evidence of degradation. The BioB control cRNA was spiked in at the detection threshold of 1.5 pM and received a 'present' detection call. Housekeeping genes had 3′–5′ detection ratios of <4. Complementary DNA synthesis, *in vitro* transcription to cRNA and hybridization were performed as per http://www.healthsystem.virginia.edu/internet/biomolec/microarray.cfm.

Raw data from the arrays was normalized at probe level using gcRMA algorithm. The detection call (Present, Marginal, Absent) for each probe set was obtained using the GeneChip Operating Software system. CBA/J vs B6 groups were compared using dChip software, whereby gene expression was deemed significantly different if the *P*-value was less than or equal to 0.05, the fold change in signal intensity was >1.75 or <−1.75, and the absolute difference in signal intensity was >100.

RT-PCR

Quantitative RT-PCR was used to validate changes in a subset of genes from microarray selection from each of the three CBA and three B6 RNA samples. RT-PCR primers were designed using Primer3 based on the published 3′ sequence as per Affymetrix. The primers used were glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): forward, GCTAAGCAGTTGGTGGTGCA and reverse, TCACCACCATGGAGAAGG; BCL2-like 11 apoptosis facilitator (*Bcl2l11*): forward, TCCTATTTCC TTCCAGTGTTGT and reverse, GGGAACACAATTGCA CAGAG; lymphocyte antigen 96 (*Ly96*): forward, CTTTTCGACGCTGCTTTCTC and reverse, CCATGGC ACAGAACTTCCTT; transformation related protein 53 binding (*Trp53bp1*): forward, AGTCCTGAGACAGA GGCCAGT and reverse, GTGTGGGTGCCACAGAGGT; transmembrane protein 87A (*Tmem87a*): forward, TGT GGTGCACACTCCAAAA and reverse, TAAGCTCCCC TTCTGAAGCA; pallidin (*Pldn*): forward, GCAGCTCA CAGCCATCTGTA and reverse, TTAGGGCTTCATCA CCTATGG; carboxy-terminal domain, RNA polymerase II, polypeptide A (*Ctdspl2*): forward, GAGAGCCCAAT GATTAGCAA and reverse, CAGCAACAAAATAATTA TAAAGTCAGA; spermatogenesis associated 5-like 1 (*Spata5l1*): forward, CGGCCTGGAAGATTAGATAAGA and reverse, CACAGTCTTGAGTGCTTCTGTG. GAPDH was used as the internal control. Real-time PCR was conducted using iQ SYBR Green SuperMix (Bio-Rad, Hercules, CA, USA) on an Bio-Rad iCycler. Target gene amplification was quantified as the reciprocal $\log_2$ of the $\Delta C(t)$ between GAPDH and the target gene.

Statistics

Genetic markers were assigned to and mapped within the chromosomes using multipoint linkage analysis with Map Maker QTXb20. Genome-wide interval mapping for amebic antigen load measured by ELISA and inflammation score was performed using QTX software to identify QTLs. All QTL mapping methods offered by QTX generate a LRS as a measure of the significance of a possible QTL.[31] The LRS approaches a χ^2-statistic with large sample sizes. Single-locus associations were tested by simple regression analysis between trait values and genotypes and the significance of each potential association was measured using the LRS. A total of 5000 permutations of the trait values were used to define the genome-wide LRS threshold required to be significant or suggestive for each specific trait. Loci exceeding the 95th percentile of the permutation distribution were defined as significant ($P<0.05$) and those exceeding the 37th percentile were suggestive ($P<0.63$).[32] Statistical significance for infection rate was determined by Fisher's exact test. Means were compared using *t*-test or Mann–Whitney test if data were not gaussian. RT-PCR differences were determined using unpaired Student's *t*-test. Fold changes between RT-PCR and microarray were compared using linear regression analysis using Pearson's R correlation.

Acknowledgements

This work was supported by research grants from NIH (AI071373, AI052444), the Crohn's and Colitis Foundation of America, the Commonwealth of Virginia Technology Research Fund. SH was supported by a grant-in-Aid for Scientific Research from the Ministry of Education, Science, Technology, Sports and Culture of Japan and by the Mochida Memorial Foundation for Medical and Pharmaceutical Research. We thank Anna Velcich for provision of the Muc2 KO mice, Craig Coopersmith for the FVB mice, William A Petri Jr, for helpful advice and David Lyerly, Techlab, for the *E. histolytica* II ELISA kits. We acknowledge the UVA Research Histology Core of the Center for Research in Reproduction and thank Sharon Hoang of the UVA Digestive Health Center of Excellence Histology Core for immunohistochemistry support, whose work was partially supported by the Morphology/Imaging Core of the National Institutes of Health-funded Silvio O Conte Digestive Diseases Research Center (P30DK56703).

Disclosures

The authors have no conflicting financial interests.

References

1 Walsh JA. Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. *Rev Infect Dis* 1986; **8**: 228–238.
2 Haque R, Duggal P, Ali IM, Hossain MB, Mondal D, Sack RB *et al*. Innate and acquired resistance to amebiasis in Bangladeshi children. *J Infect Dis* 2002; **186**: 547–552.
3 Haque R, Mondal D, Duggal P, Kabir M, Roy S, Farr BM *et al*. *Entamoeba histolytica* infection in children and protection from subsequent amebiasis. *Infect Immun* 2006; **74**: 904–909.
4 Irusen EM, Jackson TF, Simjee AE. Asymptomatic intestinal colonization by pathogenic *Entamoeba histolytica* in amebic liver abscess: prevalence, response to therapy, and pathogenic potential. *Clin Infect Dis* 1992; **14**: 889–893.
5 Blessmann J, Ali IK, Nu PA, Dinh BT, Viet TQ, Van AL *et al*. Longitudinal study of intestinal *Entamoeba histolytica* infections in asymptomatic adult carriers. *J Clin Microbiol* 2003; **41**: 4745–4750.
6 Gathiram V, Jackson TF. A longitudinal study of asymptomatic carriers of pathogenic zymodemes of *Entamoeba histolytica*. *S Afr Med J* 1987; **72**: 669–672.
7 Acuna-Soto R, Maguire JH, Wirth DF. Gender distribution in asymptomatic and invasive amebiasis. *Am J Gastroenterol* 2000; **95**: 1277–1283.
8 Houpt E, Barroso L, Lockhart L, Wright R, Cramer C, Lyerly D *et al*. Prevention of intestinal amebiasis by vaccination with the *Entamoeba histolytica* Gal/GalNac lectin. *Vaccine* 2004; **22**: 611–617.

9 Haque R, Mondal D, Shu J, Roy S, Kabir M, Davis AN *et al.* Correlation of interferon-gamma production by peripheral blood mononuclear cells with childhood malnutrition and susceptibility to amebiasis. *Am J Trop Med Hyg* 2007; **76**: 340–344.
10 Houpt ER, Glembocki DJ, Obrig TG, Moskaluk CA, Lockhart LA, Wright RL *et al.* The mouse model of amebic colitis reveals mouse strain susceptibility to infection and exacerbation of disease by CD4+ T cells. *J Immunol* 2002; **169**: 4496–4503.
11 Asgharpour A, Gilchrist C, Baba D, Hamano S, Houpt E. Resistance to intestinal *Entamoeba histolytica* infection is conferred by innate immunity and Gr-1+ cells. *Infect Immun* 2005; **73**: 4522–4529.
12 Hamano S, Asgharpour A, Stroup SE, Wynn TA, Leiter EH, Houpt E. Resistance of C57BL/6 mice to amoebiasis is mediated by nonhemopoietic cells but requires hemopoietic IL-10 production. *J Immunol* 2006; **177**: 1208–1213.
13 Belley A, Keller K, Grove J, Chadee K. Interaction of LS174T human colon cancer cell mucins with *Entamoeba histolytica*: an *in vitro* model for colonic disease. *Gastroenterology* 1996; **111**: 1484–1492.
14 Petri Jr WA, Smith RD, Schlesinger PH, Murphy CF, Ravdin JI. Isolation of the galactose-binding lectin that mediates the *in vitro* adherence of *Entamoeba histolytica*. *J Clin Invest* 1987; **80**: 1238–1244.
15 Velcich A, Yang W, Heyer J, Fragale A, Nicholas C, Viani S *et al.* Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science* 2002; **295**: 1726–1729.
16 Gottke MU, Keller K, Belley A, Garcia RM, Hollingsworth MA, Mack DR *et al.* Functional heterogeneity of colonic adenocarcinoma mucins for inhibition of *Entamoeba histolytica* adherence to target cells. *J Eukaryot Microbiol* 1998; **45**: 17S–23S.
17 Whitmore AC, Whitmore SP. Subline divergence within L.C. Strong's C3H and CBA inbred mouse strains. A review. *Immunogenetics* 1985; **21**: 407–428.
18 Miron M, Woody OZ, Marcil A, Murie C, Sladek R, Nadon R. A methodology for global validation of microarray experiments. *BMC Bioinformatics* 2006; **7**: 333.
19 Lotter H, Jacobs T, Gaworski I, Tannich E. Sexual dimorphism in the control of amebic liver abscess in a mouse model of disease. *Infect Immun* 2006; **74**: 118–124.
20 Strachan NJ, Watson RO, Novik V, Hofreuter D, Ogden ID, Galan JE. Sexual dimorphism in campylobacteriosis. *Epidemiol Infect* (in press).
21 Ge Z, Feng Y, Taylor NS, Ohtani M, Polz MF, Schauer DB *et al.* Colonization dynamics of altered Schaedler flora is influenced by gender, aging, and *Helicobacter hepaticus* infection in the intestines of Swiss Webster mice. *Appl Environ Microbiol* 2006; **72**: 5100–5103.
22 Fox JG, Wang TC, Rogers AB, Poutahidis T, Ge Z, Taylor N *et al.* Host and microbial constituents influence *Helicobacter pylori*-induced cancer in a murine model of hypergastrinemia. *Gastroenterology* 2003; **124**: 1879–1890.
23 Aebischer T, Laforsch S, Hurwitz R, Brombacher F, Meyer TF. Immunity against *Helicobacter pylori*: significance of interleukin-4 receptor alpha chain status and gender of infected mice. *Infect Immun* 2001; **69**: 556–558.
24 Kiyota M, Korenaga M, Nawa Y, Kotani M. Effect of androgen on the expression of the sex difference in susceptibility to infection with *Strongyloides ratti* in C57BL/6 mice. *Aust J Exp Biol Med Sci* 1984; **62** (Part 5): 607–618.
25 Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S *et al.* Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol* 2002; **3**: 667–672.
26 Wieland CW, Florquin S, Maris NA, Hoebe K, Beutler B, Takeda K *et al.* The MyD88-dependent, but not the MyD88-independent, pathway of TLR4 signaling is important in clearing nontypeable haemophilus influenzae from the mouse lung. *J Immunol* 2005; **175**: 6042–6049.
27 Clarke AR, Jones N, Pryde F, Adachi Y, Sansom OJ. 53BP1 deficiency in intestinal enterocytes does not alter the immediate response to ionizing radiation, but leads to increased nuclear area consistent with polyploidy. *Oncogene* 2007; **26**: 6349–6355.
28 Yan L, Stanley Jr SL. Blockade of caspases inhibits amebic liver abscess formation in a mouse model of disease. *Infect Immun* 2001; **69**: 7911–7914.
29 Cliffe LJ, Potten CS, Booth CE, Grencis RK. An increase in epithelial cell apoptosis is associated with chronic intestinal nematode infection. *Infect Immun* 2007; **75**: 1556–1564.
30 Carlstedt I, Lindgren H, Sheehan JK, Ulmsten U, Wingerup L. Isolation and characterization of human cervical-mucus glycoproteins. *Biochem J* 1983; **211**: 13–22.
31 Haley CS, Knott SA. A simple regression method for mapping quantitative trait loci in line crosses using flanking markers. *Heredity* 1992; **69**: 315–324.
32 Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* 1995; **11**: 241–247.

Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)

Am. J. Trop. Med. Hyg., 79(6), 2008, pp. 819–822
Copyright © 2008 by The American Society of Tropical Medicine and Hygiene

Short Report: Concurrent Infection with *Heligmosomoides polygyrus* Modulates Murine Host Response against *Plasmodium berghei* ANKA Infection

Kohhei Tetsutani,* Kenji Ishiwata, Motomi Torii, Shinjiro Hamano, Hajime Hisaeda, and Kunisuke Himeno

Department of Parasitology, Kyushu University Graduate School of Medicine, Fukuoka, Japan; Department of Tropical Medicine, The Jikei University School of Medicine, Tokyo, Japan; Department of Molecular Parasitology, Ehime University School of Medicine, To-on, Ehime, Japan

Abstract. We investigated whether concurrent infection with *Heligmosomoides polygyrus*, an intestinal nematode, modulated anti-malaria parasite immunity and development of experimental cerebral malaria (ECM) in mice. The C57BL/6 mice infected with *Plasmodium berghei* ANKA showed typical symptoms of ECM. Interestingly, preceding *H. polygyrus* infection did not alter ECM development, despite accelerated *P. berghei* growth *in vivo*. Our observation provides a new insight that ECM can be induced in a fashion independent of the immune responses affected by concurrent *H. polygyrus*. Differentiation between protective immunity and infection-associated host-damaging inflammatory response is urgently required for understanding the pathogenesis of cerebral malaria.

