

## $\alpha_{s1}$ -Casein-specific CD8<sup>+</sup> T Cell Clone That Inhibits Its Own Interferon- $\gamma$ Production by Producing Interleukin-10

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Received June 26, 1995

**A CD8<sup>+</sup> T cell clone specific to  $\alpha_{s1}$ -casein, one of the major allergens in milk, is shown to inhibit its own production of interferon (IFN)- $\gamma$  by producing interleukin (IL)-10. Anti-IL-10 antibodies enhanced the production of IFN- $\gamma$  induced by the antigen plus antigen-presenting cells from 12 h onward after initiating the culture. This enhancing effect was observed only when the cells were stimulated in the presence of the antigen-presenting cells. Neither IL-2 nor IL-4 abrogated this enhancing effect. This reveals a new regulating mechanism for IFN- $\gamma$  production from CD8<sup>+</sup> T cells.**

The critical role of CD8<sup>+</sup> T cells is in host defense against viral or parasitic infection, in killing tumors or grafted tissues, and in suppressing immune response. Recent findings that some CD8<sup>+</sup> T cells recognized exogenous antigens<sup>1)</sup> suggested that they participated in immune regulation more widely than had previously been expected. The activation of these CD8<sup>+</sup> T cells has been studied extensively, although little is known about their down-regulation.

In the previous study, we reported that the proliferation of CD8<sup>+</sup> T cell clones was inhibited by the addition of interleukin (IL)-10,<sup>2)</sup> which is known as a lymphokine with potent immune-inhibiting activity.<sup>3)</sup> We also showed that these CD8<sup>+</sup> T cell clones produced IL-10, which inhibited the proliferation of the CD8<sup>+</sup> T cells themselves, and that this was a novel type of regulatory mechanisms for proliferation inhibition involving the responding T cells themselves.<sup>2)</sup> In this study, we focused on the effect of endogenous IL-10 on the effector function of CD8<sup>+</sup> T cells. Among various effector functions, interferon (IFN)- $\gamma$  production is essential for several reasons. First, all known murine CD8<sup>+</sup> T cell clones with various antigenic specificity produced IFN- $\gamma$ ,<sup>2,4)</sup> the amount being comparable to that from CD4<sup>+</sup> T cell clones. Second, its biological activity is essential in some forms of CD8-related immune regulation. It has been indicated that CD8<sup>+</sup> T cells exerted their activity through the effect of IFN- $\gamma$  in protecting from parasitic infection.<sup>5)</sup> Furthermore, it has been reported that IFN- $\gamma$  induced inflammation or inhibited the production of IgE.<sup>6,7)</sup> By using a CD8<sup>+</sup> T cell clone which recognizes  $\alpha_{s1}$ -casein, we examined whether IFN- $\gamma$  production would also be auto-regulated by producing IL-10.

### Materials and Methods

**Reagents.**  $\alpha_{s1}$ -Casein was prepared from fresh raw skim milk and digested with trypsin-TPCK as previously described.<sup>2)</sup> Anti-T cell receptor (TCR)  $\beta$ -chain monoclonal antibody (mAb) H57-597, anti-IL-10 mAbs SXC-1 and 2A5, anti-IFN- $\gamma$  mAbs XMG1.2 and 6A2, and anti-mouse IL-4 mAb 11B11 were purified from ascitic fluid as previously described.<sup>2)</sup> Human

recombinant IL-2 was provided by Takeda Chemical Industries (Osaka, Japan). Mouse recombinant IL-4 was purchased from Genzyme (Boston, MA, U.S.A.), and mouse recombinant IL-10 was expressed by COS 7 cells.

