have recently revealed that $CD4^+CD25^+$ regulatory T cells play a crucial role in interfering with the initiation of protective immunity in very early stages after the infection in a rodent model (Hisaeda et al., 2004). Many issues remain to be elucidated before the establishment of an effective method for controlling malaria, including the development of effective vaccines. First of all, however, the complicated and inscrutable evasion strategies of this parasite should be elucidated.

2. Pathogenesis

2.1. Life cycle of malaria parasites and major symptoms of infection

Malaria parasites undergo a complicated life cycle (Fig. 1). Infection is initiated by inoculation of sporozoites, an infectious form of the parasite, through mosquito bites. Sporozoites multiply in the liver, a single sporozoite develops 30,000–40,000 merozoites that are released into the blood stream. Each of these can invade a red blood cell (RBC). Inside a RBC, the merozoite undergoes morphological changes into a trophozoite. This asexually divides to generate up to 32 merozoites over a period of 2 or 3 days, depending on the species of parasite. Mature parasites rupture the host cell and each merozoite continues the cycle. After invading a RBC, a small proportion of asexual parasites convert into male or female gametocytes, which are essential for transmitting the infection to other hosts through female anopheline mosquitoes.

A newly infected person has no symptoms for approximately 1–2 weeks. During this latent period, replication occurs in the liver and initial replication in the blood. All symptoms commence when the parasites undergoing an asexual blood cycle, reach a level sufficient to generate the host's pathogenic process. Fever, a hallmark of malaria, is due to parasite-derived molecules, which are released from ruptured host cells activating inflammatory cells such as macrophages. These secrete pro-inflammatory cytokines include powerful endogenous pyrogens, such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α. Typically, *Plasmodium vivax* takes 48 h (tertian malaria) and *Plasmodium malariae* takes 72 h (quartan malaria) to undergo a complete cycle in RBCs. As the cycles become synchronized, the periodic fever and other symptoms associated with

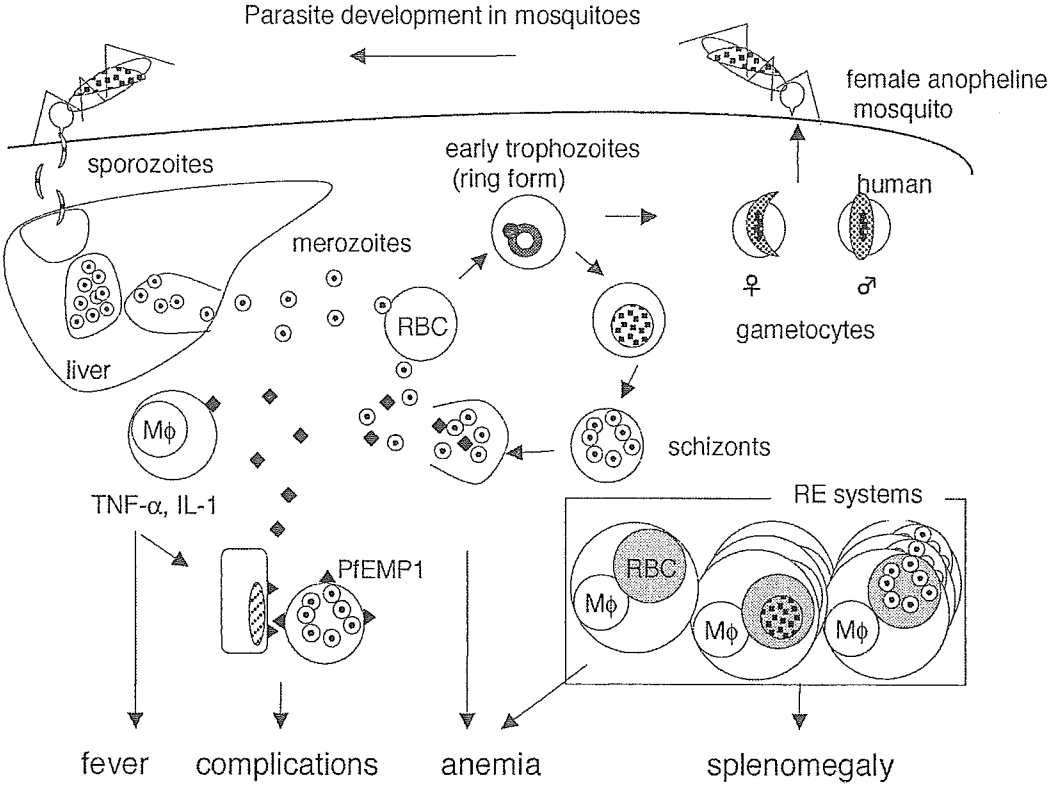

Fig. 1. Life cycle and pathogenesis of malaria parasites.

malaria paroxysms become locked into the same period, triggered by the near simultaneous rupture of the infected RBCs.

The reticuloendothelial system is activated to clear infected RBCs, resulting in remarkable splenomegaly. Patients with malaria also suffer from anemia. Several mechanisms are considered as the etiology, such as destruction of infected and normal RBCs by the activated reticuloendothelial system and bone marrow suppression.

2.2. Cerebral malaria

A major cause of death in patients with *P. falciparum* malaria is cerebral malaria resulting from occlusion of brain microvessels sequestered with infected RBCs (Miller, Baruch, Marsh, & Doumbo, 2002). The pathogenic processes are triggered by the release of immune stimulatory molecules from the infected RBCs upon rupture. Parasite-derived toxic molecules stimulate endothelial cells directly or indirectly through pro-inflammatory cytokines produced by immune cells, and the stimulation of endothelial cells induces expression of adhesion molecules, such as intercellular adhesion molecule (ICAM)-1, CD36, vascular cell adhesion molecule (VCAM)-1, and endothelial/leukocyte adhesion molecule (ELAM)-1, on the surface of the endothelium.

On the other hand, unlike other parasites, *P. falciparum* modifies the surfaces of RBCs so that infected RBCs can bind to the endothelium as asexual development progresses (Luse & Miller, 1971). As a consequence, only early trophozoites (ring forms) and gametocytes of *P. falciparum* are found in circulating blood. The surfaces of *P. falciparum* trophozoite- and schizont-infected RBCs have many knob-like processes that provide contact points with host cells and infected RBCs. Among the many parasite-molecules involved in the processes, only a single protein, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), is responsible for parasite binding to endothelial cells. Although the various endothelial cells express different and variable amounts of adhesion molecules, PfEMP1 can bind to all of the various receptors (Table 1). The binding to chondroitin sulphate A expressed by the placenta, one of the pivotal bindings of PfEMP1, results in premature labor or abortion in pregnant women (Fried & Duffy, 1996).

2.3. Host immune responses to malaria parasites

Considerable evidence has revealed that antibodies and T cells play crucial roles in protective immunity against malaria parasites (Good, Kaslow, & Miller, 1998). Antibody responses are elicited against sporozoites, inhibiting sporozoite invasion of hepatocytes. $CD8^+$ T cells exhibit cytotoxicity to hepatocytes, in which sporozoites mature. If the release of merozoites from the liver into the bloodstream is prevented, the infection could be terminated before disease onset.

$CD4^+$ T cells are indispensable for protection against blood-stage parasites by providing assistance for antibody production and by secreting interferon (IFN)-γ for activation of macrophages to promote clearance of infected RBCs. Antibodies to molecules on the merozoite surface break the blood cycle by blocking merozoite invasion of new RBCs. In addition to immunity breaking the parasite cycle, immunity to interrupt the pathogenic processes is important for protection from *falciparum* malaria. Antibodies to the glycosylphosphatidylinositol (GPI) portion of GPI-anchored proteins, one of the putative immune stimulatory molecules, could suppress the activation of macrophages, resulting in failure of production of pro-inflammatory cytokines (Schofield, Hweitt, Evans, Siomos, & Seeberger, 2002). Furthermore, antibodies to PfEMP1 on the surfaces of infected RBCs interfere with binding to endothelial cells (Giha et al., 2000). Thus, these antibodies reduce the chance of binding of infected RBCs to endothelial cells. Immune responses

Table 1
Symptoms related to PfEMP1 functions in *falciparum* malaria

Symptom/phenomenon	Pathology	Host molecules involved
Cerebral malaria	Binding to endothelium	ICAM-1, VCAM-1, CD36, etc.
Placental malaria	Binding to placenta	Chondroitin sulphate A
Immune suppression	Binding to dendritic cells	CD36
Antigenic wave of parasitemia	Clonal antigenic variation	Specific antibodies

to blood-stage parasites contribute to reduction in disease severity by eradicating the parasites and/or by preventing pathogenesis.

Hosts also develop transmission-blocking antibodies that recognize molecules expressed by gametocytes or extracellular gametes and interfere with the development of parasites in the mosquito vectors, preventing the spread of the parasite. Although this kind of immunity does not protect infected individuals, it does help reduce infection at the community level (Snewin et al., 1995).

2.4. Immune evasion of malaria parasites

2.4.1. Intracellular parasitism

Intracellular parasitism is a primitive escape mechanism of microbes. Antibodies bind to free sporozoites or merozoites, but they can no longer access them once the parasites enter the host cells. Furthermore, as RBCs express no MHC molecules on their surfaces, merozoites escape recognition by $CD8^+$ T cells.

2.4.2. Adherence of infected red blood cells to endothelial cells

Adherence of infected RBCs to endothelial cells is responsible for the severe pathogenesis as described above. This mechanism might have been originally acquired as an immune evasion strategy, since adherence may protect the parasites from entering the spleen and liver, where mature parasites are trapped and digested.

2.4.3. Antigenic diversity/polymorphism

As antibodies and T cells recognize antigen epitopes in a very strict manner, they no longer recognize the antigenically different epitopes. Therefore, changing antigenicity is a major strategy for parasites to escape from host immunity. One strategy is antigenic diversity/polymorphism, which involves the expression of antigenically different alleles of a gene in different parasite populations and presumably results from selection under the immune pressures. In the presence of specific immunity, parasites expressing mutated alleles that are not recognized by the immune system, would selectively expand. In fact, antigens eliciting immune responses often show extensive polymorphisms. This characteristic is the most troublesome hurdle to overcome in the development of effective malaria vaccines. Merozoite surface protein-1 (MSP-1), a leading vaccine candidate, has many alleles (Qari et al., 1998) and some antibodies to an MSP-1 allele do not recognize the others (Burns et al., 1989). Also, antigen polymorphism affects T cell recognition more severely, because T cell recognition depends on the primary structure (amino acid sequence) of proteins rather than protein conformation, which is often recognized by antibodies. Substitution of only one amino acid in a protective T cell epitope results in the failure to activate protective T cells (Plebanski et al., 1999).

