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Combined Action of Nucleic Acid-Sensing Toll-like Receptors and TLR11/TLR12 Heterodimers Imparts Resistance to *Toxoplasma gondii* in Mice

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SUMMARY

"Triple-defective" (3d) mice carrying a mutation in UNC93B1, a chaperone for the endosomal nucleic acid-sensing (NAS) Toll-like receptors TLR3, TLR7, and TLR9, are highly susceptible to *Toxoplasma gondii* infection. However, none of the single or even the triple NAS-TLR-deficient animals recapitulated the 3d susceptible phenotype to experimental toxoplasmosis. Investigating this further, we found that while parasite RNA and DNA activate innate immune responses via TLR7 and TLR9, TLR11 and TLR12 working as heterodimers are required for sensing and responding to *Toxoplasma* profilin. Consequently, the triple TLR7/TLR9/TLR11-deficient mice are highly susceptible to *T. gondii* infection, recapitulating the phenotype of 3d mice. Humans lack functional *TLR11* and *TLR12* genes. Consistently, human cells produce high levels of proinflammatory cytokines in response to parasite-derived RNA and DNA, but not to *Toxoplasma* profilin, supporting a more critical role for NAS-TLRs in human toxoplasmosis.

INTRODUCTION

Natural infection with *Toxoplasma gondii* has been described in more than 300 mammal and 30 avian species. While felines are the definitive hosts, mice—the cats' prey—are the natural intermediate hosts and main reservoirs of this coccidian parasite. Even though humans are considered "accidental" intermediate hosts, one-third of the world population carries a chronic and asymptomatic infection with *T. gondii* (Robert-Gangneux and Dardé, 2012). However, in immune-compromised individuals, the dormant parasite becomes highly virulent, leading to reactivation of the chronic infection and causing severe disease and lethality (Weiss and Dubey, 2009).

Host resistance to *T. gondii* infection is primarily dependent on T-cell-mediated immunity, and most attention has been focused on IFNγ-producing $CD4^+$ T helper type 1 (Th1) and $CD8^+$ T effector lymphocytes that are critical for the resolution of acute illness and to prevent reactivation of latent infection (Denkers and Gazzinelli, 1998). In addition, activation of MyD88, an universal adaptor for all Toll-like receptors (TLRs) (except TLR3) (Gazzinelli and Denkers, 2006; Takeuchi and Akira, 2010), is essential for the optimal production of IL-10, IL-12, TNF-α, and IFNγ, all of which are important mediators of host survival during primary infection with *T. gondii* (Scanga et al., 2002; Sukhumavasi et al., 2008). While the three latter cytokines (Gazzinelli et al., 1994; Suzuki, 1999) are critical to control parasite growth through activation of effector mechanisms such as inducible GTPases (Howard et al., 2011), IL-10 prevents an excessive inflammatory response that is lethal to the host (Gazzinelli et al., 1996).

As for the pathogen-associated molecular patterns (PAMPs) that activate TLRs during *T. gondii* infection, important pieces of the puzzle are still missing. Several parasite products, including glycosylphosphoinositol (GPI) anchors, and heat shock protein were shown to activate TLR2 and TLR4. Yet, mice lacking such TLRs have a rather mild or no phenotype upon *T. gondii* infection (Aosai et al., 2006; Debierre-Grockiego et al., 2007). Importantly, the *T. gondii* profilin-like protein (TgPRF) was shown to activate TLR11, and gene-target disruption of *TLR11* results in a partial defect of IL-12 production and increased number of cysts in the brain from mice infected with *T. gondii* (Plattner et al., 2008; Yarovinsky et al., 2005). However, none of these mice recapitulate the profound phenotype observed in *MyD88* knockout (KO) mice infected with *T. gondii* (Melo et al., 2010; Scanga et al., 2002; Sukhumavasi et al., 2008), suggesting that other members of the TLR family are involved.

The "triple D" (3d) mouse expresses an UNC93B1 missense mutant that is incapable of binding the nucleic-acid sensing (NAS) TLRs (i.e., TLR3, TLR7, and TLR9) (Brinkmann et al., 2007; Tabeta et al., 2006), and therefore, to mediate their translocation from the endoplasmic reticulum (ER) and consequent activation into the endolysosomes (Kim et al., 2008). We have

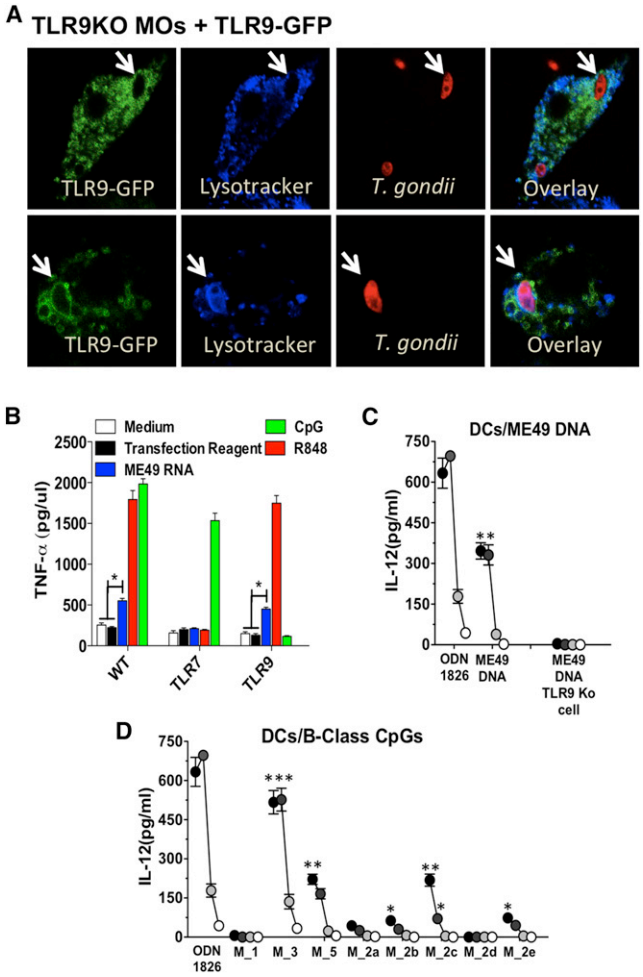

Figure 1. *T. gondii* RNA and DNA Activate Host Cells via TLRs

(A) Confocal microscopy of immortalized *TLR9*$^{-/-}$ macrophages stably expressing TLR9-GFP and infected with CMTPX-stained *T. gondii*. Acidic compartments were stained with LysoTracker White-Blue. Arrows indicate internalized parasites.

(B) Immortalized WT, TLR7 KO, and TLR9 KO macrophages were stimulated with *T. gondii* RNA at 2 μg/ml complexed with DOTAP (Roche). CpG ODN (3 μM) and R848 (2 μM) were used as positive controls.

(C) DCs were stimulated with *T. gondii* DNA complexed with DOTAP at 10, 5, 1, and 0.1 μg/ml. CpG ODN 1826 was used as positive control at the same concentrations.

(D) DCs were stimulated with CpG ODN 1826 as positive control and *T. gondii*-derived oligonucleotides containing B-class mouse-like stimulatory CpG motifs at 3 μM (black circles), 1 μM (dark gray), 0.3 μM (light gray), and 0.1 μM (white circles). Cytokine levels were measured in the tissue culture supernatants at 24 hr after stimulation.

(B–D) Data are represented as mean ± SD of three independent experiments (*0.01 < p < 0.05, **0.001 < p < 0.01, ***p < 0.001). See also Table S1.

shown that 3d mice are highly susceptible to infection with *T. gondii*, presenting a profound impairment of IL-12 and consequent delay in IFNγ production (Melo et al., 2010). In the current study, we further defined the role of endosomal TLRs during infection with *T. gondii.* Our data indicate that TLR7 and TLR9 recognize *Toxoplasma* RNA and DNA, respectively. On the other hand, we found that both TLR11 and TLR12 are required for UNC93B1-dependent cellular responses to TgPRF. We also report that the triple TLR7/TLR9/TLR11-deficient mice are highly susceptible to *T. gondii* infection, recapitulating the phenotype of 3d mice. It is noteworthy that while the mouse genome encodes 13 TLRs, the human genome lacks functional *TLR11*, *TLR12*, and *TLR13* (Roach et al., 2005). Consistently, human cells produce high levels of proinflammatory cytokines, including IL-12 and TNF-α, in response to parasite-derived RNA and DNA, but not to TgPRF. Hence, our results support the hypothesis that NAS-TLRs play an important role in human toxoplasmosis.

