half volume of FCS followed by two additional washes. Five million cells carrying each peptide were mixed and injected intravenously into immunized mice. At 12 h after injection, the spleens were harvested and single cell suspensions were prepared. CFSE-positive cells were analyzed by a flow cytometer with exclusion of dead cells with propidium iodide. For example, NP-specific killing was calculated as follows: %killing = [1 − {(number of cells carrying $NP_{366-374}$ in immunized mice (CFSE high))/(number of cells carrying $OVA_{257-264}$ in immunized mice (CFSE low))}/{(number of cells carrying $NP_{366-374}$ in normal mice (CFSE high))/(number of cells carrying $OVA_{257-264}$ in normal mice (CFSE low))}] × 100.

2.4. Influenza viruses

Influenza virus, A/Aichi/2/68 (H3N2), was propagated in the allantoic cavities of 10-day-old embryonated hen's eggs at 35 °C for 48 h. Then the viruses were purified by ultracentrifugation of allantoic fluid passed through a 10–50% sucrose density gradient. The viruses were suspended in PBS. The viruses were prepared from culture supernatant of Madin–Darby canine kidney (MDCK) cells for experimental infection [16].

2.5. Virus challenge and protection tests

B6 mice were challenged intranasally with 15 μl of 10^4 plaque-forming units (PFU) of A/Aichi/2/68 (H3N2) under anesthesia. Since A/Aichi/2/68 (H3N2) did not kill mice without systemic infection, it was adequate for assessing virus growth in mouse lungs at 5 days after challenge. Lungs were collected from three mice of each group and used for the measurement of virus titers. In the measurement of virus titers, 10% suspensions of the tissue homogenates were prepared.

2.6. Titration of virus

MDCK cells were grown in minimal essential medium (MEM) supplemented with 10% bovine calf serum. Suspensions of the lungs serially diluted from 1:10 were inoculated into confluent MDCK cell monolayers on 6-well plates and incubated at room temperature for 1 h for adsorption. After 1 h, the inoculum was removed and cells were overlaid with MEM containing 1% bacto agar (BD Diagnostic Systems, Sparks, MD) and 5 μg/ml of trypsin (BD Diagnostic Systems). After incubation at 35 °C for 2 days in 5% CO_2, the plaques were counted. The limit of detection in this assay was 0.5×10^3 PFU/g [16].

2.7. ELISA

Enzyme-linked immunosorbent assay (ELISA). 96-Well plates were coated with 50 μl of purified A/Aichi/2/68 (H3N2) (20 μg/ml) disrupted with 0.05 M Tris–HCl (pH 7.8) containing 0.5% Triton X-100 and 0.6 M KCl. After washing three times with PBS containing 0.05% Tween20, PBS with 3% BSA was added for blocking. Sera were collected 5 days after infection following subcutaneous immunization twice with NP–liposome and CpG. Serially diluted sera were incubated overnight. For measuring total IgG, horseradish peroxidase-conjugated rabbit anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA) (1/2000× 50 μl) was added after washing 5 times. For measuring IgE, biotinylated anti-mouse IgE (clone 23G3) (eBioscience) (0.5 μg/ml × 50 μl) was added. Horseradish peroxidase-conjugated Extravidin (Sigma–Aldrich) (1/3000× 100 μl) was incubated for 3 h. Horseradish peroxidase activity was assessed by 3,3′,5,5″-tetramethyl benzidine substrate (100 μl). The reaction was stopped by 1 N HCl (100 μl). Optical density was measured at 450 nm.

2.8. Statistical analyses

Statistical analyses were carried out using Student's *t*-test. p values <0.05 were considered significant.

3. Results

3.1. Comparison of CTL activation capacities of surface-linked liposomal OVA peptide and OVA peptide entrapped within the aqueous lumen of liposomes

In a previous study, we intranasally inoculated anti-CD40 antibody and multilamellar liposome containing $NP_{366-374}$ peptide, resulting in induction of protective CTL responses against influenza A virus in mouse lungs [18]. Thus, firstly we compared the capacity for activating CTL *in vivo* of liposome with chemically bound peptides on the surface (peptide–Oleoyl liposome) and that of the multilamellar liposome containing peptides inside. B6 mice were injected subcutaneously with either liposome with chemically bound $OVA_{257-264}$ on the surface or multilamellar liposome containing $OVA_{257-264}$ inside in the presence of CpG and anti-CD40 antibody, and then 7 days later CFSE-labeled target cells pulsed with $OVA_{257-264}$ were injected intravenously. Viability of the target cells in the spleen was examined at 12 h after injection. Injection of liposome with chemically bound $OVA_{257-264}$ on the surface induced CTL activity more effectively than did multilamellar liposome containing $OVA_{257-264}$ inside ($p = 0.049$) (Fig. 1A).

3.2. Comparison of CTL activation capacities of liposomes consisting of different components of surface-linked liposomal OVA peptide

Some scavenger receptors recognize phosphatidyl serine and support phagocytosis. Thus, we altered phosphatidyl choline to phosphatidyl serine of Oleoyl liposome this liposome was termed PS–Oleoyl liposome. However, we could

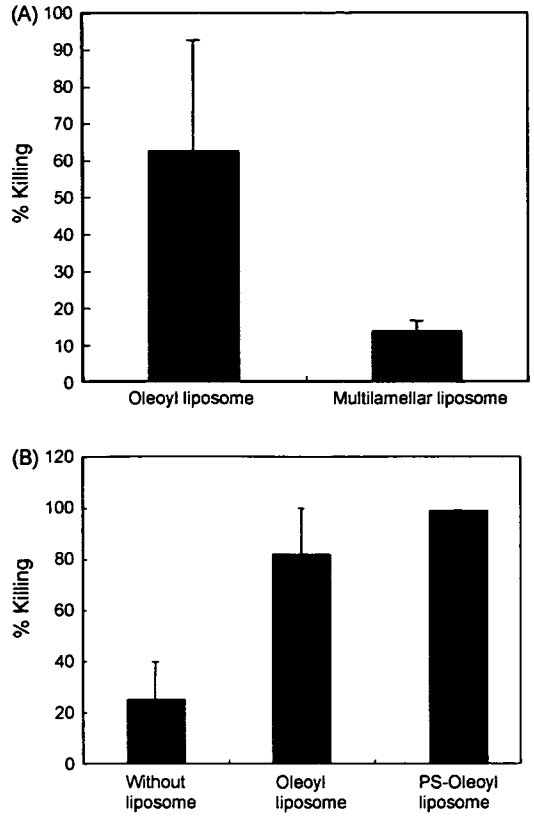

Fig. 1. Comparison of CTL activation capacities of various liposomes carrying peptides. (A) B6 mice were injected subcutaneously with either liposome with chemically bound $OVA_{257-264}$ on the surface or multilamellar liposome containing $OVA_{257-264}$ inside in the presence of CpG and anti-CD40 antibody. Composition of each liposome is described in Section 2. Seven days later, high CFSE-labeled target cells pulsed with $OVA_{257-264}$ and low CFSE-labeled target cells pulsed with $NP_{366-374}$ were injected intravenously as an *in vivo* CTL assay. Viability of the target cells in the spleen was examined at 12 h after injection. OVA specific killing was calculated as follows: %killing = [1 − {(number of cells carrying $OVA_{257-264}$ in immunized mice (CFSE high))/(number of cells carrying $NP_{366-374}$ in immunized mice (CFSE low))}/{(number of cells carrying $OVA_{257-264}$ in normal mice (CFSE high))/(number of cells carrying $NP_{366-374}$ in normal mice (CFSE low))}] × 100. Injection of liposome with chemically bound $OVA_{257-264}$ on the surface induced CTL activity more effectively than did multilamellar liposome containing $OVA_{257-264}$ inside ($p = 0.049$). (B) PS–Oleoyl liposome was generated by alteration of phosphatidyl choline to phosphatidyl serine of Oleoyl liposome. $OVA_{257-264}$–PS–Oleoyl liposome, $OVA_{257-264}$–Oleoyl liposome or $OVA_{257-264}$ unbound to liposome was inoculated with CpG and anti-CD40 antibody. Significant difference between CTL-activating capacities of $OVA_{257-264}$–PS–Oleoyl liposome and $OVA_{257-264}$–Oleoyl liposome was not observed ($p = 0.167$).

not detect a significant difference between CTL-activating capacities of between $OVA_{257-264}$–PS–Oleoyl liposome and $OVA_{257-264}$–Oleoyl liposome ($p = 0.167$), although $OVA_{257-264}$–Oleoyl liposome and $OVA_{257-264}$–PS–Oleoyl liposome induced significant CTL activity compared to that induced by peptide without liposome ($p = 0.013$ and $p < 0.001$, respectively) (Fig. 1B). On the other hand, Oleoyl liposome is preserved more easily than PS–Oleoyl liposome, because Oleoyl liposome is more stable than PS–Oleoyl liposome. Therefore, we used Oleoyl liposome in this study.

3.3. CTL activation by simultaneous inoculation of CpG and $OVA_{257-264}$ bound to Oleoyl liposome

We tested which combination among $OVA_{257-264}$–Oleoyl liposome, CpG and anti-CD40 antibody was critical for induction of CTL activity. Subcutaneous inoculation of a combination of CpG and $OVA_{257-264}$–Oleoyl liposome elicited CTL responses *in vivo*, while simultaneous inoculation of anti-CD40 and $OVA_{257-264}$–Oleoyl liposome, or $OVA_{257-264}$–Oleoyl liposome alone did not (Fig. 2). On the other hand, CTL activity elicited by CpG and $OVA_{257-264}$–Oleoyl liposome was not different from that elicited by CpG, anti-CD40 and $OVA_{257-264}$–Oleoyl liposome ($p = 0.12$). Therefore, inoculation of a combination of CpG and $OVA_{257-264}$–Oleoyl liposome was sufficient to elicit CTL responses *in vivo*.

3.4. Detection of immunoenhancers for eliciting CTL activity with peptide–Oleoyl liposome

In order to find effective immune enhancers eliciting CTL responses with peptide–Oleoyl liposome, we inoculated peptide–Oleoyl liposome simultaneously with various TLR ligands. $OVA_{257-264}$–Oleoyl liposome alone did not elicit CTL responses as shown in Fig. 2. CpG (10 μg/mouse) (TLR9 ligand) and Poly(I:C) (TLR3 ligand) (10 μg/mouse) showed sufficient adjuvant activity in combination with $OVA_{257-264}$–Oleoyl liposome compared with LPS (10 μg/mouse) (TLR4 ligand) ($p = 0.003$ and 0.012, respectively) (Fig. 3A). Administration of LPS

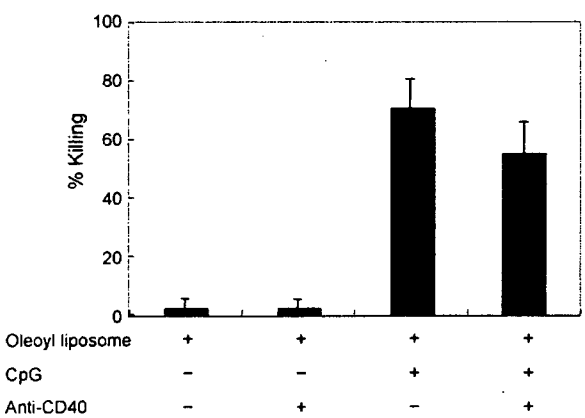

Fig. 2. CTL activation by simultaneous inoculation of CpG and $OVA_{257-264}$–Oleoyl liposome. Various combinations of CpG, anti-CD40 and $OVA_{257-264}$–Oleoyl liposome were inoculated subcutaneously into B6 mice for an *in vivo* CTL assay. Subcutaneous inoculation with CpG and $OVA_{257-264}$–Oleoyl liposome elicited CTL responses *in vivo*, but that with anti-CD40 and $OVA_{257-264}$–Oleoyl liposome ($p < 0.001$) or $OVA_{257-264}$–Oleoyl liposome alone ($p < 0.001$) did not. CTL activity elicited by CpG and $OVA_{257-264}$–Oleoyl liposome was not different from that elicited by CpG, anti-CD40 and $OVA_{257-264}$–Oleoyl liposome ($p = 0.12$).

