Production of IFN-\(\gamma\) by CD4\(^+\) T cells in response to malaria antigens is IL-2-dependent

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Abstract

T cell immune responses are critical for protection of the host and for disease pathogenesis during infection with *Plasmodium* species. We examined the regulation of CD4$^+$ T cell cytokine responses during infection with *Plasmodium berghei* ANKA (PbA). CD4$^+$ T cells from PbA-infected mice produced IFN-γ, IL-4 and IL-10 in response to TCR stimulation at levels higher than those from uninfected mice. This altered cytokine response was dependent on parasitemia. To examine the specificity of the response, mice were adoptively transferred with CD4$^+$ T cells from OT-II TCR transgenic mice, and were infected with PbA expressing OVA. Unexpectedly, CD4$^+$ T cells from the OT-II-transfected wild-type PbA-infected mice showed high levels of IFN-γ production after stimulation with OVA and the cells producing IFN-γ were not OT-II, but were host CD4$^+$ T cells. Further investigation revealed that host CD4$^+$ T cells produced IFN-γ in response to IL-2 produced by activated OT-II cells. This IFN-γ response was completely inhibited by anti-CD25 mAbs, and this effect was not due to the block of the survival signals provided by IL-2. Furthermore, IFN-γ production by CD4$^+$ T cells in response to PbA antigens was dependent on IL-2. These findings suggest the importance of IL-2 levels during infection with malaria parasites and indicate that CD4$^+$ T cells can produce IFN-γ without TCR engagement via a bystander mechanism in response to IL-2 produced by other activated CD4$^+$ T cells.
Introduction

Malaria is caused by infection of red blood cells with parasites of the *Plasmodium* species and remains one of the crucial threats to public health in the world. The critical roles of cellular immunity in protection against the blood stage of malaria infection has been demonstrated in early studies using thymectomized rats and mice depleted of B cells by anti-μ chain Ab treatment (1-3). These studies were confirmed using mice that were rendered B cell deficient by targeted disruption of Ig gene (4, 5). It was also shown that protective immunity can be transferred to naïve hosts by adoptive transfer of immune CD4+ T cells (6). IFN-γ produced by T cells as well as NK cells play a central role in regulating the protective immune response against blood-stage malaria infection (3,7-9). Mice deficient in IFN-γ or its receptor showed delayed development of protective immunity and high mortality rate after infection with *P. chabaudi* (10). However, as with other protective immunity, production of this potent cytokine is not always beneficial, but rather is harmful for the host in some cases. Pregnant IFN-γ−/− mice showed a delay in malaria-induced fetal loss relative to wild-type controls, although these mice experienced a more severe course of infection (11). Neutralization of IFN-γ with its specific mAb early after infection with *P. berghei* ANKA (PbA) prevented cerebral malaria (12). These studies point to the importance of regulation of IFN-γ levels during malaria infection in order to establish anti-parasite immune protection, while maintaining minimum pathogenesis. However, little is known about the regulation of conventional CD4+ T cell function with respect to IFN-γ production during infection with *Plasmodium* species.

We have examined the cytokine production of CD4+ T cells from mice infected with PbA. These T cells produced little IL-2 and a large amount of IFN-γ, IL-4 and IL-10 in response to TCR stimulation. Further study indicated that the production of IFN-γ by CD4+ T cells from the infected mice does not necessarily require direct TCR engagement. Rather, these CD4+ T cells
produced IFN-γ, IL-4 and IL-10 in response to IL-2. Furthermore, IFN-γ production by CD4⁺ T cells in response to malaria-antigen was dependent on IL-2.
Methods

Mice and *P. berghei* infection

OT-II transgenic mice expressing the TCR specific for OVA\(^{323-339}/I-A^b\), were kindly provided by Dr. H. Kosaka (Osaka Univ., Japan) (13), B6.SJL-Ptprc congenic (B6.Ly5.1) mice (CD45.1\(^{+}\)) by Dr. Y. Takahama (Tokushima Univ., Japan), Rag-2\(^{-/-}\) mice by Dr. Y. Yoshikai (Kyushu University, Fukuoka, Japan) (14), MyD88\(^{-/-}\) and TRIF\(^{-/-}\) mice by Dr. K. Takeda and S. Akira (Osaka Univ., Japan) (15,16). C57BL/6 (B6) mice were purchased from SLC (Hamamatsu, Japan). OT-II and B6.Ly5.1 mice were bred and offspring were intercrossed to obtain CD45.1 OT-II mice. Rag-2\(^{-/-}\) mice and CD45.1 OT-II mice were intercrossed to obtain CD45.1 Rag-2\(^{-/-}\) OT-II mice. These mice were maintained in the Laboratory Animal Center for Animal Research at Nagasaki University and were used at the age of 8-14 wks. OVA-PbA was described previously (17). Mice were infected with wild type PbA WT- or OVA-PbA by i.p. injection of parasitized RBCs (10\(^4\) infected RBC), monitored by microscopic examination of standard blood films and were sacrificed when parasitemia reached 3.7-15.0% (day 6-8). The animal experiments reported herein were conducted according to the Guidelines of the Laboratory Animal Center for Biomedical Research at Nagasaki University.

Adoptive transfer and flow cytometry

For adoptive transfer, CD4\(^{+}\) T cells were purified using anti-CD4 IMag (BD Biosciences, San Diego, CA, USA), labeled with CFSE (Invitrogen, Carlsbad, CA, USA) at the final concentration of 15mM and were injected into the tail vein of B6.Ly5.1 or B6 mice (0.5-1 X 10\(^7\) /mice). After infection with PbA and parasitemia reached 3%, spleen cells were prepared, stained with biotin-anti-CD45.2 plus streptavidin-Cy5.2 and PE-anti-CD4 mAb, and analyzed using FACScan (BD Biosciences) (Fig.3A). For all other analysis, spleen cells were stained
with PE-Cy7-anti-CD45.1 (or anti-CD45.2), PE-Cy7- or allophycocyanin-anti-CD4, PE-anti-CD69, FITC- or PE-anti-CD25, FITC-streptavidin plus biotin-anti-CD122 or PE-anti-CD122, PE-anti-CD62L, FITC-anti-CD44, FITC-anti-TCRβ or allophycocyanin-streptavidin plus biotin-anti-NK1.1 mAb and analyzed using FACSCantoII (BD Biosciences). For cell sorting, purified CD4$^+$ T cells were stained with a combination of allophycocyanin-anti-CD4, FITC-anti-CD44, and PE-anti-CD62L (Fig. 2), or by a combination of PE-anti-CD4, PE-Cy7-anti-CD45.1 and allophycocyanin-anti-CD45.2 mAb (Fig. 4) and were sorted using FACSaria II (BD Biosciences).

