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Malaria Parasites Require TLR9 Signaling for Immune Evasion by Activating Regulatory T Cells

Hajime Hisaeda,2* Kohhei Tetsutani,* Takashi Imai,* Chikako Moriya,* Liping Tu,* Shinjiro Hamano,* Xuefeng Duan,* Bin Chou,* Hidekazu Ishida,* Akiko Aramaki,* Jianying Shen,* Ken J. Ishii,§ Cevayir Coban,§ Shizuo Akira,§ Kiyoshi Takeda,3† Koji Yasutomo,‡ Motomi Torii,¶ and Kunisuke Himeno§

Malaria is still a life-threatening infectious disease that continues to produce 2 million deaths annually. Malaria parasites have acquired immune escape mechanisms and prevent the development of sterile immunity. Regulatory T cells (Tregs) have been reported to contribute to immune evasion during malaria in mice and humans, suggesting that activating Tregs is one of the mechanisms by which malaria parasites subvert host immune systems. However, little is known about how these parasites activate Tregs. We herein show that TLR9 signaling to dendritic cells (DCs) is crucial for activation of Tregs. Infection of mice with the rodent malaria parasite Plasmodium yoelii activates Tregs, leading to enhancement of their suppressive function. In vitro activation of Tregs requires the interaction of DCs with parasites in a TLR9-dependent manner. Furthermore, TLR9+/− mice are partially resistant to lethal infection, and this is associated with impaired activation of Tregs and subsequent development of effector T cells. Thus, malaria parasites require TLR9 to activate Tregs for immune escape. The Journal of Immunology, 2008, 180: 2496–2503.
FIGURE 1. Activation of Tregs during P. yoelii infection. (A) Flow cytometric analysis of Foxp3 expression in CD4+CD25+ T cells. Splenocytes from uninfected (left panels) and PyL-infected (right panels) were stained with fluorescence-conjugated anti-CD4, anti-CD25, and anti-Foxp3 mAbs. Gated CD4+ cells were separated based on the expression of Foxp3 and CD25 (bottom panels). The numbers represent percentages of all cells in each of the quadrants. The results are representative of five repeated experiments. (B) The percentages of Foxp3+CD25+ T cells among CD4+ T cells (upper panel) and the absolute number of CD4+CD25+Foxp3- cells (bottom panel) in the spleens of uninfected (open bars) and PyL-infected (filled bars) mice were quantified. Values are means ± SD of six mice. Asterisks indicate statistical significance at p < 0.05 with the Student t test. (C) Suppressive function of Tregs in mice infected with PyL. CD4+CD25+ T cells (1 × 10^4) purified from uninfected mice were stimulated with anti-CD3 mAb in the presence of T cell-depleted spleen cells as APCs, and they were mixed with splenic Tregs (upper panel) or Tregs from lymph nodes (bottom panel) obtained from uninfected (open symbols) or PyL-infected (filled symbols) mice at the indicated ratio. The proliferation of CD4+CD25+ T cells was measured by [3H]thymidine incorporation. Values are means of triplicate cultures, and SD was <10% of the mean value. Each symbol represents results from an individual mouse. Splenic Tregs from infected mice suppressed significantly more than did those from uninfected mice (p < 0.01 with the unpaired Student t test). The results are representative of six repeated experiments.

Materials and Methods

Mice and parasites

C57BL/6 mice were purchased from Kyudo; RAG2-/- mice were from the Central Laboratory of Experimental Animals (Kawasaki, Japan); Ly5.1 C57BL/6 mice were from the Sankyo Lab Service under permission of Dr. H. Nakauchi (Tokyo University), OT-II mice were provided by Dr. K. Yui (Nagasaki University), and TRIF-, MyD88-, TLR7-, and TLR9-deficient mice on C57BL/6 background were generated as previously described (21–24). TLR9-deficient mice had been backcrossed for at least 15 generations. Age- and sex-matched groups of wild-type and mutant mice were used for experiments. All experiments using mice were reviewed by the Committee for the Ethics on Animal Experiment in the Faculty of Medicine, and conducted under the control of the Guidelines for Animal Experiment in the Faculty of Medicine, Kyushu University, and the Law (no. 105) and Notification (no. 6) of the government of Japan. Blood-stage parasites of P. yoelii were prepared after fresh passage through a donor mouse 2–3 days after inoculation with a frozen stock. Parasitized RBCs (pRBCs) were prepared as previously described (3) and used as a stimulant. Mice 2–3 days after inoculation with a frozen stock. Parasitized RBCs (pRBCs) were prepared as previously described (3) and used as a stimulant. Mice were injected i.p. with 10,000 to 15,000 pRBCs i.p.