2.4.4. Molecular smokescreen

Tandem repeats are found in many malaria antigens, which provide immunodominant B cell epitopes. Individuals who have had repeated infections have high concentrations of antibodies to these regions in serum, though they are still susceptible to new infections, suggesting that antibodies that contribute to a lesser extent to protection are produced. Together with the generation of antigen diversity, it is postulated that tandem repeats have a pivotal function in immune evasion. The presence of polymorphic repeats in antigens that are not targets of protective immunity, affects affinity maturation of antibodies and thus masks the critical epitopes. Tandem repeats also induce T cell-independent B cell activation by cross-linking their surface immunoglobulins and suppressing antibody responses to important adjacent regions.

2.4.5. Clonal antigenic variation

Unlike antigens associated with intracellular parasites, PfEMP1 on the surface of infected RBCs is accessible to antibodies. This is unfavorable for the parasites, as PfEMP1 is the only protein by which essential functions, such as adhesion to endothelium, are achieved. PfEMP1 is equipped with surprising immune evasion mechanisms. Malaria parasites in mammal hosts are haploid, and only one allele per locus is expressed in blood-stage parasites. The gene encoding PfEMP1, termed *var* gene, is present in multiple loci (approximately 50) on different chromosomes and parasites are able to switch expression between different loci in blood stages (Smith et al., 1995). Antigenic variants may escape the primary antibody response to PfEMP1 expressed by a new infecting parasite clone and show second and third recrudescence, which is called antigenic distinct wave of parasitemia (Table 1).

2.4.6. Total immune suppression

Malaria patients often show reduced immune responses not only to malaria parasites but also to unrelated antigens, including vaccines (Williamson and Greenwood, 1978), suggesting that an active immune suppression mechanism may operate during the course of malaria.

Activated T cells play major roles in immune responses. Naive T cells recognize epitopes generated from parasite antigens in context with MHC molecules on antigen-presenting cells (APCs) via T cell receptors (TCRs) and the engagement of TCR provides the primary signal to T cells. To fully activate T cells, a secondary co-stimulatory signal provided by mature APCs is required. During infection, T cell responses develop effectively because infectious agents induce maturation of APCs through microbial common pattern molecules that bind to Toll-like receptors (TLRs). These are key receptor for activating the innate immune system and have evolved as sensing receptors for microbes (Takeda, Kaisho, & Akira, 2003). However, *P. falciparum* inhibits the maturation of APCs, resulting in impaired T cell responses to antigens unrelated to malaria. Binding of PfEMP1 on *P. falciparum*-infected RBCs to CD36 expressed on APCs is responsible for maturational arrest of APC (Urban et al., 1999). In a mouse malaria model, blood-stage parasites also interfered with APC function, inhibiting maturation and the capacity to initiate immune responses and inverting the protective IL-12/suppressive IL-10 secretion pattern (Ocana-Morgner, Mota, & Rodriguez, 2003).

Induction of suppressive cytokines, such as IL-10 and transforming growth factor (TGF)-β, is a major strategy of the parasites and high levels of those cytokines are found in the sera from patients with acute infections. The parasites stimulate T cells or APCs (Ocana-Morgner et al., 2003) to produce the cytokines and directly activate TGF-β. The alteration of T cell epitopes recognized by protective T cells, antagonizes T cell signaling and induces production of suppressive IL-10 instead of production of protective IFN-γ (Plebanski et al., 1999). TGF-β usually exists as an inactive latent form and the latent TGF-β is activated by limited proteolysis with proteases such as thrombospondin or calpain. Malaria homologues to thrombospondin and molecules with matrix metalloprotease activity can activate TGF-β (Omer, de Souza, Corran, Sultan, & Riley, 2003). *P. falciparum* itself also produces prostaglandins that are not cytokines but immune suppressive biological compounds that can act on APCs as well as T cells (Kilunga Kubata et al., 1998).

Among $CD4^+$ T cells, regulatory T cells compose a unique population. They constitutively express CD25, the IL-2 receptor α chain and play a crucial role in maintenance of self-tolerance by regulating the activation/proliferation of autoreactive T cells (Shevach, 2002). In addition to the important role of regulatory T cells in the prevention of autoimmunity, recent studies have demonstrated that regulatory T cells are involved in immune responses to infectious agents (Belkaid, Piccirillo, Mendes, Shevach, & Sacks, 2002). We recently proposed that malaria parasites exhibit a novel immune evasion mechanism via preferential activation of regulatory T cells (Hisaeda et al., 2004). A rodent malaria parasite, *Plasmodium yoelii*, has two substrains: one is highly virulent in mice and causes lethal infection (PyL) and the other causes a self-limiting, nonlethal infection (PyNL). Mice infected with PyNL developed parasite-specific antibodies and T cell responses at early stages of infection. In sharp contrast, mice infected with PyL showed impaired immune responses to the parasites as well as to non-specific stimulation in association with an increase in $CD4^+CD25^+$ T cells with enhanced suppressive activity. Furthermore, the depletion of regulatory T cells allowed mice to survive infection with PyL. These findings clearly indicate that the immune suppression observed in lethal malaria is associated with activation of regulatory T cells.

3. Potential for future therapy

Insights into host–parasite interaction could be applied to the development of vaccines as well as the development of therapeutic reagents. Vaccination with synthetic GPI has been shown to induce antibodies that prevent cerebral malaria in a rodent model (Schofield et al., 2002), indicating the possibility of developing an anti-toxin vaccine. Information on important molecules (or regions) that the parasites conceal from host immunity would be useful for developing vaccines. PfEMP1 is considered to be the Achilles heel of *P. falciparum*, because it is responsible for much of malaria pathology and it is

encoded by huge loci, presumably to protect its function (Table 1). Thus, this molecule is a good vaccine candidate. Indeed, vaccine trials using recombinant proteins of the conserved functional region of PfEMP1 have been extensively performed and the immunization of monkeys induced protection against infection with *P. falciparum* (Baruch et al., 2002).

4. Conclusion

Immune evasion of malaria parasites has been summarized. In the attempt to develop a malaria vaccine, immune responses to malaria parasites have been extensively examined. Further investigations should reveal the versatility of malaria parasites in evading host immunity. To develop better methods for controlling malaria, our understanding of the host–parasite relationship is critical. Two promising areas of development that come from this knowledge are the development of new drugs that inhibit the activation of suppressive cytokines by parasite proteases and the development of effective vaccines that overcome immune evasion mechanisms.

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Resistance of regulatory T cells to glucocorticoid-viduced TNFR family-related protein (GITR) during *Plasmodium yoelii* infection

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$CD4^+$ T cells are the major effector T cells against blood-stage *Plasmodium yoelii* infection. On the other hand, the lethal strain of *P. yoelii* (PyL) has acquired an escape mechanism from host T cell immunity by activating $CD4^+CD25^+$ regulatory T cells (Treg). Although the activation of Treg during PyL infection precludes the clearance of PyL from mice, it remains unclear whether activation of Treg is attributable to a specific response against PyL infection. Thus, we examined here whether Treg proliferate in an antigen-dependent manner during PyL infection. We also investigated the effector and regulatory mechanisms of Treg. Infection with PyL increased the number of $CD4^+CD25^+$ T cells, in which expression of Foxp3 mRNA is up-regulated. The Treg that were transferred into mice infected with PyL, but not with a non-lethal strain of *P. yoelii* (PyNL), proliferated during the initial 5 days following infection. The Treg from PyL-infected mice showed strong suppression compared with those from naive or PyNL-infected mice, and could suppress T cell activation by recognizing PyL- but not PyNL-derived antigens. Furthermore, the suppressive function of Treg activated in PyL-infected but not in naive mice could not be inhibited by treatment with an anti-glucocorticoid-induced TNFR family-related protein (GITR) mAb. These findings indicate that PyL infection specifically activates Treg that are specific for PyL-derived antigens. The infection also induces resistance for Treg to GITR signaling, and this eventually contributes to the escape of parasites from host T cell immunity.

Key words: Immune escape · Malaria · Regulatory T cells

Introduction

The immune system exploits clonal diversity in order to combat pathogens that produce a variety of antigens. In contrast, pathogens such as parasites and viruses have evolved mechanisms to escape from host immune systems in order to survive within the host. Many such evasion strategies have been demonstrated [1, 2]. For instance, certain viruses encode viral cytokines or chemokines that inhibit the host immune system, which

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Abbreviations: **GITR:** glucocorticoid-induced TNFR family-related protein · **MCC:** moth cytochrome c · **pRBC:** parasitized red blood cells · **PyNL:** non-lethal strain of *Plasmodium yoelii* · **PyL:** lethal strain of *Plasmodium yoelii* · **Treg:** regulatory T cells

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is then unable to clear the viruses [3]. Also, adenoviruses suppress the MHC class I antigen presentation pathway, and this eventually impairs maturation of lytic $CD8^+$ T cells to kill viral infected cells [4]. Optimization of therapeutic strategies and vaccine against pathogens will benefit from detailed knowledge of these escape mechanisms.

Malaria parasites cause approximately 2 million deaths per year [5]. Antibodies and T cells play crucial roles in protective immunity against malaria parasites [6]. Antibodies to merozoites and parasitized red blood cells (pRBC) can reduce disease severity by inhibiting new RBC infections by merozoites, or by inhibiting the cytoadherence of pRBC to the capillary endothelium [7]. $CD8^+$ T cells exhibit cytotoxicity to hepatocytes, where sporozoites mature [8]. $CD4^+$ T cells are indispensable for protection against blood-stage parasites [9]. However, it is difficult to acquire long-lasting protective immunity, despite frequent exposure to the parasite in endemic areas. There are several reasons to explain this failure. For example, antigenic diversity results in altered T cell epitopes that prevent a protective T cell response [10]. Clonal antigenic variation of molecules expressed on merozoites or pRBC hide the parasite from antibody recognition [11–14]. One recent report demonstrated that pRBC impair the maturation of dendritic cells that initiate T cell immune responses [15]. Malaria patients also show some level of global immunosuppression [16–18].

We have recently revealed that T cells expressing both CD4 and CD25 (IL-2R α chain) (regulatory T cells, Treg), which are known to suppress autoreactive T cells [19–22], inhibit the activation of effector T cells against antigens from the blood stage of the lethal strain of *Plasmodium yoelii* (PyL) [23]. This inhibition of effector T cell activation finally induces host death, while the depletion of Treg enables mice to survive [23]. These studies suggest that activation of Treg is one of the escape mechanisms for malaria parasites from host T cell immunity [23]. Although those studies have revealed the clear contribution of Treg to the inhibition of effector T cell activation, it remains unclear whether activation of Treg is attributable to the specific recognition of PyL-derived antigens.

