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Reciprocal action of interferon-γ and interleukin-4 promotes granulomatous inflammation induced by *Rhodococcus aurantiacus* in mice

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SUMMARY

An intravenous injection of *Rhodococcus aurantiacus* to mice causes granulomatous inflammation dependent on endogenous interferon-γ (IFN-γ). The present study examined the role of endogenous interleukin-4 (IL-4) on granulomatous inflammation. Endogenous IL-4 in the spleen extracts was not detected during the phase of granuloma formation by enzyme-linked immunosorbent assay (ELISA). However, IL-4 protein level was elevated during the phase of granuloma regression. IL-4 mRNA expression in the livers and spleens was also elevated during the phase of granuloma regression. In addition, IL-4 levels during the phase of granuloma formation were increased by treatment with anti-IFN-γ monoclonal antibody (mAb), suggesting that endogenous IFN-γ might inhibit IL-4 production during the phase of granuloma formation. Administration of anti-IL-4 mAb on weeks 3 and 4 after the inoculation inhibited the regression of granulomas and augumented IFN-γ level at 5 weeks. Endogenous IFN-γ was produced by $CD4^+$ T cells during the phase of granuloma regression and endogenous IL-4 was produced by both $CD4^+$ and $CD8^+$ T cells. These findings suggest that during the phase of granuloma formation endogenous IL-4 might be inhibited by IFN-γ, while during the phase of granuloma regression endogenous IL-4 might play a crucial role in the reduction of granulomas and IFN-γ production.

INTRODUCTION

Rhodococcus aurantiacus (*Tsukamurella paurometabolum*), which is a psychronic acid-fast bacterium and is closely related to members of the genera *Corynebacterium*, *Mycobacterium* and *Nocardia*.[1,2] *Rhodococcus* species, including *R. aurantiacus*, have recently been recognized as human and animal pathogens that caused pulmonary infection, granuloma formation and abscess in the skin, lung and brain.[3–6] We previously reported that an intravenous injection of *R. aurantiacus* into mice induced granuloma formation and that the histological construction of granulomas and interferon-γ (IFN-γ) production in the tissues of *R. aurantiacus*-inoculated mice resembled those seen with sarcoidosis patients.[7–9] The non-necrotic granulomas in the liver, spleen and lung develop until 3 weeks after *R. aurantiacus* inoculation and then regress until 5 weeks. In addition, granuloma formation is dependent on the biphasic production of endogenous IFN-γ which was produced by natural killer (NK) cells soon after the inoculation (within 1 day) and by $CD8^+$ T cells during the phase of granuloma formation (1–3 weeks).[8,9]

During the immune response to microbial infections, peripheral T cells develop into functionally distinct subpopulations.[10] T helper (Th)1 cells produce IFN-γ and interleukin-2 (IL-2) and induce cell-mediated immunity. Th2 cells produce IL-4, IL-5, IL-6 and IL-10 and support the regulation of humoral immune responses. IFN-γ inhibits the differentiation and effector functions of Th2 cells and can lead to a dominant Th1 response. Conversely, IL-4 and IL-10 inhibit Th1-cell proliferation and oppose the effects of IFN-γ on macrophages.[11] Therefore, reciprocal regulation occurs between Th1- and Th2-cell subsets and the balance between the secreted cytokines is important for the resistance against pathogens, such as *Leishmania major*[12] and *Schistosoma mansoni*.[13] In addition, the Th2 response seems to play an important role in ameliorating the tissue damage occurring in the course of the protective function of Th1-like cells responding to an infectious agent or in autoimmune diseases.[14]

Granuloma formation induced by *R. aurantiacus* is mediated by Th1-type cells until 3 weeks and then the developed granulomas spontaneously diminish.[8] To confirm whether IL-4 secreted from Th2-type cells participates in ameliorating granulomatous inflammation, we monitored endogenous IL-4 in the livers and spleens of *R. aurantiacus*-

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; NK, natural killer; Th, T helper.

