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Development of T_h1 and not T_h2 immune responses in mice lacking IFN-regulatory factor-4

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Abstract

IFN-regulatory factor (IRF)-4 is a member of the IRF family of transcription factors expressed in lymphocytes and macrophages. The previous studies using mice deficient in the IRF-4 gene showed profound defects in function of both B and T cells. To further investigate the role of IRF-4 in $CD4^+$ T cell function, IRF-$4^{-/-}$ mice were challenged with the intracellular pathogen *Leishmania major*. The mice were protected against *L. major* during the early phase of the infection and $CD4^+$ T cells of the infected mice produced IFN-γ in response to *L. major* antigen. However, during the late phase of infection, lymphocyte numbers were dramatically reduced in the draining lymph nodes, resulting in the deterioration of the lesion, indicating that IRF-4 was required for sustained immune responses against *L. major* infection. The function of $CD4^+$ T cells was further investigated using TCR transgenic mice lacking the IRF-4 gene. $CD4^+$ T cells from IRF-$4^{-/-}$ mice produced IFN-γ and expressed T-bet after culture under T_h1-skewing conditions *in vitro.* However, T_h2 cell development was not observed after culture under T_h2-polarizing conditions. Proliferation of $CD4^+$ T cells to IL-4 was reduced in IRF-$4^{-/-}$ mice, suggesting the defects in the responsiveness to IL-4. Furthermore, stimulation of the IRF-$4^{-/-}$ $CD4^+$ T cells with IL-4-induced activation of signal transducer and activator of transcription 6, but not expression of growth factor independent-1. Thus, development of $CD4^+$ T cell subsets differentially depends on IRF-4; induction of T_h1 response does not depend on IRF-4, while T_h2 response depends entirely on IRF-4.

Introduction

IFN-regulatory factors (IRF) constitute a family of transcription factors that commonly possess a novel helix-turn-helix DNA-binding motif and bind to the IFN-stimulated response element (ISRE) (1). Members of the IRF family are involved in diverse immune processes that include pathogen response, cytokine signaling, apoptosis, control of cell proliferation, and regulation of the development and function of the immune response (2–6). In particular, IRF-1-deficient mice fail to mount T_h1 responses and instead undergo accelerated T_h2 differentiation, suggesting that IRF-1 is required for the development of a T_h1-type immune response (5,6).

IRF-4 is a member of the IRF family of transcription factors. It was originally discovered independently as a binding factor to the Ig light chain enhancer in association with PU.1 (7) and as a new IRF family member (8). It is expressed in B cells, mature T cells and macrophages (8,9), and its expression is up-regulated by IgM or TCR cross-linking as well as co-stimulation of B cells with CD40 and IL-4 (8,10,11). Mice deficient in the IRF-4 gene exhibited an age-dependent increase of T and B lymphocytes in spleen and lymph nodes (12). These mice showed profound reduction in serum Ig concentrations, and impaired B and T lymphocyte function. They could not generate cytotoxic or anti-tumor responses, indicating a profound defect in $CD8^+$ T cell function. However, the function of $CD4^+$ T cells was not extensively studied in these mice. IRF-4 has been shown to have dual roles in the

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regulation of cytokine-regulated gene function. It is repressive over the gene activation induced by IFN stimulation (13), while it interacts with the signal transducer and activator of transcription (Stat)6 and drives the expression of IL-4 inducible genes (11). These features reminded us of the possible link between IRF-4 function and the development of $CD4^+$ T cells to T_h1 and T_h2 subsets.

In this study we determined whether IRF-$4^{-/-}$ mice were able to mount protective immune responses against infection with the intracellular pathogen *Leishmania major*, one of the best-studied models of $CD4^+$ T cell function *in vivo* (14). We found that IRF-$4^{-/-}$ mice were protected against *L. major* infection during the early phase of infection and T cells of the infected mice produced IFN-γ in response to *L. major* antigen. However, the protective immunity deteriorated during the late phase of infection, which was due to the striking reduction of lymphocytes in the draining lymph nodes. We also examined whether IRF-$4^{-/-}$ $CD4^+$ T cells can differentiate into T_h1 or T_h2 cells in response to peptide antigen *in vitro*. The results indicated that T_h1 but not T_h2 cells can develop in the absence of IRF-4. IRF-$4^{-/-}$ $CD4^+$ T cells showed reduced proliferative response to IL-4 due in part to the defective expression of growth factor independent (Gfi)-1.

Methods

Animals

IRF-$4^{-/-}$ mice were described previously (12) and were maintained by intercrossing. OT-II transgenic mice expressing the TCR specific for OVA323–339 and I-A^b (15) were provided by Dr W. R. Heath (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). These mice were maintained in the Laboratory Animal Center for Animal Research at Nagasaki University, School of Medicine. Intercross (F_2) matings were performed between OT-II and IRF-$4^{-/-}$ mice (both C57BL/6 background) to generate TCR transgenic IRF-$4^{-/-}$ (OT-II/IRF-$4^{-/-}$) mice. Mice were analyzed for transgenic TCR expression by cell surface staining of the TCR using phycoerythrin (PE)–anti-CD4 mAb and biotin–anti-TCR Vα2 mAb (PharMingen, San Diego, CA). IRF-4 genotype was determined by PCR using two pairs of oligonucleotide primers; Neo, 5′-ATTCGGCTATGACTGGGCACAACA-3′ and 5′-GAGCAAGGTGAGATGACAGGAGAT-3′; IRF-4, 5′-GCA-ATGGGAAACTCCGACAGT-3′ and 5′-CAGCGTCCTCCTCA-CGATTGT-3′. These PCR products were distinguished by electrophoresis on a 3% agarose gel. Mice that carry the Neo gene and not the IRF-4 gene were determined as IRF-$4^{-/-}$, and maintained by interbreeding thereafter. Transgenic mice were used at the age of ~10 weeks. Female C57BL/6 and BALB/c mice were purchased from SLC (Hamamatsu, Japan). The animal experiments reported herein were conducted according to the Guidelines of the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine.

Infection of mice with L. major and monitoring of the disease

L. major (MHOM/S U/73-5-ASKH strain) was provided by Dr K. Himeno (Kyushu University, Fukuoka, Japan). Mice were injected in the left hind footpad with 1×10^6 promastigotes of the *L. major* strain. The thickness of the infected and the contralateral uninfected footpad was measured once per week using a vernier caliper as described (6). The increase in footpad thickness was calculated as: [(thickness of infected footpad) – (thickness of uninfected footpad)/(thickness of uninfected footpad)] × 100. Parasite burden was determined using popliteal lymph nodes. Serial dilutions of single-cell suspensions obtained from infected mice were placed in each well of 96-well culture plates in culture medium as previously described (6). After 10 days, growth of parasites was determined and the cell dilution of the wells in which 37% of the wells were negative for parasite growth was taken to represent the original plating of one single parasite according to Poisson statistics. To determine *L. major*-specific IFN-γ production, the draining popliteal lymph node cells (1×10^5/well) were cultured *in vitro* with mitomycin C-treated syngeneic spleen cells (4×10^5/well) with or without *L. major* antigen (freeze–thawed lysates of promastigotes) for 48 h. Culture supernatants were harvested and tested for IFN-γ by ELISA. The number of lymphocytes in each lymph node was determined by counting viable cells under a microscope using Trypan blue. The ratio of lymphocyte subsets was determined by flow cytometry analysis of lymph node cells stained with PE–anti-CD4 and FITC–anti-CD8 antibody or with FITC–anti-B220 antibody. The number of each lymphocyte subset was determined by multiplying the total cell number of each lymph node with the ratio of each subset.