Malaria parasites cause the worst parasitic disease, with hundreds of millions of clinical cases annually worldwide. Severe malarial anemia and cerebral malaria are of particular importance clinically, both of which are responsible for millions of deaths. Cerebral malaria is considered to be a consequence of mechanical occlusion of the small blood vessels in the brain with parasitized red blood cells (RBCs), and/or of immunologic pathology attributed to local inflammation.[1] Several lines of evidence supporting these hypotheses have been reported, yet the precise mechanisms underlying onset of cerebral malaria remain unclear.

A good rodent model for cerebral malaria is the infection of C57BL/6 mice with *Plasmodium berghei* ANKA, where the degree of immune activation reflects the extent of neurologic complications.[2] However, intestinal helminths cause the largest number of parasitic infections among humans, but they usually cause nil to mild symptoms during their chronic infections. It has been reported that host immune responses are modulated with intestinal helminthes. Infections with these helminths increase susceptibility to viral, bacterial, or parasitic infections, and attenuate efficacy of several vaccines against infectious diseases[3]; helminthic infections moderate allergic reactions or autoimmune conditions, which result from aberrant immune responses against foreign antigens or self-constituents.[3]

In humans, the prevalence of malaria and infections with intestinal helminths overlap geographically, and the population in a given area suffers from both infections.[4,5] It is supposed that infections with helminths affect the symptoms of malaria: Thai patients who have intestinal nematode infections suffer from malaria episodes more frequently.[6] Conversely, symptoms of each episode are less severe and the frequency of cerebral malaria, pulmonary edema, or renal failure, all of which are associated with immunopathology, is lower when patients have dense helminthic infections.[7]

It has been hypothesized that infections with intestinal helminths weaken protective immunity against co-existing malaria parasites, and simultaneously, the host-damaging inflammatory responses associated with malaria. To test this hypothesis, we examined the effects of concurrent infection with *Heligmosomoides polygyrus* on protective immunity and on experimental cerebral malaria (ECM) using C57BL/6 mice infected with *P. berghei*. All experiments using mice were conducted according to the guidelines for animal experimentation of Kyushu University.

Heligmosomoides polygyrus is a mouse intestinal nematode, which resides in the upper small intestine,[8] and it is maintained through *in vivo* passage using male ICR mice. For infection, feces containing eggs were incubated on wet filter paper for a minimum of three days to allow eggs to develop infective larvae. Male C57BL/6 mice at the age of eight to ten weeks were infected orally with 200 infective larvae by gastric intubation. Production of eggs began to be detected as early as 10 days after infection and continued for longer than eight weeks (data not shown). The mice did not show any central nervous system (CNS) symptoms during infection with *H. polygyrus*. After confirmation of *H. polygyrus* infection by feces examination, 2.5×10^4 *P. berghei*-infected RBCs were injected intraperitoneally (IP), at 14 days after *H. polygyrus* infection. Co-infection with *P. berghei* increased egg production by *H. polygyrus* slightly, without statistical significance (Figure 1).

Infection of C57BL/6 mice with *P. berghei* caused high lethality with CNS symptoms, which usually developed within

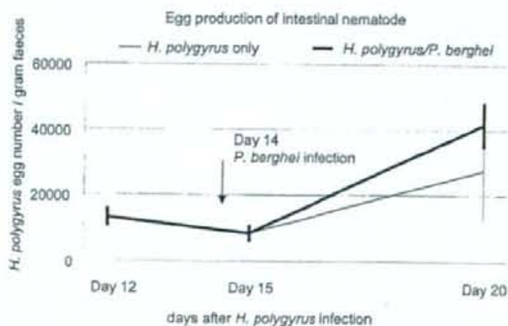

FIGURE 1. Egg production of *Heligmosomoides polygyrus*. The C57BL/6 mice were orally infected with infective larvae of *H. polygyrus*. Fecal egg number was counted at the indicated days after infection with *H. polygyrus*. Data show means ± SE of four to six mice. Experiments were repeated three times with similar results.

* Address correspondence to Kohhei Tetsutani, Department of Parasitology, Kyushu University Graduate School of Medicine, 3-1-1, Maidashi, Higashi-ku, Fukuoka, 812-0054, Japan. E-mail: tetztani@parasite.med.kyushu-u.ac.jp

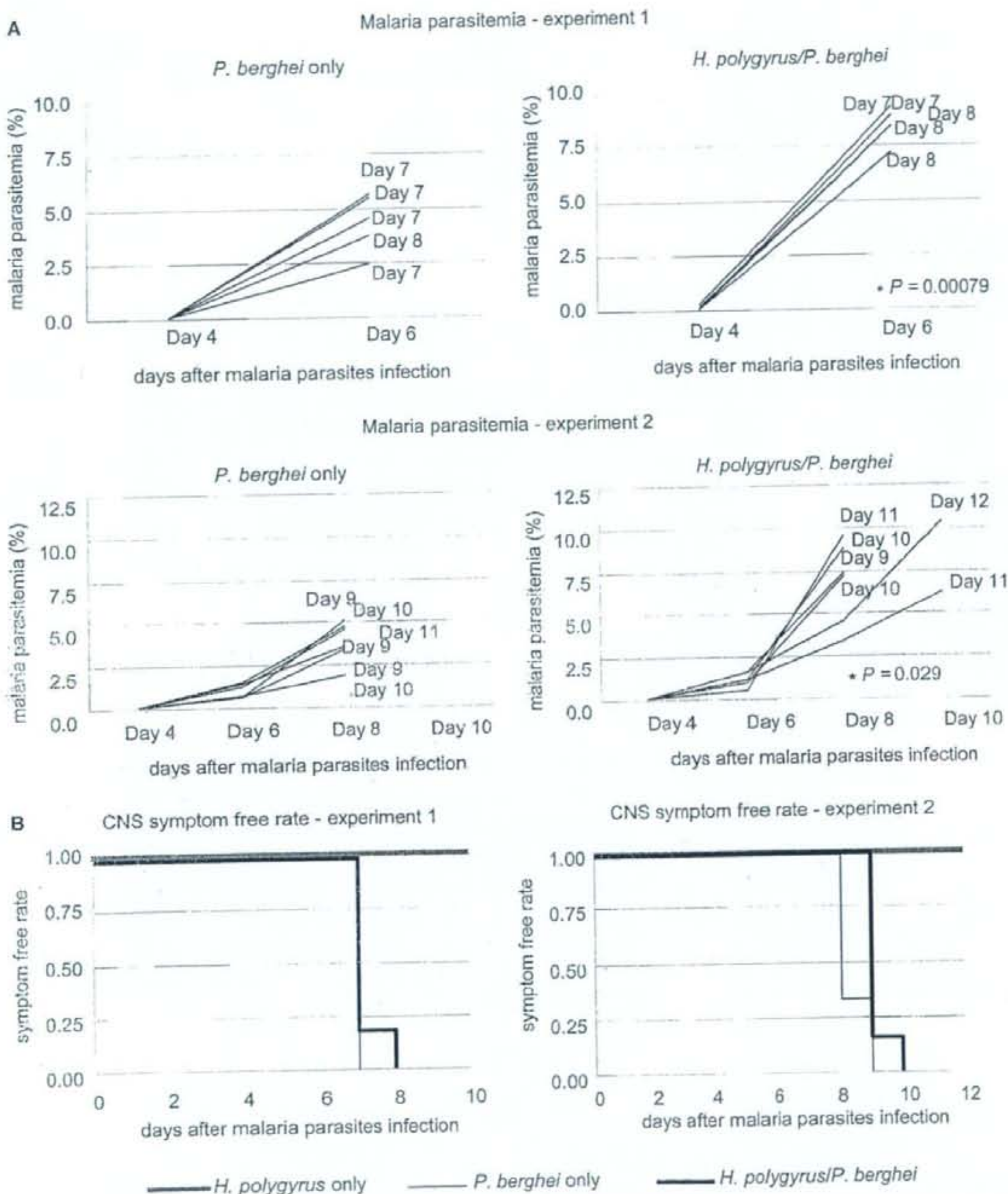

FIGURE 2. Course of infection with *Plasmodium berghei* in mice co-infected with *Heligmosomoides polygyrus*. The C57BL/6 mice were infected with *P. berghei* 14 days after *H. polygyrus* infection and were analyzed for percentage parasitemia monitored by microscopic evaluation of thin blood films stained with Giemsa solution (**A**), and for experimental cerebral malaria (ECM) incidence (**B**). Five to six animals were used in each group. Experiments were repeated three times with similar results and two of them are shown. (**A**) Each line represents data from an individual mouse, and the numbers indicate day of mouse death. An asterisk indicates statistical significance between mice infected with *H. polygyrus/P. berghei* and those only with *P. berghei* using the Student's *t* test. (**B**) *P. berghei*-infected animals were considered to have ECM when neurologic signs, described in the text, appeared. Statistical significance was not found between mice infected with *H. polygyrus/P. berghei* and those only with *P. berghei*.

10 days, despite a small parasite load in the circulation (Figure 2). *Heligmosomoides polygyrus*-infected mice showed significantly higher malaria parasitemia at the sixth to eighth day after infection (Figure 2A), which suggested that anti-malaria immunity might be suppressed, compared with the control mice infected only with *P. berghei*. But co-infected mice with *H. polygyrus* and *P. berghei* developed ECM similar to that in the control group, as evaluated by: 1) decrease in spontaneous activity; 2) loss of escape from handling; and 3) abnormal, non-abdominal body position (Figure 2B). Consequently, all mice died at around the tenth day, similar to the control mice (Figure 2A).

We observed here the rapid growth of *P. berghei* in mice co-infected with *H. polygyrus*, suggesting that concurrent *H. polygyrus* infection might have reduced anti-malaria immunity. Although we did not evaluate malaria–specific immune responses in these animals, our observations can be compatible with previous studies showing that *H. polygyrus*-harboring mice have suppressed both cellular and humoral immune responses against another rodent malaria, *Plasmodium chabaudi*.[9]

Although we have not addressed how *H. polygyrus* suppresses immunity, it has been postulated that protective Th1 responses are attenuated in the Th2-biased environment induced by concurrently infected *H. polygyrus*.[3] In addition to the classic Th1/2 balance, immunosuppressive mechanisms induced by *H. polygyrus* can explain the reduced protective immunity. *Heligmosomoides polygyrus* is now known to induce alternatively activated macrophages,[10] regulatory T cells,[11] interleukin-10, and indoleamine-2,3-dioxygenase (our unpublished observations). No matter what the mechanisms are, anti-malaria immunity is thought to be suppressed by *H. polygyrus* infection, which suggests that pathogenic processes formed by immune responses in ECM development are also attenuated.

There are some responses reported to participate in both protection and immunopathology: Interferon (IFN)-γ contributes not only to elimination of malaria parasites,[12] but also to the pathology of ECM.[13] Our preliminary results showed that *H. polygyrus* suppressed IFN-γ production from antigen–specific splenic T cells in mice after immunization with the corresponding antigen (unpublished observations). However unexpectedly, concurrent infection with *H. polygyrus* did not alter ECM development. This might be explained by the fact that immune suppression/modulation induced by concurrent *H. polygyrus* infection is not probably spread into CNS: *H. polygyrus* infection is reported to suppress experimental airway hypersensitivity,[14] although there have been no studies showing that nematodes whose life-cycle in their host are limited in alimentary tract to prevent experimental autoimmune/allergic encephalomyelitis (EAE) in CNS, although *Schistosoma mansoni*[15] and *Trichinella spiralis*,[16] which are thought to have easier access to the host blood stream, are reported to modulate EAE pathology. An alternative explanation is that protective immunity against malaria parasites is simply different from the pathogenic responses. For instance, although $CD8^+$ T cells are responsible for ECM development,[17] these cells are not supposed to contribute to protective immunity against blood-stage malaria parasites, mainly because of absence of MHC class I molecules on the surface of RBCs.[18] Finally, we cannot strictly exclude the probability that ECM pathology develops so quickly that modulations by concurrent *H. polygyrus* infection, if any, might not be obvious in our experiments. More sensitive methods of ECM diagnosis and definition are required for further research.

In conclusion, our results showed that infection with an intestinal nematode increased malaria parasite growth in vivo but did not alter immunopathology in ECM development. Differentiation between pathogen-killing immunity and self-damaging inflammatory responses is essential for a complete understanding of the pathology of cerebral malaria, and for designing effective vaccine strategies.

Received April 19, 2008. Accepted for publication August 20, 2008.

Financial support: This work was supported by the Ministry of Education, Science, Sport and Culture of Japan (Grants 20390121, 19041056), and by the Uehara Memorial Foundation.

Authors' addresses: Kohhei Tetsutani, Shinjiro Hamano, Hajime Hisaeda, and Kunisuke Himeno, Department of Parasitology, Kyushu University Graduate School of Medicine, 3-1-1, Maidashi, Higashi-ku, Fukuoka, 812-0054, Japan, Tel: +81-92-642-6117, Fax: +81-92-642-6118, E-mail: tetztani@parasite.med.kyushu-u.ac.jp. Kenji Ishiwata, Department of Tropical Medicine, The Jikei University School of Medicine, 3-25-8, Nishi-shinbashi, Minato-ku, Tokyo, Japan. Motomi Torii, Department of Molecular Parasitology, Ehime University School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan.

