Toll-like Receptor Recognition Regulates Immunodominance in an Antimicrobial CD4+ T Cell Response

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Summary

Although Toll-like Receptors (TLRs) play a major function in innate recognition of pathogens, their role in antigen processing and presentation in vivo is poorly understood. Here we establish that Toxoplasma gondii profilin, a TLR11 ligand present in the parasite, is an immunodominant antigen in the CD4+ T cell response to the pathogen. The immunogenicity of profilin was entirely dependent on both TLR11 recognition and signaling through the adaptor myeloid differentiation factor 88 (MyD88). Selective responsiveness to this parasite protein was regulated at the level of antigen presentation by dendritic cells (DC) and required both TLR signaling and major histocompatibility complex (MHC) class II recognition acting in cis. These findings support a major influence of TLR recognition in antigen presentation by DC in vivo and establish a mechanism by which TLR ligand association regulates the immunogenicity of microbial antigens.

Introduction

Toll-like receptors (TLRs) are a major class of pattern-recognition receptors involved in “self” versus “nonself” discrimination that play a major role in the initiation of the both innate and adaptive immunity to infectious agents (Takeda and Akira, 2005; Iwasaki and Medzhitov, 2004). To date, 12 different TLRs have been identified in mammals that recognize a number of distinct and highly conserved chemical structures (pathogen-associated molecular patterns, PAMP) present on nucleic acids, carbohydrates, lipids, and proteins of microbial agents (Takeda et al., 2003; O’Neill, 2006). Upon recognition of their ligands, TLRs transduce signals through two pathways involving distinct adaptor proteins containing Toll/IL-1R (TIR) domains (O’Neill et al., 2003). One of these adaptors, MyD88, is utilized by all of the known TLRs except TLR3, which instead signals through the TIR domain-containing adaptor inducing IFN-β (TRIF), also known as TIR domain-containing adaptor molecule 1 (TICAM-1) (Yamamoto et al., 2002; Oshimi et al., 2003; Hoebe et al., 2003). TLR4, the receptor for LPS, is unique in its capacity to signal through either of the two adaptors (Yamamoto et al., 2003). The end product of TLR signaling is the nuclear factor-kB (NF-kB)-dependent induction of proinflammatory cytokines and costimulatory molecules required for immune response initiation.

Dendritic cells (DC) are important mediators of TLR interaction with microbial ligands (Iwasaki and Medzhitov, 2004). These major sentinels of the immune system transport foreign antigens from the periphery to lymphoid organs where they initiate T cell responses (Banchereau and Steinman, 1998). In order to do so, DC must first be activated through TLR encounter with PAMP, resulting in the induction of inflammatory cytokines as well as migration to secondary lymphoid organs (Kaisho et al., 2001; Kaisho and Akira, 2001). Recent in vitro data suggest that TLR ligand interaction in DC also directly affects the process of antigen (Ag) presentation itself (Blander and Medzhitov, 2004, 2006; West et al., 2004). Thus, in the absence of MyD88 signaling or specific TLR, DC show impaired uptake of bacteria or LPS-coated beads and subsequent stimulation of CD4+ T cells (Blander and Medzhitov, 2004). Nevertheless, these findings have been difficult to generalize to other systems, and their possible relevance in regulating T cell activation in vivo has never been formally addressed.

In the present study we have examined the role of TLR recognition in governing the CD4+ T cell response to a pathogen-derived protein from the protozoan parasite Toxoplasma gondii. This molecule, T. gondii profilin, was recently identified by us as a TLR11 ligand that selectively activates DC belonging to the CD8α+ subset for the production of Interleukin-12 (IL-12) and other cytokines by means of an MyD88-dependent pathway (Yarovinsky et al., 2005; Yarovinsky and Sher, 2006). We demonstrate here that although previously characterized as a minor component in the parasite, profilin is an immunodominant protein in the CD4+ T cell response to a soluble extract of the tachyzoite stage of the parasite (STAg) as well as to live T. gondii infection. We further show that the immunodominance of profilin depends on TLR11 and MyD88 both in vivo and in vitro and appears to result from the enhanced and selective uptake of the protein by CD8α+ DC expressing this signaling pathway. Taken together, our findings suggest that physical association with TLR ligands can play a major role in promoting CD4+ T cell responsiveness to protein Ag and, in the case of pathogens, may be an important factor determining the selective immunogenicity of microbial components.