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inoculated mice and investigated the effect of *in vivo* administration of anti-IL-4 monoclonal antibody (mAb) to the mice. In addition, we investigated the reciprocal regulation between IFN-γ and IL-4 in the course of granulomatous inflammation. The fate of developed granulomas and elevated IFN-γ from 3 weeks after the inoculation might be regulated by endogenous IL-4.

MATERIALS AND METHODS

Animals and micro-organisms

Female ddY mice (obtained from SLC, Hamamatsu, Shizuoka, Japan), 4-weeks old, were used. *R. aurantiacus* strain 80005[2] was kindly provided by Y. Kato, Osaka Research Institute, Sawai Pharmaceutical Co. Ltd., Osaka, Japan and I. Yano, Department of Bacteriology, Osaka City University Medical School, Osaka, Japan. Mice were intravenously injected with 10^8 colony-forming units (CFU) of viable *R. aurantiacus* suspended in 0·2 ml of saline.

In vivo *depletion of T-cell subsets*

Hybridoma cell lines GK1.5 (anti-CD4, rat immunoglobulin G2a; IgG2a)[15] and 53-6.72 (anti-CD8, rat IgG2a)[16] were used. The mAbs in ascitic fluids were partially purified by 50% $(NH_4)_2SO_4$ precipitation. Normal rat globulin, prepared as described previously,[17] was used as a control. The mice were given an intravenous 400-μg injection of mAbs or normal rat globulin one day before *R. aurantiacus* inoculation and also on weeks 1 and 2 after the inoculation, and then the organs were harvested at 3 weeks. Other groups of mice were injected with mAbs or normal rat globulin on weeks 3 and 4 after the inoculation, and then the organs were harvested at 5 weeks. We checked the level of T-cell depletion in the spleens obtained from *R. aurantiacus*-inoculated mice in each experiment by flow cytometric analysis (FACScan, Becton Dickinson, Mountain View, CA). Three injections of anti-CD4 mAb or anti-CD8 mAb until 2 weeks after the inoculation could deplete >90% of either $CD4^+$ or $CD8^+$ cells at 3 weeks, respectively. Two injections of anti-CD4 mAb or anti-CD8 mAb on weeks 3 and 4 after the inoculation could deplete >90% of either $CD4^+$ or $CD8^+$ cells, respectively. Treatment with combination of anti-CD4 mAb and anti-CD8 mAb on 3 and 4 weeks after the inoculation could deplete >90% of either $CD4^+$ or $CD8^+$ cells at 5 weeks.

In vivo *depletion of endogenous IFN-γ or IL-4*

Rat anti-murine IFN-γ mAb (hybridoma R4-6A2)[18] and anti-murine IL-4 mAb (11B11, rat IgG1, purchased from the American Type Culture Collection, Rockville, MD)[19] were partially purified by 50% $(NH_4)_2SO_4$ precipitation.[20] To deplete endogenous IFN-γ or IL-4 *in vivo*, 1 mg of mAb was injected intravenously 2 hr before the inoculation and on weeks 1 and 2, or on weeks 3 and 4 after the inoculation. Normal rat globulin was injected as a control.[8]

Histology

The size of granuloma in the liver section was calculated by diameter of each granuloma measured with an ocular micrometer as described previously.[8]

Preparation of organ extracts

The organs suspended in RPMI-1640 medium containing 1% 3-((cholamidopropyl) dimethyl-ammonio)-1-propane-sulphonate (CHAPS: Wako Pure Chemicals Co., Kyoto, Japan) (10% w/v) were homogenized and then the homogenates were centrifuged at 2000 *g* for 20 min. The supernatants were stored at −70° until cytokine assays.

Assays for IFN-γ and IL-4

IFN-γ assay was carried out by a double sandwich enzyme-linked immunosorbent assay (ELISA) as described previously.[21] IL-4 assay was carried out by ELISA using purified rat anti-murine IL-4 mAb (11B11) and biotinylated rat anti-murine IL-4 mAb (BVD6-24G2) (rat IgG1) (Pharmingen, San Diego, CA). The assay was determined to signficantly detect IL-4 concentration as low as 10 pg/ml in the organ extracts.