For intracellular cytokine staining, CD4$^+$ T cells were stimulated with anti-TCR mAb (H57-597, 10 μg/ml), DC pulsed with OVA323-339 (10 μg/ml) or recombinant IL-2 (10ng/ml) for total of 12-24 h with an addition of monensin during the final 6 h. After blocking Fc receptor with mAb (2.4G2), cells were stained with PE-Cy7-anti-CD45.1, allophycocyanin-anti-CD4, FITC-anti-CD44, FITC-anti-CD25, allophycocyanin- or FITC-streptavidin, biotin-anti-CD122, biotin-anti-NK1.1 or FITC-anti-TCRβ, fixed, permeabilized, and were stained with PE-anti-Foxp3, PE-anti-IL-2, PE-anti-IL-4, FITC- or PE-anti-IL-10, Alexaflour 488- or PE-anti-IFN-γ mAb according to the manufacturer’s instructions (BD Biosciences). The ratios of viable cells were determined by staining cells with FITC-Annexin V and 7-aminoactinomycin D (7AAD).

**Cell culture and ELISA**

CD4$^+$ T cells (2-3X10$^5$) were cultured for 24-48 hr in 96-well plates coated with anti-TCR mAb (H57-597, 3-5 μg/ml), or in the presence of recombinant IL-2 (10ng/ml), IL-7 (10ng/ml), IL-12 (0.01-3ng/ml) (Pepro Tec, London, UK), or IL-18 (0.1-30ng/ml)(MBL, Nagoya, Japan). For stimulation with protein antigens, CD4$^+$ T cells (2-3X10$^5$) were cultured with DC (1-3X10$^4$)
pulsed with OVA323-339 (3 µg/ml), streptococcal enterotoxin B (SEB, 2 µg/ml)(Toxin Tec, Sarasota, FL, USA) or crude malaria Ag (6.6 x 10⁷ RBCs/ml). Crude malaria antigens were prepared using blood samples collected from PbA-infected Rag2⁻/⁻ mice (parasitemia ~45%). After washing, RBCs were suspended in PBS at a final concentration of 1 x 10⁹ RBCs/ml, and were lysed by five freeze-thaw cycles as described previously (17). DC (>95%) were prepared from B6 spleen cells using anti-CD11c MACS microbeads and autoMACS (Miltenyi Biotech, Gladbach, Germany). IL-12 and IL-18 were neutralized using specific mAbs C17.8 (10 µg/ml) and 93-10C (5 µg/ml; MBL), respectively. To prepare NK1.1⁻CD4⁺ T cells, splenocytes were depleted of NK1.1⁺ cells using autoMACS prior to CD4⁺ T cell purification.

Transwell experiments were performed in 24-well plates with pore size 0.4 µM cell culture inserts (BD Biosciences). OT-II CD4⁺ T-cells (2 x 10⁵) were cultured in the bottom chamber in the presence and absence of DC (1x10⁵) pulsed with OVA323-339 (3 µg/ml). Responder CD4⁺ T-cells (1.8 x 10⁶) were cultured in the top chamber. After 48 h in culture, supernatant was recovered from both upper and lower chambers.

In the experiments using OT-II culture supernatant, OT-II CD4⁺ T cells (1x10⁴) and DC (1x10⁴) pulsed with OVA323-339 were cultured for 48 h in 96-well plate, and the supernatant was collected. The level of IL-12 and IL-18 in the supernatant was 17.5 ± 2.3 pg/ml and 9.1±8.1 pg/ml, respectively. This supernatant (50 µl) was added in culture (the total volume of 200 µl). Thus, the final concentration of IL-12 and IL-18 contained in the supernatant was ~4.4 pg/ml and ~2.3 pg/ml, respectively. Inhibition of IL-2 receptor (CD25) was performed using a combination of 3C7 (5µg/ml) and PC61.5 (5µg/ml) (18,19).

The levels of cytokines in the supernatants were determined by a sandwich ELISA according to
the manufacturer's directions using the following mAbs; JES6-1A12 and biotin-JES6-5H4 for IL-2, 11B11 and biotin-BVD6-24G2 for IL-4, and R4-6A2 and biotin-XMG1.2 for IFN-γ (all mAbs were from e-Bioscience, San Diego, CA, USA). The levels of IL-10, IL-12p70 (e-Bioscience), IL-18 (MBL) and TGF-β (Promega, Madison, WI, USA) were determined using a manufacturer’s cytokine ELISA kits.

**Real-time RT-PCR**

The mRNA expression was determined by an automated real-time RT-PCR system using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described previously (20). RNA was converted to cDNA and amplified by PCR in the PCR reaction buffer containing the dsDNA-specific fluorescent dye SYBR Green. Sequences of primers used for IFN-γ, IL-4, T-bet, GATA3 and G3PDH are indicated (21-23). Primers for IL-10 was 5'-TGACTGGCATGAGGATCAGC-3' and 5'-AGTCCGCAGCTCTAGGAGCA-3'. The mRNA levels were determined as the ratio of the each DNA to G3PDH.