We here demonstrate that infection with PyL induces proliferation of Treg during a very early stage of infection, which is, at least partly, dependent on the recognition of PyL-derived antigens. Furthermore, the suppressive function of Treg during PyL infection could not be inhibited by anti-glucocorticoid-induced TNFR family-related protein (GITR) mAb, in contrast to complete relief of suppressive activity of Treg from naive mice. These studies have revealed that PyL infection induces rapid proliferation of Treg and enables Treg to acquire GITR-resistant mechanisms, which are responsible for the strong function of Treg during PyL infection.

Results

Infection with PyL increases $CD4^+CD25^+$ cells and their Foxp3 expression

We have previously shown that C57BL/6 mice can clear the infections with a non-lethal strain of *P. yoelii* (PyNL) by 20 days after infection, while mice die around 10 days after infection with PyL [23]. Furthermore, the injection of anti-CD25 mAb 3 and 1 day before, and 5 days after PyL infection, enabled mice to become resistant to PyL infection, and these mice finally cleared the parasites [23].

In order to address the question as to whether $CD25^+$ Treg proliferated in response to PyL-derived antigens, we first examined the kinetics of relative $CD4^+CD25^+$ T cell number during the course of infection. We found that the ratio of $CD25^+$ cells in total spleen cells or in the splenic $CD4^+$ T cell fraction was slightly higher in both PyL-infected and PyNL-infected mice compared with naive mice as early as 5 days after infection (Fig. 1a, b). Although the increase was transient in mice infected with PyNL, that in mice infected with PyL sustained until 7 days after infection (Fig. 1a, b).

Since CD25 is not a specific marker for Treg, and activated T cells also express CD25 [19], we evaluated the expression of Foxp3. The Foxp3, a forkhead transcription factor, has been identified as a gene selectively expressed in $CD4^+CD25^+$ Treg. The expression of Foxp3 is associated with the development and function of $CD4^+CD25^+$ Treg [24–26]. As previously demonstrated, among $CD4^+$ T cells purified from naive mice, $CD25^+$ cells specifically expressed Foxp3 and $CD25^-$ cells showed only a trace expression (Fig. 2a). We next analyzed $CD4^+CD25^+$ cells purified from mice after infection with the malaria parasites. $CD4^+CD25^+$ cells from mice infected with PyNL expressed slightly more Foxp3 transcripts 5 days after infection when transient increase of $CD4^+CD25^+$ cells was observed (Fig. 2b). In contrast, those from mice infected with PyL showed remarkable increase of Foxp3 5 days after infection and returned to normal level 7 days after infection (Fig. 2b).

Treg proliferated in PyL-infected mice

In order to examine whether Treg proliferate in response to PyL but not PyNL infection, $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells labeled with CFSE were transferred into C57BL/6 mice, and they were subsequently infected with each parasite strain. About 17% of $CD4^+CD25^+$

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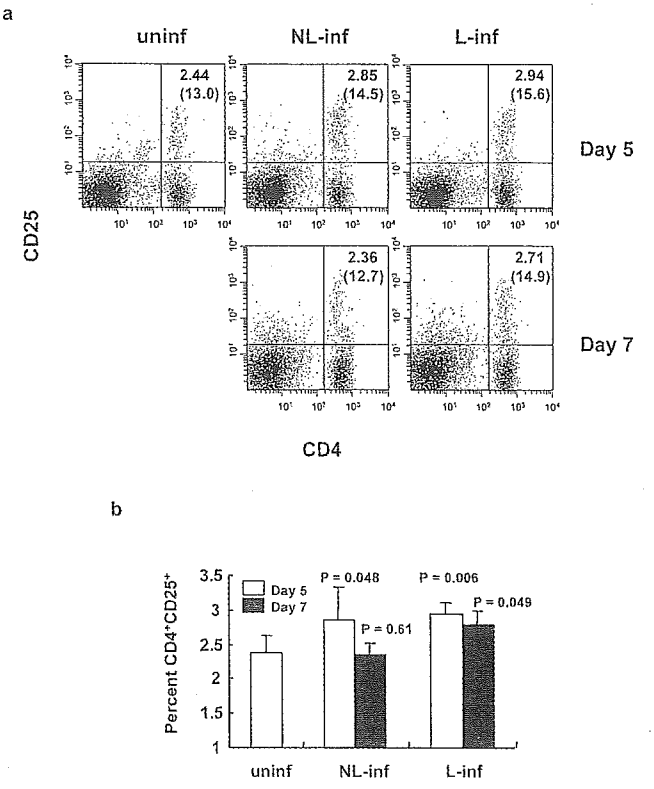

Figure 1. Increase of $CD4^+CD25^+$ T cells in mice infected with PyL. Spleen cells obtained from the indicated mice 5 and 7 days after infection were stained with a combination of fluorescent-labeled anti-CD4 and anti-CD25 mAb. (a) Flow cytometric profiles represent four individual experiments, and numbers represent percentages of the quadrant. Numbers in parentheses represent the ratio of $CD25^+$ cells to $CD4^+$ cells. (b) The data represent average percentages plus SD of $CD4^+CD25^+$ cells in total spleen from six to eight mice in each group. *p* values were calculated according to Student's *t*-test against uninfected mice based on the value of uninfected mice; PyNL (day 5): 0.048, PyNL (day 7): 0.61, PyL (day 5): 0.006, PyL (day 7): 0.048.

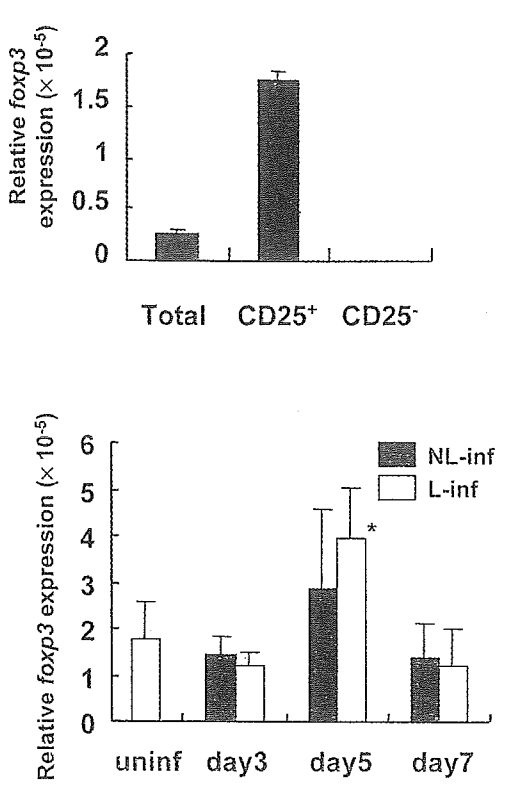

Figure 2. Enhanced expression of *foxp3* in $CD4^+CD25^+$ cells after PyL infection. Total RNA was extracted from $CD4^+$, $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from naive mice (a) or from $CD4^+CD25^+$ cells 3, 5 and 7 days after infection with PyL or PyNL (b), for quantitative RT-PCR assays. The data represent means ± SD of relative expression of *foxp3* transcripts to expression of 18S RNA from three individual experiments; $^*p<0.05$ according to the unpaired Student's *t*-test.

T cells isolated from mice 5 days after infection with PyL had divided, while PyNL infection induced few cell divisions of $CD4^+CD25^+$ T cells (Fig. 3a, b). The $CD4^+CD25^-$ T cells isolated from mice 5 days after PyL or PyNL infection did not divide significantly (Fig. 3a, b). These findings indicate that PyL infection allows $CD4^+CD25^+$ T cells to proliferate at an early phase of infection before proliferation of $CD4^+CD25^-$ T cells.

Treg induced by PyL infection show strong suppressive function

We next evaluated the functional properties of $CD25^+$ T cells obtained from PyL- or PyNL-infected mice. The $CD4^+CD25^+$ T cells from uninfected, PyL- or PyNL-infected mice were cultured with naive $CD4^+CD25^-$ T cells in the presence of anti-CD3 mAb. $CD4^+CD25^+$ T cells from naive and PyNL-infected mice similarly suppressed the anti-CD3 mAb-induced proliferation of naive $CD4^+CD25^-$ T cells in a dose-dependent manner (Fig. 4). The $CD4^+CD25^+$ T cells from PyL-infected mice had much stronger suppressive ability than those from PyNL-infected or naive mice (Fig. 4).

Treg induced by PyL infection show suppression in an antigen-specific manner

As Treg requires T cell receptor (TCR) engagement for exertion of their suppressive function [27], we next examined whether this suppression occurs in an antigen-specific manner *in vitro*. Splenic total $CD4^+$ T cells purified from mice 7 days after infection with PyL proliferated less than those isolated from PyNL-infected mice when stimulated with PyL- or PyNL-pRBC (Fig. 5a, left and right panels), as we previously described [23]. These results indicate that $CD4^+$ T cells from PyL-infected mice contain cell population able to respond to PyNL-derived antigen. When $CD25^+$ cells were removed from $CD4^+$ T cells in spleen cells of PyL-infected mice,

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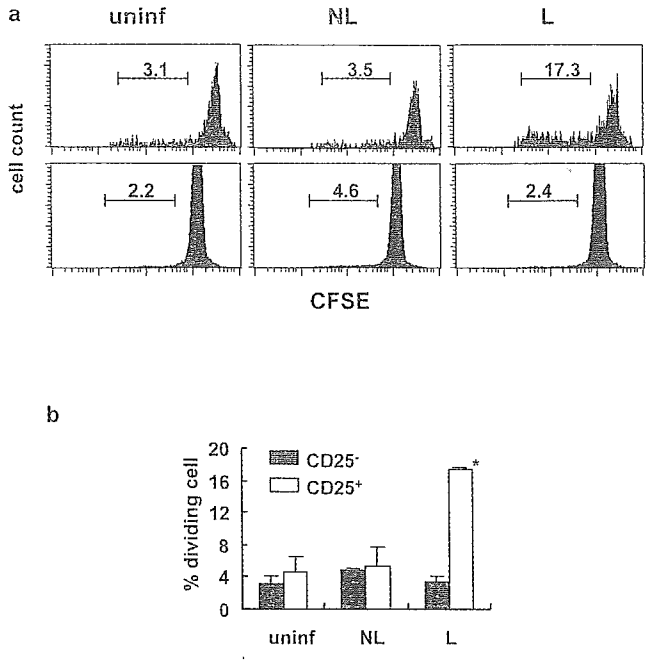

Figure 3. Preferential activation of regulatory T cells by infection with PyL. (a) Spleen cells from C57BL/6 mice that had been transferred with CFSE-labeled $CD4^+CD25^+$ (upper panels) or $CD4^+CD25^-$ T cells (lower panels) were analyzed 5 days after infection with PyL or PyNL. After excluding unlabeled cells, $CD4^+$ T cells were gated. Numbers represent percentage of divided cells in the CFSE-labeled population. (b) Means ± SD of the percentages of dividing $CD4^+CD25^-$ (open bars) or $CD4^+CD25^+$ T cells (filled bars) from two experiments. $^*p<0.05$ according to the unpaired Student's t-test.