Results

CD4+ T Cells from Mice Immunized with a Complex Parasite Extract Selectively Recognize T. gondii Profilin

It has been previously established that CD4+ T lymphocytes are rapidly activated in response to T. gondii infection and that they are necessary for host resistance to the pathogen (Suzuki and Remington, 1988; Gazzinelli et al., 1991; Araujo, 1991). We predicted that because of its dual property as a protein Ag and TLR receptor agonist that potently stimulates DC, T. gondii profilin might
be highly immunogenic and provide a better stimulus for CD4+ T cell responses than other Ag in the parasite. To test this hypothesis, we employed a previously established protocol (Jankovic et al., 2002) mimicking T. gondii infection in which mice are repeatedly immunized with an unfractionated soluble extract of the tachyzoite stage of the parasite (STAg) containing a complex mixture of microbial components (Yarovinsky et al., 2005). This procedure results in the induction of strong parasite-specific CD4+ T cell response comparable to those observed in infected animals (Jankovic et al., 2002). At 7 days after the last STAg injection, purified CD4+ T cells from mice primed in this manner were assessed for their recall proliferative responses to STAg versus recombinant profilin via irradiated splenocytes from unimmunized animals as antigen-presenting cells (APC). We found that on a weight per weight basis, purified profilin stimulated as much if not more proliferation than the unfractionated parasite extract (Figure 1A) in recall assays, suggesting that although it is a minor component in the pathogen (Yarovinsky et al., 2005), this protein is a dominant Ag in the CD4+ T cell response elucidated by T. gondii. Importantly, no measurable recall response was observed to a second recombinant protein Cyclophilin-18, a parasite molecule previously identified in STAg that functions as a CC chemokine receptor 5 (CCR5) rather than TLR ligand (Alberti et al., 2003). Similar results were obtained when IFN-γ production was measured as a read-out of CD4+ T cell reactivity (Figure 1B). The observed immunodominance of profilin could not be explained by possible endotoxin contamination of either the recombinant protein or STAg, since the same response pattern was observed in STAg-immunized TLR4-deficient mice (see Figure S1 in the Supplemental Data available online).

The Immunogenicity of Profilin Depends on Host TLR11 Expression
To determine whether the unusual CD4+ T cell stimulatory capacity of profilin stems from its property as a TLR11 ligand, we compared recall responses to profilin and STAg in parasite extract-immunized Tlr11−/− versus WT mice, in each case using WT splenocytes as APCs. Strikingly, we found that profilin failed to elicit a substantial recall response in the TLR11-deficient animals and that in addition, the response to STAg was also greatly diminished in these mice versus similarly immunized WT mice (Figure 1C versus Figure 1A). Further analysis revealed that CD4+ T cell activation as measured by CD44 expression was substantially reduced in STAg-immunized Tlr11−/− versus WT control animals (Figure 1D). The latter observation raised the possibility that the nonresponsiveness of Tlr11−/− mice to profilin stems from a generalized abrogation of APC cytokine and costimulatory signals resulting from the absence of the major TLR signal associated with the parasite extract (Yarovinsky et al., 2005). To test this hypothesis, we asked whether the inclusion of either LPS (Figure 1E) or complete Freund’s adjuvant (data not shown) in the STAg immunization protocol would rescue the profilin response. We found that while addition of either of these TLR stimuli corrected the defect in CD4+ T cell activation in the immunized TLR11-deficient mice (Figure 1F), neither stimulus restored the dose-dependent CD4+ T cell recall response to purified profilin (Figure 1E and data not shown).

We next asked whether TLR11 governs the response to profilin during natural infection with T. gondii. To do so, WT and Tlr11−/− mice were inoculated with the ME49 parasite strain, and 2 weeks postinfection, spleen cells were restimulated with either STAg or recombinant profilin, and IFN-γ-producing CD4+ T cells were quantitated by intracellular cytokine staining and flow cytometry. As shown in Figure 1G, infected WT mice mounted strong recall responses to both STAg and profilin. Consistent with the results obtained with STAg immunization (Figure 1C), the response to profilin (as well as to STAg itself) was found to be greatly diminished in the infected Tlr11−/− animals.

