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Interleukin-12 Augments the Generation of Autologous Tumor-reactive CD8$^+$ Cytotoxic T Lymphocytes from Tumor-infiltrating Lymphocytes

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Human tumor-infiltrating lymphocytes (TIL) were obtained from breast cancer, renal cancer or neuroblastoma to investigate the generation of autologous tumor-reactive CD8$^+$ cytotoxic T lymphocytes (CTL). When TIL were cultured with interleukin (IL)-2 (100 U/ml), the growth of TIL peaked around 8–10 days after the initiation of culture. In contrast, the proliferation of TIL cultured with IL-2 plus IL-12 peaked around 4–5 days after culture and tumor cells rapidly disappeared from the culture. To determine the generation of autologous tumor-reactive CD8$^+$ CTL, TIL-derived CD8$^+$ T cells were separated by FACStar. Both IL-2-activated and IL-2 plus IL-12-activated TIL-CD8$^+$ T cells showed the same level of lymphokine-activated killer activity against a variety of tumor cells. However, TIL-CD8$^+$ T cells activated with IL-2 plus IL-12 revealed greatly augmented cytotoxicity against autologous tumor cells compared with that induced by IL-2 alone. The autologous tumor cell-killing activity of TIL-CD8$^+$ CTL was significantly inhibited by the addition of F(ab)$_2$ anti-CD3 monoclonal antibody, indicating that these CTL recognize autologous tumor antigen through T cell receptor. These results imply that IL-12 is a novel cytokine which facilitates the generation of autologous tumor-reactive CD8$^+$ CTL from TIL.

Key words: IL-12 — TIL — CTL — Autologous tumor — Human

CTL[6] can recognize MHC-binding tumor-rejection antigen peptide to lyse autologous tumor cells in both mouse and human systems.[1,2] The cloning of the gene encoding tumor rejection antigen encouraged us to develop a tumor vaccine which might induce protective antitumor immunity.[3] Studies on tumor rejection antigen have been carried out using CTL reactive against autologous tumor cells which have rather high antigenicity, such as melanoma cells.[2,3] However, in general, it is very hard to induce CTL reactive against autologous tumor cells because of their low immunogenicity. If we can develop a novel method to induce autologous tumor-reactive CTL, it might be applicable to adoptive tumor immunotherapy and for the determination of various tumor rejection antigens.

IL-12 has a variety of immunoregulatory functions and facilitates the generation of antitumor effector cells, including NK, CTL, LAK and Th1 cells.[4–7] Moreover, IL-12 showed a potent antitumor activity *in vivo*.[8] Therefore, it is important to investigate the effect of IL-12 on the generation of autologous tumor-reactive CTL from TIL.

TIL were obtained from breast tumor, renal carcinoma or neuroblastoma and cultured with IL-2 (100 U/ml) alone or IL-2 plus IL-12 (100 U/ml). A typical growth pattern of TIL cultured with IL-2 alone or IL-2 plus IL-12 for 5 days is shown in Fig. 1. In the culture with medium alone (Figs. 1a and 1d), tumor cell growth was predominant and no significant growth of TIL was observed. However, culture of TIL with IL-2 plus IL-12 caused a marked proliferation of TIL and disappearance of tumor cells 5 days after the initiation of TIL culture (Figs. 1c and 1f). At this point, IL-2 induced marginal proliferation of TIL and many tumor cells still existed in the culture (Figs. 1b and 1e). The growth of TIL cultured with IL-2 alone peaked around 10–14 days after the initiation of culture (data not shown). IL-12 alone also induced no significant growth of TIL (data not shown). After expansion of TIL, TIL-derived CD8$^+$ T cells were sorted by FACStar and their cytotoxicity towards a variety of tumor cells including autologous tumor cells was measured by ^{51}Cr-release assay. As shown in Fig. 2, CD8$^+$ TIL expanded from breast cancer with IL-2 alone showed a significant LAK activity against Daudi cells, K562 cells and allogeneic breast tumor cells (MCF-7),

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[6] Abbreviations: CTL, cytotoxic T lymphocytes; MHC, major histocompatibility complex; IL-12, interleukin 12; NK, natural killer; LAK, lymphokine-activated killer; Th1, T helper type 1 cell; TIL, tumor-infiltrating lymphocytes; mAb, monoclonal antibody; TCR, T cell receptor.