Cell culture

To isolate $CD4^+$ T cells, lymph node cells were treated with anti-CD8 mAb (3.155), anti-MHC class II mAb (M5/114.15.2) and complement followed by nylon wool column enrichment. This population consisted of >95% $CD4^+$ T cells. $CD4^+$ T cells (1×10^5) were cultured in RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 5×10^{-5} M 2-mercaptoethanol, penicillin/streptomycin and 10% heat-inactivated FCS in each well of a microtiter plate. Cells were cultured in each well of a flat-bottom 96-well plate in the presence of mitomycin C-treated C57BL/6 spleen cells and varying dose of OVA323–339 peptide or with IL-4 (250 U/ml), phorbol myristate acetate (PMA; 20 ng/ml) and ionomycin (1 μM). After culture for 43 h, proliferation was assessed by a terminal pulse of 18.5 kBq [^{3}H]thymidine (NEN Life Science Products, Boston, MA) for 5 h. Alternatively, supernatant was collected from each well after culture for 48 h to determine the level of cytokines. The OVA323–339 peptide was purchased from Sawady Technology (Tokyo, Japan). In some experiments, $CD4^+$ T cells were cultured in each well of a flat-bottom 96-well plate coated with anti-TCRβ mAb (H57-597, 10 μg/ml) in the presence of anti-CD28 mAb (PV-1, 2 μg/ml) (16).

Cytokine assays

The levels of cytokines in the supernatants of T cell cultures were determined by a sandwich ELISA. IFN-γ was measured using R4-6A2 as the capture antibody and biotinylated XMG1.2 as the detecting antibody according to the manufacturer's directions (all mAb for ELISA were from BD PharMingen, San Diego, CA). IL-4 was measured using

11B11 as capture antibody and BVD6-24G2 as the detecting antibody. Recombinant mouse IFN-γ (PeproTech, London, UK) and IL-4 (Genzyme, Cambridge, MA) were used as standards. The ELISPOT assay was performed using nitrocellulose-lined 96-well microtiter plates (MAHAS45; Millipore, Bedford, MA). Plates were coated with 10 μg/ml of anti-mouse IFN-γ antibody (clone R4-6A2; PharMingen). After washing with PBS containing 0.25% Tween 20, plates were overlaid with PBS containing 5% BSA at 37°C for 30 min. $CD4^+$ T cells were prepared from popliteal lymph nodes of mice, and placed in each well of a treated microtiter plate (1×10^5/well) in the presence of various doses of *L. major* antigen and mitomycin C-treated T cell-depleted syngeneic spleen cells (4×10^5/well). After culture for 24 h, plates were washed, incubated with biotinylated anti-IFN-γ mAb, washed, and incubated with streptavidin-conjugated alkaline phosphatase and then with BCIP/NBT. Spots were counted using a stereomicroscope with a magnification of ×20.

To induce T_h1 and T_h2 development *in vitro*, purified $CD4^+$ T cells of OT-II and OT-II/IRF-$4^{-/-}$ mice (10^5/ml) were cultured in complete medium containing mitomycin C-treated C57BL/6 spleen cells (4×10^5/ml) and OVA323–339 peptide (10 μM). T_h1 conditions contained IL-2 (20 U/ml), IL-12 (20 ng/ml) and anti-IL-4 mAb (11B11; 10 μg/ml). T_h2 conditions contained IL-2 (20 U/ml), IL-4 (100 U/ml), anti-IFN-γ mAb (R4-6A2; 15 μg/ml) and anti-IL-12 mAb (C17.8; 10 μg/ml). After culture for 7 days, cells (1×10^5/well) were re-stimulated in the presence of mitomycin C-treated C57BL/6 (4×10^5/well) spleen cells and OVA323–339 peptide for 2 days. The levels of cytokines in the culture supernatant were determined by ELISA. For intracellular cytokine staining, polarized $CD4^+$ T cells were stimulated for 5 h in GolgiStop (BD PharMingen, Beverly, MA) containing PMA (50 ng/ml) and ionomycin (1 μM). Intracellular staining was performed according to the protocol of the manufacturer using CytoStain kits (PharMingen). After fixation and permeabilization, cells were stained with FITC–anti-IFN-γ mAb (R4-6A2) and PE–anti-IL-4 mAb (BVD4-1D11), and analyzed using a FACScan (Becton Dickinson, San Jose, CA). The expression of IL-4 receptor and IL-2 receptor was determined by staining cells with anti-IL-4 receptor mAb (M1; BD PharMingen) and FITC–anti-rat IgG (MBL, Nagoya, Japan) or with FITC–anti-CD25 mAb (BD PharMingen).

RT-PCR

Total RNA was extracted from $CD4^+$ T cells using Isogen (Nippon Gene, Tokyo, Japan). cDNA was generated from 1 μg RNA using random hexamers and MMLV reverse transcriptase (Sawady Technology). Each aliquot of the sample was amplified in a volume of 50 μl for 27 cycles to detect IFN-γ and IL-4 (94°C for 30 s, 55°C for 50 s, 72°C for 80 s), and for 35 cycles to detect G3PDH (94°C for 60 s, 60°C for 60 s, 72°C for 60 s) using LA Taq DNA polymerase (Takara, Tokyo, Japan). The primers used for IFN-γ were 5′-TGAACGCTACACACTGCATCTTGG-3′ and 5′-CGACTCCTTTTCCGCTTCCTGAG-3′, for IL-4 were 5′-TAGTTGTCATCCTGCTCTT-3′ and 5′-CTACGAGTAATCCATTTGC-3′, and for G3PDH were 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′. PCR analysis of T-bet, GATA-3 and Gfi-1 expression was performed as described (17,18), except that cDNA was amplified 27 cycles for T-bet and GATA-3, and 30 cycles for Gfi-1. The PCR products were size fractionated using a 3% agarose gel and visualized by ethidium bromide staining.