**Statistical analysis**

Except for the frequency or count data, comparison of the distributions of measurements among groups was based on the logarithmic values of the measurements. In comparison of two groups, \( t \)-test was used, while in comparison of three or more groups, overall comparison was first made by ANOVA (analysis of variance) for one-way or two-way data at the significance level of 0.05, and if significant, each pair of the groups was compared by \( t \)-test with the significance level determined by Bonferroni procedure controlling the familywise error rate (FEW) less than 0.05. Procedures of ANOVA and TTEST in the SAS system were used for the calculations.
Results

Up-regulation of cytokine production in CD4\(^+\) cells from PbA-infected mice

To determine the function of CD4\(^+\) T cells during infection with malaria parasites, CD4\(^+\) cells from mice uninfected or infected with PbA were stimulated with DC pulsed with superantigen, SEB, or with plate-coated anti-TCR mAb (Fig. 1A). CD4\(^+\) cells from PbA-infected mice showed increased production of IFN-\(\gamma\), IL-4 and IL-10 in response to TCR stimulation, while the production of IL-2 was severely impaired. The reduction in IL-2 production by T cells in infected mice was consistent with previous studies (24,25). To examine whether the same cells produced IFN-\(\gamma\), IL-4 and IL-10 simultaneously, we performed intracellular cytokine staining (Fig. 1B). Approximately 50% of IL-10-producing cells produced IFN-\(\gamma\), and ~80% of IL-4-producing cells produced IL-10, indicating that a significant number of cells produced multiple cytokines. We focused further studies on the mechanism of upregulation of IFN-\(\gamma\), IL-4 and IL-10 in PbA-infected mice. These CD4\(^+\) cells expressed much higher levels of IFN-\(\gamma\), IL-4 and IL-10 mRNA than those from naïve mice after anti-TCR-stimulation (Fig. 1C). However, these CD4\(^+\) cells did not express increased levels of T-bet or GATA-3 (Fig. 1C), key transcription factors of Th1 and Th2 differentiation (26), respectively, suggesting that the up-regulation of cytokine production was not due to the differentiation towards Th1 or Th2 lineages. Recently, it was shown that CD4\(^+\) T cells from post-septic model mice expressed a reduced level of IL-2 and increased levels of other cytokine genes including IFN-\(\gamma\), IL-4 and IL-10 after stimulation with anti-CD3 mAb, and that they exhibited decreased capacity to commit to either Th1 or Th2 lineages (27). Thus, this type of altered cytokine responses is not unique to PbA infection.

Next, C57BL/6 mice were infected with PbA for 0-8 days, and the levels of parasitemia as well as cytokine production by CD4\(^+\) cells in response to anti-TCR mAb were monitored in
individual mice (Fig. 2A). Parasitemia became detectable (~0.1%) 4 days after infection. After 5 days, when the level of parasitemia reached ~1%, the production of IFN-γ was greatly up-regulated. However, after 3 days of chloroquine treatment, parasitemia became undetectable and IFN-γ production by CD4+ cells was reduced (Fig. 2B), indicating that the altered cytokine response by CD4+ cells was dependent on parasitemia. We next examined whether IFN-γ producing cells were naïve or effector/memory (E/M) cells. The proportion of E/M-type cells (CD62L\textsuperscript{low}CD44\textsuperscript{high}) increased in CD4+ cells of PbA-infected mice (42.8%) when compared with those from uninfected mice (20.7%) (Fig. 2C). Naïve (CD62L\textsuperscript{high}CD44\textsuperscript{low}) and E/M-type cells were purified by cell sorting and were evaluated for their IFN-γ-production. E/M-type CD4+ T cells from PbA-infected mice produced IFN-γ at levels much higher than those from uninfected mice or naïve CD4+ T cells in response to anti-TCR mAb.

**Antigen specificity of CD4+ cells producing high levels of IFN-γ**

To determine whether the PbA-specific CD4+ T cells mediated high IFN-γ production, we used recombinant PbA that express OVA (OVA-PbA) (17). C57BL/6 mice were adoptively transferred with CFSE-labeled CD4+ cells from OVA-specific TCR transgenic mice, OT-II, and were infected with wild type PbA (WT-PbA) or OVA-PbA (Fig. 3). The proportion of OT-II cells was increased in mice infected with OVA-PbA (2.1%) when compared with mice not infected (0.3%) or infected with WT-PbA (0.4%). OT-II cells divided 2-3 times and up-regulated activation markers CD69 and CD25 in the OVA-PbA-infected host (Fig. 3B), suggesting the specific activation of OT-II cells in response to OVA-PbA. However, total CD4+ cell population from mice infected with either WT- or OVA-PbA produced IFN-γ at levels much higher than those from uninfected mice after co-culture with DC pulsed with OVA\textsubscript{323-339} (Fig. 3C). Intracellular cytokine staining showed that significant numbers of host CD4+ cells, and
not OT-II cells, produced IFN-γ in response to OVA323-339-pulsed DC (Fig. 3D). To confirm this observation, OT-II and host CD4+ cells were purified by sorting, and examined for production of IFN-γ in response to OVA323-339-pulsed DC (Fig. 4). Host CD4+ cells from infected mice did not produce significant levels of IFN-γ by themselves, indicating that these cells did not directly respond to OVA323-339. When they were mixed with OT-II cells either from infected or uninfected mice, high levels of IFN-γ were detected, suggesting that CD4+ cells from infected mice produced IFN-γ in cooperation with activated OT-II cells.

**CD4+ cells from PbA-infected mice produce high levels of IFN-γ in response to a soluble mediator produced by OT-II**

To determine whether IFN-γ response of CD4+ cells from PbA-infected mice was mediated by the cognate interaction with OT-II or by soluble factor(s), two methods were used; transwell culture and the addition of culture supernatant. When CD4+ cells from PbA-infected mice were cultured in the upper chamber and OT-II/OVA323-339-pulsed DC in the lower chamber, CD4+ cells produced IFN-γ at levels similar to co-culture (Fig. 5A). Reproducible results were observed when the cells in upper and lower chambers were exchanged (data not shown). Culture supernatant from OT-II/OVA323-339-pulsed DC stimulated IFN-γ production by CD4+ T cells from PbA-infected mice (Fig. 5B). These results suggest that CD4+ cells from PbA-infected mice produced IFN-γ without direct TCR engagement, and that this response was mediated by soluble factor(s) produced by OT-II/OVA323-339-pulsed DC.