Reagents

PE-anti-CD25 (PC61.5), allophycocyanin-anti-CD4 (RM4.4), and FITC-anti-Foxp3 (FJK-16s) staining kits, and FITC-anti-CD11c (N418), PE-anti-CD200 (RA3-6B2), allophycocyanin-anti-CD86, PE-Cy5.5-anti-CD40, FITC-anti-CD69, FITC-anti-CD26L, PE-anti-IFN-γ, purified anti-CD3 (2C11), purified anti-CD16/32 (2.4G2), and purified anti-MHC class II (M5/114.15.2) Abs were obtained from eBioscience. The CD4+ T cell separation kit, plasmacytoid DC (pDC) isolation kit, and anti-plasmacytoid DC Ag-1 (PDCA1), anti-PE, and anti-FITC microbeads (Miltenyi Biotec) were used for cell purification. mAbs to CD4 (GK1.5) or to IFN-γ (R4–6A2), purified from the ascites of hybridoma-injected athymic nude mice, were used for in vivo treatments.

Flow cytometry

For Treg cell analyses, cells in a single suspension were stained with allophycocyanin-anti-CD4 and PE-anti-CD25 followed by intracellular staining with FITC-anti-Foxp3 according to the manufacturer’s protocol. Stained cells were analyzed by FACSCalibur (BD Biosciences) and the list data were analyzed using CellQuest Pro software (BD Biosciences).

Cell purification and cultures

Single-cell suspensions were prepared from spleens or lymph nodes. To purify Tregs, CD4+ T cells were first negatively isolated using a CD4+ T cell separation kit. Then, CD4+ cells were stained with PE-anti-CD25 and labeled with anti-PE microbeads. Positively selected cells were used as Tregs, and others were used as CD4+CD25+ cells. For purification of DCs, splenic single-cell suspensions prepared using collagenase and DNase I were incubated with anti-CD16/32 and then stained with FITC-anti-CD11c, followed by staining with anti-FITC microbeads. pDCs were purified using a pDC isolation kit with slight modifications. After negative isolation of DCs (whole), pDCs were purified using anti-PDCA1 microbeads instead of PE-B220 Ab. The purity of the separated cell subset usually exceeded 92%. T cell-depleted spleen cells of uninfected mice after removal of CD4+ and CD8+ cells were used as APCs.

For activation of Tregs, typically 1.5 × 10^5 purified Tregs were cultured with 1.5 × 10^4 DCs and 2 × 10^4 pDCs for 60 h. Then, Tregs were isolated as live cells. To analyze Treg function, purified CD4+CD25+ cells from uninfected mice stimulated with soluble anti-CD3 Ab or ConA (both 2.5 μg/ml), in the presence of APCs, were cultured with a variety of freshly isolated or cultured Tregs in 200 μl of media (for 72 h) and incubated with 1 μCi/well [3H]thymidine for the last 6–8 h. Radioactivity was measured using a liquid scintillation counter.

In vivo depletion of CD4+ T cells and neutralization of IFN-γ

To deplete CD4+ T cells in vivo, mice were injected i.p. with 250 μg of anti-CD4 Ab 3 days and 1 day before infection. Depletion of CD4+ T cells was evaluated using peripheral blood, from which >95% of CD4+ T cells were depleted. To neutralize IFN-γ, mice were injected with 200 μg of anti-IFN-γ Ab 1 day before and 1 day after infection.
fected mice were mixed with CD4+/CD25+ cells from uninfected mice cultured with DCs were analyzed as in Fig. 1A, using ConA instead of anti-CD3. A, Tregs cultured with (•) or without (○) pRBCs in the presence of DCs were analyzed for their suppressive function. Freshly isolated Tregs (○) were also used. B, Tregs cultured with (circles) or without (triangles) DCs in the absence (open symbols) or presence (filled symbols) of pRBCs were analyzed for their suppressive activity. C, Tregs cultured with DCs from uninfected (circles) or PyL-infected (triangles) mice in the absence (open symbols) or presence (filled symbols) of pRBCs were analyzed. DCs were collected 5 days after infection. Values are means ± SD of triplicate cultures. Asterisks indicate statistical significance at p < 0.05 with the Student t test. These in vitro experiments were repeated at least four times.

