Immune Responses in Resistant and Susceptible Strains of CD4-Mutant Mice Infected with *Leishmania major*

Kazuo Yamakami,*,[a] Takashi Oda,[a] Naoki Watanabe,[c] Akio Matsuzawa,[d] Hideo Nariuchi,[e] Takushi Tadakuma,[b] and Nobuyuki Yoshizawa[a]

[a]Department of Public Health and [b]Department of Parasitology and Immunology, National Defense Medical College, 3–2 Namiki, Tokorozawa, Saitama 359–8513, Japan, [c]Department of Tropical Medicine, Jikei University School of Medicine, 3–25–8 Nishi-simbashi, Minato-ku, Tokyo 105–8461, Japan, and [d]Laboratory Animal Research Center and [e]Department of Allergology, Institute of Medical Science, University of Tokyo, 4–6–1 Shirokanedai, Minato-ku, Tokyo 108–8639, Japan

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We examined the immune response of CD4-mutant mice that have soluble form of CD4 in the circulation without expression of CD4 on T cell surface. We infected the CD4-mutant mice of BALB/c and C57BL/6 backgrounds with *Leishmania major* and subsequently examined parameters of disease and immune response. In contrast to wild-type (wt) BALB/c mice, mutant mice of both strains showed decreased parasite replication and an intense leishmanial antigen-specific delayed-type hypersensitivity (DTH) response. *In vitro* cytokine analysis revealed that popliteal lymph node cells (LNC) from mutant mice of both strains secreted little or no detectable interleukin-4 (IL-4) and they secreted interferon-γ (IFN-γ) at levels similar to those of wt mice. The cytokine profile shows that a lack of IL-4 production underlies the lack of T helper type 2 (Th2) response. As validation of the impaired Th2 response, infection of the mice with *Nippostrongylus brasiliensis*, a typical Th2 inducer, showed a lack of Th2 response. Furthermore, in contrast to LNC from wt mice, Th2 cells were not induced from naive LNC of mutant mice when the cells were cultured in the presence of IL-4 and anti-IL-12 antibody. These findings indicate that the lack of IL-4 production is due to a lack of CD4 on T cells and that IFN-γ production is independent of CD4 on T cells. Thus, Th1 and Th2 responses to leishmanial infection are not due to the balance of IL-4/IFN-γ that are produced, but the production of IL-4 determines whether a Th1 or Th2 response will develop.

Key words —— CD4, mutation, *Leishmania major*, cytokine, T cell, mice

INTRODUCTION

Cutaneous leishmaniasis is one of the most widespread infectious diseases and it is prevalent in many tropical and subtropical regions of the world, where it is transmitted *via* the bite of sand fly. The leishmanial disease is caused by obligate intracellular protozoa *Leishmania major* (*L. major*) and is characterized by cutaneous lesions that can be self-resolving with life-long immunity or chronic when accompanied by defective cellular immune responses.[1] Elucidation of the infectious mechanisms underlying this disease will aid in the development of strategies for preventive medicine. In this respect, clarification of the function of CD4 molecule in response to *L. major* infection will be important.

The genetic basis of resistance and susceptibility to *L. major* infection are provided in a concept of development of T helper type 1 (Th1) and T helper type 2 (Th2) responses, respectively.[2] A resistant mouse strain, C57BL/6, develops a Th1 response with high interferon-γ (IFN-γ) production, low interleukin-4 (IL-4) production, and protective responses, whereas a susceptible mouse strain, BALB/c, suffers a Th2 response with high IL-4 and low IFN-γ production, resulting in exacerbation of the disease.[3] These diverse responses are coupled with the findings that $CD4^+$ T cells play a role in the response to *L. major* infection.[4,5]

The role of CD4 molecule in the immune response has been substantiated *in vivo* in a *cd4* gene-deficient mouse model of *L. major* infection.[6] However, the genetic absence of CD4 did not alter the