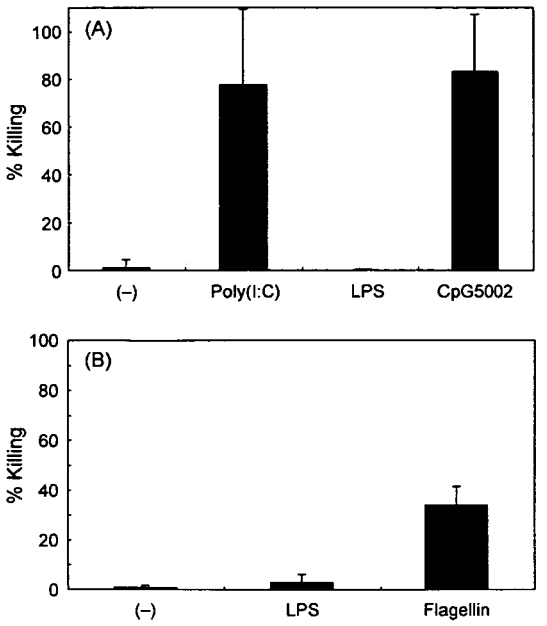

Fig. 3. Comparison of TLR ligands as adjuvants. We inoculated simultaneously with various TLR ligands and $OVA_{257-264}$– or $NP_{366-374}$–Oleoyl liposome. Seven days later, high CFSE-labeled target cells pulsed with $OVA_{257-264}$ or $NP_{366-374}$, and low CFSE-labeled target cells pulsed with VSV-NP_{52-59} (RGYVFQGL) or LCMV-$NP_{396-404}$ (FQPQNGQFI) were injected intravenously as an *in vivo* CTL assay. Viability of the target cells in the spleen was examined at 12 h after injection. Killing activity specific for $OVA_{257-264}$ or $NP_{366-374}$ was calculated as described in the legend of Fig. 1. (A) Inoculation dose of CpG, LPS or Poly(I:C) was 10 μg, respectively. CpG showed sufficient adjuvant activity in combination with $OVA_{257-264}$–Oleoyl liposome comparing with LPS (TLR4 ligand) ($p = 0.003$). Poly(I:C) (TLR3-ligand) worked as an enhancer similar to CpG ($p = 0.82$). (B) Flagellin (TLR5-ligand) (10 μg/mouse) or LPS (10 μg/mouse) was inoculated into mice with $NP_{366-374}$–Oleoyl liposome. In combination with the peptide–Oleoyl liposome, Flagellin showed significant adjuvant activity comparing with LPS ($p = 0.0027$).

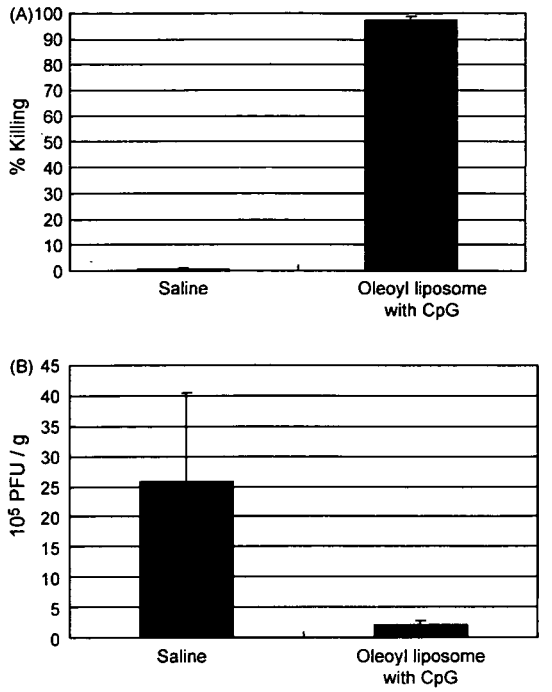

Fig. 4. Vaccination of $NP_{366-374}$ bound to Oleoyl liposome in combination with CpG. (A) B6 mice were injected twice subcutaneously with Oleoyl liposome with chemically bound $NP_{366-374}$ on the surface with CpG. Seven days later, high CFSE-labeled target cells pulsed with $NP_{366-374}$ and low CFSE-labeled target cells pulsed with $OVA_{257-264}$ were injected intravenously as an *in vivo* CTL assay. NP-specific killing was calculated as follows: %killing = [1 − {(number of cells carrying $NP_{366-374}$ in immunized mice (CFSE high))/(number of cells carrying $OVA_{257-264}$ in immunized mice (CFSE low))}/{(number of cells carrying $NP_{366-374}$ in normal mice (CFSE high))/(number of cells carrying $OVA_{257-264}$ in normal mice (CFSE low))}] × 100. Injection of $NP_{366-374}$–Oleoyl liposome with CpG induced CTL activity more effectively than that of saline ($p < 0.01$). (B) B6 mice were injected twice subcutaneously with Oleoyl liposome with chemically bound $NP_{366-374}$ on the surface with CpG. Seven days later, the immunized mice were challenged with Influenza virus, A/Aichi/2/68 (H3N2). At 5 days after challenge, lungs of the immunized mice were homogenized for use in a plaque-forming assay using MDCK cells as described in Section 2. Injection of $NP_{366-374}$–Oleoyl liposome with CpG reduced virus growth in the lungs more effectively than that of saline ($p = 0.047$).

(10 μg/mouse) and OVA protein induced *in vivo* CTL responses (around 40%killing) but that of OVA protein alone did not (data not shown). Thus, it seems that administration of 10 μg/mouse of LPS is quantitatively sufficient for working as adjuvant. Next, combination of another peptide–Oleoyl liposome and adjuvants was also tested. $NP_{366-374}$–Oleoyl liposome alone and with LPS did not elicit CTL responses, but Flagellin (TLR5 ligand) worked as an enhancer, although %killing was less than that by CpG or Poly(I:C) in combination with $OVA_{257-264}$–Oleoyl liposome (Fig. 3B).

3.5. Vaccination of NP366-374 bound to Oleoyl liposome in combination with CpG

In order to test whether peptide conjugated to Oleoyl liposome in combination with CpG works as a vaccine, we subcutaneously injected twice B6 mice with $NP_{366-374}$ conjugated to Oleoyl liposome in combination with CpG and measured virus titers in the lungs after intranasal challenge of influenza viruses. We detected CTL activity in spleens of mice inoculated with a mixture of $NP_{366-374}$–Oleoyl liposome and CpG but not in spleens of mice inoculated with saline (Fig. 4A). Similarly, inoculation of a mixture of $NP_{366-374}$–Oleoyl liposome and CpG inhibited virus growth in mouse lungs, but saline did not (Fig. 4B).

We have demonstrated that surface-linked liposomal antigens induce antigen-specific IgG antibody production but not antigen-specific IgE antibody production [10]. Herein, we evaluated again whether $NP_{366-374}$–Oleoyl liposome and CpG induce IgE production, because IgE shows detrimental effects, such as allergy. $NP_{366-374}$–Oleoyl liposome and CpG induced IgG production specific for NP protein, although antibody titer was marginal (Fig. 5A). In contrast, $NP_{366-374}$–Oleoyl liposome and CpG did not elicit any IgE production against NP protein (Fig. 5B). In conclusion,

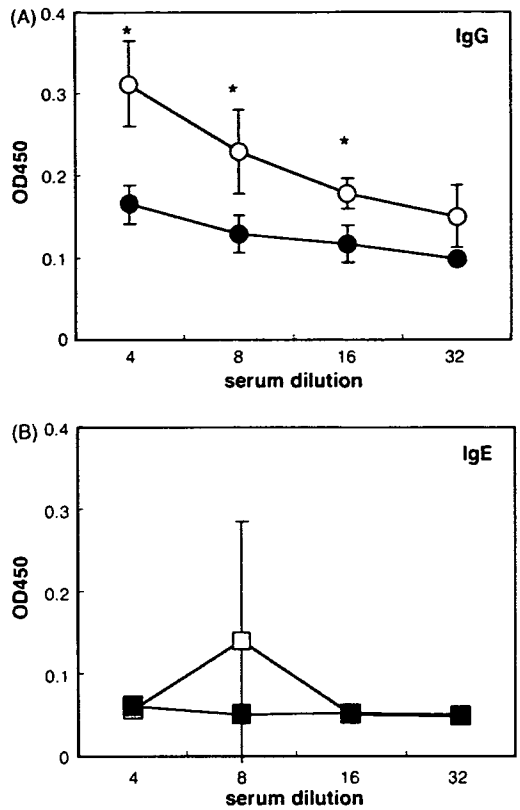

Fig. 5. Antibody production by simultaneous inoculation of CpG and $NP_{366-374}$–Oleoyl liposome. Sera were collected 5 days after infection following subcutaneous immunization twice with NP–liposome and CpG. (A) Total IgG against disrupted A/Aichi/2/68 (H3N2) in the serially diluted sera was measure with ELISA as described in the Section 2. IgG in the immunized sera (○) was subtle but significantly increased comparing control (●) (* indicates $p < 0.05$). (B) IgE against disrupted A/Aichi/2/68 (H3N2) in the immunized sera (□) or control (■) were measured with ELISA. Optical density was measured at 450 nm.

peptide–Oleoyl liposome is an effective vaccine candidate in the presence of CpG without detrimental effects in mice.

4. Discussion

It has been shown that liposomes work as carriers of vaccines and supported immune responses by vaccines [19–26]. Furthermore, alteration of lipid composition has been reported to modulate immune responses [27–33]. Indeed, the inducibility of antigen-specific IgG antibody production by antigen–liposome has been shown to vary among the liposome preparations used for the production of antigen–liposome; the greater the membrane mobility in liposomes is, the greater is antibody production induced by antigen–liposome [11]. In this study we searched for a composition of liposome eliciting CTL activity more effectively by using an *in vivo* CTL assay, in which CTL activity was detected timely in immunized animals. Subcutaneous inoculation of CpG and OVA peptide, $OVA_{257-264}$, coupled on the surface of Oleoyl liposome, consisting of dioleoyl phosphatidyl choline, dioleoyl phosphatidyl ethanolamine, cholesterol, and dioleoyl phosphatidyl glycerol acid in a 4:3:7:2 molar ratio, elicited CTL responses more vigorously than did other liposomes in the presence of CpG. Furthermore, subcutaneous inoculation of a mixture of $NP_{366-374}$–Oleoyl liposome in combination with CpG protected against infection of influenza A virus.

In a previous study, we intranasally inoculated anti-CD40 antibody and multilamellar liposome containing $NP_{366-374}$ peptide, resulting in induction of protective CTL responses against influenza A virus in mouse lungs [18]. Indeed, in MHC class I deficient mice intranasal inoculation with anti-CD40 antibody and multilamellar liposome containing $NP_{366-374}$ peptide did not protect viral growth in lungs, therefore CTL responses were presumably involved in protective responses in lungs. Herein, however, subcutaneous inoculation of anti-CD40 antibody and multilamellar liposome containing $OVA_{257-264}$ peptide did not induce CTL responses in spleen. These data suggest that simultaneous inoculation of anti-CD40 antibody and multilamellar liposome containing $OVA_{257-264}$ peptide does not elicits systemic CTL responses, but when anti-CD40 antibody and multilamellar liposome containing $OVA_{257-264}$ peptide are locally inoculated, even subtle CTL responses are able to protect infection in the areas, such as lungs.

At present, a few adjuvants, such as aluminum hydroxide and MF59, are clinically in use, although a number of candidate adjuvants have been examined in preclinical studies. However, aluminum adjuvants and MF59 are known to be effective only for the induction of humoral immunity, not for the induction of cell-mediated immunity [34]. In addition, aluminum adjuvants are also known to induce the production of IgE antibodies, which is a cause of allergic response against the vaccine [35–37]. On the other hand, some candidate adjuvants activating innate immunity via TLR support cellular immunity. CpG oligonucleotides are known to enhance maturation and antigen-presenting capacity of dendritic cells via TLR9, resulting in activation of CTL responses. In this study, support of CpG was necessary for the induction of CTL response and protection against influenza infection by peptide–Oleoyl liposome. However, CpG are reported to possess toxicity against hepatocytes and induce immune suppression after daily high-dose administrations [38]. Similarly, Poly(I:C) supported the induction of CTL responses by peptide–Oleoyl liposome. In mice CpG and Poly(I:C) worked most effectively as adjuvant in combination with peptide–Oleoyl liposome, but in human usage CpG and Poly(I:C) will be converted to inhibit induction of detrimental effects, such as toxicity.