Statistical analyses
Differences between groups were analyzed for statistical significance using Excel software with two-tailed the unpaired Student t tests. For survival curves, Kaplan-Meier plots and χ² tests were performed. Probability below 0.05 was considered to be statistically significant.

Results
Infection with malaria parasites activates Tregs
Plasmodium yoelii 17XL strain (PyL), a rodent malaria parasite, is highly virulent in mice and causes lethal infection. We previously reported that high susceptibility to this parasite correlates with severe immune suppression induced by activation of Tregs, and that depletion of Tregs before infection made mice resistant to the otherwise lethal infection; this resistance was associated with a reversal of T cell unresponsiveness against the parasite (8). As early as 5 days after infection with PyL, when parasites began to be detected in the circulation, the proportion of CD4+CD25+ T cells increased in the spleen (Fig. 1A). Because CD25 is not a specific marker for Tregs and is expressed in activated non-Treg T cells, we analyzed the expression of Foxp3. Foxp3 is a forkhead/winged-helix transcription factor specifically expressed in Tregs, and its marker for Tregs and is expressed in activated non-Treg T cells, we analyzed the expression of Foxp3. Foxp3 is a forkhead/winged-helix transcription factor specifically expressed in Tregs, and its expression is associated with the development and function of Tregs (25, 26). At this time point, most CD4+CD25+ T cells in the spleens of both PyL-infected and uninfected mice were Foxp3+ (Fig. 1A). The percentage of CD25+Foxp3+ cells among CD4+ T cells and the total number of splenic Foxp3+CD4+CD25+ cells in PyL-infected mice were significantly increased after infection (Fig. 1B). We next evaluated the suppressive function of Tregs by determining the degree of suppression of TCR-triggered T cell proliferation. Purified CD4+CD25+ T cells obtained from PyL-infected mice were mixed with CD4+CD25− T cells obtained from uninfected mice stimulated by TCR engagement in the presence of APCs, CD4+CD25+ T cells from infected mice showed remarkable suppressive activity compared with those from uninfected mice (Fig. 1C). Such alterations in suppressive function were not observed in Tregs from mesenteric lymph nodes, in which no parasite was detected during infection (Fig. 1C), suggesting that Treg activation occurs after intimate contact with parasites.

Malaria parasites interact with DCs to activate Tregs
We next analyzed how Tregs are activated during malaria infection. Some cytokines secreted by lymphocytes are reported to be important for Treg activities. To determine the cellular requirements for Treg activation, RAG2−/− mice received 1 × 10⁶ Tregs from syngeneic mice, and they were then infected with PyL. Because CD25 expressed by Tregs disappears in the inflammatory environment in lymphopenic hosts (7), CD4+ cells were purified. Approximately 1 × 10⁶ and 1.4 × 10⁶ cells were recovered from uninfected and PyL-infected mice, respectively (not significant). Tregs recovered from uninfected mice still suppressed TCR-triggered T cell proliferation in a dose-dependent manner. Infection of recipient mice with PyL significantly enhanced this suppressive function, as observed in immunocompetent mice (Fig. 2), indicating that no lymphocytes other than Tregs are required for the activation of this function of Tregs. These results suggest that the interactions between Tregs, APCs, and pRBCs are sufficient for Treg activation.

To evaluate this possibility, we tried to reproduce Treg activation in vitro. Tregs from uninfected mice cultured with pRBCs and CD11c+ splenic DCs, as APCs, were analyzed for a suppressive function. There was no evidence for Treg proliferation, even in the presence of pRBCs, as determined by the incorporation of [3H]thymidine or CFSE dilution (data not shown). Recovered Tregs maintained their suppressive function at a comparable level to freshly isolated Tregs. The addition of pRBCs promoted the suppressive activity of Tregs (Fig. 3A). This enhancement was associated with parasites, because the addition of normal RBCs did not enhance Treg activities (data not shown).

