TABLE 3. GENOTYPE FREQUENCIES FOR *IL-18* SINGLE NUCLEOTIDE POLYMORPHISMS AND ASTHMA SEVERITY

Genotype	Steps 1,2 n = 248 (%)	Steps 3,4 n = 205 (%)	Allele	Steps 1,2 n = 248 (%)	Steps 3,4 n = 205 (%)	P Value and ORs (95% CI) Genotype	Dominant	Recessive	Allelic
rs1946519									
TT	77 (31)	89 (44)	T	268 (55)	259 (63)	0.026	0.0077	0.153	0.0078
TG	114 (47)	81 (40)	G	222 (45)	149 (37)		0.59		0.69
GG	54 (22)	34 (17)					0.40–0.87		0.53–0.91
rs360718									
TT	182 (73)	159 (78)	T	427 (86)	359 (88)	0.266	0.305	0.323	0.515
TG	63 (25)	41 (20)	G	69 (14)	51 (12)				
GG	3 (1)	5 (2)							
rs5744247									
CC	87 (35)	51 (25)	C	294 (59)	200 (50)	0.014	0.024	0.012	0.0034
CG	120 (48)	98 (49)	G	202 (41)	204 (50)		1.60	1.79	1.49
GG	41 (17)	53 (26)					1.06–2.41	1.14–2.74	1.14–1.94

Definition of abbreviations: CI = confidence interval; OR = odds ratio.

inhaled corticosteroid and long-acting β_2-agonist is more efficacious in asthma than either alone (33, 34). Another study has shown synergistic suppression of virus-induced chemokines in airway epithelial cells by combination therapy (35). We next investigated the effects of DEX and SAL on the expression of *IL-18*. We examined effects of DEX and SAL on *IL-18* mRNA expression levels in NHBE. The expression of *IL-18* was not affected by DEX or SAL (Figure 2C).

IL-18 mRNA Is Highly Expressed in NK Cells, Dendritic Cells, and Monocytes and Is Highly Induced by LPS in Human Monocytes

We further investigated whether *IL-18* mRNA was up-regulated by TLR ligands in immune blood cells, using quantitative real-time PCR. *IL-18* mRNA was highly expressed in nonstimulated NK cells, dendritic cells, and monocytes as compared with $CD4^+$ or $CD8^+$ T cells and B cells (Figure 3A). After stimulation, levels of *IL-18* mRNA in monocytes were increased threefold in response to LPS (Figure 3A).

Polymorphism rs5744247 Is Associated with *IL-18* mRNA Expression Level in LPS-Stimulated Monocytes

To investigate whether the -380C>G (rs5744247) polymorphism affected the mRNA levels of *IL-18*, we measured relative mRNA expression and compared it in subjects with different genotypes. We isolated monocytes from PBMCs of a total of nine healthy volunteers, five of whose genotypes were homozygous for -380C and four of whose were homozygous for -380G, and stimulated them with 5 μg/ml LPS. After LPS stimulation, we found significant increases in both *IL-18* (-380C group, $P = 0.0044$; -380G group, $P = 0.011$) and *IL-6* (-380C group, $P = 0.0036$; -380G group, $P = 0.014$) mRNAs within each group by the Friedman test. Monocytes homozygous for the -380G allele exhibited significantly higher expression of *IL-18* mRNA in response to LPS for 1.5, 3, and 6 hours ($P = 0.014$, 0.014, and 0.033, respectively, by the Mann-Whitney *U* test); however, this genotype effect was not observed for *IL-6* mRNA induction (Figure 3B). A recent study has shown antiinflammatory effects of SAL after inhalation of LPS in humans (36). However, a combination effect of glucocorticoid and SAL on inflammatory cytokines in response to LPS has not been reported. We next investigated the effects of DEX and SAL on the induction of *IL-18* mRNA by LPS in monocytes. Although the addition of DEX with/without SAL to the medium with LPS significantly reduced induction of *IL-6* mRNA, DEX and SAL did not affect the level of induction of *IL-18* mRNA (Figure 3C).

Polymorphism rs5744247 Is Associated with Serum IL-18 Level

To evaluate whether the serum IL-18 protein level correlated with the *IL-18* genotype, we conducted ELISA assays of sera of 88 patients with asthma. Baseline characteristics among serum study participants are shown in Table E6. We compared the distribution of severity between subjects with serological studies and that with genetic studies by the Mann-Whitney *U* test. In the subgroup examined for serum IL-18, asthma tended to be more severe than in the genotyped group ($P = 0.023$; Table E7). The Jonckheere-Terpstra trend test is a nonparametric trend statistic to test for ordered differences among groups assumed to be arranged ordinally. The test is superior to the Kruskal-Wallis procedure when the conjectured ordering of the genotype effects is, indeed, appropriate (37). Serum levels of the IL-18 protein positively correlated with the *IL-18* genotype in the Jonckheere-Terpstra trend test ($P = 0.031$; Figure 4).

DISCUSSION

In this study we identified 18 polymorphisms, conducted LD mapping of the gene region, and found significant associations between polymorphisms and asthma severity. Several genetic studies have already surveyed the genes involved in the IL-18 signaling pathway as candidate genes for asthma. For the *IL-18* receptor and related molecules, a recent study has shown significant replicated associations between polymorphisms in the *IL18R1* gene and asthma, atopic asthma, and bronchial hyperreactivity (38). Another study, in which adult asthma probands aged 18.1–64.7 years were examined, has reported associations of *IL1RL1*, *IL18R1*, and *IL18RAP* gene cluster polymorphisms with asthma and atopy in a Dutch population (21). Several association studies of the *IL-18* gene have also been conducted. Five polymorphisms, rs1946518, rs187238, rs360718, rs360717, and rs360721, located in the promoter and exon 1 region, were screened in a cohort of 228 children with asthma, but no polymorphism showed a significant difference in frequency between the case and control groups (17). The association study did not contain adult subjects with asthma and did not examine associations between the SNPs and asthma severity. Among the five SNPs, we found a significant association between rs1946518 and asthma severity. The recent SAPALDIA Cohort study using a Swiss population (mean ± SD, 41.2 ± 11.4) has shown associations between the *IL-18* promoter variant -137G>C and atopic asthma (19). SNP rs187238 (-137G>C) was not associated with either adult asthma susceptibility or severity in our study. The proportion

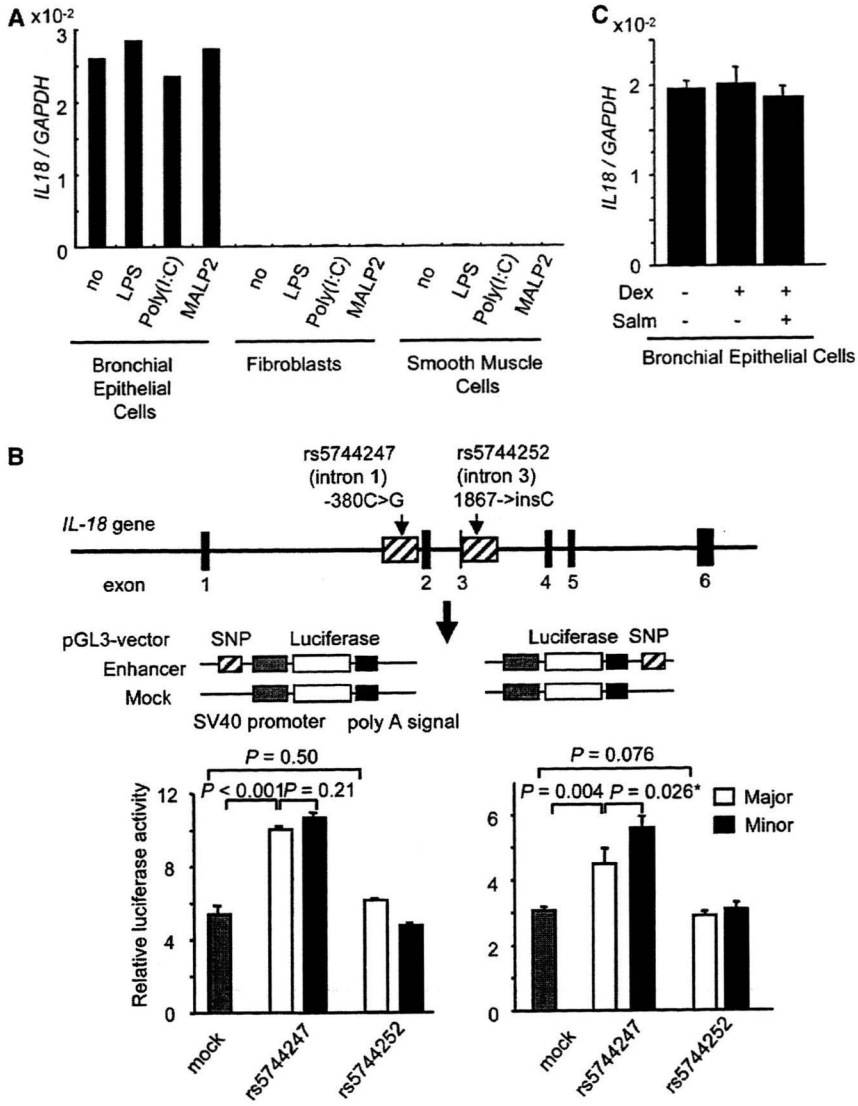

Figure 2. (*A*) *IL-18* mRNA expression analyses of normal human bronchial epithelial cells (NHBE), normal human lung fibroblasts (NHLF), and bronchial smooth muscle cells (BSMC) stimulated with 100 ng/ml LPS, 10 μg/ml poly(I:C) or 1 μg/ml macrophage-activating lipopeptide-2 for 4 hours. NHBE, NHLF, and BSMC were each derived from one individual, respectively. Data represent means of duplicate samples. Two independent experiments were performed with similar results. (*B*) Enhancer activity of *IL-18* introns 1 and 3 in NHBE, containing rs5744247 and rs5744252 variants. Schematic representation of the reporter constructs and relative luciferase activities of the two SNPs. Values represent the means ± SD of three independent experiments. The asterisk (*) indicates a significant difference between minor and major alleles ($P = 0.026$) by Student *t* test. (*C*) Effects of dexamethasone (Dex) and salmeterol (Salm) on *IL-18* mRNA expression levels in NHBE. Cells were treated with dexamethasone and salmeterol for 0.5 hours before addition of 100 ng/ml LPS. Values represent the means ± SD using NHBE derived from four individuals. GAPDH = glyceraldehyde 3-phosphate dehydrogenase; MALP = macrophage-activating lipopeptide-2; SNP = single nucleotide polymorphism.

of subjects with severe asthma in the prospective cohort study appeared to be lower than that in our study using clinic-based samples, and the difference might have affected the results. In a Japanese population, an association study of the *IL-18* gene was conducted using three polymorphisms, rs1946518, rs187238, and rs549908 (Ser35Ser), with 221 children and 276 adults with asthma and 85 adult control subjects (20). The study screened only polymorphisms in the coding and promoter regions of the *IL-18* gene, and found a significant association between rs549908 (Ser35Ser) and asthma susceptibility. Among the three SNPs, we could not find any association between the *IL-18* variants and adult asthma susceptibility; however, we found a significant association between rs1946518 and asthma severity. Association between the SNPs and asthma severity was not examined in the earlier study (20), and sample size might account for the contradictory results. Furthermore, according to the data of Hapmap (www.hapmap.org), the allele frequencies of SNP rs5744247 in Japanese, Han Chinese, Yoruba people, and the Centre d'Etude du Polymorphisme Humain population are 34%, 53%, 1%, and 9%, respectively. Thus the functional effect of the rs5744247 variant on asthma severity might be specific to the Japanese population.