ELISPOT assay

An enzyme-linked immunospot (ELISPOT) assay was performed to enumerate IL-4-producing cells in the spleens. The procedure was similar to that described by Czenskinsky *et al.*[22] Splenocytes (10^6 cells/well) were incubated with or without heat-killed *R. aurantiacus* (10^6 CFU/well) at 37° overnight on a 96-well plate coated with anti-IL-4 mAb (11B11). After washing with 0·01% Tween 20-phosphate-buffered saline (PBS), biotinylated anti-IL-4 mAb (BVD6-24G2) (Pharmingen) was added on the wells. Streptavidin–alkaline phosphatase (Dako, Denmark) was used as the secondary layer of reagents. The alkaline phosphatase reaction was developed with 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine (Wako) in 50 mM Tris-HCl, pH 7·6 containing 0·5% agar. Number of spots was determined as IL-4-producing cells.

Reverse transcription and PCR

The organ (0·1 g) was homogenized in 1 ml of solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7·0; 0·5% sarcosyl) and extracted with phenol–chloroform solution.[23] The aqueous extracts were precipitated with equal volume of isopropanol and the resultant RNA pellets were washed with 70% ethanol. The RNA was resuspended in autoclaved water, quantified by 260-nm optical density readings, and stored at −80°. cDNAs were synthesized from 1 μg of total RNA using random hexamer (USB Co., Cleveland, OH) as a primer, 1 mM dNTPs (Pharmacia, Uppsala, Sweden) and Moloney-murine leukaemia virus (MMLV)-reverse transcriptase (USB) in 20 μl of RT buffer (50 mM Tris, pH 8·3; 5 mM KCl, 3 mM $MgCl_2$). Total cDNA synthesized in 20 μl reactions were amplified for 40 cycles in 80 μl reactions using 1·25 U Ampli Taq polymerase (Pharmacia, Uppsala, Sweden), a final concentration of 0·5 mM dNTPs and the primers for IL-4 (Stratagene, La Jolla, CA). Polymerase chain reaction (PCR) cycles (Perkin Elmer Cetus, Norwalk, CT) were 93° denaturation (30 seconds), 56° annealing (29 seconds), and 72° extension (2 min). Samples were reverse transcribed and amplified using primers specific for β-actin (Stratagene, La Jolla, CA), to verify that RNA was intact and could be reverse transcribed and amplified. The amplified products were run on a 4% agarose gel.

Statistical analysis

Each experiment was repeated at least three times and accepted as valid only when similar results were obtained. All data were expressed as mean ± standard deviation (SD). Significance of difference between the values in experimental and control groups was calculated by Student's *t*-test and values of $P \leq 0{\cdot}05$ were considered significant.

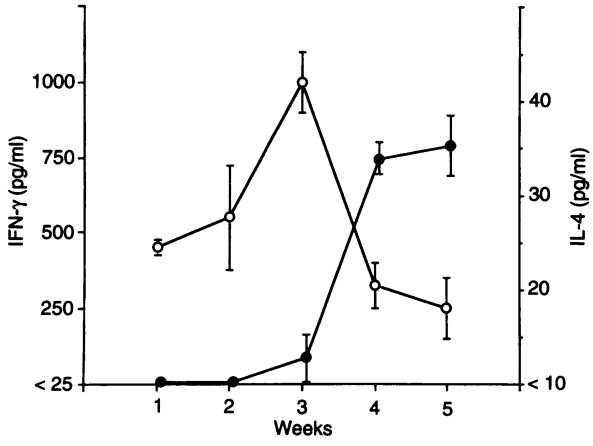

Figure 1. Kinetics of IFN-γ production (open circles) and IL-4 production (filled circles) in the spleen extracts of *R. aurantiacus*-inoculated mice. Each point represents the mean ± SD for six to 10 mice.