RESULTS

RNA and DNA Derived from *T. gondii* Tachyzoites Activate Host Cells via TLRs

NAS-TLRs are important cognate receptors for viruses, bacteria, and different protozoan parasites (Alexopoulou et al., 2001; Bartholomeu et al., 2008; Benson et al., 2009; Caetano et al., 2011; Heil et al., 2004; Hemmi et al., 2000; Parroche et al., 2007). Here, we exposed immortalized macrophages expressing transgenic TLR9-GFP with Red CMTPX-labeled tachyzoites. TLR9-GFP co-localized with intracellular parasites in the endolysosomal compartment, but not in the parasitophorous vacuole (PV), which is lysotracker negative (Figure 1A). We also demonstate that RNA (Figure 1B) and DNA (Figure 1C) extracted from highly purified tachyzoites stimulate immortalized macrophages or DCs, in a TLR7- or TLR9-dependent manner, respectively. Table S1 (available online) lists a series of (total number of 92) immunostimulatory mouse B class-like CpG motifs encoded in the *Toxoplasma* genome. They are potent activators of DCs via TLR9 (Figure 1D).

Combined Deficiency of NAS-TLRs Does Not Recapitulate the High Susceptibility of 3d Mice Infected with *T. gondii*

We found that none of the single TLR3, TLR7, or TLR9 KOs were more susceptible to infection with *T. gondii* (Melo et al., 2010). Thus, we hypothesize that a combined deficiency of NAS-TLRs would explain the dramatic phenotype observed in the 3d mice. However, none of the double TLR3/TLR7, TLR7/TLR9, TLR7/TLR8, or even the triple TLR3/TLR7/TLR9-deficient mice had an impaired IL-12 and IFNγ production (Figure 2A and data not shown), or were highly susceptible to *T. gondii* infection. Despite this apparently normal IL-12/IFN-γ response, few of the triple TLR3/TLR7/TLR9 KO mice succumbed during an early stage of infection (Figure 2B), which was associated with an increased parasitism in peritoneal cells and spleens (Figure 2C), as well as in the brain (Figure 2D).

Expression of Endosomal TLRs in DCs from Mice Infected with *T. gondii*

Because resistance to experimental infection with *T. gondii* was only slightly affected in the TLR3/TLR7/TLR9 KO mice, we investigated the role of other TLRs, whose function is also dependent on UNC93B1. We generated a molecular tree of mouse TLRs (Phylogeny.fr), which indicated that TLR12 is closely associated to TLR11 and TLR13, that are also endosomal TLRs (Shi et al., 2011; Zhang et al., 2004) (Figure S1). Amino acid sequence

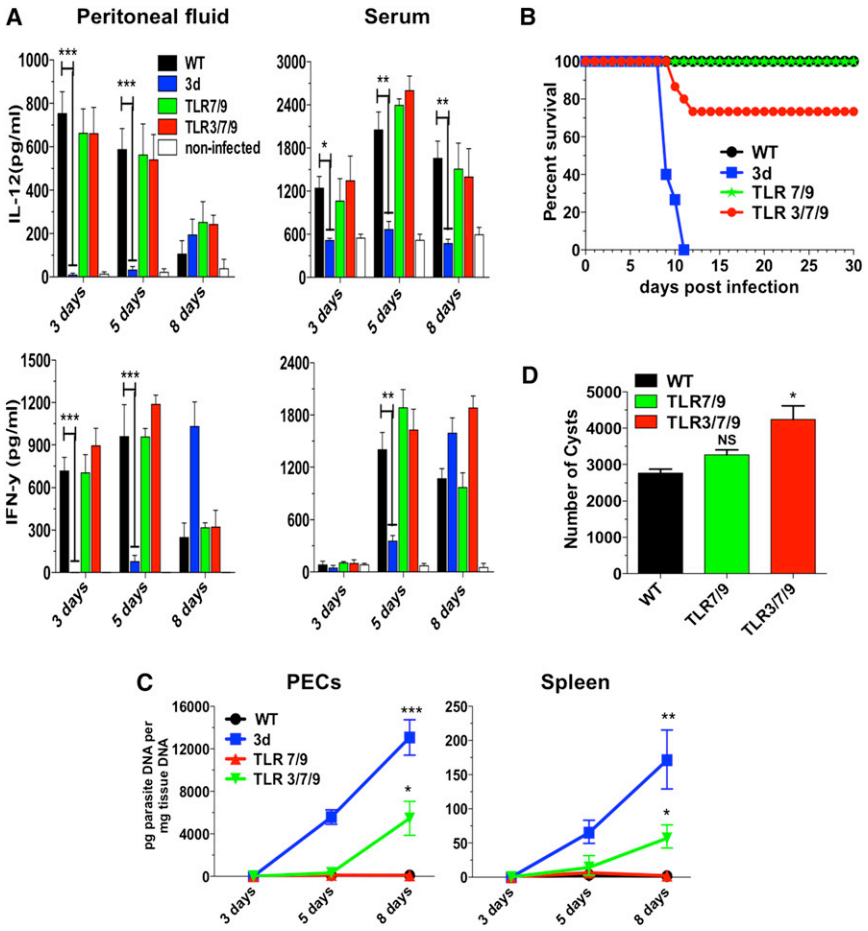

Figure 2. Mice Deficient in TLR3/TLR7/TLR9 Are Only Partially Susceptible to *T. gondii* Infection

Mice were infected intraperitoneally with 25 cysts of *T. gondii* ME49 strain.

(A) Levels of IL-12p40 and IFNγ were measured in the peritoneal cavity exudate and sera at different times postinfection.

(B) Combined survival data from WT (n = 20), 3d (n = 16), TLR7/9 (n = 20), and TLR3/7/9 (n = 20) mice from four independent experiments.

(C) Quantitative real-time PCR analysis was performed on the indicated tissues collected from animals infected with *T. gondii*. Data are the mean of three independent experiments.

(D) Cysts counts in the brain determined at 30 days postinfection are the mean from four experiments.

(A–D) Data are represented as mean ± SD. Asterisks indicate that difference is statistically significant, when comparing different mouse lineages infected with *T. gondii,* (NS, not significant; *0.01 < p < 0.05, **0.001 < p < 0.01, ***p < 0.001).

alignment for TLR11 and TLR12 shows a high degree (35%) of sequence identity, suggesting the possibility that both of them may recognize TgPRF.

As both macrophages and DCs are important sources of IL-12 during *T. gondii* infection in mice, we evaluated the expression of the endosomal TLRs mRNAs in $CD11b^+$, $CD11c^+/CD8^-$, and $CD11c^+/CD8^+$ cells. As shown in Figure 3A, mRNAs of TLR3, TLR9, TLR11, and TLR12 were expressed in higher levels in the $CD11c^+/CD8^+$ cells, consistent with the hypothesis that this DC subset is the main source of IL-12 in mice infected with *T. gondii* (Mashayekhi et al., 2011; Yarovinsky et al., 2005). However, a recent study (Goldszmid et al., 2012) indicates that the main IL-12 source in the peritoneal cavity of infected mice is the $CD11c^+CD8^-$ DCs, which we found to express high levels of TLR3, TLR7, and TLR9, and minimal levels of TLR11 or TLR12. Thus, we speculate that IL-12 production by $CD11c^+CD8^+$ DCs and $CD11c^+CD8^-$ DCs is triggered by parasite TgPRF and nucleic acids, respectively. Macrophages also expressed mRNA for the various endosomal TLRs, but in a lesser amount than DCs.