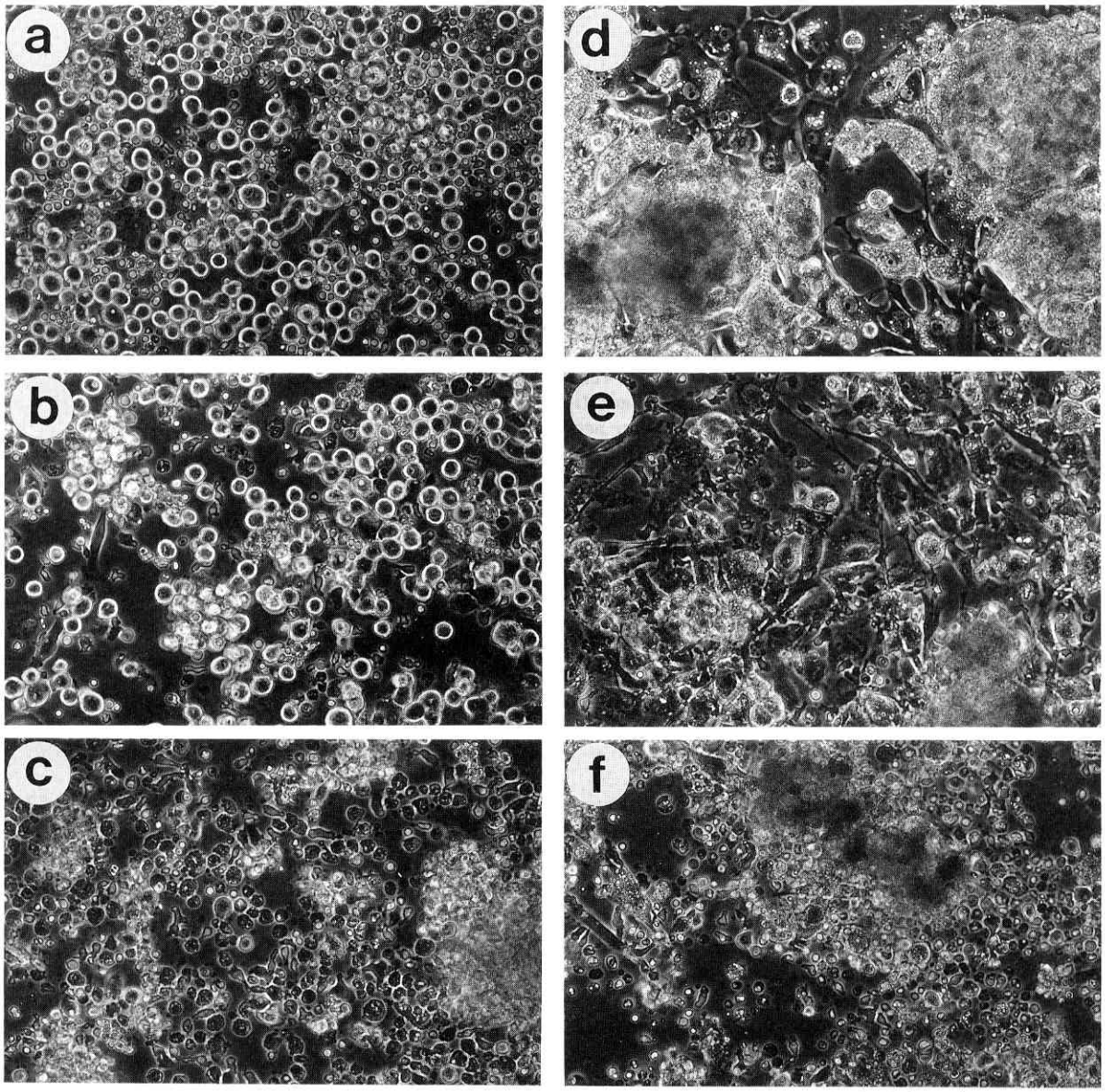

Fig. 1. Augmented proliferation of human TIL by culture with IL-2 plus IL-12. TIL were obtained from human breast (a, b, c) or renal cancer tissues (d, e, f) by mechanical treatment of tumor specimens with scissors.[16] The TIL suspension (5×10^5/ml) was cultured with medium alone (a, d), IL-2 (100 U/ml; kindly supplied by Shionogi Pharmaceutical Co., Ltd., Osaka) (b, e) or IL-2 plus IL-12 (100 U/ml; kindly donated by Genetics Institute, Boston, USA) (c, f). AIM-V medium (Gibco, NY, USA) containing 10% human serum was used for all the experiments. The photographs were taken 5 days after the initiation of culture.

but exhibited lower cytotoxicity towards autologous breast cancer cells. However, addition of IL-12 to the culture resulted in a dramatic augmentation of the cytotoxicity of TIL-CD8$^+$ T cells towards autologous tumor cells, though their LAK activity was not affected by IL-12 addition. We confirmed such an enhancing effect of IL-12 on the generation of autologous tumor-reactive TIL-derived CD8$^+$ T cells in four separate experiments using breast cancer, renal cancer and neuroblastoma (Fig. 3). The autologous tumor cell-killing activity of TIL-CD8$^+$ CTL induced by IL-2 plus IL-12 was blocked by the addition of $F(ab)_2$ anti-CD3 mAb, while IL-2-activated TIL-CD8$^+$ T cells showed disparate sensitivity to the blocking effect of anti-CD3 mAb (Fig. 4). Thus, it is suggested that TIL-derived CD8$^+$ CTL, whose activity was enhanced by IL-12, may recognize the autologous