The major cytokines that are known to promote CD4+ T cell production of IFN-γ are IL-12 and IL-18 (28, 29). A combination of these two cytokines can stimulate IFN-γ production of CD4+ T cells without TCR engagement (30-32). Although these cytokines were able to stimulate IFN-γ production of CD4+ cells from PbA-infected mice (Fig. 6), the levels of IL-12 and IL-18
detected in the culture supernatant of the OT-II/OVA\textsubscript{323-339}-pulsed DC was 17.5 pg/ml and 9.1 pg/ml, respectively, and was not sufficient to promote IFN-\(\gamma\) production (Fig. 6A, B). In addition, a saturating concentration of anti-IL-12 mAb did not inhibit IFN-\(\gamma\) production by CD4\(^+\) cells co-cultured with OT-II/OVA\textsubscript{323-339}-pulsed DC or with their supernatant. Since signal transduction through the IL-18 receptor depends on MyD88, the response of CD4\(^+\) cells from MyD88 KO mice were also examined. CD4\(^+\) cells of the PbA-infected MyD88/Trif double KO mice produced IFN-\(\gamma\) at levels similar to CD4\(^+\) cells from PbA-infected B6 mice in the presence of culture supernatant from OT-II/OVA\textsubscript{323-339}-pulsed DC. Saturating concentrations of anti-IL-12 and anti-IL-18 mAb did not have significant inhibitory effects (Fig. 6). Thus, the soluble mediator produced by OT-II/OVA\textsubscript{323-339}-pulsed DC, which stimulated IFN-\(\gamma\) production by CD4\(^+\) cells, was neither IL-12 nor IL-18.

**IL-2 stimulates IFN-\(\gamma\) production of CD4\(^+\) cells from PbA-infected mice**

Our study using different types of TCR transgenic T cells suggested that CD4\(^+\) T cells, and not CD8\(^+\) T cells, produced the stimulating factor (data not shown). Therefore, we examined whether IL-2 can stimulate IFN-\(\gamma\) production. OT-II cells produced IL-2 at a concentration of 9.76 \(\pm\) 1.26 ng/ml in the supernatant. This concentration was sufficient to induce the production of IFN-\(\gamma\), IL-4 and IL-10 in CD4\(^+\) cells from PbA-infected mice without TCR engagement, and the response was IL-2-dose dependent (Fig. 7, AB). To confirm the effect of IL-2 on cytokine production, we used cocktails of anti-CD25 mAbs to inhibit IL-2 receptor signaling, and supplemented the culture with IL-7, which supported the survival of CD4\(^+\) T cells in the absence of IL-2 signals. Anti-CD25 mAbs completely inhibited the production of IFN-\(\gamma\) by CD4\(^+\) cells from PbA-infected mice in response to the supernatant of OT-II cells or during co-culture with OT-II/OVA\textsubscript{323-339}-pulsed DC, and this effect was not due to the inhibition of survival signal, since the viability of CD4\(^+\) cells was not impaired (Fig. 7B). Other
common γ-chain family cytokines such as IL-4, IL-7 and IL-15 did not have this effect (data not shown). Therefore, we concluded that IL-2 produced by OT-II cells was responsible for IFN-γ production by CD4+ cells from PbA-infected mice.

We examined the expression of the IL-2 receptor on CD4+ spleen cells. Purified CD4+ cell population contained minor population of NK1.1+TCRβ+ NKT cells (4.9%) and NK1.1+TCRβ+ conventional T cells (92.2 %)(Fig. 8A). The proportion of NKT cells was increased to 9.2% in CD4+ cells after infection with PbA. In uninfected mice, 8.5% and 5.6% of CD4+ T (CD4+NK1.1+) cells expressed IL-2 receptor α- and β-chains, respectively. These levels increased in PbA-infected mice to 24.0% and 17.3%, respectively. Similarly, the expression of IL-2 receptor α- and β-chains increased in CD4+ NKT (CD4+NK1.1+) cells from 16.5% to 28.8% and from 18.0% to 30.6% in PbA-infected mice, respectively. The majority of CD4+CD25+ cells in uninfected mice were foxp3+ regulatory T cells, and this population did not increase in PbA-infected mice, while the proportion of CD25+foxp3+CD4+ T cells increased by ~6.5 % (Fig. 8B). These CD4+ cells were cultured in the presence of IL-2 for 24 h and IFN-γ expression was examined by intracellular cytokine staining (Fig 8C). Despite their expression of IL-2 receptor, significant numbers of CD4+ T cells and NKT cells that produce IFN-γ were not detected in uninfected mice, indicating that CD4+foxp3+ cells did not produce IFN-γ in response to IL-2. In contract, 4.8% and 10.4% of CD4+ T and NKT cells from PbA-infected mice, respectively, produced IFN-γ in response to IL-2. Since the majority of CD4+ cells are NK1.1+ conventional T cells, these cells are the main population of CD4+ cells that produce IFN-γ in response to IL-2. Taken together, CD4+foxp3+ conventional T cells, which were activated and expressed IL-2 receptor during Plasmodium-infection, produced IFN-γ in response to IL-2. In addition, we were able to show that CD4+ NK T cells in PbA-infected mice also produced IFN-γ in response to IL-2.
Finally, we examined whether IFN-γ production by CD4⁺ T cells in response to TCR ligand, was dependent on IL-2 (Fig. 9). CD4⁺ T cells (CD4⁺NK1.1⁻ cells) were purified from mice before and after infection with PbA. IFN-γ production by these CD4⁺ T cells was examined in the presence and absence of anti-CD25 mAbs and IL-7, which supported the survival. CD4⁺ T cells from uninfected mice produced IFN-γ in response to anti-TCR mAb, although the level was lower than that of infected mice. This response was almost completely inhibited by anti-CD25 mAbs. CD4⁺ T cells from PbA-infected mice produced IFN-γ in response to PbA antigen, and this response was also inhibited by anti-CD25 mAbs. The inhibitory effects of anti-CD25 mAbs were not due to the inhibition of survival signal, as shown previously (Fig. 7B). In contrast, IFN-γ production of the same CD4⁺ T cells in response to anti-TCR mAb was not inhibited by anti-CD25 mAbs. Thus, CD4⁺ T cells from PbA-infected mice produced IFN-γ in IL-2-dependent in response to malaria antigens. However, these CD4⁺ T cells produced IFN-γ in IL-2-independent manner in response to anti-TCR mAb.
Discussion

