The Journal *of* Immunology

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Therapeutic Protective Effects of IL-12 Combined with Soluble IL-4 Receptor Against Established Infections of Herpes Simplex Virus Type 1 in Thermally Injured Mice[1]

Hiroyuki Kobayashi,*[†] Makiko Kobayashi,*[†] Tokuichiro Utsunomiya,* David N. Herndon,[†] Richard B. Pollard,* and Fujio Suzuki[2]*[†]

The effect of combination therapy between IL-12 and soluble IL-4R (sIL-4R) on the established infection of HSV-1 in thermally injured mice (TI mice) was investigated. All of the TI mice infected with lethal amounts of HSV-1 died when IL-12 was given therapeutically at a dose of 500 U/mouse. However, 80% of these mice treated prophylactically with IL-12 survived compared with 0% survival of the same mice treated with saline. The therapeutic administration of IL-12 to TI mice currently infected with HSV-1 caused an 80% survival of these mice when the treatment was combined with sIL-4R. Although IL-12 did not stimulate IFN-γ production in cultures of splenic T cells from TI mice, IFN-γ was produced by stimulation with IL-12 when the producer cells were prepared from TI mice that had been treated previously with sIL-4R. After stimulation with anti-CD3 mAb, splenic T cells from TI mice with the established infection of HSV-1 produced IL-4 into their culture fluids. However, IL-4 was not produced by splenic T cells that were prepared from the same infected mice treated with IL-12 and sIL-4R in combination. The results obtained herein indicate that the efficacies of the combination therapy against the established infection of HSV-1 may result from the IFN-γ production stimulated by IL-12 in TI mice that are treated with sIL-4R for reducing burn-associated type 2 T cell responses. *The Journal of Immunology,* 1999, 162: 7148–7154.

T he majority of deaths in thermally injured patients are associated with infection rather than physical damage to the skin and/or abnormal metabolism induced by the injury (1–5). Although HSV-1 is a minor pathogen in healthy individuals, the severity of the infections induced by this pathogen markedly increase in burned patients (5). Th1 cell-associated and/or CD8^{+} type 1 T cell-associated cellular responses (type 1 T cell responses) are essential in the immunologic control of HSV-1 (6–8). The inhibition of herpesviruses is facilitated by the disruption of infected cells, and effector cells for type 1 T cell responses produce injuries in cells infected with the virus. Type 1 T cell responses are manifested by the increased production of type 1 cytokines (IL-2, IFN-γ, etc.) by type 1 T cells (Th1 cells and CD8^{+} type 1 T cells) (9–12). In contrast, Th2 cell-associated and/or CD8^{+} type 2 T cell-associated cellular responses (type 2 T cell responses) are manifested by the production of type 2 cytokines (IL-4, IL-10, IL-13, etc.) by type 2 T cells (Th2 cells and CD8^{+} type 2 T cells). Type 2 cytokines secreted from type 2 T cells have been shown to be inhibitors of the differentiation of type 1 T cells (6–11). In our previous studies using a mouse model of thermal injury, thermally injured mice (TI mice)[3] were 100 times more susceptible to HSV-1 infection compared with normal mice

(12). The susceptibility of TI mice to HSV-1 infection was increased through the generation of burn-associated CD8^{+} CD11b^{+} TCRγδ^{+} type 2 T cells (12), because the impaired resistance of TI mice to HSV-1 infection was completely transferred to normal unburned mice through the adoptive transfer of burn-associated type 2 T cells (12). In TI mice and mice inoculated with burn-associated type 2 T cells, a marked suppression was observed in the production of IFN-γ (a typical cytokine involved in type 1 T cell responses) and in the generation of CTLs (a representative effector cell in type 1 T cell responses) (13–20). These findings suggest that TI mice with sufficient levels of type 1 T cell responses may have a possibility to resist severe infection with HSV-1.

IL-12 has been shown to be an inducer of type 1 T cell responses (21, 22). Originally, IL-12 had been described as an inducer of IFN-γ from resting and activated NK cells and T cells (23, 24). However, recent papers have described that IL-12 is a pivotal cytokine in promoting the differentiation of naive T cells into type 1 T cells, and it functions as a costimulus for maximal IFN-γ production by already differentiated type 1 T cells (21, 22). In our previous studies (25), the resistance of TI mice infected with conditioned doses of HSV-1 could be improved to the levels observed in normal mice by prophylactic treatment of IL-12. In these experiments, IL-12 was administered to TI mice 2 days before HSV-1 infection, which occurred 1 day after thermal injury. However, even though the sufficient amount of IL-12 was consequently given to the mice beginning 2 days postinfection (3 days after burn injury), TI mice currently infected with HSV-1 did not survive. Type 1 T cell responses were not induced by the administrations of IL-12 in TI mice with an established infection of HSV-1. A predominance of type 2 T cell responses was detected in these mice. Therefore, to induce sufficient amounts of IFN-γ by stimulation with IL-12, regulation of type 2 T cell responses in TI mice currently infected with HSV-1 may be required. The soluble form of

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[3] Abbreviations used in this paper: TI mice, thermally injured mice; sIL-4R, soluble IL-4R; MSD, mean survival time in days.