REFERENCES

1. van der Heyde HC, Nolan J, Combes V, Gramaglia I, Grau GE, 2006. A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. *Trends Parasitol 22:* 503–508.
2. Medana IM, Chaudhri G, Chan-Ling T, Hunt NH, 2001. Central nervous system in cerebral malaria: "innocent bystander" or active participant in the induction of immunopathology? *Immunol Cell Biol 79:* 101–120.
3. van Riet E, Hartgers FC, Yazdanbakhsh M, 2007. Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiol 212:* 475–490.
4. World Health Organization, 2004. Malaria cases (per 100,000) by country, latest available data. Available at: http://gamapserver.who.int/mapLibrary/Files/Maps/global_cases.jpg. Accessed April 8, 2008.
5. World Health Organization, 2006. Soil-transmitted helminth (STH) infections are widely distributed in tropical and subtropical areas – 2006. Available at: http://www.who.int/intestinal_worms/epidemiology/map/en/index.html. Accessed April 8, 2008.
6. Nacher M, Singhasivanon P, Yimsamran S, Manibunyong W, Thanyavanich N, Wuthisen P, Looareesuwan S, 2002. Intestinal helminth infections are associated with increased incidence of *Plasmodium falciparum* malaria in Thailand. *J Parasitol 88:* 55–58.
7. Nacher M, Gay F, Singhasivanon P, Krudsood S, Treeprasertsuk S, Mazier D, Vouldoukis I, Looareesuwan S, 2000. *Ascaris lumbricoides* infection is associated with protection from cerebral malaria. *Parasite Immunol 22:* 107–113.
8. Gause WC, Urban JF Jr, Stadecker MJ, 2003. The immune response to parasitic helminths: insights from murine models. *Trends Immunol 24:* 269–277.
9. Su Z, Segura M, Morgan K, Loredo-Osti JC, Stevenson MM, 2005. Impairment of protective immunity to blood-stage malaria by concurrent nematode infection. *Infect Immun 73:* 3531–3539.
10. Anthony RM, Urban JF Jr, Alem F, Hamed HA, Rozo CT, Boucher JL, van Rooijen N, Gause WC, 2006. Memory Th2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat Med 12:* 955–960.
11. Finney CAM, Taylor MD, Wilson MS, Maizels RM, 2007. Expansion and activation of CD4+CD25+ regulatory T cells in

Heligmosomoides polygyrus infection. *Eur J Immunol 37:* 1874–1886.

12. Shear HL, Srinivasan R, Nolan T, Ng C, 1989. Role of IFN-γ in lethal and nonlethal malaria in susceptible and resistant murine hosts. *J Immunol 143:* 2038–2044.
13. Grau GE, Heremans H, Piguet PF, Pointaire P, Lambert PH, Billiau A, Vassalli P, 1989. Monoclonal antibody against interferon γ can prevent experimental cerebral malaria and its associated overproduction of tumour necrosis factor. *Proc Natl Acad Sci USA 86:* 5572–5574.
14. Wilson MS, Taylor MD, Balic A, Finney CAM, Lamb JR, Maizels RM, 2005. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J Exp Med 202:* 1199–1212.
15. La Flamme AC, Ruddenklau K, Baecktroem BT, 2003. Schistosomiasis decreases central nervous system inflammation and alters the progression of experimental autoimmune encephalomyelitis. *Infect Immun 71:* 4996–5004.
16. Gruden-Movsesijan A, Ilic N, Mostarica-Stojkovic M, Stosic-Grujicic S, Milic M, Sofronic-Milosavljevic Lj, 2008. *Trichinella spiralis*: modulation of experimental autoimmune encephalomyelitis in DA rats. *Exp Parasitol 118:* 641–647.
17. Yanez DM, Manning DD, Cooley AJ, Weidanz WP, van der Heyde HC, 1996. Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. *J Immunol 157:* 1620–1624.
18. Vinetz JM, Kumar S, Good MF, Fowlkes BJ, Berzofsky JA, Miller LH, 1990. Adoptive transfer of CD8+ cells from immune animals does not transfer immunity to blood stage *Plasmodium yoelii* malaria. *J Immunol 144:* 1069–1074.

Malaria Parasites Require TLR9 Signaling for Immune Evasion by Activating Regulatory T Cells[1]

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

Malaria is still a life-threatening infectious disease that continues to produce 2 million deaths annually. Malaria parasites have acquired immune escape mechanisms and prevent the development of sterile immunity. Regulatory T cells (Tregs) have been reported to contribute to immune evasion during malaria in mice and humans, suggesting that activating Tregs is one of the mechanisms by which malaria parasites subvert host immune systems. However, little is known about how these parasites activate Tregs. We herein show that TLR9 signaling to dendritic cells (DCs) is crucial for activation of Tregs. Infection of mice with the rodent malaria parasite *Plasmodium yoelii* activates Tregs, leading to enhancement of their suppressive function. In vitro activation of Tregs requires the interaction of DCs with parasites in a TLR9-dependent manner. Furthermore, $TLR9^{-/-}$ mice are partially resistant to lethal infection, and this is associated with impaired activation of Tregs and subsequent development of effector T cells. Thus, malaria parasites require TLR9 to activate Tregs for immune escape. *The Journal of Immunology,* **2008, 180: 2496–2503.**

Malaria caused by protozoan parasites of the genus *Plasmodium* is still one of the most life-threatening infectious diseases. Approximately 40% of people worldwide reside in areas at risk of malaria. Three hundred million people are infected every year and 2 million die. Generation of effector T cells is crucial for the development of protective immunity against malaria. Ingenious strategies for immune escape by malaria parasites, including antigenic diversity (1), clonal antigenic variation (2), and impairment of dendritic cell (DC)[4] maturation (3), prevent the development of sterile immunity, resulting in repeated symptomatic infections throughout the life of the host. Therefore, an understanding of this evasion mechanism is important for the effective control of malaria.

$CD4^+CD25^+$ regulatory T cells (Tregs) contribute to the maintenance of self-tolerance by suppressing autoreactive T cells in the periphery (4, 5). Recently, Tregs were reported to play pivotal roles in infectious diseases as well as in the suppression of autoimmunity. For instance, Tregs suppress harmful immune pathogenesis caused by infection (6), and they contribute to the establishment of chronic infection instead of the elimination of pathogens, thus maintaining exposure of memory T cells to microbial Ags (7). With use of a murine model, we and others have demonstrated that the immune escape of malaria parasites requires activation of Tregs (8, 9). Furthermore, Walther and colleagues found that up-regulation of Tregs correlates with rapid parasite growth during human malaria infection (10). These results suggest that activation of Tregs is a central mechanism by which malaria parasites subvert host immune systems. However, it remains to be elucidated how malaria parasites activate Tregs.

Several lines of evidence for the functional regulation of Tregs have been accumulated. IL-2 is crucial for maintaining Tregs in the peripheral pool of T cells (11). TGF-β maintains the suppressive functions of Tregs (12). DCs also contribute to controlling Treg cell functions by supporting the expansion of functional Tregs in an Ag-specific manner (13), and immature DCs selectively induce Tregs (14). TLR signaling in DCs blocks Treg-mediated suppression by affecting effector T cells (15) and reverses suppression of Tregs (16). Some TLRs are expressed on Tregs themselves, and these receptors are also involved in both positive and negative regulation of Treg functions (17–20). Thus, the involvement of TLRs expressed on DCs or Tregs in the regulation of Treg function appears to be controversial.

We herein show that the interaction of malaria parasites with DCs through TLR9 is required for the activation of Tregs in vitro. Additionally, infection of $TLR9^{-/-}$ mice with malaria parasites failed to activate Tregs, resulting in the activation of effector T cells. These mice showed partial resistance to infection. Our results suggest a novel mechanism for immune evasion by malaria parasites; that is, Treg activation by DCs stimulated via TLR9.

---

*Department of Parasitology, Graduate School of Medical Sciences and †Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukoka; ‡Department of Molecular Parasitology and §Department of Host Defense, Institute for Microbial Diseases, Osaka University, and Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Suita; ¶Department of Immunology and Parasitology, Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima; and ‖Department of Molecular Parasitology, Ehime University, Graduate School of Medicine, Ehime, Japan

Received for publication July 3, 2007. Accepted for publication December 10, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Ministry of Education, Science, Sport and Culture of Japan (Grants 16017278, 18590400, 19041056), and by the Uehara Memorial Foundation.

[2] Address correspondence and reprint requests to Dr. Hajime Hisaeda, Department of Parasitology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail address: hisa@parasite.med.kyushu-u.ac.jp

[3] Current address: Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, 2- Yamada-oka, Suita, 565-0871, Japan.

[4] Abbreviations used in this paper: DC, dendritic cell; pDC, plasmacytoid DC; PDCA1, anti-plasmacytoid DC Ag-1; pRBC, parasitized RBC; PyL, *Plasmodium yoelii* 17XL strain; Treg, regulatory T cell; TRIF, Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

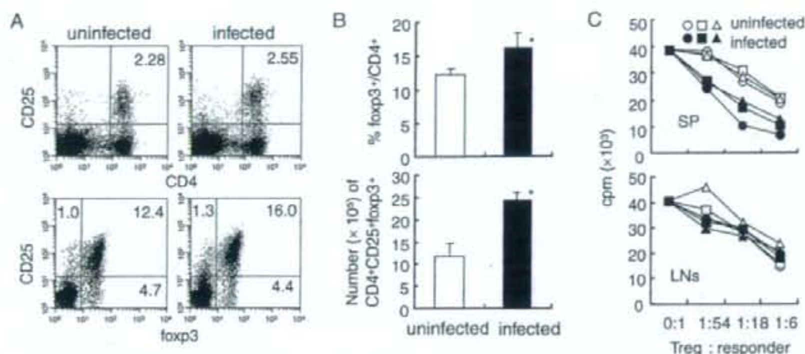

FIGURE 1. Activation of Tregs during *P. yoelii* infection. *A*, Flow cytometric analysis of Foxp3 expression in $CD4^+CD25^+$ T cells. Splenocytes obtained from uninfected (*left panels*) and PyL-infected (*right panels*) mice were stained with fluorescence-conjugated anti-CD4, anti-CD25, and anti-Foxp3 mAbs. Gated $CD4^+$ cells were separated on the basis of Foxp3 and CD25 expression (*bottom panels*). The numbers represent the percentages of all cells in each of the quadrants. The results are representative of five repeated experiments. *B*, The percentages of $Foxp3^+CD25^+$ cells among $CD4^+$ T cells (*upper panel*) and the absolute number of $CD4^+CD25^+Foxp3^+$ cells (*bottom panel*) in the spleens of uninfected (open bars) and PyL-infected (filled bars) mice were quantified. Values are means ± SD of six mice. Asterisks indicate statistical significance at $p < 0.05$ with the Student *t* test. *C*, Suppressive function of Tregs in mice infected with PyL. $CD4^+CD25^-$ T cells (1×10^5) purified from uninfected mice were stimulated with anti-CD3 mAb in the presence of T cell-depleted spleen cells as APCs, and they were mixed with splenic Tregs (*upper panel*) or Tregs from lymph nodes (*bottom panel*) obtained from uninfected (open symbols) or PyL-infected (filled symbols) mice at the indicated ratio. The proliferation of $CD4^+CD25^-$ T cells was measured by [^{3}H]thymidine incorporation. Values are means of triplicate cultures, and SD was <10% of the mean value. Each symbol represents results from an individual mouse. Splenic Tregs from infected mice suppressed significantly more than did those from uninfected mice ($p < 0.01$ with the unpaired Student *t* test). The results are representative of six repeated experiments.

Materials and Methods

Mice and parasites

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

Reagents

PE-anti-CD25 (PC61.5), allophycocyanin-anti-CD4 (RM4.4), and FITC-anti-Foxp3 (FJK-16s) staining kits, and FITC-anti-CD11c (N418), PE-anti-B220 (RA3–6B2), allophycocyanin-anti-CD86, PE-Cy5.5-anti-CD40, FITC-anti-CD69, FITC-anti-CD62L, PE-anti-IFN-γ, purified anti-CD3 (2C11), purified anti-CD16/32 (2.4G2), and purified anti-MHC class II (M5/114.15.2) Abs were obtained from eBioscience. The $CD4^+$ T cell separation kit, plasmacytoid DC (pDC) isolation kit, and anti-plasmacytoid DC Ag-1 (PDCA1), anti-PE, and anti-FITC microbeads (Miltenyi Biotec) were used for cell purification. mAbs to CD4 (GK1.5) or to IFN-γ (R4–6A2), purified from the ascites of hybridoma-injected athymic nude mice, were used for in vivo treatments.

Flow cytometry

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

Cell purification and cultures

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

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

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

To deplete $CD4^+$ T cells in vivo, mice were injected i.p. with 250 μg of anti-CD4 Ab 3 days and 1 day before infection. Depletion of $CD4^+$ T cells was evaluated using peripheral blood, from which >95% of $CD4^+$ T cells were depleted. To neutralize IFN-γ, mice were infected with 200 μg of anti-IFN-γ Ab 1 day before and 1 day after infection.

