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**Interferon regulatory factor-4 differentially regulates the production of Th2
cytokines in naïve vs. effector/memory CD4⁺ T cells**

Running title: Regulation of IL-4 production by IRF-4

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Abstract

Interferon regulatory factor (IRF)-4 is a member of the IRF family of transcription factors and plays critical roles in the development of CD4⁺ T-cells into Th2 and Th17 cells. Using the infection model of *Nippostrongylus brasiliensis*, we have confirmed the critical roles of IRF-4 in Th2 development *in vivo* using IRF-4^{-/-} BALB/c mice. However, naïve IRF-4^{-/-}CD4⁺ T-cells produced Th2 cytokines including IL-4, IL-5 and IL-10, but not IL-2 or IFN-gamma, at levels higher than wildtype BALB/c CD4⁺ T-cells in response to TCR stimulation. In contrast, effector/memory IRF-4^{-/-}CD4⁺ T-cells did not exhibit increased production of Th2 cytokines. Knockdown of IRF-4 expression using small interfering RNA promoted IL-4 production in naïve CD4⁺ T-cells, but inhibited it in effector/memory CD4⁺ T-cells. These results indicate that IRF-4 plays differential roles in the regulation of Th2 cytokine production in naïve CD4⁺ T-cells and effector/memory CD4⁺ T-cells. IRF-4 inhibits Th2 cytokine production in naïve CD4⁺ T-cells while it promotes Th2 cytokine production in effector/ memory CD4⁺ T-cells.

Introduction

CD4⁺ T-cells play critical roles in the generation of protective immunity against a variety of pathogens by dictating the type of immune response that is effective against each pathogen encountered. The three types of effector CD4⁺ T-cells; Th1, Th2 and Th17, are characterized by their ability to produce signature cytokines IFN-gamma, IL-4 and IL-17, respectively (1, 2). Th2 cells produce IL-4, IL-5, and IL-13, and are responsible for humoral immunity and host immune responses against extracellular parasites. Differentiation of helper T-cells is determined after encounter of naïve CD4⁺ T-cells with antigen (3, 4). Initiation of Th2 differentiation is potentiated by IL-4 during encounter of naïve CD4⁺ T-cells with antigen, but the early source of IL-4 that is important for Th2 differentiation under physiological conditions is unclear (5). Although innate immune cells such as basophils and NK T-cells can produce IL-4, it has been shown that Th2 cells can develop from naïve CD4⁺ T-cells independently of IL-4 produced by non-T-cells (6). Naïve CD4⁺ T-cells themselves can produce small amounts of IL-4 after antigen stimulation, which is sufficient for Th2 differentiation under certain conditions (7). Therefore, IL-4 production by naïve CD4⁺ T-cells must be tightly regulated to coordinate differentiation of effector helper CD4⁺ T-cells. Differentiation of helper CD4⁺ T-cells to Th1 or Th2 is genetically controlled and the BALB/c strain possesses a genetic predisposition toward the development of Th2 cells (8). These strain differences appear to be controlled at several different levels and the underlying mechanisms are not clearly understood (9-11).

Interferon regulatory factors (IRFs) are a family transcription factors that bind to a specific DNA motif known as the IFN-stimulated response element (ISRE) and play

critical roles in a variety of immune processes (12). One of the members, IRF-4, is expressed specifically in lymphocytes and macrophage/dendritic cells (13-17). In contrast to other IRF family members, the expression of IRF-4 in lymphocytes is induced by stimulation of the antigen receptor and plays critical roles for the differentiation of naïve lymphocytes to effectors (13, 18). In T cells, IRF-4 plays a critical role in the differentiation of CD4⁺ T-cells to Th2 and Th17 effectors (19-22). However, IRF-4 is dispensable for Th1 development of CD4⁺ T-cells, and IRF-4^{-/-} CD4⁺ T cells can develop protective immunity during the early phase of *Leishmania major* (*L. major*) infection. In these studies, IRF-4^{-/-} mice of C57BL/6 (B6) genetic background, a Th1-biased strain, were used, and it was not clear whether mice with a Th2-biased genetic background also show defects in Th2 development in the absence of the IRF-4 gene.

To examine the role of IRF-4 in effector T-cell development under a Th2-biased genetic background, we have backcrossed IRF-4^{-/-} mice to the BALB/c strain. These mice did not develop Th2 immune responses even under strong Th2-biased conditions. Surprisingly, however, naïve IRF-4^{-/-}CD4⁺ T-cells produced Th2 cytokines at levels much higher than BALB/c wild-type T-cells, suggesting that IRF-4 negatively regulates production of IL-4 in naïve CD4⁺ T-cells. Further study showed that IRF-4 plays differential roles in the regulation of Th2 cytokine production by naïve vs. effector/memory CD4⁺ T-cells. IRF4 inhibits IL-4 production in naïve CD4⁺ T-cell, while it promotes IL-4 production in effector/memory CD4⁺ T-cells.

Results

IRF-4^{-/-} mice are sensitive to *N. brasiliensis* infection.

IRF-4 KO mice were backcrossed to BALB/c mice (IRF-4^{-/-} mice) to examine the role of IRF-4 in T-cell function under a Th2-biased genetic background. We investigated the response of BALB/c and IRF-4^{-/-} mice to infection with *Nippostrongylus brasiliensis* (*N. brasiliensis*) which normally induces strong Th2-biased immune responses. In BALB/c mice, expulsion of the adult worms occurred within 2 weeks after infection. In contrast, IRF-4^{-/-} mice maintained similar numbers of intestinal worms for more than 3 weeks (Fig. 1A). In addition, IRF-4^{-/-} mice did not show any signs of eosinophilia, a hallmark of the Th2 response, during the course of *N. brasiliensis* infection, while BALB/c mice exhibited eosinophilia with a peak at 2 weeks after infection (Fig. 1B). Expulsion of intestinal adult worms is critically dependent on IL-4 and IL-13 produced by T-cells (23, 24). CD4⁺ T-cells were prepared from the draining lymph nodes of the infected mice and their ability to produce IL-4 was determined by ELISA. CD4⁺ T-cells of BALB/c mice produced IL-4 in response to *N. brasiliensis* antigen, while those from IRF-4^{-/-} mice did not (Fig. 1C). The lack of IL-4 production was not due to a defect in antigen presentation by IRF-4^{-/-} APC during the culture, since IRF-4^{-/-}CD4⁺ T-cells did not produce IL-4 when cultured with wild-type BALB/c APC pulsed with *N. brasiliensis* antigen (data not shown). In addition, IRF-4^{-/-} mice showed Th1-based protective immune responses against infection with *Leishmania. major* (SI Fig. 6). Collectively, these studies established that the Th2 response *in vivo* is critically depended on IRF-4 even in mice genetically biased to Th2.