In previous studies, inoculation of CpG and antigens elicited CTL responses independent $CD4^+$ T cells [39,40]. Indeed, CTL responses were elicited by simultaneous inoculation of CpG and $OVA_{257-264}$–Oleoyl liposome in MHC class II-deficient mice in which $CD4^+$ T cells were deleted

(data not shown). Thus, this combination could be used as a vaccine in immunocompromised hosts, such as AIDS patients or aged people who have declined antibody production due to defective $CD4^+$ T cell help. Furthermore, it has been shown that pretreatment with OVA–liposomes suppressed IgE antibody responses to CT-combined OVA, with significantly high levels of production of both nasal IgA and serum IgG antibodies. Moreover, treatment with OVA–liposomes 1 and 3 weeks after CT-combined OVA administration also suppressed IgE antibody responses [12]. Taken together, the results in the present study suggest that peptide–Oleoyl liposome conjugates in combination of some sort of immuno-potentiators without toxicity, might serve as a vaccine candidate without detrimental effects, such as allergic responses, even in immunocompromised hosts.

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Hepatology Research 2007; 37: S310–S318 doi: 10.1111/j.1872-034X.2007.00249.x

SESSION 1. IMMUNE DISORDER IN VIRAL HEPATITIS AND ITS REGULATION

Roles of the novel interleukin-12-associated cytokine, interleukin-23, in the regulation of T-cell-mediated immunity

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Interleukin (IL)-12 is a heterodimeric proinflammatory cytokine formed by a 35-kDa light chain (p35) and a 40-kDa heavy chain (p40). This cytokine is a key regulator of cell-mediated immunity, and therefore should have therapeutic potential in infectious diseases and tumors. Recently, a novel IL-12-associated cytokine, IL-23 has been discovered. IL-23 is also a heterodimer that consists of the p40 subunit of IL-12 and a novel subunit, p19. Several studies have shown that IL-23 possesses immunoadjuvant activity against tumor and infectious diseases as well as IL-12. On the other hand, there is increasing evidence that IL-12 and IL-23 have discrete roles in the regulation of T-cell-mediated immunity despite their structural similarities. IL-12 leads to the development of interferon-γ-producing T-helper type 1 (Th1) cells, whereas IL-23 amplifies and stabilizes a new $CD4^+$ T-cell subset, Th17 producing IL-17. The IL-23/Th17 axis rather than the IL-12/Th1 axis contributes to several immune-mediated inflammatory autoimmune diseases. Furthermore, IL-23/IL-17 promotes tumor incidence and growth. Therefore, IL-23 and Th17 are attracting considerable attention at present. Taken together, these findings suggest that IL-23 may be an immunoadjuvant against infectious diseases and tumors, and a viable target for the treatment of inflammatory diseases.

Key words: IFN-γ, IL-12, IL-17, IL-23, Th1, Th17

INTRODUCTION

INTERLEUKIN (IL)-12 is a heterodimeric proinflammatory cytokine formed by a 35-kDa light chain (p35) and a 40-kDa heavy chain (p40) (Fig. 1). This cytokine is a dominant factor in the differentiation of T-helper type 1 (Th1) cells which direct cellular immunity.[1] Therefore, IL-12 is considered to be a key regulator of cell-mediated immunity and to have therapeutic potential in infectious diseases and tumors. However, the first clinical trial of IL-12 for tumor therapy resulted in the death of two persons and caused severe toxic effects in 15 individuals.[2] Recently, a novel polypeptide, p19, has been discovered by searching the databases with a computationally-derived profile of IL-6.[3] The p19 polypeptide itself shows no biological activity; instead, the p19 subunit associates with the p40 of IL-12 to form a biologically active, new heterodimeric cytokine, IL-23 (Fig. 1). This cytokine is secreted primarily from activated dendritic cells.[3] Furthermore, the heterodimeric receptor for IL-23 shares one subunit, IL-12Rβ1 with the IL-12 receptor, and the other subunit is a novel IL-23R (Fig. 1).[4] As a consequence of these structural similarities, it was originally considered that IL-23, similar to IL-12, would play a critical role in the regulation of Th1-mediated immune responses. In fact, early studies pointed out that IL-23 stimulated the production of interferon (IFN)-γ and enhanced activities of cytotoxic T lymphocytes (CTL).[5–8] However, it is now apparent that IL-12 and IL-23 have discrete roles in the regulation of T-cell-mediated immunity during infection and autoimmunity.

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ADJUVANT ACTIVITY OF IL-23

THE BIOLOGICAL ACTIVITY of IL-23 was initially characterized in comparison with that of IL-12.[3] Like IL-12, IL-23 enhanced proliferation of T cells and IFN-γ production produced by human blast T cells. However, the maximum levels of IFN-γ production induced by IL-23 were always lower than those induced by IL-12. It was also demonstrated that IL-23 possessed antitumor activity as well as IL-12.[6] Several groups indicated that

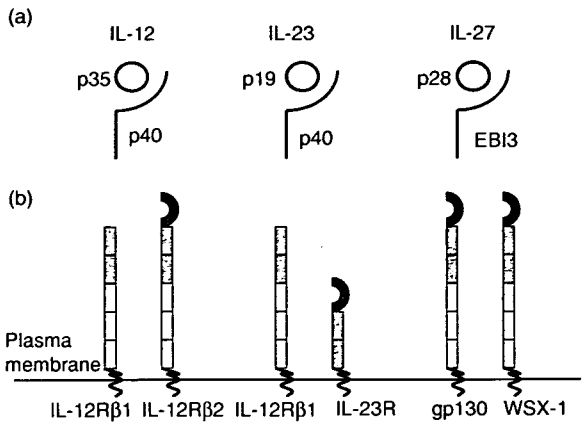

Figure 1 Schematic representation of the interleukin (IL)-12-associated cytokines and their receptors. (A) IL-12 is a covalently linked heterodimeric molecule composed of a light chain (p35) and a heavy chain (p40). The p40 subunit of IL-12 also associates with the p19 subunit to form IL-23. IL-27 is also a heterodimeric cytokine composed of Epstein–Barr virus-induced gene 3 (EBI3) and p28. (B) The IL-12 receptor consists of IL-12Rβ1 and IL-12Rβ2 which have homology to gp130. The receptor of IL-23 is formed by the association of IL-12Rβ1 and IL-23R. The IL-27 receptor is composed of gp130 and WSX-1.

IL-23 efficiently promoted the induction of $CD8^+$ CTL specific for tumors[6,9] and hepatitis C virus (HCV).[7,8] We have shown that co-administration of an IL-23 expression plasmid in a prime-boost immunization enhanced the induction of HCV-specific CTL and led to dramatic increases in numbers of IFN-γ-producing, HCV-specific $CD8^+$ T cells in human leukocyte antigen (HLA)-A*0201 transgenic mice (Fig. 2).[8] These data suggest that IL-23 might be a potent adjuvant for CTL-based vaccine against infectious pathogens such as HCV.

Vaccinia virus (VV) is a large DNA virus and a member of the *Orthopoxvirus* genus in the *Poxviridae* family. VV has been used as an effective vaccine against variola virus, which is the cause of smallpox. VV has also been used as an expression vector for foreign genes in a great number of experimental systems. Recently, the threat of bioterrorism has raised concerns over the reemergence of smallpox. It has been thought that both cellular immunity and humoral immunity play a significant role in protection against VV infection. We have previously shown that the central transcriptional factor for the Th1 subset, T-bet, was required for protection against VV infection using T-bet-deficient mice, indicating that the IL-12/Th1 pathway plays a crucial role in the host defense against VV.[10] To investigate the role of IL-23 in the defense against VV, we have engineered recombinant VV expressing IL-23 (VV-IL-23). Inoculation of VV-IL-23 into mice resulted in a rapid clearance of the virus compared to wild-type VV, suggesting that IL-23 as well as IL-12 would be a potent adjuvant for viral infection (Matsui M *et al.*, 2006, unpublished data).

INTERLEUKIN-23 AND AUTOIMMUNITY

ALTHOUGH IL-12 promotes the production of IFN-γ and thereby generates resistance to intracellular infections, it was known that the IL-12-induced Th1 responses also contribute to autoimmunity. Using IL-12p40-deficient mice and anti-p40 antibodies, it was found that p40 was required for the development of T-cell-mediated autoimmune diseases such as experimental allergic encephalomyelitis (EAE; a murine model for multiple sclerosis), collagen-induced arthritis (CIA; a murine model for rheumatoid arthritis) and inflammatory bowel disease (IBD; a murine model for Crohn's disease and ulcerative colitis). However, IFN-γ-deficient mice still remained susceptible to these diseases, suggesting that IFN-γ is not essential for these autoimmune diseases. This discrepancy between the roles of IL-12 and IFN-γ in the development of autoimmunity was dissolved by the studies of Cua *et al.*[11,12] They found that mice deficient in either the p19 subunit (lacking IL-23 alone) or the p40 subunit (lacking both IL-12 and IL-23) were resistant to EAE and CIA, whereas p35-deficient mice (lacking IL-12 only) remained susceptible to these diseases. The data indicate that IL-23, not IL-12, is essential for the development of these autoimmune diseases. The resistance to CIA was found to correlate with an absence of IL-17-producing $CD4^+$ T cells despite normal induction of collagen-specific, IFN-γ-producing Th1 cells.[12] Furthermore, it was shown that the systemic inflammatory response of IBD was driven by IL-12 and not IL-23, whereas local intestinal inflammation required the presence of IL-23 and was independent of IL-12.[13] This result demonstrates a striking dichotomy in the regulation of inflammation; that is, IL-12 promotes innate systemic responses and IL-23 directs local inflammation. Taken together, these data indicate that IL-23 rather than IL-12 contribute to several T-cell-mediated inflammatory autoimmune diseases. Therefore, specific therapeutic blockade of IL-23 may provide a promising approach for various inflammatory autoimmune diseases.

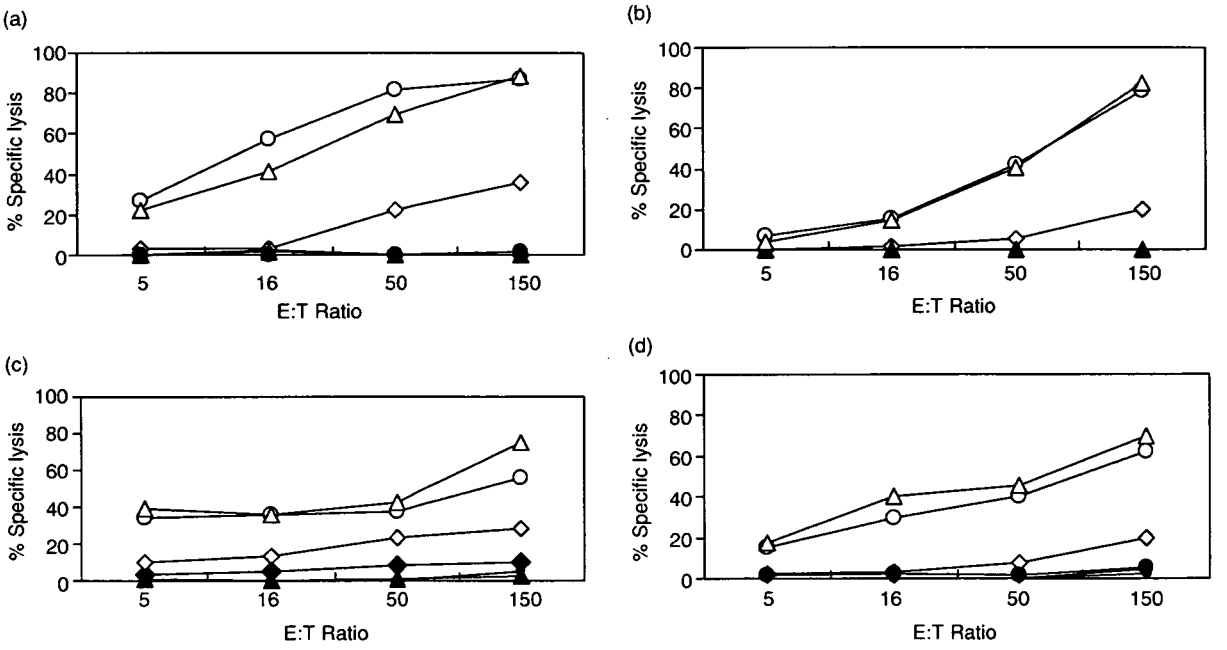

Figure 2 Enhancement of hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTL) induction of either an IL-12 expression plasmid or an IL-23 expression plasmid in the prime-boost immunization. human leukocyte antigen (HLA)-A*0201 transgenic mice were immunized as follows: priming and the first boosting with 100 μg of either a plasmid expressing HCV-derived proteins together with 100 μg of an IL-12 expression plasmid (circles), an IL-23 expression plasmid (triangles), or an empty plasmid (diamonds), followed by the second boosting with 5×10^7 plaque forming units (PFU) of replication-defective recombinant adenovirus expressing HCV-derived proteins. The interval between immunizations was 2 weeks. After 2–3 weeks following the last immunization, spleen cells were prepared and stimulated *in vitro* with syngeneic spleen cells pulsed with 10 μM of HLA-A*0201-restricted, HCV-derived peptides: (A) core-132 (DLMGYIPLV), (B) NS3-1073 (CINGVCWTV), (C) NS4-1666 (VLVGGVLAA) and (D) NS4-1769 (HMWNFISGI). After 1 week, ^{51}Cr-release assays were performed at various E : T ratios, using mouse lymphoma, RMA cells expressing HLA-A*0201 molecule pulsed with (white symbols) or without (black symbols) 10 μM of each peptide as targets.