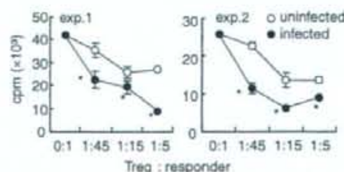

FIGURE 2. No lymphocytes other than Tregs are required for the activation of Tregs. $CD4^+$ cells purified 5 days after infection with PyL (●) from $RAG2^{-/-}$ mice that had received 1×10^6 Tregs a day before infection were analyzed for suppressive function as described in Fig. 1*C*. Those from uninfected recipients were also analyzed (○). The results of two separate experiments are shown.

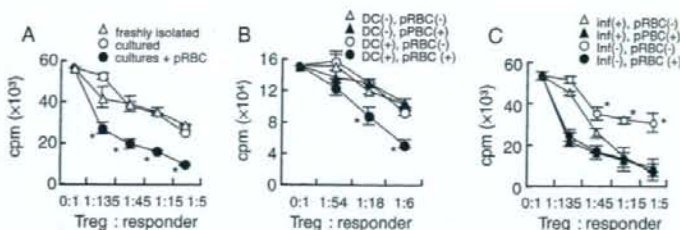

FIGURE 3. Requirement of an interaction between pRBC and DCs to activate Tregs. Suppressive functions of CD4$^+$CD25$^+$ cells from uninfected mice cultured with DCs were analyzed as in Fig. 1*A*, using ConA instead of anti-CD3. *A*, Tregs cultured with (●) or without (○) pRBC in the presence of DCs were analyzed for their suppressive function. Freshly isolated Tregs (△) were also used. *B*, Tregs cultured with (circles) or without (triangles) DCs in the absence (open symbols) or presence (filled symbols) of pRBCs were analyzed for their suppressive activity. *C*, Tregs cultured with DCs from uninfected (circles) or PyL-infected (triangles) mice in the absence (open symbols) or presence (filled symbols) of pRBCs were analyzed. DCs were collected 5 days after infection. Values are means ± SD of triplicate cultures. Asterisks indicate statistical significance at $p < 0.05$ with the Student *t* test. These in vitro experiments were repeated at least four times.

Statistical analyses

Differences between groups were analyzed for statistical significance using Excel software with two-tailed the unpaired Student *t* tests. For survival curves, Kaplan-Meier plots and χ^2 tests were performed. Probability below 0.05 was considered to be statistically significant.

Results

Infection with malaria parasites activates Tregs

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

Malaria parasites interact with DCs to activate Tregs

We next analyzed how Tregs are activated during malaria infection. Some cytokines secreted by lymphocytes are reported to be important for Treg activities. To determine the cellular requirements for Treg activation, *RAG2*$^{-/-}$ mice received 1×10^6 Tregs from syngeneic mice, and they were then infected with PyL. Because CD25 expressed by Tregs disappears in the inflammatory environment in lymphopenic hosts (7), CD4$^+$ cells were purified. Approximately 1×10^5 and 1.4×10^5 cells were recovered from uninfected and PyL-infected mice, respectively (not significant). Tregs recovered from uninfected mice still suppressed TCR-triggered T cell proliferation in a dose-dependent manner. Infection of recipient mice with PyL significantly enhanced this suppressive function, as observed in immunocompetent mice (Fig. 2), indicating that no lymphocytes other than Tregs are required for the activation of this function of Tregs. These results suggest that the interactions between Tregs, APCs, and pRBCs are sufficient for Treg activation.

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

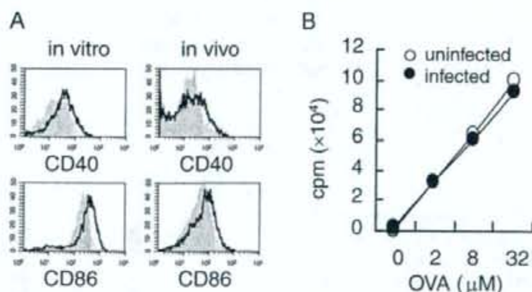

FIGURE 4. Activation status of DCs that had interacted with malaria parasites. *A*, DCs cultured with pRBCs (*left panels*) and splenic DCs from PyL-infected mice (*right panels*) were analyzed for the expression of CD40 and CD86. The expression profiles of DCs cultured with pRBCs or DCs from infected mice (solid lines) were plotted against that of DCs cultured without pRBCs or that of DCs from uninfected mice (shaded areas), respectively. *B*, CD4$^+$CD25$^-$ cells from OT-II mice were stimulated with the indicated amount of OVA in the presence of splenic DCs from mice infected with PyL (●) or with DCs from uninfected mice (○). The proliferation of OT-II T cells was measured by [^{3}H]thymidine incorporation. The results represent the means of triplicate cultures. SDs were <5% of the mean.

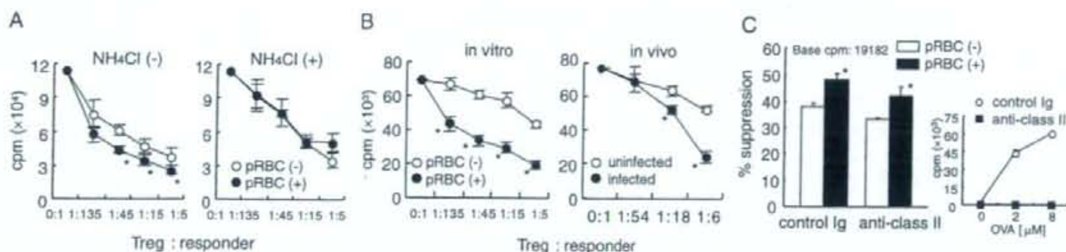

FIGURE 5. Phago-endocytic pathway-dependent, Ag-nonspecific activation of Tregs by malaria parasites. *A*, Blocking endosomal maturation inhibits Treg activation. Tregs and DCs obtained from uninfected mice were cultured in the absence (*left panel*) or presence (*right panel*) of NH_4Cl (250 μg/ml) and analyzed as in Fig. 3*A*. *B*, $CD4^+CD25^+$ cells from OT-II mice cultured with DCs and pRBCs (*left panel*) or those from OT-II mice infected with PyL (*right panel*) were analyzed for suppressive activity as in Fig. 3*A*. *C*, Treg activation with pRBCs was performed in the presence of anti-class II Ab (1 μg/ml). Suppressive activity was evaluated as the ratio of Tregs to $CD4^+CD25^-$ cells (1:5). Percentage suppression was calculated as (1 − experimental cpm/base cpm (cpm without Tregs)) × 100. Open and filled columns represent suppressive activity in the absence or presence of pRBCs, respectively. $CD4^+CD25^-$ cells from OT-II mice were cultured with OVA at the indicated concentration in the presence of anti-class II Ab (*inset*).

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

TLR9 signaling in DCs is required for Treg activation

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

Foreign Ags phagocytosed by DCs are proteolytically processed and undergo Ag presentation to MHC class II molecules (28). The necessity for engulfment of pRBCs by DCs for Treg activation might be explained by the ability of DCs to activate Ag-specific Tregs. To address this issue, we used Tregs isolated from OVA-specific TCR-transgenic OT-II mice (29). Stimulation of these Tregs with pRBCs resulted in an enhancement of their suppressive function. Additionally, infection of OT-II mice with PyL also activated Tregs (Fig. 5*B*). We could not exclude the possibility of an involvement of remnant Tregs with non-OT-II TCR in the enhancement of Treg function, because RAG-deficient OT-II mice could not be used owing to requirement of TCR rearrangement for the development of Tregs (30). To further confirm Ag-nonspecific Treg activation, we used an anti-MHC class II Ab in Treg cultures with pRBCs. The enhancement of the suppressive function of Tregs by adding pRBCs was not altered, even in the presence of the anti-class II Ab, which completely blocked the OVA-specific proliferation of $CD4^+CD25^-$ cells isolated from OT-II mice (Fig. 5*C*).

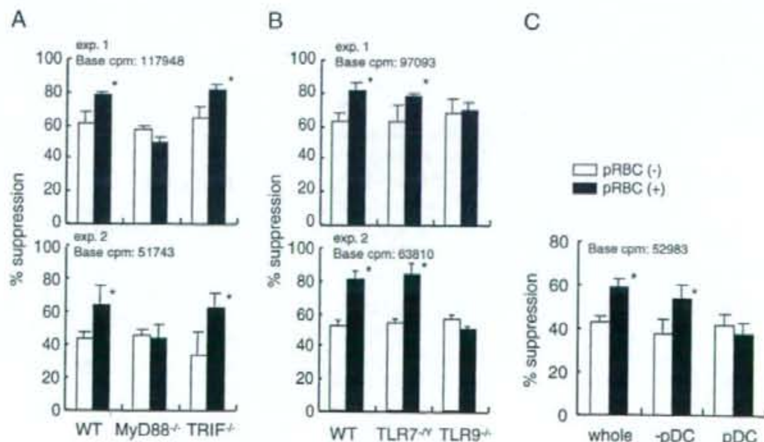

FIGURE 6. Essential role of TLR9 signaling in DCs for the activation of Tregs. $CD4^+CD25^+$ cells from uninfected WT mice were cultured with pRBCs in the presence of DCs from the indicated mutant mice (*A* and *B*) or with the indicated DCs from WT mice (*C*). These cells were then evaluated as in Fig. 5*C*. Values are means ± SD of triplicate cultures. Asterisks indicate statistical significance at $p < 0.05$ with the Student *t* test. The results of two separate experiments of four experiments are shown.

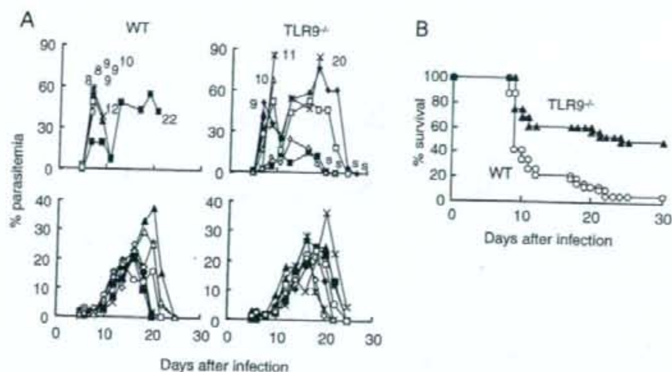

FIGURE 7. Resistance of $TLR9^{-/-}$ mice to PyL infection. *A*, The kinetics of parasitemia in WT or $TLR9^{-/-}$ mice infected with PyL (*upper panels*) or with PyNL (a nonlethal variant of PyL; *lower panels*) was monitored by microscopic evaluation of Giemsa-stained thin blood films. Each symbol represents a value from an individual mouse. The numbers represent days of mouse death and "s" shows mice that survived. Similar results were obtained from at least three experiments. *B*, The cumulative mortality rate of infection with PyL in WT (○) or $TLR9^{-/-}$ mice (▲) is shown. $p < 0.001$ for the percentage survival of WT vs $TLR9^{-/-}$ mice by χ^2 test.

To activate Tregs, phagocytosed pRBCs might not be displayed as Ags, but they were thought to provide signals to DCs. We hypothesized that TLRs are involved in this interaction. To investigate the possible roles of TLRs, we used DCs obtained from mice lacking MyD88 or TRIF, both of which are essential adaptor molecules for TLR signaling (21, 22). As shown in Fig. 6*A*, DCs from $TRIF^{-/-}$ mice were capable of activating Tregs as well as those from wild-type (WT) mice. By contrast, Tregs were not activated when cultured with DCs from $MyD88^{-/-}$ mice. The dependency of Treg activation on MyD88 and the endosomal localization allowed us to deduce that the TLRs likely to be involved are TLR7 and TLR9. TLR7 recognizes single-stranded RNA from viruses (23), while TLR9 recognizes DNA containing unmethylated CpG motifs (24). Recently, some reports have found that malaria parasites express molecules that are recognized by TLR9 (31, 32), suggesting that TLR9 is a much more likely candidate. To confirm this, we conducted studies using mice lacking TLR7 or TLR9. As expected, DCs from $TLR7^{-/Y}$ mice, but not those from $TLR9^{-/-}$ mice, were able to activate Tregs (Fig. 6*B*). TLR9 is expressed predominantly by pDCs, which are reported to be involved in Treg induction (33). However, our experiments denied the involvement of pDCs in Treg activation during malaria infection. Purified $PDCA1^{+}$ pDCs could not activate Tregs in vitro, presumably due to an inability to ingest pRBCs, whereas DCs depleted of pDCs could (Fig. 6*C*). These results indicate that TLR9 plays a critical role in the interaction between pRBCs and myeloid DCs, which underlies the activation of Tregs, and thus prompted us to examine the susceptibility of $TLR9^{-/-}$ mice to infection with PyL.