The Immunogenicity of T. gondii Profilin Depends on Signaling through MyD88
To determine whether TLR11 governs the selective immunogenicity of profilin by simply promoting interaction of the antigen with APC or whether immunogenicity of profilin also requires signal transduction, we analyzed the response of Myd88−/− mice to either STAg or profilin immunization. As shown in Figure 1H, STAg-immunized Myd88−/− mice failed to mount a detectable recall response to profilin and displayed a diminished response to the parasite extract itself, findings that closely mirrored those obtained with Tlr11−/− mice (Figure 1C).

Taken together, the above data indicated that the immunodominance of profilin depends on both its recognition by TLR11 and on the generation of an MyD88-dependent signal.

The Immunogenicity of Profilin Depends on Dual Expression of MyD88 and MHC Class II Molecules in the Same Bone Marrow-Derived Cell Population
Although the CD4+ T cell response to profilin was greatly impaired in MyD88-deficient mice, it was possible that this defect is due to a generalized lack of TLR-induced cytokine production in vivo (Takeda et al., 2003). To address whether MyD88 expression is required within antigen-presenting cells, bone marrow chimera experiments were performed involving WT recipients reconstituted with a 1:1 mixture of H2-Ab1−/− (MHC class II-deficient) and Myd88−/− BM cells (Figure 2). As expected, chimeras reconstituted with WT or MHC class II-deficient BM cells and injected with STAg produced high amounts of serum IL-12 measured at 6 hr whereas the chimera reconstituted with Myd88−/− BM failed to respond. Also as expected, chimeras reconstituted with a mixture of Myd88−/− and MHC class II-deficient BM cells mounted a vigorous cytokine response (Figure 2A). CD4+ T cell recall responses to profilin were then analyzed by intracellular IFN-γ staining in the same animals after repeated immunization with STAg as described above. As indicated in Figures 2B and 2C, the mixed chimeras reconstituted with both Myd88−/− and MHC class II-deficient BM displayed greatly diminished CD4+ T cell IFN-γ responses to STAg or profilin in comparison with the control WT into WT chimeras despite their unimpaired IL-12 production (Figure 2A). This reduction in IFN-γ CD4+ T cells was not due to their conversion into an IL-4-positive (Th2) population (Figures 2B and 2C). Similar results were obtained when proliferative responses were assayed in
the same cultures (data not shown). The above observations argued that the unusual immunogenicity of profilin depends on the dual expression of MyD88 and MHC class II on the same BM-derived cell population and is not simply the result of the potent systemic TLR-dependent cytokine response triggered by the protein.
Antigen Presentation of Profilin by DC Is MyD88 Dependent and Limited to the CD8α+ Subset

To characterize the MyD88-dependent cell population required for the immunogenicity of profilin, ex vivo experiments were performed in which splenic APC from STAg-injected mice were used to stimulate a pool of different profilin-specific CD4+ T cell clones. Initial experiments (data not shown) indicated that only the CD11c+ spleen cell fraction was able to trigger proliferation of these T lymphocytes. Interestingly, in kinetic experiments, peak antigen presentation by the CD11c+ population was observed at 6 hr postinjection (Figure 3A), a time corresponding to that previously described as the period of maximal IL-12 production by DC after STAg injection (Reis e Sousa et al., 1997; Schulz et al., 2000). Similarly, as reported previously for DC IL-12 production, the antigen-presentation function of DC primed in vivo by STAg was restricted to the CD8α+ subset (Figures 3B and 3C). Despite their similar requirements, STAg-induced IL-12 production and profilin-specific antigen presentation were shown to be independent functions since DC from IL-12p40-deficient mice showed unimpaired APC function in our assay (Figures 3B and 3C). Importantly, DC stimulation of profilin-specific CD4+ T cells in common with in vivo responsiveness to the protein and is not due solely to its function in DC activation (Figure S2).

The requirements for TLR11 and MyD88 in presentation of profilin to CD4+ T cells were also evident when mice were infected with live tachyzoites (the rapidly growing stage of the T. gondii parasite). While CD8α+ splenic DC recovered from WT mice 6 hr after parasite inoculation potently stimulated proliferation of profilin-specific CD4+ T cells, DC from similarly infected MyD88- or TLR-deficient animals were highly defective in promoting this response (Figure S3).