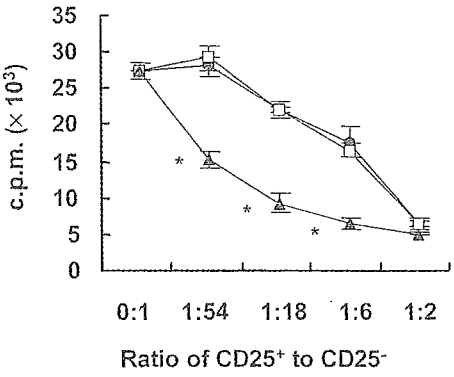

Figure 4. Strong suppressive function exerted by $CD4^+CD25^+$ cells after PyL infection. $CD4^+CD25^-$ T cells (2×10^5) purified from naive mice were stimulated with anti-CD3 mAb plus APC in the presence of the indicated number of $CD25^+$ T cells from uninfected (open squares), PyNL-infected (filled circles), or PyL-infected mice (filled triangles) 5 days after infection. $^*p<0.05$ compared with other groups according to the unpaired Student's t-test. $CD4^+CD25^+$ cells from PyL- or PyNL-infected mice were also cultured without $CD4^+CD25^-$ T cells, and the incorporations were less than 4000 cpm.

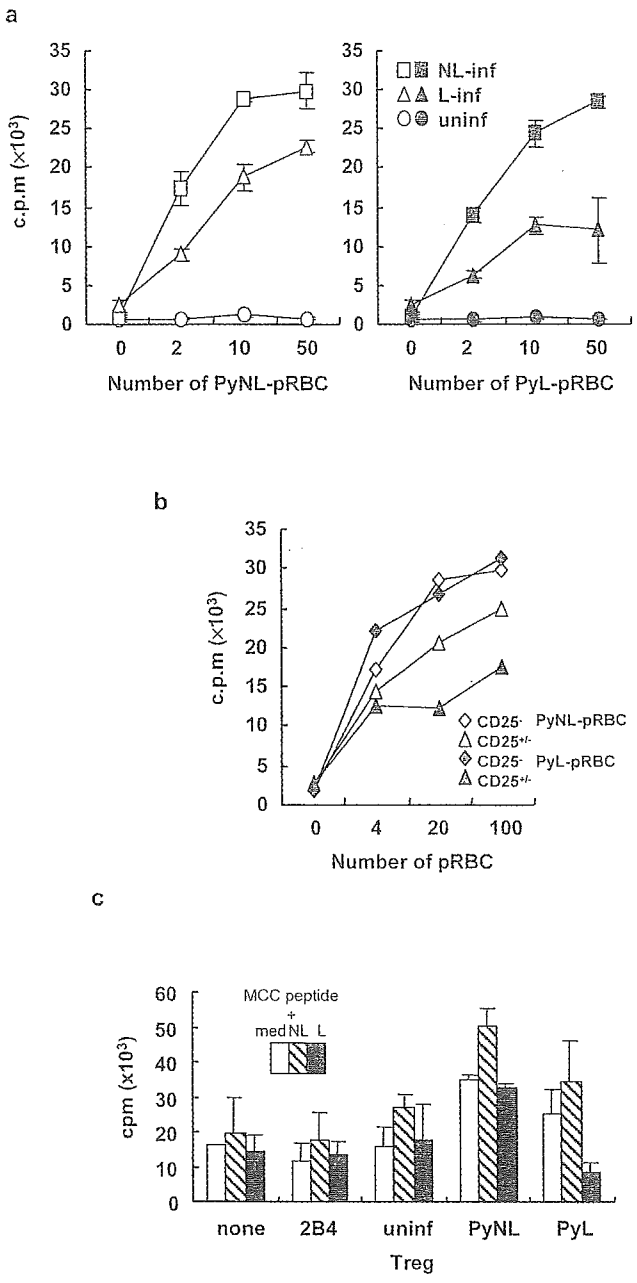

Figure 5. Suppression by Treg specific for PyL. (a) $CD4^+$ T cells (1×10^5) purified from mice 7 days after infection were cultured with PyL-pRBC (left panel) or PyNL-pRBC (right panel) in the presence of APC (1×10^5). Data are means ± SD of triplicate culture. (b) $CD4^+CD25^-$ cells (1×10^5) from PyL-infected mice with (triangles) or without (diamonds) $CD4^+CD25^+$ cells (2×10^4) of same origin were stimulated with PyL-pRBC (filled symbols) or PyNL-pRBC (open symbols) in the presense of APC (1×10^5). The data are means of duplicate cultures. SD are less than 15% of the means. (c) $CD4^+CD25^-$ T cells (1×10^5) purified from 2B4 TCR-transgenic mice were cultured with APC (1×10^5 each of H-2^k and H-2^b) and 1 μM of MCC peptide. $CD25^+$ cells from the indicated mice were added and cultured in the presence of PyNL-pRBC (striped bars) or PyL-pRBC (filled bars) or in the absence of pRBC (open bars). $CD25^+$ cells were obtained 5 days after infection. Data represent averages of duplicate cultures. Ratio of $CD25^+$ cells to the responder was 1:8.

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the resultant cells could proliferate in response to PyL-pRBC comparably to PyNL-pRBC-derived antigens (Fig. 5b). Furthermore, the addition of $CD25^+$ cells back to $CD4^+CD25^-$ cells from PyL-infected mice remarkably inhibited the response against PyL-pRBC compared with that against PyNL-pRBC-derived antigens (Fig. 5b).

In order to further confirm the antigen specificity of the Treg, we performed the experiments using 2B4 TCR-transgenic mice that have only T cells bearing TCR specific for moth cytochrome c (MCC) peptide presented by $I\text{-}E^k$ MHC class II molecules [28]. The co-culture of Treg from PyL-infected mice and 2B4 T cells reduced the proliferative responses of 2B4 T cells against MCC peptide in the presence of PyL-pRBC (Fig. 5c). Taken together, these results indicate that Treg from PyL-infected mice are able to exert suppressive activity by recognizing antigens preferentially expressed in PyL-pRBC.

The anti-GITR mAb could block the suppressive function of Treg from naive mice but not from PyL-infected mice

Several reports have shown that the suppressive function of $CD25^+$ Treg is mediated by cytokines as well as by direct cell contact [19]. We examined whether $CD25^+$ Treg from PyL-infected mice have similar suppressive mediators to those from naive mice. The $CD4^+CD25^-$ T cells from naive mice and $CD25^+$ T cells from PyL-infected or naive mice were stimulated with anti-CD3 mAb in the presence or absence of anti-IL-10 and anti-TGF-β mAb, and total T cell proliferation was measured. The anti-IL-10 and TGF-β mAb could not inhibit the suppressive ability of Treg from both naive and PyL-infected mice against anti-CD3 mAb-treated T cell proliferation (Fig. 6a). The addition of anti-IL-10 and anti-TGF-β mAb could not also affect Treg activity against PyL-derived antigen-specific T cell proliferation (Fig. 6b).

Recent studies have shown that GITR signaling in Treg inhibits the suppressive function of Treg [29, 30]. Thus, we next examined whether GITR signaling inhibits the suppressive function of Treg from PyL-infected mice. Treating Treg from naive mice with anti-GITR mAb completely inhibited the suppressive function of Treg, while the same treatment could not inhibit the suppressive function of Treg from mice infected with PyL (Fig. 6a). Anti-GITR mAb also inhibited suppression of $CD25^+$ cells from PyNL-infected mice (data not shown). Similarly, the anti-GITR mAb could not inhibit the suppressive function of Treg that were activated by PyL-pRBC (Fig. 6b). To confirm the resistance of Treg from PyL-infected mice to GITR stimulation, we also examined the dose response to anti-GITR mAb. The anti-GITR mAb dramatically increased the T cell proliferation when Treg from naive mice were used, while only slight T cell proliferation was observed when Treg from PyL-infected mice were used with all mAb concentrations (Fig. 6c). We finally checked the expression level or pattern of GITR on $CD4^+$ T cells from naive or PyL-infected mice. Splenic $CD4^+CD25^-$ cells expressed significant level of GITR and expressed $CD25^+$ fraction at much higher level. There was no difference in expression profiles between naive and PyL-infected mice (Fig. 6d).

GITR is expressed not only on Treg but also on non-Treg T cells and several reports demonstrated that anti-GITR antibodies act on non-Treg cells by providing costimulation [31, 32]. Thus, the outcome of treatment with anti-GITR in mixed culture with Treg and effector cells depends on the potency of Treg and on the strength of the effector T cell proliferation responses. Although Treg from PyL-infected mice are more efficient in suppression than naive Treg (Fig. 6a), it is possible that resistance to reversal of suppression induced by anti-GITR is due to the quantity of Treg potential. To test this possibility, we titrated effector cell-to-Treg ratios in the presence or absence of anti-GITR antibodies.

The addition of anti-GITR antibodies to cultures mixed with $CD4^+CD25^-$ T cells and $CD25^+$ cells from naive mice increased the baseline proliferation and Treg could not inhibit the proliferation as much as 1:6 $CD4^+CD25^-$ T cell-to-$CD25^+$ T cell ratio. However, the suppression began to be observed in cultures containing half number of naive Treg-to-effector cells (Fig. 6e, left panel). In contrast, when $CD25^+$ cells from PyL-infected mice were used, the addition of anti-GITR antibodies did not reverse the suppression at the all ratios of $CD25^+$ cells to effector cells (Fig. 6e, right panel). It should be noted that 1:54 ratio of $CD25^+$ cells from PyL-infected mice suppressed the proliferation equivalently to that used in 1:6 ratio of $CD25^+$ cells from naive mice in the absence of anti-GITR antibodies, and that remarkable suppression was observed in the former but not in the latter cultures in the presence of anti-GITR antibodies (Fig. 6e). Taken together, these findings suggest that Treg from PyL-infected mice have different regulatory mechanisms from those by Treg from naive mice.