Western blot analysis

$CD4^+$ T cells were resuspended in 50 μl lysis buffer (1% Triton X-100, 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1 mM PMSF). After centrifugation, the lysate was mixed with 2 × SDS–PAGE sample buffer and size fractionated on 8% SDS–PAGE. After transfer to a PVDF membrane, the blot was incubated in blocking buffer (TBS containing 5% skim milk powder and 0.1% Tween 20) and probed with anti-phospho Stat6 or anti-Stat6 antibody (Cell Signaling Tech, Beverly, MA). After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit Ig antibody (1:1000), washed and analyzed using ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Results

Protection of IRF-$4^{-/-}$ mice against infection with L. major

One of the best-studied models of $CD4^+$ T cell function *in vivo* is murine cutaneous Leishmaniasis, induced by inoculation of *L. major* (14). In the resistant mice, parasite-specific T_h1 cells activate parasite-killing macrophages to contain the infection, while in susceptible mice T cells shift to T_h2 development resulting in a high parasite burden. To study whether a protective T_h1 response can develop in the absence of IRF-4 against *L. major in vivo*, BALB/c (susceptible strain), C57BL/6 (resistant strain) and IRF-$4^{-/-}$ mice were infected s.c. with *L. major*, and the course of disease was monitored (Fig. 1A). BALB/c mice developed a strong continuous increase in footpad thickness, while C57BL/6 mice showed only a mild and transient increase. The footpad thickness of IRF-$4^{-/-}$ mice was lower than that of C57BL/6 mice up to 5 weeks after infection, but did not diminish thereafter. Instead, it increased gradually. The parasite burden was determined 7 weeks after infection (Fig. 1B). The number of *L. major* in the draining lymph nodes of IRF-$4^{-/-}$ mice was much lower than that of BALB/c, but higher than that of C57BL/6 mice, which was consistent to the increased footpad swelling in IRF-$4^{-/-}$ mice during the late stage of infection.

The number of lymphocytes in the draining lymph nodes of the infected mice was determined during the course of infection (Fig. 2). In both BALB/c and C57BL/6 mice, the number of $CD4^+$ T cells, $CD8^+$ T cells and B cells dramatically increased during infection, while those in the non-draining lymph nodes were constant. In IRF-$4^{-/-}$ mice, however, the number of lymphocytes in the draining lymph nodes increased during the first 3 weeks of infection and dramatically declined during the later period of infection. This reduction was specific for the draining lymph nodes, since the number of each lymphocyte subset in the non-draining lymph nodes was much higher than normal mice and generally increased during the period of infection corresponding to aging as reported previously (12). To determine whether specific T_h1 cells develop in *L. major*-infected mice, T cells from the draining lymph nodes were isolated 3 or 7 weeks after infection and

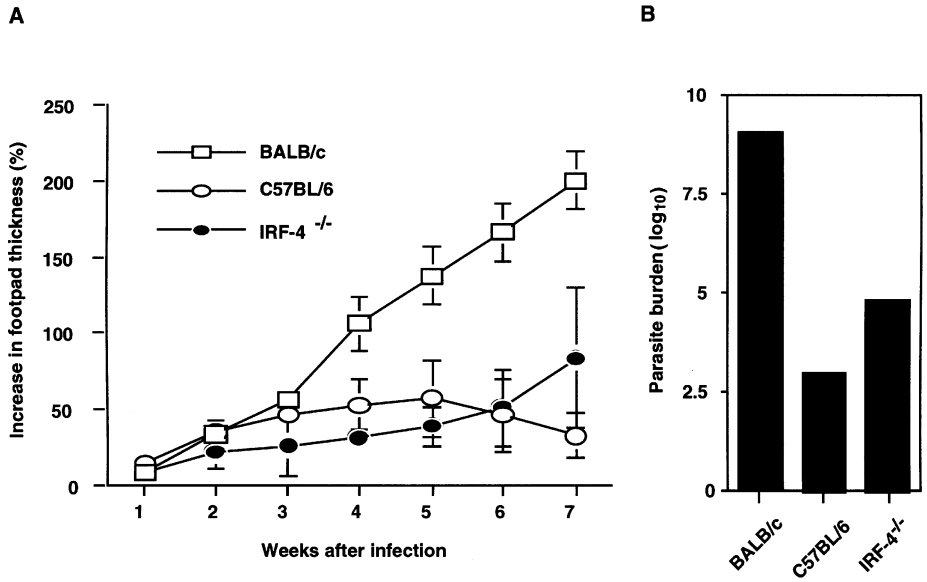

Fig. 1. Increase in lesion size and parasite burden in *L. major*-infected mice. (A) BALB/c (squares), C57BL/6 (open circles) and IRF-4$^{-/-}$ (solid circles) mice were infected with *L. major* promastigotes, the thicknesses of infected (left) and uninfected (right) footpads measured, and the increase in footpad thickness (%) calculated. The data represent means ± SD of five mice in each group. The data represent three separate experiments with similar results. (B) Parasite burden of the draining popliteal lymph nodes was assessed 7 weeks after *L. major* infection. Serial dilutions of single-cell suspensions were cultured in 96-well plates for 10 days and the growth of parasites was determined. The dilution containing 37% negative wells was taken as the original plating of a single parasite per well. Results are given as the log of the cell number plated per well in this dilution.

cultured for 2 days in the presence or absence of *L. major* antigen. Culture supernatants of the lymph node cells were tested for IFN-γ production by ELISA (Fig. 3A). CD4$^+$ T cells from both C57BL/6 and IRF-4$^{-/-}$ mice but not BALB/c mice showed specific IFN-γ production in response to *L. major* antigen. The level of IFN-γ produced by T cells of IRF-4$^{-/-}$ mice was equivalent to that of C57BL/6 mice 3 weeks after infection and reduced to approximately half of that of C57BL/6 mice 7 weeks after infection, consistent with the level of footpad swelling. Next, we determined the numbers of IFN-γ-producing cells in the draining lymph nodes of each mouse by the ELISPOT assay (Fig. 3B). The proportion of CD4$^+$ T cells producing IFN-γ in response to *L. major*-antigen in IRF-4$^{-/-}$ mice was approximately half of the C57BL/6 mice, consistent with the result of ELISA. Thus, when the production of IFN-γ was monitored on a per cell basis, each CD4$^+$ T cell in IRF-4$^{-/-}$ mice produced IFN-γ at levels similar to C57BL/6 mice. However, since the number of CD4$^+$ T cells in the draining lymph node of IRF-4$^{-/-}$ mice (2.5×10^6/lymph node) was much lower than C57BL/6 mice (6.7×10^7/lymph node) at 7 weeks after infection (Fig. 2), the total number of IFN-γ-producing CD4$^+$ T cells in each draining lymph node of IRF-4$^{-/-}$ mice ($\sim 1.2 \times 10^3$) was much lower than C57BL/6 mice ($\sim 8.1 \times 10^4$). Taken together, the results indicated that IRF-4 is not required for the induction of the protective T_h1 immune response against cutaneous infection of *L. major*, but is required to sustain the protective T_h1 response.