*To whom correspondence should be addressed: Department of Public Health, National Defense Medical College, 3–2 Namiki, Tokorozawa, Saitama 359–8513, Japan. Tel.: +81-4-2995-1575; Fax: +81-4-2996-5196; E-mail: yamakami@cc.ndmc.ac.jp

innate response in a resistant mouse strain.[7] A deficient in surface CD4 on T cells was first identified in the C57BR/cdJ strain, in which no $CD4^+$ cell but soluble CD4 in the circulation was detected.[8] Mutation of the junctional sequence between exon VIII and intron VIII of the *cd4* gene results in loss of the entire transmembrane domain and formation of a soluble form of CD4. The mice, termed as CD4-mutant mice, demonstrate an impaired delayed-type hypersensitivity (DTH) and antibody production against T-dependent antigen.[8] However, mutant mice express normal numbers of $CD8^+$ T cells and produce antibodies against T-independent antigens.[8] No mouse models of a similar phenotype have been studied with respect to the response to *L. major* infection, with the exception of induced mutants lacking expression of CD4.[9] The present study was designed to analyze the immune response of CD4-mutant mice of BALB/c and C57BL/6 backgrounds. We found that CD4-mutant mice challenged with *L. major* were resistant to infection even though the mice on BALB/c background. Furthermore, the response was coupled with a lack of IL-4 production and an intense DTH response.

MATERIALS AND METHODS

Mice and Parasites —— A mutant *cd4* gene, characterized by the production of soluble but not of membrane-bound CD4 has been reported in C57BR/cdJ mice.[8] This mutant gene was introduced into BALB/c and C57BL/6 mice (CLEA Japan, Inc., Tokyo, Japan) by 5 backcrossings and the use of intermediate expression of CD4 on lymphocytes as a selection marker.[8,10] *cd4* gene-deficient mice of C57BL/6 background, termed CD4-knockout mice, were obtained from The Jackson Laboratory (Bar Harbor, ME, U.S.A.).

Leishmanial infections were induced with stationary-phase promastigotes of *L. major* (MHOM/SU73/5HSKH) grown at 27°C in Schneider medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 20% heat-inactivated fetal calf serum (FCS) (HyClone Laboratories, Inc., Logan, UT, U.S.A.). Female mice of designated groups (6 weeks of age, $n = 6$) were injected into the left hind footpad with 1×10^6 stationary-phase promastigotes, and the course of the disease was monitored by measurement of footpad thickness with a dial-gauge caliper. The mice were maintained under pathogen-free conditions. Soluble leishmanial antigen (SLA) was prepared from the promastigotes by 4 freeze-thaw cycles in phosphate-buffered saline followed by centrifugation at $20000 \times g$ for 10 min. The antigen was passed through a 0.2-μm filter and stored at –80°C until use.[11]

Infective third-stage larvae of *Nippostrongylus brasiliensis* (*N. brasiliensis*) were isolated from experimentally infected rats, and 500 worms/mouse were injected subcutaneously into designated groups of mice (6 weeks of age, $n = 5$). Serum IgE levels were quantified at 15 days post-infection by enzyme-linked immunosorbent assay (ELISA).

Quantitative Parasite Cultures —— Viable *L. major* in infected tissues was quantified by limiting-dilution assay. Draining lymph nodes obtained from individual mice were suspended at 4×10^5 cells/0.2 ml Schneider medium with supplements, and serial 5-fold dilutions of the cell suspensions were plated in flat-bottomed 96-well plates in triplicate and maintained at 27°C for 7 days. Wells containing motile parasites were identified by microscopy. The data reported are the geometric means and standard errors of dilution factors of the last positive well multiplied by the numbers of original lymph node cells (LNC).

***In Vitro* Stimulation of LNC** —— Popliteal LNC were prepared from individual mice 12 weeks after infection with *L. major*. Syngenic spleen cells γ-irradiated at 2000 rad were used as antigen-presenting cells (APC). LNC (2×10^6 cells) from individual mice and APC (6×10^6 cells) were dispensed in triplicate onto 24-well plates and cultured in 1 ml of RPMI-1640 with 10% FCS in the presence of 50 μg/ml SLA. Seventy-two hr after culture at 37°C in 5% CO_2, the supernatants were collected and assayed for cytokines. The levels of IFN-γ and IL-4 in the supernatants were quantified with a commercial ELISA kit (Endogen, Rockford, IL, U.S.A.).

