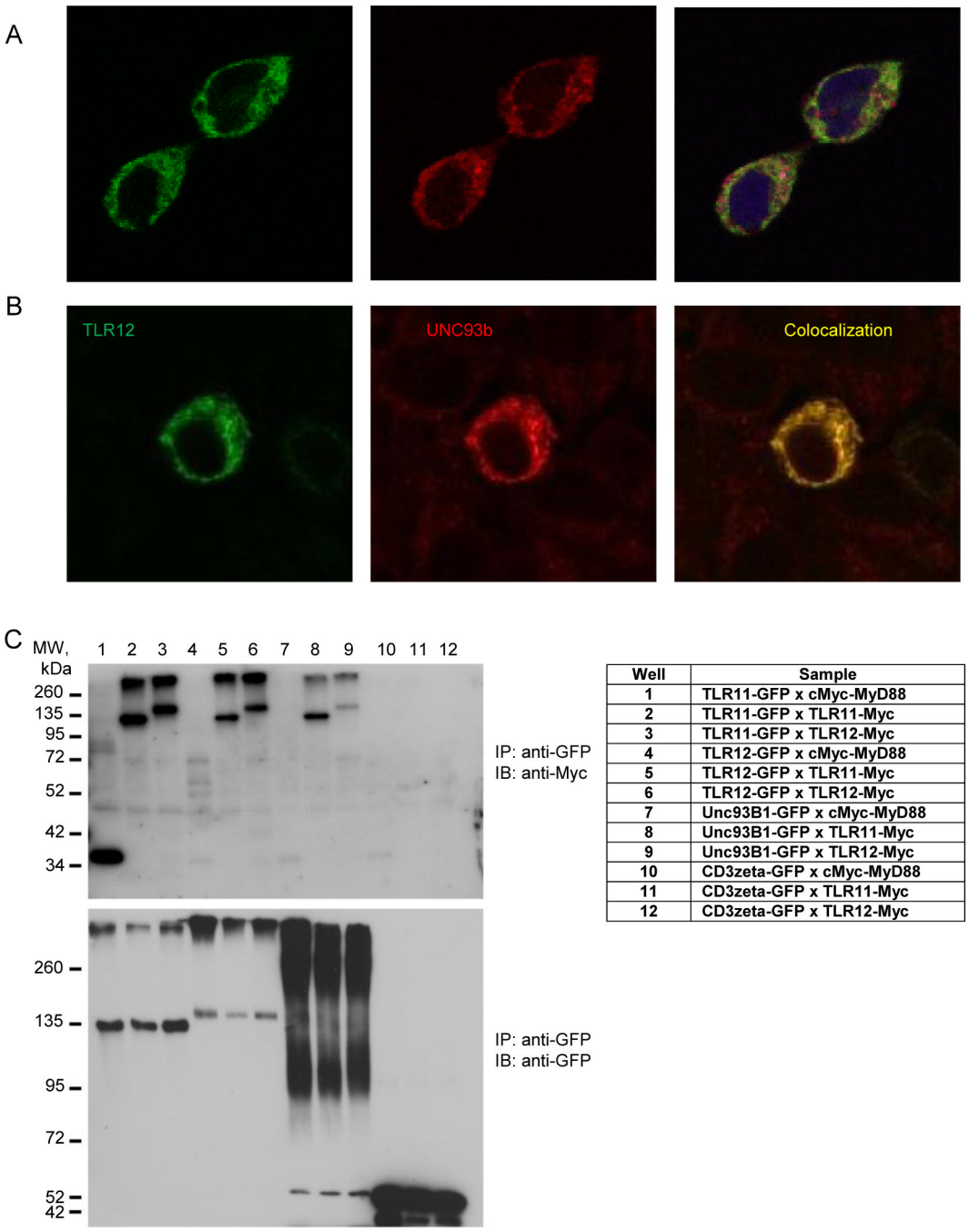
48. Harris TH, Wilson EH, Tait ED, Buckley M, Shapira S, Caamano J, Artis D, Hunter CA. NF-kappaB1 contributes to T cell-mediated control of Toxoplasma gondii in the CNS. J Neuroimmunol. 2010; 222:19–28. [PubMed: 20156658]

49. Mason N, Aliberti J, Caamano JC, Liou HC, Hunter CA. Cutting edge: identification of c-Rel-dependent and -independent pathways of IL-12 production during infectious and inflammatory stimuli. J Immunol. 2002; 168:2590–2594. [PubMed: 11884420]