The Role of MyD88 in Profilin-Induced CD4+ T Cell Responses Is DC Intrinsic

To further address the role of MyD88 signaling in the stimulation of CD4+ T cell responses by profilin-primed DC, we asked whether this requirement is intrinsic to the APC itself or depends on the expression of the adaptor molecule in unrelated cells in the host environment. To do so, we prepared mixed chimeric animals in which WT mice were reconstituted with a 1:1 ratio of WT and MyD88-deficient BM cells expressing either CD45.1 or CD45.2, respectively. These, as well as control chimeras, were then injected with STAg and the spleens recovered 6 hr later and separated into CD11c+ populations of Myd88<sup>−/−</sup> and WT origin by means of the presence of the appropriate CD45 allelic markers (Figures 4A–4C). The sorted DC populations were then tested for their ability to stimulate profilin-specific CD4+ T cells. WT (Myd88<sup>+</sup> CD45.1<sup>+</sup>) DC isolated from mice reconstituted
The experiment shown is representative of three preparations performed. The data shown are the mean of three mice per group and are representative of at least two experiments shown in (D)–(G) involved DC recovered and pooled from purified 6 hr post STAg inoculation and tested as above. The experiments involving the DC populations tested in terms of their ability to stimulate profilin-specific CD4+ T cell clones for 72 hr. T lymphocyte proliferation was then measured by H^3-thymidine incorporation. Figure 3. Stimulation of Profilin-Specific CD4+ T Cells by In Vivo Primed DC Is TLR11 and MyD88 Dependent and Limited to the CD8+ Subset

C57BL/6 mice (3 per group) were i.p. injected with 10 μg of STAg or PBS (empty bars), and spleen cells were recovered and pooled from these animals at 6 hr (gray bars), 24 hr (dark gray bars), and 48 hr (black bars) after antigen inoculation. (A–C) Bulk CD11c+ (A) as well as CD11c+CD8α+ (B) or CD11c+CD8α− (C) DC were then purified from these samples and incubated with a mixture of profilin-specific CD4+ T cell clones for 72 hr. T lymphocyte proliferation was then measured by H^3-thymidine incorporation. (D and E) The experiment shown is representative of three performed CD8α+ (D) and CD8α− (E) DC were sort-purified from WT (black bars) or IL-12p40-deficient animals (white bars) 6 hr post STAg inoculation and tested for their ability to stimulate profilin-specific CD4+ T cells as above. (F and G) CD11c+ (F) and CD8α− (G) DC from WT (black bars), MyD88-deficient animals (white bars), or Tlr11−/− (gray bars) were sort-purified 6 hr post STAg inoculation and tested as above. The experiments shown in (D)–(G) involved DC recovered and pooled from three mice per group and are representative of at least two experiments performed. The data shown are the mean ± SD.

either with WT BM alone or the mixture of WT and Myd88−/− BM cells induced potent CD4+ T cell proliferation (Figure 4D). In contrast, Myd88−/−DC45.1+ DCs recovered from either the mixed chimera or a control chimera reconstituted with MyD88-deficient BM cells alone failed to stimulate significant CD4+ T cells responses (Figure 4D). These results confirmed that the requirement for MyD88 in DC priming by profilin is cell intrinsic and is not the result of indirect maturation signals dependent on this adaptor molecule.

In Vitro Stimulation of Profilin-Specific CD4+ T Cells Is Partially Dependent on TLR11 and MyD88 Expression in DC

We next addressed whether the same requirement for TLR signaling in activation of profilin-specific CD4+ T cell responses that we observed in vivo also occurs with isolated DC directly exposed to the protein in vitro in the absence of other interacting cell populations. To do so, we sort-purified splenic DC from naive WT, Myd88−/−, or Tlr11−/− mice and cocultured them with profilin-specific T cells in the presence of increasing concentrations of STAg or profilin. Proliferative responses were then measured 72 hr later. As expected, both antigen preparations induced potent responses in the cultures containing WT DC. In contrast, at most doses tested, reduced proliferation was observed in cultures containing Myd88−/− or Tlr11−/− DC (Figure 4S). Nevertheless, this dependency on both MyD88 and TLR11 was overcome at high antigen concentration, suggesting that while TLR signaling facilitates triggering of profilin-specific CD4+ T cells, it is not a mandatory requirement.