To evaluate whether TLR11 and TLR12 are associated with other endosomal TLRs, HEK293T cells were transiently cotransfected with different Flag-tagged TLRs and TLR11-HA, and TLR11 immunoprecipitated as bait. As shown (Figure 3B), TLR3, TLR7, TLR11, and TLR12, but not TLR4, coimmunoprecipitated with TLR11. UNC93B1 has been reported to physically interact with TLR3, TLR7, TLR9, TLR11, and TLR13 (Brinkmann et al., 2007; Kim et al., 2008; Pifer et al., 2011; Tabeta et al., 2006). Thus, the interpretation of these results is that when pulling down TLR11, coimmunoprecipitation of TLR3, TLR7, TLR11, and TLR12 is observed because all the endosomal TLRs are bound to UNC93B1.

Next, we purified $CD11c^+$ cells from the spleens of 3d as well as double TLR7/TLR9, triple TLR3/TLR7/TLR9, and single TLR11 KO mice. DCs from 3d mice were responsive to LPS, but not to R848 (TLR7 agonist), CpG ODN (TLR9 agonist), STAg, ME49, or recombinant TgPRF (rTgPRF). The lack of TLR11, but not TLR3/TLR7/TLR9, had a major impact on IL-12 production by splenic DCs exposed to STAg, ME49, or rTgPRF. Nevertheless, compared to rTgPRF, DCs from TLR11 KO or TLR12 KO mice still produced significant amounts of IL-12 when exposed to either STAg or ME-49 (Figures 3C and 3D). As expected, DCs from TLR3/TLR7/TLR9 KOs did not respond to R848 or CpG ODN, whereas DCs from TLR11 KOs produced high levels of IL-12 in response to these TLR agonists (Figure 3C). Treatment with Proteinase K, but not with RNase or DNase, destroyed the ability of rTgPRF to activate CD11c+ cells (Figure 3D).

Colocalization and Heterodimerization of TLR11 and TLR12

Macrophages were genetically engineered to stably express color-tagged TLR11 or TLR12 and used to analyze their subcellular distribution by confocal microscopy. We found that in macrophages, TLR11 and TLR12 colocalize with ER tracker, but not with cholera toxin (a cell-surface membrane marker) (Figure 4A). We also transfected HEK293T cells with different combinations of plasmids encoding fluorescent protein-tagged UNC93B1, TLR11, or TLR12, and they all colocalized in the ER (Figure 4B). As control, we transfected HEK293T cells with

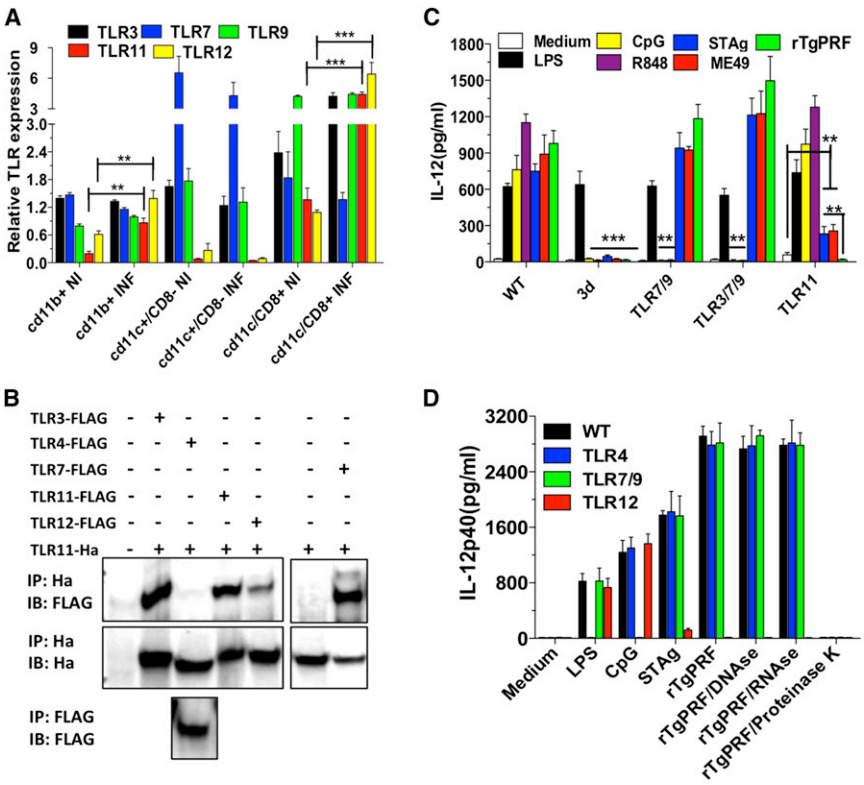

Figure 3. Endosomal TLRs Are Highly Expressed in CD8α⁺ DCs and Upregulated upon *T. gondii* Infection

(A) Real-time PCR was performed to determine the relative levels of TLR3, TLR7, TLR9, TLR11, and TLR12 mRNA expressed by CD11b⁺, CD11c⁺/CD8α⁺, and CD11c⁺/CD8α⁻ cells sorted from splenocytes from uninfected controls as well as infected (5 days postinfection) WT mice. TLR mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Data are represented as mean ± SD of three experiments.

(B) HEK293T cells were transfected with different pairs of plasmids, total lysates immunoprecipitated (IP) with anti-hemagglutinin (anti-HA, top) or anti-Flag (bottom), and analyzed by immunoblot (IB) with anti-Flag (top and bottom). The top membrane was then stripped and reprobed with anti-HA to ensure expression of hemagglutinin-tagged TLR11 (middle).

(C) CD11c⁺ cells were purified from spleen of WT, 3d, TLR7/TLR9, TLR3/TLR7/TLR9, and TLR11 KO mice and stimulated with LPS (100 ng/ml) ODN CpG 1826 (1 μM) or R848 (2 μM), STAg (10 μg/ml), rTgPRF (10 ng/ml), or infected with ME49 tachyzoites (moi 3:1).

(D) CD11c⁺ cells purified from WT, TLR4, TLR7/TLR9, and TLR12 KO mice were stimulated with LPS (100 ng/ml), ODN CpG 1826 (1 μM), STAg (10 μg/ml), or rTgPRF (10 ng/ml); left untreated; or treated with DNase (100 U/ml), RNase (10 μg/ml), or Proteinase K (10 μg/ml). IL-12 levels were measured in the supernatant at 24 hr after stimulation.

(C–D) Data are represented as mean ± SD of four experiments. Asterisks indicate that difference is statistically significant when comparing cytokines levels from WT to different KO mice, infected or not infected with *T. gondii* (*0.01 < p < 0.05, **0.001 < p < 0.01, ***p < 0.001). See also Figure S1.

a color-tagged TLR4. The pattern of cellular distribution for TLR4 was distinct from TLR11 and TLR12 (Figure 4B). As expected (Latz et al., 2004), TLR4 was primarily expressed at the surface membrane of the transfected cells.

Importantly, immortalized macrophages produced high levels of IL-12 in response to STAg or ME49 tachyzoites when they were stably transfected with both *TLR11* and *TLR12*, but not with either *TLR11* or *TLR12* alone (Figure 4C). As dimerization appears to be required for PAMP recognition and activation of TLRs (Latz et al., 2007; Leonard et al., 2008), we used fluorescence resonance energy transfer (FRET) to evaluate the intermolecular distance between the TIR domains of TLRs. HEK293T cells were transfected with different combinations of fluorescent TLRs fused to cerulean (Donor) or citrine (Acceptor) and protein-protein interaction evaluated. A strong FRET signal was observed in cells cotransfected with TLR11 and TLR12, but not with either TLR11/TLR11, TLR12/TLR12, or TLR11/TLR9 stimulated with STAg (Figure 4D) or rTgPRF (Figure 4D and data not shown). After stimulation with CpG, we observed a strong FRET signal for TLR9/TLR9 homodimers. Based on the results described above, we hypothesized that TLR11 and TLR12 work as heterodimers and that deficiency of either TLR11 or TLR12 results in impaired IL-12 production during *T. gondii* infection. Whether formation of TLR11 and TLR12 heterodimers is necessary for recognition of bacterial components (e.g., flagellin) by TLR11 (Mathur et al., 2012; Zhang et al., 2004) remains to be defined.