Recent studies have shown important roles of bronchial epithelial cells as both mediators and regulators of innate immune responses and adaptive immune responses (39), and constitutive expression of IL-18 protein was observed within airway epithelial cells (40, 41). In this study, human *IL-18* was highly expressed by airway epithelial cells, and we could confirm the enhancer-like effects of the rs5744247 variant and significantly greater transcriptional activity of the susceptible G allele in NHBE. Although the majority of individuals with asthma are well controlled with current therapies, the existent therapeutic strategies are inadequate for those with severe asthma (42). Approximately 5% to 10% of the asthmatic population is in the severe end of the disease spectrum and it is difficult to control the asthma with maximal inhaler therapy (43, 44). Combination therapy with long-acting β_2-agonists and inhaled corticosteroids reduces exacerbation frequency in asthma, and it is also efficacious as intervention therapy for exacerbation of the disease (33, 34, 45). The suppressive effect

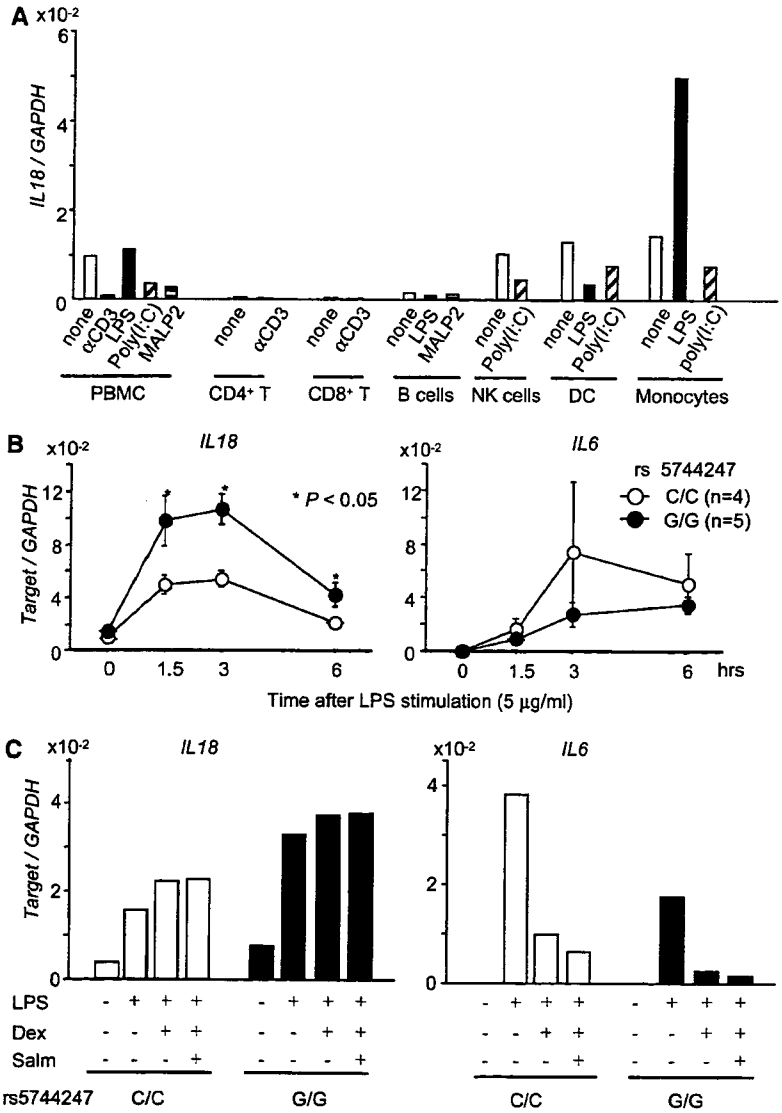

Figure 3. (*A*) Quantitative reverse transcriptase-polymerase chain reaction assays of *IL-18* in immune cells. The expression levels were normalized with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression. Freshly isolated immune cells were stimulated using 1 μg/ml plate-bound anti-CD3 monoclonal antibody (mAb) with 1 μg/ml soluble anti-CD28 mAb, 5 μg/ml LPS, 25 μg/ml poly(I:C), or 1 μg/ml macrophage-activating lipopeptide (MALP)-2 for 5 hours. Data were averaged among duplicate samples and are representative of two independent experiments. Similar results were obtained using immune cells from two individuals. (*B*) Relationship of rs5744247 genotype to *IL-18* mRNA expression level. The *IL-18* and *IL-6* mRNA expression levels were measured in monocytes stimulated with 5 μg/ml LPS for the indicated time. The asterisks (*) represent $P = 0.014$, 0.014, and 0.033, respectively, by the Mann-Whitney *U* test. (*C*) Effects of dexamethasone (Dex) and salmeterol (Salm) on *IL-18* and *IL-6* mRNA expression levels in monocytes stimulated with 5 μg/ml LPS. Data are means in duplicate from an individual. Two independent experiments each using human monocytes, homozygous for C ($n = 4$) and homozygous for G ($n = 3$), were performed with similar results.

of steroids mediated by glucocorticoid response elements within the *IL-6* promoter has been reported (46). In the present study, we could confirm the effects of DEX and SAL on *IL-6* expression in LPS-stimulated monocytes; however, DEX and SAL were not able to suppress *IL-18* mRNA expression in either monocytes or NHBE cells. A recent study has shown that corticosteroid-resistant (CR) asthma is associated with classic antimicrobial activation of airway macrophages, and higher endotoxin levels are detected in bronchoalveolar lavage fluid from subjects with CR asthma (47). The study implies that prolonged exposure to LPS might contribute to CR asthma (47). In human monocytes, we here demonstrated that the mRNA expression of *IL-18* was highly induced by LPS and identified a -380C>G (rs5744247) SNP that had an allele-specific effect on mRNA expression. Primary monocytes from subjects homozygous for the susceptible -380G allele exhibited significantly high expression of *IL-18* mRNA in response to LPS. In addition, the induction of *IL-18* mRNA in monocytes was not suppressed by DEX and SAL. IL-18 is involved in severe asthma through functional polymorphism and might contribute to enhanced innate immunity and both Th1- and Th2-driven immune responses. It is likely that targeting IL-18 itself might be therapeutically efficacious as a new treatment for severe asthma.

Recent studies have reported elevated circulating levels of IL-18 in patients with allergic diseases (22–25). Serum IL-18 levels are higher in patients with acute asthma than in control subjects and the IL-18 level has a tendency to inversely correlate with peak expiratory flow (22, 23). In patients with atopic dermatitis, serum IL-18 levels are elevated and the levels are correlated with disease severity and with the number of eosinophils in peripheral blood (25). In another study, IL-18 secretion from mononuclear cells of patients with bronchial asthma and atopic dermatitis was significantly higher than that in nonallergic controls (48). In this study, we found a positive correlation between the serum IL-18 level and *IL-18* rs5744247 genotype. Although we randomly recruited 88 subjects with asthma who provided serum samples, in the subgroup examined for serum IL-18, asthma tended to be more severe than in the genotyped group. There might be a subpopulation selection bias with regard to disease severity, and the bias might influence the positive correlation between serum levels of the IL-18 protein

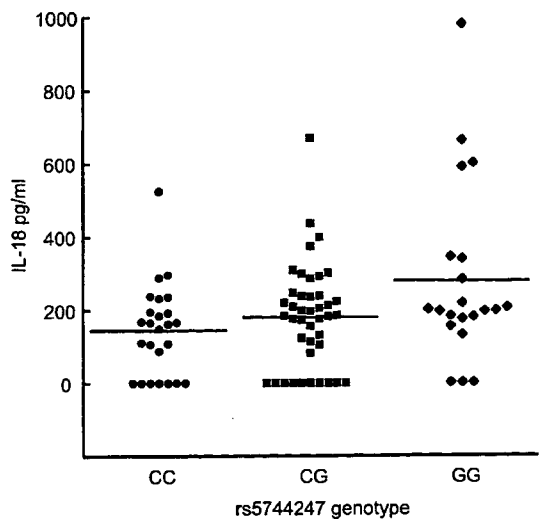

Figure 4. Relationship between rs5744247 genotype and serum IL-18 level. Serum levels of the IL-18 protein positively correlated with rs5744247 genotype by the Jonckheere-Terpstra trend test (P = 0.031).

and the *IL-18* genotype. Thus, validation studies of the connection between the serum IL-18 protein level and genotype are needed in a large number of samples.

We concluded that a genetic variant in the *IL-18* gene appears to influence the serum level of IL-18 and the asthma severity, putatively by altered enhancer activity in NHBE and increased *IL-18* mRNA expression in monocytes in response to LPS. Further investigations of IL-18 function would be helpful to understand the pathophysiology of inflammatory diseases whose development and progression are affected by microbial infections.

Conflict of Interest Statement: M.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.Y. received up to $1,000 from Boehringer Ingelheim in lecture fees and $5,001–$10,000 from Novartis Foundation Japan in grants. Y.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.D. received up to $1,000 from Ono Pharmaceutical Co., Ltd., up to $1,000 from Sanofi Aventis, and up to $1,000 from Kyorin Pharmaceutical Co., Ltd. in lecture fees. A.M. received up to $1,000 from GlaxoSmithKline and up to $1,000 from Banyu Pharmaceutical Co., Ltd. in lecture fees. K.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.E. received $1,001–$5,000 from Sanofi Aventis, $5,001–$10,000 from Kyowa Hakko Kirin Pharma, $1,001–$5,000 from Boehringer Ingelheim, $1,001–$5,000 from Dainippon Sumitomo Pharma, and up to $1,000 from Ono Pharmaceutical Co., Ltd. in lecture fees. M.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.N. received $1,001–$5,000 in consultancy fees from Abbott, $1,001–$5,000 from Nippon Boehinger Ingelheim, $1,001–$5,000 from Nippon Boehinger Ingelheim, $1,001–$5,000 from Astellas Pharm, $1,001–$5,000 from Kyowa Kirin, and $1,001–$5,000 from Dainippon Sumitomo Seiyaku in lecture fees. Y.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.T. received up to $1,000 from Kyorin Pharmaceutical Co., Ltd. and up to $1,000 from GlaxoSmithKline in lecture fees.

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Critical role for the immunoproteasome subunit LMP7 in the resistance of mice to *Toxoplasma gondii* infection

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Proteasome-mediated proteolysis is responsible for the generation of immunogenic epitopes presented by MHC class I molecules, which activate antigen-specific CD8$^+$ T cells. Immunoproteasomes, defined by the presence of the three catalytic subunits LMP2, MECL-1, and LMP7, have been hypothesized to optimize MHC class I antigen processing. In this study, we demonstrate that the infection of mice with a protozoan parasite, *Toxoplasma gondii*, induced the expression of LMP7 mRNA in APC and increased the capacity of APC to induce the production of IFN-γ by antigen-specific CD8$^+$ T cells. *In vitro* infection of a DC cell line with *T. gondii* also induced the expression of LMP7 and resulted in enhanced proteasome proteolytic activity. Finally, mice lacking LMP7 were highly susceptible to infection with *T. gondii* and showed a reduced number of functional CD8$^+$ T cells. These results demonstrate that proteasomes containing LMP7 play an indispensable role in the survival of mice infected with *T. gondii*, presumably due to the efficient generation of CTL epitopes required for the functional development of CD8$^+$ T cells.