MyD88/TLR11 Regulates Uptake of Profilin by DC In Vivo

The above data indicated that MyD88- and TLR11-dependent presentation of profilin is dose dependent and therefore may reflect a role of TLR in Ag uptake by DC distinct from their function in DC maturation. This hypothesis is also compatible with the more pronounced affects of TLR11 signaling in regulating profilin responses in vivo as opposed to in vitro, since Ag concentration is more limiting in the former situation. To assess the role of enhanced Ag uptake as an explanation of the data, we injected mice with fluorescent-labeled profilin (Alexa 488-profilin) and examined its incorporation by both CD8α+ and CD8α− DC11c+ DC and then assessed the ability of Alexa 488+ versus Alexa 488− DC to activate profilin-specific CD4+ T cells. The Alexa 488-profilin conjugate was confirmed to be fully active in its ability to trigger TLR11-dependent cytokine production (Figure S5). As shown in Figure 5A, uptake of the labeled Ag was observed in WT CD8α+, but not in CD8α− DC. Moreover, in the former population, DC from WT animals showed a greater than 5-fold increase in Alexa 488+ cells compared with either MyD88- or TLR11-deficient DC, with the latter displaying no substantial enhancement in fluorescence with respect to cells from uninjected mice. Consistent with these observations, Alexa 488+CD8α+ DC from WT mice were the most effective of any of the Alexa 488+ or Alexa 488− DC populations tested in terms of their ability to stimulate profilin-specific CD4+ T cells (Figure 5B).

Fusion of Profilin or CpG with Ovalbumin Results in Markedly Enhanced OVA-Specific CD4+ T Cell Priming

The results presented above demonstrated that TLR recognition and MyD88-dependent signaling regulate the CD4+ T cell response to T. gondii profilin. Nevertheless, most TLRs recognize chemical structures that are
and their respective flow-cytometric profile were as follows: WT+/− producing CD4+ T cell responses at a level comparable to protein retained its profilin-specific immunogenicity in TLR11-dependent IL-12-inducing activity of the profilin OVA fusion protein. Fusion with OVA did not impair the TLR agonist activity of the parasite protein. To do so, we constructed a recombinant Profilin-OVA fusion protein. 8 weeks later, the animals were injected with STAg (10 μg) and CD11c+CD45.1+ (WT) and CD11c+CD45.1− (Myd88−/−) were sort-purified from spleens as shown in density plots.

Figure 4. The Role of MyD88 in In Vivo APC Priming Is DC Intrinsic

(A–C) Irradiated C57BL/6.SJL (CD45.1+) mice were reconstituted with WT (CD45.1+) (A), Myd88−/− (CD45.1−) (B), or a mixture of WT and MyD88-deficient (C) BM cells. 8 weeks later, the animals were injected with STAg (10 μg) and CD11c+CD45.1+ (WT) and CD11c+CD45.1− (Myd88−/−) were sort-purified from spleens as shown in density plots.

(D) The different DC populations were then tested for their ability to stimulate profilin-specific CD4+ T cell proliferation. The populations studied and their respective flow-cytometric profile were as follows: WT → WT reconstitution (gray bars, quadrant 1); Myd88−/− → WT reconstitution (white bars-panel, quadrant 2); WT+ Myd88−/− → WT reconstitution (black bars, WT DC; hatched bars, Myd88-deficient DCs; quadrant 3 and 4, respectively). The data shown are the mean ± SD, the results are representative of two independent chimera experiments performed, each involving at least six animals per group of which spleens from three mice were pooled for cell separation.

### Discussion

The results presented here identify a function for TLR signaling in regulating the immunogenicity of pathogen-associated protein antigens and elucidate a pathway through which innate recognition can influence the adaptive immune response. We observed that TLR11 recognition of T. gondii profilin renders this molecule into a highly immunodominant Ag in the CD4+ T cell response to the parasite, a property that appears to stem from the enhanced uptake and presentation of the protein as a consequence of its activity as a TLR ligand.