Quadruple TLR3/TLR7/TLR9/TLR11 KO Mice Are Highly Susceptible to *T. gondii* Infection

Despite a significant impairment on IL-12 response, TLR11-deficient mice still produced higher levels than the 3d mice, being sufficient to induce IFNγ (Figure 5A) and protect mice from death during acute phase of infection (Figure 5B). Nevertheless, TLR11 KOs showed a 4-fold increase in cyst numbers (Figure 5C). These data left us with the observation that although the NAS-TLRs and TLR11 are all required for optimal host responses to *T. gondii*, neither seemed essential for survival. Hence, we generated triple TLR7/TLR9/TLR11- and quadruple TLR3/TLR7/TLR9/TLR11-deficient mice. In contrast to DCs from TLR11 KO, CD11c⁺ cells purified from spleens of either TLR7/TLR9/TLR11 or TLR3/TLR7/TLR9/TLR11 KO mice did not respond to either STAg or live tachyzoites (ME49) (Figure 6A). As shown in (Figure 6B and Figure S2A), the quadruple KO mice had a major defect in IL-12, IL-6, and MCP1 production, at levels similar to those of 3d mice. MCP1 is an important chemokyne for the recruitment of inflammatory monocytes and host resistance to *T. gondii* (Dunay et al., 2008). As presented in Figure S2B, by day 3 after infection, WT mice have an increased number of inflammatory monocytes, which was not observed in 3d or quadruple KO mice. The influx of inflammatory monocytes at 3 days post-challenge was partially reduced in TLR3/TLR7/TLR9 KO mice and may explain the slight enhancement on susceptibility to infection (Figure 2). An impairment in IL-12 production by

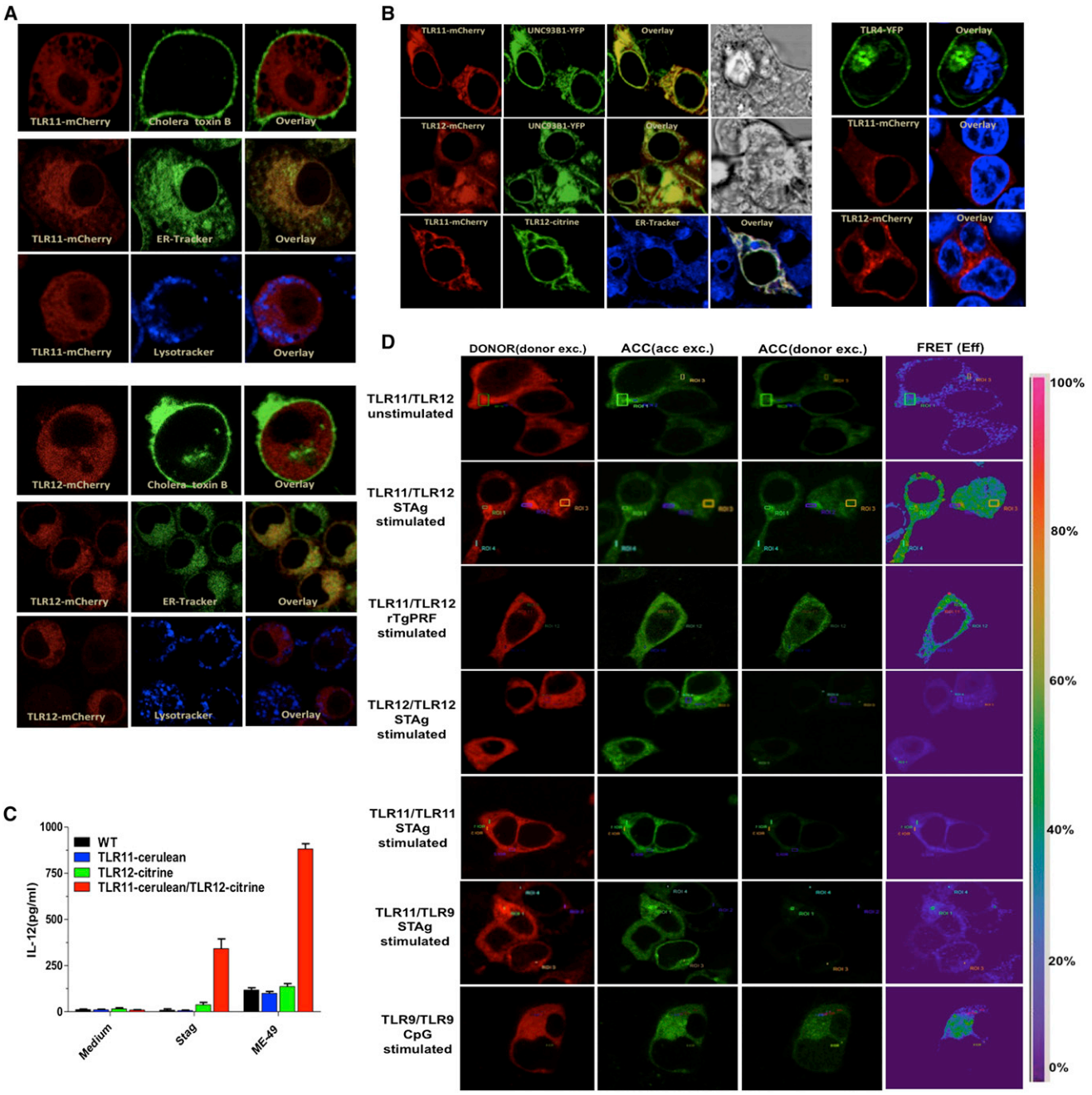

Figure 4. Colocalization and Heterodimerization of TLR11 and TLR12

(A) Immortalized macrophages were stably transfected with *TLR11-mcherry* or *TLR12-mcherry* and imaged poststaining with Cholera toxin B subunit FITC conjugate, LysoTracker White-Blue, or ER-Tracker White-Blue.

(B) HEK293T cells transfected with TLR11-mCherry or TLR12-mcherry with UNC93B1-YFP or TLR11-mcherry and TLR12-citrine (left panel) or with TLR4-YFP and stained with Hoechst 33342 as nuclear marker (right panel).

(C) Immortalized macrophages stably expressing either TLR11, TLR12, or both were stimulated in vitro with STAg (10 μg/ml) or exposed to live tachyzoites (moi 3:1) of the ME49 strain and levels of IL-12p40 measured in the supernatants at 24 hr poststimulation. Data are represented as mean ± SD of three experiments.

(D) HEK293T cells were transfected with the plasmids encoding the proteins indicated in the figure; 48 hr after transfection, cells were left unstimulated or were stimulated with STAg (10 μg/ml), rTgPRF (100 ng/ml), or CpG 1826 (1 μM) for TLR9/TLR9. FRET between the respective proteins was calculated by measuring sensitized emission (SE) fluorescence using the FRET SE wizard on the Leica SP2 confocal laser-scanning microscope. For each plasmid combination, cerulean (represented in red) was used as donor and citrine (represented in green) as acceptor. Data are from one representative experiment of four.

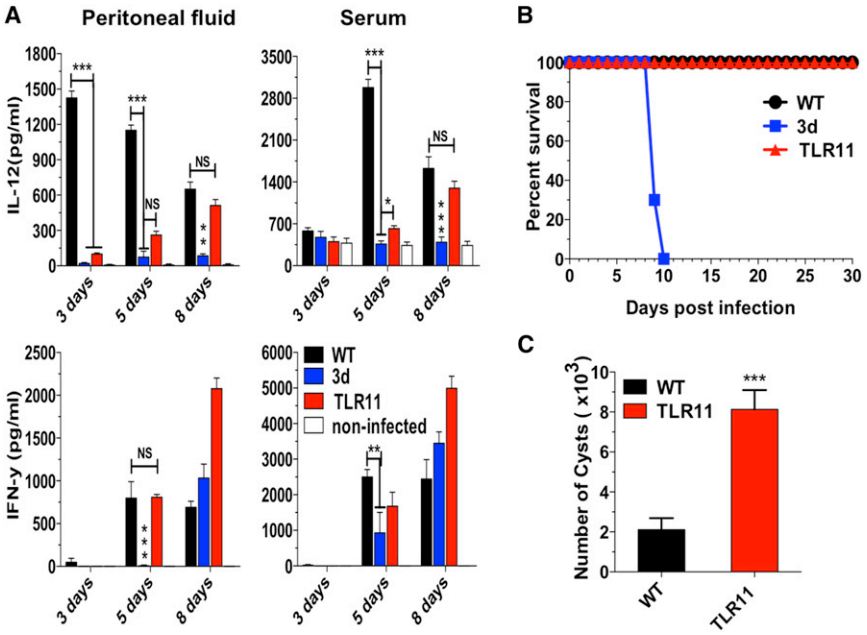

Figure 5. TLR11 Mice Are Resistant to *T. gondii* Infection

Mice were infected intraperitoneally with 25 cysts ME49 strain of *T. gondii*.