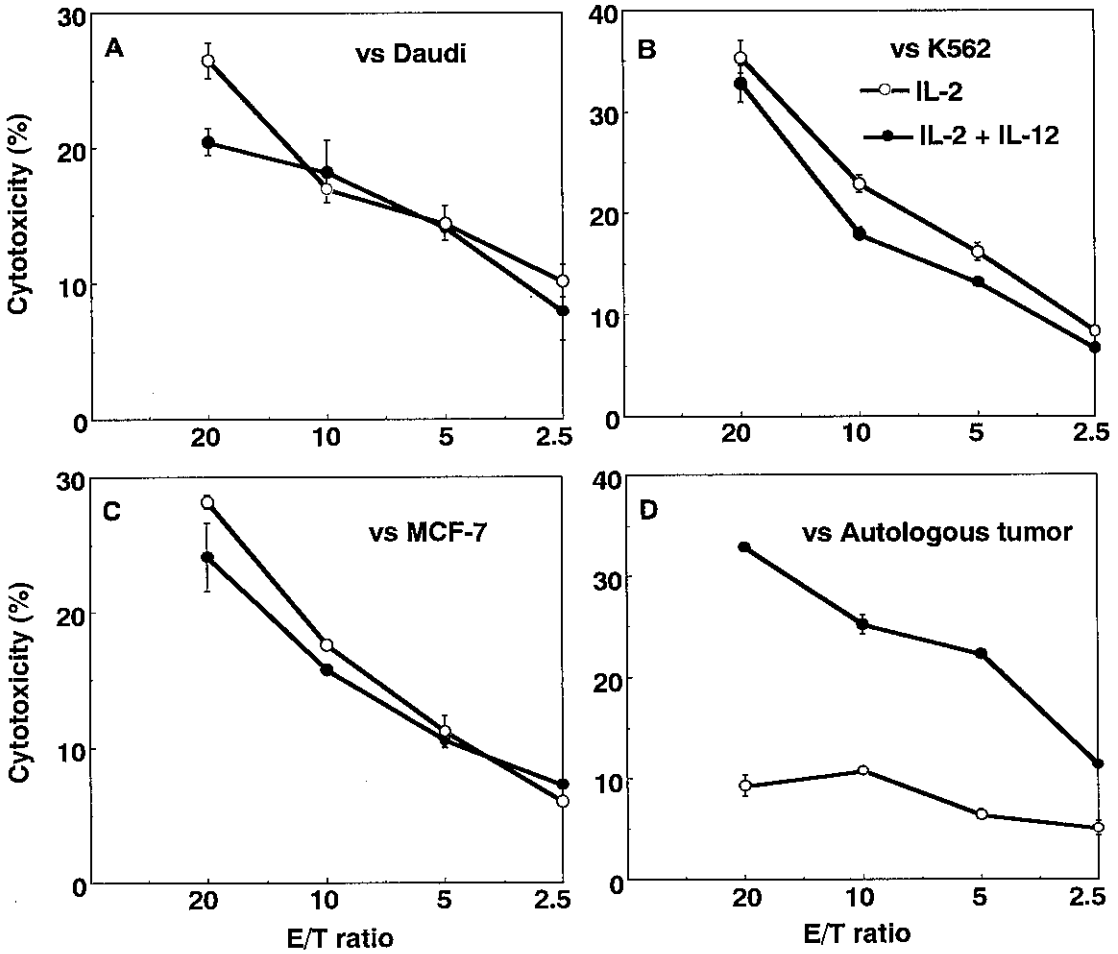

Fig. 2. Augmented generation of CD8+ CTL reactive against autologous tumor cells. TIL isolated from breast cancer were cultured with IL-2 or IL-2 plus IL-12 for 14 days. Then, TIL-CD8+ T cells were isolated by FACStar to determine their cytotoxicity. The LAK activity of TIL-CD8+ T cells was determined using Daudi B lymphoma cells, K562 chronic myelogenous leukemia cells or MCF-7 breast cancer cells. The cytotoxicity of TIL-CD8+ T cells to autologous tumor cells was also measured by 4-h ^{51}CR-release assay at various effector-to-target ratios. Autologous tumor cell lines were established by culturing small tumor tissue specimens with medium alone. (open circles), TIL-CD8+ T cells cultured with IL-2; (closed circles), TIL-CD8+ T cells cultured with IL-2 plus IL-12. The bars represent mean±SE of triplicate samples.

tumor antigen via TCR. These results demonstrated that IL-12 is a useful cytokine to induce autologous tumor-reactive CD8+ CTL from TIL in combination with a low dose of IL-2.

It has been demonstrated that IL-12 stimulated the growth of activated T cells and NK cells.[1,9] IL-12 was also shown to induce the expression of IL-2 receptor on T cells.[10] Moreover, IL-12 enhanced B7-mediated costimulatory signaling, which is essential for antigen recognition by T cells.[11] Therefore, it is reasonable to assume that IL-12 acts synergistically with IL-2 in the preferential stimulation of autologous tumor-reactive CD8+ T cells. Our results are not inconsistent with those of other groups, who reported that IL-12 stimulated the proliferation and generation of TIL-derived killer cells which could lyse autologous tumor cells.[12,13] However, they did not separate TIL into NK-type and T cell type killer cells, so that it remains unclear whether autologous tumor cell-killing activity was derived from TIL-CD8+ T cells or TIL-NK-type cells. To our knowledge, this is the first paper which demonstrates that IL-12 has the capability of enhancing the induction of autologous tumor-reactive CD8+ CTL from TIL. We believe the culture methods described herein may make it possible to apply autologous tumor-specific CTL to adoptive tumor immunotherapy. Because antigen-reactive CTL specifically mi-