Thus, an in vitro system can be used to study Treg activation. First, we cultured Tregs with DCs in the absence of pRBCs or with pRBCs in the presence of DCs. As shown in Fig. 3A, the addition of pRBCs promoted the suppressive activity of Tregs. These results suggest that Tregs from infected mice showed remarkable suppressive activity compared with those from uninfected mice (Fig. 1C). Such alterations in suppressive function were not observed in Tregs from mesenteric lymph nodes, in which no parasite was detected during infection (Fig. 1C), suggesting that Treg activation occurs after intimate contact with parasites.

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reproduce Treg activation during infection in vivo. Using this system, further analyses were performed. First, to pinpoint the cellular interactions of pRBCs with Tregs and DCs, Tregs were stimulated with pRBCs in the absence of DCs. The addition of pRBCs did not augment Treg function in this setting (Fig. 3B). Furthermore, DCs from PyL-infected mice activated Tregs, even in the absence of pRBCs (Fig. 3C). These results demonstrate that malaria parasites interact with DCs to activate Tregs. Malaria parasites have been reported to inhibit the maturation of DCs (3), and immature DCs preferentially activate Tregs (13), suggesting that PyL suppresses DC maturation. Thus, we checked the status of pRBC-interacting DCs capable of activating Tregs. The exposure of DCs to malaria parasites slightly enhanced the expression of CD40 and CD86, both in vitro and in vivo (Fig. 4A). Moreover, the Ag processing/presenting capacity of these DCs to activate OVA-specific OT-II CD4+ T cells was identical with that of untreated DCs (Fig. 4B). These results exclude the possibility that malaria parasites down-regulate DC activities.

**TLR9 signaling in DCs is required for Treg activation**

Parasite-derived molecules usually contact DCs after being processed in phag-endosomes (27). In contrast, pRBCs express some molecules derived from the parasites on their surfaces, and this enables parasites to interact with the surfaces of DCs without phagocytosis (3). We next examined whether endocytic pathways are required to make DCs competent for Treg activation. Inhibition of endosomal maturation with ammonium chloride, a reagent that blocks endosomal acidification, precluded enhancement of the suppressive function of Tregs (Fig. 5A).

Foreign Ags phagocytosed by DCs are proteolytically processed and undergo Ag presentation to MHC class II molecules (28). The necessity for engulfment of pRBCs by DCs for Treg activation might be explained by the ability of DCs to activate Ag-specific Tregs. To address this issue, we used Tregs isolated from OVA-specific TCR-transgenic OT-II mice (29). Stimulation of these Tregs with pRBCs resulted in an enhancement of their suppressive function. Additionally, infection of OT-II mice with PyL also activated Tregs (Fig. 5B). We could not exclude the possibility of an involvement of remnant Tregs with non-OT-II TCR in the enhancement of Treg function, because RAG-deficient OT-II mice could not be used owing to requirement of TCR rearrangement for the development of Tregs (30). To further confirm Ag-nonspecific Treg activation, we used an anti-MHC class II Ab in Treg cultures with pRBCs. The enhancement of the suppressive function of Tregs by adding pRBCs was not altered, even in the presence of the anti-class II Ab, which completely blocked the OVA-specific proliferation of CD4+CD25+ cells isolated from OT-II mice (Fig. 5C).
To activate Tregs, phagocytosed pRBCs might not be displayed as Ags, but they were thought to provide signals to DCs. We hypothesized that TLRs are involved in this interaction. To investigate the possible roles of TLRs, we used DCs obtained from mice lacking MyD88 or TRIF, both of which are essential adapter molecules for TLR signaling (21, 22). As shown in Fig. 6A, DCs from TRIF−/− mice were capable of activating Tregs as well as those from wild-type (WT) mice. By contrast, Tregs were not activated when cultured with DCs from MyD88−/− mice. The dependency of Treg activation on MyD88 and the endosomal localization allowed us to deduce that the TLRs likely to be involved are TLR7 and TLR9. TLR7 recognizes single-stranded RNA from viruses (23), while TLR9 recognizes DNA containing unmethylated CpG motifs (24). Recently, some reports have found that malaria parasites express molecules that are recognized by TLR9 (31, 32), suggesting that TLR9 is a much more likely candidate. To confirm this, we conducted studies using mice lacking TLR7 or TLR9. As expected, DCs from TLR7−/− mice, but not those from TLR9−/− mice, were able to activate Tregs (Fig. 6B). TLR9 is expressed predominantly by pDCs, which are reported to be involved in Treg induction (33). However, our experiments denied the involvement of pDCs in Treg activation during malaria infection. Purified PDCA1+ pDCs could not activate Tregs in vitro, presumably due to an inability to ingest pRBCs, whereas DCs depleted of pDCs could (Fig. 6C). These results indicate that TLR9 plays a critical role in the interaction between pRBCs and myeloid DCs, which underlies the activation of Tregs, and thus prompted us to examine the susceptibility of TLR9−/− mice to infection with PyL.