IRF-4^{-/-} CD4⁺ T cells produce high levels of Th2 cytokines.

During the course of the study, we evaluated the ability of CD4⁺ T-cells to produce IL-4 in response to anti-TCR signals. Unexpectedly, CD4⁺ T-cells from IRF-4^{-/-} mice produced IL-4 at levels higher than that produced by CD4⁺ T-cells from BALB/c mice when stimulated with anti-TCR mAb despite the lack of their antigen-specific IL-4 production (Fig. 1C). Therefore, we examined whether conventional CD4⁺ T-cells expressing alpha/beta TCR that recognize MHC/peptide antigens mediate the high IL-4 responses. CD4⁺ T-cells from BALB/c and IRF-4^{-/-} mice were stimulated with APC pulsed with staphylococcal enterotoxin B (SEB) or with plate-coated anti-TCR mAb, and their ability to produce cytokines was determined (Fig. 2A). IRF-4^{-/-} CD4⁺ T cells produced IL-2 and IFN-gamma at levels lower than BALB/c CD4⁺ T cells, consistent with previous studies (18, 20, 21). These T-cells, however, produced IL-4 and IL-5 at levels much higher than BALB/c CD4⁺ T cells when stimulated with SEB-pulsed APC or anti-TCR mAb (Fig. 2A). To determine whether conventional CD4⁺ T-cells or NK T-cells produce high levels of IL-4 and IL-5, we stimulated these cells with staphylococcal enterotoxin A (SEA), that stimulates T-cells expressing Vbeta1, 3 10, 11, 12 and 17; or with alpha-galactocylceramide (alpha-GalCer) that stimulates Valpha14⁺ NK T-cells to determine whether conventional CD4⁺ T-cells or NK T-cells produce high levels of IL-4 and IL-5 (Fig. 2B) (25). IRF-4^{-/-}CD4⁺ T-cells produced higher levels of IL-4 and lower levels IL-2 and IFN-gamma in response to SEA compared to BALB/c CD4⁺ T cells. To confirm that IRF-4^{-/-} naïve conventional CD4⁺ T-cells produce higher levels of IL-4, we have backcrossed DO11.10 TCR transgenic mice to IRF-4^{-/-}BALB/c mice and examined the cytokine production of naïve DO11.10 IRF-4^{-/-} CD4⁺ T-cells in response to APC pulsed with OVA323-339 peptide. These T-cells

produced higher levels of IL-4 and reduced levels of IFN-gamma in response to OVA323-339 peptide (Fig. 2C), indicating that conventional IRF-4^{-/-}CD4⁺ T cells produced IL-4 at high levels in response to TCR stimulation. In contrast, total CD4⁺ cells from IRF-4^{-/-} mice produced IL-4, IL-2 and IFN-gamma at reduced levels in response to alpha-GalCer (Fig. 2B). The proportion of CD4⁺NK T-cells (DX5⁺ cells) in IRF-4^{-/-} mice was lower than BALB/c mice (SI Fig. 7), partly explaining the reduced IL-4 production of CD4⁺ cells from IRF-4^{-/-} mice in response to alpha-GalCer (SI Fig. 7). Purified CD4⁺DX5⁺ cells from IRF-4^{-/-} mice, however, produced equivalent or higher levels of IL-4 in response to alpha-GalCer than those from BALB/c mice (SI Fig. 7), suggesting that IRF-4^{-/-} CD4⁺DX5⁺ NK T cells are able to produce sufficient levels of IL-4 in response to alpha-GalCer.

IL-4 production by naïve vs. effector/ memory IRF-4^{-/-} CD4⁺ T cells.

Peripheral CD4⁺ T-cells contain both naïve and effector/memory type T-cells that can be distinguished by their cell surface phenotype such as CD62L. Naïve lymphocytes have higher levels of CD62L expression than effector and effector memory T cells (26). The proportion of CD62L⁺ cells in CD4⁺ T-cells was not significantly different between BALB/c and IRF-4^{-/-} mice (data not shown). To determine which cell type produces higher levels of Th2 cytokines in IRF-4^{-/-} mice, we prepared naïve (CD62L⁺) and effector/memory (CD62L⁻) CD4⁺ T-cells, and examined their cytokine production in response to anti-TCR mAb (Fig. 3A). Naïve IRF-4^{-/-}CD4⁺ T-cells produced IL-4 and IL-5 at levels higher than BALB/c naïve CD4⁺ T-cells, and IL-2 at reduced levels. Effector/memory CD4⁺ T-cells from IRF-4^{-/-}BALB/c mice, however, produced IL-4, IL-5 and IL-2 at levels lower than BALB/c CD4⁺ T-cells. CD62L⁺CD4⁺ T-cell

population may contain central memory CD4⁺ T-cells (CD62L⁺CD44⁺) in addition to naïve CD4⁺ T-cells (CD62L⁺CD44⁻). To confirm that naïve CD4⁺ T-cells produce higher levels of IL-4, we purified CD62L⁺CD44⁻CD4⁺ T-cells by sorting and cultured in the presence of anti-TCR mAb (Fig. 3B). CD62L⁺CD44⁻CD4⁺ T-cells from IRF-4^{-/-} mice produced IL-4 at levels higher than those BALB/c mice and IL-2 at reduced levels. Thus, we concluded that naïve IRF-4^{-/-}CD4⁺ T-cells rather than effector/memory IRF-4^{-/-}CD4⁺ T-cells were responsible for the high levels of Th2 cytokines produced. We also determined the expression of Th2 cytokine mRNAs by naïve CD4⁺ T-cells (SI Fig. 8). The expression levels of cytokine mRNA was detected basically in parallel to their protein production. Also, upregulation of GATA3 mRNA expression was observed in naïve IRF-4^{-/-}CD4⁺ T-cells and not in BALB/c CD4⁺ T-cells. Taken together, these results suggest that IRF-4 plays an inhibitory role in the expression of Th2 cytokines in naïve T-cells. This inhibitory effect, however, was not seen in effector/memory type CD4⁺ T-cells.