50. Koblansky AA, Jankovic D, Oh H, Hieny S, Sungnak W, Mathur R, Hayden MS, Akira S, Sher A, Ghosh S. Recognition of Profilin by Toll-like Receptor 12 Is Critical for Host Resistance to Toxoplasma gondii. Immunity. 2013; 38:119–130. [PubMed: 23246311]

51. Andrade WA, Souza Mdo C, Ramos-Martinez E, Nagpal K, Dutra MS, Melo MB, Bartholomeu DC, Ghosh S, Golenbock DT, Gazzinelli RT. Combined action of nucleic acid-sensing Toll-like receptors and TLR11/TLR12 heterodimers imparts resistance to Toxoplasma gondii in mice. Cell Host Microbe. 2013; 13:42–53. [PubMed: 23290966]

52. Pepper M, Dzierszinski F, Wilson E, Tait E, Fang Q, Yarovinsky F, Laufer TM, Roos D, Hunter CA. Plasmacytoid dendritic cells are activated by Toxoplasma gondii to present antigen and produce cytokines. J Immunol. 2008; 180:6229–6236. [PubMed: 18424745]



Well	Sample
1	TLR11-GFP x cMyc-MyD88
2	TLR11-GFP x TLR11-Myc
3	TLR11-GFP x TLR12-Myc
4	TLR12-GFP x cMyc-MyD88
5	TLR12-GFP x TLR11-Myc
6	TLR12-GFP x TLR12-Myc
7	Unc93B1-GFP x cMyc-MyD88
8	Unc93B1-GFP x TLR11-Myc
9	Unc93B1-GFP x TLR12-Myc
10	CD3zeta-GFP x cMyc-MyD88
11	CD3zeta-GFP x TLR11-Myc
12	CD3zeta-GFP x TLR12-Myc

Figure 1. TLR12 localizes intracellularly and associates with TLR11 and UNC93B1

(A) HEK293 cells were plated on glass coverslips and co-transfected with TLR11-GFP and TLR12-mCherry. After 24 hours, the cells were imaged with a Leica SPE microscope equipped with a 63× objective. (B) HEK293 cells were plated on glass coverslips and co-transfected with TLR12-GFP and UNC93B1-mCherry. After 24 hours, the cells were imaged with a Leica SPE microscope equipped with a 63× objective. (C) HEK293 cells were co-transfected 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)

CD3z-GFP and myc-MyD88, (11) CD3z-GFP and myc-TLR11, or (12) CD3x-GFP and myc-TLR12. Thirty-six hours after transfection, the cell lysates were immunoprecipitated (IP) with anti-GFP antibody, and the immunoprecipitated proteins were analyzed by immunoblotting (IB) with anti-myc or anti-GFP antibodies to detect myc- or GFP-tagged proteins, respectively. A representative result from five experiments is shown.