INTERLEUKIN-23 AND A NEW CD4$^+$ T-CELL SUBSET, TH17

INTERLEUKIN-17 IS a potent proinflammatory cytokine produced by CD4$^+$ T cells, and has pleiotropic activities including the induction of a variety of proinflammatory cytokines, chemokines and adhesion molecules on various cell types. IL-17 is detected in serum of patients with rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus and asthma, suggesting that it is involved in the development of various human autoimmune diseases.[14] In addition, it was reported that IL-17-deficient mice were resistant to the development of CIA[15] and EAE,[16] suggesting a pathogenic role of IL-17 in these autoimmune diseases. Thus, CD4$^+$ T cells producing IL-17 were likely to be associated with autoimmunity. However, the precise mechanism of their development was undefined. Naive CD4$^+$ T-helper precursor was originally thought to differentiate into either classical Th1 or Th2 cells (Fig. 3). Th1 cells mainly produce IFN-γ and direct cell-mediated protective immunity, whereas Th2 cells secrete IL-4 and promote humoral immune responses. Th1 cells and the IL-17-producing CD4$^+$ T cells were initially believed to arise from a common Th precursor. However, Harrington *et al.*[17] have found that IL-23 stimulates naive precursor cells to differentiate into a new CD4$^+$ T-cell subset, termed Th17 (Fig. 3). Th17 cells produce IL-17, IL-17F, IL-6 and tumor necrosis factor (TNF)-α, but not IFN-γ or IL-4, and therefore this subset is distinct from the classical Th1 or Th2 subset.[17,18] Because the IL-23-dependent Th17 subset is critical in driving tissue

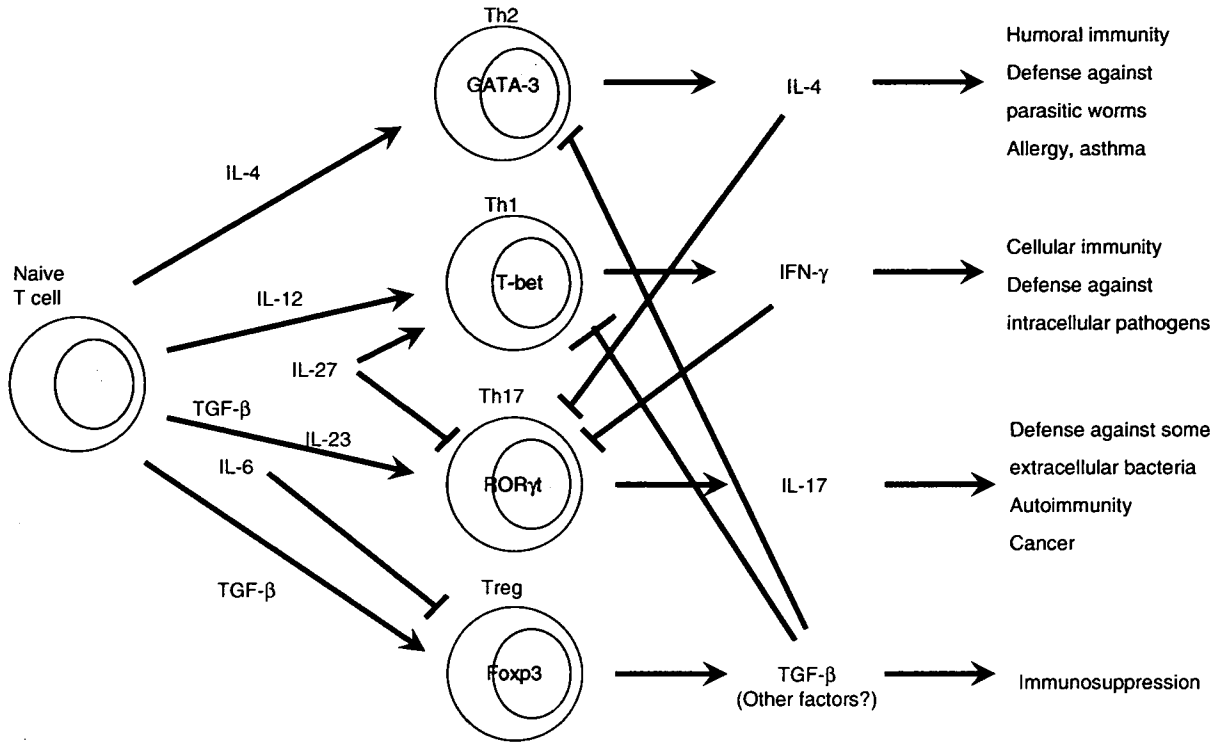

Figure 3 Differentiation of CD4⁺ T-cell lineages. IL-12 and IL-4 prompt to differentiate naive T cells into the T-helper (Th)1 and Th2 lineages, respectively. transforming growth factor (TGF)-β in the presence of IL-6 initiates the development of Th17 cells, and IL-23 amplifies and stabilizes Th17. On the other hand, interferon (IFN)-γ, IL-4 and IL-27 inhibit the differentiation of Th17 cells. TGF-β also promotes the development of some type of regulatory T (Treg) cells. T-bet, GATA-3, RORγt and Foxp5 are central transcription factors that regulate the differentiation of Th1, Th2, Th17 and Treg, respectively.

inflammatory responses, the IL-23/IL-17 pathway is attracting considerable attention at present.

Recently, three reports[19-21] have demonstrated that IL-23 is not necessary for development of Th17 cells, but transforming growth factor (TGF)-β and IL-6 are required for the differentiation of Th17 cells. However, when naive T cells were cultured with TGF-β and IL-6 to induce Th17 cells, restimulation with IL-23 was needed to maintain the Th17 phenotype.[21] It is known that IL-17 is essential for host defense against *Citrobacter rodentium*. However, even with the presence of Th17 cells, IL-23-deficient mice succumbed to the infection with *C. rodentium*.[19] Taken together, these data indicate that although TGF-β and IL-6 are required for the lineage commitment of Th17 cells, IL-23 is essential for Th17 cells to acquire full effector function (Fig. 3). It has recently been shown that Th17 cells express IL-22 which is involved as an IL-10 family member.[22] Similar to IL-17, IL-22 production is initiated by TGF-β in the presence of IL-6, and expanded by IL-23. IL-22 synergizes with IL-17 to regulate genes associated with skin innate immunity. Interestingly, IL-6 is capable of inhibiting TGF-β-dependent Foxp3⁺ regulatory T (Treg) cells (Fig. 3), suggesting that Th17 cells are closely related with Treg cells.[20] Because TGF-β is also known as a cytokine that promotes the differentiation of Treg cells, there should be a reciprocal development pathway for the generation of pathogenic Th17 cells and suppressive Treg cells (Fig. 3). Therefore, Th17 cells and Treg cells may work together in the inflammation. These findings should offer promising novel approaches for the treatment of T-cell-mediated inflammatory diseases.

In addition to TGF-β and IL-6, Sutton *et al.*[23] have shown a crucial role for IL-1 in the induction of Th17 cells. It was observed that IL-1 receptor type I-deficient (IL-1RI$^{-/-}$) mice did not develop EAE following immunization with myelin oligodendrocyte glycoprotein (MOG) and adjuvant. In addition, stimulation of spleen

cells of IL-1RI$^{-/-}$ mice with the MOG peptide resulted in reduced IL-17 production. These data indicate a crucial role for IL-1 in the differentiation of Th17 cells. However, the relative roles of IL-23, IL-6, TGF-β and IL-1 in the differentiation and survival of Th17 cells require further investigation.

T-bet, GATA-3 and Foxp3 are known to be central transcription factors that regulate the differentiation of Th1, Th2 and Treg, respectively (Fig. 3). Until recently, however, the lineage-specific transcription factor for Th17 cells was not identified. Most recently, Ivanov *et al.*[24] have demonstrated that the orphan nuclear receptor, RORγt is the key transcription factor orchestrating the differentiation of Th17 cells (Fig. 3) and has a potential to be a therapeutic target in inflammatory diseases.

INTERLEUKIN-27 SUPPRESSES THE DEVELOPMENT OF TH17

IN ADDITION TO IL-23, another IL-12-associated cytokine, IL-27 has recently been discovered.[25] Like IL-12 and IL-23, IL-27 is a heterodimeric cytokine composed of the *Epstein–Barr virus-induced gene 3* (EBI3) subunit and a new subunit, p28 (Fig. 1). The EBI3 and p28 subunits are IL-12p40 and IL-23p35 homologs, respectively. The IL-27 receptor consists of WSX-1 and gp130, and is closely related to the IL-12 and IL-23 receptors (Fig. 1). Although early studies demonstrated that IL-27 promotes the differentiation of Th1 cells, it has become apparent that IL-27 has both proinflammatory and anti-inflammatory properties.[26] Recently, two independent studies have evaluated the possible role of IL-27 signaling in autoimmune inflammation by using mice lacking the WSX-1 subunit of the IL-27 receptor.[27,28] To assess T-cell-dependent immune responses in the IL-27R-deficient mice, the two groups induced neuroinflammation by different methods. Battern *et al.* immunized IL-27R-deficient mice and wild-type mice with MOG peptide for induction of EAE.[27] In the other study by Stumhofer *et al.*,[28] IL-27R-deficient mice were chronically infected with *Toxoplasma gondii* to develop severe Th17-dependent neuroinflammation. Both of the groups have concluded that the absence of IL-27-mediated signaling exacerbates neuroinflammation due to an increased number of Th17 cells in inflamed tissue. They found that *in vitro* treatment of naive primary T cells with IL-27 suppresses the development of Th17 cells induced by TGF-β and IL-6 (Fig. 3), and STAT1 is required for the suppressive effect of IL-27. These data suggest that IL-27 may be a useful tool for treating inflammatory diseases mediated by Th17 cells.

INTERLEUKIN-23 PROMOTES INCIDENCE OF TUMORS

BECAUSE IL-23 induces inflammation, which is closely associated with incidence of malignancy, it was thought that IL-23 might promote the growth of tumors. To test this hypothesis, Langowski *et al.*[29] employed a comprehensive examination of mRNA expression within various human tumors. Surprisingly, p19 mRNA was significantly upregulated in the majority of carcinoma samples derived from various human organs when compared with their adjacent normal tissues. In contrast, p35 mRNA was not upregulated in tumors. These data indicate that the expression of IL-23, but not IL-12, is increased in various human tumors. It was also found that the expression of IL-17 was significantly elevated in human tumors, consistent with the activation of IL-23-induced processes. Furthermore, p35-deficient mice developed significantly increased numbers of papillomas compared to control mice, whereas p19-deficient mice were resistant to tumor induction. In addition, genetic deletion of p19 or antibody-mediated elimination of IL-23 led to increased infiltration of CTL, rendering a protective effect against cancer. Growth of transplanted tumors was limited in p19-deficient mice or IL-23 receptor-deficient mice. These data show a link between cancer and inflammation, and suggest that anti-IL-23 therapy may prove efficient for tumor treatment.