To examine the mechanisms underlying the differential effect of IRF-4 in naïve and effector/memory T-cells, we compared the expression levels of IRF-4 in BALB/c CD4⁺ T-cells after stimulation with anti-TCR mAb. Naïve (CD62L⁺) CD4⁺ T-cells expressed IRF-4 protein at a level significantly higher than effector/memory (CD62L⁻) CD4⁺ T-cells (Fig. 4A). We examined the kinetics of IRF-4 expression after T-cell activation both at the RNA and protein levels (Fig. 4B,C). The expression of IRF-4 was induced after activation with TCR stimulation in both naïve and effector/ memory CD4⁺ T-cells. When compared with effector/memory CD4⁺ T-cells, however, naïve CD4⁺ T-cells showed higher levels of IRF-4 mRNA expression at early hours after TCR stimulation.

Similarly, intracellular staining of IRF-4 protein indicated that the expression of IRF-4 was induced at higher levels in naïve CD4⁺ cells than effector/memory CD4⁺ cells during the early time point after activation (Fig. 4C). In contrast, the expression of IRF-1 was not induced in naïve CD4⁺ T-cells and was only transiently increased in effector/memory CD4⁺ T-cells (Fig. 4B). These results indicate that the expression pattern of IRF-4 after T-cell activation is distinct in naïve and effector/ memory CD4⁺ cells.

Regulation of Th2 cytokine production by IRF-4

To determine whether the ability of CD4⁺ T cells to express Th2 cytokines is directly regulated by IRF-4, we reconstituted the IRF-4 gene in IRF-4^{-/-} CD4⁺ T cells by transfection. CD4⁺ T-cells reconstituted with IRF-4 produced reduced levels of IL-4 and IL-5 in response to anti-TCR mAb (SI Fig. 9). We next used the siRNA technique to inhibit expression of IRF-4 in CD4⁺ T-cells. Naïve CD4⁺ T-cells were prepared by depletion of CD44^{high} cells from total CD4⁺ T-cells. After transfer of IRF-4 siRNA by electroporation, cells were stimulated with anti-TCR mAb. Naïve CD4⁺ T-cells expressing greatly decreased levels of IRF-4 produced IL-4 at levels much higher than control CD4⁺ T-cells in response to TCR stimulation at both the RNA and protein levels (Fig. 5A, B). We also evaluated the effect of inhibiting IRF-4 expression in effector/memory CD4⁺ T-cells, since these cells normally produce IL-4 at levels higher than IRF-4^{-/-} effector/memory CD4⁺ T-cells. Effector/memory CD4⁺ T-cells were prepared by depletion of CD62L⁺ cells from total CD4⁺ T-cells, transfected with IRF-4 siRNA, and stimulated with anti-TCR mAb. The production of IL-4 by effector/memory CD4⁺ T-cells was significantly inhibited by IRF-4-specific siRNA at

both protein and RNA levels (Fig. 5C, D). These results suggest that IRF-4 expressed in CD4⁺ T-cells differentially regulates Th2 cytokine production in naïve and effector/memory CD4⁺ T-cells. IRF-4 is inhibitory to IL-4 production in naïve CD4⁺ T-cells and is stimulatory in effector/memory CD4⁺ T-cells.

Discussion

We have shown a complete lack of Th2-type immune responses *in vivo* in IRF-4^{-/-} mice of BALB/c genetic background. IRF-4^{-/-} mice were unable to expel *N. brasiliensis* *in vivo* due to the lack of Th2 type effector cells. These results are consistent with our previous reports using IRF-4 knock-out mice on a B6 background, in which we and others showed *in vitro* that Th2 responses of IRF4^{-/-} CD4⁺ B6 T-cells were impaired (19-21). Rengarajan et al. described that IRF-4 physically interacts with NFATc2 and enhances transcriptional activation of the IL-4 promoter (19). In human studies, Hu et al. reported that stable expression of IRF-4 in the Jurkat human T-cell line lead to production of Th2 cytokines and that IRF-4 binds the IL-4 promoter element triggering the transcription of IL-4 (27). These studies utilized immortalized cells, which may represent effector/memory cell types, to evaluate the effect of IRF-4 on IL-4 production, and are consistent with our observation that IRF-4^{-/-}CD4⁺ T cells are defective in Th2 development and that IL-4 production by effector/memory IRF-4^{-/-}CD4⁺ T-cells is much lower than BALB/c CD4⁺ T-cells. This study, however, has added a novel finding that IRF-4 has an inhibitory effect on the production of Th2 cytokines by naïve CD4⁺ T-cells in response to TCR stimulation. These results are seemingly paradoxical but are consistent with the results of IRF-4 knock-down experiments using siRNA in naïve vs.

effector/memory type CD4⁺ T-cells; the inhibition of IRF-4 expression in naïve CD4⁺ T-cells conferred the ability to produce IL-4 on naïve CD4⁺ T-cells, while inhibition of IRF-4 expression in effector/memory CD4⁺ T-cells down regulated IL-4 expression.

