IL-12 Is Not Required for Induction of Type 1 Cytokine Responses in Viral Infections

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IL-12 Is Not Required for Induction of Type 1 Cytokine Responses in Viral Infections

Annette Oxenius, Urs Karrer, Rolf M. Zinkernagel, and Hans Hengartner

To investigate the physiological role of IL-12 in viral infections in terms of T cell cytokine responses involved in virus-specific Ig isotype induction and in antiviral protection, immune responses elicited upon infection of IL-12-deficient mice with lymphocytic choriomeningitis virus (LCMV) or vesicular stomatitis virus (VSV) were studied. Infection of IL-12-deficient mice with LCMV induced a virus-specific type 1 cytokine response as determined by in vitro cytokine secretion patterns as well as by in vivo intracellular cytokine staining of LCMV-specific CD4+ TCR transgenic T cells that had clonally expanded in LCMV-infected IL-12-deficient recipient mice. In addition, LCMV- and VSV-specific IgG responses exhibited normal serum IgG2a/IgG1 ratios, demonstrating again virus-specific CD4+ T cell induction of type 1 phenotype in IL-12-deficient mice upon viral infection. LCMV and VSV immune mice were found to be protected against challenge immunization with recombinant vaccinia viruses expressing either the LCMV- or the VSV-derived glycoprotein, respectively. This protection is known to be mediated by T cell-secreted type 1 cytokines IFN-γ and TNF-α. In contrast, IL-12-deficient mice showed impaired abilities to control infection with the facultative intracellular bacterium Listeria monocytogenes at early time points after infection. However, at later time points of infection, IL-12-deficient mice were able to clear infection. These findings may indicate that viruses are able to induce type 1 T cell responses in the absence of IL-12 as opposed to some bacterial or parasitological infections that are crucially dependent on the presence of IL-12 for the induction of type 1 immune responses. The Journal of Immunology, 1999, 162: 965–973.
grown on BHK-21 (ATCC CRL 8544) cells infected at low multiplicity of infection and plaqued on Vero cells.

Vaccinia virus expressing the glycoprotein of VSV was a generous gift of Dr. B. Moss (Laboratory of Viral Diseases, National Institutes of Health, Bethesda, MD) (17). Recombinant vaccinia virus expressing LCMV GP has been described (18). Recombinant viruses were grown at low multiplicity of infection on BSC cells and plaqued on BSC cells.

The recombinant baculovirus expressing the LCMV nucleoprotein has been previously described (19). The recombinant baculovirus was derived from nuclear polyhedrosis virus and was grown at 28°C in Spodoptera frugiperda cells in spinner cultures in TC-100 medium. Recombinant proteins were produced as previously described (20).

Listeria monocytogenes was originally obtained from B. Blanden (Canberra, Australia). It was cultured in trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD), and overnight cultures were titrated on tryptose blood agar plates (Difco Laboratories, Detroit, MI).

**T cell proliferation**

Mice were immunized i.v. with 200 pfu LCMV-WE. Thirteen days later, CD4+ T cells were purified from spleen cell suspensions by MACS-sorting according to the protocol of the supplier (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4+ T cells (1 x 10^6) were incubated in 96-well plates with 3-fold serial dilutions of live LCMV (highest concentration, MOI = 1), peptide P13 (highest concentration, 5 μg/ml), peptide P61 (highest concentration, 5 μg/ml) or medium only in the presence of 6 x 10^3 irradiated (2000 cGy) C57BL/6 spleen cells for 3 days. Proliferation was assessed by incorporation of [3H]thymidine (1 μCi/well). P13 and P61 represent I-A<sup>+</sup>-restricted T cell epitopes of the glycoprotein and nucleoprotein of LCMV that has been described elsewhere (21).

**Cytokine analysis**

Supernatants of proliferation assays as described above were analyzed for IFN-γ content and IL-4 content (72 h after restimulation). IFN-γ and IL-4 were assessed by ELISA as described (12).

**Adoptive transfer of naive TCR transgenic CD4<sup>+</sup> T cells followed by challenge with live or UV-inactivated LCMV**

CD4<sup>+</sup> T cells were purified by MACS-sorting (Miltenyi Biotec) from spleen cell suspensions of naive TCR transgenic mice (Smarta) (22), and 5 x 10<sup>5</sup> CD4<sup>+</sup> T cells were adoptively transferred into naive IL-12-deficient or control C57BL/6 recipients. Three days later recipient mice were infected intravenously with 200 pfu of LCMV. Control mice were not infected. Spleen cells were harvested 9 days after infection and either stained for the presence of TCR transgenic CD4<sup>+</sup> T cells or stained for intracellular cytokine production.