Discussion

Treg play a vital role in the induction and maintenance of peripheral self tolerance [19, 21, 22]. These professional regulatory cells prevent the activation and proliferation of potentially autoreactive T cells that have escaped thymic deletion or that recognize extra-thymic antigens [19]. Several recent studies have demonstrated that Treg regulate effector T cell re-

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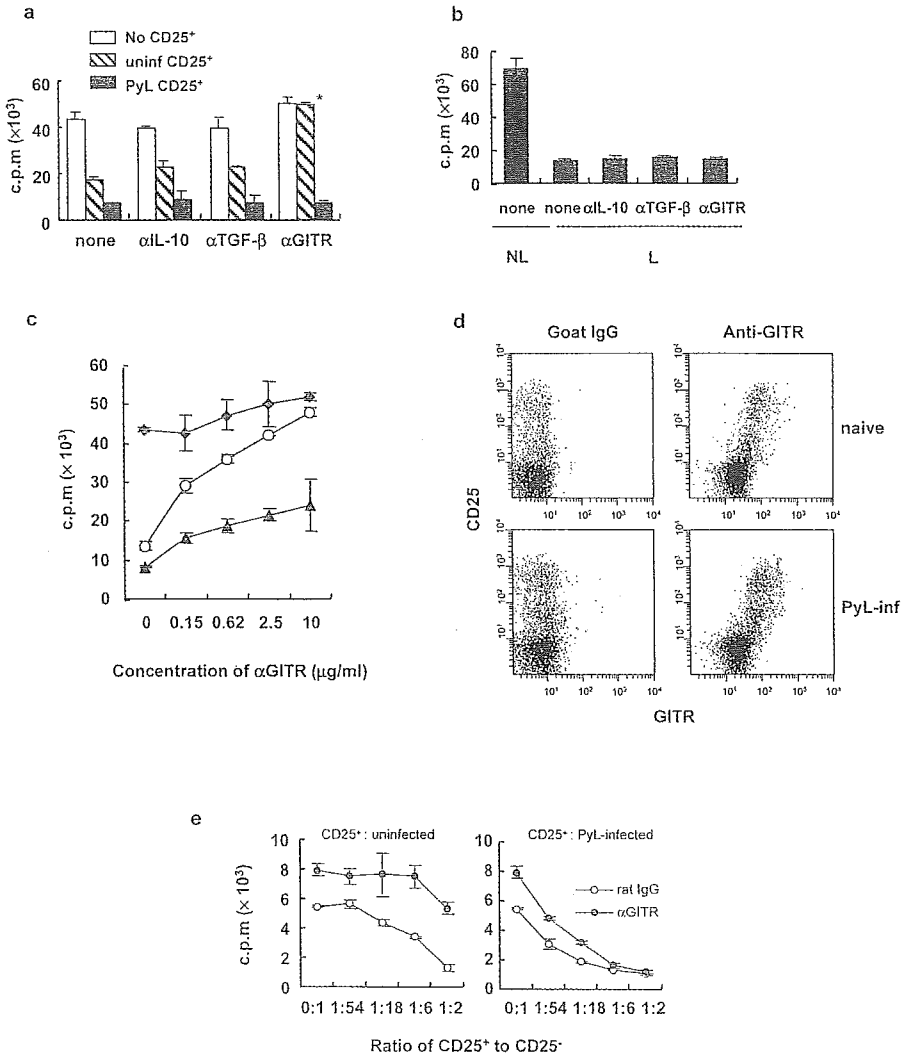

Figure 6. Resistance to GITR signaling in suppression by PyL-induced $CD4^+CD25^+$ cells. (a) $CD4^+CD25^-$ T cells (1×10^5) purified from naive mice stimulated with soluble anti-CD3 plus APC were co-cultured with $CD25^+$ cells (2.5×10^4) from naive mice (striped bars), PyL-infected mice (filled bars), or cultured without Treg (open bars) in the presence of 10 μg/mL of anti-IL-10, 20 μg/mL of anti-TGF-β or 10 μg/mL of anti-GITR antibody. Asterisk indicates significant elevation after addition of the antibody. (b) $CD4^+$ T cells purified from mice 7 days after infection were stimulated with PyL-pRBC plus APC in the presence of antibodies used above. (c) Graded amount of anti-GITR mAb were used in culture similar to (a) except for 5×10^4 $CD25^+$ cells from naive (open circles) or PyL-infected mice (filled triangles). $CD4^+CD25^-$ T cells alone were also cultured (closed circles). (d) Spleen cells obtained from mice 5 days after infection with PyL were stained with goat anti-GITR (right panels) or irrelevant goat IgG (left panels), fluorescent anti-CD4 and anti-CD25, followed by staining with fluorescent anti-goat IgG. The $CD4^+$ cells were analyzed for expression of CD25 and GITR. (e) $CD4^+CD25^-$ T cells (1×10^5) purified from naive mice and various numbers of $CD4^+CD25^+$ cells from naive (left panel) or PyL-infected mice were stimulated with soluble anti-CD3 plus APC (1×10^5) in the presence (filled symbols) or the absence (open symbols) of anti-GITR antibodies (2.5 μg/mL).

sponses against infectious organisms [33–37]. We also have revealed that activation of Treg is one of the escape mechanisms of malaria parasites from host immunity [23]. Here, we provide evidence that PyL infection resulted in Treg that proliferated in a PyL-specific manner at a very early stage of infection, although Treg are known to be anergic *in vitro* [38]. Furthermore, the activation of Treg during PyL infection enabled the Treg to become resistant to GITR signaling that generally makes Treg losing their suppressive function [29, 30].

We here found that Treg proliferated significantly after infection with PyL, but not PyNL, suggesting that PyL infection specifically activated Treg *in vivo*. There are at least three possible explanations for these findings. One explanation for the increased proliferation of Treg in PyL-infected mice *in vivo* is that PyL secretes soluble factors that cause proliferation of Treg. Our preliminary experiments, however, indicate that this explanation is unlikely, because the addition of parasite lysate or pRBC did not affect the proliferative response

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of Treg *in vitro* (data not shown). This also could not explain the difference between PyL and PyNL, because PyL, a variant derived from PyNL [39], is supposed to be identical in its genetic and antigenic background to PyNL.

The second possibility is that the qualitative or quantitative properties of antigen displayed by MHC class II are different between PyL- and PyNL-infected mice. Such differences might contribute to the preferred activation of Treg in PyL-infected mice. Treg are thought to have TCR with a relatively high affinity against self antigens compared with conventional $CD4^+$ T cells [40, 41]. Thus, the TCR repertoire might differ between the two populations. PyL might enable the escape mechanism by expressing peptides on MHC class II that have high affinity against TCR on Treg rather than on conventional T cells. The final possibility is that the parasite or pRBC directly interact with Treg or non-T cells, thus augmenting Treg activation. Indeed, recent findings have shown that Toll-like receptors expressed on Treg regulate Treg function even in the absence of TCR signaling [42]. In any case, the mechanisms that activate Treg in PyL infection would be the next logical step to study.

We here observed that $CD4^+CD25^+$ T cells from PyL-infected mice have stronger suppressive activity than those from naive or PyNL-infected mice. Furthermore, the expression of Foxp3 was higher in $CD4^+CD25^+$ T cells from PyL- than PyNL-infected mice. However, it remains unclear whether the relative percentage of Treg is increased in $CD4^+CD25^+$ T cells, or suppressive effector ability in individual Treg is augmented in PyL-infected mice. These issues may be solved by comparing Foxp3 expression in $CD4^+CD25^+$ T cells from PyL- and PyNL-infected mice by flow cytometry.

The effectors involved in the suppressive function of Treg and inducers of the suppressive mechanism of Treg remain controversial. As for effectors of Treg, Nakamura *et al.* [43] reported that TGF-β plays a role in the suppressive function of Treg, but other groups do not support this concept [44]. Also, the blockade of IL-10 *in vitro* does not affect Treg function [43] while IL-10 has been shown to block Treg function in some *in vivo* studies [45]. We also examined whether TGF-β or IL-10 is responsible for the suppressive function of Treg from naive or PyL-infected mice. Using blocking antibodies, we found that neither cytokine could suppress Treg function.

As a regulator of Treg function, the signaling through GITR, which is highly expressed on Treg, is known to inhibit the suppressive function of Treg [29, 30]. The stimulation of GITR by an antibody could inhibit the suppressive function of Treg from naive mice, as reported [29, 30]. In contrast, the same stimulation did not affect the function of Treg from PyL-infected mice, indicating that Treg acquire GITR-resistant mechanisms during PyL infection. Since we have not observed such a loss of GITR effect in Treg that were activated during tumor invasion (H.H. *et al.*, unpublished observation), the GITR resistance in Treg is somehow specific for PyL infection. In addition the GITR signaling enhances the proliferative responses of effector T cells [31, 46]. Given these findings it will be interesting to examine whether the observed resistance against GITR signaling reflects defective GITR signaling in Treg, or the acquisition of strong suppressive function of Treg overriding the GITR-mediated costimulation to effector T cells. Either way, GITR resistance in Treg function is likely to play a role in the escape of parasites from T cell immunity. It would be interesting to examine whether TCR signaling through the recognition of PyL-derived antigens, or a TCR independent effect on Treg, contribute to GITR resistance.

Collectively, our data provide a new perspective to evaluate mechanisms by which Treg are specifically activated during PyL infection by acquiring GITR resistance. Further studies to address the activator of Treg and inhibitors of GITR signaling will be important for understanding molecular mechanisms of parasite escape mechanisms from host immunity, as well as regulatory pathways of naturally occurring Treg.

Material and Methods

Mice and parasites

Female C57BL/6 mice (6–8 wk old) purchased from SLC (Hamamatsu, Japan) and RAG2-deficient mice from CLEA (Kawasaki, Japan) were utilized. 2B4 TCR-transgenic mice [28] were a generous gift from Dr. Fukui (Kyushu University, Japan) under permission of Dr. Davis (Stanford University, CA). Blood-stage parasites of PyL or PyNL (generous gifts from Dr. Torii, Ehime University, Japan) were obtained after fresh passage through a donor mouse 3–7 days after inoculation with frozen stock. Mice were infected with 10 000 to 15 000 pRBC intraperitoneally. To purify pRBC for use as a stimulant, blood collected by heart puncture from infected mice was passed through a CF11 column to eliminate host white cells, followed by discontinuous gradient centrifugation on 55% Percoll (Pharmacia, Uppsala, Sweden). The interphase fraction was collected as pRBC.