ROLES OF IL-23 IN INFECTIOUS DISEASES

ALTHOUGH THE IL-23/IL-17 axis is associated with the development of autoimmunity, it seems unlikely that the main physiological function of IL-23 is to promote autoimmunity. Several studies have shown a certain role for IL-23 in resistance to infection with several extracellular pathogens. However, thus far, IL-23 seems to be required for host protection against only two pathogens, *Klebsiella pneumoniae*[30] and *C. rodentium*.[19]

Klebsiella pneumoniae and *C. rodentium*

It was shown that p40-deficient mice were exquisitely sensitive to *K. pneumoniae*, and that p19-, p35- and IL-17R-deficient mice were also susceptible to this infection.[30] These data indicate that both of the IL-12/IFN-γ and IL-23/IL-17 pathways are critical for host defense

against *K. pneumoniae*. Time course of p19, p40 and p35 gene expression in bronchoalveolar lavage cells following *K. pneumoniae* infection indicated that IL-23 was induced rapidly and sooner than IL-12. In addition, p19-deficient mice dramatically reduced IL-17 production and showed substantial mortality from a normally sublethal dose of bacteria, despite normal IFN-γ production. Administration of recombinant IL-17 restored bacterial control in p19-deficient mice, suggesting that IL-17 plays a significant role in the host defense. Thus, the IL-23/IL-17 axis plays a critical role in early host resistance to *K. pneumoniae* independently of IL-12, indicating divergent roles of IL-12 and IL-23 in this host defense.[30] IL-23 is also required for host protection against *C. rodentium*. [19] It was shown that even with the presence of Th17 cells, IL-23-deficient mice succumb to infection with *C. rodentium*. These findings indicate that IL-23 is needed for the full differentiation of Th17 cells.

Toxoplasma gondii

Toxoplasma gondii is an important opportunistic pathogen in patients with primary or acquired T-cell deficiencies. Because p40 is required for the development of the Th1-type response, which is necessary for resistance to *T. gondii*, Lieberman *et al.*[31] assessed the role of IL-23 in comparison with IL-12 for the host defense against this pathogen. When gene-targeted mice lacking either p40 or p35 were infected with *T. gondii*, p40-deficient mice and p35-deficient mice succumbed to the infection. Administration of recombinant IL-23 to p40-deficient mice resulted in a decreased parasite burden. However, when p19-deficient mice were infected with *T. gondii*, these mice controlled parasite replication to the same extent as wild-type mice. Taken together, these findings indicate that IL-12, not IL-23, plays a dominant role in resistance to this parasite, but, in the absence of IL-12, IL-23 can provide a limited mechanism of resistance to this infection.

Mycobacterium tuberculosis

Tuberculosis is the most prevalent infectious disease worldwide. It is known that IL-12-induced IFN-γ is required to control the growth of *M. tuberculosis*. p40-deficient mice were very sensitive to *M. tuberculosis*. In contrast, mice lacking the p35 subunit were less susceptible to this infection, suggesting that IL-23 may play a crucial role in resistance to *M. tuberculosis*. However, Khader *et al.*[32] have shown that p19-deficient mice controlled mycobacterial growth and there was no diminution in the number of antigen-induced, IFN-γ-producing $CD4^+$ T cells or local IFN-γ mRNA expression. Conversely, there was an almost total loss of both IL-17-producing antigen-specific $CD4^+$ T cells and local IL-17 mRNA expression in these mice. These data indicate that IL-23 is dispensable for the protection against *M. tuberculosis* although IL-23 is required to provide a moderate level of protection in the absence of IL-12. This result is quite similar to the case of *T. gondii* infection. This protection by IL-23 is associated with the IL-12-independent induction of IFN-γ-producing $CD4^+$ T cells.

Cryptococcus neoformans

Cryptococcus neoformans is an encapsulated yeast-like, facultative intracellular organism and causes meningoencephalitis in immunodeficient hosts. IL-12 and IFN-γ play a central role in protective immunity to this pathogen. To investigate the role of IL-23 in host resistance during chronic fungal infection, p40-, p35- and p19-deficient mice were infected with *C. neoformans*.[33] It was shown that p40-deficient mice demonstrated higher mortality than p35-deficient mice. Reconstitution of p40-deficient mice with recombinant IL-23 prolonged their survival to levels similar to p35-deficient mice, suggesting a certain role of IL-23 in the host defense against *C. neoformans*. p19-deficient mice showed a moderately reduced survival time and delayed fungal clearance in the liver. Although IFN-γ production was similar in wild-type and p19-deficient mice, IL-17 production was strongly impaired in p19-deficient mice. These results indicate that IL-23 complements the more dominant role of IL-12 in protection against a chronic fungal infection by an enhanced inflammatory cell response and distinct cytokine regulation, although IL-23 is not sufficient for complete protection in the absence of IL-12.

CONCLUSIONS

ALTHOUGH IL-23 was initially supposed to be an analog of IL-12 due to the structural similarities, several studies have shown that IL-23 is functionally distinct from IL-12; that is, IL-12 leads to the development of IFN-γ producing Th1 cells, whereas IL-23 amplifies and stabilizes a new $CD4^+$ T-cell subset, Th17, which produces a proinflammatory cytokine, IL-17 (Fig. 3). Importantly, the IL-23/Th17 axis rather than the IL-12/Th1 axis is likely to contribute to a number of T-cell-mediated inflammatory autoimmune diseases. In fact, levels of IL-23 and IL-17 are increased in human diseases such as multiple sclerosis, Crohn's disease, psoriasis, ulcerative colitis, cystic fibrosis, asthma and rheumatoid arthritis. Furthermore, the IL-23/Th17 axis

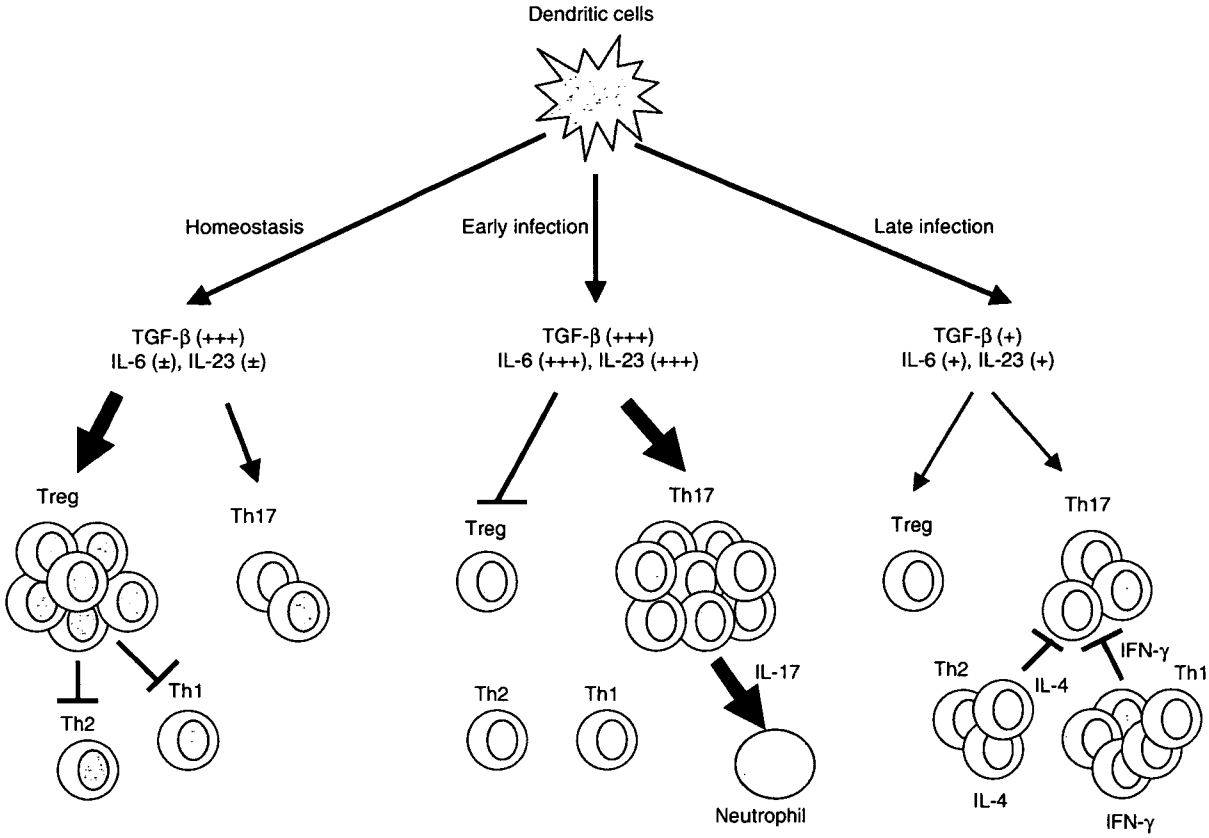

Figure 4 The balance between regulatory Treg and Th17 cells during infection. In homeostasis, TGF-β promotes the differentiation of Treg cells. Therefore, a large number of Treg cells suppress Th1 and Th2 cells. During the early phase of mucosal infection, dendritic cells activated via toll-like receptors produce large amounts of IL-6 and IL-23. TGF-β and IL-6 enhance the differentiation of Th17, whereas IL-6 inhibits the differentiation and function of Treg cells. IL-23 then amplifies and stabilizes Th17 cells. IL-17 released by Th17 cells recruits neutrophils to protect the host from pathogens. During the late phase of infection, Th1 and Th2 cells perform final clearance of infection. IL-4 and IFN-γ produced by them inhibit Th17 differentiation and function. After infection and inflammation, TGF-β-dependent Treg cells bring homeostasis again.

promotes tumor incidence and growth, demonstrating a link between inflammation and tumor. These findings suggest that IL-23 might be a viable target for the treatment of T-cell-mediated autoimmune diseases and tumors. For example, antagonist of p19 might be able to repress such inflammatory diseases while leaving the IL-12/IFN-γ axis intact. However, the main physiological function of IL-23 seems unlikely to promote autoimmunity or tumor. Several groups have indicated that IL-23 plays a crucial role in resistance to infection with certain extracellular bacteria. Thus far, however, the involvement of IL-23 is likely to be required for the host protection against only two pathogens, *K. pneumoniae*[30] and *C. rodentium*.[19] It is not obvious why it is so difficult to find a function for IL-23 in protection against specific pathogens. Cue *et al.* suggested in their review[34] that the IL-23/Th17 axis has not been evolved to provide host protection against specific classes of pathogens but has been designed for the induction of immediate inflammatory response against catastrophic breaches of pathogens in the mucosal barrier of the lung and gut. They have proposed a model whereby Th17 cells and Treg cells may work together to elicit or restrain tissue inflammation (Fig. 4). During homeostasis, TGF-β induces a local large population of Treg cells which maintain the homeostasis by suppressing Th1 and Th2 cells. During the initial phase of mucosal infection, dendritic cells are triggered via toll-like receptors to

secrete large amounts of IL-6 and IL-23. As a consequence, a number of Th17 cells produce IL-17 which recruits neutrophils to protect the host from pathogens. During the late phase of infection, microbe-specific Th1 and Th2 cells enter the inflamed mucosa, and clear the infection.

Whatever the physiological role of IL-23 turns out to be, the adjuvant activity of IL-23 might be useful for the prophylactic and therapeutic strategies against infectious pathogens. Especially, a combination of IL-12 and IL-23 should lead to a synergistic adjuvant effect even at a low dose, and thereby might avoid side-effects such as the IL-12-associated toxicity. However, it is essential to take into consideration that cytokines are pleiotropic, and therefore, influence many elements of the immune system for better or worse. It is important to understand more details concerning the molecular mechanisms of the IL-23-mediated immune responses for the development of new therapies for infectious diseases.

ACKNOWLEDGMENTS

I AM GRATEFUL to all my collaborators: S. Ohno, S. Kohyama, A. Isoda, H. Hayashi (Josai University); T. Yoshimoto (Tokyo Medical University); and O. Moriya, T. Akatsuka (Saitama Medical University).

CONFLICT OF INTEREST

NO CONFLICT OF interest has been declared by M. Matsui.