RESULTS

Detection of endogenous IL-4 and IFN-γ

The spleen extracts obtained from the *R. aurantiacus*-inoculated mice were used for detection of IL-4 by ELISA. IL-4 level in the spleen extracts of normal mice was lower than 10 pg/ml. Endogenous IL-4 level was lower than 10 pg/ml until 2 weeks after the inoculation and then raised from 3 weeks (Fig. 1). Endogenous IFN-γ in the spleen extracts reached a peak at 3 weeks after the inoculation and the result was verified as described in a previous paper.[8]

PCR analysis of IL-4 mRNA expression in the organs after *R. aurantiacus* inoculation

We investigated IL-4 mRNA expression at time points in the host response to *R. aurantiacus* inoculation. IL-4 mRNA expressions in the livers and spleens of normal mice were not detected. Low levels of IL-4 mRNA expression were detected in the livers and spleens of *R. aurantiacus*-inoculated mice at 2 and 3 weeks after the inoculation. IL-4 mRNA expression in both tissues was increased from 4 weeks after the inoculation (Fig. 2). The results of Figs 1 and 2 show that endogenous IL-4 is produced during the phase of granuloma regression.

Effect of treatment with anti-IFN-γ mAb on endogenous IL-4 during the phase of granuloma formation

To examine whether high amounts of endogenous IFN-γ inhibit the production of endogenous IL-4 until 3 weeks after the inoculation, *R. aurantiacus*-inoculated mice were administered with anti-IFN-γ mAb or normal rat globulin 2 hr before the inoculation and on weeks 1 and 2 after the inoculation, and then endogenous IL-4 in the spleen extracts was determined by ELISA at 3 weeks after the inoculation. IL-4 level in anti-IFN-γ mAb-treated mice was significantly increased ($P < 0{\cdot}01$) (Table 1). We previously reported that endogenous IFN-γ at 1 to 3 weeks after the inoculation was secreted from $CD8^+$ T cells.[8] Therefore we examined the effect of treatment with mAbs against T-cell subsets on IL-4 production. Endogenous IL-4 was augmented by depletion of $CD8^+$ T cells ($P < 0{\cdot}01$), but not by depletion of $CD4^+$ T cells (Table 1).

In addition, ELISPOT assay was carried out using the splenocytes stimulated *in vitro* with or without heat-killed *R. aurantiacus*. As compared with number of IL-4 producing cells in the splenocytes obtained from normal rat globulin-treated mice, the number of IL-4 producing cells in anti-IFN-γ mAb-treated mice was significantly increased ($P < 0{\cdot}01$) (Table 1).

Effect of treatment with mAb against IFN-γ or IL-4 on the cytokine secretion during the phase of granuloma regression

During the phase of granuloma regression, endogenous IL-4 was increased, whereas endogenous IFN-γ was decreased. To

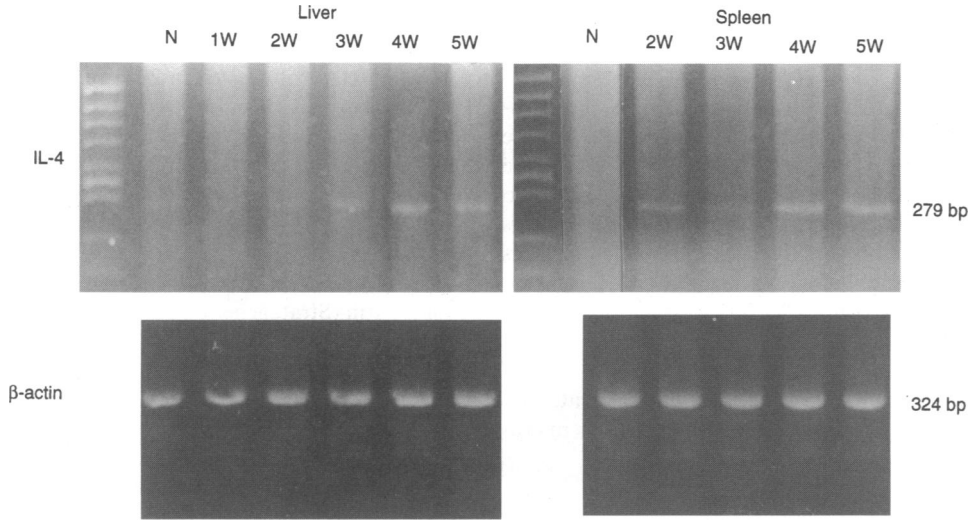

Figure 2. IL-4 and β-actin mRNA profiles in the livers obtained from normal mice (lane N) and *R. aurantiacus*-inoculated mice from 1 to 5 weeks (lane 1W–5W), in the spleens obtained from *R. aurantiacus*-inoculated mice from 2 to 5 (lane 2W–5W) weeks and normal mice (lane N). The extracts of livers and spleens were taken from three mice.