Antibodies

Purified anti-CD25 (PC61.5) was purchased from eBioscience (San Diego, CA). Purified anti-CD16/32, anti-CD11b, anti-MHC class II, anti-DX5, PE-anti-CD25 (PC61.5), allophycocyanin-anti-CD4 (RM4-4) and anti-IL-10 (JES5–16E3) mAb were purchased from BD PharMingen (San Diego, CA). Anti-rat IgG microbeads were purchased from Miltenyi (Auburn, CA). Anti-

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rat IgG Dynabeads were purchased from Dynal AS (Oslo, Norway). The goat anti-GITR polyclonal antibody was purchased from R&D Systems (Minneapolis, MN). The anti-CD3 (2C11), anti-CD4 (GK1.5), anti-CD8 (53.6.7), anti-CD25 (7D4), anti-B220 (RA3–6B2), anti-GITR (YGITR 765.4.16) [31] and anti-TGF-β (1D11) mAb were purified from ascites of mice injected with hybridomas.

Flow cytometric analysis

Cells in single suspension were stained with allophycocyanin-anti-CD4, PE-anti-CD25 in combination with various antibodies. Cells were analyzed by FACS Calibur (Becton Dickinson, Mountain View, CA) and data were analyzed using CellQuest software (Becton Dickinson).

Cell purification and culture

Spleens of mice were prepared as single suspensions. To remove non-T cells, the suspensions were incubated with anti-B220, anti-CD11b, anti-CD16/32, anti-MHC class II and anti-DX5 mAb, followed by incubation with anti-rat IgG Dynabeads. To purify $CD4^+CD25^+$ T cells, enriched T cells were incubated with anti-CD25 (PC61.5) followed by incubation with anti-rat IgG microbeads. Positive selection was performed according to the manufacturer's protocol. $CD4^+CD25^-$ T cells were purified by depletion of $CD8^+$ and $CD25^+$ cells from the enriched T cell fraction. The purity of each cell subset usually exceeded 85%. Purified cells were stimulated with soluble anti-CD3 antibody at a concentration of 2.5 μg/mL or with pRBC (up to 1×10^6) in the presence of APC in 0.2 mL of media for 72 h and incubated with 1 μCi/well of [^{3}H]thymidine for the final 6 h. Radioactivity was measured by a liquid scintillation counter. In some experiments, anti-IL-10 (10 ng/mL), anti-TGF-β (20 ng/mL) or anti-GITR (20 ng/mL) mAb was added to the culture.

Real-time RT-PCR

Total RNA was extracted from purified $CD4^+CD25^+$ T cells and then was reverse-transcribed to cDNA. cDNA was analyzed for the expression of *foxp3* mRNA by SYBR Premix Ex Taq (Takara, Tokyo, Japan) using a Perkin-Elmer ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR was performed according to manufacturer's instruction. Quantity of *foxp3* mRNA was expressed as ratio to expression of 18S rRNA. The sequences of PCR primers for *foxp3* were 5′–CCCAGGAAAGACAGCAACCTT-3′ and 5′–TTCTCACAAC-CAGGCCACTTG-3′, and those for 18S rRNA were 5′–GTAACCCGTTGAACCCCATT-3′ and 5′–CCATCCAATCG-GTAGTAGCG-3′.

Cell division analysis

Purified cells were incubated with 5 μM of CFSE (Molecular Probes, Eugene, OR) at a concentration of 1×10^7/mL for 10 min at 37°C. After three washes, labeled cells were transfused intravenously into recipient mice 1 day prior to infection. Transferred cells were recovered from mice from 5 to 8 days after infection with *P. yoelii* and were stained with anti-CD4 and anti-CD25 mAb.

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RESEARCH ARTICLE

A novel DNA vaccine based on ubiquitin–proteasome pathway targeting 'self'-antigens expressed in melanoma/melanocyte

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Cancer vaccine that targets 'self'-antigens expressed at high levels in tumor cells is a potentially useful immunotherapy, but immunological tolerance often defeats this strategy. Here, we describe the use of a naked DNA vaccine encoding a self tumor antigen, tyrosinase-related protein 2, to whose N-terminus ubiquitin is fused in a 'nonremovable' fashion. Unlike conventional DNA vaccines, this vaccine broke the tolerance and induced protective immunity to melanoma in C57BL/6 mice, as evaluated by tumor growth, survival rate and lung metastasis. The protective immunity was cancelled in the proteasome activator PA28α/β knockout mice. Moreover, this vaccination exhibited therapeutic effects on melanoma implanted before vaccination. Our findings provide evidence for the first time that naked DNA vaccines encoding a ubiquitin-fused self-antigen preferentially induce the main effector CD8⁺ T cells through efficient proteolysis mediated by the ubiquitin–proteasome pathway, and lead the way to strategies aimed at targeting tissue differentiation antigens expressed by tumors.
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Introduction

Genetic immunization strategies have become attractive for the development of melanoma vaccines, because a number of antigens recognized by cellular components of the immune system have been identified at the molecular level in melanoma patients.[1] These melanoma antigens include normal cellular proteins, such as tyrosinase-related protein 2 (TRP-2) involved in melanin synthesis, which are constitutively expressed in melanocyte and melanoma cells.[2] Previous reports showed poor immunogenicity of conventional plasmids encoding non-mutated autologous melanocyte/melanoma antigens.[3–5] Furthermore, genetic immunization of C57BL/6 (B6) mice with cDNA encoding autologous murine melanocyte/melanoma protein, such as murine TRP-2 (mTRP-2), failed to break peripheral T-cell tolerance to self-antigens.[6] Subsequently, some investigators have attempted to break the tolerance using genes encoding heterologous melanocytic antigens such as human TRP-2 or chimeric genes encoding green fluorescent protein (GFP) fused to TRP-2.[5,7] Clinical application of these systems, however, can result in certain predictable side effects or risks, especially after repeated vaccinations. For example, neutralizing antibodies for heterologous gene products or peptides may interfere with the vaccination effect. Furthermore, anaphylactic shock caused by homocytotropic antibodies or systemic inflammation mediated by CD4⁺ T cells specific for those heterologous gene products/peptides may occur.

Autoreactive T cells are not completely deleted within the thymus, but such T cells become tolerant to self-antigens in peripheral lymphoid tissues.[8] Self-tolerance is broken in certain conditions. One case is that self-antigens are effectively presented by professional antigen-presenting cells (APC) or dendritic cells (DC) to those tolerant T cells.[9] Furthermore, some cytokines such as interleukin 12 (IL-12) break the peripheral tolerance of CD8⁺ T cells specific for autologous tumor antigen, probably through activation of DC,[10,11] although IL-12 is not always suitable for clinical trials presently due to its side effects.

Antigen presentation to CD8⁺ T cells is mediated by MHC class I molecule expressed on cells. Primarily, CD8⁺ T cells recognize MHC class I-associated peptides derived from endogenous antigens, such as oncogene products or viral antigens, located in the cytosol. Prior to antigen presentation by MHC class I molecules, antigens must be ubiquitinated and then processed to antigenic peptides by the proteasome.[12–14]

Immunization with DNA vaccine containing ubiquitin gene fused with minigenes encoding cytotoxic T

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lymphocyte (CTL) epitopes of melanoma differentiation antigens broke self-tolerance for the tumor antigen through effective antigen processing and presentation.[15] Here, we constructed a chimeric DNA encoding a fusion protein linking murine ubiquitin (UB) to the N-terminus of the full-length mTRP-2, designated pUB-TRP-2, and then immunized B6 mice using a gene gun containing the chimeric naked DNA, before or after inoculation of B16 melanoma cells obtained from B6 mice. This strategy is based on the rapid destruction of cellular proteins through the UFD (UB fusion degradation) pathway, a virtual route of the ubiquitin–proteasome system.[16] By using this DNA vaccine technique, we succeeded in inducing antitumor immunity mediated by $CD8^+$ T cells in the host mice. Intriguingly, the antitumor immunity induced by this DNA immunization was almost completely cancelled in mice deficient in the proteasome activator PA28α/β.[17] Taken together, our results indicate that ubiquitin fused to mTRP-2 plays an essential role in introducing mTRP-2 protein into the proteasome-dependent degradation and creating epitopes presented by MHC class I, resulting in the preferential activation of mTRP-2-specific $CD8^+$ T cells.

Results

Rapid degradation of ubiquitin-fused TRP-2 by the proteasome

Most endogenously synthesized peptides such as mutant and viral antigens are processed by a cytosolic protease, the proteasome, followed by ubiquitination of those antigens, and then presentation to MHC class I molecules, resulting in activation of corresponding $CD8^+$ T cells.[12–14] Accordingly, artificially ubiquitinated mTRP-2 should be directed to the proteasome and effectively presented to MHC class I molecules. To confirm this scenario, we prepared the expression vectors of pTRP-2 encoding the full-length TPR-2 protein tagged with HA epitope at its C-terminus (TRP-2-HA) and pUB-TRP-2 encoding modified ubiquitin (G76A) in-frame with TRP-2-HA so as not to be cleaved by ubiquitin C-terminal hydrolases in cells (Figure 1a). To analyze expression of TRP-2 and its degradation by proteasomes, COS-7 cells were transfected with pTRP-2 or pUB-TRP-2 in the presence or absence of a proteasome inhibitor, MG-132 (Figure 1b). The expression of the UB-TRP-2 fusion protein was considerably lower than that of TRP-2 alone in the absence of MG-132. When the transfected cells were treated with MG-132, however, the level of UB-TRP-2 recovered to the same level as that in TRP-2. To further confirm, pulse–chase experiments were performed (Figure 1c). In COS-7 transfected with pTRP-2, TRP-2 was detected up to 20 min irrespective of the existence of MG-132. In contrast, in those transfected with pUB-TRP-2, TRP-2 was completely degraded within 20 min. Addition of MG-132 showed prolonged detection of the fusion protein. These results suggest that ubiquitin-fused TRP-2 protein is unstable compared to unfused TRP-2 and is rapidly degraded by the proteasome.