Studies on the function of IRF-4 indicated that it could function as either a transcriptional activator or repressor depending upon the context of the DNA-binding sequences and/or protein-interacting partners (14, 19, 28-31). IRF-4 contains an amino-terminal DNA-binding domain and a carboxyl terminal IRF association domain (IAD) which can interact with a variety of proteins including IRFs. IRF-4 interacts with Ets family member PU.1, transcription factor E47, Stat6, Bcl-6 or NFATc2 to synergistically enhance transcriptional activity of a variety of genes including the Ig light chain gene in B cells and IL-4 in T cells (14, 19, 28, 29). However, IRF-4 has been shown to repress gene activation induced by other IRFs, and does not appear to require association with other proteins (30, 31). We have previously shown that IRF-4 inhibits IRF-1-dependent transcription of genes including TRAIL, and that the IRF association domain of IRF-4 was dispensable for this inhibition, suggesting that IRF-4 acts as a natural antagonist of IRF-1 and inhibits its transactivation (31). There are at least three IRF-binding sites located in the IL-4 promoter region, some of which correspond with sequences that negatively regulate IL-4 promoter activity (32, 33). Elser et al. showed that IRF-1 and IRF-2 bind to these sites and inhibit IL-4 promoter activity and that naïve IRF-1^{-/-}CD4⁺ T-cells produce IL-4 at levels higher than wild type (33). Since IRF-1 and IRF-2 are induced by IFN-gamma, these IRFs were believed to mediate the inhibitory role of IFN-gamma on expression of the IL-4 gene. Our study shows that naïve IRF-4^{-/-}CD4⁺ T-cells produce higher levels of IL-4 upon TCR

stimulation. Therefore, both IRF-4 and IRF-1 appear to be required for optimum inhibition of IL-4 production in naïve CD4⁺ T-cells. Unlike IRF-1 and IRF-2, which are constitutively expressed in T-cells, IRF-4 expression is induced by antigen receptor-mediated stimuli (Fig. 4B) (13). Thus, IRF-1 and IRF-4 may cooperate temporally to tightly regulate the induction of IL-4 in naïve CD4⁺ T-cells. Taken together, these studies suggest that IRF-4 plays a dual function in the production of IL-4 in CD4⁺ T-cells; IRF-4 promotes IL-4 production in effector/memory CD4⁺ T-cells in association with NFATc2, and inhibits IL-4 transcription in naïve CD4⁺ T-cells. The precise mechanisms underlying the inhibition of IL-4 transcription by IRF-4 remain to be determined.

The question, then, is how the same transcription factor can have two opposing functions in naïve vs. effector/memory CD4⁺ T-cells. One possibility lies in the differential expression levels of IRF-4 in naïve and effector/memory type CD4⁺ T-cells. IRF-4 expression is induced after activation of T-cells through TCR signaling and reaches higher levels in naïve CD4⁺ T-cells than in effector/memory CD4⁺ T-cells. The early and strong induction of IRF-4 may be required for silencing of IL-4 expression. Second, IRF-4 might affect the signaling of TCR or threshold of T cell activation, which ultimately dictate the differential production of cytokines. This possibility may also explain the lack of Th2 differentiation in IRF-4 KO mice, since the strength of TCR signaling appear to affect the generation of Th1/Th2 cells (34). We have previously reported that IRF-4 regulates the TLR signaling pathway including the activation of MAP kinases and NF- κ B in macrophages (17), and it is possible that IRF-4 affects TCR signaling in T-cells. Third, IRF-4 might interact with partners

distinct from NFAT, which dictates the repressive function of IRF-4 on the IL-4 promoter in naïve CD4⁺ T-cells. However, IL-4 expression is controlled not only by the IL-4 promoter, but by several other regulatory elements including enhancers, silencers and locus control regions which become targets of transcription factors and chromatin remodeling factors (3, 4). There are IRF-binding sites within the silencer region (35). Therefore, IRF-4 may affect not only promoter activity of the target genes, but also the chromatin environment of the target genes in regulating gene expression (36). Our study indicates that IRF-4 is involved in regulating the expression of the majority of Th2 cytokines including IL-4 and IL-5, supporting the view that IRF-4 is involved in the coordinated expression of Th2 cytokines through influences on chromatin structure. Control of Th2 cytokine expression is brought about by the coordinated actions of IRF-4 and a variety of transcriptional elements as well as chromatin structure influences.

Taken together, our findings suggested that IRF-4 plays dual roles in Th2 cytokine production in CD4⁺ T cells. IRF-4 is a negative regulator of Th2 cytokines during the early activation phase of naïve CD4⁺ T cells, while it promotes Th2 development and Th2 cytokine production in effector/memory CD4⁺ T-cells. Th2 T-cells are critical for protection against extracellular parasites and for the pathogenesis of allergic immune responses. Our study reveals critical dual roles of IRF-4 in the regulation of Th2 function.

Materials and Methods

Mice and *N. brasiliensis*

IRF-4 KO mice were initially mated to C57BL/6 mice as described previously (17, 21). IRF-4 KO mice of BALB/c background (IRF-4^{-/-} mice) were generated by backcrossing IRF-4 KO mice onto BALB/c for ten generations and were maintained by intercrossing. The genotype was determined by PCR as described (21). DO11.10 mice, provided by Dr. M. Kubo (Riken, Yokohama, Japan), were crossed with IRF-4^{-/-} mice to generate IRF-4^{-/-}DO11.10 mice. These mice were maintained by intercrossing in the Laboratory Animal Center for Animal Research at Nagasaki University. C57BL/6 (B6) and BALB/c mice were purchased from SLC. Mice were used at 6-8-wks old. All animal experiments were conducted with approval from the Nagasaki University Institutional Animal Care Committee. *N. brasiliensis* was provided by Dr. K. Ishiwata (Jikei Univ., Sch. Med., Tokyo, Japan). Third-stage larvae (L3) of *N. brasiliensis* were injected into mice subcutaneously (500/mouse) at the base of tail as described (37). Worm burden was analyzed after longitudinal dissection of the small intestine. Blood smear was stained using Diff-Quick (Sysmex) and eosinophils were counted under a microscope. *N. brasiliensis* antigens were prepared by freezing and thawing.