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

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

Infection with PyL failed to activate Tregs in $TLR9^{-/-}$ mice

To relate the partial resistance against PyL infection to the impairment of Treg activation in $TLR9^{-/-}$ mice, we analyzed Treg function in $TLR9^{-/-}$ mice. Splenic $Foxp3^{+}CD4^{+}CD25^{+}$ cells were increased in $TLR9^{-/-}$ mice, similarly to those in WT mice, 5 days

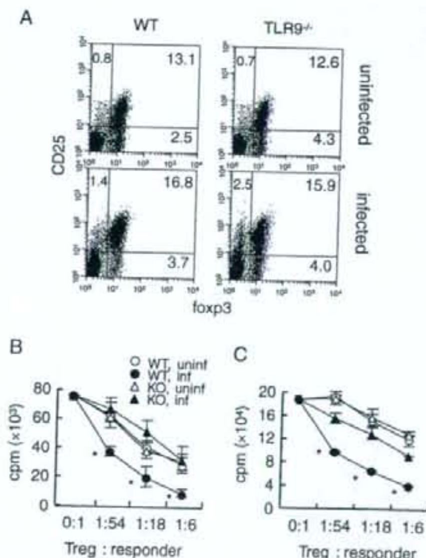

FIGURE 8. Impaired Treg activation in $TLR9^{-/-}$ mice after infection with PyL. *A*, Flow cytometric analysis of Foxp3 expression in $CD4^{+}CD25^{+}$ T cells. Splenocytes obtained from WT (*left panels*) or $TLR9^{-/-}$ (*right panels*) mice with (*bottom panels*) or without (*top panels*) infection with PyL were analyzed as in Fig. 1*A*. Results are representative of five experiments. *B*, Suppressive function of Tregs in $TLR9^{-/-}$ mice infected with PyL. Splenic Tregs obtained from uninfected (open symbols) or PyL-infected (filled symbols), WT (circles) or $TLR9^{-/-}$ (triangles) mice were mixed with $CD4^{+}CD25^{-}$ T cells at the indicated ratio. *C*, Transferred $Ly5.1^{+}$ Tregs recovered from WT (circles) or $TLR9^{-/-}$ (triangles) recipients before (open symbols) or after (filled symbols) PyL infection were analyzed for their suppressive activity. Values are means ± SD of triplicate cultures. Asterisks indicate statistical significance at $p < 0.05$ with the Student *t* test. These experiments were repeated at least three times.

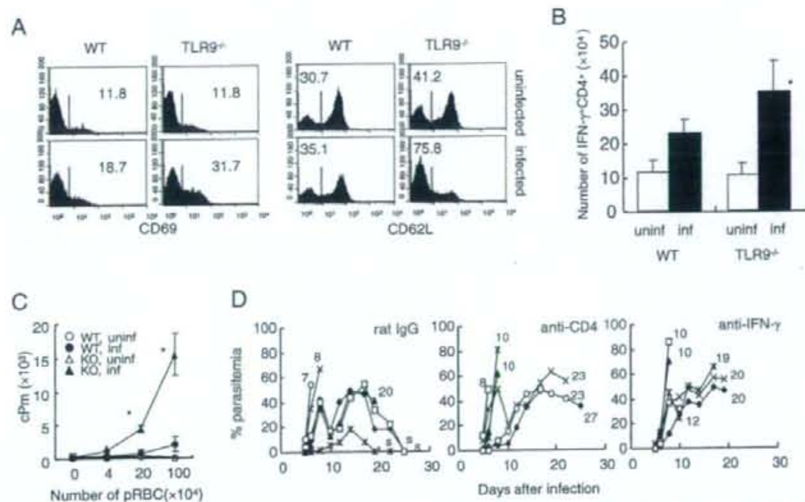

FIGURE 9. Activation of $CD4^+$ T cells is required for resistance in $TLR9^{-/-}$ mice. *A* and *B*, Spleen cells from WT or $TLR9^{-/-}$ mice 5 days after infection (*A*, *bottom panels*) or uninfected mice (*A*, *upper panels*) were stained with combinations of fluorescence-conjugated anti-CD4 and Abs to the indicated molecules, followed by flow cytometric analyses. *A*, Histograms show expression of CD69 or CD62L on gated $CD4^+$ T cells. Numbers indicate the percentages of $CD4^+$ T cells comprising the $CD69^{high}$ or $CD62L^{low}$ populations. *B*, The absolute number of $CD4^+IFN\text{-}\gamma^+$ cells in the spleen was calculated. Values are means ± SD of four mice. Asterisks indicate statistical significance at $p < 0.05$ with the Student *t* test. *C*, Proliferation of splenic $CD4^+$ T cells isolated from uninfected (open symbols) or PyL-infected (filled symbols) WT (circles) or $TLR9^{-/-}$ (squares) mice. $CD4^+$ T cells were cultured with the indicated number of pRBCs in the presence of APCs. Values are means ± SD of triplicate cultures. Asterisks indicate statistical significance at $p < 0.05$ with the Student *t* test. These experiments were repeated at least three times. *D*, $CD4^+$ T cells and IFN-γ are essential for protection against malaria parasites in $TLR9^{-/-}$ mice. The kinetics of parasitemia in $TLR9^{-/-}$ mice treated with the indicated Ab were evaluated as in Fig. 7*A*. Each symbol represents a value from an individual mouse. The numbers represent days to mouse death and "s" shows mice that survived. A similar result was obtained from another experiment.

after infection (Fig. 8*A*). Infection of WT mice with PyL again enhanced the suppressive function of Tregs, and infection of $TLR9^{-/-}$ mice failed to do so, although there was no difference in the suppressive efficacy between $TLR9^{-/-}$ and WT Tregs before infection (Fig. 8*B*). This failure might be attributable to the inclusion of $Foxp3^-$ cells among $CD4^+CD25^+$ cells, because $TLR9^{-/-}$ mice had more $CD4^+CD25^+Foxp3^-$ cells, which are thought to be effector cells originating from the pool of $CD4^+CD25^-$ cells (Fig. 8*A*). To exclude this possibility, we infected $TLR9^{-/-}$ mice into which Tregs from Ly5.1 mice had been adoptively transferred, and $Ly5.1^+$ cells recovered from the infected mice were analyzed for suppressive activity. $Ly5.1^+$ Tregs recovered from WT mice showed enhanced suppression after infection. By contrast, no enhancement of Treg function was observed in $TLR9^{-/-}$ recipients (Fig. 8*C*). These results confirm that Tregs are not activated in $TLR9^{-/-}$ mice, regardless of the expression of TLR9 on Tregs themselves.

Finally, we analyzed immune responses in PyL-infected $TLR9^{-/-}$ mice. $CD4^+$ T cells and their product IFN-γ are known to be important for protection against blood-stage malaria (34, 35). Flow cytometric analyses revealed that infection of $TLR9^{-/-}$ mice with PyL increased activated ($CD62L^{low}$, $CD69^{high}$) and $IFN\text{-}\gamma^+$ populations among splenic $CD4^+$ T cells, compared with infection of WT mice (Fig. 9, *A* and *B*). Furthermore, $CD4^+$ T cells from $TLR9^{-/-}$ mice proliferated in response to pRBCs, while those from WT mice showed only a marginal response (Fig. 9*C*). We depleted $CD4^+$ T cells or neutralized the IFN-γ from these mutant mice. These manipulations completely abolished the partial protection observed in $TLR9^{-/-}$ mice (Fig. 9*D*), indicating that $CD4^+$ T cells were efficiently activated under conditions in which Tregs are not activated in $TLR9^{-/-}$ mice.

Discussion

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

It is generally thought that TLRs are crucial for the induction of innate and acquired immunity (39). Some reports have shown that mice deficient in a TLR show higher susceptibility to pathogens recognized by the corresponding TLR (40, 41). By contrast, recent reports have shown TLR-mediated immune suppression. Upon stimulation through TLRs, on the one hand, DCs decrease the susceptibility of effector T cells to suppression mediated by Tregs (15). Stimulation of Tregs via TLR8 or TLR2 reverses the suppressive function of Tregs (17, 18). These behaviors drive toward the development of immunity. In contrast, systemic or excessive activation of TLRs in DCs has been reported to induce several types of immune suppression (38, 42). Stimulation with a large amount of LPS, a TLR4 ligand, and the TLR5 ligand flagellin

directly activate Tregs as determined by their enhanced suppressive function (19, 20). Thus, TLRs contribute to controlling the balance between Tregs and effector T cells by affecting both DCs and Tregs. Furthermore, TLR signaling provides negative feedback mechanisms for preventing immunopathogenesis when stimulation is saturated. Because TLR9 recognizes endogenous ligands and is involved in the development of autoimmune diseases (43, 44), this type of signal could have powerful regulatory functions, such as activation of Tregs, which are suppressors of autoimmunity. Indeed, TLR9 signaling plays a protective role in the development of autoimmunity by modulating Treg activity in autoimmune-prone MRL mice (45). Furthermore, common polymorphisms of TLR9 are reported to be associated with the clinical manifestation of malaria during pregnancy (46). It is quite possible that malaria parasites cleverly exploit this machinery by providing a large amount of TLR9 stimulant.

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

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

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

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

Disclosures

The authors have no financial conflicts of interest.

References

1. Plebanski, M., K. L. Flanagan, E. A. M. Lee, W. H. H. Reece, K. Hart, C. Gelder, G. Gillespie, M. Pinder, and A. V. S. Hill. 1999. Interleukin 10-mediated immunosuppression by a variant CD4 T cell epitope of *Plasmodium falciparum*. *Immunity* 10: 651–660.
2. Smith, J. D., C. E. Chitnis, A. G. Craig, D. J. Roberts, D. E. Hudson-Taylor, D. S. Peterson, R. Pinches, C. I. Newbold, and L. H. Miller. 1995. Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* 82: 101–110.
3. Urban, B. C., D. J. P. Ferguson, A. Pain, N. Willcox, M. Plebanski, J. M. Austyn, and D. J. Roberts. 1999. *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature* 400: 73–77.
4. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155: 1151–1164.
5. Shevach, E. M. 2002. $CD4^+CD25^+$ supperssor T cells: more questions than answers. *Nat. Rev. Immunol.* 2: 389–400.
6. Hori, S., T. L. Carvalho, and J. Demengeot. 2002. $CD25^+CD4^+$ regulatory T cells suppress $CD4^+$ T cell-mediated pulmonary hyperinflammation driven by *Pneumocystis carinii* in immunodeficient mice. *Eur. J. Immunol.* 32: 1282–1291.
7. Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks. 2002. $CD4^+CD25^+$ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* 420: 502–507.
8. Hisaeda, H., Y. Maekawa, D. Iwakawa, H. Okada, K. Himeno Kishihara, S.-I. Tsukumo, and K. Yasutomo. 2004. Escape of malaria parasites from host immunity requires $CD4^+CD25^+$ regulatory T cells. *Nat. Med.* 10: 29–30.
9. Long, T. T., S. Nakazawa, S. Onizuka, M. C. Huaman, and H. Kanbara. 2003. Influence of $CD4^+CD25^+$ T cells on *Plasmodium berghei* NK65 infection in BALB/c mice. *Int. J. Parasitol.* 33: 175–183.
10. Walther, M., J. E. Tongren, L. Andrews, D. Korbel, E. King, H. Fletcher, R. F. Andersen, P. Bejon, F. Thompson, S. J. Dunachie, et al. 2005. Upregulation of TGF-β, FOXP3, and $CD4^+CD25^+$ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 23: 287–296.
11. Setoguchi, R., S. Hori, T. Takahashi, and S. Sakaguchi. 2005. Homeostatic maintenance of natural $Foxp3^+CD25^+CD4^+$ regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 201: 723–735.
12. Marie, J. C., J. J. Letterio, M. Gavin, and A. Y. Rudensky. 2005. TGF-β1 maintains suppressor function and Foxp3 expression in $CD4^+CD25^+$ regulatory T cells. *J. Exp. Med.* 201: 1061–1067.
13. Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson, K. Velinzon, K. Inaba, and R. M. Steinman. 2003. Direct expansion of functional $CD25^+CD4^+$ regulatory T cells by antigen-processing dendritic cells. *J. Exp. Med.* 198: 135–147.
14. Mahnke, K., Y. Qian, J. Knop, and A. H. Enk. 2003. Induction of $CD4^+CD25^+$ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* 101: 4862–4869.
15. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of $CD4^+CD25^+$ T cell-mediated suppression by dendritic cells. *Science* 299: 1033–1036.
16. Kubo, T., R. D. Hatton, J. Oliver, X. Liu, C. O. Elson, and C. T. Weaver. 2004. Regulatory T cell suppression and anergy are differentially regulated by proinflammatory cytokine produce by TLR-activated dendritic cells. *J. Immunol.* 173: 7249–7258.
17. Peng, G., Z. Guo, Y. Kiniwa, K. S. Voo, W. Peng, T. Fu, D. Y. Wang, Y. Li, H. Y. Wang, and R.-F. Wang. 2005. Toll-like receptor 8-mediated reversal of $CD4^+$ regulatory T cell function. *Science* 309: 1380–1384.
18. Sutmuller, R. P. M., M. H. den Brok, M. Kramer, E. J. Bennink, W. L. Toonen, B.-J. Kullberg, L. A. Joosten, S. Akira, M. G. Netea, and G. J. Adema. 2006.