Antitumor immunity against B16 melanoma

CTL epitopes including mTRP-$2_{181–188}$ are generated by the ubiquitin–proteasome pathway.[17] Therefore, we expected that antigen presentation of MHC class I-associated TRP-2 peptides to $CD8^+$ T cells becomes significantly effective following vaccination with the ubiquitin-fused TRP-2. C57BL/6 mice were immunized with pTRP-2 or pUB-TRP-2 three times at 1-week intervals using a gene gun and a total dose of 18 μg plasmid per mouse. A week after the last immunization, mice were challenged subcutaneously with 2×10^5 B16F1 cells. Control mice and mice immunized with pTRP-2 showed rapid tumor growth, whereas 85% of mice immunized with pUB-TRP-2 were free of tumors and the remaining 15% of the mice exhibited almost complete suppression of tumor growth (Figure 2a). Strictly, all mice immunized with pUB-TRP-2 survived over 80 days after implantation of tumor cells, although all mice of the other two groups died within 60 days (Figure 2b). Furthermore, immunization with pUB-TRP-2 was also effective in suppressing the growth of B16F10 melanoma cells, a more virulent type of melanoma (Figure 2c). A total of 43% of mice immunized with pUB-TRP-2 survived over 45 days after implantation with B16F10 tumor cells, whereas all mice of the other two groups were dead within 35 days (Figure 2d). The number of lung metastatic tumors with B16F10 cells was significantly lower in pUB-TRP-2-immunized mice than in control or pTRP-2-immunized mice (Figure 2e). On the other hand, immunization with pUB-TRP-2 in B6 mice did not affect tumor formation of 3LL lung carcinoma cells, which do not express TRP-2 antigens (Figure 2f), indicating that the antitumor immunity acquired by pUB-TRP-2 vaccine is a specific event for melanoma cells. These results show that forced expression of ubiquitin-fused TRP-2 proteins *in vivo* induces antitumor immunity against B16 melanoma more effectively than that of native TRP-2 proteins, as evaluated by tumor growth, survival rate and lung metastasis.

Effector cells in the pUB-TRP-2 induce antitumor immunity

We speculated that immunization with pUB-TRP-2 preferentially activated antigen-specific $CD8^+$ T cells through efficient antigen processing of the encoded protein by the proteasome. To determine the effector cells in the observed protective immunity, we treated pUB-TRP-2-immunized mice with anti-CD4 or anti-CD8 antibody to deplete the corresponding T-cell subset, and then the mice were implanted with B16F1 cells. As expected, anti-CD8 treatment completely abolished the antitumor immunity induced by pUB-TRP-2 immunization (Figure 3). In contrast, treatment with control IgG, anti-CD4 antibody, did not attenuate the tumor growth. The crucial role of $CD8^+$ T cells was confirmed also by intracellular IFN-γ staining assay (Figure 4a). These T cells had the potential to generate IFN-γ but $CD4^+$ T cells did not. These results indicated that the antitumor immunity provoked by pUB-TRP-2 immunization is mediated by tumor-specific $CD8^+$ T cells that recognize the TRP-2 epitope presented on MHC class I molecules. To confirm the existence of antigen-specific CD8 T cells, lymph node cells obtained from vaccinated mice were stained with the tetramer of MHC class I molecule and dominant CTL epitope TRP-$2_{181–188}$. Antigen-specific CD8 T cells significantly increased in mice vaccinated with pUB-TRP-2 but not with pTRP-2 (Figure 4b).

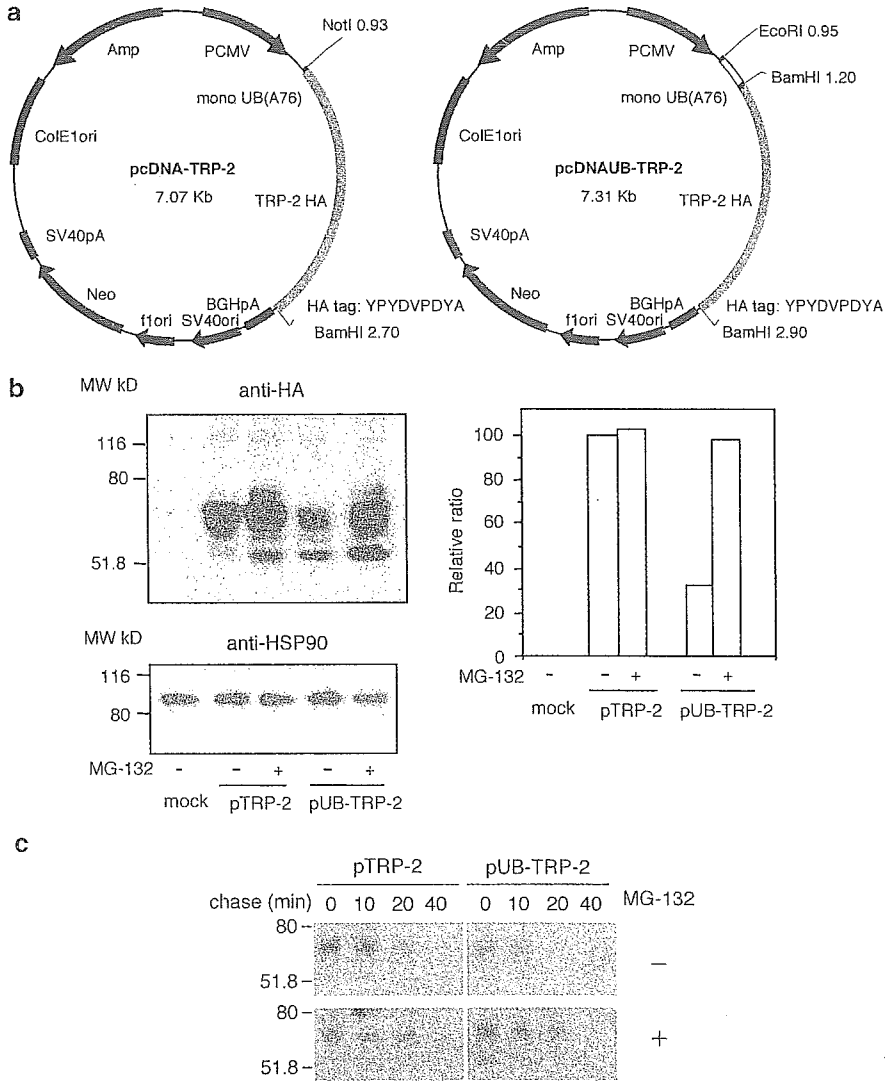

Figure 1 Plasmid construction and expression of TRP-2 in vitro. (a) Schematic representation of TRP-2 and UB-TRP-2. Each protein has an HA tag at the C-terminus. Note that the ubiquitin moiety of the generated UB-TRP-2 protein is not cleavable in the cells because the C-terminal Gly of ubiquitin was replaced by Ala residue (designated as UB(G76A)). (b) Instability of UB-TRP-2 fusion protein. COS-7 cells were transfected with pcDNA (mock), pTRP-2 or pUB-TRP-2 and cultured for 48 h. MG-132 (10 μM) was treated for 24 h before harvesting the cells. Cell lysates (10 μg) were analyzed by immunoblotting with an anti-HA (upper panel) or anti-HSP90 (lower panel) antibody. Relative expression of UB-TRP-2 to that of HSP90 was calculated densitometrically (right panel). (c) Rapid degradation of UB-TRP-2 fusion protein by proteasomes. The transfected cells were metabolically labeled with ^{35}S-labeled-amino acids for 30 min 18 h after transfection. Labeled cells were washed and chased for the indicated period, and then they were harvested. Some cells were treated with 10 μM of MG-132 when they were labeled and chased. An immunoprecipitation analysis was performed with anti-HA monoclonal antibody, and the precipitants were separated with SDS-PAGE.

Defective induction of antitumor immunity by immunization with pUB-TRP-2 in PA28αβ$^{-/-}$ mice

In order to clarify the role of the proteasome in antitumor immunity induced by pUB-TRP-2, we used PA28αβ$^{-/-}$ mice, because TRP-2$_{181-188}$, one of the major antigenic peptides, is generated by the proteasome activated by PA28.[17] There was no significant difference in tumor growth between PA28αβ$^{-/-}$ mice and wild-type mice after subcutaneous challenge with B16F10 (Figure 5a). Immunization of wild-type mice with pUB-TRP-2, again, suppressed tumor growth. In contrast, PA28αβ$^{-/-}$ mice immunized with pUB-TRP-2 were markedly susceptible to B16F10 tumor challenge compared with wild-type B6 mice immunized with the same plasmid (Figure 5a). And the level of tumor growth in PA28αβ$^{-/-}$ mice immunized with pUb-TRP-2 was comparable to that in PA28αβ$^{-/-}$ mice immunized with pTPR-2 (Figure 5a). Coincident with the result of tumor growth, CTL activity against TRP-2$_{181-188}$ epitope was lower in PA28αβ$^{-/-}$ mice immunized with pUB-TRP-2 than in wild-type mice (Figure 5b). These results prove that the proteasome activated by PA28 plays an important role in the induction of mTRP-2-specific CTL and antitumor immunity following DNA vaccination with pUB-TRP-2. It should be noted that PA28αβ$^{-/-}$ mice are not always immunocompromised hosts even in the level of CD8$^+$ T cells as well as CD4$^+$ T cells. When these deficient mice have been immunized with plasmid DNA encoding GFP by the gene gun, similar levels of antigen-specific IgG are produced in these mice compared with those in