Cell culture, ELISA and Flow cytometry

Mesenteric lymph node cells (1×10^5) were cultured in the presence of *N. brasiliensis* antigen. In other T-cell responses, lymphocytes were collected from a mixture of lymph nodes (mesenteric and inguinal) and spleen. CD4⁺ T-cells (>98%) were prepared using anti-CD4 IMag (BD Biosciences). CD62L⁺CD4⁺ T cells were purified by sorting (purity; 97~99%) using FACS Aria (BD Biosciences)(Fig. 3A) or separated using the

CD4⁺CD62L⁺ isolation kit (purity; 70-80 %) (Miltenyi Biotec)(Fig. 4, 5). CD62L⁻CD4⁺ T-cells were purified by sorting (purity; >99 %, Fig. 3A) or were prepared by treating the negative fraction of CD4⁺CD62L⁺ isolation kit with complement at 37° C for 30 min to deplete the remaining CD62L⁺ cells (purity; 93-97 %, Fig. 4, 5). T-cells were stimulated with plate-bound anti-TCRbeta mAb (H57) or mitomycin C treated T-depleted spleen cells pulsed with alpha-Galactosylceramide (KRN7000, Kirin Brewery), Staphylococcal enterotoxin A (SEA)(Toxin Technology) or Staphylococcal enterotoxin B (SEB). Supernatant was collected 48 hrs after stimulation and the levels of cytokines in the supernatants were determined by sandwich ELISA. IL-2, IL-4, and IFN-gamma levels were determined as described previously (17, 21). IL-5 levels were determined by ELISA kit (R&D). We did not find any significant differences in the cytokine profiles between CD62L⁺CD4⁺ or CD62L⁻CD4⁺ T-cells that were purified by sorting and those purified by CD4⁺CD62L⁺ isolation kit (data not shown).

For intracellular staining of IRF-4, CD62L⁺CD4⁺ T-cells and CD62L⁻CD4⁺ T-cells were purified using CD4⁺CD62L⁺ isolation kit, stimulated with plate coated anti-TCR mAb, fixed and permeabilized using Cytofix/Cytoperm kits (BD Pharmingen), and were stained with anti-IRF-4 Ab (Santa-Cruz biotech), biotin-anti-goat IgG Ab and PE-streptavidin. Cells were analyzed using FACScan (BD Biosciences) (Fig. 4C).

Realtime PCR.

RNA was prepared from cells using Isogen (Nippon gene). Total RNA (500ng) was reverse-transcribed to cDNA using random hexamers and real-time PCR was performed

as described previously (17). The mRNA expression was determined as the ratio of each DNA to G3PDH. The sequence of primers for GATA3 and Tbet was described previously (21). The sequences of other primer pairs are shown in Table 1 published as supporting information on the PNAS web site.

Western blotting.

Cells were washed and resuspended in sample buffer. The lysate was size fractionated on 12.5% SDS-PAGE and transferred to a PVDF membrane. The blot was blocked with 5% milk TBS-tween, washed twice with PBS-tween and incubated with anti-IRF-4 Ab (Santa-Cruz). The membrane was incubated with HRP-anti-goat Ig Ab (MBL), washed and visualized using ECL reagent (Amersham Pharmacia). The same blot was stripped and reprobed with anti-actin Ab (Sigma).

Transfection and RNA interference assay

Full length mouse IRF-4 cDNA was cloned into pcDNA3 (Invitrogen). CD4⁺ T-cells were prepared using CD4⁺ T-cell isolation kit (Milteny Biotec) and AutoMACS, and were transfected with the plasmid DNA (30microgram) using an Amaxa Nucleofector apparatus (Amaxa) in a 2.0 mm electroporation cuvette according to the protocol X-01. Three hrs later, cells (5×10^5) were washed and seeded in 96-well flat bottom plates coated with anti-TCR Ab and cultured for 48 h.

Double-stranded RNA for IRF-4 and control (22) were purchased from Takara bio. CD4⁺ T-cells were prepared using the CD4⁺ T-cells isolation kit (Milteny Biotec). Naive and effector/memory CD4⁺ T-cells were prepared from these cells by negative

selection using biotin-anti-CD44 mAb plus streptavidin-microbeads and FITC-anti-CD62L plus anti-FITC microbeads, respectively, followed by AutoMACS. Cells were transfected with FITC-labeled dsRNA (100pmol) in a 2.0 mm electroporation cuvette according to the protocol X-01 using an Amaxa Nucleofector apparatus, and were cultured on plates coated with anti-TCR mAb for 4-48 h. The proportion of cells that incorporated the dsRNA was ~97% as assessed by flow cytometry.

Statistics

Significance levels were determined by Mann-Whitney's *U* test for unpaired observations. Results were considered significant when $P < 0.05$.

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Figure legends

Figure 1

IRF-4^{-/-} mice are susceptible to *N. brasiliensis* infection.

(A) BALB/c (open bar) and IRF-4^{-/-} mice (dark bar) were infected with *N. brasiliensis* (500 organisms) subcutaneously at the base of the tail. Worm burden was analyzed after longitudinal dissection of the small intestine. The data represents the mean \pm SD with 3 mice per group. (B) Eosinophils in the peripheral blood were counted under a microscope. (C) Mesenteric lymph node cells (1×10^5) were collected on the indicated days after infection, and were cultured in the presence of *N. brasiliensis* antigen (upper panel) or on plates coated with anti-TCR mAb (lower panel) for 48 h. The IL-4 levels in the supernatant were determined by ELISA. Representative results of three independent experiments are shown.

Figure 2.

Conventional CD4⁺ T cells from IRF-4^{-/-} mice produce IL-4 at levels higher than wild type CD4⁺ T cells.

(A) Splenic CD4⁺ T cells (1X10⁵) from BALB/c (open circle) or IRF-4^{-/-} (closed circle) mice were cultured with mitomycin C-treated T-depleted spleen cells (5X10⁵) pulsed with SEB or on plates coated with anti-TCR Ab for 48 h at the indicated concentrations. The cytokine levels in the supernatant were determined by ELISA.

(B) Splenic CD4⁺ T cells (2X10⁵) from BALB/c (open circle) or IRF-4^{-/-} (dark circle) mice were cultured in the presence of mitomycin C-treated T-depleted spleen cells (5X10⁵) and SEA or alpha-GalCer at the indicated concentrations for 48h.

(C) Naïve CD4⁺ T-cells (1X10⁵CD62L⁺CD4⁺ T cells) from DO11.10 (open circle) or IRF-4^{-/-}DO11.10 (closed circle) mice were stimulated with mitomycin C-treated T-depleted spleen cells (4X10⁵) in the presence of OVA₃₂₃₋₃₃₉ peptide (0-10microgram/ml) for 48 h. Representative results of three independent experiments are shown.