IL-4R (sIL-4R) binds IL-4 and inhibits its specific interaction with cell-associated IL-4R (26). sIL-4R has been described as prolonging the survival of an allograft in a mouse model of allotransplantation (27). Also, sIL-4R does induce protective immunity in susceptible mice after infection with *Candida albicans* or *Leishmania major* (28, 29) and prevents allergic airway hyperresponsiveness (30). In our preliminary study, minimal levels of type 2 T cell responses were demonstrated in TI mice treated previously with sIL-4R. Therefore, in the present study, the effect of a combination therapy between IL-12 and sIL-4R on the resistance of TI mice with the established infection of HSV-1 was investigated. Along with the elimination of type 2 cytokines by sIL-4R, an optimal level of the IFN-γ production was induced in these mice by stimulation with IL-12. Also, the mortality rate of TI mice with the established infection of HSV-1 was greatly reduced after the combination therapy between IL-12 and sIL-4R. This combination therapy may have the potential to control current HSV-1 infections in burned patients who routinely carry a predominance of type 2 T cell responses.

Materials and Methods

Animals

Eight-week-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were used in the experiments. All procedures using animal experiments were approved by the Animal Care and Use Committee (ACUC) of The University of Texas Medical Branch at Galveston (ACUC approval number 95-04-039).

Thermal injury

TI mice were produced according to our previous descriptions with minor modifications (31). Before being subjected to burn injury, mice were anesthetized with an i.p. administration of pentobarbital (40 mg/kg). The mice were weighed, and their hair was removed on their back from groin to axilla with electric clippers. A custom insulated mold with a 2.5 × 3.5-cm window was pressed firmly against the shaved back and the area was exposed for 9 s to a gas flame from Bunsen burner equipped with a flame dispersing cap. This procedure produced a third-degree burn of ~15% total body surface area on 26-g mice (31). Immediately after the injury, 4 ml of physiologic saline per mouse was administered i.p. for fluid reconstitution, and the animals were housed until used in the experiments. As controls, mice with their back hair shaved received 4 ml of saline without being exposed to a gas flame.

Reagents, viruses, media, and cells

Murine rIL-12 was kindly provided by Dr. Maurice K. Gately (Hoffmann-La Roche, Nutley, NJ). Murine sIL-4R, rIL-4, and rIFN-γ were obtained from Genzyme (Cambridge, MA). Anti-IFN-γ and anti-IL-4 mAbs for ELISA were purchased from PharMingen (San Diego, CA). Vero cells were serially maintained in minimum essential medium supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics. The KOS strain of HSV-1 was propagated in Vero cells and stored at −70°C until used for infection (12, 25). The titer of the virus stock solution was 1.8 × 10⁷ PFU/ml as assayed by the plaque method on Vero cells cultured in maintenance medium (minimum essential medium supplemented with 2% FBS, 2 mM L-glutamine, and antibiotics). RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, antibiotics, 30 mM HEPES, and 5×10^{-5} M 2-ME (complete media) was used for the cultivation of murine spleen cells. As described previously (12, 25), mononuclear cells were prepared from the spleens of mice by Ficoll-Hypaque sedimentation. Spleens were removed from normal and TI mice treated with or without IL-12 and/or sIL-4R. T cells were purified from mononuclear cells using T cell enrichment columns (R&D Systems, Minneapolis, MN) (32). The purity of the T cells harvested was >96%, as described previously (32).

Viral isolation from spleen and liver

Spleens and livers were removed from three mice 5 and 7 days after infection with 5×10^2 PFU/mouse of HSV-1. Homogenates of these organs (20% suspension, w/v) were subjected to freezing and thawing three times and subsequently centrifuged at $400 \times g$ for 15 min. The supernatants obtained were assayed for HSV-1 in Vero cells by a standard plaque method (33).

Detection of IFN-γ and IL-4

Type 1 T cell responses were manifested as the amounts of IFN-γ produced by appropriate cells after stimulation with IL-12 (10 U/ml, 48 h) or anti-CD3 mAb (2.5 μg/ml, 48 h). Type 2 T cell responses were manifested as the amounts of IL-4 produced by appropriate cells after stimulation with anti-CD3 mAb (2.5 μg/ml, 48 h). Splenic T cells suspended in complete media at a cell density of 2×10^6 cells/ml were stimulated with IL-12 for the production of IFN-γ in vitro. These cell preparations were also stimulated in vitro with anti-CD3 mAb or UV-inactivated HSV-1 (HSV-1 Ag) for the production of IFN-γ or IL-4. Culture fluids harvested at 48 h post-stimulation were assayed for IFN-γ or IL-4 by ELISA according to the manufacturer's protocol. In addition, the sera of mice at various days after thermal injury were assayed for IFN-γ and IL-4 by ELISA. Serum specimens were prepared from TI mice treated with or without rIL-12 and/or sIL-4R. In our assay system, the detection limit for respective cytokines was 5–50 pg/ml. The assay was performed three times, and the results were expressed as the mean of these three tests.