Toll-like receptor 2 controls expansion and function of regulatory T cells. *J. Clin. Invest.* 116: 485–494.
19. Caramalho, I., T. Lopes-Carbalho, D. Ostler, S., Zelenay, M. Haury, and J. Demengeot. 2003. Regulatory T cells selectively express Toll-like receptors and are activated by lipopolysaccharide. *J. Exp. Med.* 197: 403–411.
20. Crellin, N. K., R. V. Carcia, O. Hadisfar, S. E. Allan, T. S. Steiner, and M. K. Levings. 2005. Human CD4$^+$ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4$^+$CD25$^+$ T regulatory T cells. *J. Immunol.* 175: 8051–8059.
21. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11: 115–122.
22. Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* 301: 640–643.
23. Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and R. Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303: 1529–1531.
24. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408: 740–745.
25. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
26. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat. Immunol.* 4: 330–336.
27. Ing, R., M. Segura, N. Thawani, N. Tam, and M. M. Stevenson. 2005. Interaction of mouse dendritic cells and malaria-infected erythrocytes: uptake, maturation, and antigen presentation. *J. Immunol.* 176: 441–450.
28. Germain, R. N. 1994. MHC-dependent antigen processing and peptide presentation: providing ligand for T lymphocyte activation. *Cell* 76: 278–289.
29. Barnden, M. J., J. Allion, W. R. Heath, and R. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using dDNA-based α- and β-chain gene under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76: 34–40.
30. Hori, S., M. Haury, A. Coutinho, and J. Demengeot. 2002. Specificity requirements for selection and effector function of CD25$^+$4$^+$ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc. Natl. Acad. Sci. USA* 99: 8213–8218.
31. Pichyangkul, S., K. Yongvanitchit, U. Kum-arb, H. Hemmi, S. Akira, A. M. Krieg, D. G. Heppner, V. A. Stewart, H. Hasegawa, S. Looareesuwan, et al. 2004. Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. *J. Immunol.* 172: 4926–4933.
32. Coban, C., K. J. Ishii, T. Kawai, H. Hemmi, S. Sato, S. Uematsu, M. Yamamoto, O. Takeuchi, S. Itagaki, N. Kumar, et al. 2005. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J. Exp. Med.* 201: 19–25.
33. Moseman, E. A., W. Liang, A. J. Dawson, A. Panoskaltsis-Mortari, A. M. Krieg, Y.-U. Liu, B. R. Blazar, and W. Chen. 2004. Human plasmacytoid dendritic cells activate by CpG oligodeoxynucleotides induce the generation of CD4$^+$CD25$^+$ regulatory T cells. *J. Immunol.* 173: 4433–4442.
34. Good, M. F., D. C. Kaslow, and L. H. Miller. 1998. Pathways and strageties for developing a malaria blood-stage vaccine. *Annu. Rev. Immunol.* 16: 57–87.
35. Matsumoto, S., H. Yukitake, H. Kanbara, and T. Yamada. 1998. Recombinant *Mycobacterium bovis* bacillus Calmette-Guerin secreting merozoite surface pfotein 1 (MSP1) induces protection against rodent malaria parasite infection depending on MSP1-stimulated interferon γ and parasite-specific antibodies. *J. Exp. Med.* 188: 845–854.
36. Ocana-Morgner, C., M. M. Mota, and A. Rodriguez. 2003. Malaria blood stage supperession of liver stage immunity by dendritic cells. *J. Exp. Med.* 197: 143–151.
37. Perry, J. A., C. S. Olver, R. C. Burnett, and A. C. Avery. 2005. Cutting edge: The acquisition of TLR tolerance during malaria infection impacts T cell activation. *J. Immunol.* 174: 5921–5925.
38. Wilson, N. S., G. M. N. Behrens, R. J. Lundie, C. M. Smith, J. Waithman, L. Young, S. P. Forehan, A. Mount, R. J. Steptoe, K. D. Shortman, et al. 2005. Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. *Nat. Immunol.* 7: 165–172.
39. Takeda, K., and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21: 335–376.
40. Bafica, A., C. A. Scanga, C. G. Feng, C. Leifer, A. Cheever, and A. Sher. 2005. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to *Mycobacterium tuberculosis*. *J. Exp. Med.* 202: 1715–1724.
41. Yarovinsky, F., D. Zhang, J. F. Andersen, G. L. Bannenberg, C. N. Serhan, M. S. Hayden, S. Hieny, F. S. Sutterwala, R. A. Flavell, S. Ghosh, and A. Sher. 2005. TLR11 activation of dendrtic cells by a protozoan profiling-like protein. *Science* 308: 1626–1629.
42. Wingender, G., N. Garbi, B. Schumak, F. Jungerkes, E. Endl, D. von Bubnoff, J. Steitz, J. Striegler, G. Moldenhauer, T. Tutting, et al. 2006. Systemic application of CpG-rich DNA suppresses adaptive T cell immunity via induction of IDO. *Eur. J. Immunol.* 36: 1–9.
43. Leadbetter, E. A., I. R. Rifkin, A. M. Hohlbaum, B. C. Beaudette, M. J. Shlomchik, and A. Marshal-Rothstein. 2002. Chromatin-IgG complexes activate B cell by dual engagement of IgM and Toll-like receptors. *Nature* 416: 603–607.
44. Barrat, F. J., T. Meeker, J. Gregorio, J. H. Chan, S. Uematsu, S. Akira, B. Chang, O. Duramod, and R. L. Coffman. 2005. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J. Exp. Med.* 202: 1131–1139.
45. Wu, X., and S. L. Peng. 2006. Toll-like receptor 9 signaling protects against murine lupus. *Arthritis Rheum.* 54: 336–342.
46. Mockenhaupt, F. P., L. Hamann, C. van Gaertner, G. Bedu-Addo, C. von Kleinsorgen, R. R. Schumann, and U. Bienzle. 2006. Common polymorphisms of Toll-like receptors 4 and 9 are associated with the clinical manifestation of malaria during pregnancy. *J. Infect. Dis.* 194: 184–188.
47. Amante, F. A., A. C. Stanley, L. M. Randall, Y. Zhou, A. Haque, K. McSweeney, A. P. Waters, C. J. Janse, M. F. Good, G. R. Hill, and C. R. Engwerda. 2007. A role for natural regulatory T cells in the pathogenesis of experimental cerebral malaria. *Am. J. Pathol.* 171: 548–559.
48. Coban, C., K. J. Ishii, S. Uematsu, N. Arisue, S. Sato, M. Yamamoto, T. Kawai, O. Takeuchi, H. Hisaeda, T. Horii, and S. Akira. 2007. Pathological role of Toll-like receptor signaling in cerebral malaria. *Int. Immunol.* 19: 67–79.
49. Togbe, D., R. Schofield, G. E. Grau, B. Schnyder, V. Boissay, S. Charron, S. Rose, B. Beutler, V. F. J. Quesniaux, and B. Ryffel. 2007. Murine cerebral malaria development is independent of Toll-like receptor signaling. *Am. J. Pathol.* 170: 1640–1648.
50. Millington, O. R., C. Di Lorenzo, R. S. Phillips, P. Garside, and J. M. Brewer. 2006. Suppression of adaptive immunity to heterologous antigens during *Plasmodium* infection through hemozoin-induced failure of dendritic cell function. *J. Biol.* 5: 5.
51. Parroche, P., F. N. Lauw, N. Goutagny, E. Latz, B. G. Monks, A. Visintin, K. A. Halmen, M. Lamphier, M. Olivier, D. C. Bartholomeu, et al. 2007. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. *Proc. Natl. Acad. Sci. USA* 104: 1919–1924.
52. Hisaeda, H., S. Hamano, C. Mitoma-Obata, K. Tetsutani, T. Imai, H. Waldmann, K. Himeno, and K. Yasutomo. 2005. Resistance of regulatory T cells to glucocorticoid-induced TNFR-family related protein during *Plasmodium yoelii* infection. *Eur. J. Immunol.* 35: 3516–3524.
53. Wilson, M. S., M. D. Taylor, A. Balic, C. A. M. Finney, J. R. Lamb, and R. M. Maizels. 2005. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J. Exp. Med.* 202: 1199–1212.
54. Tetsutani, K., H. To, M. Torii, H. Hisaeda, and K. Himeno. 2007. Malaria parasite induces tryptophan-related immune suppression in mice. *Parasitology* 134: 923–930.

npg
Genes and Immunity (2008) 9, 302–308
© 2008 Nature Publishing Group All rights reserved 1466-4879/08 $30.00
www.nature.com/gene

ORIGINAL ARTICLE

Onecut transcription factor OC2 is a direct target of T-bet in type-1 T-helper cells

K Furuno[1,2], K Ikeda[2], S Hamano[3], K Fukuyama[1], M Sonoda[1], T Hara[2], T Sasazuki[4] and K Yamamoto[1]
[1]Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan; [2]Department of Pediatrics, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan; [3]Department of Parasitology, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan and [4]International Medical Center of Japan, Tokyo, Japan

T-box transcription factor, T-bet, has a central role in the differentiation of T-helper (Th) progenitor cells to Th1 or Th2 effector cells, partly by regulating the expression of genes such as interferon-γ (IFN-γ). However, the direct target genes, especially those mediating the transcriptional network initiated by T-bet, are not yet fully understood. By combining chromatin immunoprecipitation from Th1 cells with human cytosine-phosphate-guanine-island array analysis, Onecut 2 (OC2), which encodes a member of the ONECUT class of transcriptional activators, was identified as a direct target gene of T-bet. OC2 is expressed in Th1 but not Th2 cells and reporter assays showed that T-bet transactivates OC2 transcription through putative T-bet half-sites locating −451 to −347 of OC2 promoter region. Moreover, we found that OC2 binds and transactivates human T-bet promoter. These results suggest that not only cell-extrinsic regulation via the IFN-γ/STAT1 pathway, but also cell-intrinsic transcriptional positive feedback loop between T-bet and OC2 could be involved in Th1 development.
Genes and Immunity (2008) **9**, 302–308; doi:10.1038/gene.2008.18; published online 17 April 2008

Keywords: *T-bet; Onecut 2; type-1 T-helper cell; transcription*

Introduction

The differentiation of naive CD4+ T cells to either T-helper 1 (Th1) or 2 (Th2) effector cells is a critical process during immune responses, with broad implications both in responses to pathogens and in autoimmune and allergic disorders. Transcription factors including T-bet and GATA3 have appeared to play a key role in this process by regulating expression of cytokine genes such as interferon-γ (IFN-γ) and interleukin-4 (IL-4) that are characterized as dominant factors guiding the development of Th1 and Th2 cells.[1,2] T-bet belongs to a family of transcription factors, the members of which share a highly conserved DNA-binding domain T-box, flanked by diverse non-DNA-binding domains, which are involved in transactivation or repression.[3–6] A series of studies *in vitro* and *in vivo* showed that T-bet is induced by the T-cell receptor and the IFN-γR/STAT1 signaling pathway in antigen-recognizing naive CD4+ T cells. The elevated T-bet induces IL-12Rβ2 and IFN-γ expression, allowing IL-12R/STAT4 signaling to optimize IFN-γ expression, and thereby amplifying and establishing the effector commitment of Th1 cells.[7–9] In addition, T-bet induces the expression of homeobox transcription factor, Hlx, and both proteins cooperate to transactivate IFN-γ and T-bet expression.[10] Thus, the central roles of T-bet in the cell-extrinsic and -intrinsic pathways involved in Th1 lineage commitment have been well established. However, the direct target genes of T-bet are not yet fully understood.

Global analysis of the *in vivo* effects of protein–DNA interactions on gene expression can be achieved by hybridizing the DNA fragments isolated by a protein-specific antibody from the cell against a microarray constructed from sequences containing regulatory elements. Taking advantage of the strong association between CpG islands (CGIs) and gene regulatory regions,[11] one type of microarray constructed with CGIs has been established to identify transcription factor target genes.[12–14] To identify genes that are directly regulated by T-bet in Th1 cells, we employed combinatory analysis of chromatin immunoprecipitation (ChIP) and CGI microarray. We described here that ONECUT class of transcriptional activator OC2 is a target gene of T-bet. T-bet is associated with OC2 promoter and transactivates the expression. Furthermore, there exist OC2-binding elements in human T-bet promoter and OC2 transactivates T-bet expression. Our finding suggests that transcriptional positive feedback loop between T-bet and OC2 could be involved in Th1 development.