Response of T cells from IRF-4$^{-/-}$ mice to TCR engagement in vitro

To monitor the response of CD4$^+$ T cells of the IRF-4$^{-/-}$ genotype in response to peptide–MHC, OT-II TCR transgenic mice expressing a TCR specific for OVA323–339 and I-A^b were bred to IRF-4$^{-/-}$ mice (OT-II/IRF-4$^{-/-}$ mice). The flow cytometric analysis of T cell populations in the thymus and peripheral lymphoid tissue indicated that the distribution of T cell subpopulations in OT-II/IRF-4$^{-/-}$ mice was not significantly different from that of OT-II mice with a marked shift to CD4$^+$CD8$^-$ T cells as described (15). The proportions of CD4$^+$CD8$^-$ T cells in total lymph node cells were ~42% in OT-II and ~52% in OT-II/IRF-4$^{-/-}$ mice, and CD4$^-$CD8$^+$ T cells were ~6% in OT-II and ~7% in OT-II/IRF-4$^{-/-}$ mice. Purified CD4$^+$ T cells from OT-II and OT-II/IRF-4$^{-/-}$ mice were cultured in the presence of mitomycin C-treated C57BL/6 spleen cells and a range of antigenic peptide (OVA323–339 0–10 μM) (Fig. 4A). OT-II T cells showed an antigen-specific proliferative response in a dose-dependent manner. OT-II/IRF-4$^{-/-}$ T cells also showed a similar antigen dose-dependent proliferation, although the level of the response was reduced when compared with OT-II T cells. We also used ELISA to examine the production of IFN-γ in response to the antigenic peptide (Fig. 4B). Both OT-II and OT-II/IRF-4$^{-/-}$ T cells exhibited comparable levels of IFN-γ secretion in response to OVA323–339.

Differentiation of IRF-4$^{-/-}$ T cells to T_h1/T_h2 in vitro

Since CD4$^+$ T cells from IRF-4$^{-/-}$ mice were able to respond to a specific antigen, we investigated whether they could differentiate into T_h1 and T_h2 cells *in vitro*. Purified CD4$^+$ T cells from OT-II and OT-II/IRF-4$^{-/-}$ mice were cultured *in vitro* with antigenic peptide and C57BL/6 spleen cells in the presence of IL-2, IL-12 and anti-IL-4 mAb to generate T_h1 cells or in the presence of IL-2, IL-4, anti-IL12 mAb and anti-IFN-γ mAb to generate T_h2 cells. After 1 week in culture, cells were

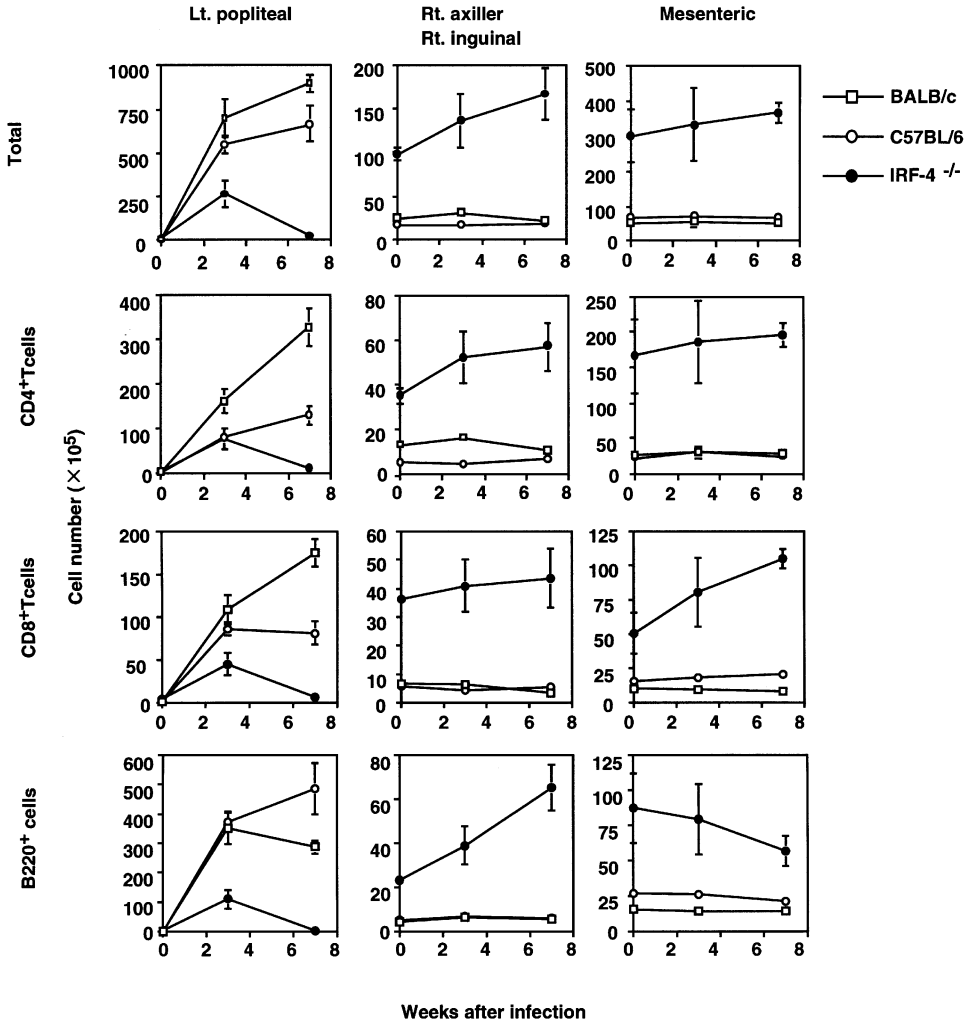

Fig. 2. The kinetic change of the lymphocyte numbers in draining lymph nodes during *L. major* infection. The number of lymphocytes in the draining (left popliteal) and non-draining lymph nodes (mixture of right axillary and right inguinal, and mesenteric) of BALB/c (squares), C57BL/6 (open circles) and IRF-4$^{-/-}$ (solid circles) mice were counted 3 and 7 weeks after inoculation of *L. major* in the left footpad. The numbers of lymphocytes in each lymph node in naive mice (day 0) were also counted. The lymph node cells were stained with anti-CD4, anti-CD8 and anti-B220 mAb, and the proportion of each subset was determined using flow cytometry. The number of each lymphocyte subset was calculated by multiplying the total cell number with the proportion of each subset. The data shown are means ± SD obtained from three mice at each time point.

re-stimulated with OVA323–339 peptide and C57BL/6 spleen cells, and the levels of IFN-γ and IL-4 in the culture supernatant of each T cell subset were determined by ELISA (Fig. 5A). CD4$^+$ T cells from OT-II and OT-II/IRF-4$^{-/-}$ mice produced similar levels of IFN-γ after they were cultured under T_h1 conditions. No significant IL-4 production was detected in these cultures. When CD4$^+$ T cells were cultured in T_h2 skewing conditions, cells from OT-II mice produced IL-4 and not IFN-γ, whereas those from OT-II/IRF-4$^{-/-}$ mice produced neither IL-4 nor IFN-γ. Next, we determined cytokine production by individual T cells by intracellular cytokine staining of IFN-γ and IL-4 after 1 week of polarizing culture (Fig. 5B). CD4$^+$ T cells from OT-II mice showed significant development to both T_h1 and T_h2 cells after culture in each condition. About 49.3% became IFN-γ producers under T_h1 conditions and ~18.1% became IL-4 producers under T_h2 conditions. A similar proportion of CD4$^+$ T cells from OT-II/IRF-4$^{-/-}$ mice (~45.8%) exhibited IFN-γ production under T_h1 conditions, and the levels of IFN-γ in each cell were equivalent to those of OT-II cells. Therefore, T_h1 development of CD4$^+$ T cells *in vitro* was not impaired in IRF-4$^{-/-}$ mice. In contrast, very little development of IL-4 producers (~2.9%) was observed under T_h2 conditions, suggesting that T_h2 development *in vitro* was impaired in the mice lacking the IRF-4 gene.