**Cytokine analyses**

The following mAbs were used for analysis: biotinylated 7G8, specific for Vp8.3 (a gift from Dr. I. Förster, Ref. 23); phycoerythrin-conjugated B20.1, specific for V<sub>β</sub>2 (purchased from Pharmingen, San Diego, CA); fluorescence-conjugated anti-IFN-γ or anti-IFN-α (purchased from Pharmingen), and phycoerythrin-conjugated anti-IL-4 (purchased from Pharmingen). Tricolor-conjugated streptavidin or anti-CD4 were purchased from Caltag Laboratories (South San Francisco, CA). Flow cytometry was performed on a FACStar Plus flow cytometer (Becton Dickinson, Mountain View, CA).

Intracellular cytokine stainings were performed according to the instructions of the supplier (Pharmingen).

**ELISA**

The LCMV nucleoprotein-specific ELISA has been described previously (19). Ninety-six-well plates (Petrax Plastik, Chur, Switzerland) were incubated with LCMV nucleoprotein (0.01 μg/well) in 0.1 M NaH<sub>2</sub>PO<sub>4</sub> (pH 9.4) at 4°C. Plates were then preincubated with 2% BSA in PBS for 2 h and washed, and serial dilutions of serum samples (30-fold prediluted) were added to the wells and incubated for 90 min. Plates were washed and incubated with horseradish peroxidase-labeled goat anti-mouse IgG2a or IgG1 (Zymed, San Francisco, CA). After 90 min, plates were washed and developed with ABTS (5 mg of 2,2'-azino-di-3-ethyl-benzthiazolinsulfonate and 20 μl of H<sub>2</sub>O<sub>2</sub> in 50 ml of NaHCO<sub>3</sub> (pH4.0). Optical densities were determined at 405 nm.

For the VSV-specific IgG subclass analysis, ELISA plates were coated with purified VSV (10 μg/ml), serial dilutions of 30-fold prediluted serum samples were added, and VSV-bound IgG was detected with horseradish peroxidase-labeled goat anti-mouse IgG1, IgG2a, or IgG2b Abs (Zymed).

**Serum neutralization test**

Neutralizing titers of sera were determined as described (24). Sera were preincubated 40-fold in supplemented MEM and heat-inactivated for 30 min at 56°C. Serial 2-fold dilutions were mixed with equal volumes of virus diluted to contain 500 pfu/ml. The mixture was incubated for 90 min at 37°C in an atmosphere containing 5% CO<sub>2</sub>. One hundred microliters of the serum-virus mixture were transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. The monolayers were then overlaid with 100 μl of DMEM containing 1% methyl cellulose. After incubation for 24 h at 37°C, the overlay was removed and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of the serum that reduced the number of plaques by 50% was taken as the neutralizing titer. To determine IgG titers, undiluted serum was first pretreated with an equal volume of 0.1 M 2-ME in saline (25).

**Protection of mice from replication of recombinant vaccinia virus**

Mice were immunized with 200 pfu of LCMV or with 2 x 10<sup>5</sup> pfu of VSV and challenged i.p. 20 days later with 5 x 10<sup>5</sup> pfu of recombinant vaccinia virus expressing LCMV glycoprotein (Vac-G2) or expressing VSV-glycoprotein (Vac-INd). Vaccinia titers in ovaries were determined 5 days later as described previously (13). Titers are shown as log<sub>10</sub> pfu per animal.

**LCMV-specific CTL activity and determination of titers**

IL-12-deficient and control mice were infected i.v. with 200 pfu of LCMV, and 8 days later spleen cell suspensions were prepared and tested for cytotoxic activity on peptide-pulsed (gp33, 1 μM) EL4 (H-2<sup>b</sup>) cells (26).

LCMV titers were determined in the spleen, liver, kidney, and blood of IL-12-deficient or control mice 4 and 8 days after i.v. infection with 200 pfu of LCMV as described previously (27).

**Determination of bacterial titers**

On the indicated days after inoculation the whole spleen or one lobe of the liver were harvested and homogenized. Bacterial titers were determined by plating out four serial 10-fold dilutions of organ suspensions on tryptose blood agar plates.

**NK response**

Ex vivo NK activity of spleen cells was determined 2 days after infection of IL-12-deficient mice or control mice with 1 x 10<sup>6</sup> pfu of LCMV-WE i.v. Spleen cell suspensions were tested on NK-sensitive YAC-1 target cells at the indicated effector to target ratios in a standard 5-h <sup>51</sup>Cr release assay (26).

**In vivo depletion of NK cells/in vivo neutralization of IFN-γ**

Depletion of NK cells was performed by i.v. administration of the NK-depleting Ab TMJ81 (28) or i.p. administration of anti-NK1.1 mAb (29) 1 day before LCMV infection. In vivo neutralization of IFN-γ was performed by daily i.p. injections of anti-IFN-γ sheep serum (30). As a control, mice were treated daily with normal sheep serum.