Key words: Antigen processing · CD8$^+$ T cells · Parasitology · Proteasomes

Introduction

CD8$^+$ T cells play crucial roles in immunity against intracellular pathogens, including *Toxoplasma gondii* [1, 2]. Classical CD8$^+$ T cells recognize antigenic peptides after MHC class I-associated antigen processing, involving the proteolytic digestion of cytosolic antigens by proteasomes [3]. Processed peptides are transported to the ER through a TAP, where they associate with newly synthesized MHC class I molecules before migrating to the cell surface [4]. Proteasomes are responsible for intracellular protein degradation and play pivotal roles in vital cellular functions, such as cell cycle control, stress responses, intracellular signaling, and MHC class I antigen processing [5]. Under certain conditions, such as exposure to IFN-γ, three catalytic subunits, namely LMP2, MECL-1, and LMP7, are coordinately expressed. These inducible subunits replace the constitutive catalytic subunits, resulting in the formation of so-called immunoproteasomes [6]. Immunoproteasomes appear to have enhanced capabilities for generating MHC class I-binding peptides compared with constitutive proteasomes. They cleave proteins more efficiently after basic or hydrophobic amino acids that provide the preferred C-terminal anchors for most CTL epitopes, firmly binding them to the antigenic groove of MHC class-I molecules [7, 8]. Indeed, mice deficient in LMP2 and LMP7 show reduced presentation of certain MHC class I antigens [9, 10]. In contrast, the presentation of most CD8$^+$ T-cell epitopes is unaffected or rather enhanced in these mutant mice [11, 12]. Thus the role of immunoproteasomes in MHC class I-associated antigen processing is still controversial.

T. gondii is an intracellular protozoan parasite that infects a broad range of vertebrates, including humans. It causes opportunistic infections, such as toxoplasmic encephalitis in immunocompromised hosts, although in immunocompetent hosts it causes latent asymptomatic infections. The tachyzoite stage of *T. gondii* invades a variety of nucleated cells and replicates within them.

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Invasion of APC by this parasite induces immune responses, such as the secretion of inflammatory cytokines and antigen presentation to T cells [13, 14]. A previous study has shown that *T. gondii* preferentially invades immature DC and inactivates them, resulting in the failure to activate naïve $CD4^+$ T cells [15]. In contrast, it is reported that the ability to activate $CD8^+$ T cells is preserved in nonprofessional and professional APC infected with *T. gondii* [16, 17].

We previously reported that the vaccination of mice with DNA-encoding SAG1, a leading *T. gondii* vaccine candidate, induced protective $CD8^+$ T cells in an LMP7-dependent manner [18]. These results led us to hypothesize that immunoproteasomes are required for the protective response to *T. gondii*. In this study, we observed that the expression of LMP7, the catalytic activity of proteasomes, and the ability to present antigens to $CD8^+$ T cells were up-regulated in APC in response to infection with *T. gondii in vitro* and *in vivo*. Furthermore, mice lacking LMP7 were highly susceptible to infection with *T. gondii* and showed impairment in the development of functional $CD8^+$ T cells. This is the first demonstration of the critical role of immunoproteasomes in mice resistance to infectious agents.

Results

Infection with *T. gondii* enhances the expression of LMP2 and LMP7

Infection with *T. gondii* elicits $CD8^+$ T-cell responses that contribute to protection [3, 17], suggesting that antigen processing/presentation to $CD8^+$ T cells is augmented during infection. Thus we analyzed the changes in the expression of mRNA encoding the catalytic subunits of proteasomes after infection with *T. gondii*. C57BL/6 mice were intraperitoneally inoculated with 1×10^4 *T. gondii* tachyzoites. Four days later, peritoneal exudate cells (PEC) were collected, and total RNA extracted for quantitative RT-PCR. Although the amount of mRNA encoding the catalytic subunits of constitutive proteasomes, β1 and β5, was not altered, that encoding those of immunoproteasomes was remarkably increased (Fig. 1A). In association with the increase in mRNA, both LMP7 and LMP2 protein levels were increased in peritoneal macrophages in response to infection with *T. gondii* (Fig. 1B). Similar results were obtained when using purified splenic DC (data not shown). These data suggest that the number of immunoproteasomes, but not constitutive proteasomes, increased in mice infected with *T. gondii*.

Infection with *T. gondii* improves the ability of DC to activate $CD8^+$ T cells

We next analyzed whether the increase in immunoproteasome numbers was directly linked to efficient antigen presentation to $CD8^+$ T cells. Splenic $CD11c^+$ DC obtained from mice infected with *T. gondii* were cultured with $CD4^+$ or $CD8^+$ T cells purified from OVA-specific TcR-transgenic mice, OT-II or OT-I [19] respectively, in the presence of OVA protein. As we expected, DC from *T. gondii*-infected mice induced enhanced proliferation of OT-I $CD8^+$ T cells over those from uninfected mice (Fig. 1C). In contrast, infection with *T. gondii* impaired the ability of DC to activate OT-II $CD4^+$ T cells (Fig. 1C) as has been previously reported [20, 21]. When DC were pulsed with OVA epitopes instead of the whole protein, both OT-I $CD8^+$ T cells and OT-II $CD4^+$ T cells were activated, regardless of the source of DC (Fig. 1C). These results, together with the failure of DC to activate $CD4^+$ T cells after infection with *T. gondii*, excluded the possibility that the enhanced ability of DC to activate $CD8^+$ T cells was due to the up-regulation of MHC or co-stimulatory molecule expression due to activation of DC themselves during infection. Furthermore, the differences in their ability to induce $CD8^+$ T-cell IFN-γ production were much more obvious (Fig. 1D).

T. gondii promotes proteolysis of DC through immunoproteasome induction

To confirm the relationship between immunoproteasome induction and the enhanced ability of DC to activate $CD8^+$ T cells during infection with *T. gondii*, we tried to evaluate proteasomal proteolytic activity. We used a DC cell line, DC2.4 cells [22], because it was hard to obtain enough protein from DC for this analysis in *ex vivo* experiments. DC2.4 cells infected with *T. gondii* tachyzoites at an MOI of 1 were periodically analyzed. As observed in mice infected with *T. gondii*, *in vitro* infection of DC2.4 with *T. gondii* induced the expression of LMP2 and LMP7 mRNA 24 h later, in contrast to the stable expression of those encoding β1 and β5 during the observation period (Fig. 2A). Infection of DC2.4 cells resulted in increased expression of the LMP7 protein (Fig. 2B). As expected, chymotrypsin-like activity mediated by β5/LMP7 and LMP2, supposedly favorable for the generation of CTL epitopes, was enhanced in association with increased levels of LMP2 and LMP7 mRNA (Fig. 2C). Whole cell lysates were used to measure proteasomal activity. To exclude the possibility that proteases with chymotrypsin activity, other than proteasomes, accounted for the enhanced activity, a proteasome inhibitor, MG-132, was utilized. Addition of MG-132 at micromolar concentrations to the lysates completely abolished chymotrypsin-like activity (Fig. 2D). As *T. gondii* is known to have proteasomes [23, 24], there was a possibility that the enhanced activity was due to increased activity of *T. gondii* proteasomes. Therefore, we collected *T. gondii* from each preparation but could not detect proteolytic activity in the parasites (Fig. 2E).

To further confirm that enhancement in the enzymatic activities was due to induction of LMP2 and LMP7 in response to infection with *T. gondii*, we used siRNA to specifically suppress their expression. Incubation of DC2.4 cells with the siRNA prior to infection with *T. gondii* suppressed the expression of mRNA encoding the corresponding subunit without interfering with mRNA expression of the irrelevant subunit (Fig. 3A). The siRNA for LMP7 inhibited both *de novo* LMP7 synthesis (Fig. 3B) and the *T. gondii*-induced up-regulation in chymotrypsin-like activity (Fig. 3C). Inhibition of LMP2 did not affect the chymotrypsin-like activity, indicating that augmentation of this activity was

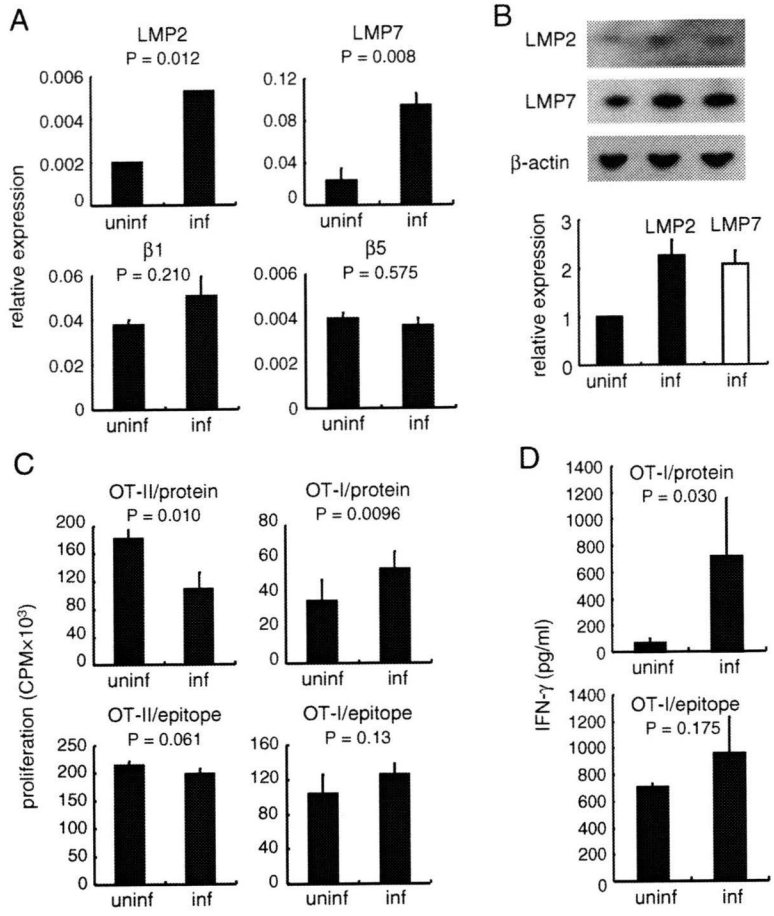

Figure 1. Infection with *T. gondii* augments the ability of APC to activate $CD8^+$ T cells. (A) Induction of immunosubunits after infection with *T. gondii*. mRNA encoding the indicated subunits in total RNA extracted from PEC 4 days after infection of mice with *T. gondii* (inf) was quantified using real-time RT-PCR. The samples from uninfected mice (uninf) were also analyzed. Values represent the relative quantities of mRNA encoding the indicated subunit to that encoding β-actin. (B) Expression of LMP7 and LMP2 was also analyzed with Western blotting. Values in the bottom panel represent the relative amount of LMP2 (closed bars) and LMP7 (open bars) to those from uninfected mice. (C, D) Activation of antigen-specific T cells by DC obtained from mice infected with *T. gondii*. OT-II $CD4^+$ (left panels) or OT-I $CD8^+$ T cells (right panels) were stimulated with OVA proteins (upper panels) or the responsive OVA epitopes (lower panels) in the presence of splenic $CD11c^+$ DC obtained from mice 4 days after infection. The proliferation of OVA-specific T cells was assessed by $[^3H]$-thymidine incorporation (C), and the concentration of IFN-γ in the culture supernatants from OT-I cells (D) is shown. In the absence of OVA, incorporation of $[^3H]$-thymidine was less than 500 cpm, and the concentration of IFN-γ produced by OT-I cells was 36.3 or 18.7 pg/mL in the presence of DC from uninfected or infected mice, respectively. Values show the mean+SD of three mice. These experiments were repeated three times with similar results. *p*-Values using an unpaired Student's *t*-test comparing uninfected and infected mice are indicated.

primarily due to LMP7 expression. These results clearly indicate that infection with *T. gondii* enhances the chymotrypsin-like activity in DC through the induction of LMP7.