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The Journal of Immunology

IL-23 Enhances Host Defense against Vaccinia Virus Infection Via a Mechanism Partly Involving IL-17[1]

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To investigate roles of IL-23 in viral infection, we have engineered recombinant vaccinia virus (VV) expressing IL-12 (VV-IL-12) and expressing IL-23 (VV-IL-23). We found VV-IL-23 was less virulent in BALB/c mice than wild-type VV (VV-WT), indicating that IL-23 enhances resistance to VV. VV-specific CTL activity in VV-IL-23-infected mice was slightly higher than activity in VV-WT-inoculated mice, although antiviral Ab production and NK activity were not increased. IL-12/23p40-deficient mice survived the infection with VV-IL-23, indicating that IL-23 promotes VV resistance independently of IL-12. The mechanism of the IL-23-mediated resistance was distinct from that of the IL-12-regulated resistance because IFN-γ-deficient mice did not eliminate VV-IL-12, but did eradicate VV-IL-23. These data indicate that IFN-γ is essential for the IL-12-mediated resistance, but dispensable for the IL-23-regulated resistance. Because IL-17 is a key in the IL-23-regulated resistance to bacteria, we hypothesized an involvement of IL-17 in the resistance to VV. Treatment with an anti-IL-17 mAb resulted in a significant increase of viral titers in VV-IL-23-infected IFN-γ-deficient mice. In addition, VV-IL-17 was less virulent than VV-WT in BALB/c mice, and IL-17-deficient mice were more sensitive to VV-WT than control mice. However, the effect of neutralization with an anti-IL-17 mAb was limited, and IL-17-deficient mice survived the infection with VV-IL-23. Taken together, these data suggest that the IL-23/IL-17 axis plays a certain but subdominant role in the IL-23-mediated resistance to VV. Unveiling of an alternative pathway in the IL-23-regulated resistance might provide a novel strategy against infectious pathogens without side effects of autoimmunity. ***The Journal of Immunology*****, 2007, 179: 3917–3925.**

Interleukin-12 is a heterodimeric proinflammatory cytokine composed of a 35-kDa L chain (p35) and a 40-kDa H chain (p40). IL-12 leads to the development of IFN-γ-producing Th1 cells, and therefore, this cytokine is considered to be a key regulator of cell-mediated immunity (1). A novel IL-12-associated cytokine, IL-23 is also a heterodimer formed by the p40 subunit of IL-12 and a new subunit, p19 (2). Furthermore, the receptor for IL-23 consists of one subunit of the IL-12 heterodimeric receptor, IL-12Rβ1, and a new receptor subunit, IL-23R (3). As a consequence of the structural similarity, it was originally considered that IL-23 would regulate Th1-mediated immune responses as well as IL-12. In fact, early studies demonstrated that IL-23 stimulated the production of IFN-γ and enhanced the activity of cellular immunity including CTLs (4–6). However, there is now increasing evidence that IL-23 is functionally distinct from IL-12 in the regulation of T cell-mediated immunity.

Although IL-12 promotes the differentiation of IFN-γ-producing Th1 cells and thereby generates resistance to intracellular infections, it was previously believed that IL-12-induced Th1 cells also triggered autoimmune diseases such as experimental allergic encephalomyelitis (EAE)[3] and collagen-induced arthritis. However, Cua and colleagues (7, 8) have demonstrated that mice deficient in either the IL-23p19 subunit or the IL-12/23p40 subunit are resistant to EAE and collagen-induced arthritis, whereas IL-12p35-deficient mice remain susceptible to these diseases. These data indicate that IL-23, not IL-12, is essential for the development of these autoimmune diseases. Furthermore, it was initially shown that IL-23 stimulated naive precursor cells to differentiate into a new $CD4^+$ T cell subset termed Th17 in the absence of IFN-γ and IL-4 (9). However, recent papers (10–12) have revealed that TGF-β and IL-6 drive the differentiation of naive T cells into Th17 cells. Although IL-23 does not induce the Th17 differentiation, this cytokine is essential for Th17 cells to expand and acquire full effector function (10–12). Because IL-23R is not expressed on naive T cells, TGF-β and IL-6 act to up-regulate IL-23R expression on them, and thereby conferring responsiveness to IL-23. Th17 cells produce IL-17 (IL-17A), IL-17F, IL-6, and TNF-α but not IFN-γ or IL-4 and, therefore, this subset is different from the classical Th1 or Th2 subset (9, 13). IL-17 has pleiotropic activities including the induction of a variety of proinflammatory cytokines, chemokines, and adhesion molecules on various cell types. IL-17 is detected in serum of patients with rheumatoid arthritis, multiple

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Received for publication June 14, 2007. Accepted for publication July 2, 2007.

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[1] This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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[3] Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; VV, vaccinia virus; VV-IL-12, VV expressing IL-12; VV-IL-23, VV expressing IL-23; VV-IL-17, VV expressing IL-17; VV-WT, wild-type VV; sc, single chain; KO, knockout; MOI, multiplicity of infection.

sclerosis, systemic lupus erythematosus, and asthma, suggesting that its involvement in the development of various human autoimmune diseases (14). In addition, IL-17-deficient mice are resistant to the development of collagen-induced arthritis (15) and EAE (16), suggesting a pathogenic role of IL-17 in these autoimmune diseases. Thus, the IL-23/Th17/IL-17 axis rather than the IL-12/Th1/IFN-γ axis is likely to be associated with T cell-mediated autoimmune diseases. However, the physiological function of IL-23 remains largely unknown.

Several groups have investigated a role of IL-23 in resistance to infection with several pathogens. It was shown that IL-12/23p40-, IL-23p19-, IL-12p35-, and IL-17R-deficient mice were sensitive to *Klebsiella pneumonia* (17), indicating that both of the IL-12/IFN-γ and IL-23/IL-17 axes are critical for the host defense against *K. pneumoniae*. However, IL-23 was induced sooner than IL-12 in this infection. Furthermore, IL-23p19-deficient mice dramatically reduced IL-17 production and showed substantial mortality from a normally sublethal dose of *K. pneumoniae*, despite normal IFN-γ production. In addition, administration of recombinant IL-17 restored bacterial control in IL-23p19-deficient mice, suggesting that IL-17 induced by IL-23 plays a significant role in the early host defense against *K. pneumoniae* (17). The IL-23/IL-17 axis is also necessary for host protection against *Citrobacter rodentium* (11). Thus, IL-23 seems to be important for host protection against several bacteria, and IL-17 plays a critical role in the IL-23-mediated resistance. However, it is unknown whether the IL-23/IL-17 axis is required for host defense against viral infection.

In the present study, we have engineered recombinant vaccinia virus (VV) expressing IL-12 (VV-IL-12) and IL-23 (VV-IL-23), and investigated a role of the IL-23/IL-17 axis for host defense against virus in comparison with the IL-12/IFN-γ axis.

Materials and Methods

Cell lines

The mouse mastocytoma cell line P815 (H-2^d), the Moloney murine leukemia virus-induced lymphoma YAC-1, the mouse fibroblast cell line NIH 3T3, and the human embryonic kidney cell line HEK 293T were obtained from the ATCC. The African green monkey-derived kidney cell lines CV-1 and BS-C-1, and the human osteosarcoma, thymidine kinase-defective cell line C143 were provided by Dr. T. Shioda (Osaka University, Osaka, Japan). These cell lines were cultured in DMEM with 10% FCS.

Construction of recombinant viruses

VV-IL-12, VV-IL-23, and VV expressing murine IL-17 (VV-IL-17) were generated as described (18). In brief, murine IL-17 cDNA without its leader sequence was generated by RT-PCR from total RNA of Con A-activated spleen cells of BALB/c mice (sense) 5′-AAGCTTGCGGCTA CAGTGAAGGCA-3′ (antisense) 5′-TCTAGATTAGGCTGCCTGGCG GACA-3′ and was subcloned into the pCR2.1 vector (Invitrogen Life Technologies). Following digestion with *Hin*dIII and *Xba*I, murine IL-17 cDNA was cloned into the p3xFLAG-CMV-9 (Sigma-Aldrich) vector (p3xFLAG-IL-17). This expression vector encodes the preprotrypsin signal peptide and the 3xFLAG-epitope-tag sequence upstream of the multiple cloning region and, hence, expresses a secreted N-terminal 3xFLAG fusion protein in mammalian cells. Conversely, the murine IL-12 (p3xFLAG-IL-12) and murine IL-23 (p3xFLAG-IL-23) expression plasmids, which include murine single-chain (sc)IL-12 and murine scIL-23 genes, respectively, had previously been constructed (6). The scIL-12 and scIL-23 genes are composed of p40 genetically fused with p35 and p19, respectively (6). The biological activity of each single-chain cytokine was shown to be almost similar to that of the native heterodimeric cytokine (4, 5).

Murine IL-17, scIL-12, and scIL-23 genes linked to a nucleotide sequence comprised of the preprotrypsin signal sequence and the 3xFLAG-tag sequence were isolated by PCR amplification from p3xFLAG-IL-17, p3xFLAG-IL-12, and p3xFLAG-IL-23, respectively. Primers used include the following: murine IL-12, IL-17, and IL-23 (sense) 5′-GGATCCGC CACCATGTCTGCACTTCTGATCCTAGCT-3′ and (antisense for murine IL-12) 5′-CTCGAGTCAGGCGGAGCTCAGATAGC-3′, (antisense for murine IL-17) 5′-CCCGGGTTAGGCTGCCTGGCGGACA-3′, and (antisense for murine IL-23) 5′-GAGTCAAGCTGTTGGCACTAA GGGCTC-3′. The amplified genes were then inserted into the transfer vector pNZ68K2 (18). VV-IL-12, VV-IL-17, and VV-IL-23 were then generated by homologous recombination between wild-type VV (VV-WT) (WR strain) and the transfer vector, purified by three cycles of plaque cloning with C143 cells in the presence of BrdU, and propagated in CV-1 cells (18).

Expression of scIL-12 and scIL-23 fusion proteins

293T cells were infected with VV at a multiplicity of infection (MOI) of 3 for 1.5 h, and incubated for 2 days at 37°C. Fusion proteins in the culture supernatants were then immunoprecipitated using the anti-FLAG M2 mAb (Sigma-Aldrich) and protein G-Sepharose (Amersham Biosciences), as previously described (6). Following immunoprecipitation, Western blotting of immunoprecipitated proteins was performed (6). Briefly, the fusion proteins were separated by electrophoresis on a 12% SDS-PAGE under reducing conditions, and blotted onto a nitrocellulose membrane. The blot was stained with 5 μg/ml M2 mAb (Sigma-Aldrich) for 1 h at room temperature, followed by secondary staining with peroxidase-conjugated anti-mouse IgG Ab. The protein bands were developed by the BCIP/NBT phosphatase substrate system (Kirkegaard & Perry Laboratories).

Tyrosine phosphorylation of STAT4 triggered by either murine IL-12 or murine IL-23 was detected as described before (6). In brief, 293 T cells expressing either murine IL-12Rβ1 and murine IL-12Rβ2, or murine IL-12Rβ1 and murine IL-23R, were stimulated for 45 min with the culture supernatant of 293 T cells infected with VV at various concentrations of 0.2, 2.0, and 20%. Cells were then subjected to Western blotting using anti-STAT4 (Santa Cruz Biotechnology) and anti-phosphorylation-STAT4 (Zymed Laboratories) Abs.

Preparation of recombinant IL-12 and IL-23 proteins

Recombinant murine scIL-12 and recombinant scIL-23 were prepared as described (19). In brief, human embryonic kidney 293F cells were transiently transfected with p3xFLAG-IL-12 or p3xFLAG-IL-23 by using 293 fection (Invitrogen Life Technologies). After 3 days, culture supernatants were harvested and 3xFLAG-tagged recombinant scIL-12 and scIL-23 were purified by affinity chromatography using anti-FLAG affinity gel (Sigma-Aldrich). The concentration of 3xFLAG-tagged recombinant scIL-12 was determined using murine recombinant IL-12 as a standard in ELISA. Protein concentration of 3xFLAG-tagged recombinant scIL-23 was determined by titration in Western blotting with anti-FLAG (M2) mAb using 3xFLAG-tagged scIL-12 prepared as a standard.

Mice and infection

BALB/c and C57BL/6 mice were purchased from Japan Charles River Breeding Laboratory. IL-12/23p40 gene knockout (KO) mice (20) of the BALB/c background were purchased from The Jackson Laboratory. IFN-γ gene KO mice (21) and IL-17 gene KO mice (22) were previously described and were backcrossed eight generations to C57BL/6 and BALB/c, respectively. Six- to 8-wk-old mice were used for all experiments. Mice were housed in appropriate animal care facilities at Saitama Medical University (Saitama, Japan), and handled according to international guidelines for experiments with animals.

Each mouse was infected i.p. with 5×10^6 PFU of VV for the ovary VV titer assay or 2×10^7 PFU of VV for the cytotoxic assay, the intracellular cytokine staining assay, and detection of VV-specific Abs. To plot survival curves, 6–12 mice in each group were infected i.p. with either 2×10^8 or 5×10^8 PFU of VV, and were examined daily for weight loss and mortality.