CD4+ T cells of E/M phenotype produced high levels of IFN-γ, IL-4 and IL-10 in response to TCR occupancy during infection with PbA. Using adoptive transfer of OT-II cells, we unexpectedly found that CD4+ T cells from PbA-infected mice, not OT-II cells, produced high levels of IFN-γ in response to OVA323-339-pulsed DC. Further investigation revealed that CD4+ T cells from PbA-infected mice produced IFN-γ, IL-4 and IL-10 in response to IL-2 without TCR occupancy (Fig. 10). To our knowledge, this is the first report that IL-2 can stimulate the production of IFN-γ by conventional αβT cells. The IL-2 receptor is expressed on two types of CD4+ T cells, recently activated CD4+ cells and foxp3+ regulatory T cells. It is likely that CD4+ T cells that were activated and induced to express IL-2 receptor by malaria antigens during infection produced IFN-γ in response to IL-2 in vitro, since ligation of the IL-2 receptor expressed on CD4+foxp3+ T cells of uninfected mice did not induce IFN-γ production. IL-2 receptor expression in conventional CD4+ T cells, however, is not sufficient for IL-2-dependent IFN-γ production. CD4+CD25+foxp3+ T cells that were pre-activated in vitro with anti-TCR mAb to induce the expression of IL-2 receptor did not produce IFN-γ in response to IL-2, consistent with previous studies (data not shown) (33). It is intriguing that some cells produce IFN-γ in response to IL-2 and others do not. One possibility is that CD4+ T cells have different components of the IL-2 receptor α-, β- and γ-chains forming high and intermediate affinity receptors, in which some of the α-chains (CD25) are located in microdomains of the plasma membrane (34). Signaling events induced by IL-2 may vary depending on the structure of the IL-2 receptor as well as association with other components such as lipid rafts (35). Alternatively, the signaling module of CD4+ T cells may vary between types of T cells and may depend on the nature of stimulation. For example, IL-12 and IL-18 induce GADD45β, an activator of MEKK4, which leads to p38 MAPK activation (32). In combination with other signaling pathways, the activation of this GADD45β-MEKK4-p38 MAPK pathway leads to
IFN-γ production in CD4+ T cells without TCR engagement (30-32). Further study is required to investigate the expression of GADD45β or other signaling molecules in the IFN-γ activation pathway by CD4+ T cells from PbA-infected mice.

In addition to CD4+ T cells, we have shown that CD4+ NKT cells from PbA-infected mice also produced IFN-γ in response to IL-2. It was previously reported that IL-2 induces IFN-γ production by human NK cells, Vγ9Vδ2 T cells and NKT cells (33,36). In these studies, human cells were purified and stimulated in vitro with their appropriate ligands to induce expression of CD25. Unlike typical IL-2 signaling pathway, the addition of IL-2 activated not only Stat3 and Stat5 pathways, but also Stat4 leading to the induction of IFN-γ production in NK, Vγ9Vδ2 T and NKT cells. NK cells are important sources of IFN-γ during early response to Plasmodium infection (7). In human studies, it was shown that NK cells from malaria-naïve donors produced IFN-γ in response to P. falciparum-infected RBCs in vitro (37). Recently, it was shown that this NK response is dependent on IL-2 produced by CD4+ T cells (38). In this study, we have shown that mouse CD4+ T and NKT cells are able to produce IFN-γ in response to IL-2 under certain conditions. It will be intriguing to investigate whether CD4+ T cells from PbA-infected mice utilize signaling pathway similar to human NK, Vγ9Vδ2 T and NKT cells (33,36) in producing IFN-γ in response to IL-2.

We have also shown that production of IFN-γ by CD4+ T cells from the infected mice in response to PbA was almost completely inhibited by anti-CD25 mAbs, indicating that these PbA-specific T cells produced IFN-γ in an IL-2 dependent manner. The effect of anti-CD25 mAbs as not due to the inhibition of survival signal, since the culture was supplemented with IL-7, and we did not detect any significant differences between CD4+ T cells culture with and without anti-CD25 mAbs. Interestingly, CD4+ T cells showed differential dependencies on
IL-2 when stimulated with anti-TCR mAb. IFN-γ production by CD4+ T cells in response to anti-TCR mAb was IL-2 prior to PbA-infection, but was IL-2 dependent during infection. Thus, the IL-2-dependency of the IFN-γ response by CD4+ T cells varied depending on the source of T cells as well as the nature of the TCR ligand. It is possible that the requirement of IL-2 for IFN-γ production differs between T cell types; effector CD4+ T cells that were induced during the active infection produce IFN-γ in an IL-2 independent manner, while naïve or memory CD4+ T cells produce IFN-γ in an IL-2 dependent manner. Alternatively, the in vivo environment may influence the IL-2-dependency of CD4+ T cell response. During PbA-infection, innate immune responses are activated which produce a variety of cytokines. This inflammatory environment may compensate for the IL-2 requirement and condition the IFN-γ response of T cells.