**TLR9-deficient mice were partially resistant to lethal infection with PyL**

As reported previously, rapid growth of the parasite occurred in WT mice, and these mice succumbed to infection within 2 wk (Fig. 7A); the overall mortality was >90% (30 of 32, Fig. 7B). Surprisingly, TLR9−/− mice were partially resistant to infection. Some mice were able to tolerate the second peak of parasitemia and ultimately survived, while other mice had only low levels of parasitemia (Fig. 7A). Cumulatively, 14 of 28 TLR9−/− mice survived (Fig. 7B). One possibility that needed to be excluded was that the WT mice died of hyperinflammation, such as CpG shock induced by the infection, and that the loss of TLR9 signaling merely reduced the immunopathology. Therefore, we measured the levels of proinflammatory cytokines in the sera of WT and TLR9−/− mice, but we were not able to detect IL-6, IL-12p70, or TNF-α, even after infection. The resistance of TLR9−/− mice was observed only when those mice were infected with PyL that activates Tregs. Infection of TLR9−/− mice with PyNL strain, which does not cause Treg activation, did not alter the course of infection (Fig. 7A). These results indicate that the loss of TLR9 might affect Treg activation but not immune responses unrelated to Treg activation.

**Infection with PyL failed to activate Tregs in TLR9−/− mice**

To relate the partial resistance against PyL infection to the impairment of Treg activation in TLR9−/− mice, we analyzed Treg function in TLR9−/− mice. Splenic Foxp3+CD4+CD25+ cells were increased in TLR9−/− mice, similarly to those in WT mice, 5 days after infection. A similar increase was observed only when those mice were infected with PyL that activates Tregs. Infection of TLR9−/− mice with PyNL strain, which does not cause Treg activation, did not alter the course of infection (Fig. 7A). These results indicate that the loss of TLR9 might affect Treg activation but not immune responses unrelated to Treg activation.

**FIGURE 7. Resistance of TLR9−/− mice to PyL infection.** A, The kinetics of parasitemia in WT or TLR9−/− mice infected with PyL (upper panels) or with PyNL (a nonlethal variant of PyL; lower panels) was monitored by microscopic evaluation of Giemsa-stained thin blood films. Each symbol represents a value from an individual mouse. The numbers represent days of mouse death and “s” shows mice that survived. Similar results were obtained from at least three experiments. B, The cumulative mortality rate of infection with PyL in WT (○) or TLR9−/− mice (▲) is shown. p < 0.001 for the percentage survival of WT vs TLR9−/− mice by χ² test.