Proteins generally are not targets for TLR recognition. Bacterial flagellin and apicomplexan profilins are the only well-defined examples of nonself proteins seen by the TLR system (Hayashi et al., 2001; Yarovinsky et al., 2005). Both molecules are also microbial antigens, and previous studies with flagellin, which is recognized by TLR5, have indicated that this protein is an immunodominant Ag in the CD4+ T cell response to Salmonella typhimurium (Cookson and Bevan, 1997; McSorley et al., 2000), and large numbers of flagellin-specific CD4+ T cells can be recovered from animals with chronic intestinal inflammation presumably as a result of sensitization by gut flora (Lodes et al., 2004). In the present study, we observed that profilin, recognized by TLR11, displays a similar immunodominance in the response to T. gondii and formally demonstrated that this property depends on TLR recognition and signaling at the level of the same APC that is presenting the antigen. The latter contention is based on the evidence that in
WT+Myd88−/− chimeric animals, only the WT DC are able to activate profilin-specific CD4+ T cells and that in MHC class II-deficient chimeras, priming of CD4+ T lymphocytes with the same specificity depends on joint expression of MyD88 and MHC class II in the same APC compartment. Furthermore, nonspecific maturation signals delivered by a heterologous TLR ligand in themselves failed to render profilin into an immunogenic molecule. These data argue that the TLR11 signal delivered by profilin influences its processing and presentation by DC in a cis fashion within the same DC. We speculate that this mechanism may also account for the previously described immunodominance of the TLR5 ligand containing protein flagellin.

A role for TLR signaling in the regulation of Ag processing and presentation has been suggested in several previous reports involving in vitro culture of DC with TLR2 or TLR4 stimuli. In one of these studies, LPS stimulation was shown to induce a rapid but transient enhancement of the uptake of FITC-labeled dextran by these cells (West et al., 2004). More relevant to the work presented here was the demonstration by Blander and Medzhitov that MyD88-dependent TLR recognition promotes the phagocytosis of Gram-negative or Gram-positive bacteria (or particles covered with LPS or PGN, respectively) while inducing phagosome maturation (Blander and Medzhitov, 2004). In a subsequent study (Blander and Medzhitov, 2006), it was shown that TLR recognition regulates the ability of DC to present experimental Ag to CD4+ T cells after phagocytosis of E. coli.

Our findings extend these earlier observations by demonstrating that TLR signaling can directly promote the ability of APC to take up and present soluble protein Ag to CD4+ T cells in vivo and that this effect depends on the physical association between the TLR ligand and the protein in question.

Multiple mechanisms are likely to regulate CD4+ T cell activation by profilin primed DC in vivo. Our studies on DC interaction with profilin reveal that TLR11- and MyD88-expressing DC have an enhanced capacity to

Figure 5. Uptake of Profilin by DC In Vivo Is Dependent on MyD88/TLR11

WT, Myd88−/−, or Tlr11−/− mice (three per group) were injected i.p. with 10 μg of Alexa 488-profilin, and 6 hr later the animals were sacrificed and spleens cells prepared and pooled for each group. The cells were then stained with CD11c-APC and CD8α-PE and analyzed for each of the latter fluorochromes as well as Alexa 488.

(A) The results shown are histograms of Alexa 488 staining gated on either the CD8α+ (PE+) or CD8α− (PE−) CD11c+ cell populations. The horizontal bars indicate the percentage of Alexa 488+ cells for each analysis based on comparison with the background staining pattern observed with cells prepared from the uninjected WT animals.

(B) Ability of Alexa 488+CD8α+(fluorescence intensity < 103) and Alexa 488+CD8α+(fluorescence intensity ≥ 103) cell populations identified in (A) to stimulate profilin-specific CD4+ cells as described in previous figures. The experiment shown is representative of three performed.
incorporate the protein when compared with DC lacking these TLR components. We speculate based on these results that TLR11, in addition to the promoting the involvement of TLR ligands in determining the immunodominance of specific CD4+ T cell responses against pathogens during infection. In addition to the example of bacterial flagellin discussed above, other microbial Ag may incorporate TLR ligands as part of their structure. For instance, the lipid moieties of pathogen-derived lipoproteins can serve as TLR2 agonists (Takeda et al., 2003), and a number of different microbial lipoproteins have been demonstrated to be highly immunogenic (Ghielmetti et al., 2005). Further work is needed to determine whether these molecules represent immunodominant Ag in the response to the pathogens from which they are derived and, if so, whether the observed immunogenicity is TLR and MyD88 dependent. If indeed our observations on the role of TLR signaling in the CD4+ T cell response to T. gondii profilin can be further generalized to other TLR ligand-associated microbial Ag, this mechanism may deserve consideration as an important factor biasing the specificity of the T cell repertoire directed against pathogens and as a strategy exploited by either host or pathogen in promoting the outcome of infection.