IFN-γ plays important roles in regulating the protective immune response against blood-stage malaria infection and in the pathogenesis of cerebral malaria (3,39). The data presented here indicate that the level of IFN-γ production by CD4+ T cells is critically dependent on the level of IL-2 production during infection with malaria parasites. However, we and others have shown that the production of IL-2 in response to TCR occupancy was severely impaired in CD4+ T cells in mice infected with PbA and in patients with acute P. falciparum infection in Thailand (this study, 24,25). While molecular mechanisms underlying the reduction in IL-2 production are not clearly understood, regulation of the levels of IFN-γ production during severe malaria infection may be important to reduce the pathogenesis caused by excessive IFN-γ. Regulation of IFN-γ may also be beneficial for the parasite, since it can escape immune attack by activated immune effector mechanisms, including macrophages. In fact, it is reported that treatment of P. yoelii infected mice with exogenous IL-2 restored IFN-γ and other cytokine expression and was associated with diminution of parasitemia, although the survival rate did not improve in this
study (40). Finally, it is likely that this type of IL-2-dependency of IFN-γ production is also induced during infection with *P. falciparum*, the human malaria parasite, as well as in other infectious diseases or immune disorders. Increased understanding of the molecular mechanisms underlying regulation of the cytokine network during infection should suggest methods to prevent the pathogenesis of malaria infection as well as other immune disorders and also enhance the protective immune response.
Abbreviations:

PbA, *Plasmodium berghei* ANKA;

OVA-PbA, *Plasmodium berghei* ANKA that expresses OVA;

WT-PbA, Wild type *Plasmodium berghei* ANKA;

SEB, streptococcal enterotoxin B;

E/M, effector/memory
Funding

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Figure legends

Figure 1

CD4⁺ T cells from PbA-infected mice produce high levels of IFN-γ, IL-4 and IL-10 in response to TCR stimulation.

(A) CD4⁺ T cells from mice not infected or infected with PbA for 7 days (parasitemia 6.4-7.8 %) were cultured in the presence of DC (1x10⁴) pulsed with SEB or on plates coated with anti-TCR mAb. The levels of IL-2, IFN-γ, IL-4, IL-10, and TGF-β in the supernatant were determined by ELISA. A significant difference was observed for all parameters between uninfected and infected mice (p < 0.005). Representative data of 2 similar results are shown.

(B) CD4⁺ T cells from mice not infected or infected with PbA for 6 days (parasitemia 5.1-8.0 %) were stimulated with plate-coated anti-TCR mAb for 12 h and the expression of IL-2, IFN-γ, IL-4, and IL-10 was determined by intracellular cytokine staining. Numbers in each quadrant represent percentages of cells.

(C) CD4⁺ T cells (5x10⁵/well) from mice not infected or infected with PbA for 7 days (parasitemia 3.7-5.6 %) were stimulated with plate-coated anti-TCR mAb for 0-48 hrs. The levels of mRNA in uninfected (○) and PbA-infected mice (●) were determined by real-time RT-PCR. Representative data of 2 similar experiments are shown.
Figure 2

Correlation between parasitemia levels and production of IFN-γ by CD4+ T-cells.

(A) Mice (3 mice/group) were infected with PbA and the level of parasitemia (▲) was determined. CD4+ T cells prepared from each mouse were stimulated with plate coated anti-TCR mAb and IFN-γ levels in the supernatant were determined by ELISA (●). Values shown are the means ± SD from each mouse.

(B) After 7 days of infection (parasitemia 7.4-8.8%), mice were treated daily with chloroquine for 6 days. The levels of parasitemia (▲) and IFN-γ production of CD4+ T cells (●) were determined. Representative data of 2 similar results are shown.

(C) Naïve (CD62L^{high}CD44^{low}) and effector/memory (CD62L^{low}CD44^{high}) CD4+ T cells were purified from uninfected (open bar) and infected for 7 days (parasitemia 8.6%) (closed bar) B6 mice by cell sorting, and were stimulated on plates coated with anti-TCR mAb. The levels of IFN-γ in the supernatant were determined by ELISA. A significant difference in the levels of IFN-γ was observed between uninfected and infected mice for whole and effector/memory cells (p < 0.05), while no difference was observed for naïve cells. Either for uninfected or infected mice, the levels of IFN-γ was significantly higher in whole and effector/memory cells than in naïve cells, while no significant difference was observed in the levels of IFN-γ between whole and effector/memory cells. Representative data of 3 similar results are shown.
Figure 3

Proliferation and cytokine production of CD4+ T cells in mice after infection with OVA-PbA. B6.Ly5.1 (CD45.1+) (A) or B6 (B-D) mice were adoptively transferred with CFSE-labeled OT-II (A, CD45.2+, 1x10^7) or CD45.1 OT-II cells (B-D, 3x10^7), respectively, and were uninfected or infected with WT- or OVA-PbA.

(A) Seven days later (parasitemia 13.6%-15.0%), spleen cells were analyzed using flow cytometry. The numbers in the right upper corner represent the proportions of OT-II (CD45.2+CD4+) cells. Representative data of 4 similar results are shown.

(B) Seven days after adoptive transfer of CD45.1 OT-II cells (parasitemia 3.8-4.2%), spleen cells were stained with allophycocyanin-anti-CD4, PE-Cy7-anti-CD45.1, PE-anti-CD69, FITC-anti-CD25, PE-anti-CD62L and FITC-anti-CD44 mAbs. FACS profiles of OT-II (CD45.1+CD4+) and host B6 (CD45.1-CD4+) -gated populations are shown. The number in each quadrant represents the proportion of each population.

(C) CD4+ T cells were stimulated with dendritic cells pulsed with OVA323-339. The proportion of OT-II in total CD4+ T cells was 3.7-5.9%. The levels of IFN-γ were significantly higher both in WT-PbA-infected mice and in OVA-PbA-infected mice than in uninfected mice (p < 0.05). Representative data of 6 similar results are shown.

(D) After 8 days of infection (parasitemia 9.0%), CD4+ T cells were purified, stimulated with DC pulsed with OVA323-339 for 24h, and the production of IFN-γ was analyzed by intracellular cytokine staining of CD45.1+CD4+ (OT-II cells, left panels) and CD45.2+CD4+ (B6 host cells, right panels) gated populations. Representative data of 3 similar results are shown.
Figure 4

Cooperation of CD4⁺ T cells from PbA-infected mice and OT-II cells in producing IFN-γ.