**FIGURE 8. Impaired Treg activation in TLR9−/− mice after infection with PyL.** A, Flow cytometric analysis of Foxp3 expression in CD4+CD25+ T cells. Splenocytes obtained from WT (left panels) or TLR9−/− (right panels) mice with (bottom panels) or without (top panels) infection with PyL were analyzed as in Fig. 1A. Results are representative of five experiments. B, Suppressive function of Tregs in TLR9−/− mice infected with PyL. Splenic Tregs obtained from uninfected (open symbols) or PyL-infected (filled symbols), WT (circles) or TLR9−/− (triangles) mice were mixed with CD4+CD25+ T cells at the indicated ratio. C, Transferred Ly5.1+ Tregs recovered from WT (circles) or TLR9−/− (triangles) recipients before (open symbols) or after (filled symbols) PyL infection were analyzed for their suppressive activity. Values are means ± SD of triplicate cultures. Asterisks indicate statistical significance at p < 0.05 with the Student t test. These experiments were repeated at least three times.
after infection (Fig. 8A). Infection of WT mice with PyL again enhanced the suppressive function of Tregs, and infection of TLR9−/− mice failed to do so, although there was no difference in the suppressive efficacy between TLR9−/− and WT Tregs before infection (Fig. 8B). This failure might be attributable to the inclusion of Foxp3− cells among CD4+CD25+ cells, because TLR9−/− mice had more CD4+CD25+Foxp3− cells, which are thought to be effector cells originating from the pool of CD4+CD25− cells (Fig. 8A). To exclude this possibility, we infected TLR9−/− mice into which Tregs from Ly5.1 mice had been adoptively transferred, and Ly5.1+ cells recovered from the infected mice were analyzed for suppressive activity. Ly5.1+ Tregs recovered from WT mice showed enhanced suppression after infection. By contrast, no enhancement of Treg function was observed in TLR9−/− recipients (Fig. 8C). These results confirm that Tregs are not activated in TLR9−/− mice, regardless of the expression of TLR9 on Tregs themselves.

Finally, we analyzed immune responses in PyL-infected TLR9−/− mice. CD4+ T cells and their product IFN-γ are known to be important for protection against blood-stage malaria (34, 35). Flow cytometric analyses revealed that infection of TLR9−/− mice with PyL increased activated (CD62Llow, CD69high) and IFN-γ populations among splenic CD4+ T cells, compared with infection of WT mice (Fig. 9, A and B). Furthermore, CD4+ T cells from TLR9−/− mice proliferated in response to pRBCs, while those from WT mice showed only a marginal response (Fig. 9C). We depleted CD4+ T cells or neutralized the IFN-γ from these mutant mice. These manipulations completely abolished the partial protection observed in TLR9−/− mice (Fig. 9D), indicating that CD4+ T cells were efficiently activated under conditions in which Tregs are not activated in TLR9−/− mice.

Discussion

We herein demonstrate that malaria parasites activate Tregs through TLR9 engagement in DCs. PyL did not exert its full virulence in TLR9−/− mice, indicating that TLR9-mediated Treg activation is an important strategy used for immune escape by this parasite. The alterations observed in TLR9−/− mice, a failure of Treg activation and subsequent effector T cell activation, are dependent on TLR9-deficient DCs, because T cells, including Tregs, do not express TLR9 (17, 19). Among a variety of immune evasion mechanisms, DCs, which play central roles in establishing immunity, are the major target for malaria parasites. For instance, malaria parasites interfere with the maturation of DCs (3, 36, 37) or prevent Ag cross-presentation (38), both of which result in the failure to directly activate protective/effector T cells. Unlike these observations, our findings propose a novel interaction of malaria parasites with DCs via TLR9 that affects Tregs rather than protective T cells.

It is generally thought that TLRs are crucial for the induction of innate and acquired immunity (39). Some reports have shown that mice deficient in a TLR show higher susceptibility to pathogens recognized by the corresponding TLR (40, 41). By contrast, recent reports have shown TLR-mediated immune suppression. Upon stimulation through TLRs, on the one hand, DCs decrease the susceptibility of effector T cells to suppression mediated by Tregs (15). Stimulation of Tregs via TLR8 or TLR2 reverses the suppressive function of Tregs (17, 18). These behaviors drive toward the development of immunity. In contrast, systemic or excessive activation of TLRs in DCs has been reported to induce several types of immune suppression (38, 42). Stimulation with a large amount of LPS, a TLR4 ligand, and the TLR5 ligand flagellin...
directly activate Tregs as determined by their enhanced suppressive function (19, 20). Thus, TLRs contribute to controlling the balance between Tregs and effector T cells by affecting both DCs and Tregs. Furthermore, TLR signaling provides negative feedback mechanisms for preventing immunopathogenesis when stimulation is saturated. Because TLR9 recognizes endogenous ligands and is involved in the development of autoimmune diseases (43, 44), this type of signal could have powerful regulatory functions, such as activation of Tregs, which are suppressors of autoimmune disease. Indeed, TLR9 signaling plays a protective role in the development of autoimmunity by modulating Treg activity in autoimmune-prone MRL mice (45). Furthermore, common polymorphisms of TLR9 are reported to be associated with the clinical manifestation of malaria during pregnancy (46). It is quite possible that malaria parasites cleverly exploit this machinery by providing a large amount of TLR9 stimulant.