**Results**

**Proliferative responses and cytokine secretion by LCMV-specific CD4<sup>+</sup> T cells**

To evaluate the proliferative capacity of LCMV-specific CD4<sup>+</sup> T cells induced in IL-12 P40 chain-deficient mice (P40<sup>−/−</sup>) and in control C57BL/6 mice, CD4<sup>+</sup> T cells were purified from spleen cell suspensions 13 days after LCMV infection. CD4<sup>+</sup> T cells were restimulated in vitro with LCMV, with the LCMV GP-derived helper epitope peptide P13 or the LCMV NP-derived helper epitope peptide P61 (21). Stimulation indices are shown in Fig. 1A and were equivalent for CD4<sup>+</sup> T cells from LCMV-infected IL-12-deficient or control mice. To analyze the cytokine secretion patterns of LCMV-specific CD4<sup>+</sup> T cells, supernatants of the above described proliferation assays were collected at 24 h (for IL-2 content) and 72 h (for IFN-γ and IL-4 contents) after in vitro stimulation. Both LCMV-specific CD4<sup>+</sup> T cells from IL-12-deficient and control mice secreted similar amounts of IFN-γ, whereas no IL-4 could be detected in both cases (Fig. 1B). IL-2 secretion was also equivalent for LCMV-specific CD4<sup>+</sup> T cells from LCMV-infected IL-12-deficient and control mice (data not
day 9 after LCMV infection (40% V), 13 days later LCMV-specific proliferation of purified CD4 T cells was determined using either LCMV (WE), peptide 13 (P13) or peptide 61 (P61) as LCMV-specific Ags (concentrations of stimulating Ags are described in Materials and Methods). P13 and P61 are LCMV GP- or NP-derived peptides recognized by I-Ab-restricted CD4 T cells. Stimulation indices were calculated in relation to proliferation in medium control. Background cpm counts in medium control were around 3000 cpm. Two of four comparable experiments are shown.

In vivo analysis of cytokine patterns produced by LCMV-specific CD4 T cells upon adoptive transfer into LCMV-infected IL-12-deficient or normal mice

To visualize cytokine production by CD4 T cells activated specifically by LCMV, TCR transgenic T cells exhibiting defined specificity for the LCMV GP-derived epitope P13 (22) were activated in vivo in IL-12-deficient or IL-12-competent hosts upon LCMV infection. When CD4 T cells from naive transgenic mice were adoptively transferred into naive C57BL/6 recipients, they clonally expanded and gained cytokine expression only after activation by viral infection (22). Clonally expanded TCR transgenic LCMV-specific CD4 T cells can be analyzed directly ex vivo for their cytokine production by means of intracellular cytokine staining. Purified CD4 T cells (5 x 10⁶) originating from naive Smarta TCR transgenic mice (22) with specificity for the LCMV GP-derived I-Ab-binding epitope P13 were adoptively transferred into naive IL-12-deficient or normal C57BL/6 mice. Three days later, recipient mice were infected i.v. with LCMV. Control recipients were left untreated. Nine days after challenge, percentages of TCR transgenic CD4 T cells in spleens were determined by FACS analysis. Cytokine production by TCR transgenic CD4 T cells was assessed by intracellular cytokine staining for IFN-γ, TNF-α, and IL-4; results are summarized in Table I. The first row shows percentages of TCR transgenic T cells of total CD4 T cells in the spleen of immunized versus control recipients. TCR transgenic CD4 T cells had clonally expanded to significant levels at day 9 after LCMV infection (40% Vα2⁺ Vβ8.3⁺ T cells of total CD4 T cells). The lower three rows of Table I show the percentages of cytokine-producing CD4 Vα2⁺ T cells. In both shown). These findings indicate that IFN-γ production by LCMV-specific CD4 T cells occurs independently of IL-12 upon LCMV infection in vivo as opposed to immunizations with proteins where IL-12 was shown to be instrumental for IFN-γ production (1).

Table I. Intracellular cytokine production of TCR transgenic CD4⁺ T cells upon adoptive transfer into LCMV-infected IL-12-deficient or normal mice

<table>
<thead>
<tr>
<th></th>
<th>P40⁻/⁻</th>
<th>B6</th>
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<tbody>
<tr>
<td></td>
<td>Spleen (d9)</td>
<td>Spleen (ctrl.)</td>
</tr>
<tr>
<td>% Vα2⁺ Vβ8.3⁺ of total</td>
<td>38.4 ± 4.8</td>
<td>2.7 ± 1.5</td>
</tr>
<tr>
<td>% IFN-γ⁺ of CD4⁺ Vα2⁺</td>
<td>40.5 ± 3.5</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>% TNF-α⁺ of CD4⁺ Vα2⁺</td>
<td>27.7 ± 5.3</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>% IL-4⁺ of CD4⁺ Vα2⁺</td>
<td>0.4 ± 0.3</td>
<td>1.8 ± 1.5</td>
</tr>
</tbody>
</table>

Representative stainings: cells were gated on CD4 Vα2⁺ T cells.