Infection experiments

At 1 day after thermal injury, mice were infected i.p. with HSV-1. An inoculum of 1×10^4 PFU/mouse in normal mice and 1×10^2 PFU/mouse in TI mice has been determined to be equivalent to 1 LD_{50}. A 5 LD_{50} of HSV-1 (5×10^2 PFU/mouse in TI mice and 5×10^4 PFU/mouse in normal unburned mice) was infected to mice in all experiments. Mice were treated i.p. with IL-12 (31–500 U/mouse) once daily for 5 days beginning 3 days postinfection (4 days after thermal injury). sIL-4R (50 ng/mouse) was administered to mice 3 and 5 days postinfection (4 and 6 days after thermal injury). In experiments for the combination therapy, mice were treated with IL-12 and sIL-4R under the conditions shown in the administration schedule of each agent. The antiviral effects of the combination therapy in TI mice were evaluated using the following criteria: 1) the mean survival time in days (MSD) of tested groups compared with the MSD of controls (mice treated with saline, IL-12 alone, or sIL-4R alone), 2) the survival percentage of tested groups at 3 wk postinfection compared with that of the three control groups shown in 1), and 3) viral growth in organs of treated groups compared with that of control groups. All experiments were performed two times, and the figures and table were displayed by mean values of the results shown by these two experiments.

Statistical analysis

The survival of mice exposed to the pathogen was analyzed by log rank test. Other data expressed as mean ± SD were statistically analyzed by ANOVA followed by Fisher's protected least significant difference test. If a p value was <0.05, the result obtained was considered significant.

Results

Protective activity of IL-12 combined with sIL-4R in TI mice that have established infection of HSV-1

The effects of IL-12 on HSV-1 infection in TI mice were first examined. In this experiment, TI mice were treated i.p. with various doses of IL-12 once daily for 5 days beginning 1 day before the infection (the same day of burn injury, prophylactic treatment). Following the same schedule, a control group of TI mice was treated with saline. As shown in Fig. 1*A*, 100% of TI mice treated with saline died within 17 days after HSV-1 infection (MSD, 9.8 days), whereas 80% and 30% of TI mice treated with 500 or 125 U/mouse of IL-12 survived for >21 days, respectively (MSD: 500 U/mouse, >19.3 days; 125 U/mouse, >12.6 days). However, the protective effect of IL-12 against HSV-1 infection was not demonstrated in TI mice when IL-12 was administered to mice at the dose of 500 U/mouse once daily for 5 days beginning 3 days after HSV-1 infection (4 days after burn injury, therapeutic treatment) (Fig. 1*B*). TI mice treated with IL-12 or control mice treated with saline died within 15 days of the infection in the same fashion. Next, the effect of sIL-4R on the morbidity and mortality of TI mice exposed to the same amount of HSV-1 was examined. When infected mice were treated with sIL-4R at a dose of 50, 12, or 3 ng/mouse at 3 and 5 days after HSV-1 infection (4 and 6 days after burn injury, therapeutic treatment), 10%, 0%, and 0% of them
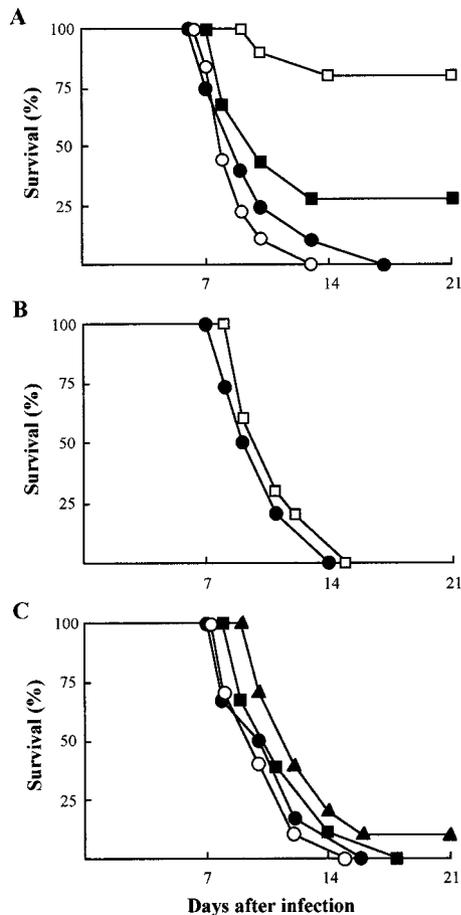
FIGURE 1. Effect of IL-12 or sIL-4R on HSV-1 infection in TI mice. At 1 day after thermal injury, groups of 18–20 mice were infected with 5 LD_{50} of HSV-1 and treated i.p. with IL-12 (□, 500 U/mouse; ■, 125 U/mouse; ○, 31 U/mouse) once daily for 5 days beginning 1 day before (*A*) or only IL-12 (□, 500 u/mouse) at 3 days after (*B*) infection. *C*, TI mice exposed to HSV-1 were also treated with sIL-4R (▲, 50 ng/mouse; ■, 12 ng/mouse; ○, 3 ng/mouse) at 3 and 5 days postinfection. As controls, TI mice exposed to HSV-1 were treated with saline (0.2 ml/mouse) (●, *A–C*) by appropriate schedules.
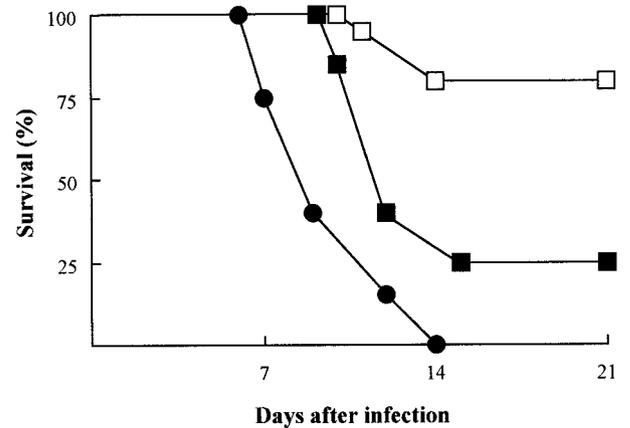