Figure 3. Th2 cytokine production by naïve vs. effector/memory CD4⁺ T-cells is differentially regulated in IRF-4^{-/-} mice.

(A) Naïve (CD62L⁺) or effector/ memory (CD62L⁻) CD4⁺ T-cells (1X10⁵/well) from BALB/c (open circle) or IRF-4^{-/-} (closed circle) mice were purified by sorting and were cultured with plates coated with anti-TCR mAb for 48h. The percentage of CD62L⁺ cells and CD62L⁻ cells within BALB/c and IRF-4^{-/-} CD4⁺ T-cells was 97-99 and >99%, respectively. Cytokine levels in the supernatant were determined by ELISA. Representative results of three independent experiments are shown. (B) CD62L⁺CD44⁻CD4⁺ T-cells (2X10⁵) from BALB/c (open circle) or IRF-4^{-/-} (closed circle) mice were purified by sorting and were cultured on plates coated with anti-TCR mAb for 48h. The purity of CD62L⁺CD44⁻CD4⁺ T-cells was >92 %.

Figure 4.

Induction of IRF-4 expression in naïve and effector/memory CD4⁺ T-cells after TCR stimulation.

(A) CD62L⁺ and CD62L⁻CD4⁺ T-cells were prepared from BALB/c mice and were cultured on plates coated with or without anti-TCR mAb (1microgram/ml for A and B, 10microgram/ml for C). After culture for 16 h, cells were lysed, separated on 12.5% SDS-PAGE, blotted and probed with anti-IRF-4 Ab. The membrane was stripped, and re-probed with anti-actin Ab. (B) After culture for 0-24 h, total RNA was prepared and the levels of IRF mRNA were determined by real-time PCR. The mRNA expression was expressed as the ratio of DNA to G3PDH. * Mann-Whitney's *U* test between naïve and effector/memory: $P < 0.05$ (C) After culture for 0-72 h, cells were fixed, permeabilized and stained with anti-IRF-4 Ab, biotin-anti-goat IgG, and PE-streptavidin. Gray shadow represents staining of the cells at each time point with biotinylated anti goat-IgG and streptavidin-PE only without anti-IRF-4 Ab. Relative mean fluorescent intensity (MFI) of IRF-4 staining was expressed as the ratio of MFI between the staining with and without anti-IRF-4 Ab. Representative results of three independent experiments are shown. *: $P < 0.05$ between naïve and effector/memory

Figure 5.

The levels of IRF-4 expression in CD4⁺ T-cells affect the levels of Th2 cytokine production.

Naïve CD4⁺ T-cells (CD44^{low}) (A, B) and Effector/memory CD4⁺ T-cells (CD62L⁻) (C, D) from BALB/c mice were transfected with IRF-4-specific (IRF-4) or control (Scr) siRNA (100pmol). Cells were stimulated with plate-bound anti-TCR mAb for 48 h (A), 60 h (C) or 4 h (B, D). The expression of IRF-4 protein was determined by Western-blot analysis using cells stimulated with anti-TCR mAb at the concentrations of 0 (-) and 2.5 (+) microgram/ml (A, C). The blot was stripped and reprobed with anti-actin Ab. The IL-4 levels in the supernatant were determined by ELISA (A, C). After culture for 4 h on plates coated with anti-TCR mAb (3microgram/ml), RNA was prepared and the levels of IL-4 were determined by real-time PCR (B, D).

Figure 1.

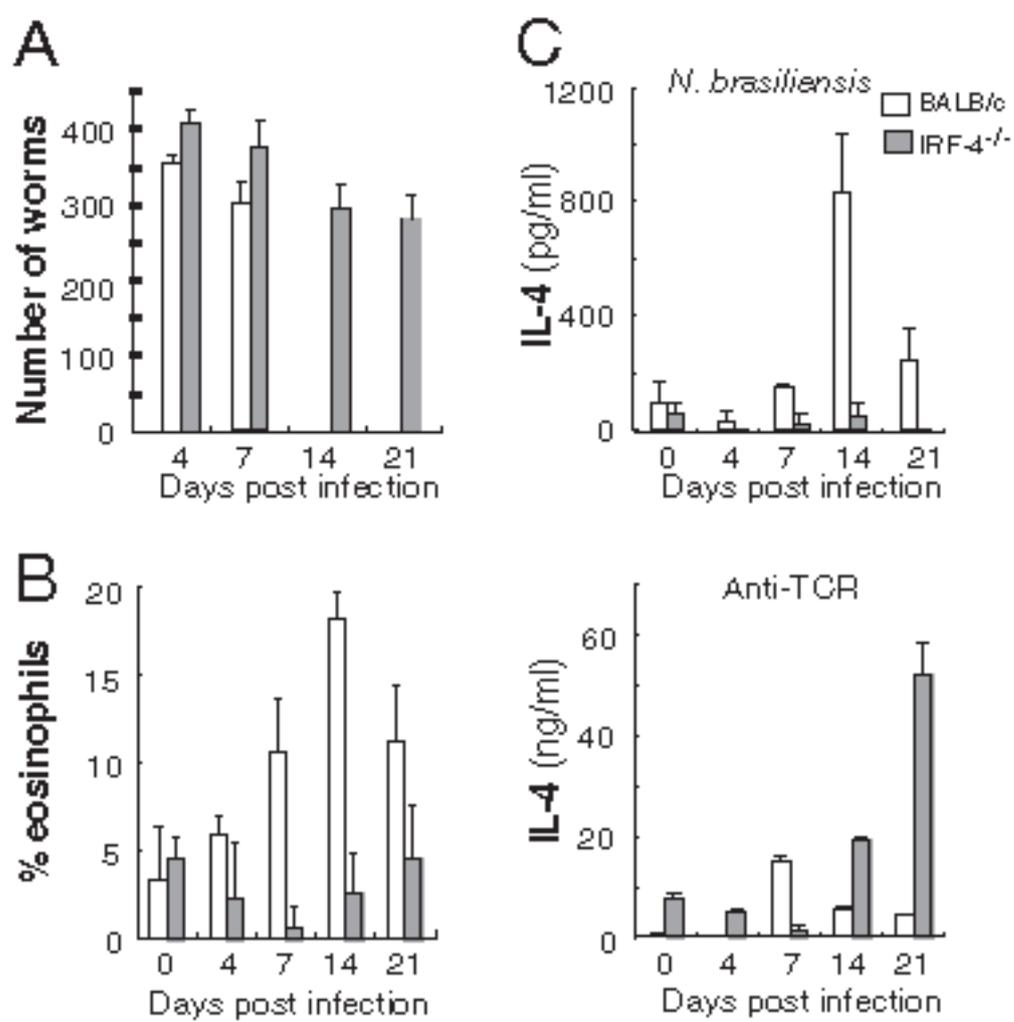


Figure 2.