**Experimental Procedures**

**Animals**

C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). MyD88−/− and Tlr4−/− mice on a C57BL/6 background were generously provided by Dr. S. Akira (Osaka University, Japan). TLR11-deficient animals on a mixed C57BL/6×129 background were kindly provided by Drs. S. Ghosh and D. Zhang (Yale University, New Haven, CT). WT (Tlr11+/+ or Tlr11+/−) littermates from the same breeding stock gave responses to T. gondii profilin and STag that were indistinguishable from those of C57BL/6 mice in the major experiments shown. C57BL/6.SJL (CD45.1), H-2bAb1−/−, and IL-12p40-deficient mice were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) Animal Supply Contract at Taconic Farms (Germantown, NY). All animals were maintained at an American Association of Laboratory Animal Care-accredited NIAID animal facility, and 5- to 12-week-old female mice were employed in all experiments. To generate bone marrow chimeric animals, mice were exposed to 950 RADs in a GammaCell 40 cesium irradiator and reconstituted on the same day with 1×10^6 bone marrow cells from the donor mouse strain(s). The animals were then

**Figure 6. Covalent Association with ISS or Profilin Dramatically Enhances the Induction of In Vivo CD4+ T Cell Responses by Ovalbumin via TLR/MyD88-Dependent Mechanism**

(A and B) WT (A), Tlr9−/−, and Mydb8−/− (B) mice (2–3 per group) were injected subcutaneously with 25 µg of either unmodified OVA protein (open triangles), OVA, conjugated with 1018 ISS (filled circles), or OVA mixed with 1018 ISS (filled circles), or OVA conjugated with control oligonucleotides (filled triangles). After 7 days, CD4+ T cells from the donor mouse strain(s) were restimulated with titrated doses of OVA in the presence of irradiated WT splenocytes as APC. CD4+ T cell proliferation was then measured by incorporation of H3-thymidine as in previous figures. (C) WT mice (3 per group) were immunized i.p. with 10 µg of either unmodified OVA protein (open triangles), Profilin-OVA fusion protein (filled circles), OVA mixed with profilin (1:1 ratio, open circles), or profilin alone (filled triangles). Splenic CD4+ T cell responses to titrated doses of profilin were then assayed as in (A) and (B) above. (D) WT and Tlr11−/− mice were injected with either Profilin-OVA fusion protein, OVA mixed with profilin, or OVA alone. CD4+ T cell responses to titrated doses of OVA fragment (311–348) containing the OT-II CD4+ T epitope were then assayed in (A)–(C) above. In preliminary studies, s.c. injection of CpG and i.p injection of profilin were found to be the optimal routes for immunization. The experiments shown are representative of three performed.
maintained on acidified water for 4 wk and used in experiments 2–4 weeks later.

Parasite Antigens and Immunological Reagents
STAg (solute tachyzoite antigen) was prepared from tissue-culture-derived tachyzoites of the RH88 strain as previously described (Reis e Sousa et al., 1997). Recombinant T. gondii profilin was generated and purified as outlined previously (Yarovinsky et al., 2005). Ovalbumin fraction VI (Sigma, cat #A2512) and LPS 0111:B4 (Sigma, cat #L3024) were purchased from Sigma (St. Louis, MO). 1018 ISS and 1040 ISS were synthesized and conjugated with OVA by a coupling procedure described elsewhere (Cho et al., 2000). OVA-profilin fusion protein was generated by inserting the OVA311-348 fragment (containing the OT-II CD4+ T cell epitope) in-frame with profilin via an Xhol restriction site located at the N terminus of the profilin gene and cloning the resulting construct into pET-14b vector (Novagen). The recombinant Profilin-OVA fusion was then expressed in BL21DE3pLysE. coli by the same protocol employed in the expression of the unmodified profilin gene. Alexa 488-labeled profilin was prepared by reacting the purified protein with the fluorochrome and purifying the product with a commercial kit from Invitrogen (Carlsbad, CA). Flourescin isothiocyanate (FITC)-labeled CD8α, CD11b, B220, and CD45.1 and phycoerythrin (PE)-labeled CD11c monoclonal antibodies were purchased from BD Biosciences (San Diego, CA).