B6 mice were adoptively transferred with CD45.1 Rag-2⁻/⁻ OT-II cells (1x10⁷) and were infected or not infected with OVA-PbA. After 7 days (parasitemia 6.7%), OT-II and host B6 CD4⁺ T cells were purified by cell sorting from uninfected (open bar) and infected (closed bar) mice, and were cultured in the presence of DC (1x10⁴) pulsed with OVA₃₂₃-₃₃₉. The numbers of OT-II (2x10⁴) and host CD4⁺ T cells (1.8x10⁵) in culture were determined to reflect the original in vivo ratio of these cells. Addition of OT-II cells from uninfected or infected mice showed a significantly larger effects in increasing IFN-γ levels in infected B6 cells than in uninfected B6 cells (p < 0.05). Representative data of 3 similar results are shown.
**Figure 5**

CD4⁺ T cells from PbA-infected mice produce IFN-γ in response to a soluble factor produced by OT-II / OVA323-339-pulsed DC.

(A) CD4⁺ T cells from uninfected (open bar) or PbA-infected for 8 days (parasitemia 5.8-8.8 %, closed bar) mice or no cells (gray bar) were placed in the upper or lower chamber of a Transwell plate, and OT-II cells were placed in the lower chamber in the presence and absence of DC pulsed with or without OVA323-339. In the group with OT-II/OVA-DC in lower well, the levels of IFN-γ in CD4⁺ T cells were significantly higher in those from infected mice than in those from uninfected mice irrespective of whether T cells were in upper well or lower well (p < 0.05). Representative data of 3 similar results are shown.

(B) CD4⁺ T cells from uninfected (open bar) or infected for 6 days (parasitemia 4.0-5.7 %, closed bar) mice or no cells (gray bar) were cultured in the presence (OT-II/OVA-DC) and absence (-) of OT-II cells and DC pulsed with OVA323-339, or in the presence of supernatant from OT-II cells stimulated with OVA323-339-pulsed DC (SUP). Either in group with OT-II/OVA-DC or with OVA-DC, the levels of IFN-γ were significantly higher in CD4⁺ T cells from infected mice than in those from uninfected mice (p < 0.05). Representative data of 4 similar results are shown.
Figure 6

The soluble mediator that stimulates IFN-γ production of CD4+ T cells from PbA-infected mice is not IL-12 or IL-18.

(A, B, left) CD4+ T cells from the infected for 7 days (parasitemia 5.2-7.7%) mice were cultured in the presence of varying concentrations of recombinant IL-12 or IL-18 with (open bar) and without (closed bar) anti-IL-12 mAb or anti-IL-18 mAb, respectively. In CD4+ T cells from the infected mice, IFN-γ was produced significantly depending on the levels of rIL-12 ($p < 0.05$), while the production was blocked by anti-IL-12 mAb. Although a similar dose-response was suggested for rIL-18, the level of IFN-γ at 1000 (ng/ml) was significantly higher ($p < 0.05$) compared to that at 100 or 0 (ng/ml). (A, right) CD4+ T-cells from uninfected or infected mice were cultured in the presence and absence of supernatant of OT-II/OVA323-339-pulsed DC (SUP) with (open bar) and without (closed bar) anti-IL-12 mAb. In the presence of SUP, no significant difference was observed in the levels of IFN-γ between CD4+ T-cells with or without anti-IL-12 mAb. Representative data of 2 similar results are shown. (B, right) CD4+ T-cells from uninfected or infected B6 or MyD88−/−TRIF−/− double knock-out mice were cultured in the presence and absence of SUP with (open bar) and without (closed bar) anti-IL-18 mAb. In both of CD4+ T cells from B6 and DKO mice, the levels of IFN-γ significantly increased in the presence of SUP irrespective of whether the mice were infected or uninfected and of with or without anti-IL-18 ($p < 0.05$). However, no significant decrease with anti-IL-18 mAb was observed in the levels of IFN-γ. (C) CD4+ T-cells from uninfected and infected for 7 days (parasitemia 7.6%) B6 mice were cultured in the presence (+) and absence (-) of SUP with (closed bar) and without (open bar) anti-IL-12, anti-IL-18 mAb or both. In CD4+ T cells either from uninfected or infected mice, the levels of IFN-γ significantly increased with SUP ($p < 0.05$). However, single or simultaneous effects of anti-IL-12 and anti-IL-18 mAbs in blocking the increase were not significant. Representative data of 2 similar results are shown.
Figure 7

IL-2 stimulates CD4⁺ T cells from PbA-infected mice to produce IFN-γ, IL-4 and IL-10.

(A) CD4⁺ T cells from infected (8 days, parasitemia 9.8 %, closed bar) and uninfected (open bar) mice were stimulated with the supernatant from the OT-II/OVA323-339-pulsed DC (SUP), recombinant IL-2 (rIL-2) or co-cultured with OT-II and OVA323-339-pulsed DC (OT-II/OVA-DC) in the presence (+) and absence (-) of anti-CD25 mAbs. In CD4⁺ T cells from PbA-infected mice, addition of SUP, rIL-2 or OT-II/OVA-DC significantly increased the levels of IFN-γ, IL-4 and IL-10 (p < 0.05), and such an increase was significantly blocked by anti-CD25 mAbs (p < 0.05). Representative data of 3 similar results are shown.

(B) CD4⁺ T cells from infected (7 days, parasitemia 6.6%, closed bar) and uninfected (open bar) mice were cultured in the presence of varying concentrations of recombinant IL-2. The levels of IFN-γ in the culture supernatant were determined by ELISA. In CD4⁺ T cells from infected mice, the levels of IFN-γ significantly increased with IL-2 equal to or exceeding 1.25 ng/ml (p < 0.05).