FIGURE 2. Therapeutic effects of combination therapy in TI mice infected with HSV-1. At 1 day after thermal injury, groups of 20 mice were infected with 5 LD_{50} of HSV-1 and treated i.p. with IL-12 (□, 500 U/mouse; ■, 125 U/mouse) once daily for 5 days beginning 3 days postinfection. These mice were also treated with sIL-4R (50 ng/mouse) at 3 and 5 days postinfection. As a control, TI mice infected with HSV-1 were treated with saline (●).

survived for >21 days postinfection, respectively (Fig. 1*C*). According to these results, therapeutic treatment with IL-12 alone or sIL-4R alone provided no significant protective effect against HSV-1 infection in TI mice.

To examine the therapeutic effects of IL-12 combined with sIL-4R on HSV-1 infection, TI mice were treated with IL-12 and sIL-4R in combination after HSV-1 infection. In this experiment, TI mice were treated with 500 or 125 U/mouse of IL-12 once daily for 5 days beginning 3 days after the infection of 5 LD_{50} of HSV-1 in combination with the sIL-4R treatment (50 ng/mouse, 3 and 5 days postinfection). Whereas control mice treated with saline died within 14 days postinfection (MSD, 10.7 days), 80% and 25% of TI mice treated with 500 or 125 U/mouse of IL-12 in combination with sIL-4R survived for >21 days postinfection, respectively (MSD: 500 U/mouse, >19.0 days; 125 U/mouse, >13.6 days) (Fig. 2). Also, the viral growth in the spleens and livers of TI mice that were exposed to HSV-1 and treated with IL-12 and/or sIL-4R was examined. When TI mice were infected with 5×10^2 PFU/mouse of HSV-1 (5 LD_{50} in TI mice; 0.05 LD_{50} in normal BALB/c mice), 2.2×10^4 PFU/spleen and 7.5×10^4 PFU/liver of HSV-1 were detected in these mice at 5 days postinfection; a total of 8×10^3 PFU/spleen and 2.8×10^4 PFU/liver of HSV-1 were detected

in these mice at 7 days postinfection. When TI mice were exposed to the same amount of HSV-1 and treated with IL-12 (500 U/mouse) or sIL-4R (50 ng/mouse) alone, amounts of HSV-1 in organs were not significantly reduced. However, amounts of HSV-1 in organs were reduced in TI mice treated with IL-12 and sIL-4R in combination (day 5, 7.2×10^2 PFU/spleen and 4.5×10^3 PFU/liver; day 7, 6.8×10 PFU/spleen and 4.8×10^2 PFU/liver) (Fig. 3). These results suggest that the antiviral resistance of TI mice to HSV-1 infection was improved after the combination treatment. When the treatment with the constant amount of IL-12 (500 U/mouse) was combined with various doses of sIL-4R (3–50 ng/mouse), the maximal protective effect against the established infection of HSV-1 was produced by IL-12 combined with sIL-4R at doses of 50 ng/mouse (Table I). These results, which are shown in Figs. 1 and 2 and Table I, suggest that TI mice currently infected with HSV-1 may be controlled therapeutically by the combination treatment between IL-12 and sIL-4R, whereas therapeutic treatment of TI mice with either IL-12 or sIL-4R alone did not produce any protective effects against the established HSV-1 infection.