**Cell line.**  $\alpha_{s1}$ -Casein-specific CD8<sup>+</sup> T cell clone 6B1 was maintained by weekly stimulation with trypsinized  $\alpha_{s1}$ -casein in the presence of irradiated syngeneic spleen cells, which were used as antigen-presenting cells (APC), in a conditioned RPMI 1640 medium as previously described. 6B1 produced IFN- $\gamma$  and IL-10, but not IL-4, upon antigenic stimulation.<sup>2)</sup>

**T cell stimulation assay.** T cells ( $2 \times 10^4$ ) were stimulated with the antigen ( $1 \mu\text{M}$  of trypsinized  $\alpha_{s1}$ -casein) in the presence of  $2 \times 10^5$  cells of APC in  $200 \mu\text{l}$  of the medium in 96-well microtiter plates. To assess the IFN- $\gamma$  production,  $50 \mu\text{l}$  of the culture supernatant was harvested 24 h after initiating the culture, the amount of IFN- $\gamma$  being determined by an enzyme-linked immunosorbent assay (ELISA).<sup>1)</sup> In some experiments, the T cells were stimulated with a plate-coated anti-TCR antibody as previously described.<sup>8)</sup> The results are expressed as the arithmetic mean with SD calculated from three independent cultures.

### Results

The effect of exogenous IL-10 on the production of IFN- $\gamma$  was initially assessed. 6B1 produced 26 U/ml of IFN- $\gamma$  when stimulated with the antigen plus APC, which was reduced to 15 U/ml by the addition of 10 U/ml of IL-10. No IFN- $\gamma$  was detected in the supernatant when APC alone was cultured (below 8 U/ml). This result indicates that murine IL-10 inhibited the production of IFN- $\gamma$  from the CD8<sup>+</sup> T cell clone.

As previously reported, 6B1 produced IL-10 upon antigenic stimulation.<sup>2)</sup> We next assessed the effect of anti-IL-10 antibodies, which would neutralize the possible inhibitory effect of endogenous IL-10, on the production of IFN- $\gamma$  by 6B1. The supernatant was harvested after a 24 h culture, in which the amount of IFN- $\gamma$  was measured by ELISA. As shown in Fig. 1, two anti-IL-10 antibodies with different epitopes significantly enhanced the production of IFN- $\gamma$ , while isotype-matched control antibodies had no effect. This result indicates that the CD8<sup>+</sup> T cells inhibited their own IFN- $\gamma$  production in an auto-regulatory manner by producing IL-10.

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Abbreviations: APC, antigen-presenting cell; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; TCR, T cell receptor.

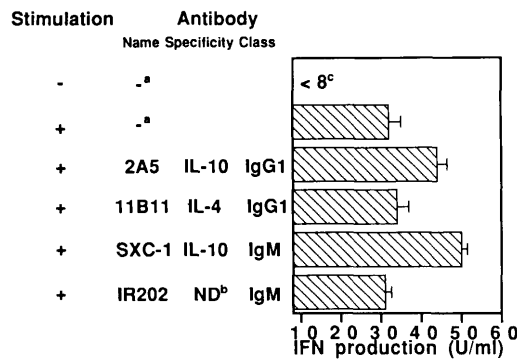


**Fig. 1.** Enhancing Effect of Anti-IL-10 Antibodies on IFN- $\gamma$  Production from 6B1.

T cells ( $2 \times 10^4$ ) were stimulated by  $1 \mu\text{M}$  of trypsinized  $\alpha_1$ -casein in the presence of irradiated syngeneic spleen cells. A monoclonal antibody,  $2 \mu\text{g}/\text{ml}$  of 2A5 (anti-IL-10, rat IgG) or 11B11 (anti-IL-4, rat IgG), or  $20 \mu\text{g}/\text{ml}$  of SXC-1 (anti-IL-10, rat IgM) or IR202 (control rat IgM, Genzyme) was added at the initiation of the culture. The amount of produced IFN- $\gamma$  was assessed after 24 h of culture. The detection limit was 8 U/ml.

a, no antibody was added; b, not determined; c, below detectable limit.