Recently it was reported that Tregs contribute to the pathogenesis of cerebral malaria by suppressing antimalarial immunity during infection with Plasmodium berghei ANKA (47). Tregs appeared to be activated in this model. It would be of interest to analyze whether TLR signaling is involved in this Treg activation, although the roles of TLRs in the development of cerebral malaria are controversial (48, 49).

Although we have not identified any TLR9 ligands derived from malaria parasites, the quantity of TLR9 signaling might be a key factor in the activation of Tregs. TLR9+/− heterozygous mice had a similar phenotype to TLR9−/− mice, but not to WT mice, in terms of a high resistance to malaria and an absence of Treg activation after infection (data not shown). Additionally, we postulate that the quality of parasite-derived TLR9 ligands is also important in the activation of Tregs for the following reasons. First, CpG-triggered DCs did not activate Tregs (data not shown). Second, stimulation of DCs with pRBCs did not induce production of IFN-α, which is secreted from DCs activated by CpG (data not shown). Given these findings, hemozoin, a known parasite ligand for TLR9, is a possible candidate ligand. Hemozoin is abundant in pRBCs, and it does not induce IFN-α production upon stimulation of DCs (31). Indeed, hemozoin-related immune suppression has been previously reported (50). Recently, the concept of malaria hemozoin stimulating TLR9 has been revised to malarial DNA presented by hemozoin (51). Careful investigations are required to identify the molecule(s) responsible for TLR9 stimulation.

Another issue to be clarified is how DCs that have interacted with malaria parasites activate Tregs. Infection of WT mice with PyL activated Tregs in terms of proliferation and suppressive functions. Cultivation of Tregs with DCs and pRBCs enhanced suppression, but did not induce proliferation. By contrast, infection of TLR9−/− mice increased the number of Tregs but did not enhance suppression. These results suggest that the regulation mechanisms for the augmentation of suppression might be different from those for proliferation. The failure of cultivation to increase the number of Tregs suggests that factors derived from other cell types, such as IL-2, are required for Treg proliferation. We postulate that parasite-simulated DCs up-regulate Foxp3 expression in Tregs, because flow cytometric analyses (Figs. 1A and 8A) show a slight increase in Foxp3 protein level in Tregs from infected mice, and because we found that the level of Foxp3 mRNA was increased in Tregs from mice infected with PyL (52). Ag-nonspecific augmentation of the suppressive activity of natural Tregs observed here has been reported after TLR5 ligation in human Tregs in vitro (20) or after infection of mice with helminth (53), both of which are associated with enhanced Foxp3 expression. TGF-β is important for Foxp3 expression, and both the increase in the number of Foxp3+ Tregs and the production of TGF-β are associated with higher rates of parasite growth in human falciparum malaria (8), suggesting that TGF-β is likely to be responsible for Treg activation. However, our preliminary experiments showed that although the neutralization of TGF-β during PyL infection in mice augmented protective immunity, it did not attenuate Treg activation. Recently we found that malaria parasites induce DCs with a suppressive phenotype expressing IDO (54), which is known to be induced in DCs stimulated with systemic CpG injection (42). It would be interesting to analyze the involvement of this suppressive enzyme in Treg activation. Indeed, clarification of the molecular basis of Treg activation is our next objective.

In conclusion, we propose a novel model for the functional regulation of Tregs as well as for the immune escape of malaria parasites, which may enable us to establish new approaches to developing effective immunity against malaria or preventing autoimmunity by correcting the balance between Tregs and effector/pathogenic T cells.

Disclosures
The authors have no financial conflicts of interest.

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