* Each number represents the percentage of cells that fulfill the criteria indicated on the left column. One of three similar experiments is shown.
* Spleen cells were isolated 9 days after LCMV infection.
* Spleen cells were isolated 9 days after transfer into naive recipient mouse.
LCMV-infected IL-12-deficient and control mice, significant numbers of TCR transgenic CD4<sup>+</sup> T cells produced IFN-γ (40% IFN-γ-producing CD4<sup>+</sup> Vα2<sup>+</sup> T cells) or TNF-α (25% TNF-α-producing CD4<sup>+</sup> Vα2<sup>+</sup> T cells), whereas no IL-4-producing transgenic T cells could be detected (<1% IL-4-producing CD4<sup>+</sup> Vα2<sup>+</sup> T cells). In the lower section of Table 1 representative FACS stainings are shown.

Gating on nontransgenic CD4<sup>+</sup> T cells in the same experimental setup did not reveal any differences in IFN-γ staining between IL-12-deficient and control mice; i.e., about 6–9% of TCR nontransgenic CD4<sup>+</sup> T cells stained positive for IFN-γ, which is close to background levels. Thus, LCMV-specific TCR transgenic CD4<sup>+</sup> T cells were specifically activated in an IL-12-deficient environment to produce type 1 but not type 2 cytokines.

**LCMV- and VSV-specific humoral responses in IL-12-deficient mice**

To correlate the type 1 cytokine response elicited in IL-12-deficient mice upon LCMV infection with the isotype patterns of LCMV-specific IgG responses, IL-12-deficient and control mice were infected with LCMV, and 20 days later LCMV NP binding IgG2a and IgG1 Abs were measured by ELISA (Fig. 2). A predominant LCMV NP-specific IgG2a response was induced in both IL-12-deficient and control mice, reflecting the induction of a type 1 CD4<sup>+</sup> T cell response. However, if IL-12-deficient or control mice were immunized s.c. with noninfectious UV-inactivated LCMV in CFA, the predominant isotype of the LCMV NP-specific IgG response was IgG1 in IL-12-deficient and control mice, indicating that the phenotype of the CD4<sup>+</sup> T cell response induced after immunization with nonreplicating LCMV is different from the one after infection with live LCMV. This is in contrast to observations made for mouse hepatitis coronavirus (MHV) infection where infectious and nonreplicating MHV were both predominantly inducing IgG Abs of the IgG2a isotype (31).

**Antiviral protection mediated by type 1 cytokines in IL-12-deficient mice**

In some viral infections, T cell-secreted cytokines can directly exhibit antiviral protective effector functions. Vaccinia virus infection for example is resolved in mice by the direct antiviral effects

**FIGURE 2.** LCMV NP-specific IgG1 and IgG2a response in IL-12-deficient and control mice upon LCMV infection or upon immunization with nonreplicating UV-inactivated LCMV in CFA. IL-12-deficient mice (upper panels, circles) and normal C57BL/6 control mice (lower panels, circles) were immunized with 200 pfu of LCMV i.v. or with 20 mg purified UV-inactivated LCMV in CFA s.c., and sera were collected 20 days after immunization. In addition, normal C57BL/6 serum was included as a negative control (squares). LCMV NP-specific IgG1 and IgG2a Abs were determined by ELISA in 30-fold prediluted sera. Each line represents one individual mouse. One of three comparable experiments is shown.

**FIGURE 3.** VSV-neutralizing Ab response. IL-12-deficient and C57BL/6 mice were immunized with VSV. A, VSV-neutralizing IgM titers (squares) and VSV-neutralizing IgG titers (triangles) were determined from 40-fold prediluted sera 4, 8, 12 and 20 days after infection. Each line represents one individual mouse. Variations were smaller than two dilution steps. One of three comparable experiments is shown. B, Isotypes of VSV-binding IgG Abs were determined 12 days after VSV infection of IL-12-deficient and normal mice. Thirty-fold prediluted sera were tested on VSV-coated ELISA plates. Average values of five individual mice are shown.
In the case of VSV-primed mice, VSV G-specific CD4 T cell responses were induced in the absence of IL-12, IL-12-deficient mice and control mice were infected with either LCMV or VSV and 20 days later they were challenged with Vacc-G2 or Vacc-INDG, respectively (13, 35). In the case of LCMV-immune mice, LCMV GP-specific CD4 T cells, naive TCR transgenic CD4 T cells, and control mice were infected with either LCMV or VSV and 20 days later they were challenged with Vacc-G2 or Vacc-INDG, respectively (13, 35). In both experimental systems, protection is mediated by T cell-secreted cytokines rather than by direct T cell-mediated cytolytic mechanisms (13, 35, 36). Thus, to address the question whether antivirally protective type 1 T cell responses were induced in the absence of IL-12, IL-12-deficient mice and control mice were infected with either LCMV or VSV and 20 days later they were challenged with Vacc-G2 or Vacc-INDG, respectively. Vaccinia titers in ovaries were determined 5 days after challenge infection. Each symbol represents one individual mouse. One of two experiments is shown.