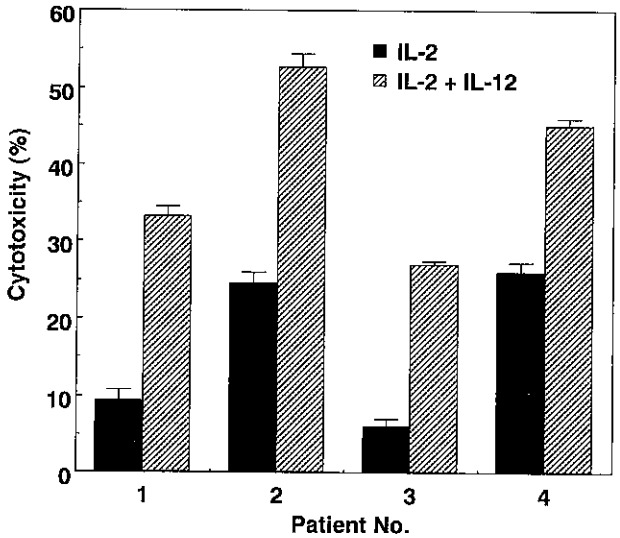

Fig. 3. IL-12 augmented the generation of autologous tumor-reactive CD8$^+$ CTL from TIL in various tumor patients. TIL were isolated from four different patients (patient 1, breast cancer; patient 2, renal cancer; patient 3 and patient 4, neuroblastoma). The expansion and separation of TIL-derived CD8$^+$ T cells was carried out by the same protocal described in the legend to Fig. 2. The cytotoxicity of TIL-derived CD8$^+$ T cells cultured with IL-2 (closed bars) or IL-2 plus IL-12 (hatched bars) was determined by 4-h ^{51}Cr-release assay. The effector-to-target ratio was 20:1. The bars represent mean±SE of triplicate samples.

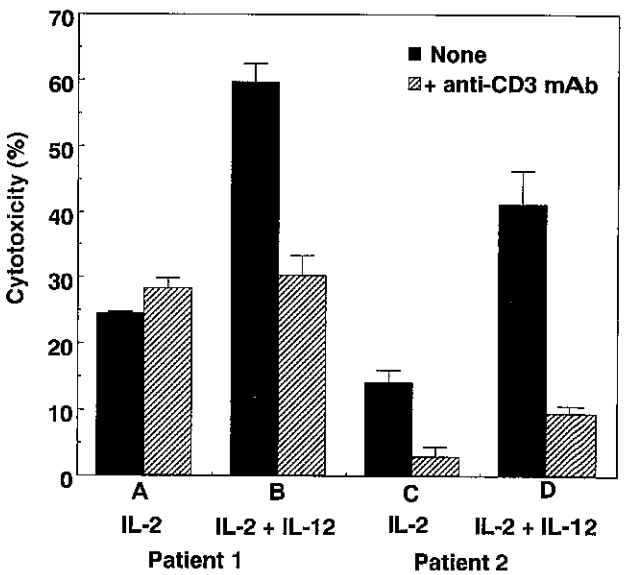

Fig. 4. The F(ab)$_2$ anti-CD3 mAb blocked autologous tumor cell-killing activity of TIL-derived CD8$^+$ T cells induced by IL-2 plus IL-12. TIL obtained from patient 1 (breast cancer) or patient 2 (renal cancer) were isolated and cultured with IL-2 or IL-2 plus IL-12 by the same protocol described in the legend to Fig. 3. After separation of TIL-CD8$^+$ T cells by FACStar their cytotoxicity towards autologous tumor cells was determined in the presence (hatched bars) or absence (closed bars) of F(ab)$_2$ anti-CD3 mAb (OKT3 mAb; 25 μg/ml). A, IL-2-activated TIL-CD8$^+$ T cells obtained from patient 1; B, IL-2 and IL-12-activated TIL-CD8$^+$ T cells obtained from patient 1; C, IL-2-activated TIL-CD8$^+$ T cells obtained from patient 2; D, IL-2 and IL-12-activated TIL-CD8$^+$ T cells obtained from patient 2. The bars represent mean±SE of triplicate samples.

grate to the tumor site through TCR, adoptive tumor immunotherapy using autologous tumor-reactive CTL may provide a more physiological and more efficient therapeutic effect compared with LAK therapy[14] or targeting therapy using bispecific antibody.[15] We are also trying to characterize the nature of HLA-binding tumor rejection antigen peptide using autologous tumor-reactive CD8$^+$ CTL induced from TIL by IL-12 and IL-2.

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