Table 1. Effect of treatment with mAb against IFN-γ or T-cell subsets on endogenous IL-4 production

Globulin injected*	ELISA† IL-4 (pg/ml)	ELISPOT assay† (spots/10^6 cells) Stimulation without	with heat-killed *R. aurantiacus*
Normal rat globulin	13 ± 3	5 ± 2	10 ± 5
Anti-IFN-γ mAb	885 ± 35‡	90 ± 20‡	115 ± 2‡
Anti-CD4 mAb	10 ± 3	ND§	ND
Anti-CD8 mAb	606 ± 19‡	ND	ND

* Mice were injected with anti-IFN-γ mAb 2 hr before *R. aurantiacus* inoculation and on weeks 1 and 2 after the inoculation, or with anti-CD4 mAb or anti-CD8 mAb 1 day before the inoculation and on weeks 1 and 2 after the inoculation.

† The spleens were taken at 3 weeks after the inoculation for ELISA and ELISPOT assay. Each result represents the mean ± SD for six mice per group.

‡ Significantly different from the value for normal rat globulin-treated group ($P < 0.01$).

§ Not determined.

determine the reciprocal relationship between IFN-γ and IL-4, *R. aurantiacus*-inoculated mice were injected with normal rat globulin or mAb against IFN-γ or IL-4 on weeks 3 and 4 after the inoculation, and then the mice were killed at 5 weeks. IFN-γ level in anti-IL-4 mAb-treated mice was significantly increased ($P < 0.01$) (Fig. 3a), whereas IL-4 production was not significantly affected by treatment with anti-IFN-γ mAb (Fig. 3b).

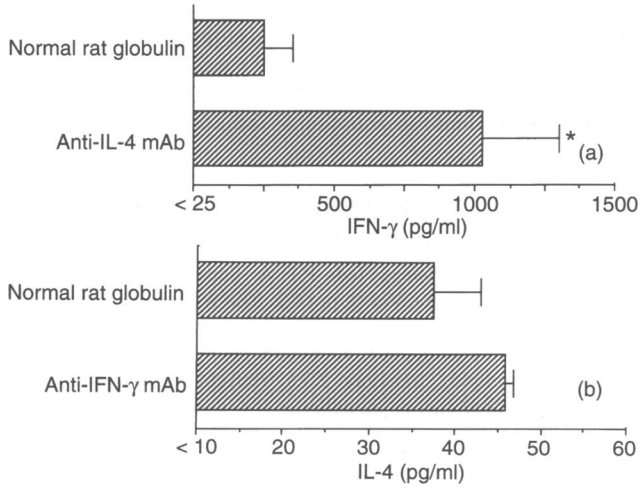

Figure 3. Effect of treatment with mAb against IL-4 or IFN-γ on IFN-γ production (a) or IL-4 production (b), respectively. Mice were injected with the globulins on weeks 3 and 4 after the inoculation and the spleens were taken at 5 weeks for assays of IFN-γ and IL-4. Each value represents the mean ± SD for a group of six mice. Symbol: *significant difference from the value for normal rat globulin-treated mice (control) at $P < 0.01$.