***In Vitro* Th1/Th2 Cell Differentiation** —— LNC from CD4-mutant mice on BALB/c background and wild-type (wt) mice (6 weeks of age, $n = 3$) were cultured at 10^6 cells/well in RPMI-1640 supplemented with 10% FCS on 24-well plates precoated with anti-CD3 mAb (2C11, 1 μg/ml). Th1 development was driven by IL-12 (2 ng/ml; PeproTech, Inc., London, U.K.) and anti-IL-4 mAb (11B11, 0.2 μg/ml), and Th2 development was driven by IL-4 (200 units/ml, PeproTech, Inc.) and anti-IL12 mAb (C17.8.20, 1 μg/ml). After 72 hr, cells were washed, transferred to uncoated wells, and cultured for 7 days without stimulation. After driving of Th1 or Th2 development 3 times, designated groups of cells were

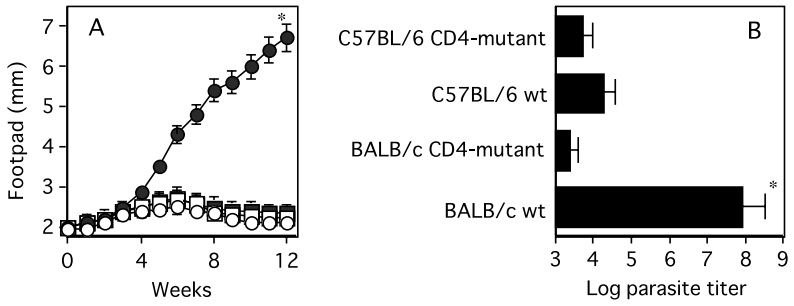

Fig. 1. Resistance to *L. major* Infection in CD4-Mutant Mice of BALB/c and C57BL/6 Strains

(A) Groups of mice were infected with *L. major* in the left hind footpad, and the disease was monitored by measurement of footpad swelling. The mice included C57BL/6 CD4-mutant mice (open squares), BALB/c CD4-mutant mice (open circles), wt C57BL/6 mice (closed squares), wt BALB/c mice (closed circles). Footpad thickness is represented as the mean value ± SEM of 6 mice per group. $*p < 0.01$ for wt BALB/c mice versus CD4-mutant mice by Dunnett's *t*-test. (B) A limiting dilution assay was performed 12 weeks post-infection on cells isolated from popliteal lymph nodes and cultured for 7 days at serial 5-fold dilutions. The numbers of viable parasites were determined from the well with the highest dilution. The bar represents the average score ± SEM of 6 mice per group. $*p < 0.01$ for wt BALB/c mice versus CD4-mutant mice.

transferred to wells precoated with anti-CD3 mAb. Cell-free supernatants were collected after 72 hr of culture. Levels of IFN-γ and IL-4 in the supernatants were quantified by ELISA.

Assay of Delayed-Type Hypersensitivity Response —— DTH response was assessed by injecting 50 μg of SLA into the right hind footpad of mice at 10 weeks after infection. The increase in footpad thickness was measured for up to 72 hr at 24-hr intervals with a dial-gauge caliper.[12)]

Statistical Analysis —— All values are expressed as the mean ± SEM for each group. Dunnett's *t*-test was used to compare means with the use of the General Linear Models mode in a Statistical Analysis System (SAS Institute Inc., Cary, NC, U.S.A.). Reported *p*-values are two-sided, and *p*-values of less than 0.01 were regarded as statistically significant.

RESULTS

CD4-Mutant Mice on BALB/c and C57BL/6 Backgrounds were Resistant to *L. major* Infection

CD4-mutant mice on C57BL/6 and BALB/c backgrounds and respective wt mice were infected with *L. major*, and footpad swelling was measured as an assessment of susceptibility (Fig. 1A). Mutant mice of both strains showed mild footpad swelling, with a peak at week 6 and a decrease thereafter, as was shown in wt C57BL/6 mice. However, wt BALB/c mice showed a high degree of footpad swelling that persisted until termination of the experiments. Further experiments were performed to determine whether CD4-mutant mice were resistant to infection as assessed by viability of *L. major* (Fig. 1B). A limiting dilution assay of popliteal LNC from mutant mice of BALB/c strain showed a 10^4-fold reduction in the parasite burden compared to its wt mice. However, mutant and wt mice of C57BL/6 strain showed a comparable level of parasite burden. Thus, CD4-mutant mice of both strains were resistant to infection with *L. major* as assessed by suppression of footpad swelling as well as marked parasite clearance.