Ovary VV titer assay

Mice were sacrificed on certain days after infection with 5×10^6 PFU of VV, and viral titers in ovaries were measured as described before (23). Six to twelve mice were used in each group. All titrations were performed in duplicates, and the average PFU per mouse was calculated. Some mice were treated in vivo by i.p. injection with 70 μg of neutralizing anti-mouse IL-17 mAb (clone 50104.11; R&D Systems) or a relevant isotype control at days 0, 2, and 7 postinfection with VV.

Cytotoxic assay

At the indicated time points following infection with 2×10^7 PFU of VV, mice were sacrificed, and spleen cells were prepared for effector cells. Cytotoxic activities of VV-specific CTLs and NK cells were measured in standard ^{51}Cr release assays. For preparation of virus-infected targets in CTL assays, P815 cells were infected with VV at an MOI of 3 for 1.5 h at 37°C, washed three times, and incubated in RPMI 1640 containing 10%

FCS overnight at 37°C. For detection of NK cell-mediated lysis, YAC-1 cells were used for targets. Target cells (1×10^6 cells) were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 30 min at 37°C. After washing three times, the labeled target cells were plated in wells of a round-bottom 96-well plate at 1×10^4 cells/well with or without effector cells at various E:T ratios. After a 4-h incubation at 37°C, supernatant from each well was harvested and the radioactivity was counted. The results were calculated as the mean of a triplicate assay. The percentage of specific lysis was calculated according to the formula: Percentage of specific lysis = $((cpm_{sample} - cpm_{spontaneous})/(cpm_{maximum} - cpm_{spontaneous})) \times 100$, where spontaneous release represents the radioactivity released by target cells in the absence of effectors, and maximum release represents the radioactivity released by target cells lysed with 5% Triton X-100. At least three mice per group were used in each experiment. The experiment was repeated three times.

Intracellular cytokine staining

Intracellular cytokine staining was performed as previously described (23). Briefly, spleen cells of three to five mice per group infected with VV were pooled and resuspended in RPMI 1640 containing 10% FCS. In each well of a 96-well round-bottom plate, 2×10^6 spleen cells were incubated with 1×10^5 cells of either VV-infected P815 or naive P815 in the presence of 0.2 μl/well brefeldin A (GolgiPlug; BD Biosciences) for 5 h at 37°C. The cells were then washed once and incubated for 10 min at 4°C with the rat anti-mouse CD16/CD32 mAb (Fc Block; BD Biosciences) at a concentration of 1 μg/well. Following incubation, cells were stained with FITC-conjugated rat anti-mouse CD8α mAb (clone 53-6.7; BD Biosciences) at a concentration of 0.5 μg/well for 30 min at 4°C. After washing twice, the cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), and stained with PE-conjugated rat anti-mouse IFN-γ (clone XMG1.2; BD Biosciences) mAb. After washing, flow cytometric analyses were performed. The experiment was repeated three times.

Detection of VV-specific Abs

Titers of VV-specific serum Abs were determined by ELISA as described before (23). In brief, each well of a 96-well flat-bottom plate (model no. 3590; Costar) was coated with VV-infected cell lysate diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 5×10^7 PFU/ml. The plate was then fixed with 2% paraformaldehyde and washed three times with PBS containing 0.05% Tween 20 (PBS-Tween 20). After blocking, 100 μl of diluted mouse serum was added to each well, and the plate was incubated for 1 h at 37°C. After washing three times with PBS-Tween 20, HRP-conjugated goat anti-mouse IgM, IgG1, or IgG2a Ab (Sigma-Aldrich) diluted 1/5000 in blocking buffer was added, and the plate was incubated for 1 h at 37°C. After washing five times with PBS-Tween 20, 100 μl of *o*-phenylenediamine dihydrochloride substrate (Sigma-Aldrich) was added to each well. The reaction was stopped with 50 μl/well of 6 N H_2SO_4 per well and the plate was read at 492 nm. Four to six mice per group were used in the experiments.

Cytokine ELISA

Spleen cells of naive mice were infected in vitro with VV at an MOI of 3, and cultured in wells of a 96-well round-bottom plate at 1×10^6/well for 2 days at 37°C. In some experiments, naive spleen cells were cultured with either murine recombinant IL-12 or murine recombinant IL-23 at various concentrations for 2 days at 37°C. Culture supernatants were then harvested and screened for the presence of murine IFN-γ and murine IL-17 by ELISA as described (23). Capture Abs, biotinylated detection Abs, and recombinant cytokines were purchased from BD Biosciences. Quantitative ELISA for murine IFN-γ and murine IL-17 was performed using paired mAbs specific for corresponding cytokines according to the manufacturer's instruction. Briefly, an ELISA plate was coated with a capture Ab for each cytokine and incubated overnight at 4°C. The plate was washed with PBS-Tween 20 and blocked with 10% calf serum in PBS for 2 h at room temperature. After washing, serially diluted samples and a recombinant standard were added to the plate and incubated at 4°C overnight. The plate was washed four times followed by the addition of cytokine-specific detection Abs. After 1 h of incubation at room temperature, HRP-conjugated avidin (BD Biosciences) was added. The color was developed by adding *o*-phenylenediamine dihydrochloride substrate, and the reaction was stopped with H_2SO_4. The concentration of each cytokine was calculated by reading the plates at 492 nm. Data represent three to five mice per group and are given as mean values ± SEM.

Detection of IL-17 secreted from VV-IL-17-infected cells

The 293 T cells were infected with either VV-IL-17 or VV-WT at an MOI of 3 for 1.5 h, and incubated for 2 days at 37°C. Murine IL-17 in the culture supernatants was then quantitated by ELISA as described. The biological activity of murine IL-17 was also measured by its ability to induce murine IL-6 production by NIH 3T3 as also described (24). Briefly, NIH 3T3 cells (2.5×10^6/well) were cultured with the culture supernatants of VV-IL-17- or VV-WT-infected 293T cells in wells of a 24-well plate. After incubation for 24 h at 37°C, 50 μl of supernatant was removed and assayed for murine IL-6 using ELISA as described. Capture and biotinylated detection Abs and recombinant murine IL-6 were purchased from BD Biosciences.

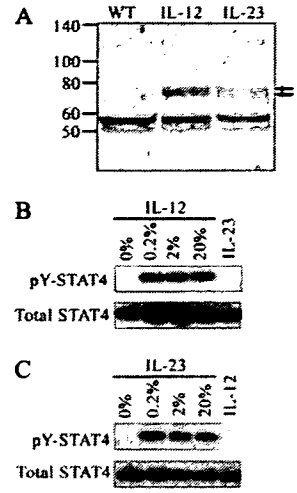

FIGURE 1. Detection of the murine scIL-12 and scIL-23 fusion proteins secreted from VV-infected cells. *A*, 293 T cells were infected with VV-WT (WT), VV-IL-12 (IL-12), or VV-IL-23 (IL-23). Proteins secreted in the supernatants were immunoprecipitated with the anti-FLAG mAb. The immunoprecipitated proteins were then subjected to Western blot analysis with the anti-FLAG mAb. The positions of protein molecular mass markers in kilodaltons are shown. Arrows indicate the bands of the murine scIL-12 (76 kDa) and scIL-23 (74 kDa) fusion proteins. *B* and *C*, STAT tyrosine phosphorylation assay. 293T cells expressing either murine IL-12Rβ1 and IL-12Rβ2 (*B*), or murine IL-12Rβ1 and IL-23R (*C*) were stimulated for 45 min with the culture supernatant containing either murine scIL-12 (IL-12) (*B*) or scIL-23 (IL-23) (*C*) at final concentrations of 0, 0.2, 2, and 20%. As negative controls, the cells were stimulated for 45 min with the culture supernatant containing either murine scIL-23 (IL-23) (*B*) or scIL-12 (IL-12) (*C*) at a final concentration of 20%. The cells were then subjected to Western blotting using anti-STAT4 (Total STAT4) and anti-phosphorylation-STAT4 (pY-STAT4) Abs.

Statistical analyses

Statistical analyses were performed with Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

Results

Secretion of scIL-12 and scIL-23 from VV-infected cells

VV-IL-12 and VV-IL-23 were generated by homologous recombination between VV-WT and the transfer vector. The 293T cells were then infected with VV-WT, VV-IL-12, or VV-IL-23, and culture supernatants were harvested. Fusion proteins secreted into the culture supernatants were immunoprecipitated and subjected to Western blot analysis using the anti-FLAG mAb. As shown in Fig. 1*A*, the scIL-12 and scIL-23 fusion proteins were detected in lanes as single polypeptide bands of 76 and 74 kDa, respectively. Furthermore, STAT4 in 293T cells expressing the IL-12R composed of IL-12Rβ1 and IL-12Rβ2 was phosphorylated on tyrosine in response to scIL-12, but not by scIL-23 (Fig. 1*B*). In contrast, scIL-23 induced STAT4 tyrosine phosphorylation in 293 T cells expressing the IL-23R consisting of IL-12Rβ1 and IL-23R (Fig. 1*C*). These data indicate that scIL-12 and scIL-23 proteins secreted from VV-infected cells are functional.

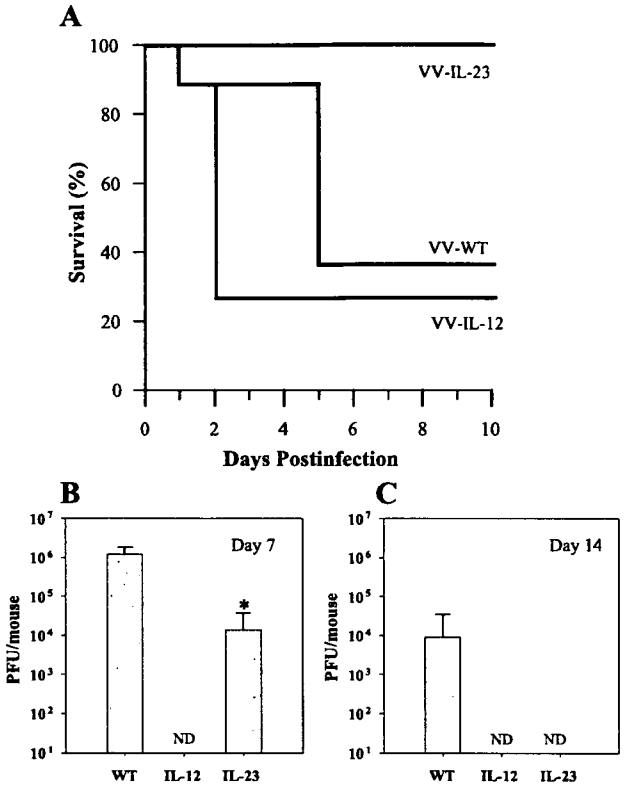

FIGURE 2. IL-23 delivered by VV-IL-23 enhances resistance to VV infection in BALB/c mice. BALB/c mice were infected i.p. with 2×10^8 PFU (*A*) or 5×10^6 PFU (*B* and *C*) of VV-WT, VV-IL-12, or VV-IL-23. *A*, Eight mice per group were monitored daily for mortality. *B* and *C*, BALB/c mice inoculated with VV-WT (WT), VV-IL-12 (IL-12), or VV-IL-23 (IL-23) were sacrificed at days 7 (*B*) and 14 (*C*) postinfection, and viral titers in ovaries were measured. From 8 to 12 mice were used in each group, and all titrations were performed in duplicates. Data are representative of one of three independent experiments, and results are shown as the mean PFU ± SEM. ND, Not detected. *, $p < 0.001$ compared with VV-WT.