LMP7-deficient mice are highly susceptible to infection with *T. gondii*

Our results suggest that immunoproteasome induction causes efficient activation of *T. gondii*-specific protective $CD8^+$ T cells. To confirm this, we infected LMP2- or LMP7-deficient mice with *T. gondii*. All LMP7-deficient mice died after infection with 1×10^3 tachyzoites, whereas WT mice all survived at this level of infection and LMP2-deficient mice did not exhibit a significant reduction in survival (Fig. 4A). However, LMP2-deficient mice were also susceptible to infection with increased dose of *T. gondii* though to a lesser degree than LMP7-deficient mice (Fig. 4B).

Impaired development of functional $CD8^+$ T cells in LMP7-deficient mice

To relate the higher susceptibility to *T. gondii* infection in LMP7-deficient mice to the failure to activate effector $CD8^+$ T cells, we first analyzed the ability of DC to activate OVA-specific OT-I cells. As shown in Fig. 5A, DC obtained from WT and LMP2KO mice infected with *T. gondii* induced vigorous proliferation of OT-I cells compared with those from uninfected mice. In contrast,

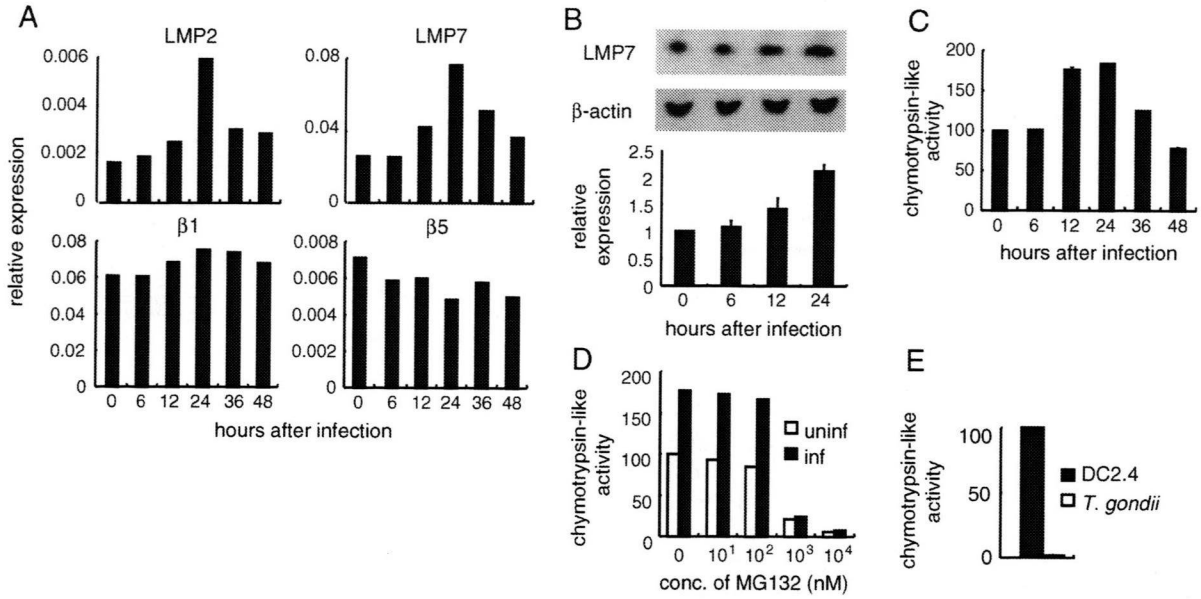

Figure 2. Infection with *T. gondii* enhances proteolysis in DC by induction of LMP7. Total RNA or cell lysates extracted from DC2.4 cells at the indicated time points after infection with *T. gondii* were analyzed for mRNA encoding the immunosubunits of proteasomes (A), expression of LMP7 (B), and proteasomal proteolysis (C). (A) The amount of mRNA encoding the indicated subunit was measured as in Fig. 1A. Data show the mean of duplicate cultures. (B) LMP7 protein was detected using Western blotting as in Fig. 1B. Data show the mean+SEM from three independent experiments. (C) Chymotrypsin-like activity was assessed with fluorogenic substrates. The relative activities to those before infection are represented. (D) Chymotrypsin-like activities in cell lysates from DC2.4 before (open columns) and 24 h after infection (closed columns) were analyzed in the presence of a proteasome inhibitor, MG-132, at the indicated concentrations. (E) Proteasomal activity of purified *T. gondii* was also analyzed. *T. gondii* was isolated from DC2.4 cells 24 h after infection. Values represent the relative activity to that of DC2.4 without infection (closed bars). Three independent experiments showed similar results.

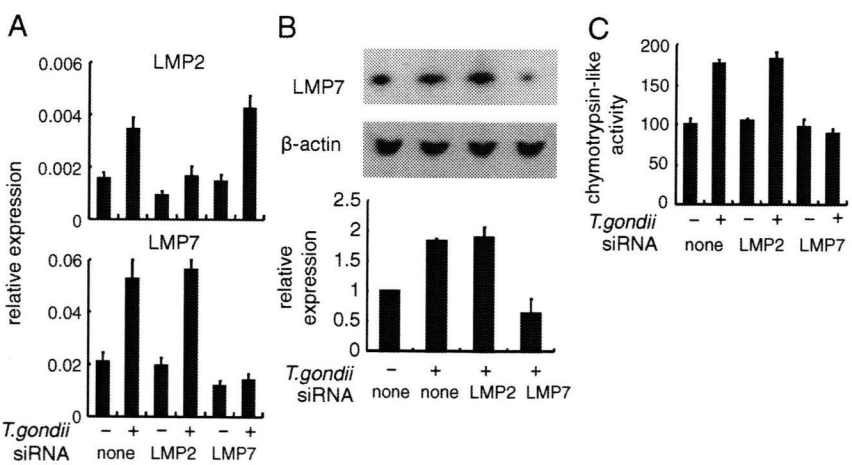

Figure 3. Suppression of LMP7 expression reversed proteolytic activities. DC2.4 cells were incubated with siRNA against an immunosubunit prior to infection with *T. gondii*. The expression of mRNA encoding LMP7 or LMP2 (A), the LMP7 protein (B), and proteolytic activity (C) were analyzed in DC2.4 transfected with the indicated siRNA 24 h after infection.

infection of LMP7KO mice did not alter the ability of DC to activate OT-I cells (Fig. 5A). We next analyzed the activation status of splenic $CD8^+$ T cells 7 days after infection with *T. gondii*. Infection with *T. gondii* increased $CD69^+CD62L^-$ activated $CD8^+$ T cells in WT mice. Unexpectedly, LMP7-deficient mice contained a comparable number of activated $CD8^+$ T cells (Fig. 5B). We next analyzed the functional properties of these $CD8^+$ T cells. Protective immune responses exerted by $CD8^+$ T cells have been shown to correlate well with IFN-γ production and cytolytic behavior. In WT mice infected with *T. gondii*, IFN-γ^+CD8^+ T cells were substantially increased (Fig. 5C), but in LMP7-deficient mice far fewer cells were able to secrete this cytokine following

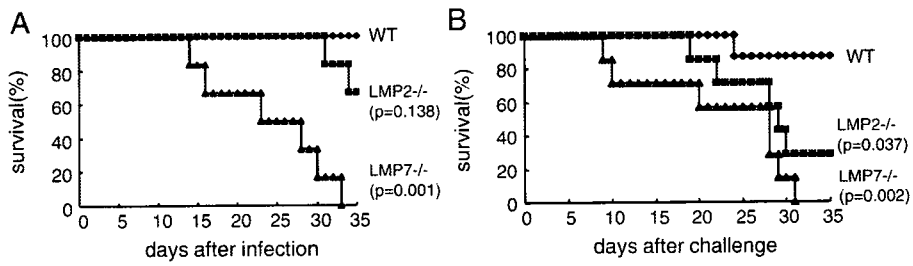

Figure 4. LMP7-deficient mice are susceptible to *T. gondii* infection. Survival curves of WT (diamonds), LMP2- (squares), and LMP7-deficient mice (triangles) infected with 1×10^3 (A) or 1×10^4 (B) *T. gondii* are shown. Each group comprised six or seven mice. These experiments were repeated three times with similar results. *p*-Values from the log-rank test for survival rates between WT and the mutant mice are indicated.

infection. This was in contrast to resistant LMP2-deficient mice that showed a slight increase in IFN-γ^+CD8$^+$ T cells. The partial failure to develop IFN-γ-producing cells was limited to CD8$^+$ T cells because the lack of LMP7 did not affect the number of IFN-γ^+CD4$^+$ T cells (Fig. 5C).

We next evaluated molecules associated with cytolytic activity. It is well known that T cells are able to lyse target cells using two pathways: the Fas-FasL pathway and the granule exocytosis pathway [25, 26]. The expression of FasL on CD8$^+$ T cells was enhanced in response to infection with *T. gondii*, but significant differences were not found among WT, LMP2-, and LMP7-deficient mice (Fig. 5B). Granzyme B (GrzB) is a key molecule mediating granule-dependent cytotoxicity and activates apoptosis-related caspases after entering the target cells through a perforin pore [26, 27]. Infection of WT mice with *T. gondii* remarkably increased the number of CD8$^+$ T cells containing GrzB (Fig. 5C). As in the case of IFN-γ, a deficiency in LMP7, but not LMP2, suppressed the development of GrzB$^+$CD8$^+$ T cells (Fig. 5C). These results indicate that potentially functional CD8$^+$ T cells could not be activated in susceptible LMP7-deficient mice.

The data presented here do not demonstrate that LMP7 is involved in the processing of *T. gondii*-derived antigens, because processing of OVA (Fig. 5A) and IFN-γ/GrzB produced by CD8$^+$ T cells using a TCR-bypassing stimulus (Fig. 5C) were analyzed. Thus, we finally hypothesized that LMP7 is required for the induction of CD8$^+$ T cells specific for *T. gondii* and for the presentation of the parasite antigens. To test this, CD8$^+$ T cells obtained from LMP7KO mice were stimulated with freeze-thawed tachyzoites in the presence of DC isolated from uninfected WT mice, and their production of IFN-γ was measured. CD8$^+$ T cells from WT mice infected with *T. gondii* produced IFN-γ in response to parasite antigens, and this response was decreased in LMP7KO CD8$^+$ T cells, although IFN-γ production from CD4$^+$ T cells from LMP7KO mice was comparable to that from WT mice (Fig. 6A). This indicated that CD8$^+$ T cells specific for *T. gondii* did not develop in the absence of LMP7. Furthermore, potentially responsive CD8$^+$ T cells obtained from WT mice infected with *T. gondii* could not be activated when stimulated with *T. gondii* antigens in the presence of LMP7KO DC (Fig. 6B). Those DC were still capable of inducing IFN-γ production by CD4$^+$ T cells (Fig. 6B). It should be noted that molecules other than LMP7 seem responsible for the generation of some epitopes because CD8$^+$ T cells from LMP7KO mice and WT CD8$^+$ T cells stimulated in the presence of LMP7KO DC still produce IFN-γ. These results clearly demonstrated that the absence of LMP7 affects the generation of CD8$^+$ T cells specific for *T. gondii* and indicate that the effect of LMP7 is most likely based on processing of *T. gondii*-derived antigens in DC.