(A) Levels of IL-12p40 and IFNγ were measured in the peritoneal cavity exudate and sera from uninfected as well as infected mice.

(B) Combined survival data from WT (n = 15), 3d (n = 12), and TLR11 (n = 15) mice from three independent experiments.

(C) The cyst numbers in the brain were counted at 30 days postinfection and presented as mean from the three experiments.

(A–C) Data are represented as mean ± SD. Asterisks indicate that difference is statistically significant, when comparing to unstimulated controls (NS, not significant; *0.01 < p < 0.05, **0.001 < p < 0.01, and ***p < 0.001).

DCs and inflammatory monocytes was observed both in 3d and quadruple TLR3/TLR7/TLR9/TLR11 KO mice (Figure 6C), causing a delay in IFNγ production (Figure 6D). After challenge with ME49 strain, quadruple KOs were as susceptible as 3d mice, as indicated by the survival curve and increased parasite burden in peritoneal cells, spleen, and liver (Figures 6E and 6F). We assume that the influx of neutrophils, inflammatory monocytes, and DCs in the highly susceptible mutant/KO mice was too late, and thus, not able to control parasite replication (Figure S2B). Importantly, as shown in Figure 6G, all 3d and TLR3/TLR7/TLR9/TLR11 KO mice treated with recombinant IL-12 (rIL-12) survived the experimental infection with *T. gondii*.

We also generated the triple TLR7/TLR9/TLR11-deficient mice, which were as susceptible as the 3d and quadruple KO mice infected with *T. gondii* (Figure 6D). As TRIF and type I IFN are, respectively, the main adaptor molecule and outcome of TLR3 activation, we performed experimental infections in TLR3 KO, TRIF KO, and Type I IFN receptor KO mice. Our results show that when compared to WT mice, none of these mice displayed enhanced susceptibility to *T. gondii* infection (Figures S2C and S2D). Consistently, we were unable to detect any increase in the production of IFN-α protein in peritoneal fluids, splenocyte cultures, or sera from WT mice infected with *T. gondii* (data not shown).

Human Peripheral Blood Mononuclear Cells Produce High Levels of IL-12 in Response to *T. gondii* DNA and RNA, but Not rTgPRF

TLR11 and TLR12 are not expressed in human cells (Roach et al., 2005; Zhang et al., 2004). This raised the hypothesis that NAS-TLRs are the key TLRs sensing *T. gondii* parasites in human cells. The results presented in Figure 7A indicate that when compared to R848 (agonist for human TLR8) and LPS (agonist for TLR4), STAg is a poor stimulator of IL-12p70, TNF-α, and IL-1β production by human PBMCs. Furthermore, we were unable to detect production of any cytokine (including IL-12p70) in response to rTgPRF (Figure 7B). Nevertheless, we found that parasite RNA and DNA elicited the production of proinflammatory cytokines, including TNF-α, IL-12p40, and IL-12p70, which was more pronounced when PBMCs were primed with IFNγ (Figure 7B). We also searched for immunostimulatory CpG motifs (Table S2) that activate human TLR9. In the whole genome, we found 363 human B class CpG motifs and 67 human C class motifs. Synthetic B and C class-like immunostimulatory oligonucleotides derived from *T. gondii* genome induced NF-κB activation in HEK cells (Figure 7C), as well as proinflammatory cytokines, and in special IL-12p40/IL-12p70, when human PBMCs were primed with IFNγ (Figure 7D).

DISCUSSION

We hypothesized that a combined deficiency of NAS-TLRs was responsible for the observed phenotype of 3d mice infected with *T. gondii*. Our current report indicates that the extreme susceptibility of 3d mice is due to a combined defect on endosomal TLRs, but not simply a deficiency of the NAS-TLRs, also including TLR11 and TLR12. The combined deficiency of endosomal TLRs results in a profound impairment of IL-12 production by both dendritic cells and macrophages, and a subsequent reduction in the levels of IFNγ.

In our previous study (Melo et al., 2010), we suggested, as an alternative mechanism, that UNC93B1 was directly mediating control of tachyzoite replication in the PV. However, induction and translocation of iGTPases to the PV are normal, and IFNγ-activated macrophages from 3d mice effectively controlled parasite replication (Melo et al., 2010). In addition, macrophages lacking functional TLRs, including NAS-TLRs, are not more permissive to parasite growth in vitro. To test this hypothesis in vivo, we generated mixed chimeras and observed that in vivo parasite replication was equal in cells from WT and 3d mice (data not shown). UNC93B1 is also involved in antigen crosspresentation (Tabeta et al., 2006),