CD4-Mutant Mice Showed Impaired Th2 Response to *L. major* Infection

Given the resistance of CD4-mutant mice on BALB/c background to *L. major* infection, we analyzed cytokine production in LNC *in vitro*. LNC isolated from draining lymph nodes were cultured and restimulated with SLA, and production of IL-4 and IFN-γ was quantified for assessment of the Th1/2 response (Fig. 2). LNC from wt BALB/c mice predominantly showed production of IL-4, and LNC from wt C57BL/6 mice showed high levels of IFN-γ. LNC from CD4-mutant mice of both strains expressed IFN-γ at levels similar to those of the respective wt mice and produced little or no IL-4. Thus, CD4 mutation did not alter the ability of C57BL/6 mice to mount a Th1 response. However, the cytokine profiles of CD4-mutant BALB/c mice indicated an impaired Th2 response.

CD4-Mutant Mice Previously Infected with *L. major* Showed an Intense Delayed-Type Hypersensitivity Response

Th1 and Th2 immune responses correlate with the soluble leishmanial antigen-specific DTH response in infected mice. We studied whether the

control of *L. major* infection in mutant mice was associated with the development of DTH response to *L. major*. CD4-mutant mice of both strains showed increased footpad swelling compared to wt BALB/c mice (Fig. 3). The intensity of the DTH response in mutant mice was almost identical to that of wt C57BL/6 mice. These findings were consistent with the results of resistance to leishmanial infection and IFN-γdominant profile in cytokine assay. Thus, challenge of CD4-mutant mice with *L. major* results in an intense DTH response in a $CD4^+$ T cell-independent manner.

CD4-Mutant Mice Showed an Impaired Th2 Response *in Vivo*

CD4-knockout mice infected with *L. major* are incapable of Th2 development.[7] To study the ability of CD4-mutant mice to mount a Th2 response, CD4-mutant C57BL/6 mice were infected with *N. brasiliensis*, a potent inducer of theTh2 response.[6] In this experiment, we used the C57BL/6 strain, because this strain shows a Th1 response but no Th2 response to infection with *L. major*. Wild-type C57BL/6 mice showed robust Th2 responses 15 days after infection with marked elevations in serum IgE, as the typical indicator in model of *N. brasiliensis* infection (Fig. 4). However, CD4-mutant C57BL/6 mice as well as CD4-knockout C57BL/6 mice showed markedly abrogated IgE responses. Thus, mice that do not express CD4 on T cells were incapable of mounting a Th2 response to infection with *N. brasiliensis*.

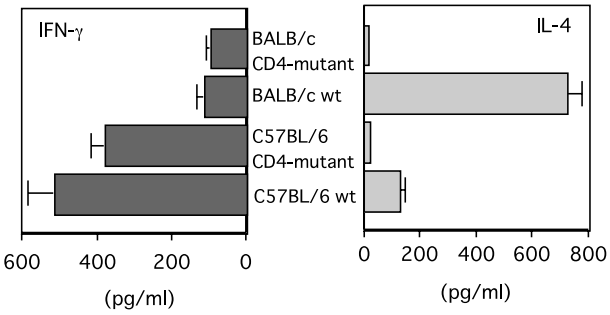

Fig. 2. An Impaired IL-4 Response to *L. major* Infection in CD4-Mutant Mice of C57BL/6 and BALB/c Strains

Single-cell suspensions from the popliteal lymph nodes of individual mice in the designated groups were prepared 12 weeks after infection. Cells in triplicate were cultured for 72 hr in the presence of soluble leishmanial antigen and γ-irradiated splenocytes from syngenic mice as antigen-presenting cells. Supernatants were assayed for IFN-$\gamma$and IL-4 by ELISA. Each bar represents the mean ± SEM for 6 mice per group.