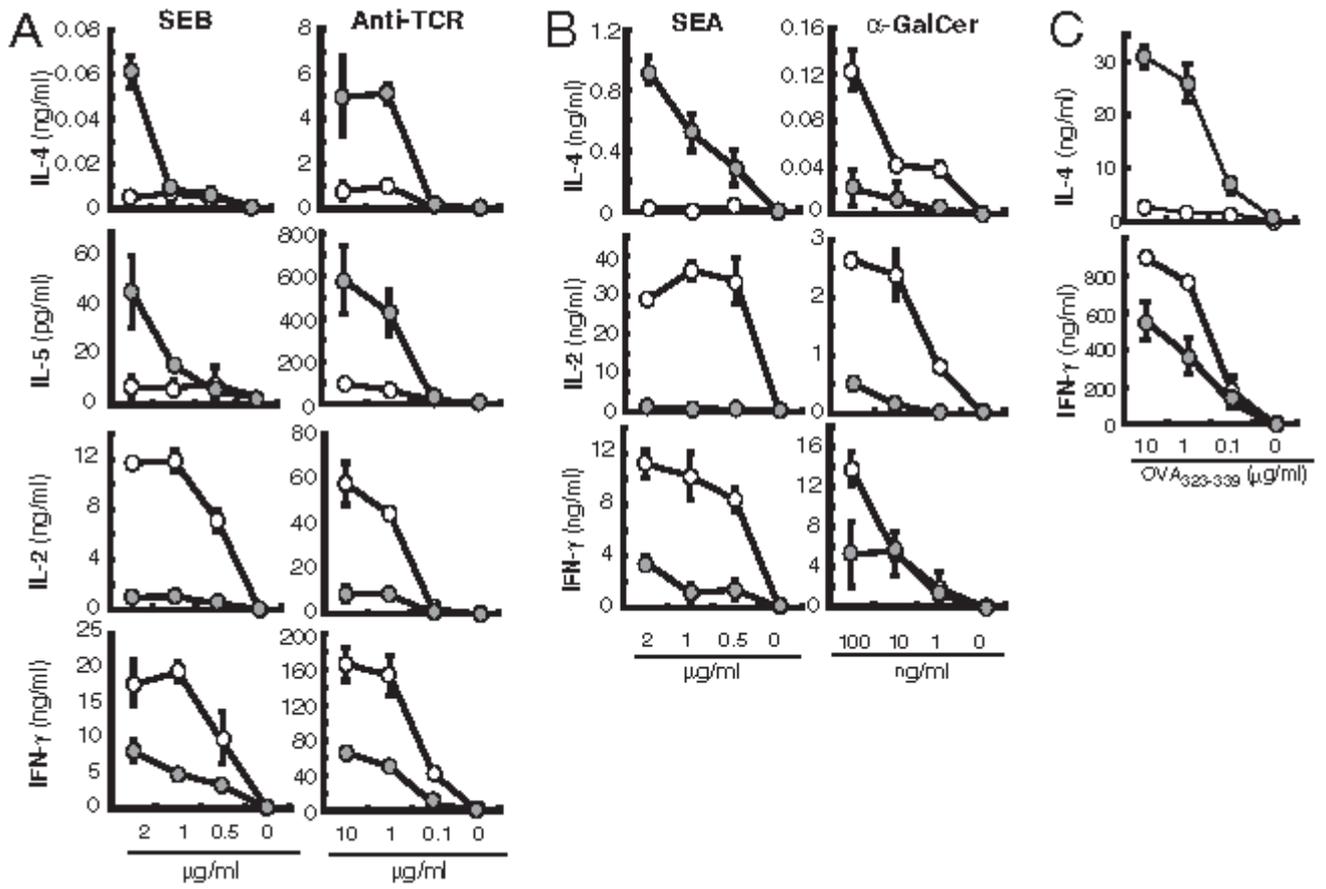


Figure 3.