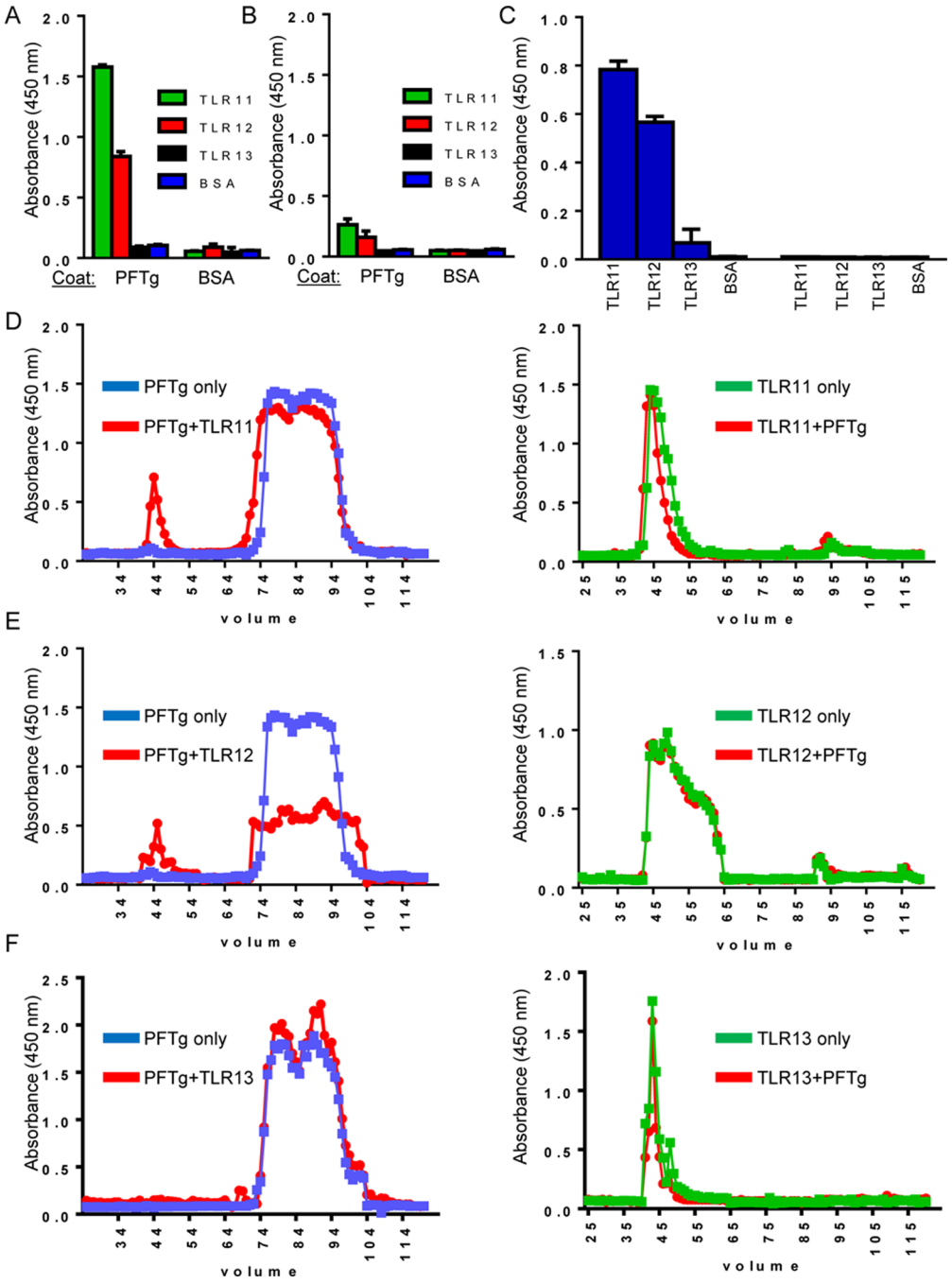


Figure 2. *T. gondii* profilin directly interacts with TLR11 and TLR12

(A) ELISA plates were coated with *T. gondii* profilin (PFTg, 10μ/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 of purified TLR11 (green), TLR12 (red), TLR13 (black) or BSA (blue). Binding of the TLR ectodomains was detected with a DED-specific monoclonal antibody. All ELISA steps were performed at pH 6.0. (B) ELISA plates were coated with *T. gondii* profilin (PFTg, 10μ/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 of purified TLR11 (green), TLR12 (red), TLR13 (black) or BSA (blue). Binding of the TLR ectodomains was detected with a DED-specific monoclonal

antibody. All ELISA steps were performed at pH 8.0. (C) The 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 antibody. All ELISA steps were performed at pH 6.0. A representative result from at least ten experiments is shown. (D) *T. gondii* profilin (2 μM) alone (blue) or pre-incubated with 0.2 μM purified TLR11 ectodomain (red). Biochemical fractionation of *T. gondii* profilin (PFTg) or PFTg +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 PFTg+TLR11 fractions (red) were additionally tested for TLR11 compared to TLR11 alone (green) and analyzed according to the same gel filtration chromatography procedure with a Superdex 200 10/300 GL chromatographic separation column. (E) PFTg alone (blue), PFTg+TLR12 (red), or TLR12 alone were analyzed by gel filtration chromatography as described in C. Each fraction was tested for *T. gondii* profilin (left) or TLR12 (right) by ELISA. The chromatograms are representative of three independent experiments. (F) PFTg alone (blue), PFTg+TLR13 (red), or TLR13 alone were analyzed by gel filtration chromatography as described in D and E. Each fraction was tested for *T. gondii* profilin (left) or TLR13 (right) by ELISA. The chromatograms are representative of three independent experiments.