The transcription factors T-bet and GATA-3 are putative master regulators of T_h1 and T_h2 development respectively (19,20). The expression of T-bet and GATA-3 is induced under T_h1 and T_h2 conditions respectively, and correlates to their production of IFN-γ and T_h2 cytokines. When expressed ectopically, T-bet and GATA-3 are sufficient to induce expression of IFN-γ and IL-4 synthesis respectively. Thus, we determined whether these transcription factors are

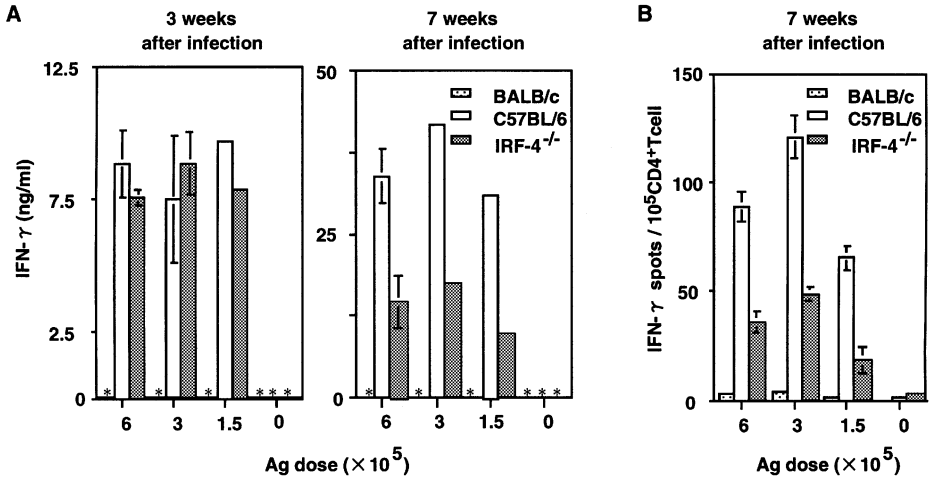

Fig. 3. Production of IFN-γ in response to *L. major* antigen by lymphocytes from the draining lymph nodes. (A) Mice were sacrificed 3 and 7 weeks after infection. Lymphocytes (1×10^5/well) prepared from the draining popliteal lymph nodes were cultured with various doses of *L. major* antigen and mitomycin C-treated syngeneic spleen cells (4×10^5/well) for 48 h. The levels of IFN-γ in the supernatant were determined by ELISA. The data represent the means ± SD of triplicate wells. (B) CD4+ T cells (1×10^5/well) from the popliteal lymph node 7 weeks after infection with *L. major* were cultured with various doses of *L. major* antigen and mitomycin C-treated T cell-depleted syngeneic spleen cells (4×10^5/well). After 24 h, the frequency of IFN-γ-secreting cells was determined by ELISPOT assay. The data represent the means ± SD of the triplicate experiments. *, Not detectable.

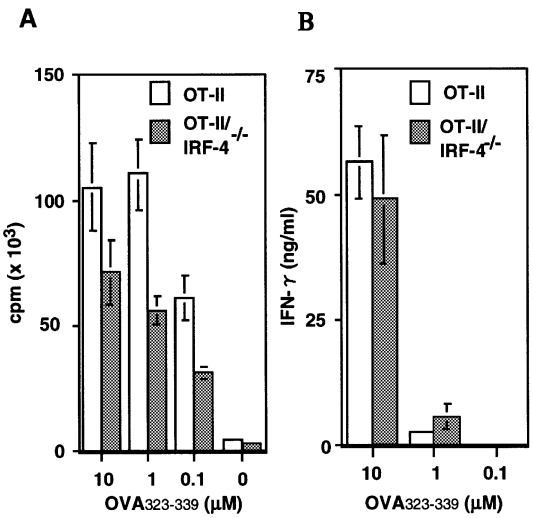

Fig. 4. Proliferation and IFN-γ secretion of CD4+ T cells from OT-II and OT-II/IRF-$4^{-/-}$ mice in response to OVA323–339 peptide. CD4+ T cells (1×10^5) were cultured in wells of a flat-bottom 96-well plate in the presence of mitomycin C-treated C57BL/6 spleen (4×10^5) cells and varying doses of OVA323–339 peptide. (A) After culture for 43 h, the proliferation was assessed by a terminal pulse of [^{3}H]thymidine for 5 h. (B) After culture for 48 h, supernatant was collected from each well and the levels of IFN-γ were determined by ELISA. The data represent means ± SD of triplicate experiments. The data represent three separate experiments with similar results.

expressed in CD4+ T cells of IRF-$4^{-/-}$ mice after culture *in vitro* (Fig. 6). RT-PCR analysis was performed using RNA extracted from non-stimulated CD4+ T cells, CD4+ T cells cultured under T_h1 conditions and those cultured under T_h2 conditions. IFN-γ mRNA was detected in CD4+ T cells from both OT-II and OT-II/IRF-$4^{-/-}$ mice, while IL-4 mRNA was detected in CD4+ T cells from OT-II and not OT-II/IRF-$4^{-/-}$ mice. The expression of T-bet and GATA-3 mRNA in each T cell population was consistent with their production of IFN-γ and IL-4. T-bet expression was induced under T_h1 conditions in CD4+ T cells from both OT-II and OT-II/IRF-$4^{-/-}$ mice, while GATA-3 mRNA was detected under T_h2 conditions in CD4+ T cells from OT-II mice and not OT-II/IRF-$4^{-/-}$ mice. Taken together, CD4+ T cells from IRF-$4^{-/-}$ mice are able to differentiate into T_h1 cells, but are impaired in their ability to differentiate into T_h2 cells.