Discussion

In this study, we demonstrated that an immunoproteasome subunit, LMP7, was important for the resistance of mice to *T. gondii* infection. Infection of WT mice with *T. gondii* enhanced the ability of DC to activate OVA-specific CD8$^+$ T cells. This was attributed to the induction of immunoproteasomes and they were able to induce the development of functional/protective CD8$^+$ T cells responsible for protection to this infection, in addition to CD4$^+$ T cells. Indeed, LMP7-deficient DC showed no augmentation in activation of *T. gondii*-specific as well as OVA-specific CD8$^+$ T cells. Moreover, mice lacking LMP7 could not mount CD8$^+$ T-cell specific responses to *T. gondii* and were highly susceptible to infection. These results strongly suggest that LMP7 contributes to the induction of protective CD8$^+$ T cells by generating CTL epitopes.

Several studies have reported that LMP7 deficiency is unable to present one single antigen in particular [9, 10]. However, it is hard to elucidate the role of LMP7 in antigen processing during infections, mainly because infectious agents display numerous microbial antigens, some of which might be processed in an LMP7-independent manner. Moreover, there is little information about CTL epitopes in *T. gondii* antigens. Our previous findings revealed that vaccination with SAG1 protected mice against infection with *T. gondii*, and that induction of protective CD8$^+$ T cells specific for SAG1 was completely dependent on LMP7 [18]. We actually tried to analyze SAG1-specific CD8$^+$ T cells during infection, but the frequency of SAG1-specific CD8$^+$ T cells was too small to analyze. Thus, it would be interesting to use genetically engineered *T. gondii* expressing OVA to evaluate this issue. Fortunately, however, the usage of freeze-thawed *T. gondii* allowed us to demonstrate that LMP7 is involved in processing of parasite antigens. It has been reported that a *T. gondii*-derived immunodominant antigen eliciting protective CD8$^+$ T-cell responses is secreted from live parasites in parasitophrous vacuoles after the invasion of host cells [28]. Thus, our system using dead parasites can be

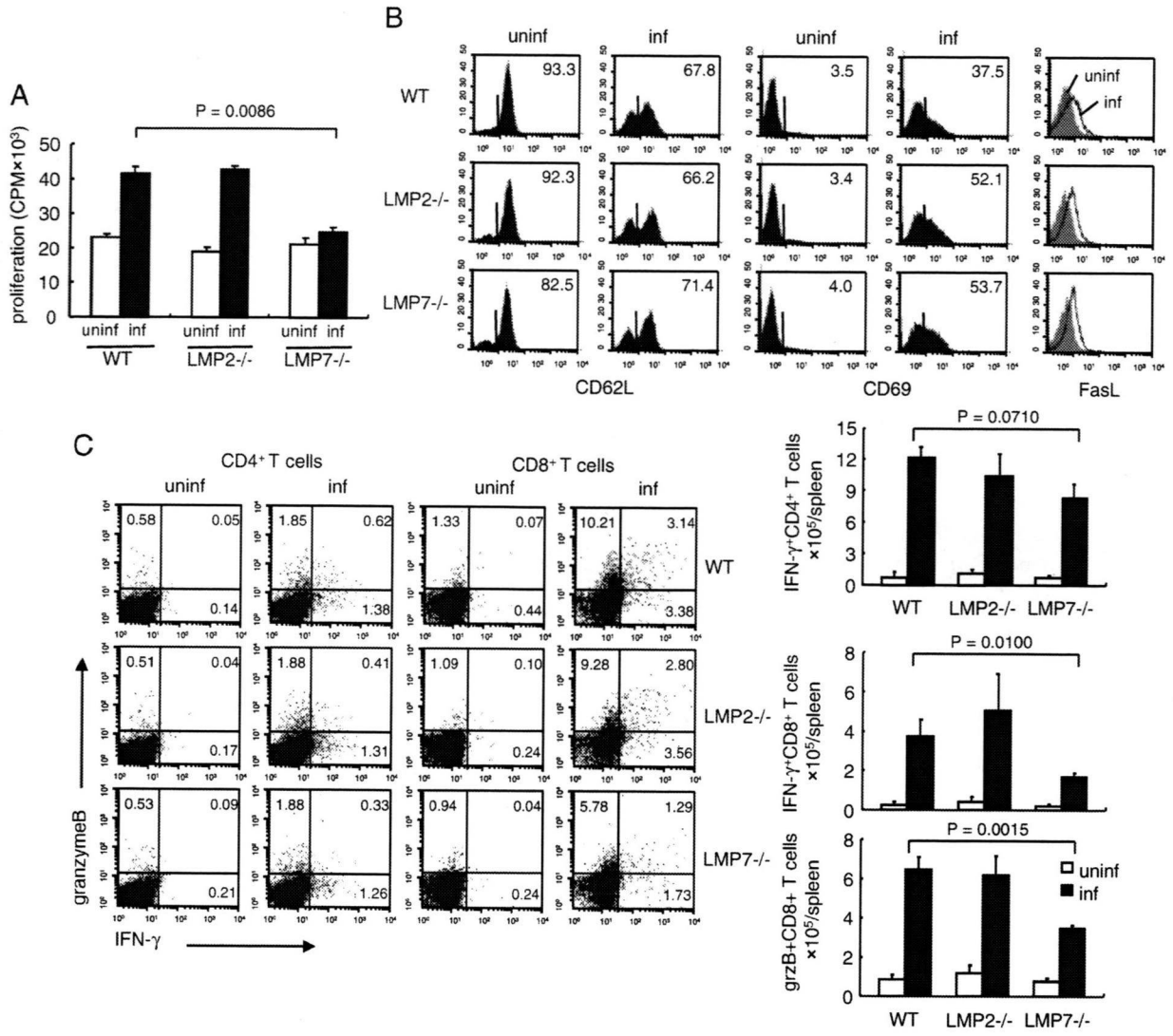

Figure 5. Development of functional CD8$^+$ T cells is suppressed in LMP7-deficient mice. (A) DC from the indicated mice 4 days after infection with 1×10^4 tachyzoites *T. gondii* were evaluated for their ability to activate OT-I CD8$^+$ T cells as in Fig. 1CB. (B) Activation of splenic CD8$^+$ T cells was evaluated by determination of the expression of the indicated cell surface markers in mice 7 days after infection. The numbers in histograms showing the expression of CD62L and CD69 indicate the percentage of positive cells among the gated CD8$^+$ T cells. In histograms showing FasL, shaded areas or lines indicate the expression in uninfected or infected mice, respectively. (C) Production of IFN-γ and GrzB in splenic CD4$^+$ (left panels) and CD8$^+$ T cells (right panels) of the indicated mice 7 days after infection was analyzed. Dot plots show the expression of those molecules, and numbers indicate the percentage in each quadrant among the gated cells. The absolute number of IFN-γ$^+$ or GrzB$^+$ T cells in the spleen was calculated and shown as bar graphs. Values are the mean+SD of three mice. These experiments were repeated three times with similar results. *p*-Values were calculated using the student's *t*-test.

considered artificial. We have tried using *T. gondii*-infected DC as APC but failed to observe optimal activation of CD8$^+$ T cells. Further investigation into this is required.

We hypothesize that LMP7 is essential for the induction of protective CD8$^+$ T cells by mediating effective antigen processing during infection. Protective effector mechanisms exerted by CD8$^+$ T cells include the production of IFN-γ [29] and killing of *T. gondii*-infected cells *via* the granule exocytosis pathway [30]. Although CD8$^+$ T cells were activated to some degree (in terms of the CD69$^+$ CD62^{lo} activated phonotype and FasL expression) even in the absence of LMP7, their functional development appears to require at least some quantity and quality of CTL epitopes generated by immunoproteasomes containing LMP7. We postulate that LMP7 deficiency impairs the development of functional CD8$^+$ T cells after encountering the infectious microbe. Our previous report demonstrated that LMP7-deficient mice showed a similar distribution of CD8$^+$ T cells in the periphery to that in WT mice [10, 18], suggesting that the thymic ontogeny of CD8$^+$ T cells is not affected in the absence of LMP7. However, it is possible that existing CD8$^+$ T cells have an incomplete repertoire, lacking *T. gondii*-specific

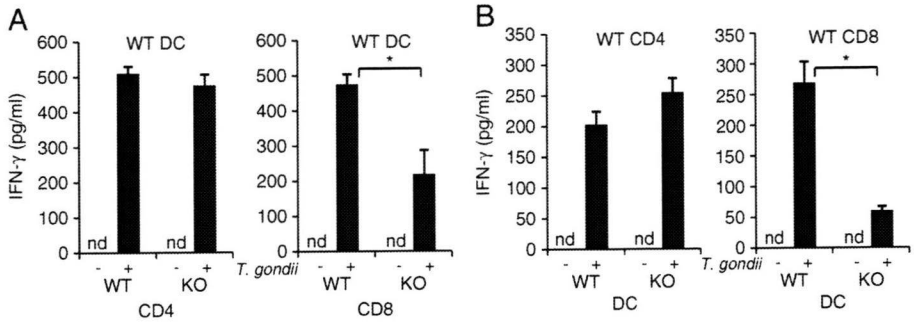

Figure 6. Involvement of LMP7 in the processing of *T. gondii*-derived antigens. $CD4^+$ or $CD8^+$ T cells (1×10^5) were stimulated with (+) or without (–) 1×10^6 freeze-thawed tachyzoites of the PLK parasite strain. (A) T cells obtained from WT and LMP7KO (KO) mice infected with *T. gondii* were cultured with 2×10^4 $CD11c^+$ DC obtained from uninfected WT mice. (B) T cells obtained from WT mice infected with *T. gondii* were cultured with DC obtained from uninfected WT or KO mice. (A,B) Culture supernatants were collected after 48 h and analyzed for IFN-γ by ELISA. In the absence of *T. gondii* antigens, the production of IFN-γ was not detected in any preparations (nd). T cells from uninfected mice did not produce IFN-γ even in the presence of *T. gondii* antigens (data not shown). Values are the mean + SD of three mice. These experiments were repeated three times with similar results. *indicate statistical significance at $p<0.0001$ using an unpaired Student's t-test.

$CD8^+$ T cells due to impaired thymic positive selection. Recently, Murata *et al.* demonstrated that the β5 thymus-specific subunit is responsible for positive selection of $CD8^+$ T cells [31], supporting the hypothesis that LMP7-deficiency impacts on antigen processing to fully activate $CD8^+$ T cells instead of affecting repertoire formation.

However, other possibilities for the effects of LMP7-deficiency remain to be excluded. For instance, LMP7 deficiency is postulated to cause the impairment of other immunosubunits, LMP2 and MECL-1, due to their interdependent incorporation into the proteasome [32]. Thus, the phenotype of susceptibility in LMP7-deficient mice might not be simply explained by deficiency in the single subunit. Indeed, LMP2-deficient mice were susceptible to increased numbers of *T. gondii*, a feature which is supposed to result from a lack of LMP7-containing immunoproteasomes. However, a lack of LMP2 seemed not to alter the function of LMP7 because the siRNA for LMP2 did not suppress chymotrypsin-like activity *in vitro*, presumably resulting in the comparable activation of $CD8^+$ T cells in LMP2-deficient mice to WT mice. In the absence of LMP2, antigen processing exerted by LMP7 might be achieved by proteasomes composed of combinations of immunosubunits and constitutive subunits [33], in this case LMP7 and β1 instead of LMP2.