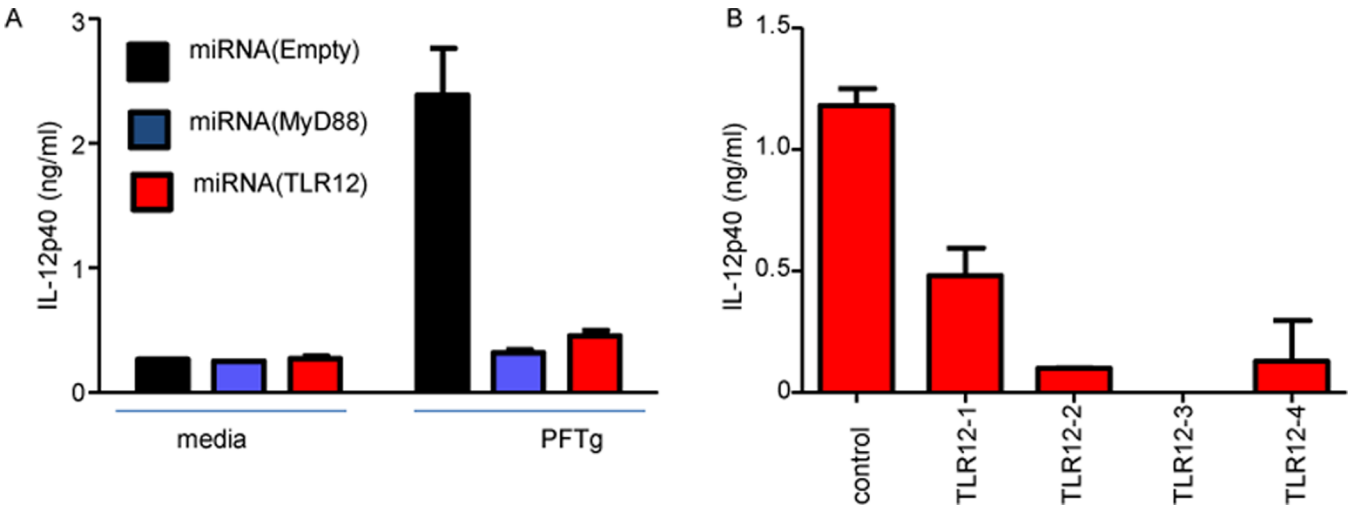


Figure 3. TLR12 participates in the regulation of IL-12 responses triggered by *T. gondii* profilin

(A) Flt3L-derived DCs were transduced with lentiviral constructs expressing miRNA against MyD88 (blue) or TLR12 (red). After 48 hours, the DCs were stimulated with 1 μg/ml *T. gondii* profilin, and IL-12/23p40 production was measured by ELISA twenty hours after stimulation. (B) Flt3L-derived DCs were transfected with control or TLR12-targeting siRNA constructs and stimulated with *T. gondii* profilin. IL-12/23p40 production was measured by ELISA twenty hours after stimulation. The data shown are representative of four experiments.