The proliferative response to IL-4 of CD4+ T cells from IRF-$4^{-/-}$ mice

Since IL-4 is critical for the development of T_h2 cells, we investigated the possibility that IL-4-induced CD4+ T cell activation is defective in IRF-$4^{-/-}$ mice. CD4+ T cells from IRF-$4^{-/-}$ and control C57BL/6 mice were stimulated in the presence of IL-4, and their proliferative response was assessed by [^{3}H]thymidine incorporation (Fig. 7). The proliferative response of CD4+ T cells from IRF-$4^{-/-}$ mice in response to IL-4 was reduced when compared with C57BL/6 T cells. These cells, however, proliferated at equivalent levels in response to PMA and ionomycin. These results suggested that CD4+ T cells from IRF-$4^{-/-}$ mice have defects in the responsiveness to IL-4. This can be caused by the reduced expression of IL-4 receptor or defects in the subsequent signaling events. Thus, the expression of IL-4 receptor on naive and activated CD4+ T cells was examined by flow cytometry (Fig. 8A). IL-4 receptor α chain was expressed on CD4+ T cells from IRF-$4^{-/-}$ mice at levels similar to control mice. After the activation of CD4+ T cells with anti-TCR and anti-CD28 mAb for 40 h, the level of IL-4 receptor expression was similar to naive T cells in both C57BL/6 and IRF-$4^{-/-}$ and mice, while the expression of IL-2 receptor was strongly induced. Stat6 is rapidly tyrosine phosphorylated following stimulation of T cells with IL-4 (21), which is essential for signal transduction of IL-4 receptor (22–24). Also, it was previously reported that IRF-4 interacts with Stat6 and drives the expression of IL-4-inducible genes in

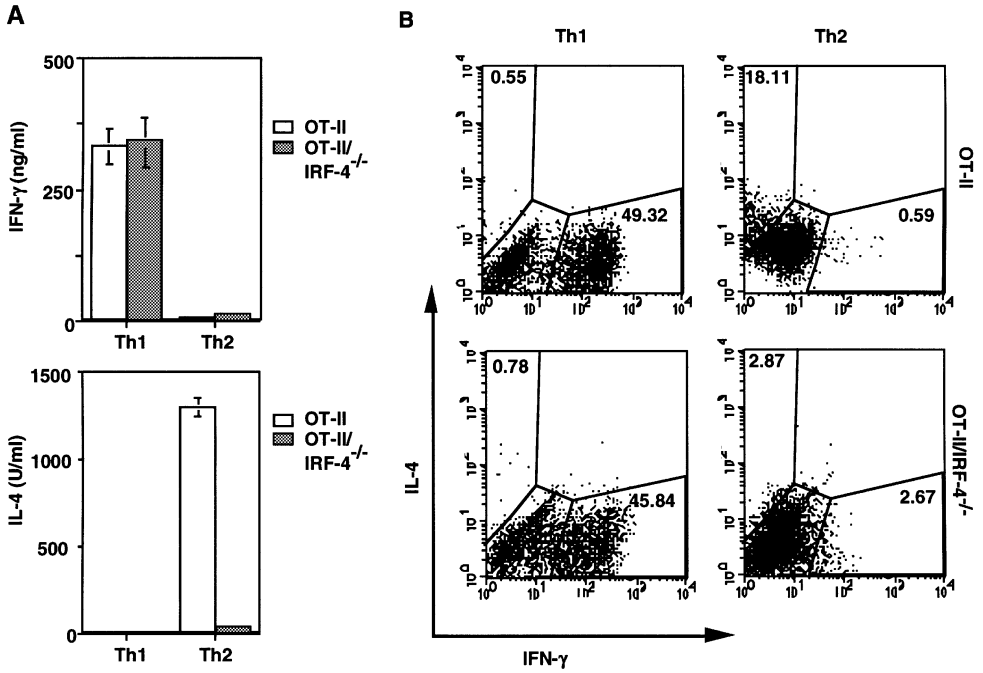

Fig. 5. T_h1 and T_h2 development of CD4+ T cells from OT-II and OT-II/IRF-4$^{-/-}$ mice *in vitro*. CD4+ T cells (1×10^5/ml) were cultured in T_h1 (IL-2, IL-12 and anti-IL-4 mAb)- or T_h2 (IL-2, IL-4, anti-IL-12 mAb and anti-IFN-γ mAb)-promoting conditions in the presence of mitomycin C-treated C57BL/6 spleen cells (4×10^5/ml) and OVA323–339 peptide (10 μM). (A) After culture for 7 days, T cells were collected and re-stimulated in wells of a 96-well plate (1×10^5) with mitomycin C-treated C57BL/6 spleen cells (4×10^5) and OVA323–339 peptide (10 μM). Two days later, supernatant was collected from each well to determine the levels of cytokines by ELISA. The data represent means ± SD of triplicate experiments. (B) Seven days after polarizing culture, cells were stimulated for 5 h in GolgiStop containing PMA and ionomycin. Intracellular cytokine content was measured by staining these cells with FITC–anti-IFN-γ mAb and PE–anti-IL-4 mAb. The percentage of cells that secrete each cytokine is indicated. The data represent three separate experiments with similar results.

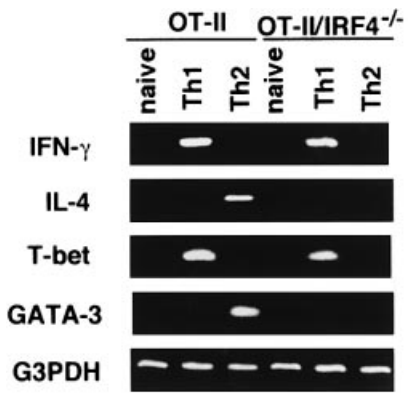

Fig. 6. RT-PCR analysis of IFN-γ, IL-4, T-bet and GATA-3 gene expression. CD4+ T cells from OT-II and OT-II/IRF-4$^{-/-}$ mice were cultured under T_h1 and T_h2 conditions as described in the legend of Fig. 2. RNA was extracted from non-stimulated CD4+ T cells (naive) as well as T cells cultured under T_h1- and T_h2-promoting conditions for 7 days. RT-PCR analysis of IFN-γ, IL-4, GATA-3 and G3PDH was performed as described in Methods. G3PDH was used as an internal control to normalize the amount of PCR templates. PCR products were size fractionated using a 3% agarose gel and visualized by ethidium bromide staining. The data represent four separate experiments with similar results.

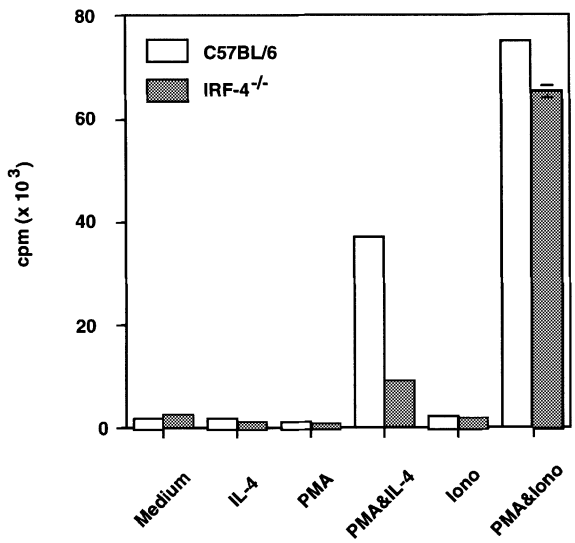

Fig. 7. Proliferation of CD4+ T cells from C57BL/6 and IRF-4$^{-/-}$ mice in response to IL-4. CD4+ T cells were cultured in medium, IL-4 (250 U/ml), PMA (20 ng/ml), ionomycin (1 μM) and their combinations. After 48 h, proliferation was assessed by [^{3}H]thymidine incorporation. Values represent means ± SD of triplicate wells. The data represent three separate experiments with similar results.