IL-4 in sera of TI mice treated with IL-12

In the series of our previous studies, the increased susceptibility of TI mice to HSV-1 infection has been shown to be produced by type 2 cytokines released from burn-associated type 2 T cells (12, 25, 32). Therefore, we examined the level of IL-4 in the sera of TI mice treated with IL-12 once daily for 5 days beginning on the same day as burn injury or once daily for 3 days beginning 4 days after burn injury (see Fig. 1*B*). IL-4 at an amount of 28 ng/ml was detected in the sera of control mice 7 days after thermal injury. In contrast, IL-4 was not demonstrated in the sera of TI mice 7 days after burn injury when IL-12 was given to TI mice once daily for 5 days beginning on the same day as thermal injury. IL-4 was detected in the sera of TI mice when the treatment with IL-12 was started 4 days after burn injury (Fig. 4). These results indicated that the production of IL-4 in TI mice was inhibited when the IL-12 treatment was started before burn injury. However, the production of IL-4 was not inhibited in the sera of TI mice when the IL-12 treatment was started 4 days after burn injury.
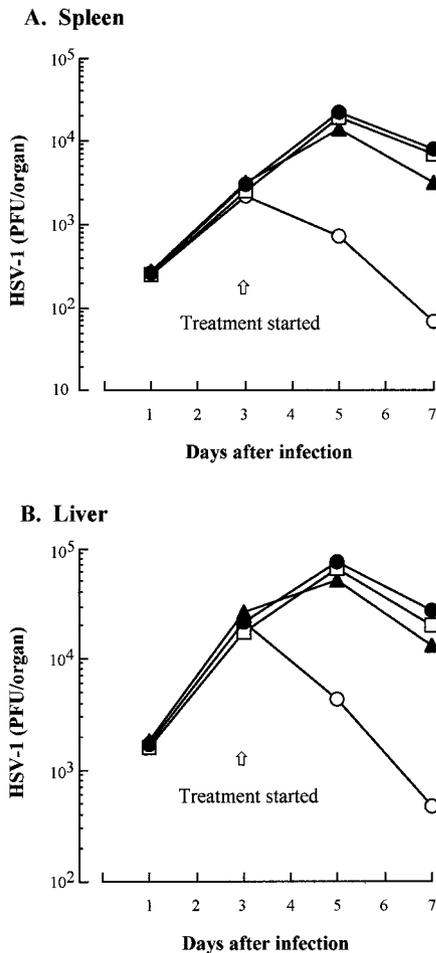
A. Spleen

B. Liver

FIGURE 3. Growth of HSV-1 in spleens and livers of TI mice treated with IL-12 and sIL-4R in combination. At 1 day after thermal injury, 48 mice were infected with 5 LD_{50} of HSV-1 and divided into four equal groups. These groups of mice were treated i.p. with IL-12 ($\square$, 500 U/mouse) once daily for 5 days beginning 3 days postinfection, with sIL-4R ($\blacktriangle$, 50 ng/mouse) 3 and 5 days postinfection, or with both ($\bigcirc$). As a control, TI mice infected with HSV-1 were treated with saline ($\bullet$). At various days postinfection, three mice from each group were killed and organs were removed. The HSV-1 titers in the tissue homogenates of spleens (*A*) and livers (*B*) were measured in Vero cells.

Production of IFN-γ by splenic T cells from TI mice treated with sIL-4R

Type 1 T cell responses have been shown to be essential for the defense of the host against HSV-1 infection. Because TI mice with the established HSV-1 infection survived after the combination therapy between IL-12 and sIL-4R, type 1 T cell responses might be developed in these mice. Therefore, we attempted to induce type 1 T cell responses by IL-12 in cultures of splenic T cells from TI mice that were previously treated with sIL-4R. Type 1 T cell responses were manifested as the amount of IFN-γ produced by splenic cells. sIL-4R was administered twice (4 and 6 days postburn) to these mice, and splenic T cells were prepared from mice 7 days after thermal injury. Culture fluids harvested 48 h after the stimulation with 10 U/ml of IL-12 were assayed for IFN-γ by ELISA. As shown in Fig. 5, naive splenic T cells stimulated with IL-12 produced 520 pg/ml of IFN-γ into their culture fluids. The production of IFN-γ by splenic T cells from mice 7 days after thermal injury was not elicited after the IL-12 stimulation. However, splenic T cells from mice treated with sIL-4R (50 ng/mouse) 7 days after burn injury produced 511 pg/ml of IFN-γ into their

Table I. *Effect of various doses of sIL-4R on the efficacy of combination therapies with IL-12 in T1 mice that have an established infection of HSV-1[a]*

TI Mice Treatment	No. of Mice	MSD ± SD (days)[b]	Survival (%)[b]
Saline, 0.2 ml/mouse	20	9.4 ± 2.2	0
A. IL-12 alone, 500 U/mouse	20	11.0 ± 2.3	0
B. sIL-4R alone, 3 ng/mouse	20	9.5 ± 2.2	0
C. sIL-4R alone, 12 ng/mouse	18	10.9 ± 3.0	0
D. sIL-4R alone, 50 ng/mouse	20	>12.2 ± 3.6	10
Combination therapy			
A + B	20	11.2 ± 2.4	0
A + C	20	>13.5 ± 3.7	30
A + D	20	>19.0 ± 3.8[c]	80[d]

[a] At 1 day after thermal injury, mice were infected with 5 LD_{50} of HSV-1. These mice were treated i.p. with IL-12 (500 U/mouse), sIL-4R (3–50 ng/mouse), or both. IL-12 was administered to TI mice once daily for 5 days beginning 3 days postinfection. sIL-4R was given to TI mice at 3 and 5 days postinfection. In the combination therapy, IL-12 and sIL-4R were administered to these mice according to the schedules shown above.