LNC from CD4-Mutant Mice Showed Impaired Th2 Differentiation *in Vitro*

IL-4 induces differentiation of naive Th cells to Th2 cells *in vitro*, whereas Th1 development is driven by IL-12. Because BALB/c CD4-mutant mice showed an impaired Th2 response to *L. major* infection, the ability of cultured LNC to differentiate to the Th1 or Th2 phenotype was investigated. LNC from BALB/c CD4-mutant and wt mice were cultured with anti-CD3 mAb and induced to the Th1 or Th2 phenotype. Before induction, the LNC produced little or no IFN-γ or IL-4. LNC from wt BALB/c mice showed Th1 and Th2 differentiation as indicated by the production of high levels of IFN-γ and IL-4, respectively (Fig. 5). LNC from CD4-mutant mice showed a Th1 phenotype as assessed by IFN-γ production. In contrast, LNC from CD4-mutant mice showed impaired differentiation to the Th2 phenotype as determined by lack of IL-4 production. Thus,

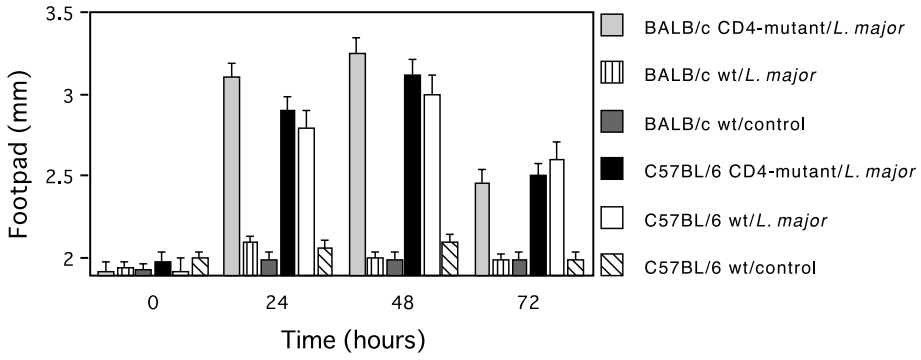

Fig. 3. An Intense Delayed-Type Hypersensitivity Response to *L. major* Infection in CD4-Mutant Mice of C57BL/6 and BALB/c Strains

The groups of mice were injected with soluble leishmanial antigen into the right hind footpad 10 weeks after being infected with *L. major*, and the increase in footpad size was measured at 24-hr intervals. Each bar represents the mean ± SEM for 6 mice per group.

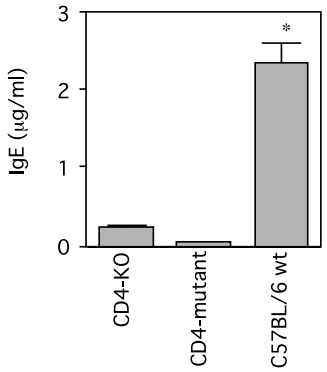

Fig. 4. An Impaired Th2 Response as Assessed by IgE Production in CD4-Mutant Mice Previously Infected with *N. brasiliensis*

C57BL/6 CD4-mutant mice, CD4-knockout (KO) mice, and wt controls were infected with *N. brasiliensis*, and serum IgE levels of each mouse were assayed at 15 days post-infection. Each bar represents mean ± SEM of 5 mice per group. *Significantly lower compared to wt control mice ($p < 0.01$).

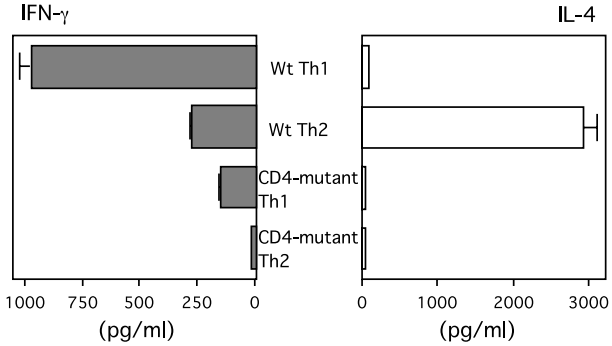

Fig. 5. An Impaired *in Vitro* Th2 Differentiation of Lymph Node Cells from CD4-Mutant Mice

Naive LNC from BALB/c CD4-mutant or wt mice were cultured with anti-CD3 mAb. T helper development was promoted by a combination of IL-12 and anti-IL-4 mAb to generate Th1 cells, or by IL-4 in the presence of anti-IL-12 mAb to generate Th2 cells. The induction protocols were performed 3 times. Respective cells plated on anti-CD3 mAb-coated wells without additional stimuli were cultured for 72 hr, and IFN-γand IL-4 levels in the supernatants were determined. Each bar represents mean ± SEM from triplicate cultures.