Infection with *T. gondii* enhanced antigen processing/presentation to $CD8^+$ but not $CD4^+$ T cells, although $CD4^+$ T cells are activated during the infection. This might be explained by strategies adopted by *T. gondii* for parasitism and immune evasion. Within infected cells, *T. gondii* inhibits fusion of parasitophorous vacuoles (where the parasite lives) to lysosomes that are rich in proteases critical for antigen processing *via* MHC class-II. Our results indicated that the processing of OVA in DC obtained from *T. gondii*-infected mice was decreased (Fig. 1C). Furthermore, DC infected with *T. gondii* were refractory to activation signals through microbial sensory receptors, resulting in the failure to stimulate $CD4^+$ T cells [15]. The expression of MHC class II in macrophages was also reported to be down-regulated during infection with *T. gondii* [34]. In contrast, antigens escaping from the vacuole [35] and those transferred from the vacuoles to the ER [36] could be processed and presented to $CD8^+$ T cells. Thus the parasitism of *T. gondii* seems to severely impair antigen presentation to $CD4^+$ T cells, and activation of $CD4^+$ T cells is unlikely to be antigen-specific, as observed in $CD8^+$ T cells in LMP7-dificient mice, but instead presumably due to cytokines produced by immune cells.

We have not yet addressed the issue of how infection with *T. gondii* induces immunoproteasomes. In mice infected with *T. gondii*, several types of cell secrete IFN-γ [37–39], which is known to be the most potent inducer of the immunosubunits. Among the cells secreting IFN-γ, DC themselves [40] are most likely to be involved because *in vitro* infection of DC could also induce immunosubunit expression in the absence of other cell types. It is most likely that IFN-γ secreted immediately after infection might be crucial for the induction of immunoproteasomes. This cytokine subsequently induces the expression of numerous transcription factors, one of which, IRF-1, is reported to mediate up-regulation of LMP7 [41]. Another possibility is that innate immune responses directly enhance the expression of immunoproteasomes. Analysis of the contribution of TLR to the expression of immunoproteasomes would be interesting because *T. gondii* could stimulate several TLR [42–44] and signals downstream of TLR appear to link to those of IFN-γ [45, 46].

In conclusion, we have reported that an immunosubunit, LMP7, plays a critical role in resistance to *T. gondii* infection presumably due to the generation of potent CTL epitopes that fully activate $CD8^+$ T cells. Our results provide substantial evidence for the theoretical roles of immunoproteasomes in efficient antigen processing using infection with *T. gondii*.

Materials and methods

Mice and parasites

C57BL/6 mice (purchased from Kyudo) were used for experiments at 8–10 wk of age. OT-I and OT-II TcR-transgenic mice

were provided by Dr. K. Yui (Nagasaki University), and LMP2- or LMP7-deficient mice were generated as previously described [10, 12]. These mice had been backcrossed for at least ten generations. Age- and sex-matched groups of mutant and WT mice were used. All experiments using mice were reviewed by the Committee for the Ethics on Animal Experiment in the Faculty of Medicine, and carried out under the control of the Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University, the Law (No. 105) and Notification (No. 6) of the Government. Tachyzoites of the PLK strain of *T. gondii* were maintained through *in vitro* infection of Vero cells cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (complete medium). To purify tachyzoites, the cultures were washed and passed through a 27-gauge needle and a 5.0 μm-pore filter (Millipore). Mice were infected with 1 to 10×10^3 tachyzoites.

Reagents

FITC-conjugated monoclonal antibodies (to CD11c, CD4, and CD8), PE-conjugated anti-GrzB, PE-cy5-conjugated anti-CD8 and APC-conjugated anti-IFN-γ were purchased from eBiosciences. Anti-FITC and anti-PE Microbeads were purchased from Miltenyi Biotech. OVA was purchased from Sigma. OVA peptides were synthesized at Hokkaido systems. Duplexed stealth siRNA for LMP2 and LMP7 were purchased from Hayashi Kasei. The 21-mer siRNA sequences were as follows: LMP2, 5′-guaccgugaggacuuguuagc-3′ and 5′-uaacaaguccucacgguacuu-3′; LMP7, 5′–caucuucagucuuaggaaaaa-3′ and 5′-uaacaaguccucacgguacuu-3′. These siRNA were introduced using RNAiMAX (Invitrogen) according to the manufacturer's protocols.

Cell purification and culture

PEC were prepared from peritoneal exudates obtained from the peritoneal cavity by lavage with 5 mL of complete medium. For purification of DC, single-cell suspensions generated from spleens using collagenase and DNase I were incubated with FITC-anti-CD11c, followed by staining with anti-FITC microbeads. $CD8^+$ or $CD4^+$ T cells were purified from OT-I or OT-II mice, respectively. $CD8^+$ or $CD4^+$ T cells were also purified from mice 7–8 days after infection with *T. gondii* in a similar manner to DC. Finally, cells were positively selected using an MACS column according to the manufacturer's instructions. The purity of the separated cell subset usually exceeded 90% for DC or 95% for T cells.

For OVA presentation, 5×10^4 OT-I $CD8^+$ or OT-II $CD4^+$ T cells were cultured with 2×10^4 DC in the presence of 32 ng/mL OVA protein or OVA peptides in 200 μL of complete medium for 60 h. Culture supernatants collected were analyzed for IFN-γ production, and T-cell proliferation was assessed by incorporation of [^{3}H]-thymidine after an additional incubation for 12 h with 1 μCi/well of the radioisotope.

Quantitative RT-PCR

Total RNA extracted from the purified cells using a total RNA isolation reagent (Invitrogen) was subjected to cDNA synthesis using superscript III reverse transcriptase (Invitrogen). Quantification of mRNA was performed with a real-time PCR system GeneAmp7000 thermal cycler (Applied Biosystems) using SYBR premix Ex Taq (Takara) based on the detection of the SYBR Green double-stranded DNA-binding dye. The following primers were used: for β-actin sense and antisense primers were 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′, respectively; for LMP2 sense and antisense primers were 5′-TACCAGCTGGAGCTACACGGG-3′ and 5′-AGTGATGGCATCTGTGGTGAA-3′, respectively; for LMP7 sense and antisense primers were 5′-CGGAATGGGGAACGCATCTCC-3′ and 5′-GTTGTCTCTGTGGGTAGCATA-3′, respectively; β1 sense and antisense primers were 5′-GACAAGACAGTAATTGGCTGC-3′ and 5′-AGGCTGCAGCATGGCACTTGC-3′, respectively; β5 sense and antisense primers were 5′-CACAGCAGGTGCTTATATTGC-3′ and 5′-CTGTTCCCCTCGCTGTCTACG-3′, respectively.

Western blotting

Cell lysates prepared from PEC and DC2.4 cells infected with *T. gondii* were separated by SDS-PAGE. Proteins were transferred onto PVDF membrane using the iBlot electrotransfer system (Invitrogen), before probing with anti-mouse LMP2 or anti-mouse LMP7 conjugated with horseradish peroxidase. Bound antibodies were visualized with ECL chemiluminescence under a LAS system (Fuji Film). The quantity of LMP7 was estimated based on the intensities relative to those of β-actin.

ELISA

IFN-γ in the culture supernatants was measured using a DuoSet sandwich ELISA (R&D Systems) according to the manufacturer's instructions.

Proteasome function assays

Proteolytic activity of proteasomes was measured as described previously [47, 48]. Briefly, confluent cell layers of DC2.4 cells were washed with PBS, then with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP), and pelleted. Glass beads (< 106 microns, acid washed; Sigma) equivalent to the volume of the cellular pellet were added, followed by the same volume of homogenization buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 250 mM sucrose). Cells were vortexed for 1 min. Beads and cell debris were removed by centrifugation at $1000 \times g$ for 5 min, followed by $10\,000 \times g$ for 20 min. Protein concentrations were determined using the BCA protocol (Pierce Chemical). Ten micrograms of protein from each sample were diluted to 100 μL in 50 mM Tris, pH 7.4.

Peptidase activity was measured using the following fluorescence-labeled peptide substrate: suc-LLVY-7-amino-4-methylcoumarin (AMC) from Sigma for the analysis of β5, LMP7, and LMP2 activity of proteasomes. The substrate was added in a final concentration of 80 μM in 1% DMSO. The samples were incubated at 37°C for 30 min and the reaction was quenched with 1% SDS. Fluorescent-free AMC was assessed using a fluorescence plate reader (Perkin-Elmer ARVO SX).

Flow cytometry

Full splenocytes were stained with combinations of fluorochrome-labeled antibodies. To detect intracellular molecules, cells were stimulated with 50 ng/mL PMA and 1 μg/mL calcium ionophore in the presence of 1 ug/mL brefeldin A, in complete medium at 37°C for 5 h. These cells were then stained with surface markers, followed by fixation with 4% paraformaldehyde and permeabilization with 0.1% saponin. The cells were then stained with a second set of fluorochrome-labeled antibodies (IFN-γ and GrzB). Stained cells were analyzed using a FACSCalibur cytometer (Becton Dickinson).

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Abbreviations: **GrzB:** granzyme B · **PEC:** peritoneal exudate cell

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Concurrent infection with *Heligmosomoides polygyrus* suppresses anti-*Plasmodium yoelii* protection partially by induction of $CD4^+CD25^+Foxp3^+$ Treg in mice

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Malaria and intestinal nematode infection are widespread and co-infection frequently occurs. We investigated whether co-infected intestinal nematodes modulate immunity against co-existing malaria parasites. Infection of C57BL/6 mice with *Plasmodium yoelii* 17XNL (Py) was transient and self-limiting, but preceding infection with *Heligmosomoides polygyrus* (Hp), a mouse intestinal nematode, exacerbated malaria resulting in higher parasite burdens and poor survival of the mice. Co-infection with Hp led to reduced Py-responsive proliferation and IFN-γ production of spleen cells, and higher activation of $CD4^+CD25^+Foxp3^+$ Treg. *In vivo* depletion of Treg recovered anti-Py immunity and rescued co-infected mice from exacerbated malaria. However, we did not observe any obvious *ex vivo* activation of Treg by either Hp products or living worms. Our results suggest that intestinal nematodes moderate host immune responses during acute malaria infection by aggressive activation of Treg. Elucidation of the mechanisms of Treg activation *in situ* is a target for future analyses.

Key words: Adaptive immunity · Malaria · Intestinal nematode · Regulatory T cell

Introduction

Malaria is the most widespread and deadliest parasitic disease, and it causes hundreds of millions of clinical cases and millions of deaths annually worldwide. The severity of the disease is strongly related to the malaria parasite species, parasite density and immune responses of the host. Protective immunity against malaria develops very slowly, and individuals living in endemic areas suffer from repeated infections. The major reason why immunity to malaria is difficult to develop is that malaria parasites effectively evade host immunity in several ways. Antigenic diversity/variation allows parasites to escape immune recognition [1, 2]. They also actively suppress immunity *via* induction of Treg [3, 4], effector T-cell apoptosis [5] or dysfunctions of APCs [6]. These immune evasion mechanisms also make it difficult to develop effective vaccines against malaria.

Environmental factors, such as exposure to infective vectors [7], nutritional status [8], medical/public health interventions [9] and concurrent infections with other pathogens [10], also affect the outcome of the disease. Among infections, chronic but mostly asymptomatic infection with intestinal helminths is the most

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prevalent in malarial areas [11, 12] (http://gamapserver.who.int/mapLibrary/Files/Maps/global_cases.jpg, http://www.who.int/intestinal_worms/epidemiology/map/en/index.html) and the population in a given area tends to suffer from both infections. Generally, as well as schistosomes [13] or filarias [14], intestinal helminths are known to modulate, and mainly suppress, host immune responses [15]. Indeed, studies in Thailand showed that infection with intestinal helminths increased the frequency of malaria episodes, but decreased malaria-associated serious inflammations, such as cerebral malaria, acute renal failure or pulmonary edema [16, 17]. These observations suggest that intestinal helminths suppress host responses of both protective immunity and inflammation during malaria infection. Therefore, it is important to understand the relationship between co-infection and host immune responses for effective control of malaria.