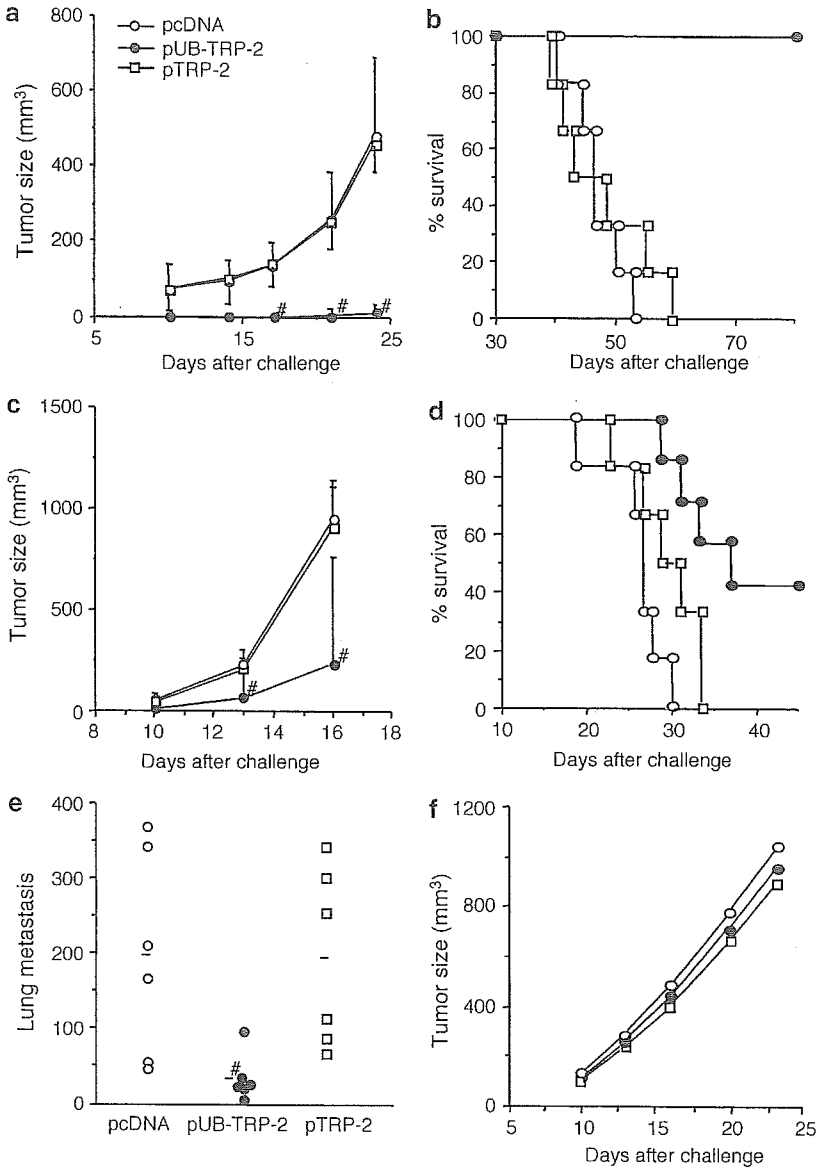

Figure 2 Induction of antigen-specific antitumor immunity by vaccination with pUB-TRP-2. C57BL/6 mice were immunized with plasmids pcDNA (open circles), pUB-TRP-2 (closed circles) or pTRP-2 (open squares) and challenged with B16F1 (a, b), B16F10 (c–e) melanoma or irrelevant 3LL lung carcinoma (f) cells. Antitumor effects were evaluated by tumor growth (a, c, f), survival rate (b, d) and frequency of lung metastasis (e). Data are mean ± s.d. of six mice in each group. #P <0.01 compared with the other two groups by unpaired Student's t-test. Each experiment was repeated at least three times.

wild-type mice, indicating that CD4+ T cells are intact in those mice.[17] Further, we confirmed that these mice develop the potential to mount MHC class I-restricted CD8+ T cells specific for OVA and influenza antigens.[18]

Therapeutic effect for melanoma by immunization with the plasmid DNAs

From the point of view of clinical application, it is more important to examine the therapeutic effects of this DNA vaccination method against melanoma implanted before vaccination. To test this effect, B6 mice were implanted with B16F1 melanoma cells before vaccination (day 0). Treatment with pTRP-2, pUB-TRP-2 or mock plasmid commenced on day 1 and was then repeated twice a week in the following 2 weeks. As shown in Figure 6, immunization of mice with pUB-TRP-2 resulted in marked suppression of tumor growth compared with mice immunized with mock or pTRP-2 plasmid. These results strongly indicate that immunization with DNA plasmid is effective even after development of the tumor, thus confirming of its potential clinical effectiveness against melanomas.

Discussion

Several melanoma antigens capable of activating immune responses have been defined.[19,20] Among them, the melanocyte lineage differentiation antigens (MDAs) are most prominent in the induction of immune responses. TRP-2 is one of the MDAs expressed in both normal and malignant melanocytes in humans and mice.[2,21] CTLs play a major role in antitumor immunity.[22] Several epitopes recognized by human CD8+ T cells are included in the TRP-2 protein.[23] Bloom *et al*[21] identified a major murine CTL epitope, TRP-$2_{181-188}$, presented on the

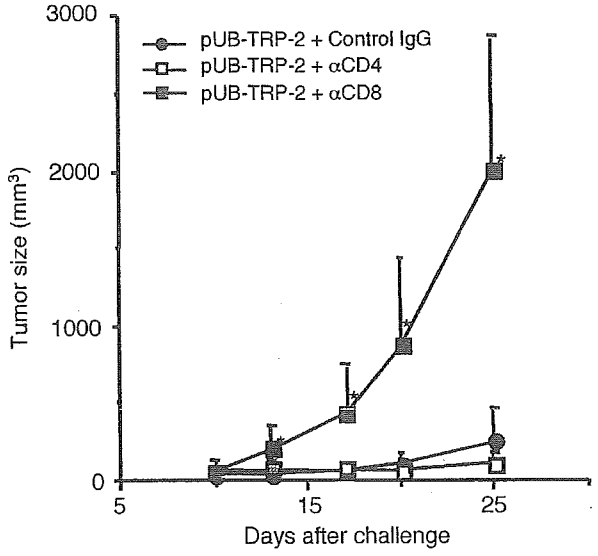

*Figure 3 Requirement of CD8⁺ T cells for antitumor immunity induced by pUB-TRP-2 vaccination. C57BL/6 mice vaccinated with pUB-TRP-2 were injected with rat IgG (closed circles), anti-CD4 (open squares) or anti-CD8 antibody (closed squares), and then challenged with B16F1 melanoma cells. Data are mean±s.d. of six mice in each group. *P<0.05 compared with the other two types by unpaired Student's t-test. This experiment was repeated three times.*

H-2K^b MHC class I molecule, and reported that passive transfer of TRP-2$_{181-188}$-specific CTLs into C57BL/6 (B6) mice had a therapeutic effect against established B16 lung metastasis. Although TRP-2 is a self-antigen, a significant number of CTL precursors potentially reactive to TRP-2 evade intrathymic selection. However, it is generally accepted that T cells specific for autologous murine melanocyte proteins such as TRP-2 fall into tolerance in the peripheral lymphoid system. Accordingly, immunization with this autologous antigen including genetic immunization could not break peripheral T-cell tolerance to the self-antigens, and thus failed to provide protective immunity against B16 melanoma cells.[3–6] Overcoming these tolerance limitations is crucial to the development of effective immunotherapy for melanoma.

It is well established that 8- to 9-mer of antigenic peptides must be presented on MHC class I molecules of target cells to be recognized by specific CTLs. The proteasome is responsible for the proteolysis of intracellular proteins including tumor antigens to generate MHC class I ligands.[24–26] Prior to degradation by the proteasome, polyubiquitin chains should be covalently attached to the substrates by a multienzymatic system.[27] Interestingly, it has been shown that fusion of a viral protein with the ubiquitin moiety at its N-terminus facilitates the formation of CTL epitopes, resulting in immunity against viral infection.[28] It is notable that proteins fused with ubiquitin whose C-terminal glycine residue is replaced with another amino acid, so as not to be cleaved and removed by the cytosolic ubiquitin C-terminal hydrolase, are degraded more rapidly than nonfused proteins in a ubiquitin–proteasome system-dependent manner. This system is particularly called the UFD pathway.[16] Thus, fusion of ubiquitin to the protein requiring degradation by the proteasome seems to be a useful strategy to effectively create CTL epitopes.

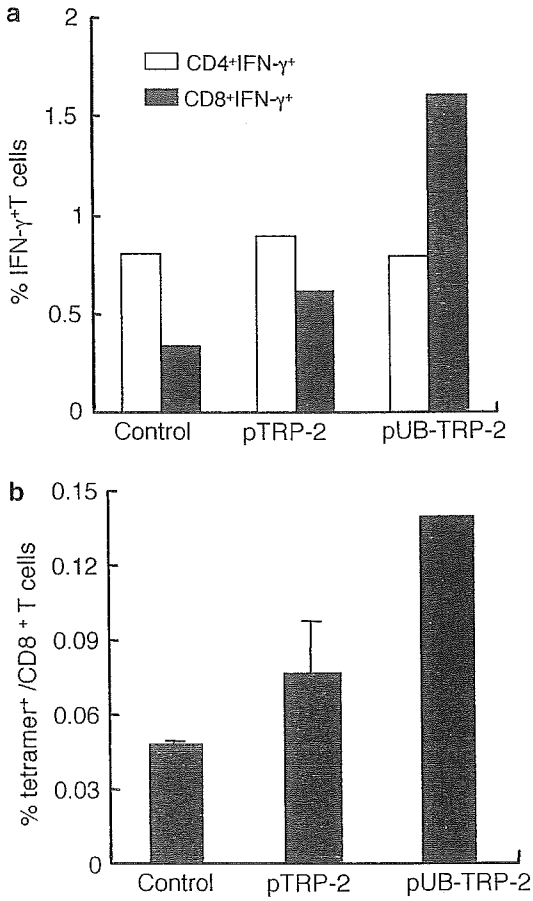

Figure 4 Functional analyses of CD8⁺ T cells in mice vaccinated with pUB-TRP-2. C57BL/6 mice were immunized with plasmids pcDNA (as control), pTRP-2 or pUB-TRP-2. (a) Intracellular IFN-γ production by CD4⁺ and CD8⁺ T cells. Splenocytes isolated from the mice were stimulated in vitro and intracellular IFN-γ production was detected by flow cytometry. Percentages of CD4⁺ T cells (open bar) or CD8⁺ T cells (closed bar) producing IFN-γ are shown. (b) Induction of antigen-specific CD8⁺ T cells in mice vaccinated with pUB-TRP-2. Lymphocytes were isolated from the inguinal and axillary lymph nodes and stained with the MHC class I tetramer. Results represent percentage of tetramer positive cells in CD8⁺ T cells.

In the case of melanoma, the biggest problem that has hampered successful immunization is that the tumor rejection antigen is a self-antigen. We assumed that tolerance against self-antigen might be broken by increasing the amount of presented epitopes. For this purpose, we applied artificially potentiated UFD pathway, constructing a naked DNA encoding a 'non-removable' fusion protein between ubiquitin and the full-length mTRP-2. As expected, ubiquitin-fused TRP-2 proteins converted into an excellent substrate for the ubiquitin–proteasome pathway (Figure 1b). Further, potent antitumor immunity was evoked against both low-virulent B16F1 and high-virulent B16F10 melanoma cells, as estimated by suppression of tumor growth, survival rate and lung metastasis (Figure 2). The depletion of CD8⁺ T cells (Figure 3) and deficiency of the proteasome activator PA28αβ (Figure 5), which is a prerequisite for proteasome-dependent processing of the major TPR-2 epitope, TRP-2$_{181-193}$,[18] counteracted the effect of vaccination. Thus, this protective immunity can be attributed to induction of melanoma-specific CTL