CD4-mutant mice were incapable of Th2 differentiation.

DISCUSSION

We studied whether lack of CD4 on T cells but presence of CD4 in circulation affects the ability of mice to mount an altered immune response to *L. major* infection. CD4-mutant mice of BALB/c strain, which are originally susceptible to the infection, showed resistance to *L. major* infection. In contrast, resistant to the infection was observed both in CD4-mutant and in wt mice on C57BL/6 background. Thus, CD4 affects to drive Th2 development in *L. major*-infected mice. Similar conclusions were drawn in previous studies that showed an impaired Th2 response to *L. major* in CD4-knockout C57BL/6 mice.[6,13] In addition, blocking of CD4 in BALB/c mice with anti-CD4 mAb resulted in loss of the Th2 response as assessed by inability of IL-4 production.[14] Similarly, blocking of IL-4 in BALB/c mice with anti-IL-4 mAb reversed the response to resistance to *L. major*.[15,16] These reports support our finding that lack of CD4 on T cells is associated with inability of IL-4 production. We found that challenge of CD4-mutant BALB/c mice with *L. major* did not upregulate the production of antigen-specific IgG1, despite the upregulation of IgG1 in wt mice (data not shown). The inability of mutant mice to mount a Th2 response was supported by the resistance to *N. brasiliensis* infection and *in vitro* experiment of inability of naive LNC to Th2 differentiation.

With respect to the immune response of CD4-mutant mice to *L. major*, the question arises as to the mechanism responsible for $CD4^+$ T cell-independent DTH given the expression of the soluble form of CD4. We observed that infection of C57BL/6 CD4-knockout mice with *L. major* also showed an intense DTH response as has shown in response of mutant mice (data not shown). Thus, on the DTH response to *L. major* infection, the effect of soluble CD4 can be ruled out. In a previous report, an absence of DTH response was observed in the mutant mice immunized with ovalbumin. However, injection of anti-CD4 mAb restored DTH response, suggesting that the soluble CD4 is responsible for suppression.[17] Similarly, soluble CD4 prevented a host resistance DTH response to infection with *Cryptococcus neoformans* in mutant mice.[18] Thus, soluble CD4 suppresses DTH response in these experimental models. In contrast, in our infection model of mutant mice, a $CD4^+$ T cell-independent DTH response was observed, regardless of the presence of soluble CD4. This difference might be due to the persistence of chronic stimulation with *L. major*. In any case, our findings imply that combination factors of characteristics of antigens and microbes used as well as host's backgrounds affect the establishment of DTH response. It is possible that there is an intrinsic bias of non-$CD4^+$ T cells toward DTH.[19] The response of CD4-mutant mice to leishmanial infection suggests the induction of a compensatory factor that can

replace the essential function of CD4.[20,21] However, a typical factor for resistance to leishmanial infection, such as upregulation of iNOS or NK activity, was not observed in these mice (data not shown).

The results of this study indicate that Th2 response to *L. major* requires $CD4^+$ T cells, but Th1 response is independent on $CD4^+$ T cells. In addition, soluble CD4 does not affect the response against *L. major*. In model of leishmanial infection, therefore, the production of IL-4 determines whether a Th1 or Th2 response develops. Further analysis of the bias for production of IFN-γ in a T cell-independent manner and the lack of IL-4 production following *L. major* infection will contribute to the understanding of the mechanism underlying Th1 or Th2 responses. The present findings will provide insight into host mechanisms that may contribute to the development of subclinical infections and active cutaneous disease in *L. major* infection. These would also improve prevention of serious infectious diseases as well as provide for preventive medicine.

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