[b] Each group of mice was observed daily for 21 days to determine the MSD and survival rates.

[c] ANOVA, $p < 0.001$ compared with a group treated with IL-12 alone.

[d] Log rank test, $p < 0.001$ compared with a group treated with IL-12 alone.

culture fluids when they were stimulated with 10 U/ml of IL-12. This finding suggests that IFN-γ could be induced by IL-12 in cultures of splenic T cells from TI mice treated with sIL-4R.

Effect of combination therapy on cytokine production by splenic T cells from TI mice with established HSV-1 infection

The IFN-γ-producing capability of splenic T cells from TI mice with established HSV-1 infection was examined after these mice were treated with IL-12 and sIL-4R in combination. Mice were exposed to 5 LD_{50} of HSV-1 at 1 day after thermal injury, and these mice were treated with IL-12 and sIL-4R in combination at the dose and schedule shown in Fig. 2. For the induction of IL-4 and IFN-γ, the cultures of splenic T cells (2×10^6 cells/ml) from these infected mice 7 days after thermal injury were stimulated with anti-CD3 mAb (2.5 μg/ml). Culture fluids harvested at 48 h poststimulation were assayed for IL-4 or IFN-γ by ELISA. IL-4 production was stimulated with anti-CD3 mAb in cultures of splenic T cells from currently HSV-1-infected TI mice that were
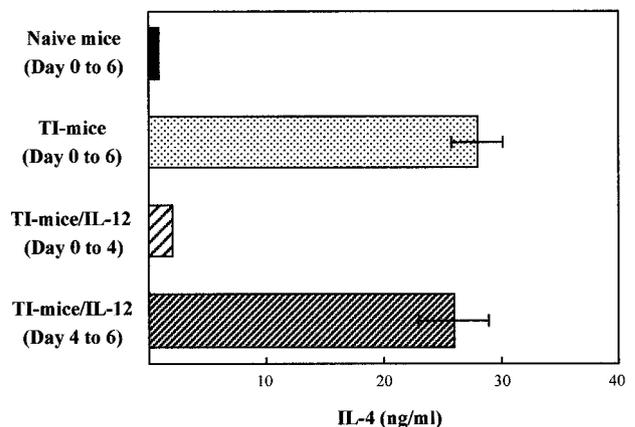

FIGURE 4. IL-4 in sera of TI mice treated with IL-12. Groups of five mice were treated with IL-12 (500 U/mouse) once daily for 5 days beginning the same day of thermal injury or once daily for 3 days beginning 4 days after thermal injury. As controls, normal mice and TI mice were treated with saline once daily for 7 days beginning the same day of thermal injury. Serum specimens prepared from these mice at 7 days after thermal injury were assayed for IL-4 by ELISA.
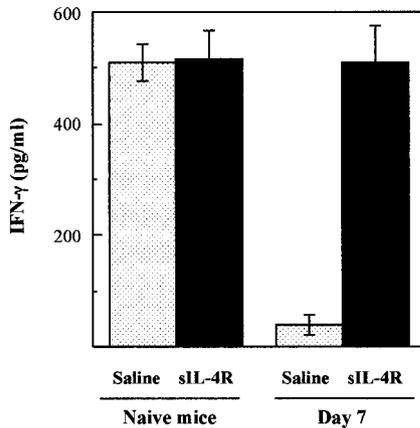
FIGURE 5. IFN-γ-inducing activity of IL-12 in cultures of splenic T cells from TI mice that were treated with sIL-4R. Groups of five TI mice were treated with sIL-4R at a dose of 50 ng/mouse at 3 and 5 days post-burn. As controls, normal mice were treated with saline or sIL-4R 2 and 4 days before sacrifice. Splenic T cells (2×10^6 cells/ml) from naive mice or mice at 7 days after thermal injury were stimulated in vitro with IL-12 (10 U/ml) for IFN-γ production. Culture fluids harvested at 48 h poststimulation were assayed for IFN-γ by ELISA.

treated with IL-12 alone, sIL-4R alone, or saline, whereas IFN-γ was not produced by these cells stimulated with anti-CD3 mAb. Alternatively, the production of IFN-γ was induced by anti-CD3 mAb in cultures of splenic T cells from currently HSV-1-infected TI mice that were treated with IL-12 and sIL-4R in combination.

Also, IL-4 was not produced by these splenic T cells even though they were stimulated with anti-CD3 mAb (Fig. 6).