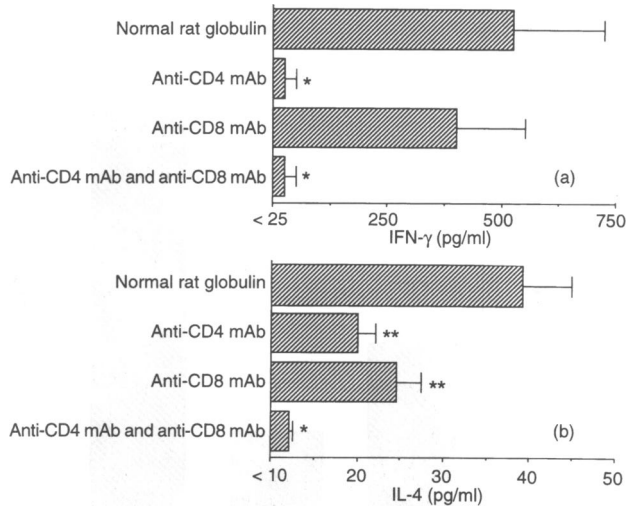

Figure 4. Effect of treatment with mAb against T-cells on IFN-γ (a) and IL-4 (b) production. Mice were injected with the globulins on weeks 3 and 4 after the inoculation and the spleens were taken at 5 weeks. Each value represents the mean ± SD for a group of six mice. Symbol: * and **, significant difference from the value for normal globulin-treated mice at $P < 0.01$ and $P < 0.05$, respectively.

Effect of treatment with mAb against T-cell subsets on IFN-γ and IL-4 production

To identify T-cell subsets which regulate the production of IFN-γ or IL-4 during the phase of granuloma regression, mice were injected with normal rat globulin or mAb against T-cell subsets on weeks 3 and 4 after the inoculation and then endogenous IFN-γ and IL-4 in the spleen extracts were determined at 5 weeks. Endogenous IFN-γ was inhibited by treatment with anti-CD4 mAb or the combination of anti-CD4 mAb and anti-CD8 mAb ($P < 0.01$) (Fig. 4a). Endogenous IL-4 was partially decreased by treatment with anti-CD4 mAb or anti-CD8 mAb ($P < 0.05$), and IL-4 was strongly decreased by treatment with the combination of anti-CD4 mAb and anti-CD8 mAb ($P < 0.01$) (Fig. 4b).

Effect of mAb against IFN-γ or IL-4 on granuloma regression

To assess the role of IFN-γ and IL-4 in granuloma regression, *R. aurantiacus*-inoculated mice were injected with normal rat globulin or mAb against IFN-γ or IL-4 on weeks 3 and 4 after the inoculation and then the livers were taken at 5 weeks for histological findings. Granuloma size in anti-IFN-γ mAb-treated mice at 5 weeks after the inoculation was slightly diminished as compared with that in the control mice. Treatment with anti-IL-4 mAb resulted in increased granuloma size at 5 weeks after the inoculation ($P < 0.01$) (Fig. 5). The granulomas in the livers of anti-IL-4 mAb-treated mice were composed of epithelioid cells and they were similar to granulomas which were routinely observed at 3 weeks after the inoculation.

DISCUSSION

In this study, we demonstrate that granulomas induced by

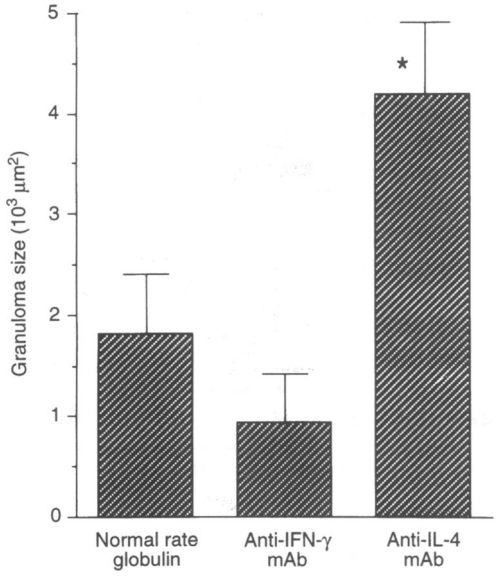

Figure 5. Effect of treatment with mAb against IFN-γ or IL-4 on granuloma regression in the livers of *R. aurantiacus*-inoculated mice at 5 weeks after the inoculation. Each value represents the mean ± SD for a group of six mice. Symbol: * significant difference from the value for normal globulin-treated mice (control) at $P < 0.01$.