Several researchers have studied the effects of co-infection with intestinal helminths on the course of malaria using mouse models and reported that co-infection causes rapid growth of malaria parasites *in vivo* [18–20]. A good experimental model is the mouse intestinal nematode *Heligmosomoides polygyrus* (Hp), which resides in the upper small intestine for a long time and is known to modulate host responses through various mechanisms [21, 22]. Su *et al.* [18] described that proliferation of immune cells in response to the malaria Ag or production of anti-malaria Ab was suppressed with the induction of regulatory cytokines during co-infection with Hp and *Plasmodium chabaudi*. Unlike the results from human studies in Thailand, Hp did not attenuate inflammation-associated experimental cerebral malaria, but it did suppress anti-parasite immunity during infection with *P. berghei* ANKA [20]. These observations are compatible with our findings from studies on mice infected with Hp [21]. However, the interactions between nematodes inside the intestine and immune systems are still not well understood.

Here, we examined the effects of co-infection with Hp on the course of infection with the rodent malaria parasite *P. yoelii* 17XNL (Py) by focusing on $CD4^+CD25^+Foxp3^+$ Treg as key factors linking nematodes and suppressed host protection. We found that Hp and Py co-infection induced strongly activated Treg, which suppressed anti-Py effector mechanisms, increased malaria parasite growth *in vivo* and deteriorated survival of mice.

Results

Preceding infection with Hp deteriorates Py infection

First, we infected Hp-harboring mice with the non-lethal malaria strain Py. As previously reported [4], infection with Py alone exhibited transient elevation of parasitemia and a spontaneous cure within 3–4 wk (Fig. 1A). Co-infection with Hp caused rapid growth of Py at the early and late phases of infection (Fig. 1A), and all the mice died (Fig. 1B). Unlike the rodent parasite *P. berghei*, Py is widely considered not to induce fatal host-damaging inflammations, such as liver injury [23]. Therefore, the cause of death of the co-infected mice was supposed to be failure to eradicate the parasites, suggesting that the presence of Hp suppressed host immune responses against Py.

Co-infection with Hp suppresses adaptive immune responses against Py

Next, we analyzed the immune responses to Py in Hp-harboring mice after infection with Py. Splenocytes isolated from co-infected mice were stimulated with Py-parasitized RBC (pRBC) and analyzed for their proliferative responses (Fig. 2A). Whole spleen cells from mice infected with Py alone showed remarkable proliferation in response to pRBC. However, preceding infection with Hp significantly suppressed this proliferation. Similar results were obtained when $CD4^+CD25^-$ spleen cells were examined. The suppression was not specific for Py Ag because responses to ConA were also suppressed. We also analyzed production of IFN-γ, one of the indispensable effector molecules against malaria parasites [24]. Consistent with the proliferative responses, production of IFN-γ by spleen cells of mice infected

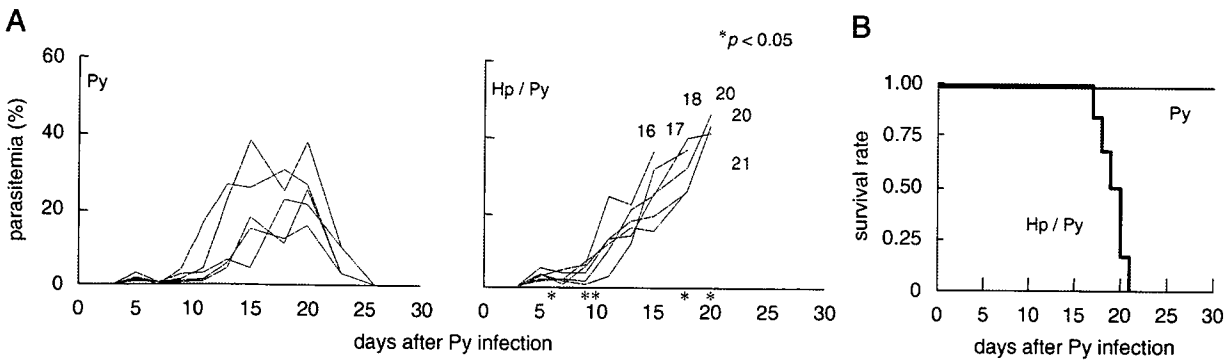

Figure 1. *H. polygyrus* and *P. yoelii* NL co-infection in C57BL/6N male mice. Mice ($n = 5–7$) were infected with Hp larvae orally and then i.p. infected with Py pRBC (2.5×10^4 cells/mouse) 2 wk later. Parasitemia (A) and survival (B) of the mice were monitored daily. Each line shows the parasitemia curve of an individual mouse, and the numbers show the death day of each individual (A). Parasitemia was analyzed statistically by Student's *t*-test. Asterisks show significant differences at the indicated *p* value. The experiment was repeated three times with similar results.

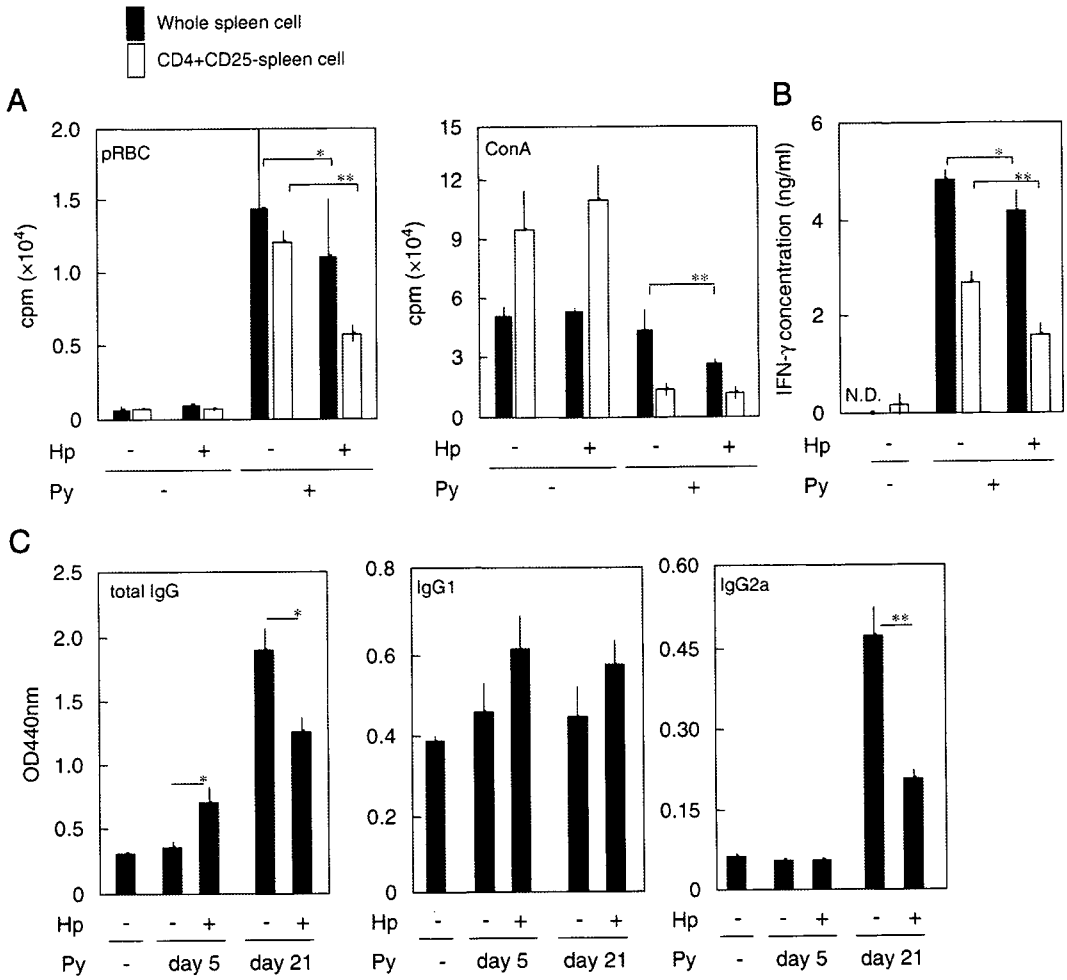

Figure 2. Effects of co-infection with Hp on immune responses against Py. Spleen cells or $CD4^+CD25^-$ spleen cells were isolated from mice co-infected with Hp and Py at 5 days after Py infection. Whole spleen cells (2×10^5; closed bars) or $CD4^+CD25^-$ cells (1×10^5) and $CD11c^+$ cells (1×10^4) from uninfected mice (open bars) were cultured with pRBC (2×10^5) or ConA (2.5 μg/mL). (A) Proliferation was analyzed by ^{3}H-thymidine uptake. (B) IFN-γ concentrations in the culture supernatants with pRBC were measured by ELISA. (C) Anti-Py IgG, IgG1 or IgG2a Ab in sera taken at the indicated days after Py infection were measured by ELISA using Py Ag. (D) $CD11c^+$ cells (1×10^4) were isolated from mice co-infected with Hp and Py at 5 days after Py infection and cultured with $CD4^+CD25^-$ cells (1×10^5) from Py-infected mice and pRBC (2×10^5). Proliferation was analyzed by ^{3}H-thymidine uptake. Data represent the means ±SE of triplicate samples in a representative of 3–6 repeated experiments. $^*p<0.05$, $^{**}p<0.01$; between the samples linked with horizontal lines, Student's t-test.

with Py was significantly suppressed by preceding infection with Hp (Fig. 2B). These results clearly indicate that co-infection with Hp suppresses cellular immune responses. Humoral immunity, represented by IgG specific for malaria parasites, was subsequently analyzed (Fig. 2C). Infection with Py alone led to the development of IgG2a-dominant Ab responses at 21 days after infection, but had no effect at 5 days after infection. Despite the higher parasitemia at the early phase, co-infection with Hp rather enhanced the production of IgG1. Conversely, it remarkably reduced the production of IgG2a in curable mice singly infected with Py, suggesting that the Ab responsible for protection at the late phase may be IgG2a. These results demonstrate that the increased Py parasitemia at the early stage of infection is due to suppression of cellular responses, rather than Ab responses. Therefore, we subsequently focused on the suppression of cellular responses at the early phase.