For the production of IFN-γ and IL-4, splenic T cells prepared from mice exposed to 5 LD_{50} of HSV-1 were stimulated with HSV-1 Ag. Inactivation procedures of HSV-1 and the amount of HSV-1 Ag used for the stimulation have been described previously (33). Culture fluids harvested at 48 h poststimulation were assayed for cytokines by ELISA. IL-4 (570–880 pg/ml) but not IFN-γ was detected in the culture fluids of splenic T cells from HSV-infected TI mice treated with IL-12, sIL-4R, or saline. However, IFN-γ (2380 pg/ml) but not IL-4 was detected in the culture fluids of splenic T cells from HSV-infected TI mice treated with IL-12 and sIL-4R in combination (Fig. 6). These results suggest that, after the combination therapy between IL-12 and sIL-4R, HSV-1-specific type 1 T cells may be generated in TI mice infected with HSV-1.

Discussion

Type 1 T cell responses are essential for the defense of the host against certain intracellular pathogens, such as *Listeria monocytogenes*, *Toxoplasma gondii*, *L. major*, *C. albicans*, *Cryptococcus neoformans*, and herpesviruses (34–39). These type 1 T cell responses inhibit the spreading of intracellular pathogens through inflammatory-tissue injury (8). Because cytokines released from type 2 T cells inhibit the differentiation of type 1 T cells from naive T cells (9–11) and the production of type 1 cytokines by the differentiated type 1 T cells (9–11), sufficient levels of type 1 T cell responses are not easily developed in burned mice that routinely carry a predominance of type 2 T cell responses. IL-12 is a cytokine that is known to play central role in the regulation and differentiation of immune responses through its ability to serve as a

FIGURE 6. Cytokine production by splenic T cells from currently HSV-1-infected TI mice that received combination therapy. At 1 day after thermal injury, groups of five mice were infected with 5 LD_{50} of HSV-1. These mice were treated with IL-12 and/or sIL-4R at the dose and schedule modified in Fig. 2. At 7 days after thermal injury, the cultures of splenic T cells (2×10^6 cells/ml) from these mice were stimulated with anti-CD3 mAb (2.5 μg/ml) or HSV-1 Ag (at a multiplicity of infection of 0.1 for live virus). Culture fluids harvested at 48 h poststimulation were assayed for IL-4 (*A*) or IFN-γ (*B*) by ELISA.

A. IL-4

B. IFN-γ
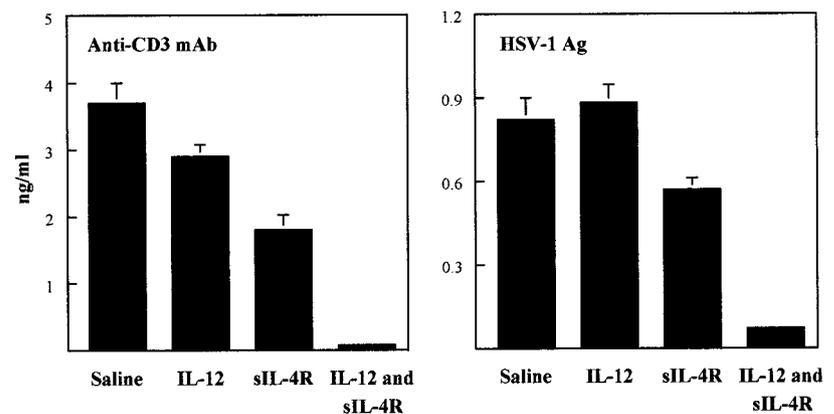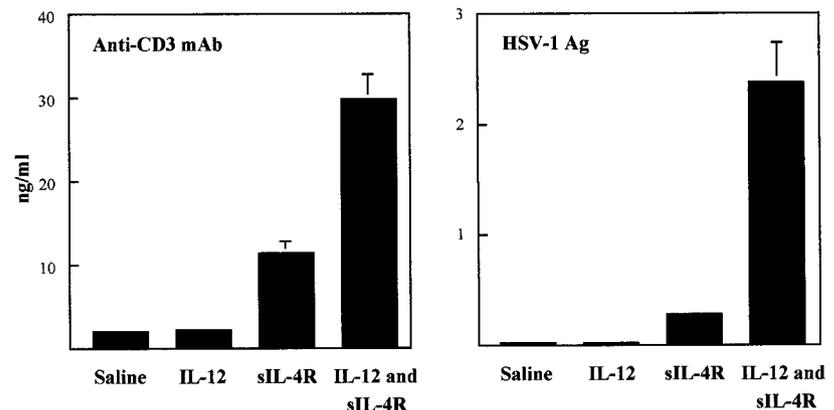
potent inducer of IFN-γ production in T cells and NK cells (23, 24). Thus, IL-12 amplifies type 1 T cell responses through its ability to clonally expand T cells (21, 22), regulate IFN-γ expression (21), and down-regulate type 2 T cell responses (21). These facts show that the immunologic effect of IL-12 might be exerted preferentially on those subsets of T cells capable of producing IFN-γ. However, in this study, the protective effect of IL-12 against HSV-1 infection was not demonstrated in TI mice when it was administered therapeutically, although the mortality rates of TI mice infected with HSV-1 were greatly decreased when IL-12 was given to them prophylactically. A predominance of burn-associated type 2 T cell responses, manifested by the amount of IL-4, were not changed in TI mice treated therapeutically with IL-12. Also, type 1 T cell responses, manifested by the amount of IFN-γ, were not elicited in these mice or in cultures of splenic T cells from these mice. In these experiments, the treatment of IL-12 was given to mice with or without infection 4 days after thermal injury. However, when IL-12 was given to TI mice prophylactically, splenic T cells from these TI mice produced IFN-γ into their culture fluids. These results suggest that, for the protection of TI mice against the established infection of HSV-1, the induction of type 1 T cell responses in these mice may be required. In our previous studies, the susceptibility of TI mice to infection with HSV-1 was shown to be 100 times greater than that of normal mice (12). Burn-associated $CD8^+ CD11b^+ TCR\gamma\delta^+$ type 2 T cells were identified as the type 2 T cells responsible for the increased susceptibility of TI mice to infection (12). The impaired resistance of TI mice to HSV-1 infection recovered when burn-associated type 2 T cell responses were reduced by treatment with 1) mAbs directed against type 2 cytokines (31), 2) antagonistic cells for type 2 T cells (12), or 3) inducers of antagonistic cells for type 2 T cells (32). However, the mortality rate of TI mice with established HSV-1 infection was not effectively reduced by these three treatments. Also, type 1 T cell responses were not elicited in these mice or in cultures of their splenic cells stimulated with anti-CD3 mAb. Because type 2 cytokines (IL-4 and IL-10) inhibit the generation of type 1 T cells, a predominance of type 1 T cell responses is not developed in currently HSV-1-infected TI mice that have a predominance of type 2 T cell responses.