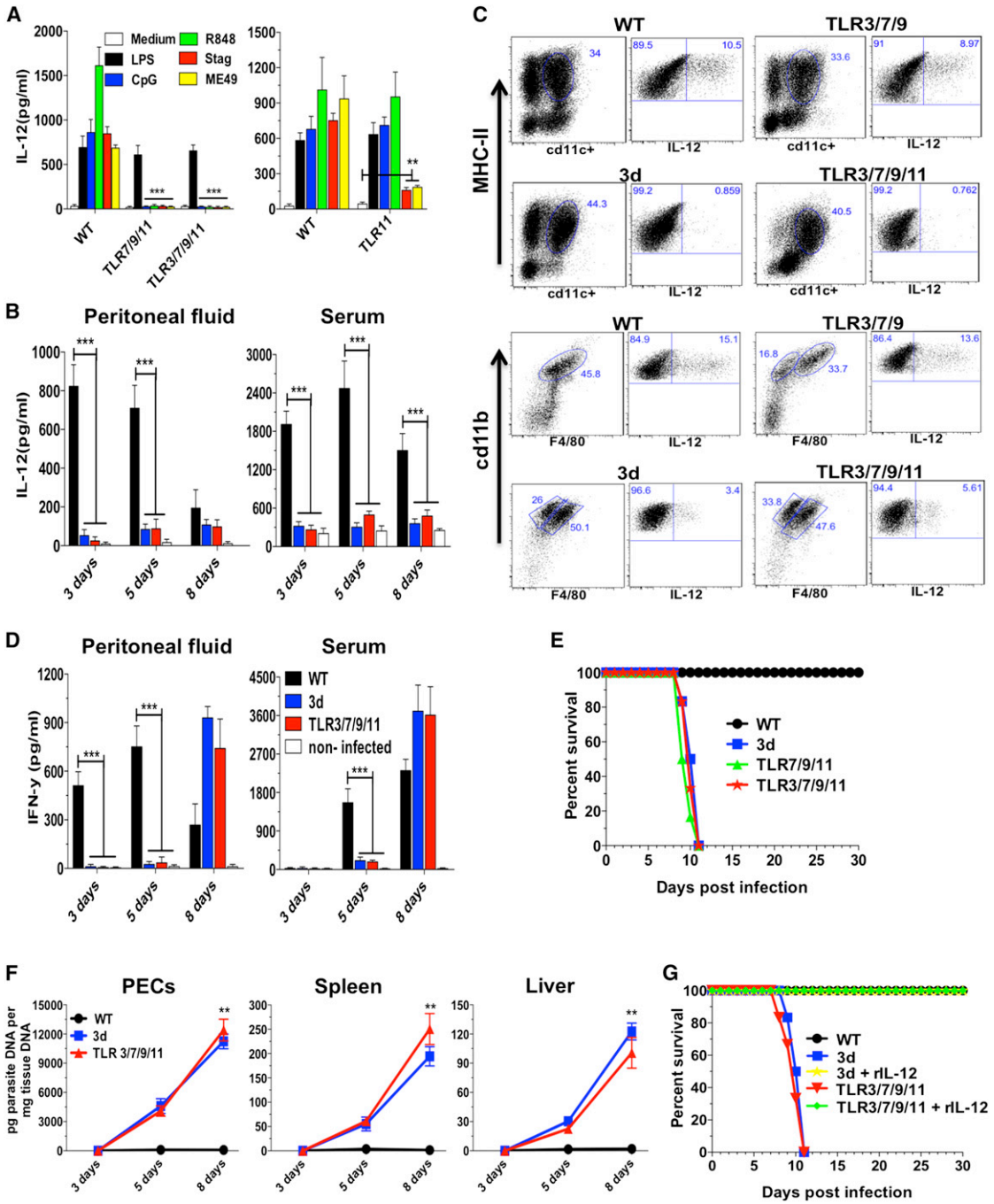

Figure 6. Quadruple Deficient Mice Have an Impaired IL-12 and Early IFNγ Production and Are Highly Susceptible to *T. gondii* Infection

(A) CD11c$^+$ cells were purified from spleen of WT, TLR7/TLR9/TLR11, TLR3/TLR7/TLR9/TLR11, and TLR11 KO mice and stimulated with LPS (100 ng/ml), ODN CpG 1826 (1 μM), R848 (2 μM), STAg (10 μg/ml), or infected with ME49 tachyzoites (moi 3:1). Data are represented as mean ± SD of two experiments.

(B) Levels of IL-12p40 were measured in the peritoneal cavity exudate and sera from uninfected controls and infected mice. Data are represented as mean ± SD of four experiments.

(C) Mice infected with *T. gondii* were sacrificed at 5 days postinfection and peritoneal cells analyzed for IL-12 cellular source by intracellular cytokine staining. Dendritic cells (top) were gated for CD11c$^+$/MHC-II$^+$. Inflammatory monocytes (bottom) were gated first for GR1$^+$ and then for CD11b$^+$/F4/80$^+$. Both populations were stained for IL-12p70. Data are from one representative experiment of three that yielded similar results.

(D) Levels of IFNγ present in the peritoneal cavity exudate and sera from uninfected controls and infected mice. Data are represented as mean ± SD of four experiments.

(E) Combined survival data from WT (n = 15), 3d (n = 10), TLR7/9/11 KO (n = 10), and TLR3/7/9/11 KO (n = 15) mice from at least two independent experiments.

(F) Quantitative real-time PCR analysis was performed on the indicated tissues collected from animals infected with *T. gondii*. Data are represented as mean ± SD of three independent experiments.

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but we have no indication that activation of $CD8^+$ T lymphocytes is impaired in 3d mice infected with intracellular protozoan parasites (Caetano et al., 2011; Melo et al., 2010). Hence, we propose that the main cause of the enhanced susceptibility of 3d mice to *T. gondii* infection is the defective activation of endosomal TLRs, impaired IL-12 production, and inadequate development of protective immunity.

As observed for other intracellular protozoan parasites (Bartholomeu et al., 2008; Caetano et al., 2011; Parroche et al., 2007), we report that *Toxoplasma*-derived DNA and RNA induce the production of proinflammatory cytokines by macrophages and DCs. However, the triple TLR3/TLR7/TLR9 KO mice are only slightly more susceptible to infection with *T. gondii*. In addition, mice deficient in TLR11, which is activated by TgPRF in an UNC93B1-dependent manner (Pifer et al., 2011), survive the acute infection with *T. gondii* (Yarovinsky et al., 2005). This complicated scenario convinced us to generate the quadruple TLR3/TLR7/TLR9/TLR11 and triple TLR7/TLR9/TLR11 KO mice that upon infection with *T. gondii* recapitulated the 3d phenotype, resulting in a profound impairment of the IL-12/IFN-γ axis, and unhampered parasite growth.

Importantly, two studies reported that TLR9 is partially responsible for the development of an optimal antiparasite Th1 response and intestinal pathology, when mice are per-orally infected with cysts of the ME49 strain of *T. gondii* (Benson et al., 2009; Minns et al., 2006). The interpretation of these results is that DNA of the intestinal microflora serves as a natural adjuvant for mucosal immunity (Hall et al., 2008). However, the adjuvant effect of the intestinal microflora is not observed when mice are infected intraperitoneally (Benson et al., 2009). Since our main interest was to identify the innate immune receptors for *T. gondii*, we used the intraperitoneal infection to avoid the interference of the gut microbiota. Hence, collectively, our results indicate that in addition to TLR11, TLR7, and TLR9 are also important sensors of *Toxoplasma* tachyzoites.

The high similarity of *TLR11* and *TLR12* found in a phylogenetic analysis led us to query if they had similar function, as previously observed for *TLR1*, *TLR2*, and *TLR6* (Roach et al., 2005; Triantafilou et al., 2006). Indeed, TLR11 and TLR12 colocalized with UNC93B1 in transfected HEK293T cells, and FRET between TLR11/TLR12 was induced upon activation with *Toxoplasma* extracts or rTgPRF. This result was not observed when the TLRs were expressed alone, suggesting that homodimerization does not occur. Together, our biochemical, cellular, and immunological assays strongly suggest that both TLR11 and TLR12 are endosomal TLRs and act as heterodimers in the recognition of *Toxoplasma* molecules. Thus, we propose that in mice the primary role of UNC93B1 in host resistance to *T. gondii* infection is to mediate translocation and function of TLR7, TLR9, TLR11, and TLR12.

The endosomal localization of TLR11 and TLR12, in contrast to the surface membrane TLRs that recognize cell wall components of bacteria (i.e., TLR1, TLR2, TLR4, TLR5, and TLR6), is an important finding. The current report further emphasizes that despite the presence of ligands for surface membrane TLRs (Gazzinelli and Denkers, 2006), endosomal TLRs are the critical ones for the in vivo sensing of intracellular protozoan parasites. The protozoan ligands that are recognized by endosomal TLRs appear to reside inside the protozoan parasite, DNA, RNA, and TgPRF, all of which are released from the parasite when it is killed in the phagolysosome. As shown for TLR9, it is reasonable to speculate that both TLR11 and TLR12 translocate from the ER and recognize the parasite components in the endolysosomes and not in the PV, which avoids fusion with host cell endocytic and exocytic vesicular trafficking pathways (Mordue et al., 1999). While activation of DCs by TgPRF, STAg, or live tachyzoites is mediated by UNC93B1, additional experiments are necessary to confirm the cellular location of TLR11/TLR12 activation.

Another intriguing aspect of the TLR11 and TLR12 biology is their species specificity. *TLR11* in humans is a pseudogene (Zhang et al., 2004), whereas *TLR12* is not present in the human genome. As mice are the natural intermediate host and highly exposed to *T. gondii* infection, we speculate that *TLR11* was not only maintained as a functional receptor but also duplicated as an important mechanism of host resistance to infection. Although TLR11 and TLR12 are not involved in recognition of *T. gondii* by human cells, one can imagine that they may play an indirect role in human disease because of the role that mouse has in transmitting the parasite to cats, which can then transmit to humans.

It is worth mentioning that in mice TLR8 has no known ligands, whereas TLR7 is widely expressed and functions as a receptor for ssRNA (Heil et al., 2004). Complicating matters, human mDCs and monocytes respond to ssRNA via TLR8. Human TLR7 is active, however, in pDCs' (which do not express TLR8) response to RNA viruses (Forsbach et al., 2008). Thus, in consideration of the lack of expression of TLR11 and TLR12 in humans, these data led us to speculate that TLR7, TLR8, and TLR9 are the key TLRs in human toxoplasmosis. Importantly, human PBMCs from uninfected healthy donors produce significant levels of IL-1β and TNF-α when stimulated with parasite-derived DNA and RNA, as well as oligonucleotides containing CpG motifs derived from *Toxoplasma* genome. Furthermore, when primed with IFNγ, human PBMCs produced high levels of IL-12p40/70 upon stimulation with parasite-derived nucleic acids, but not with STAg or rTgPRF. The precedent for the importance of IFNγ priming in the production of IL-12 also exists in the rodent model of toxoplasmosis (Gazzinelli et al., 1994; Goldszmid et al., 2012).