IL-23 delivered by VV-IL-23 enhances resistance to VV infection in mice

To investigate the effect of IL-23 on resistance to VV infection in comparison with IL-12, BALB/c mice were infected i.p. with a high dose (2×10^8 PFU) of VV-WT, VV-IL-12, or VV-IL-23 and were monitored daily for mortality. As shown in Fig. 2*A*, five of eight VV-WT-injected mice died by day 5 postinfection, whereas all mice ($n = 8$) survived the infection with VV-IL-23. These findings demonstrate that IL-23 enhances host defense against VV in mice. In contrast, six of eight mice succumbed to the infection with 2×10^8 PFU of VV-IL-12 by day 2 postinfection (Fig. 2*A*), presumably due to the IL-12-associated toxicity at a high concentration of IL-12 (25). We next examined viral load in ovaries of mice at days 7 (Fig. 2*B*) and 14 (Fig. 2*C*) after inoculation with a sublethal dose (5×10^6 PFU) of VV. As shown in Fig. 2*B*, VV-IL-23-infected mice harbored nearly 100-fold less PFU of virus than VV-WT-infected mice at day 7 postinfection. Furthermore, VV-IL-23-injected mice completely cleared VV from ovaries at day 14 postinfection, although VV-WT-infected mice still held high virus titers on the same day (Fig. 2*C*). These data indicate IL-23 delivered by VV-IL-23 leads to rapid clearance of the virus from infected mice. Conversely, no virus was detected in ovaries of mice inoculated with a sublethal dose (5×10^6 PFU) of VV-IL-12 even at day 7 postinfection (Fig. 2*B*), indicating the potent activity of IL-12 in the clearance of VV.

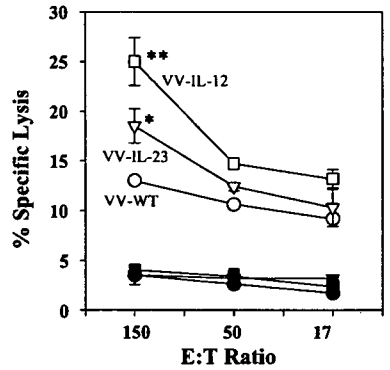

FIGURE 3. Activity of VV-specific CTLs in mice infected with VV-IL-12 and VV-IL-23. Spleen cells were prepared from mice at 2 wk after infection with 2×10^7 PFU of VV-WT (circles), VV-IL-12 (squares), or VV-IL-23 (inverted triangles). ^{51}Cr release assays were then performed to detect VV-specific CTL activity in spleen cells at various E:T ratios, using P815 cells infected with (open symbols) or without (filled symbols) VV-WT as targets. Data are representative of three independent and reproducible experiments. Results are shown as the mean ± SEM of triplicate wells. *, $p < 0.05$ compared with VV-WT; **, $p < 0.05$ compared with VV-IL-23.

Effect of IL-23 on cellular and humoral immunity to VV

Because $CD8^+$ CTLs play a critical role in the host defense against VV (26, 27), we tested the cytolytic activity of VV-specific CTLs in mice infected with 2×10^7 PFU of VV-WT, VV-IL-12, or VV-IL-23. As expected, the activity of VV-specific CTLs in VV-IL-12-infected mice was significantly higher than activity in VV-WT-injected mice (Fig. 3*A*), indicating that IL-12 enhanced the induction of VV-specific CTLs. In contrast, IL-23 delivery by VV resulted in a slight increase of VV-specific CTL induction (Fig. 3). We next examined the number of Ag-induced intracellular IFN-γ-positive $CD8^+$ T cells in spleen cells of mice infected with 2×10^7 PFU of VV (Fig. 4). Because spleen cells were stimulated in vitro with VV-WT-infected P815 cells for only 5 h, the possibility of substantial in vitro expansion of responder cells is precluded (28). At day 7 postinfection, the frequency of IFN-γ-positive $CD8^+$ T cells in mice infected with VV-IL-12 (11.94%) was ~2-fold higher than that in mice infected with-VV-WT (5.69%). In contrast, percentage of IFN-γ-producing $CD8^+$ T cells in VV-IL-23-infected mice (7.69%) was modestly increased when compared with that in VV-WT-infected mice (5.69%). These data suggest that IL-12 delivery by VV is likely to be more efficient for the induction of VV-specific CTLs than IL-23 delivery. However, these data also suggest that the VV-specific CTL activity slightly enhanced by IL-23 may be at least partly responsible for the attenuation of virulence of VV-IL-23.

It was indicated that humoral immunity was essential to protect against VV infection (26, 27). Therefore, antiviral IgM and IgG Abs were monitored in sera of mice after infection with a sublethal dose of VV-WT, VV-IL-12, or VV-IL-23 (Fig. 5, *A–C*). Levels of VV-specific IgG1 and IgG2a Abs in VV-IL-23-infected mice were almost equal to those in VV-WT-inoculated mice at days 7 (data not shown) and 14 (Fig. 5, *A* and *B*) postinfection. In contrast, VV-IL-12-inoculated mice produced much less VV-specific IgG1 Ab than VV-WT-injected mice (Fig. 5*A*), whereas there was no major difference in the production of VV-specific IgG2a Ab between the two groups (Fig. 5*B*). These data suggest that the effect of IL-23 on Ab production is likely to be different from that of

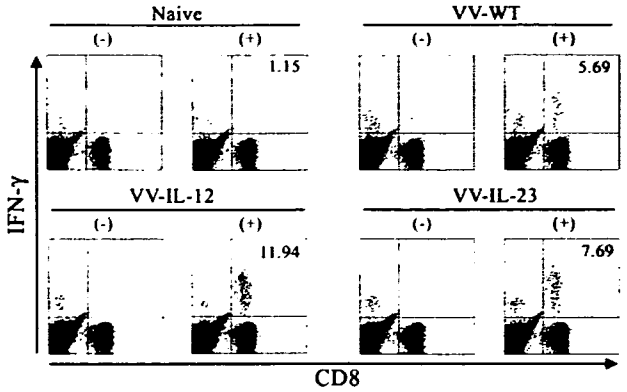

FIGURE 4. Intracellular IFN-γ staining of VV-specific $CD8^+$ T cells in mice infected with VV-WT, VV-IL-12, or VV-IL-23. BALB/c mice were infected with 2×10^7 PFU of either VV-WT, VV-IL-12, or VV-IL-23, and spleen cells were prepared at day 7 postinfection. Spleen cells of noninfected mice (Naive) were used as a negative control. Spleen cells were then stimulated with VV-infected P815 (+) or noninfected P815 (−) cells for 5 h. After stimulation, cells were stained for their surface expression of CD8 (*x*-axis) with FITC-conjugated mAb and for their intracellular expression of IFN-γ (*y*-axis) with PE-conjugated mAb. All lymphocytes were gated and analyzed on a FACScan flow cytometer using CellQuest software (BD Biosciences). Values shown in *upper right quadrant* indicate the percentage of $CD8^+$ cells that are positive for intracellular IFN-γ. Each experiment used 3–5 mice per group, and spleen cells of mice per group were pooled. Data shown are representative of three independent and reproducible experiments.

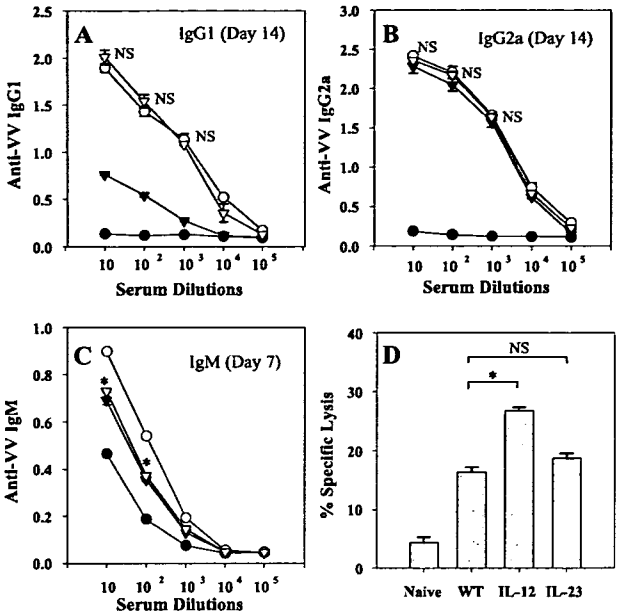

FIGURE 5. VV-specific IgG and IgM Ab production and NK activity in mice infected with either VV-WT, VV-IL-12 or VV-IL-23. *A–C*, BALB/c mice were infected i.p. with 2×10^7 PFU of VV-WT (○), VV-IL-12 (▼), or VV-IL-23 (▽), and serum of each mouse was collected at days 7 (*C*) and 14 (*A* and *B*) postinfection. Serum of noninfected mice (●) was used as negative controls. Titers of anti-VV IgG1 (*A*), IgG2a (*B*), and IgM (*C*) in serially diluted sera were determined by a solid-phase ELISA. Data are shown as the average ± SEM of four to six mice per group. The experiment was repeated twice with similar results. *, $p < 0.05$ compared with VV-WT. *D*, NK cell-mediated cytolytic activity. BALB/c mice were infected i.p. with 2×10^7 PFU of either VV-WT (WT), VV-IL-12 (IL-12), or VV-IL-23 (IL-23). Spleen cells were prepared at day 2 postinfection and used as effector cells in standard ^{51}Cr YAC-1 cell-killing assays. Spleen cells of noninfected mice (Naive) were used as a negative control. Data are shown as the mean ± SEM. The experiment was repeated three times with similar results, and at least three mice per group were used in each experiment. *, $p < 0.05$ compared with VV-WT.

IL-12 (29). Conversely, levels of anti-VV IgM Ab in both VV-IL-12-infected mice and VV-IL-23-infected mice were slightly lower than those in VV-WT-inoculated mice (Fig. 5*C*). Taken together, these data suggest that the increased resistance to VV caused by either IL-12 or IL-23 could not be explained by humoral immunity.

Because NK cells contribute to the recovery from VV infection (30), NK cell-mediated lysis was examined. The cytolytic capacity of splenic NK cells in VV-IL-12-inoculated mice was higher than that in VV-WT-infected mice. However, a significant increase of splenic NK activity was not observed in VV-IL-23-inoculated mice when compared with VV-WT-injected mice (Fig. 5*D*), suggesting that the attenuation of VV-IL-23 is likely to be independent of the activity of NK cells.

VV-IL-23-infected cells produce both IFN-γ and IL-17

It is well known that IFN-γ is a key factor in the IL-12-mediated antiviral response (1). In contrast, it has been reported that IL-17 plays a critical role in the IL-23-dependent resistance to some bacteria such as *K. pneumoniae* (17) and *C. rodentium* (11). Therefore, we wished to evaluate roles of IFN-γ and IL-17 in resistance to VV-IL-12 and VV-IL-23. Firstly, we tested whether VV-IL-12 and VV-IL-23 could stimulate lymphocytes to produce IFN-γ and/or IL-17. Naive spleen cells of BALB/c mice were infected in vitro with VV-WT, VV-IL-12, or VV-IL-23 and cultured for 2 days at 37°C. Culture supernatants were then screened for the presence of IFN-γ and IL-17 by ELISA (Fig. 6, *A* and *B*). VV-IL-12-infected cells secreted large amounts of IFN-γ (21.8 ng/ml), whereas VV-IL-23-infected cells produced much less but significant amounts of IFN-γ (1.4 ng/ml) (Fig. 6*A*). In contrast, VV-IL-12-infected cells did not produce IL-17 at all, but IL-23 delivered by VV-IL-23 led to a high production of IL-17 (760 pg/ml) (Fig. 6*B*). Similar patterns were observed when spleen cells were incubated with various concentrations of recombinant scIL-12 and scIL-23 (Fig. 6, *C* and *D*). As shown in Fig. 6*C*, large amounts of IFN-γ were secreted from cells incubated with recombinant scIL-12 even at a low concentration of 10 pg/ml, whereas recombinant scIL-23 stimulated IFN-γ production much less effectively than recombinant scIL-12. In contrast, recombinant scIL-23 induced IL-17 production in a dose-dependent manner, whereas recombinant scIL-12 did not stimulate secretion of IL-17 at all (Fig. 6*D*).

Effect of IL-23 on resistance to VV in IL-12/23p40-deficient mice

We next examined the effect of IL-23 on resistance to VV in IL-12/23p40-deficient mice. Firstly, BALB/c mice and IL-12/23p40 KO mice of the BALB/c background were infected i.p. with a high dose (2×10^8 PFU/mouse) of either VV-WT or VV-IL-23 and were monitored for mortality. As shown in Fig. 7*A*, all IL-12/23p40 KO mice ($n = 6$) succumbed to the infection with VV-WT by day 7 postinfection, whereas three of seven BALB/c mice survived the infection with VV-WT, demonstrating that IL-12/23p40 KO mice are more susceptible to the infection with VV-WT than are BALB/c mice. However, five of six IL-12/23p40 KO mice survived the infection with VV-IL-23, indicating that IL-23 promotes host defense against VV infection even in the absence of IL-12. Secondly, viral titers in ovaries of IL-12/23p40-deficient