Therefore, we hypothesized that the established HSV-1 infection in TI mice might be controlled by the induction of type 1 T cell responses along with the elimination of burn-associated type 2 T cell responses. sIL-4R has been described as a direct inhibitor for IL-4 (26). It has been reported that Ia and CD23 cell surface Ag expression and secretion of IgE and IgG1 from B cells, which were stimulated with IL-4, were inhibited by sIL-4R (40). Also, the proliferation of T cells stimulated with IL-4 was suppressed by sIL-4R (40). Therefore, for the regulation of the established HSV-1 infection in TI mice, we attempted to use sIL-4R as an inhibitor of type 2 T cell responses, and IL-12 was used as an inducer of type 1 T cell responses. TI mice currently infected with HSV-1 were treated with these two agents in combination. Thus, these mice received the IL-12 therapy starting 3 days postinfection (4 days after thermal injury) and the sIL-4R therapy at 3 and 5 days postinfection (4 and 6 days after thermal injury). Splenic T cells from these mice produced IFN-γ after stimulation with anti-CD3 mAb. However, IL-4 was not produced by these cells after the same stimulation. Also, the mortality rates of these mice were greatly reduced. Alternatively, IL-4 (but not IFN-γ) was produced by anti-CD3 mAb-stimulated splenic T cells from currently HSV-1-infected TI mice that were treated with IL-12 alone, sIL-4R alone, or saline, whereas similar mortality rates in these three groups of TI mice were demonstrated.

In our series of studies, neither therapeutic treatment with IL-12 nor sIL-4R showed significant effects on the survival of TI mice infected with HSV-1. The reason for this will be explained through the action of IL-4 secreted from burn-associated type 2 T cells. In our previous studies, type 2 T cells were demonstrated in mice 3–10 days after thermal injury (12). Papers published recently have described that IL-4 dominates the type 1 T cell response-inducing capacity of IL-12 (41–43). When naive $CD4^+$ T cells are stimulated by comparable concentrations of both IL-12 and IL-4, the effect of IL-12 on type 1 T cell differentiation is not displayed (41–43). The suppression of type 1 T cell differentiation by IL-4 is expressed based on the extinction of IL-12 signaling in the developing type 2 T cells (43). Type 2 T cells do not respond to IL-12 because of a lack of IL-12-inducing phosphorylation of STAT4, a transcription factor that is typically involved in IL-12 signaling (43). In addition, IL-12 has no inhibitory effect on IL-4-induced type 2 T cell differentiation, but rather enhances the secondary production of IL-4 after restimulation of the T cells (44, 45). Therefore, IL-12 could not induce protective immunity (type 1 T cell responses) in TI mice against HSV-1 infection. Although sIL-4R effectively inhibited IL-4 production in the spleens of TI mice, sIL-4R itself may be insufficient to elicit protective immunity against infection of HSV-1. Scott and Farrell reported (46) that $CB6F_1$ mice (a less susceptible strain) with chronic cutaneous leishmaniasis were protected by combination treatment with IL-12 and anti-IL-4 mAb, whereas treatment by either substance individually had a lesser influence on the subsequent course of the infection. This finding supports our results shown herein that the therapeutic combination treatment between an inhibitor of type 2 T cell responses (sIL-4R) and an inducer of type 1 T cell responses (IL-12) may have the potential to control current HSV-1 infections in thermally injured individuals. The combination treatment between sIL-4R and IL-12 may provide a new therapeutic strategy that could influence the outcome of HSV-1 infections in thermally injured individuals bearing a predominance of type 2 T cell responses.

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