**FIGURE 4.** T cell-dependent, cytokine-mediated protection against challenge infection with recombinant vaccinia viruses. IL-12-deficient and normal C57/BL6 mice were immunized with 200 pfu of LCMV or with 2 × 10^6 pfu of VSV, and 20 days later these primed mice as well as naive control mice were challenged i.p. with 5 × 10^6 pfu of Vacc-G2 (A) or Vacc-INDG (B). Vaccinia titers in ovaries were determined 5 days after challenge infection. Each symbol represents one individual mouse. One of two similar experiments is shown.

of the type 1 cytokines IFN-γ and TNF-α (33, 34). Thus, T cell-secreted cytokine-mediated antiviral protection can experimentally be assessed by challenge immunization of either LCMV- or VSV-primed mice with recombinant vaccinia viruses expressing either the LCMV GP (Vacc-G2) or the VSV G (Vacc-INDG), respectively (13, 35). In the case of LCMV-immune mice, LCMV GP-specific CD8 T cells mediate protection against challenge immunization with Vacc-G2 (Dr. T. Kündig, unpublished data), whereas in the case of VSV-primed mice, VSV G-specific CD4 T cells confer protection against challenge immunization with Vacc-INDG (35). In both experimental systems, protection is mediated by T cell-secreted type 1 cytokines rather than by direct T cell-mediated cytolytic mechanisms (13, 35, 36). Thus, to address the question whether antivirally protective type 1 T cell responses were induced in the absence of IL-12, IL-12-deficient mice and control mice were infected with either LCMV or VSV and 20 days later they were challenged with Vacc-G2 or Vacc-INDG, respectively. Vaccinia titers were determined 5 days after challenge in the ovaries (Fig. 4). Protection against challenge with recombinant vaccinia virus indicated that the priming infection with LCMV or VSV had induced protective type 1 T cell responses in both IL-12-deficient and control mice.

**NK responses in LCMV infected IL-12-deficient mice**

To test for NK cell activation after LCMV infection, IL-12-deficient mice and control mice were infected with 1 × 10^6 pfu of LCMV WE, and 2 days later ex vivo NK activity was determined by lysis of the NK-sensitive target cell line YAC-1 (Fig. 5). NK effectors from IL-12-deficient mice and from control C57BL/6 mice showed comparable levels of YAC-1 cell lysis.

To address the question whether NK cell-produced IFN-γ was possibly involved in the generation of the Th1 phenotype of LCMV-specific CD4 T cells, naive TCR transgenic CD4 T cells were adoptively transferred into IL-12-deficient recipients or control recipients that were either depleted of NK cells or left untreated. NK cell depletion was functionally tested by the absence of YAC-1 killing at day 2 after infection with high doses of LCMV-WE (data not shown). Two different Abs were independently used for in vivo NK cell depletion: Tmβ1 and anti-NK1.1 (29). Recipients were infected with LCMV, and the cytokine production pattern of the clonally expanded TCR transgenic CD4 T cells was analyzed by intracellular cytokine staining and FACS analysis. Also, in the absence of NK cells, LCMV TCR transgenic CD4 T cells produced significant amounts of IFN-γ and TNF-α by activation by LCMV in an IL-12-deficient recipient (Table II). These results suggest that neither IL-12 nor NK cell-produced IFN-γ are required for the induction of a type 1 phenotype in LCMV-specific CD4 T cells after LCMV infection.

To analyze whether IFN-γ is required for the induction of LCMV-specific type 1 CD4 T cells, IL-12-deficient mice and control mice were challenged i.p. with 1 × 10^6 pfu of LCMV and 2 days after NK cell activity was assessed in the spleens by 51Cr-release assay using YAC-1 target cells. Each line represents a single mouse. One of two similar experiments is shown.

**FIGURE 5.** NK cell activation in IL-12-deficient and control mice after high dose LCMV infection. IL-12-deficient and control mice were infected i.v. with 1 × 10^6 pfu of LCMV and 2 days after NK cell activity was assessed in the spleens by 51Cr-release assay using YAC-1 target cells. Each line represents a single mouse. One of two similar experiments is shown.

### Table II. Intracellular cytokine production of TCR transgenic CD4 T cells upon adoptive transfer into NK cell-depleted LCMV-infected IL-12-deficient or normal mice

<table>
<thead>
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<th>Spleen (d9) ^a</th>
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<th>Spleen (d9) ^b</th>
<th>Spleen (ctrl.) ^b</th>
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<tr>
<td>% Vα2 ^a Vβ8.3 ^a of total</td>
<td>38.2 ± 6.9</td>
<td>2.1 ± 0.6</td>
<td>35.8 ± 5.7</td>
<td>2.0 ± 0.5</td>
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<td>% IFN-γ ^a of CD4 ^a Vα2 ^a</td>
<td>74.3 ± 3.4</td>
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<td>67.1 ± 10</td>
<td>4.9 ± 1.3</td>
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<tr>
<td>% TNF-α ^a of CD4 ^a Vα2 ^a</td>
<td>47.1 ± 9.7</td>
<td>5.2 ± 1.3</td>
<td>40 ± 6.8</td>
<td>4.9 ± 1.3</td>
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<tr>
<td>% IL-4 ^a of CD4 ^a Vα2 ^a</td>
<td>0.6 ± 0.1</td>
<td>2.1 ± 1.2</td>
<td>1.0 ± 0.4</td>
<td>1.8 ± 1.1</td>
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Representative stainings: cells were gated on CD4 ^a Vα2 ^a T cells.