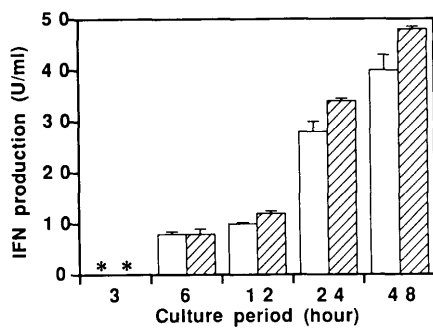


**Fig. 2.** Effect of the Anti-IL-10 Antibody on the Kinetics of IFN- $\gamma$  Production.

6B1 was stimulated with the antigen and APC, either with (hatched bars) or without (white bars)  $2 \mu\text{g}/\text{ml}$  of anti-IL-10 mAb 2A5. The culture supernatant was harvested after the indicated culture period, and the amount of IFN- $\gamma$  in each sample was measured.

\* Below detectable limit.

**Table I.** IFN- $\gamma$  Production of 6B1 Stimulated with Various Factors

| IFN- $\gamma$ production (U/ml) |      |                       |                          |                  |      |
|---------------------------------|------|-----------------------|--------------------------|------------------|------|
| Cell-stimulation factor         |      |                       |                          |                  |      |
| 2A5                             | None | Coated anti-TCR alone | Coated anti-TCR plus APC | Antigen plus APC | IL-2 |
| -                               | <8   | 60 $\pm$ 6            | 96 $\pm$ 12              | 32 $\pm$ 4       | <8   |
| +                               | <8   | 64 $\pm$ 8            | 124 $\pm$ 4              | 44 $\pm$ 4       | <8   |

6B1 was stimulated with the plate-coated anti-TCR antibody with or without APC, the antigen plus APC or by IL-2 (20 U/ml). Anti-IL-10 mAb 2A5 was added at a concentration of  $2 \mu\text{g}/\text{ml}$ .

To assess the enhancing effect of the anti-IL-10 antibody on the kinetics of the response, the supernatant was harvested after various culture periods, the amount of IFN- $\gamma$  produced being measured. The production of IFN- $\gamma$  was detected 6 h after initiating the culture, the enhancing effect of the anti-IL-10 antibody being obvious from 12 h (Fig. 2).

To further analyze the negative regulation by endogenous IL-10, the cells were activated by various stimuli, and the effect of the anti-IL-10 antibody was examined. As shown in Table I, the anti-IL-10 antibody did not affect IFN- $\gamma$  production when cells were stimulated by the coated anti-

**Table II.** Lack of Effect of IL-2 and IL-4 on the Inhibition of Endogenous IL-10

| 2A5 | IFN- $\gamma$ production (U/ml) |             |             |
|-----|---------------------------------|-------------|-------------|
|     | Addition to culture             |             |             |
|     | None                            | IL-2        | IL-4        |
| -   | $40 \pm 10$                     | $64 \pm 4$  | $72 \pm 12$ |
| +   | $64 \pm 4$                      | $152 \pm 4$ | $168 \pm 8$ |

6B1 was stimulated with the antigen plus APC with or without  $2 \mu\text{g}/\text{ml}$  of anti-IL-10 antibody 2A5.  $20 \text{ U}/\text{ml}$  of IL-2 or IL-4 was added at the initiation of the culture. IFN- $\gamma$  production without the antigen was below 8 U/ml under each condition.

TCR antibody in the absence of APC. On the other hand, IFN- $\gamma$  production induced by stimulation in the presence of APC, either by the antigen or coated anti-TCR antibody, was enhanced by adding the anti-IL-10 antibody. No detectable amount of IFN- $\gamma$  was produced by IL-2. This result indicates that endogenously produced IL-10 inhibited the production of IFN- $\gamma$  only in the presence of APC.