Results and discussion

A microarray containing duplicates of 9216 human CGIs was generated and probed with genome fragments isolated by ChIP using an antibody specific to T-bet or an antibody-free control from Th1-polarized human T cells (Figure 1a). We detected 50 spots at which the

Correspondence: Dr K Yamamoto, Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail: kyama@bioreg.kyushu-u.ac.jp
Received 17 December 2007; revised 21 February 2008; accepted 21 February 2008; published online 17 April 2008

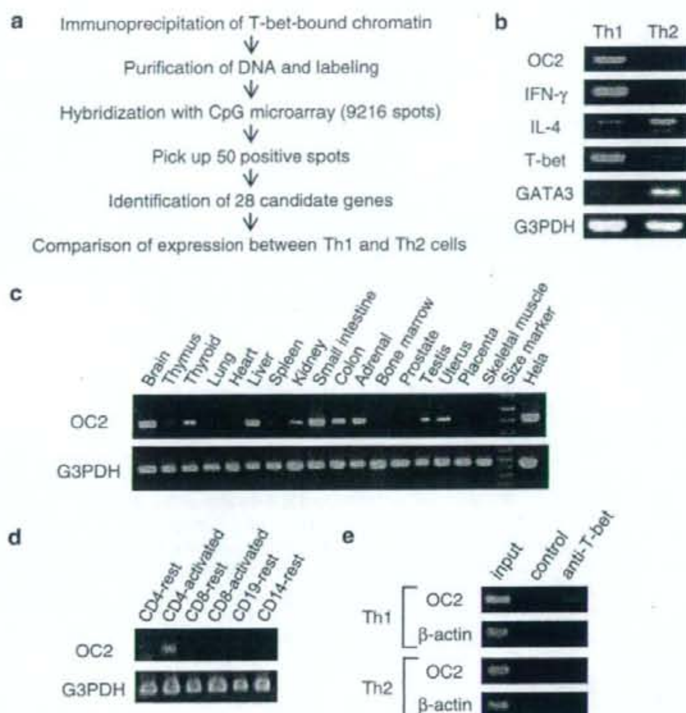

Figure 1 Identification of Onecut 2 (OC2) as T-bet target gene. (**a**) A flow chart describing the methodology used to identify T-bet targets. Human naive CD4$^+$ T cells, which were isolated from cord blood, were stimulated in Th1 condition. A total of four individual chromatin immunoprecipitation (ChIP) assays were performed with anti-T-bet antibody or no antibody, using approximately 10^7 cells for each experiment. The immunoprecipitates were pooled and then labeled to probe the CpG islands (CGI) island microarray. A total of 28 genes were selected based on the genomic location of the DNA sequences of positive spots, and the expression in Th1 and Th2 cells was analyzed by reverse transcription (RT)-PCR. (**b**) The expression of OC2 in Th1 cells. cDNAs from Th1 and Th2 cells were amplified by specific primers to the indicated genes. (**c**, **d**) The expression pattern of OC2 in adult organs and peripheral blood cells. OC2 transcripts were detected by RT-PCR. (**e**) T-bet binds the OC2 promoter in Th1 cells. ChIP assays were performed with no antibody (lane 2) and anti-T-bet antibody (lane 3). Data representative of the four independent assays are shown.

signal obtained with T-bet-associated chromatin was at least twofold higher than that obtained by probing with the control chromatin. The end reads of the positive CGI clones were aligned to the human genome sequence using the Blast-like alignment tool obtained from the University of California Santa Cruz Genome Bioinformatics Web site (http://www.genome.ucsc.edu/). The size of the chromatin in the ChIP was approximately 1–3 kb. As a result, 28 genes located within 5 kb of the CGIs were selected for further comparison of their expression in Th1 and Th2 cells by reverse transcription-polymerase chain reaction (RT-PCR) excluding false-positive clones due to repeated elements and/or nonspecific precipitation.

We found higher expression level of the gene encoding the OC2 in Th1 cells compared with Th2 cells (Figure 1b). The expression patterns of OC2 in hematopoietic lineages, including lymphocytes, have remained unclear, although a previous study reported high expression levels in the brain, testis, liver and skin.[15] We examined the expression of OC2 in various tissues and found the transcript in the thymus and spleen (Figure 1c). OC2 transcripts were not detected in resting CD4$^+$, CD8$^+$, CD19$^+$, CD14$^+$ or activated CD8$^+$ cells using RT-PCR (Figure 1d). Hence, OC2 seems to be specifically expressed in activated CD4$^+$ T cells, especially in Th1 cells (Figure 1b and d).

We next investigated whether T-bet is physically associated with the OC2 promoter *in vivo* by ChIP experiments using PCR primers specific for OC2 promoter region from −618 to −270. As shown in Figure 1e, genomic DNA fragment including OC2 promoter was coimmunoprecipitated with T-bet from the cell extract of Th1 but not Th2 cells. Thus, OC2 can be a target gene of T-bet in Th1 cells.

To determine whether T-bet directly activates the OC2 gene, a human genome fragment including the OC2 promoter region from −1905 to +124 was examined to induce reporter transcription in the presence of T-bet or GATA3 as a control. We found that overexpressed T-bet, but not GATA3, transactivated the reporter through the OC2 promoter region in a dose-dependent manner (Figure 2a). A similar level of transactivation by T-bet was observed when the OC2 promoter region from −451 to +124 was tested, whereas transactivation was markedly decreased in the OC2 promoter region from −347 to +124 (Figure 2b). We did not find the typical consensus-binding sequence for T-box in the region

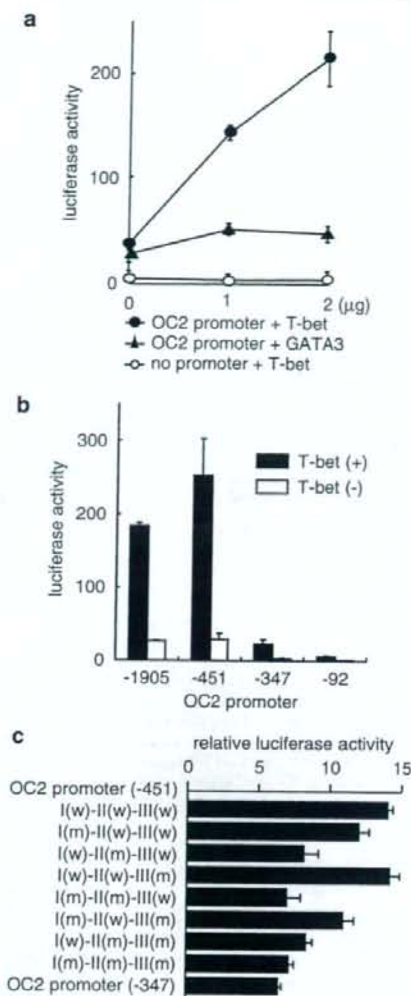

Figure 2 Onecut 2 (OC2) is a direct target gene of T-bet. (**a**) T-bet transactivates the OC2 promoter. The reporter activities of human OC2 promoter (−1905 to +124) were examined in the presence of T-bet or GATA3 in COS7 cells. All experiments were performed in triplicate and the mean ± s.d. is presented. (**b**) The OC2 promoter region between −451 and −347 is required for transactivation by T-bet. Reporter activities driven by the indicated OC2 promoter regions were examined in COS7 cells. (**c**) Putative T-box half-sites of the OC2 promoter region between −451 and −347 affect transactivation by T-bet. Three putative T-box half-sites (designated as I, II and III; Supplementary Figure S1) were mutated in the reporter containing OC2 promoter (−451 to +124) and the reporter activities of each construct, as well as the wild-type (top) and OC2 promoter (−347 to +124, bottom), were examined in the presence or absence of T-bet in COS7 cells. The relative luciferase activities were calculated by dividing the normalized activities in the presence of T-bet by those in the absence of T-bet.

between −451 and −347; however, there are at least three putative consensus half-sites (Supplementary Figure S1) that might be functionally similar to a proximal promoter and a distal enhancer of the IFN-γ gene.[16,17] We could not detect the *in vitro* binding of T-bet to these sequences (data not shown). However, the introduction of mutations in the first and second half-sites of the OC2 promoter region between −451 and +124 reduced the T-bet-mediated transactivation to a similar level as that seen in the OC2 promoter region from −347 to +124 in reporter assays (Figure 2c). Together with the ChIP experiments showing that OC2 promoter −618 to −270 region was coimmunoprecipitated with T-bet (Figure 1e), our data suggested that OC2 gene was transactivated in conjunction with these sites in cells.

We next examined the effects of truncated mutations of T-bet on transcriptional activation of OC2 promoter in reporter assays. It was found that the transactivation required the T-box DNA-binding domain (Figure 3a). We also observed that both the N- and C-terminal regions of T-bet were involved in transactivation, although previous reports suggested that the C-terminal alone was an activation domain.[10,18] Consistent with this observation, when the heterologous GAL4 DNA-binding domain (GAL4DBD) was fused to each region of T-bet, both the N- and C-terminal parts showed transactivation (Figure 3b). These results revealed that T-box is flanked by two potent transactivation domains in T-bet. Taken together, our findings indicate that T-bet binds the OC2 promoter via T-box for transactivation in Th1 cells.

Members of the ONECUT class of transcription factors are characterized by a bipartite DNA-binding domain composed of a single cut domain associated with a divergent homeodomain.[19] In mammals, three ONECUT factors, OC1 (HNF6), OC2 and OC3, have been identified so far. The molecular dissection of OC1 revealed that the cut domain and the homeodomain act together to determine DNA-binding affinity, specificity and transactivation.[20] The sequence conservation in these domains among the three paralogues suggests that OC2 binds a similar DNA consensus sequence to OC1. Indeed, many of the OC1 targets are also recognized by OC2.[21] However, the optimal binding sequence for OC2 has not yet been identified. The identification of the target genes of OC2 is essential for elucidating its functional roles in Th1 cells. Thus, we determined the consensus-binding sequence of OC2 through an *in vitro* selection experiment using a random sequence oligonucleotide and a truncated OC2 protein containing both the cut domain and the homeodomain (Supplementary Figure S2a). Five rounds of binding reactions in combination with PCR enriched the DNA species that might interact with OC2 (Figure 4a). Subsequently, we cloned the PCR products amplified in the final round and analyzed the sequences of 96 clones. We identified 20 different sequences, 16 of which were significantly bound to OC2 in an *in vitro* band-shift assay (Supplementary Figure S2b). The alignment of these sequences revealed the consensus, 5′-AATCG(A)ATA(C)-3′ (Figure 4b), which overlapped significantly with those of the cut domain of human CDP (5′-ATCGAT-3′) and OC1 (5′-ATCAAT-3′).[20,22]

Based on the consensus-binding sequence of OC2, we searched for candidate target genes encoding proteins related to Th1 function by checking the genome sequences of the promoter region. We found tandem repeats of the consensus sequence 5′ upstream of the human T-bet gene, 5′-ATCAATAAAGATCGAT-3′, in the region from −490 to −475 (the consensus sequences are underlined and designated as 'site 1' and 'site 2',

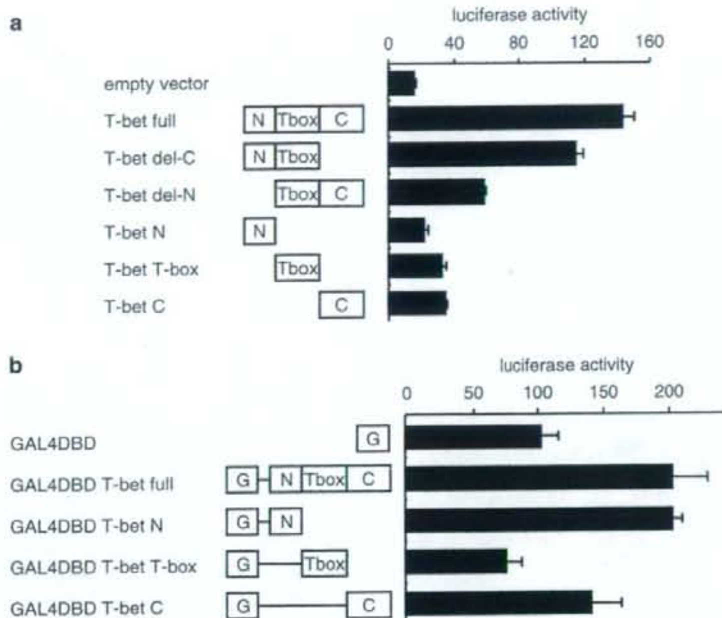

Figure 3 Activation domains of T-bet and association of T-bet with Onecut 2 (OC2) promoter in cells. (**a**) T-box is indispensable for T-bet-induced OC2 promoter activity. Reporter activities of OC2 promoter (−451 to +124) induced by truncated mutants of T-bet were examined in COS7 cells. The mutants of T-bet express truncated T-bet as follows: T-bet del-C; N-terminal part and T-box (amino acids 1–334), T-bet del-N; T-box and C-terminal part (amino acids 132–535), T-bet N; N-terminal part (amino acids 1–131), T-bet T-box; T-box (amino acids 132–334), T-bet C; C-terminal part (amino acids 335–535). Schematic representation of the deletion mutants has been shown on the left of the data column. (**b**) T-bet contains transactivation domains in the N- and C-terminal parts. The N-/C-terminal part and T-box were fused to the GAL4 DNA-binding domain (GAL4DBD). The transcriptional activities of each part were examined using pGL3-17m4-tk-luc as a reporter in the 293 cells. Schematic representation of the GAL4DBD-fused deletion mutants has been shown on the left of the data column.

respectively) (Figure 4c). OC2 significantly bound this sequence and the interaction was, at best, partially competed by a cold probe containing mutations in site 1 and/or site 2 (Figure 4d), indicating that both sites were involved in binding to OC2 *in vitro*.

To examine whether the OC2 transactivates the T-bet promoter, we performed reporter assays using reporter constructs containing T-bet or IFN-γ promoter. We found that the reporter gene that contains T-bet promoter (−976 to +27), but not the IFN-γ promoter, was transactivated by OC2 (Figure 5a). Furthermore, a ChIP experiment using an OC2-specific antibody, which is able to immunoprecipitate formaldehyde-fixed OC2 protein (Supplementary Figure S3), revealed an association between OC2 and the T-bet promoter in Th1 cells (Figure 5b). Thus, these results suggest that T-bet can be a direct target gene of OC2 and is transactivated by this transcription factor in Th1 cells.