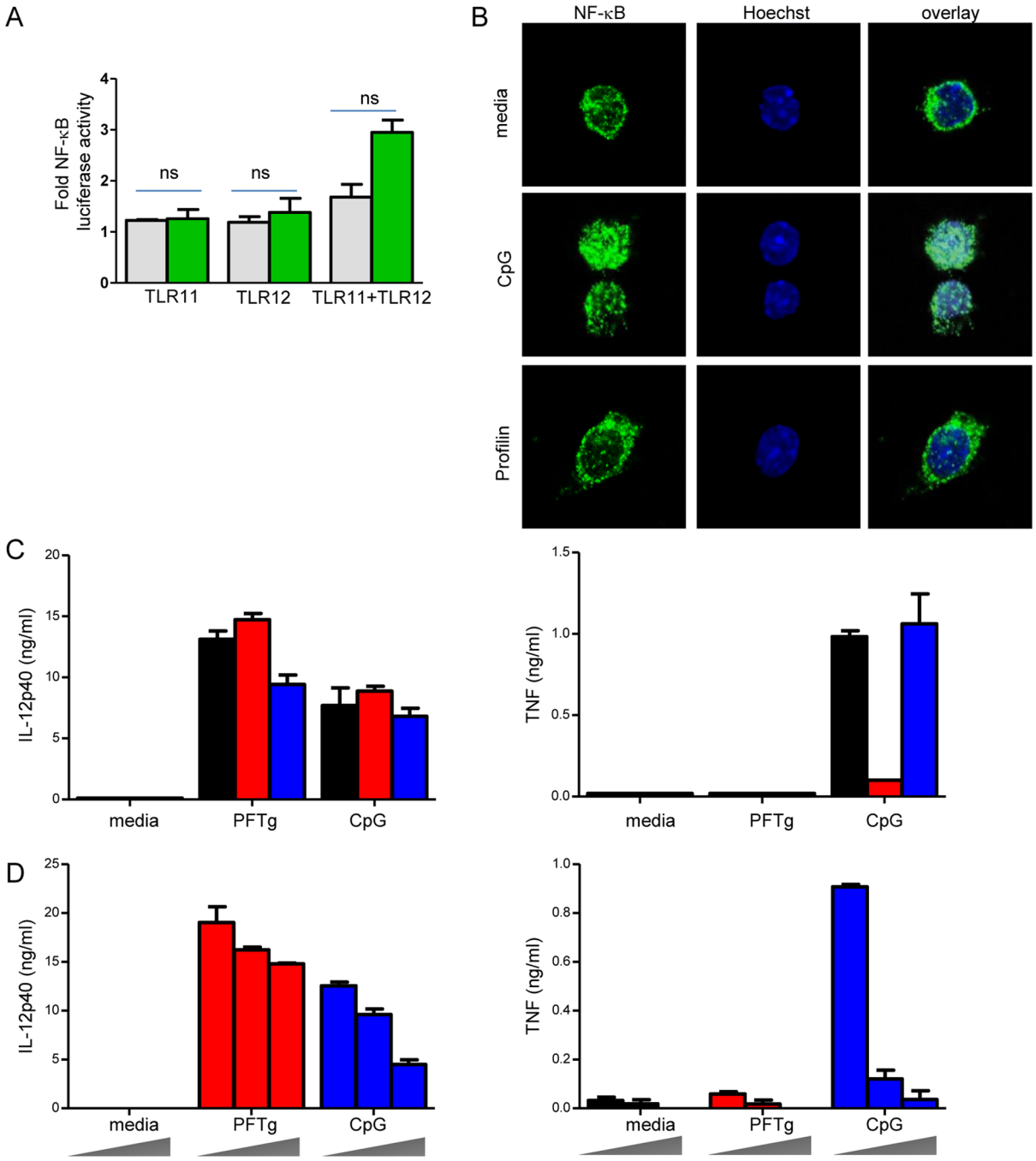


Figure 4. TLR12 and TLR11 did not induce detectable NF-κB activation

(A) HEK 293 cells were transfected with a plasmid encoding TLR11, TLR12, or TLR11 and TLR12, in addition to a 5×-NF-κB-luciferase reporter. After overnight stimulation with 1 μg/ml of *T. gondii* profilin (green) or media alone (grey), the cells were lysed and assayed for luciferase activity. The error bars represent the standard deviation. (B) Purified CD8+ DCs were stimulated with 1 μM CpG or 1 μg/ml PFTg, and NF-kBp65 was analyzed six hours after stimulation. At least 100 CD8+ DCs were analyzed in each of the three experiments. (C) Splenic DCs were isolated from WT (black), NF-κB1−/− (red), or NF-κB2−/− (blue) mice and stimulated with 1 μg/ml PFTg or 1 μM CpG. IL-12/23p40 and TNF productions were measured by ELISA twenty hours after stimulation. The data shown are

representative of two experiments. (D) Splenic DCs were isolated from WT mice and stimulated with 1 μg/ml PFTg (red) or 1 μM CpG (blue) in the presence of increasing concentrations of a pan-NF-kB inhibitor BAY-11-708 (0 uM, 1 uM, and 5 uM). IL-12/23p40 and TNF productions were measured by ELISA twenty hours after stimulation. The data shown are representative of three experiments.