Finally, although it is considered an "accidental" intermediate host, one-third of the human population in the world is chronically infected with *T. gondii* (Robert-Gangneux and Dardé, 2012). While one can imagine the evolutionary pressures that gave rise to TLR11 in mice, it remains unclear why it was downgraded to a noncoding gene in humans. Hence, our findings have

(G) WT, 3d, and TLR3/TLR7/TLR9/TLR11 KO were infected i.p. with the ME49 strain of *T. gondii* (n = 5 per group). Mice were treated with 100 ng of recombinant IL-12p70 or vehicle for 6 consecutive days, and mortality was evaluated. Data are from one representative experiment of two that yielded identical results. Asterisks indicate that difference is statistically significant, when comparing different mouse lineages infected with *T. gondii*. (**$0.001 < p < 0.01$, and ***$p < 0.001$). See also Figure S2.

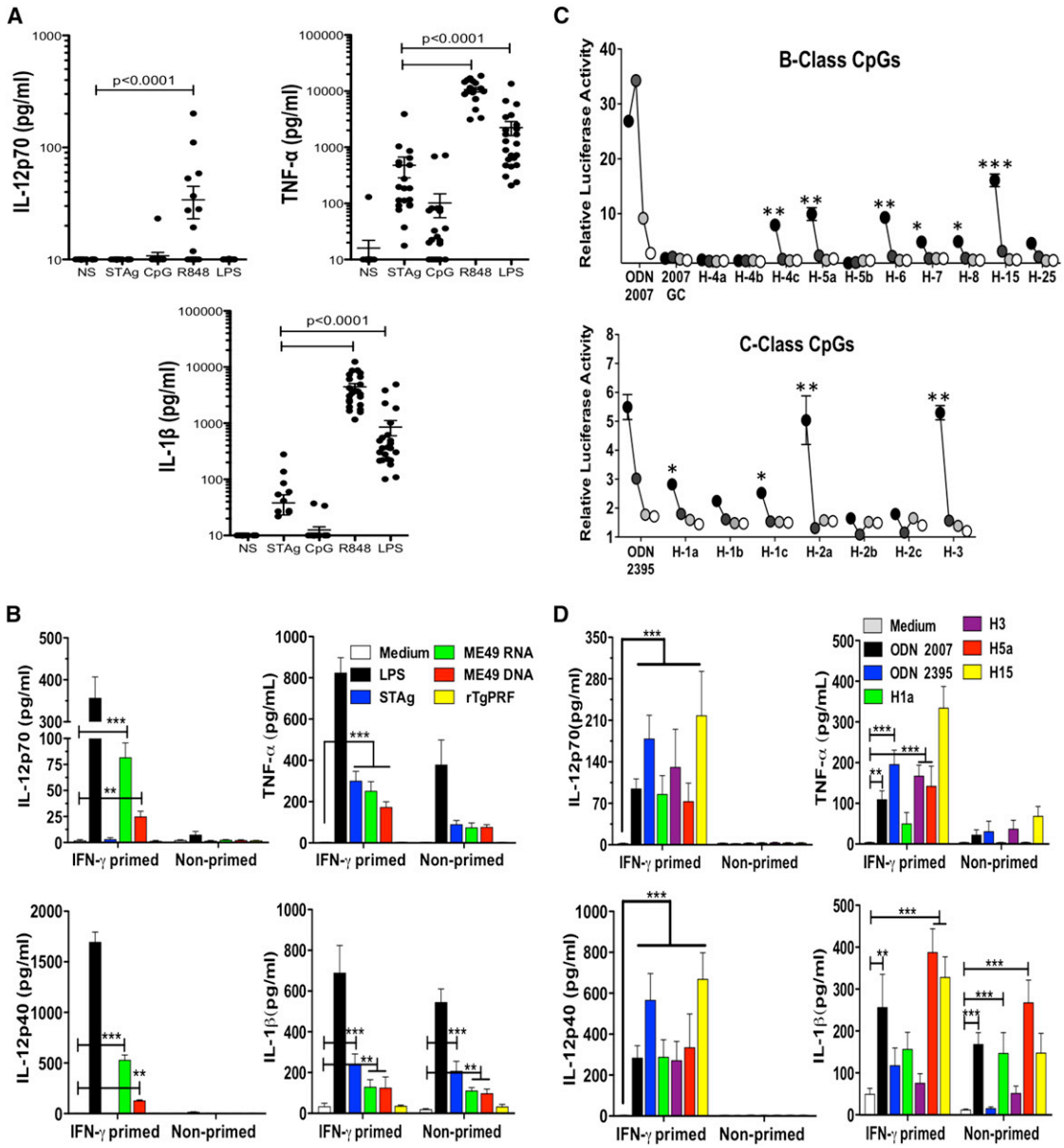

Figure 7. DNA and RNA from *T. gondii* Activate Human Peripheral Blood Mononuclear Cells

(A) PBMCs purified from blood of clinically healthy donors were stimulated in vitro with STAg (10 μg/ml), CpG ODN 2007 (1 μM), R848 (2 μM), or LPS (100 ng/ml). Data are represented as mean ± SD.

(B) PBMCs primed or not with recombinant human IFNγ (200 U/ml) were stimulated with LPS (100 ng/ml), STAg (10 μg/ml), ME49 RNA (2 μg/ml), ME49 DNA (5 μg/ml), or rTgPRF (1 μg/ml). Parasite RNA and DNA were complexed with DOTAP (Roche).

(C) HEK293T cells were stimulated with either CpG ODN 2007 (B class) or 2395 (C class) as positive controls, or *T. gondii*-derived oligonucleotides containing B or C class human-like stimulatory CpG motifs at 3 μM (black circles), 1 μM (dark gray), 0.3 μM (light gray), and 0.1 μM (white circles). Asterisks indicate that differences are statistically significant when comparing stimulated cells with negative control—ODN 2007GC (top panel).

(D) PBMCs primed or not with recombinant human IFNγ (200 U/ml) were stimulated in vitro with either CpG ODN 2007 (B class) or 2395 (C class) as positive controls, or *T. gondii*-derived oligonucleotides containing B and C class human-like stimulatory CpG motifs (5 μM). Cytokine levels were measured in the supernatants at 18 hr after stimulation.

(B–D) Data are represented as mean ± SD of three independent experiments (*0.01 < p < 0.05, **0.001 < p < 0.01, and ***p < 0.001). See also Table S2.

important implications for human disease. One has to assume that alternative TLRs are responsible for detecting the parasite, triggering innate immunity and initiating acquired immunity during acute toxoplasmosis in humans. The data presented here show that human PBMCs produce high levels of proinflammatory cytokines, including IL-12, in response to *T. gondii* RNA and DNA, but not rTgPRF. Finally, a recent study reports an association of a *TLR9* single-nucleotide polymorphism and development of ocular toxoplasmosis (Peixoto-Rangel et al., 2009). Hence, we propose that NAS-TLRs, as well as the signaling

pathways they activate, are important determinants of resistance to infection and the clinical outcome of human toxoplasmosis.

EXPERIMENTAL PROCEDURES

Ethics Statement

All experiments involving animals were performed in accordance with guidelines set forth by the American Association for Laboratory Animal Science (AALAS) and were approved by the Institutional Animal Care and Use Committee (IACUC A-1817) at the University of Massachusetts Medical School (UMASSMED). The protocols and consent forms for experiments with human PBMCs were approved by the Institutional Research Board from UMASSMED (IRB-UMMS H-12328) and from Centro de Pesquisas René Rachou–Fundação Oswaldo Cruz (CEP-CPqRR 11/2006), as well as by the National Ethical Committee (CONEP 13.368) from the Ministry of Health in Brazil.