Next, we assessed whether endogenous IL-10 would inhibit IFN- $\gamma$  production in the presence of IL-2 or IL-4. As shown in Table II, the addition of  $20 \text{ U}/\text{ml}$  of IL-2 or IL-4 significantly enhanced the production of IFN- $\gamma$ . Under each condition, the anti-IL-10 antibody enhanced the production of IFN- $\gamma$ . This result indicates that, even with an excess amount of IL-2 or IL-4, endogenously produced IL-10 was effective in suppressing IFN- $\gamma$  production from the CD8<sup>+</sup> T cell clone.

## Discussion

We assessed in this study the effect of endogenously produced IL-10 on the production of IFN- $\gamma$ , and found that CD8<sup>+</sup> T cells inhibited their own IFN- $\gamma$  production by themselves through the action of IL-10. This reveals a new role for murine IL-10 as an auto-regulator in the IFN- $\gamma$  production of cloned CD8<sup>+</sup> T cells and a new regulating mechanism for lymphokine production by responding T cells themselves. Although the inhibition of IFN- $\gamma$  production by IL-10 may be indirect through the inactivation of APC (see below), we use the term 'auto-regulation' here in the sense that IL-10 secreted by the T cells finally inhibited the production of IFN- $\gamma$  by the responding cells. Murine CD4<sup>+</sup> T cells are divided into two subsets, Th1 and Th2, which produce IFN- $\gamma$  and IL-10, respectively.<sup>9)</sup> On the other hand, CD8<sup>+</sup> T cell clones produced both lymphokines.<sup>2)</sup> Nanda *et al.* have reported that double-positive T cell clones, which were rare in peripheral organs such as lymph nodes and spleen, produced both IL-10 and IFN- $\gamma$ .<sup>10)</sup> Although the regulating mechanism for IFN- $\gamma$  production was not examined in their study, these double-positive clones might also inhibit their own IFN- $\gamma$  production by producing IL-10. Therefore, the auto-inhibitory regulation of IFN- $\gamma$  production by IL-10 is a unique characteristic of the CD8<sup>+</sup> T cell population and probably of some special T cells. This auto-regulation, as well as that with helper-type lymphokines from CD4<sup>+</sup> T cells, must have controlled the IFN- $\gamma$  production from CD8<sup>+</sup> T cells. Considering the biological significance of IFN- $\gamma$ , the phenomenon reported in this study is of importance to immune regulation.

We can assume the biological role of IL-10 produced by CD8<sup>+</sup> T cells. The inhibitory effect of exogenous IL-10 on the production of IFN- $\gamma$  by 6B1 was not strong, suggesting that the major role of IL-10 is to inhibit a more sensitive population such as CD4<sup>+</sup> Th1. Indeed, we have reported that IL-10 produced from the CD8<sup>+</sup> T cell clone inhibited the proliferation of the CD4<sup>+</sup> Th1 clone.<sup>11)</sup> The phenomenon reported here may be more important in CD8<sup>+</sup> T cells which produce a larger amount of IL-10.

It was observed that when T cells were stimulated with the anti-TCR antibody, IFN- $\gamma$  production was enhanced by the addition of APC. This was probably through their costimulatory function, which has been proposed by Williams and Unanue.<sup>12)</sup> Endogenous IL-10 inhibited the response of the CD8<sup>+</sup> T cells only in the presence of APC, suggesting that endogenous IL-10 inhibited the costimulatory function of APC. Consistent with this, several groups have reported that IL-10 inactivated APC, which resulted in a lack of costimulatory molecules such as B7 or IL-12,<sup>13,14)</sup> rather than major histocompatibility complex molecules.

The anti-IL-10 antibody enhanced both the proliferation and IFN- $\gamma$  production, but its effect on the kinetics was different. The enhancing effect of the anti-IL-10 antibody on IFN- $\gamma$  production was obvious from 12 h onwards after initiating the culture, while no enhancing effect on proliferation was observed until 3 days after culture initiation by the same experimental procedure.<sup>2)</sup> This apparent discrepancy might have been due to the different inhibitory mechanism exerted by IL-10 on IFN- $\gamma$  production and proliferation. Proliferation and IFN- $\gamma$  production possibly need different types of costimulatory signals at different time points. Alternatively, but not exclusive of the above possibility, the lack of costimulatory molecules on APC might have resulted in the inhibition of IFN- $\gamma$  production from 12 h onwards, while more time was required to manifest the inhibitory effect on proliferation. The fact that the addition of the anti-IL-10 antibody 24 h after culture initiation did not enhance the proliferation<sup>2)</sup> supports this hypothesis.