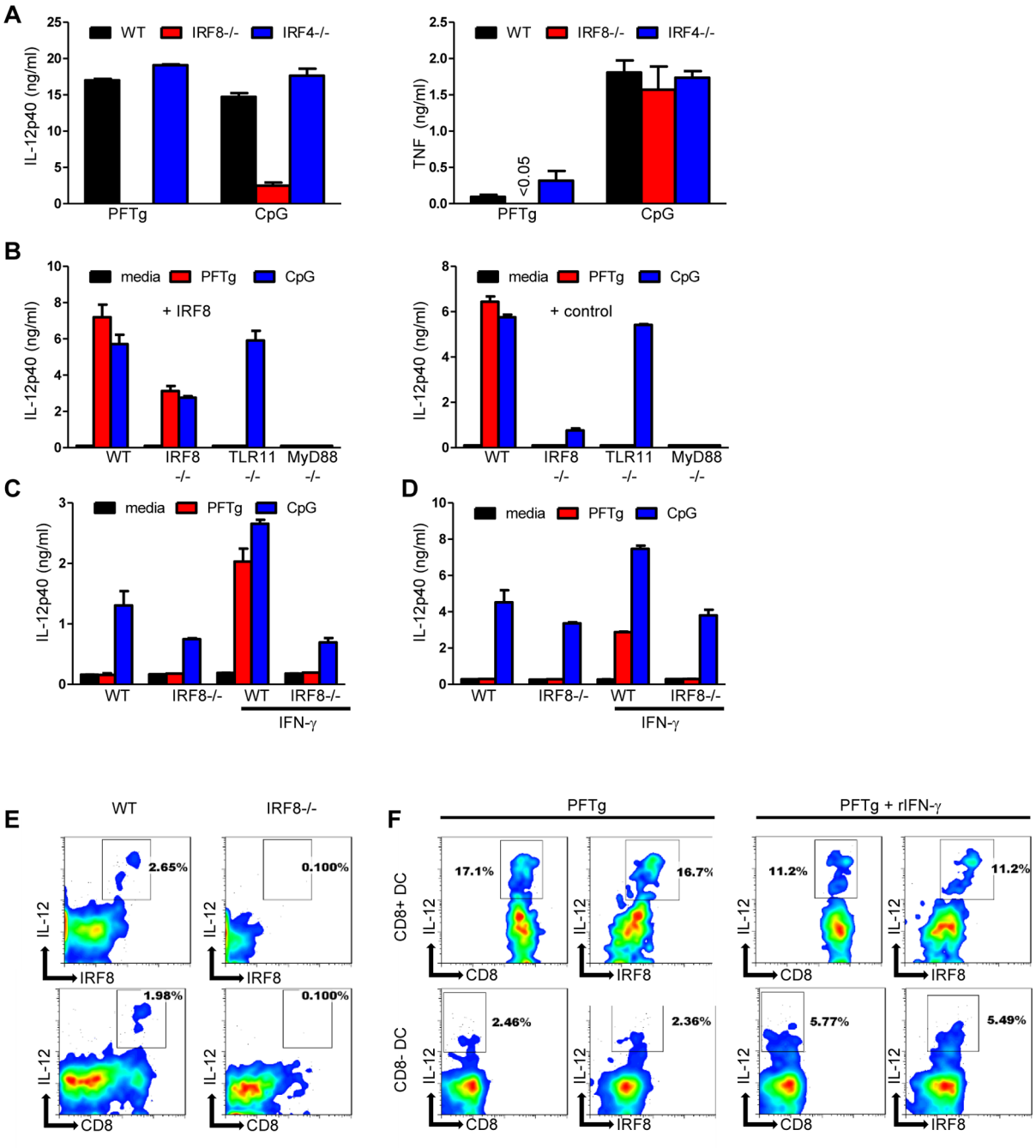


Figure 5. IRF8 regulates the TLR12- and TLR11-dependent IL-12 responses of DCs triggered by *T. gondii* profilin

(A) Splenic CD11c+ DCs were prepared from WT, IRF8−/−, and IRF4−/− mice and stimulated with 1 μg/ml of *T. gondii* profilin (PFTg) or 1 μM CpG for 20 hours. IL-12/23p40 (left) and TNF (right) production were measured by ELISA. (B) Flt3L-derived DCs were generated from bone marrow cells prepared from WT, IRF8−/−, TLR11−/−, and MyD88−/− mice and were retrovirally transduced with the IRF8 (left) or control (right) vectors. Both IRF8-rescued and control DCs were stimulated with 1 μg/ml PFTG or 1 μM CpG for twenty hours. IL-12/23p40 productions were analyzed by ELISA. The data shown are representative of four experiments. (C) WT or IRF8−/− bone-marrow macrophages or

(D) sort-purified CD11c+CD8−CD11b+ DC were incubated with PBS or rIFN-γ (10ng/ml) overnight and then were stimulated with 1 μg/ml PFTg or 1 μM CpG. IL-12/23p40 production was analyzed by ELISA twenty hours later. The data shown are representative of four experiments. (E) WT and IRF8−/− mice were injected with *T. gondii* profilin (10 μg/mouse) and IL-12/23p40 production by splenic DCs was analyzed by intracellular staining four hours later by intracellular staining for IL-12/23p40 and IRF8. (F) WT mice were injected with PBS or rIFN-γ (1μg/mouse) overnight. The next day mice were injected with *T. gondii* profilin (10 μg/mouse) and IL-12/23p40 production by splenic CD8+ and CD8− DCs was analyzed by intracellular staining. The data shown are representative of two experiments.