(C) CD4⁺ T cells from the infected (7 days, parasitemia 5.2-7.7%) mice were cultured with (gray bar) and without (closed bar) anti-CD25 mAbs for 24-48 h. The proportions of viable (Annexin V⁻/7AAD⁻) cells were determined by staining with Annexin V and 7AAD. The levels of IFN-γ in the culture supernatant were determined by ELISA. At each time point, no significant difference was observed in the frequency of viable cells between cells with and without anti-CD25 mAbs for either with or without rIL-2. Representative data of 4 similar results are shown.
Figure 8

CD4+ T cells from PbA-infected mice express high levels of IL-2 receptor.

(A) Spleen cells from B6 mice uninfected or infected for 8 days with PbA (parasitemia 6.7-13.1%) were stained with PE-Cy7-anti-CD4, allophycocyanin-streptavidin plus biotin-anti-NK1.1, FITC-anti-TCRβ and PE-anti-CD25 or PE-anti-CD122 mAbs. The expression of CD25 and CD122 are shown for CD4+NK1.1+TCR+ and CD4+NK1.1+TCR+ gated populations.

(B) Spleen cells from B6 mice uninfected or infected for 7 days with PbA (parasitemia 4.4-4.9%) were stained with PE-Cy7-anti-CD4, FITC-anti-CD25, FITC-streptavidin plus biotin-anti-CD122, and with PE-anti-Foxp3 mAbs, and the profiles of CD4+ gated populations are shown (Left). The number represents the proportion of cells in each region within total CD4+ cells from uninfected (open bar) and PbA-infected (closed bar) mice. The experiments were repeated 5 times and the mean ± SD of each subpopulation within CD4+ cells are shown (Right). The proportions of Fox3’CD25+ cells and Fox3’CD122+ cells both significantly increased in infected mice than in uninfected mice.

(C) CD4+ cells from B6 mice infected or uninfected with PbA were stimulated with IL-2 for 24 hrs, stained with PE-Cy7-anti-CD4, allophycocyanin-streptavidin plus biotin-anti-NK1.1, FITC-anti-TCRβ, and with PE-anti-IFN-γ mAb. Numbers in the right quadrant represent the proportion of IFN-γ producing cells in total CD4+ cells for CD4+NK1.1+TCR+ and CD4+NK1.1+TCR+ gated populations (Left). Representative data of 4 similar results are shown. The number of IFN-γ producing cells in each spleen was calculated from 4 mice in each group (Right). The number of IFN-γ producing cells was significantly larger in CD4+NK1.1+TCR+ gated population than in CD4+NK1.1+TCR+ gated population (p < 0.001).
**Figure 9**

IFN-γ production of CD4⁺ T cells from PbA-infected mice in response to malaria antigen is IL-2-dependent.

CD4⁺NK1.1⁻ T cells from mice uninfected or infected for 7 days (parasitemia 10.6 %) were cultured in the absence (-) and presence of IL-2 (rIL-2), DC (DC), DC pulsed with soluble malaria antigen (PbA/DC) or anti-TCR mAb (αTCR) with (open bar) and without (closed bar) anti-CD25 mAbs (A). In T cells from uninfected mice, the level of IFN-γ was significantly lower for PbA/DC and anti-TCR mAb in those with anti-CD25 mAbs than in those without anti-CD25 mAbs ($p < 0.05$). On the other hand, in T cells from infected mice, although the level of IFN-γ was significantly lower for rIL-2 and PbA/DC in those with anti-CD25 mAbs than in those without anti-CD25 mAbs ($p < 0.05$), no such significant difference was observed for anti-TCR mAb. Representative data of 3 similar results are shown.

**Figure 10**

A model of the production of IFN-γ, IL-4 and IL-10 by CD4⁺ T cells from PbA-infected mice.

CD4⁺ T cells from B6 mice that were transferred with OT-II cells and were infected with PbA produced IFN-γ, IL-4 and IL-10 in response to DC pulsed with OVA *in vitro*. 
Figure 1

A

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<td>IL-10 (ng/ml)</td>
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B

Uninfected | Infected
---|---
IL-2 | 0.2 | 0.8
IFN-γ | 0.2 | 0.3
IL-4 | 0.4 | 1.1
IL-10 | 0.6 | 6.8
IFN-γ | 0.6 | 7.1
T-bet |

C

IFN-γ | T-bet
---|---
Relative mRNA

IL-4 | GATA3
---|---
Relative mRNA

IL-10
---
Relative mRNA
Figure 2

A

IFN-γ (ng/ml)

Parasitemia (%)

Days after infection

B

IFN-γ (ng/ml)

Parasitemia (%)

Duration of treatment

C

Uninfected

Naive

Effector / Memory

Infected

CD62L

CD44

IFN-γ (ng/ml)

Whole

Naive

Effector / Memory
Figure 3

A

OT-II Tg (CD45.2^+^)

↓

OT-II CD4^+^ T cells

↓

B6.Ly5.1 (CD45.1^+^)

~7 days

Uninfected

WT-PbA

OVA-PbA

B

Uninfected

WT-PbA

OVA-PbA

OT-II

B6 host

OT-II

B6 host

C

IFN-γ (ng/ml)

0 20 40 60 80 100

0.3 0.4 2.1

D

CD4

IFN-γ

0.8 0.2 1.0 4.4

CD8

CD25

CD44
Figure 6

A

B

C
Figure 7

A

B

C

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IFN-γ (ng/ml)

IL-4 (pg/ml)

IL-10 (pg/ml)

rIL-2 (ng/ml)
Figure 8

(A) Uninfected vs. Infected NK1.1+ and CD122+ cells.

(B) Proportions in CD4+ cells (%).

(C) Number of IFN-γ+ cells (x 10^5).
Figure 9

Uninfected

Infected

IFN-γ (ng/ml)

-  rIL-2  -/DC  PbΔ/ DC  αTCR

Figure 10

Dendritic cells

MHC II

TCR

OVAp223-339

OT-II cells

IL-2

Infected B6 host CD4+ T cells

IL-10

IL-4

IL-2R

IFN-γ