^a Each number represents the percentage of cells that fulfill the criteria indicated on the left column. One of three similar experiments is shown.

^b Spleen cells were isolated 9 days after LCMV infection.
cells upon adoptive transfer into LCMV-infected anti-IFN-γ-deficient mice but not in anti-IFN-γ-treated C57BL/6 mice suggests that IFN-γ plays an important role for the induction of LCMV-specific type 1 CD4+ T cells in the absence of IL-12. However, in the presence of IL-12 (C57BL/6 mice) anti-IFN-γ treatment does not abrogate commitment of LCMV-specific CD4+ T cells towards the type 1 phenotype after LCMV infection. The source of IFN-γ after LCMV infection in IL-12-deficient mice was shown to be neutrophils and macrophages (37).

We analyzed whether impaired or delayed virus clearance due to suboptimal CTL induction or due to enhanced viral replication was observed in IL-12-deficient mice. Thus, IL-12-deficient and normal mice were infected with LCMV and viral titers were determined in blood, spleen, liver, and kidney 4 and 8 days later. No LCMV was detectable in blood, liver, and kidney at day 4 or day 8 after infection (not shown). However, LCMV titers in the spleen were comparable at day 4 and day 8 after infection (Fig. 6A). Similarly, day 8 effector CTLs were comparably induced in IL-12-deficient and control mice (Fig. 6B). This is in agreement with previously published observations where it was shown that viral replication and CTL induction after LCMV infection were unaltered after in vivo neutralization of IL-12 (37).

**L. monocytogenes clearance is impaired in IL-12-deficient mice**

In the early phase of primary *L. monocytogenes* infection in mice it has been shown that neutrophils and macrophages play an important role to restrict bacterial replication and that the cytokines IFN-γ and TNF-α as well as reactive oxygen intermediates produced by IFN-γ-activated macrophages are essential for protection (14, 15, 38). In contrast, later phases of primary infections as well as secondary infections are mainly controlled by specific (memory) CD8+ T cell responses (16, 39). It was of interest to compare the role of IL-12 in primary *L. monocytogenes* infection as opposed to the above-described viral infections, especially since IL-12 has been attributed an important indirect role in primary LCMV infection in IL-12-deficient mice (37).

**ROLE OF IL-12 FOR Th CELL INDUCTION IN VIRAL INFECTIONS**

**Table III. Intracellular cytokine production of TCR transgenic CD4+ T cells upon adoptive transfer into LCMV-infected anti-IFN-γ-treated IL-12-deficient or normal mice**

<table>
<thead>
<tr>
<th>Recipients Treated with</th>
<th>P40+/− anti IFNγ</th>
<th>B6 anti IFNγ</th>
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<tr>
<td></td>
<td>Anti-IFN-γ Sheep Serum (d9)</td>
<td>Control Sheep Serum (d9)</td>
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<tr>
<td>% IFN-γ+ of CD4+ Va2+</td>
<td>27.5 ± 2.6</td>
<td>63 ± 4.5</td>
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<tr>
<td>% TNF-α+ of CD4+ Va2+</td>
<td>47.3 ± 5.0</td>
<td>65.5 ± 6.5</td>
</tr>
<tr>
<td>% IL-4+ of CD4+ Va2+</td>
<td>31 ± 1.5</td>
<td>4.4 ± 1.0</td>
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</tbody>
</table>

Representative stainings: cells were gated on CD4+ Va2+ T cells.

* Each number represents the percentage of cells that fulfill the criteria indicated on the left column. One of three equivalent experiments is shown.
* Spleen cells were isolated 9 days after LCMV infection. Recipients were treated daily with anti-IFN-γ sheep serum.
* Spleen cells were isolated 9 days after LCMV infection but from recipient mice that had been treated daily with normal sheep serum.