Co-infection with Hp and Py induces aggressively suppressive Treg

In a previous report, we demonstrated that activation of Treg occurs during infection with a lethal strain of *P. yoelii* [4]. In addition, it has been reported that the functions of Treg are altered in mice infected with Hp [25]. These observations led us to analyze the Treg behaviors in co-infected mice. As early as 5 days after infection with Py, the proportion of $CD4^+CD25^+$ $Foxp3^+$ cells was increased in the spleen, as well as MFI of either

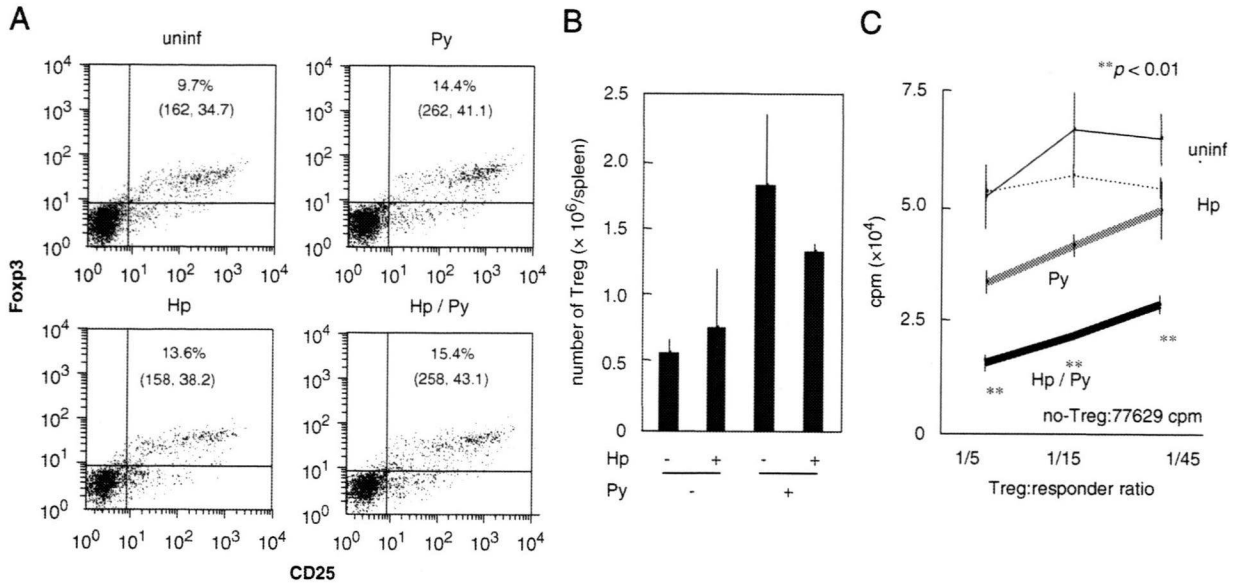

Figure 3. $CD4^+CD25^+Foxp3^+$ cells in Hp+Py co-infection. Spleen cells stained with fluorochrome-conjugated anti-CD4, anti-CD25 and anti-Foxp3 Ab were analyzed by flow cytometry at 5 days after infection with Py. (A) The dot plots represent the expression levels of CD25 and Foxp3 in CD4-gated cells from the indicated mice. Percentage inside of each plot shows the frequency of $CD4^+CD25^+Foxp3^+$ cells in total $CD4^+$ cells. The number inside of brackets shows the mean fluorescent intensity of CD25 (left) and Foxp3 (right). (B) The $CD4^+CD25^+Foxp3^+$ cell populations in individual spleens were calculated. (C) The suppressive functions of Treg from co-infected mice were analyzed at 5 days after infection with Py. Splenic Treg from the indicated mice were cultured with $CD4^+CD25^-$ cells (1×10^5) as responders and $CD11c^+$ cells (1×10^4) obtained from the spleens of uninfected mice in the presence of ConA (2.5 μg/mL) at the indicated frequencies. The proliferation of responders was analyzed by ^{3}H-thymidine uptake. Data are presented as the means ±SE of three samples in a representative of three repeated experiments. Asterisks show significant differences by Student's *t*-test between Treg from Py-infected and Hp+Py-infected mice at the indicated *p* value.

CD25 or Foxp3 (Fig. 3A). The absolute number of $CD4^+CD25^+Foxp3^+$ cells in individual spleens, calculated from the cell frequency by flow cytometry, was also increased (Fig. 3B). The amount of Foxp3 expressed in Treg may influence their functions [26], suggesting that Treg with the higher Foxp3 MFI observed in co-infection may have enhanced suppressive function. Thus, the suppressive function of Treg was determined by analyzing the degree of suppression of TCR-triggered T-cell proliferation at this time point. Co-infection with Hp enhanced the suppressive activity of Treg, but did not affect the number of Treg (Fig. 3B, C). These results suggest that co-infection with Hp and Py induces Treg more aggressively than single infection with Py, resulting in deteriorated malaria infection associated with suppressed cellular responses against Py.

In vivo depletion of Treg partially abolishes the suppressed protection against Py

To confirm that induction of Treg in mice co-infected with Hp and Py is responsible for the deteriorated infection with Py, we depleted Treg by i.p. application of an anti-CD25 Ab. This treatment depleted 85% of the $CD4^+CD25^+Foxp3^+$ Treg in the spleen, as evaluated by Foxp3 intracellular flow cytometry, at 5 days after infection (Fig. 4A). However, the depletion effect was transient and Treg were detected in mice treated with the anti-CD25 Ab at similarly high levels to those in control mice at 2 wk after the treatment (data not shown). As previously shown, infection with Py was self-limiting, and again mice co-infected with Hp suffered from higher parasitemia and finally succumbed to infection with Py (Fig. 4B, C). Depletion of Treg significantly decreased the parasitemia at the early phase of infection in co-infected mice. Furthermore, 40% of the co-infected mice depleted of Treg were able to limit the parasitemia and they survived (Fig. 4B, C).

The anti-malarial effector mechanisms were altered by depletion of Treg. The suppression of IFN-γ production by splenic $CD4^+$ T cells in response to pRBC observed in co-infected mice was clearly reversed after removal of Treg (Fig. 4D). Alterations in Ab responses due to co-infection with Hp, namely suppression of IgG and IgG2a at the later phase and enhancement of IgG at the early phase, were all offset by the removal of Treg (Fig. 4E). These results suggest that the deteriorated malaria infection in mice co-infected with Hp and Py was partially due to aggressive activation of Treg.

Hp worm products and living worms do not directly activate Treg *in vitro*

The preceding existence of Hp activates Treg in terms of enhanced suppressive functions during infection with Py. To investigate how co-infection with Hp induces stronger Treg, we performed *in vitro* Treg activation assays using various Hp products. Splenic Treg from Py-infected mice were stimulated

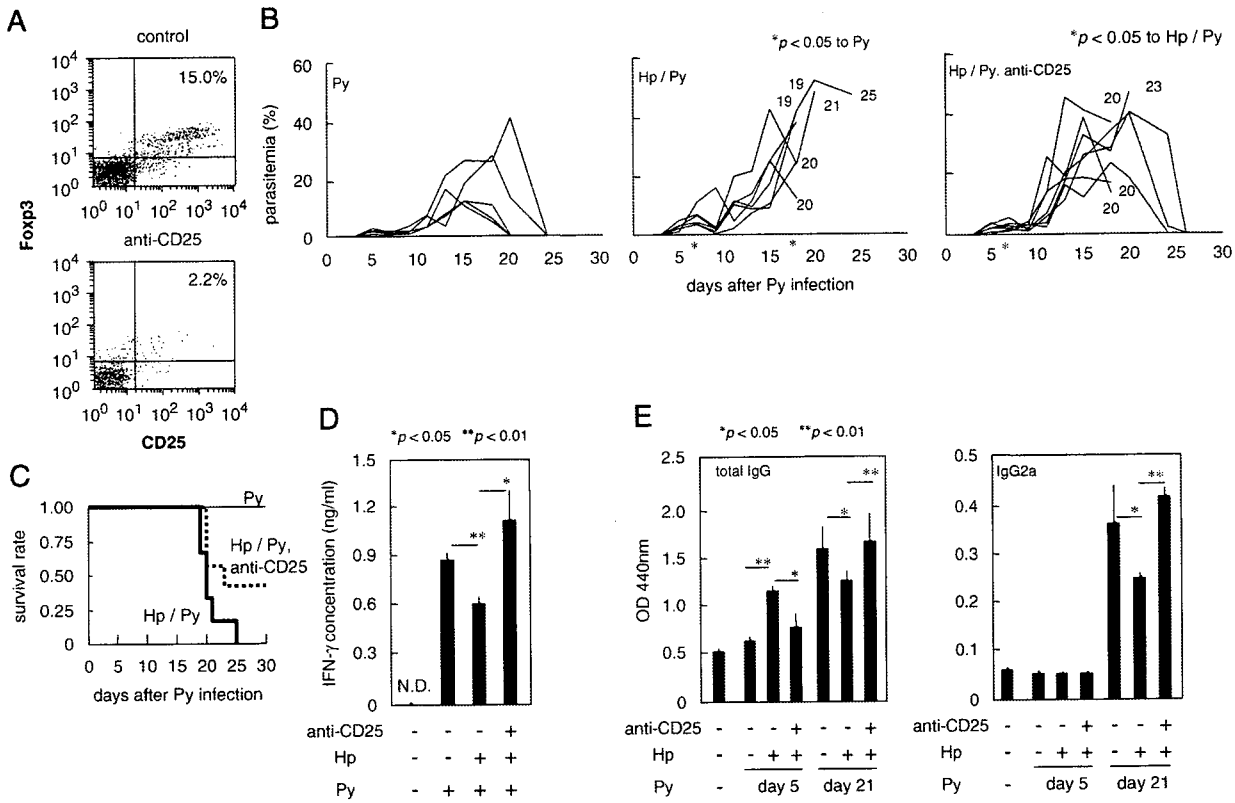

Figure 4. Effects of depletion of Treg in mice co-infected with Hp and Py. Spleen cells from mice depleted of Treg were analyzed as described in the legend for Fig. 3A. (A) The treatment depleted 85% of Treg in the spleen, as evaluated by flow cytometry on day 5 after Py infection. (B,C) Py infection in mice with or without depletion of Treg was monitored by the parasitemia (B) and survival (C) as described in the legend for Fig. 1. The infection experiments were repeated three times with similar results. (D, E) Immune responses and IFN-γ production in $CD4^+$ cells in response to pRBC (D) and serum levels of anti-Py IgG (E) were analyzed as described in the legend for Fig. 2. Data are presented as the means ±SE of three samples in a representative of 3–6 repeated experiments. Asterisks show significant differences at the indicated *p* values by Student's t-test.

with excretory/secretory (ES) Ag of Hp, an extract of adult Hp worms or living Hp worms in the presence of $CD11c^+$ DCs (Fig. 5). The cultured Treg were then analyzed for their suppressive activities. Treg cultured with medium alone still exhibited suppressive functions, although their effects were lower than those of freshly isolated Treg. However, none of the stimulatory conditions changed the suppressive abilities of the cultured Treg. The same tendency was observed when Treg were cultured without DC (data not shown) and when Treg from uninfected mice were stimulated with Hp preparations in combination with DC and pRBC, as well as without DC (data not shown). These results indicate that Hp worms residing in the intestine do not directly activate Treg.

Discussion

In the present study, we have demonstrated that a mouse intestinal nematode, Hp, was able to suppress anti-malaria protection *via* induction of Treg. Infection with malaria parasites is already known to activate Treg in humans and experimental models [3, 4], which allows rapid growth of malaria parasites. As previously reported [4], the non-lethal malaria parasite Py used in the present study does not strongly activate Treg and its low pathogenicity/virulence is closely linked to its failure to activate Treg. Therefore, it seems likely that activation of Treg in the presence of Hp converted the pathogenic behaviors of Py, as observed for a highly virulent *P. yoelii* strain [4]. Furthermore, the involvement of Treg activation for conversion to high virulence was clearly confirmed by the partial reversal of high parasitemia and mortality in mice depleted of Treg.

However, anti-CD25 treatment did not rescue all of the co-infected mice, partially because the depletion of Treg did not last longer than 2 wk. This finding may be supported by a previous observation that anti-CD25 treatment is insufficient because of a rapid expansion of Treg after infection with *P. yoelii* [27]. Interestingly, depletion of Treg recovered the suppression of presumably protective IgG responses at the late phase when Treg themselves reappeared comparably with the control Treg-sufficient mice. Therefore, besides activation of Treg, we cannot exclude various mechanisms supposed to cause the global immune suppression by intestinal helminths, regardless of the importance of Treg. For instance, the elevation of IgG1 observed at the early phase of infection suggests that Th2