Reagents

All cell culture reagents were obtained from Mediatech. LPS derived from *Escherichia coli* strain 0111:B4 was purchased from Sigma and re-extracted by phenol chloroform to remove lipopeptides as described (Hirschfeld et al., 2000). R848, a synthetic small molecule agonist for TLR7, was provided by 3M Pharmaceuticals. Phosphorothioate-stabilized unmethylated DNA oligonucleotide-bearing CpG ODN 1826, ODN 2007, and ODN 2395, as well as ODNs containing CpG motifs identified in *T. gondii* genome and qPCR primers, were obtained from Integrated DNA Technologies. Purified rTgPRF (Skillman et al., 2012) was kindly provided by Dr. David Sibley (Washington University, St. Louis, MI). Mouse and Human Recombinant Interferon-γ and rIL-12p70 were purchased from eBioscience. ER-Tracker Blue-White dpx, Lysotracker Blue-White dpx, and Cell Tracker Red CMTPX were obtained from Molecular Probes and Cholera Toxin B Subunit FITC conjugate from Sigma. Hoechst 33342 to stain the nucleus was bought from Thermo Scientific. Proteinase K was obtained from Ambion and DNase and RNase from Promega.

Mice and Parasites

C57BL/6 mice were obtained from The Jackson Laboratory. UNC93B1 mutant (3d) mice were kindly provided by Bruce Beutler at The Scripps Research Institute, La Jolla, CA (Tabeta et al., 2006). The single TLR3, TLR7, TLR9, and TLR11 KO mice were kindly provided by Shizuo Akira (Osaka University, Japan) and Richard Flavell (Yale University), respectively. TLR12 KO mice were provided by Sankar Ghosh (Columbia University, New York, NY). Interbreeding single KO animals generated TLR3/7-, TLR7/9-, TLR7/8-, TLR3/7/9-, TLR7/9/11-, and TLR3/7/9/11-deficient mice. Age-matched (6 to 8 weeks old) and female groups of WT and KO mice were used in all experiments. The ME49 strain was maintained in C57BL/6 mice by serial inoculation of brain homogenate-containing cysts. ME49 tachyzoites were maintained in human foreskin fibroblast cells (Hs27) (Lock, 1953) and used to prepare STAg as previously described (Melo et al., 2010). DNA and RNA were extracted by employing DNeasy Blood and Tissue Kit and RNesy Mini Kit from QIAGEN.

Genome-wide Scanning for TLR9-Stimulatory Sequences

Both mouse B-like class and human-like B and C class CpG motifs were searched in the DNA strands of the assembled contigs of *T. gondii* ME49 genome downloaded from the ToxoDB website (http://toxodb.org/common/downloads/), as previously described (Bartholomeu et al., 2008).

Cell Purification

CD11c+ cells were purified using the EasySep mouse CD11c-positive selection kit according to the manufacturer's protocol. In some experiments, CD11c+ cells, purified in magnetic beads, were stained with anti-CD8APC and sorted in a FACSAria for PE (CD11c+) or PE/APC (CD11c+/CD8+). Cell purity was checked by FACS.

Measurement of Cytokine Levels

The levels of mouse TNF-α, IFN-γ, IL-12, IL-6, and MCP1 were measured by using DuoSet ELISA kits (R&D Systems). IL-12p70, IL-1β, and TNF-α levels were measured in PBMC culture supernatants by using the Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (BD Bioscience) or ELISA for IL-12p40 (R&D Systems).

Flow Cytometry

Cells were stained with conjugated antibodies against the surface markers CD11b, CD11c, GR1, F4/80, and MHC-II (eBioscience). For intracellular measurement of cytokines, cells cultivated for 8 hr in the presence of GolgiPlug (BD Bioscience), surface stained, fixed with 4% formaldehyde, permeabilized with PBS + Tween 20 0.5% and incubated with Phycoerythrina anti-IL-12p70 (BD Biosciences). Subsequently cells were washed and analyzed by flow cytometry in an LSRII cytometer (BD Biosciences).

Quantitative Real-Time PCR

Total DNA from peritoneal exudate cells, spleen, and liver was used for amplification of *T. gondii* B1 gene (Melo et al., 2010). Relative quantification was performed using standard curve analysis of purified parasite DNA. For TLR expression, the following primers were used: TLR3 forward, 5′-ATAAAATCCTT GCGTTGCGAAGT−3′; TLR3 reverse, 5′-TGTTCAAGAGGAGGGCGAATAA-3′; TLR7 forward, 5′-TCTTTGGGTTTCGATGGTTTCC-3′; TLR7 reverse, 5′-GCAGT CCACGATCACATGGG-3′; TLR9 forward, 5′-ACGGGAACTGCTACTACAAG A-3′; TLR9 reverse, 5′-CCCAGCTTGACAATGAGGTTAT-3′; TLR11 forward, 5′-AGAGCTGGCTGGTATGTTCC-3′; TLR11 reverse, 5′-GTGTTCTTGTCAGGT CCAGAATC-3′; TLR12 forward, 5′-CCAGGACTGCACCTTTTGG-3′; TLR12 reverse, 5′-GTGACACTGGTTGTACGCAAT-3′.

Immunoprecipitation

HEK293T cells were cotransfected with bait and prey constructs. Forty-eight hours posttransfection, cells were lysed in Ripa lysis buffer (Sigma) with Complete Mini Protease Inhibitors (Roche). An equal amount of cell lysate was used for immunoprecipitation with monoclonal anti-HA (Sigma) bound to protein A agarose (Invitrogen). Eluted proteins were electrophoresed by SDS-PAGE, transferred to nitrocellulose (Bio-Rad), and blotted using monoclonal antibody anti-HA or anti-Flag (Sigma).

Plasmid Construction and Viral Transduction

TLR11 and *TLR12* from InvivoGen plasmids were cloned into mCherry-, Cerulean-, and Citrine-tagged vectors that were modified from the original pCLXSN backbone from Imgenex. All tags were fused in the C-terminal portion of the TLRs. Recombinant retroviruses were produced as previously described (Mann et al., 1983; Melo et al., 2010).

Confocal Microscopy

We used an inverted Leica LSM TSC SP2 AOBS microscopy and a 1.4 NA 63× plan apochromat objective (Zeiss). Cells were cultured on glass-bottom 35 mm tissue culture dishes (Matek), and dual or triple color images were acquired by consecutive scanning. For fluorescence resonance energy transfer (FRET) experiments, HEK293T cells were transiently transfected with the indicated plasmids and 48 hr later stimulated for 4 hr with STAg or rTgPRF, or for 1 hr with CpG 1826. FRET between the respective proteins was calculated by measuring sensitized emission (SE) fluorescence using the FRET SE wizard on the Leica SP2 confocal laser-scanning microscope. In each case, cerulean-tagged protein acted as the donor fluorophore, whereas the citrine-tagged protein functioned as the acceptor fluorophore. Excitation wavelengths for the donor and acceptor were 405 nM and 514 nM, respectively. The FRET efficiency is shown as a color-coded scale of values between 0 and 100%.

Experiments with Human Peripheral Blood Mononuclear Cells

PBMCs were enriched onsite by gradient centrifugation over Ficoll-Paque plus (GE-Healthcare). Cells were plated at 3×10^5 cells/well and cytokines measured 18 hr poststimulation with STAg, rTgPRF, CpG oligonucleotides, R848, LPS, ME49 DNA, or RNA.

Luciferase Assay

HEK293 cells stably expressing human TLR9 and a luciferase gene under the control of the pELAM promoter-containing NF-κB sites were used for testing the activity of *T. gondii*-derived ODNs. We also used pRL vector expressing

a *Renilla* luciferase gene (Promega) for constitutive protein expression, and assays revealed by Dual luciferase reporter assay system (Promega) substrate (Bartholomeu et al., 2008; Latz et al., 2007).

Statistical Analysis

All data were analyzed using an unpaired, two-tailed Student's t test with a 95% confidence interval (Prism; GraphPad Software, Inc.). All data are represented as means ± SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and two tables and can be found with this article at http://dx.doi.org/10.1016/j.chom.2012.12.003.

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