**FIGURE 6.** LCMV titers and CTL response in IL-12-deficient and control mice. A, IL-12-deficient and control mice were infected i.v. with 200 pfu of LCMV, and viral titers were determined in the spleen 4 and 8 days after infection. Each symbol represents a single mouse. B, IL-12-deficient mice and control mice were infected i.v. with 200 pfu of LCMV, and day 8 effector CTLs were tested on LCMV-infected (filled circles) and on uninfected (open squares) MC57 target cells. Each line represents a single mouse.
role in the activation process of macrophages (2). Thus, IL-12-deficient and control mice were inoculated intravenously with 600 live bacteria and bacterial titers were determined in the spleen and in the liver 5 or 14 days later (Fig. 7B). Listeria titers at day 5 after inoculation were significantly higher in organs of IL-12-deficient mice. In the spleen a 10-fold difference and in the liver a 100-fold difference was observed between IL-12-deficient and control mice. However, 14 days after inoculation IL-12-deficient mice as well as C57BL/6 mice had cleared the bacteria from both the spleen and the liver, suggesting that IL-12 played an important role in controlling bacterial burden at early phases of infection but was not necessary for eventual clearance of infection. To rule out the possibility that the observed differences in bacterial titers 5 days after infection were due to an altered dissemination of the bacteria in IL-12-deficient mice or to differences in early infection with L. monocytogenes, IL-12-deficient and C57BL/6 control mice were inoculated i.v. with high doses of bacteria $(7 \times 10^5 \text{ CFU})$ and bacterial titers were determined in the blood 5 min later and in the spleen and liver 1 h later. At these very early time points after inoculation both IL-12-deficient and control mice exhibited comparable bacterial titers (Fig. 7A). These results demonstrate that IL-12 seems to play an important role in the macrophage activation process required for resolution of primary infection with L. monocytogenes.

**FIGURE 7.** Listeria titers in organs of IL-12-deficient or C57BL/6 control mice. A, IL-12-deficient or control C57BL/6 mice were inoculated i.v. with $7 \times 10^5 \text{ CFU}$ of L. monocytogenes, and Listeria titers were determined in the blood 5 min after infection and in the spleen or in the liver 1 h after infection. Groups of three mice were analyzed. Each symbol represents one mouse. One representative experiment of two is shown. B, IL-12-deficient or control C57BL/6 mice were inoculated intravenously with 600 CFU of L. monocytogenes, and Listeria titers were determined in the spleen or in the liver 5 days later. Groups of three mice were analyzed. Each symbol represents one mouse. One representative experiment of two is shown.

**Discussion**

IL-12 has been described as a cytokine promoting some, but not all types of cell-mediated immune responses. Whereas induction of MHC class I-restricted CTL responses and IL-2 production by activated T cells occurred in the absence of IL-12 (1), priming of type 1 Th cell responses was shown to be crucially dependent on the presence of IL-12 (1–4). In this report the physiological role of IL-12 during antiviral cellular and humoral immune responses was analyzed. The phenotype of virus-specific CD4+ T cells induced after infection of IL-12-deficient mice with LCMV was of the Th1 phenotype as determined by Ag-induced IFN-γ secretion in vitro and was thus identical to the one observed in normal C57BL/6 mice after LCMV infection (12). In line with this, in vivo analysis of cytokine production by LCMV-specific TCR transgenic CD4+ T cells upon adoptive transfer into LCMV-infected IL-12-deficient recipients showed that the type 1 cytokines IFN-γ and TNF-α but not IL-4 were produced by the transferred CD4+ T cells. Ig isotype patterns of virus-specific Ab responses after infection of IL-12-deficient mice with either LCMV or VSV were predominantly of the IgG2a isotype, reflecting again the induction of a type 1 T cells response after infection of IL-12-deficient mice with these viruses. Antiviral protection mediated by secretion of type 1 cytokines by virus-primed T cells did also not depend on the presence of IL-12. The IL-12-independent induction of IFN-γ-secreting CD4+ T cells upon viral infections is thus in contrast to results obtained with nonviral immunizations where IL-12 seemed to play a critical role in this respect (1–9). The results described in this report fully confirm previous findings by Orange and Biron (37), which demonstrated that early IL-12 production is not measurably induced upon LCMV infection and that in vivo neutralization of IL-12 has no effect on early LCMV replication, on induction of early NK cell cytotoxicity and on viral clearance on days 7 and 9 after infection. These earlier results suggested the existence of IL-12-independent mechanisms for IFN-γ production and for T cell-mediated control of viral infection (37). In addition, Schijns et al. (31) demonstrated that IL-12-deficient mice are able to mount polarized Th1 CD4+ T cell responses upon infection with MVH, suggesting that at least certain viruses may selectively induce IFN-γ production and type 1 cytokine responses in the absence of IL-12. Apparently, protein immunizations or some infections with nonviral microorganisms seem to require more subtle mechanisms controlling induction of the phenotype of specific T cells. IL-12 production is normally observed in APCs such as macrophages, B cells or dendritic cells (40,41). It was shown for macrophages that IL-12 production was only induced after preactivation with IFN-γ, whereby IFN-γ is governing IL-12 production on the transcriptional level by activating the IL-12 p35 and p40 promoters (42). Macrophage-secreted IL-12 seems to operate then in a positive feedback mechanism enhancing IFN-γ production by NK and T cells (43). This IFN-γ-producing positive feedback mechanism mediated by IL-12 is most probably not required in viral infections because viruses are usually potent inducers of IFN-γ in T cells and NK cells. The NK cells are usually one of the first sources of IFN-γ production after viral infection (44) and at later phases of infection activated virus-specific T cells normally also produce IFN-γ (45). Taking into account the observation that in vivo depletion of NK cells in IL-12-deficient mice did not hamper the induction of LCMV-specific CD4+ T cells of type 1 phenotype suggested that NK cells are not a relevant source of early IFN-γ upon LCMV infection. This is in agreement with the findings by Orange and Biron (37) which indicated that it has not been possible to demonstrate IFN-γ production on protein level by LCMV-induced NK cells (37) although LCMV-induced NK cells express detectable levels of IFN-γ.
mRNA (46). However, in vivo neutralization of IFN-γ in IL-12-deficient mice resulted in induction of significant numbers of LCMV-specific CD4+ T cells producing IL-4. This finding suggests that IFN-γ produced after infection with LCMV is a crucial cytokine for the Th cell phenotype commitment in the absence of IL-12. This finding is in contrast to observations made in MHV-infected IL-12-deficient mice where virus-induced type 1 cytokine patterns were not reversed by in vivo neutralization of IFN-γ (31). Thus in the case of LCMV infection it seems likely that the IFN-γ present at early time points after infection is sufficient to control the induction of a type 1 phenotype T cell response after a viral infection. One possible source of IFN-γ during LCMV infection could be activated LCMV-specific CD8+ T cells which have been shown to produce IFN-γ (37, 45). However, it is difficult to experimentally address the hypothesis that IL-12-independent IFN-γ production by CD8+ T cells might be involved in Th1 development in IL-12-deficient mice. Since depletion of CD8+ T cells would interfere with virus clearance and thus result in drastically elevated Ag levels, a comparison to normal mice would be uninterpretable. Alternatively, other cytokines or host factors could possibly compensate for the lack of endogenous IL-12 for IFN-γ production. IFN-α, characteristically induced upon most viral infections, has been suggested to favor the development of type 1 Th cells (47), although contrasting results have been reported by Werner et al. (48), who showed that IFN-α is unable to induce Th1 development in a TCR transgenic system. Moreover, IFN-αβ production has been shown to inhibit endogenous IL-12 synthesis and associated IFN-γ production (49).