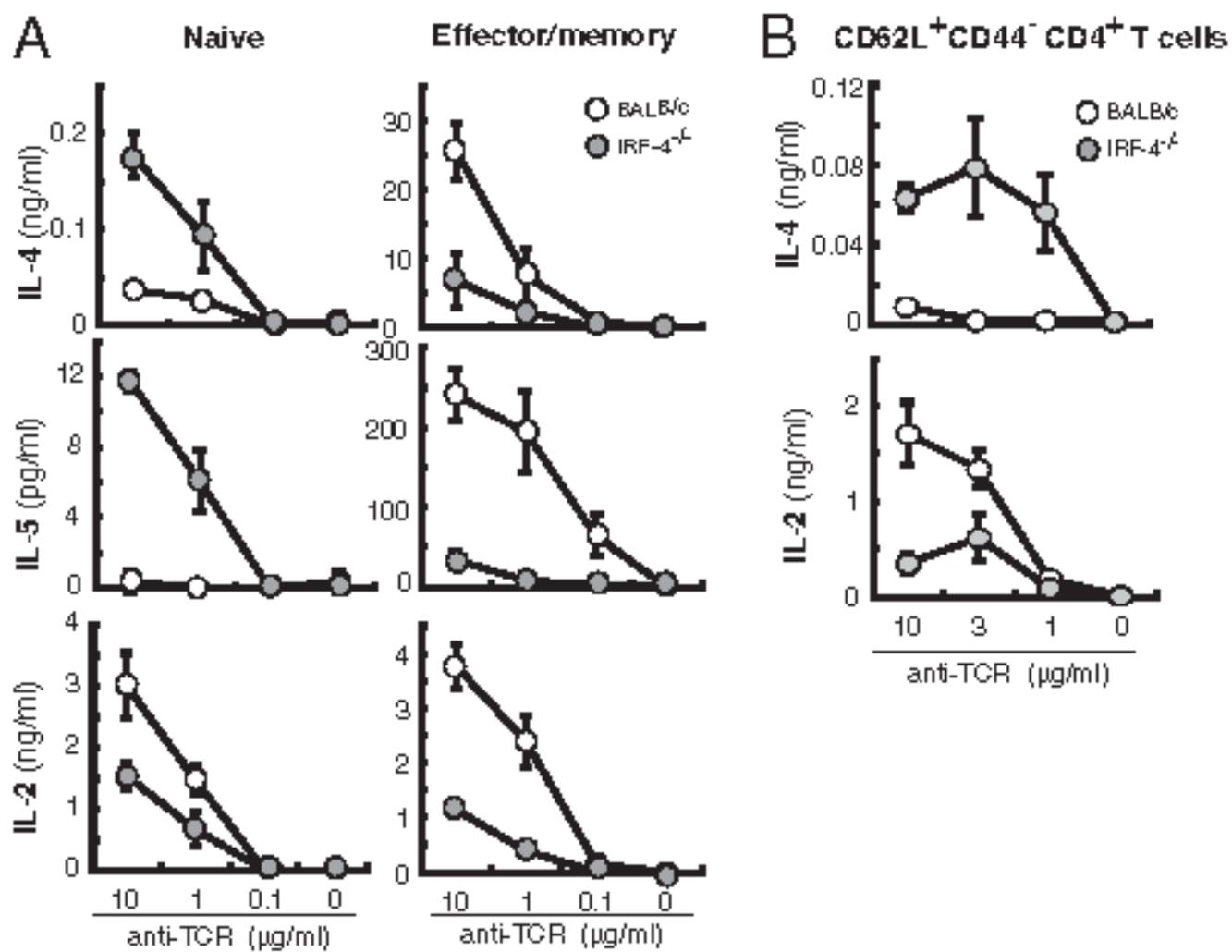


Figure 4.

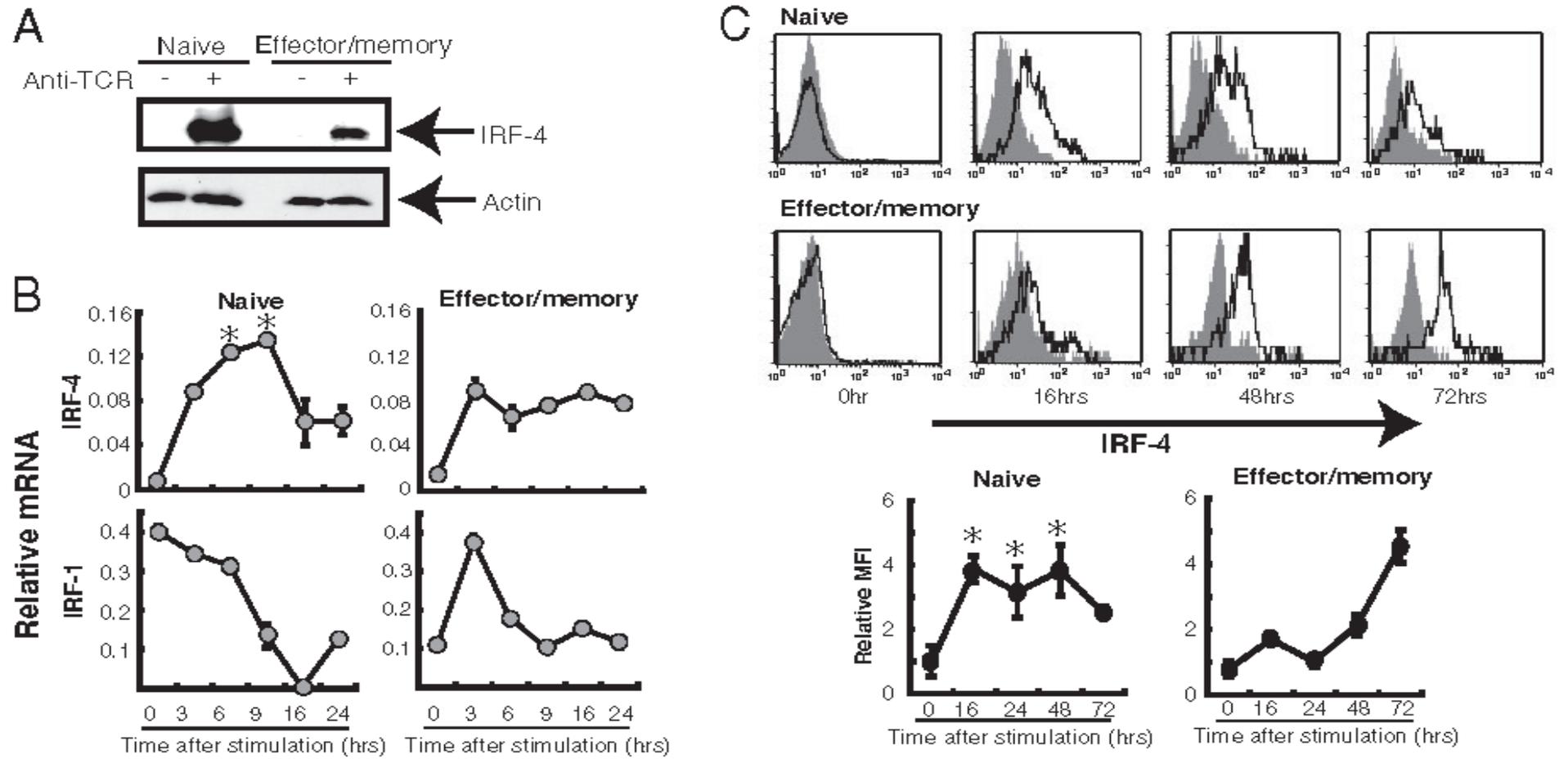


Figure 5.