Our present study suggests a transcriptional positive feedback loop between T-bet and OC2 in Th1 cells. The endogenous expression of T-bet was shown to be induced by the forced expression of ectopic T-bet,[8] which implied the autoactivation of T-bet, similar to GATA-3. However, a subsequent report revealed that a cell-extrinsic regulatory circuit involving IFN-γR signaling via STAT1 largely maintained the high-level expression of T-bet in developing Th1 cells, rather than a cell-intrinsic pathway of T-bet autoactivation.[9] Because CD4+ T cells from STAT1-deficient mice still expressed a low level of T-bet and could differentiate into Th1 cells,[23,24] it is possible that the OC2-mediated transactivation could be involved in the expression of T-bet in a different pathway from STAT1. Alternatively, OC2 could contribute to T-bet expression at some later time during Th1 development, when the stable expression of T-bet is already driven by the IFN-γR/STAT1 pathway. We should also consider the effects of other target genes of OC2 that were not determined in the present study. The studies using OC2-deficient mouse revealed the important roles of this transcription factor in liver and pancreas development,[25–27] suggesting its broad range of contribution to cellular development.

In conclusion, the present work revealed a new target gene of T-bet, OC2, in Th1 helper T cell, and implies that transcriptional network between T-bet and OC2 coexists with an exocrine mechanism in the complex cellular processes in Th1 development.

Materials and methods

Plasmid constructs

Details of the individual constructs, which were all verified by sequencing, are available upon request. Constructs for mammalian cell transfection were based on pSG5, pCMV-myc (Clontech Laboratories, Mountain View, CS, USA), or pG4MpolyII. The human promoter regions of the T-bet, OC2 and IFN-γ gene were cloned to

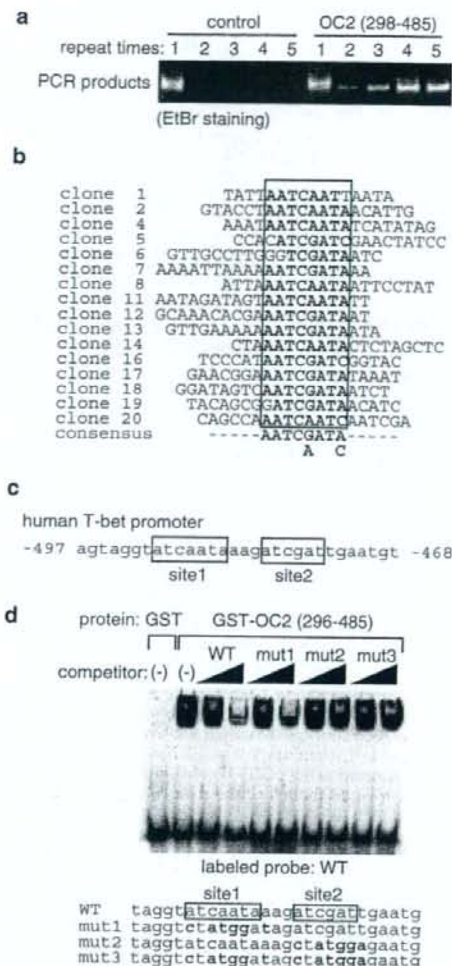

Figure 4 Identification of Onecut 2 (OC2)-binding element. (**a**) The *in vitro* selection of an OC2-binding consensus sequence. The PCR products of each round of selection are shown. The PCR products from final round of selection (lane 10) were cloned for sequencing. (**b**) A total of 16 out of the 20 sequences significantly bound to OC2 *in vitro* (Supplementary Figure S2b). These sequences were compiled and the consensus sequences are shown. (**c**) The OC2 consensus sequences in the human T-bet promoter region. The two boxed consensus sequences were designated as site 1 and site 2. (**d**) OC2 binds the fragment of the T-bet promoter *in vitro*. Glutathione *S*-transferase (GST) or GST-OC2 (296–485) were subjected to band-shift assay with a labeled probe of the T-bet promoter region from −497 to −468 (wild-type, WT) in the presence or absence of the indicated nonlabeled competitor. The mut1, mut2 and mut3 probes contained mutations in site 1, site 2 and both sites, respectively.

pGL3-basic (Clontech). The pSG5 and pG4MpolyII were kindly provided by P Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). The pGEX-2T (Amersham, Buckinghamshire, UK) and pGAD-T7 (Clontech) were used to express glutathione *S*-transferase (GST) fusion and *in vitro* translated hemagglutinin (HA)-tagged proteins, respectively.

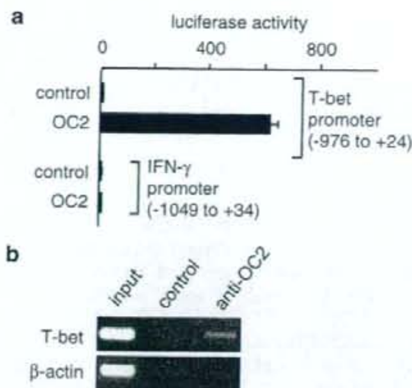

Figure 5 T-bet is a direct target gene of Onecut 2 (OC2). (**a**) OC2 transactivates the T-bet promoter. The reporter activities of human T-bet and the interferon-γ (IFN-γ) promoter were examined in the presence or absence of OC2 in COS7 cells. (**b**) OC2 binds the T-bet promoter in Th1 cells. Chromatin immunoprecipitation (ChIP) assays were performed with no antibody (lane 2) and anti-OC2 antibody (lane 3) that was generated against human OC2-derived peptide 44-ASPSPHHARR-53 (Supplementary Figure S3). Data representative of the four independent assays are shown.

Isolation of naive CD4$^+$ T cells from cord blood

Human naive T cells were prepared from cord blood cells of full-term neonates, who had no hereditary disorders, hematological abnormalities or infectious complications, by density-gradient centrifugation. Written informed consent was obtained from all mothers. This study was approved by the ethics committee of Kyushu University. CD4$^+$ cells were isolated using anti-CD4 monoclonal antibody-coated magnetic beads and an MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). The expression levels of CD4 and CD45RA were examined by flow cytometry (EPICS XL, Immunotech Coulter, Miami, FL, USA) in each preparation and most cells (>95%) were doubly positive.

T-cell stimulation

For the CGI microarray, RT-PCR analysis and ChIP assay, CD4$^+$CD45RA$^+$ cells (5×10^5) were stimulated with 1 μg ml^{-1} PHA (Wako Pure Chemical Industries, Osaka, Japan) and 50 U ml^{-1} recombinant human IL-2 (Shionogi, Osaka, Japan) in the presence of either 10 ng ml^{-1} recombinant human IL-12 (R&D Systems, Minneapolis, MN, USA) and 10 μg ml^{-1} anti-human IL-4 (R&D Systems), or 20 ng ml^{-1} human IL-4 (Pepro Tech, Rocky Hill, NJ, USA) and 10 μg ml^{-1} anti-human IL-12 (R&D Systems), for Th1 or Th2 differentiation, respectively. After 3 days of culture, the cells were restimulated with 20 nM of PMA (Sigma-Aldrich, St Louis, MO, USA) and 1 μM of ionomycin (Sigma) for 4 h, and then harvested for the experiments.

ChIP assay

The ChIP assay was performed as described previously[28] using a ChIP Assay Kit (Upstate, Lake Placid, NY, USA). Approximately 10^7 cells were used for each assay. Anti-T-bet (4B10, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-OC2 antibodies produced against human OC2-derived peptide, 44-ASPSPHHARR-53, were used.

The PCR primers are as follows: OC2, 5′-GGCATCTTT CACCGAATCTC-3′ and 5′-CGTCTTCTGTTTGGGTGA GC-3′; T-bet, 5′-TTGTCCATCAGGTTCCAGGT-3′ and 5′-ATGGCCCAAGTGGGACTC-3′; β-actin, 5′-GGGACT CAAGGCGCTAACT-3′ and 5′-GGCCGCGTTATTACCA TAAA-3′.

CGI microarray
The human CGI library was purchased from the UK Human Genome Mapping Project Resource Centre. A total of 9216 clones were organized individually in 96-well plates. The CGI inserts were amplified by PCR using the 5′-CGGCCGCCTGCAGGTCGACCTTAA-3′ and 5′-AACGCGTTGGGAGCTCTCCCTTAA-3′ primers.[29] The PCR products were purified using Magnesil (Promega, Madison, WI, USA) and then mixed with dimethyl sulfoxide (DMSO) to a final concentration of 10%. Array Spotter Generation III (Molecular Dynamics, Sunnyvale, CA, USA) was used to assemble the PCR/dimethyl sulfoxide samples on microarray slides (Type 7 Star; Amersham) as microdots (spot diameter = 250 μl and spot buffer = 30 μl). A total of four individual ChIP assays were performed with anti-T-bet antibody or no antibody, using approximately 10^7 cells for each experiment. The combined test or control samples were labeled according to the protocols developed by DeRisi *et al.* (Microarrays.org, http://www.derisilab.ucsf.edu/data/microarray/index.html) and hybridized with microarray slides using an automated slide processor (Amersham). The slides were scanned with the GenePix 4000A (Axon) and the images were analyzed with GenePix Pro 3.0 software. Spots showing a Cy5/Cy3 ratio in duplicate > 2 were selected as positive.[12]

RT-PCR
The BD Premium RNAs (Clontech) were used to examine the expression of OC2 in adult organs and peripheral blood cells. The primers for RT-PCR are as follows: OC2, 5′-ATGATGTCGCACCTGAACG-3′ and 5′-TGGGAATTGTTCCTGTCTTTG-3′; IFN-γ, 5′-TGCAG GTCATTCAGATGTAGC-3′ and 5′-CAGTTCAGCCATC ACTTGGA-3′; IL-4, 5′-ACAAGTGCGATATCACCTTAC-3′ and 5′-CAACGTACTCTGGTTGGCT-3′; T-bet, 5′-CCAA CAATGTGACCCAGATG-3′ and 5′-ATCTCCCCCAAG GAATTGAC-3′; GATA3, 5′-AACTGTCAGACCACCAC AACCACAC-3′ and 5′-GGATGCCTTCCTTCTTCATAG TCAGG-3′; G3PDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′.

Reporter assay
Reporter plasmids (1 μg), phRL-CMV (50 ng, internal control for transfection efficiency) and effector plasmids (2 μg) were used in all reporter assays except that indicated amounts of effector were transfected in the assay shown in Figure 2a. Firefly and Renilla luciferase activities were measured sequentially using a dual luciferase reporter-assay system (Promega). Transfection efficiencies were normalized to the Renilla luciferase activity.

In vitro *binding site selection*
HA-tagged OC2 protein containing the cut domain and the homeodomain was generated by an *in vitro* transcription/translation system (Promega) and purified by immunoprecipitation with anti-HA antibody coupled to protein G. Double-stranded oligonucleotides containing 20-nt random sequences were generated based on oligo N20 extended by primer B and Klenow DNA polymerase (Supplementary Figure S2a). The immunoprecipitates were preincubated with 0.1 μg ml^{-1} of poly(dI-dC) in binding buffer (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5% glycerol and 1 mM DTT), and approximately 1 μg of double-stranded oligo N20 was added for further incubation. The protein G beads were washed and the bound DNA was purified by phenol/chloroform extraction. The purified DNA was amplified by 15 cycles of PCR using primers A and B (Supplementary Figure S2a). The PCR products were used for the next round of incubation. This reaction was repeated five times and the final PCR products were cloned for sequencing.

Band-shift assay
Purification of the GST and GST-OC2 (296–485) proteins were performed as previously described.[30] Approximately 50 ng of protein was preincubated with 0.1 μg ml^{-1} of poly(dI-dC) for 5 min in binding buffer, and 0.3 pmol of the indicated oligonucleotide probe labeled with biotin at the 5′ end of one of strand was added for further incubation. For the competition assay, 0.3 or 1.5 pmol of nonlabeled competitor was added to the reaction mixture before incubation with the labeled probe. The samples were loaded onto a 10% polyacrylamide gel, transferred onto a nylon membrane (Hybond-N+; Amersham) and visualized using avidin peroxidase by standard protocol for western blotting.

Acknowledgements

We thank T Tanaka, T Akinaga, M Goto and M Ohnish for technical help. This work was supported by a Grant-in-Aid for Scientific Research in the priority area of Genome Biology, and by a grant from the 21st Center of Excellence Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1 Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. Molecular mechanisms regulating Th1 immune responses. *Annu Rev Immunol* 2003; **21**: 713–758.
2 Murphy KM, Reiner SL. The lineage decisions of helper T cells. *Nat Rev Immunol* 2002; **2**: 933–944.
3 Wilson V, Conlon FL. The T-box family. *Genome Biol* 2002; **3**: reviews 3008.1–3008.7.
4 Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000; **100**: 655–669.
5 Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH *et al.* Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 2002; **295**: 336–338.
6 Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 2002; **295**: 338–342.
7 Lighvani AA, Frucht DM, Jankovic D, Yamane H, Aliberti J, Hissong BD *et al.* T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci USA* 2001; **98**: 15137–15142.