Mice deficient for IFN-γ-regulatory factor 1 (IRF1) exhibit strongly reduced Th1 responses (50); furthermore, the recently described IFN-γ-induced factor is a strong IL-12-independent inducer of IFN-γ and of Th1 development (51).

However, once virus-specific T cells are activated and start to clonally expand, they are certainly an important source of IFN-γ by themselves, which in turn may influence the phenotype of newly primed virus-specific T cells. Interestingly, virus-specific type 1 CD4+ T cells seem to preserve their phenotype independently of the presence of IL-12 in vivo, suggesting that the IL-12β2-chain-mediated Th1 phenotype selection and phenotype conservation might not be as crucial in vivo as it has been demonstrated in vitro (52). It is unclear whether IFN-γ can directly promote differentiation of Th1 cells. In vitro it has been shown that in Con A-activated cultures cellular IFN-γ production was enhanced in the presence of IFN-γ (53). Some in vivo experiments provided strong evidence for a role of IFN-γ in regulating priming for IFN-γ-producing T cells. Treatment of mice with anti-IFN-γ mAbs at the time of infection with Leishmania major resulted in diminished production of IFN-γ and increased IL-4 production when cells from draining lymph nodes were restimulated in vitro (54, 55). However, other experimental findings question the relevance of IFN-γ for the development of Th1 responses (56).

In contrast to viral infections, IL-12 was shown to be important for the control of infection with the intracellular bacterium L. monocytogenes. Neutrophils, granulocytes, γδ T cells, and above all macrophages are important factors during the early phase of the immune response, and the type 1 cytokines IFN-γ and TNF-α are essential for protection (38). Especially, IFN-γ inhibits evasion of L. monocytogenes from the phagosome into the cytoplasm (57). Apparently, L. monocytogenes infection in IL-12-deficient mice did not seem to adequately induce type 1 cytokine responses that could efficiently activate macrophages to be as listeriocidal as in control mice at early time points after infection. However, despite this reduced capability of IL-12-deficient mice to initially control L. monocytogenes infection, IL-12-deficient mice are able to clear infection within 2 wk.

In conclusion, the in vivo relevance of IL-12 seems to depend on the nature of the infecting/immunizing agent: viruses seem to depend much less on the presence of IL-12 for the induction of (protective) type 1 T cell responses as compared to bacteria, parasites, and soluble protein Ags that seem to depend on more subtle T cell activation pathways involving the presence of IL-12.

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References

of neutralizing antiviral antibody responses by virus specific cytotoxic T cells.