R. aurantiacus developed and regressed as a consequence of the balance between endogenous IFN-γ and IL-4. Endogenous IL-4 was not detected during the phase of granuloma formation (Figs 1 and 2). However, IL-4 was increased by treatment with anti-IFN-γ mAb or anti-CD8 mAb during the phase of granuloma formation and the number of IL-4-producing cells was also increased by treatment with anti-IFN-γ mAb (Table 1). Endogenous IL-4 during the phase of granuloma formation is thought to be strongly inhibited by $CD8^+$ T cells or IFN-γ secreted from $CD8^+$ T cells.[8] In a murine model of cutaneous leishmaniasis, administration of anti-IFN-γ mAb to genetically resistant mice was shown to interfere with the development of a Th1-type response and to promote differentiation of a Th2-type response.[24,25] Endogenous IFN-γ plays an important role in the resistance against intracellular pathogens, e.g. *Listeria monocytogenes*, and also promotes early immunological and inflammatory responses.[20] The early IFN-γ is though to inhibit IL-4 production and anti-inflammatory properties via Th2-type cells.[26] In the host response to *R. aurantiacus*, IFN-γ may inhibit the development of Th2-type cells and cause granulomatous inflammation to progress until 3 weeks after the inoculation.

IFN-γ production and granuloma size reached to the peaks at 3 weeks after the inoculation and then decreased.[8] Conversely, IL-4 mRNA expression in the livers and spleens was increased at 4 and 5 weeks after the inoculation (Fig. 2) and the protein levels of IL-4 in the spleen extracts were also increased at 4 and 5 weeks (Fig. 1). Endogenous IL-4 during the phase of granuloma regression inhibited IFN-γ production, because the IFN-γ level at 5 weeks after the inoculation was increased by treatment with anti-IL-4 mAb (Fig. 3). However, treatment with anti-IFN-γ mAb from 3 weeks after the inoculation did not inhibit IL-4 production at 5 weeks, suggesting that IL-4 was not regulated by IFN-γ during the phase of granuloma regression. A Th2-type cytokine's dominant response was observed during the phase of granuloma regression, whereas a Th1-type cytokine's dominant response was observed during the phase of granuloma formation. A shift from a type 1- to type 2-cytokine profile, similar to that in individuals infected with human immunodeficiency virus during progression toward acquired immune deficiency syndrome (AIDS), was observed in *R. aurantiacus*-infected mice.[27,28]

Although the cells producing IFN-γ are $CD8^+$ T cells at 1 to 3 weeks after the inoculation,[8] IFN-γ during the phase of granuloma regression, appears to be produced by $CD4^+$ T cells (Fig. 4). Endogenous IL-4 appears to be produced by both $CD4^+$ and $CD8^+$ T cells (Fig. 4). Recent studies demonstrated that IL-4 is produced, not only by $CD4^+$ T cells, but also by $CD8^+$ T cells.[29,30] In addition, the presence of IL-4 during effector cell generation can promote $CD8^+$ T cells to develop Th2-type effector functions which produce IL-4 and IL-5 and to stop producing IFN-γ.[31–33] Experiments using rat and murine models of experimental autoimmune encephalomyelitis support the view that transforming growth factor β and IL-4 secreted from $CD8^+$ T cells might indeed be important to prevent animals from developing encephalomyelitis.[34] In this regard, $CD8^+$ T cells that produce Th2-type cytokines may provide an anti-inflammatory function that is beneficial to the host.[33] In the model of *R. aurantiacus*-inoculated mice, $CD8^+$ T cells produce IFN-γ during the phase of granuloma formation and then produce IL-4 during the phase of granuloma regression. Th2-type $CD8^+$ T cells, together with Th2-type $CD4^+$ T cells, may contribute to the anti-inflammatory function through the secretion of IL-4.

The diminution of granulomas at 5 weeks after the inoculation was inhibited by treatment with anti-IL-4 mAb. The failure of granuloma regression may be due to increased IFN-γ production caused by treatment with anti-IL-4 mAb. An important function of the Th2-type response is to ameliorate the tissue-damaging effects of immune responses mediated by Th1-type cells.[14] Endogenous IL-4, secreted from both $CD4^+$ and $CD8^+$ T cells, might play a role in stopping the granuloma formation which is dependent on IFN-γ and healing lesions. In conclusion, reciprocal actions of IFN-γ and IL-4 may function to enhance and diminish granulomas induced by *R. aurantiacus*.

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