B cells (11). Therefore, we next examined whether Stat6 can be activated by IL-4 in the absence of IRF-4. Naive and activated CD4+ T cells were stimulated with IL-4, and the activation of Stat6 was examined by Western blotting using anti-phophoStat6 antibody (Fig. 8B). Tyrosine phosphorylation

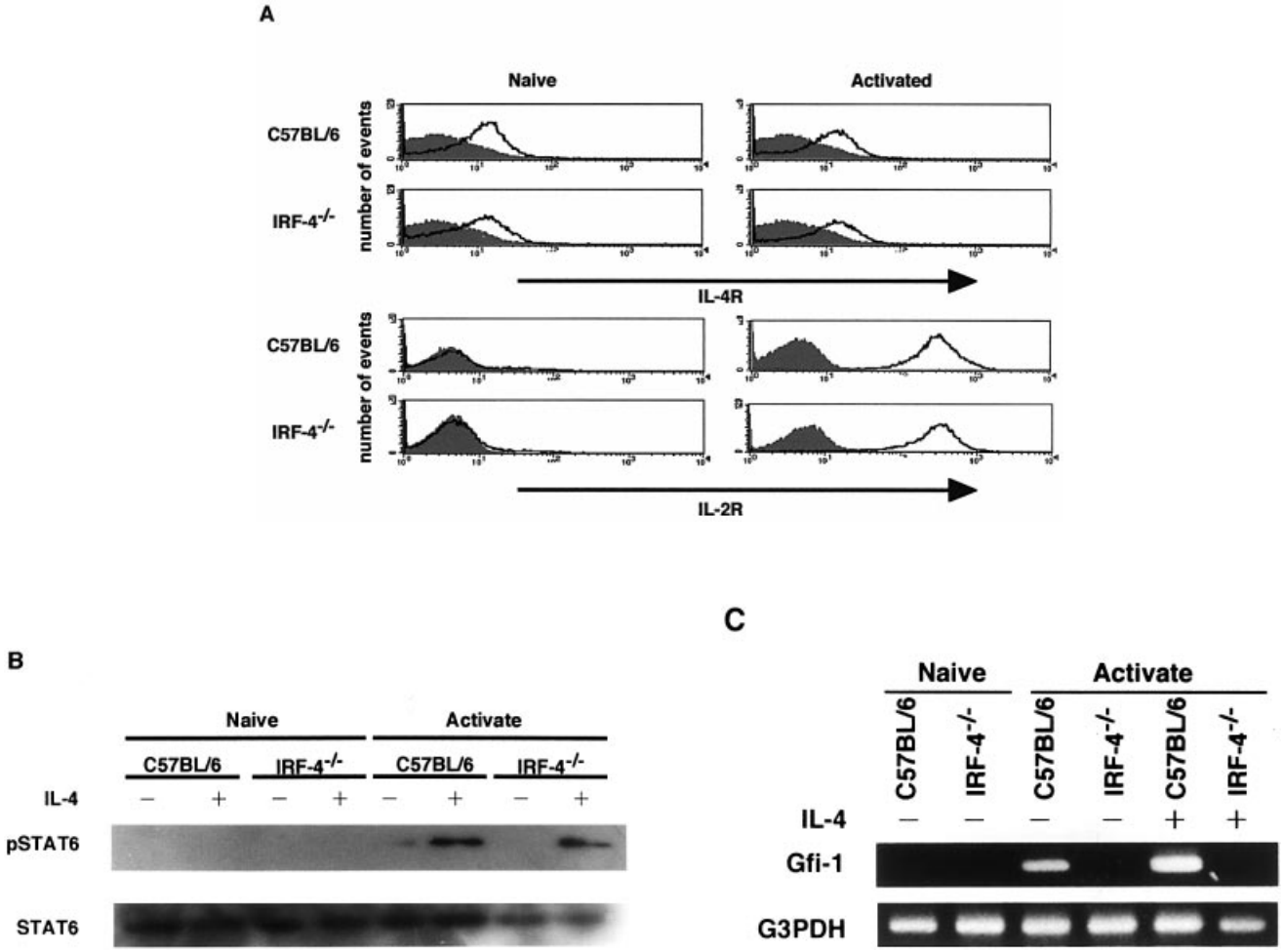

Fig. 8. The expression of IL-4 receptor, and IL-4-mediated activation of Stat6 and Gfi-1 in IRF-4$^{-/-}$ CD4$^+$ T cells. (A) CD4$^+$ T cells from C57BL/6 and IRF-4$^{-/-}$ mice were cultured on microtiter plates coated with anti-TCR mAb (10 μg/ml) in the presence of anti-CD28 mAb (2 μg/ml) for 48 h. Naive and activated CD4$^+$ T cells were stained with anti-IL-4 receptor antibody and anti-CD25 mAb, and were analyzed using flow cytometry. The gray shadow indicates the staining with FITC-conjugated anti-rat Ig antibody alone. The data represent three separate experiments with similar results. (B) CD4$^+$ T cells were stimulated with anti-TCR plus anti-CD28 mAb. After 40 h, cells were washed with fresh medium and cultured at 37°C for an additional 10 h for starvation. Naive and activated CD4$^+$ T cells (2×10^6) were cultured in the presence or absence of IL-4 (200 ng/ml) for 10 min. Total cell lysates were separated on 8% SDS–PAGE, blotted and probed with anti-phosphoSTAT6 antibody (upper panel). The same blot was stripped and reprobed with anti-STAT6 antibody (lower panel). (C) CD4$^+$ T cells were stimulated with anti-TCR plus anti-CD28 mAb for 48 h. Naive and activated CD4$^+$ T cells were cultured at 37°C in the presence or absence of IL-4 (200 ng/ml) for 4 h. At the end of culture, RNA was extracted, and RT-PCR analysis of Gfi-1 and G3PDH was performed. PCR products were size fractionated using a 2% agarose gel and visualized by ethidium bromide staining.

of Stat 6 was induced by IL-4 in activated CD4$^+$ T cells of both C57BL/6 and IRF-4$^{-/-}$ mice, indicating that activation of Stat6 is not impaired in IRF-4$^{-/-}$ mice. It was recently reported that growth factor independent (Gfi)-1, a transcriptional repressor, is induced by IL-4 in a Stat6-dependent manner, and strikingly promotes proliferation of T_h2 and T_hN cells (18). Thus, we examined the expression of Gfi-1 in CD4$^+$ T cells of both C57BL/6 and IRF-4$^{-/-}$ mice by RT-PCR (Fig. 8C). Gfi-1 was expressed in activated CD4$^+$ T cells of C57BL/6 mice and its expression was up-regulated by stimulation with IL-4. However, the expression was not detected in activated CD4$^+$ T cells from IRF-4$^{-/-}$ mice even after stimulation with IL-4. The result suggested that the reduced proliferation of IRF-4$^{-/-}$ CD4$^+$ T cells in response to IL-4 was at least in part due to the defective expression of Gfi-1.