It is known that  $\alpha_{s1}$ -casein is one of the major allergens

in milk. Considering that IFN- $\gamma$  strongly inhibits IgE synthesis, IFN- $\gamma$  might play a critical role in the activity of CD8<sup>+</sup> T cells in inhibiting the type I allergy. Our study suggests that increasing IFN- $\gamma$  production is achieved by the neutralization or reduction of IL-10. One possibility to do this is an alteration of antigenic peptides, for recent reports have indicated that each T cell response of a single T cell clone can be regulated independently by substituted antigenic peptides.<sup>15,16)</sup> We believe the results of our study will be helpful in understanding immune regulation and in curing food allergy.

**Acknowledgments.** We thank Dr. T. Tada and Y. Asano for kindly providing mAbs, and Dr. Y. Minai for critically reading the manuscript.

## References

- 1) T. Hisatsune, K. Nishijima, M. Kohyama, H. Kato, and S. Kaminogawa, *J. Immunol.*, **154**, 88–96 (1995).
- 2) K. Nishijima, T. Hisatsune, Y. Minai, M. Kohyama, and S. Kaminogawa, *Cell. Immunol.*, **154**, 193–201 (1994).
- 3) K. W. Moore, A. O'Garra, R. de Waal Malefyt, P. Vieira, and T. R. Mosmann, *Annu. Rev. Immunol.*, **11**, 165–190 (1993).
- 4) T. A. T. Fong and T. R. Mosmann, *J. Immunol.*, **144**, 1744–1752 (1990).
- 5) L. Schofield, J. Villaquiran, A. Ferreira, H. Schellekens, R. Nussenzweig, and V. Nussenzweig, *Nature*, **330**, 664–666 (1987).
- 6) M. A. Farrar and R. D. Schreiber, *Annu. Rev. Immunol.*, **11**, 571–611 (1993).
- 7) C. M. Snapper and W. E. Paul, *Science*, **236**, 944–947 (1987).
- 8) T. Hisatsune, A. Enomoto, K. Nishijima, Y. Minai, Y. Asano, T. Tada, and S. Kaminogawa, *J. Immunol.*, **145**, 2421–2426 (1990).
- 9) T. R. Mosmann and R. L. Coffman, *Annu. Rev. Immunol.*, **7**, 145–173 (1989).
- 10) N. K. Nanda, E. E. Sercarz, D.-H. Hsu, and M. Kronenberg, *Int. Immunol.*, **6**, 731–737 (1994).
- 11) T. Hisatsune, Y. Minai, K. Nishijima, A. Enomoto, K. W. Moore, T. Yokota, K. Arai, and S. Kaminogawa, *Lymphokine Cytokine Res.*, **11**, 87–93 (1992).
- 12) I. R. Williams and E. R. Unanue, *J. Immunol.*, **145**, 85–93 (1990).
- 13) L. Ding, P. S. Linsly, L. Y. Huang, R. L. Germain, and E. M. Shevach, *J. Immunol.*, **151**, 1224–1234 (1993).
- 14) A. D'Andrea, M. Aste-Amezaga, N. M. Valiante, X. Ma, M. Kubin, and G. Trinchieri, *J. Exp. Med.*, **178**, 1041–1048 (1993).
- 15) B. D. Evavold and P. M. Allen, *Science*, **252**, 1308–1310 (1991).
- 16) B. D. Evavold, J. Sloan-Lancaster, B. L. Hsu, and P. M. Allen, *J. Immunol.*, **150**, 3131–3140 (1993).