In Vivo Immunization with Protein Antigens
To generate T. gondii-specific CD4+ T cell responses, WT or gene-targeted mice were injected i.p. with STAg (10 μg per animal) 5 times at 4 day intervals as previously described (Jankovic et al., 2002) and utilized in experiments at 7–10 days after the last immunization. The effect of profilin fusion with OVA was assessed by immunizing mice with means of the same protocol except that either OVA alone, OVA-profilin fusion protein, or OVA mixed with profilin (1:1 weight ratio) at a dose of 10 μg per injection. The effect of ISS (CpG oligonucleotides) conjugation on OVA-specific CD4+ T cell responses was determined by s.c. immunization with either OVA alone (25 μg), OVA conjugated with 1018 ISS (1018-OVA, 25 μg), OVA conjugated with 1040 ISS (1040-OVA, 25 μg), or OVA (25 μg) admixed with 1018 ISS (25 μg) for each injection.

Toxoplasma gondii Infections
For studies of immune responses to live T. gondii infection, two different protocols were used. In the first, mice were inoculated i.p. with an average of 20 tissue cysts of the avirulent Me49 strain prepared from brain as described previously (Yarovinsky et al., 2005). In the second procedure, mice were infected i.p. with 107 tachyzoites obtained from brain as described previously (Yarovinsky et al., 2005).

Ex Vivo Measurement of Antigen-Specific CD4+ T Cell Responses
To assay the response of animals immunized with the above antigen preparations or infected with T. gondii, spleens (or in the case of ISS/OVA-injected animals, inguinal lymph nodes) were harvested from mice and pooled for each experimental group. The CD4+ populations were then purified by cell sorting (FACSVantage SE, BD Biosciences), and aliquots (105 cells each) were then mixed at a 1:4 ratio with irradiated WT splenocytes (as a source of APC) in 96-well U-bottom plates. After addition of the indicated amounts of antigen, the cultures were incubated for 48 hr and H3 thymidine (1 μCi) was added to each well. Incorporation of the isotope was then determined after an additional overnight incubation. The data shown are the means of duplicate or triplicate cultures.

Profilin-Specific CD4+ T Cells
Profilin-specific CD4+ T cells were generated from C57BL/6 mice immunized repeatedly with STAg (Jankovic et al., 2002) as in Figure 1. In brief, FACS-purified CD4+ T lymphocytes restimulated with STAg were cloned by limiting dilution and maintained in long-term culture as previously described (Jankovic et al., 2009). Pools containing equal numbers of five different profilin-specific CD4+ T cell clones (identified by their response to the recombinant protein [Yarovinsky et al., 2005]) were used to assess the ability of APC populations to present profilin.

Dendritic Cell Isolation
Dendritic cells were isolated from pooled (≥3) spleens of naive mice or animals injected 6 hr, 24 hr, or 48 hr previously with 10 μg of STAg or 6 hr previously with live tachyzoites. For purification, single-cell suspensions were incubated on ice for 15 min with either CD11c–PE alone or a combination of CD11c and FITC-labeled CD8α–FITC or CD45.1 antibodies. The cells were then washed and sorted on a FACSVantage SE (BD Biosciences) as described previously (Yarovinsky et al., 2005). In one set of experiments, the CD11c–CD8α–Alexa 488– and CD11c–CD8α–Alexa 488– cells were sort-purified from animals injected 6 hr previously with 1 μg of Alexa 488-labeled profilin alone (APC-labeled CD11c and PE-labeled CD8α antibodies were used for staining in this procedure rather than FITC conjugates).

Assay of CD4+ T Cell Stimulatory Activity of DC
To measure the capacity of DC to activate profilin-specific CD4+ T lymphocytes, the purified cell populations described above were cocultured with profilin-specific CD4+ T cells at the ratios indicated and proliferation measured H3 thymidine incorporation. In one set of experiments CD8α+ DC from naive WT, Thr11+/-, or Myd88+/- mice were used to stimulate profilin-specific CD4+ T cells in the presence of different concentrations of exogenously added STAg or profilin.

Supplemental Data
Five Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/25/4/655/DC1.

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