Discussion

Mice deficient in IRF-4 gene are severely defective in antibody production, cytotoxic T cell development and cytokine production by T cells, indicating the critical role of IRF-4 in B and T lymphocyte function (12). In this study, we showed that CD4$^+$ T cells from IRF-4$^{-/-}$ mice were able to mount protective T_h1 immune responses against *L. major* infection. The protective

immunity was particularly effective during early stages of the infection. The level of footpad swelling in IRF-4$^{-/-}$ mice was even less than control C57BL/6 mice and CD4$^+$ T cells from IRF-4$^{-/-}$ mice produced IFN-γ in response to the parasite antigen at levels similar to C57BL/6 mice 3 weeks after infection. The footpad swelling of the infected IRF-4$^{-/-}$ mice, however, began to increase 6 weeks after infection when it began to reduce in C57BL/6 mice. In parallel, the number of lymphocytes in the draining lymph nodes was dramatically reduced. Thus, the number of IFN-γ-producing T cells of IRF-4$^{-/-}$ mice was dramatically reduced when measured at 7 weeks after the infection, although each CD4$^+$ T cell produced IFN-γ at levels similar to control C57BL/6 mice. The striking reduction in the lymphocyte number occurred only in the draining lymph nodes, and was observed in all lymphocyte types including CD4$^+$ T cells, CD8$^+$ T cells and B cells. Therefore, IRF-4 is required for sustained protective immune responses against *L. major* infection. The reason for this reduction of the lymphocytes in the draining lymph nodes is not clear. One possibility is the migration of the lymphocytes out of the draining lymph nodes. Alternatively, these lymphocytes may undergo apoptosis in the draining lymph nodes. These possibilities require further investigation.

CD4$^+$ T cells from OT-II/IRF-4$^{-/-}$ mice produced IFN-γ at levels similar to control CD4$^+$ T cells *in vitro*, suggesting that IFN-γ production is not impaired in CD4$^+$ T cells of IRF-4$^{-/-}$ mice (Figs 4,5). This seemed contradictory to the previous report showing that cytokine secretion by T cells from IRF-4$^{-/-}$ mice was defective when they were stimulated with anti-CD3 mAb. It may be due to the differences in the T cell subsets that secrete IFN-γ: CD8$^+$ T cells were the main source of IFN-γ-secreting cells when T cells were stimulated with anti-CD3 mAb (data not shown), while CD4$^+$ T cells of OT-II mice produced IFN-γ in response to OVA323–339. Alternatively, the difference might be due to the nature of antigenic stimulation used in both studies: T cells were stimulated with peptide–MHC on antigen-presenting cells in this study, while they were stimulated with anti-CD3 antibody alone in the previous study (12). After culture under T_h1 conditions, CD4$^+$ T cells from IRF-4$^{-/-}$ mice expressed T_h1-specific transcription factor, T-bet, and were able to produce IFN-γ at levels similar to control T cells, indicating that they differentiated into T_h1 cells *in vitro*. Intracellular staining of IFN-γ confirmed that individual T cells produce IFN-γ at levels comparable to control T cells. Taken together, these studies indicated that T_h1 development and function was not impaired in CD4$^+$ T cells of IRF-4$^{-/-}$ mice.

T_h2 development was impaired in CD4$^+$ T cells from IRF-4$^{-/-}$ mice. The defect in T_h2 development was observed by both the defects in IL-4 production and the expression of GATA-3 after culture of CD4$^+$ T cells under T_h2 conditions. This observation is consistent with the recent report by Rengarajan *et al.* (25) showing similar defects in T_h2 cytokine production by CD4$^+$ T cells of IRF-4$^{-/-}$ mice after culture under T_h2 conditions. They also showed that IRF-4 interacted with NFATc2 to enhance NFATc2-driven transcriptional activation of the IL-4 promoter, suggesting that IRF-4 was important for the production of IL-4. Our study of the T_h2 defect in these mice is distinct from theirs in two points. First, we stimulated T cells with the natural ligand, peptide–MHC, while they used anti-CD3 and anti-CD28 mAb to induce T_h cell differentiation. Second, our study suggested that the defect in T_h2 development is due to the defective response of IRF-4$^{-/-}$ CD4$^+$ T cells to IL-4. Since the T_h2-skewing condition of CD4$^+$ T cell culture contains exogenous IL-4, the defect in T_h2 development could not be solely explained by the defect in the IL-4 production. In fact, we found that the response to exogenous IL-4 was defective in CD4$^+$ T cells from IRF-4$^{-/-}$ mice (Fig. 7). The expression of IL-4 receptor and the phosphorylation of Stat6 by IL-4 were not impaired, but Gfi-1 expression was defective in CD4$^+$ T cells from IRF-4$^{-/-}$ mice after activation. Gfi-1 is induced by T cell activation and IL-4. Gfi-1 promotes proliferation and diminishes apoptosis of T_h2 cells in cooperation with Gata-3. Thus, the reduced growth of CD4$^+$ T cells from IRF-4$^{-/-}$ mice is at least in part mediated by the impaired induction of Gfi-1. Taken together, these results suggest that IRF-4 is involved in both transcriptional activation of IL-4 production (25) and the responsiveness of CD4$^+$ T cells to IL-4.

The critical role of IRF-1 in the development of T_h1 immune responses is well established. Mice lacking IRF-1 failed to mount T_h1 responses and instead underwent accelerated T_h2 differentiation (5,6). On the other hand, we showed here that T_h1 but not T_h2 cells can develop in mice lacking IRF-4. Therefore, IRF-1 and IRF-4 appear to have crucial functions in the development of two opposing subsets of CD4$^+$ T cells, T_h1 and T_h2 respectively. These two members of an IRF family might coordinately regulate the development of CD4$^+$ T cells by acting on the production and responsiveness to key cytokines. IRF-1 is important for IL-12 production by macrophages as well as responsiveness of CD4$^+$ T cells to IL-12 (5), while IRF-4 is required for the production and responsiveness to IL-4. Alternatively, it is also possible that these members of the IRF family, which bind to a similar DNA motif, compete for the same DNA binding sequence of a key regulatory gene that determines T_h1/T_h2 development.

Finally, this and previous studies using mice lacking IRF-4 gene showed that IRF-4 is critical for the development and function of many lymphocyte types including B cells, CD8$^+$ T cells and T_h2 cells. There might be a key regulatory gene that is commonly required for the development of these cell types and whose expression is regulated by IRF-4. T_h1 appears to be an exception of this regulation, although IRF-4 is required for sustained T_h1 response. It is intriguing to find such regulatory mechanisms of lymphocyte development and homeostasis by IRF-4.

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Abbreviations

Gfi	growth factor independent
IRF	IFN-regulatory factor
ISRE	IFN-stimulated response element
PE	phycoerythrin
PMA	phorbol myristate acetate
Stat	signal transduction and activation of transcription

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Note added in proof

After submission of the manuscript, another group reported similar study on the differentiation of helper T cells after infection of IRF-4-deficient mice with *L. major*. Lohoff, M., Mittrucker, H.-W., Prechtl, S., Bischof, S., Sommer, F., Kock, S., Ferrick, D.A., Duncan, G.S., Gressner, A. and Mak, T.W. 2002. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. Proc. Natl Acad. Sci. USA 99:11808.