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Cytokines Required for Induction of Histocompatibility Leukocyte Antigen-class I-restricted and Tumor-specific Cytotoxic T Lymphocytes by a SART1-derived Peptide

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Although there have been several reports on peptides of human tumor-rejection antigens capable of inducing histocompatibility leukocyte antigen (HLA)-class I-restricted and tumor-specific cytotoxic T lymphocytes (CTLs), it is not yet clear which cytokines are required for CTL induction. This study has investigated the cytokine combinations required for optimal induction of CTLs by SART1$^{690–698}$ peptide, which is capable of inducing HLA-A24-restricted and tumor-specific CTLs in peripheral blood mononuclear cells (PBMCs). Pretreatment of PBMCs as a source of antigen-presenting cells (APCs) with interferon (IFN)-γ, or to some extent with IFN-α, but not with any of the other cytokines tested, augmented the peptide-induced CTL activity in HLA-A24 heterozygotes, but not in HLA-A24 homozygotes. This IFN-γ-mediated augmentation was inhibited by either interleukin (IL)-4 or IL-10. IL-2 alone in culture, along with weekly stimulation by peptide-pulsed APCs, was sufficient for the differentiation and proliferation of CTLs for the initial several weeks of culture. This IL-2-mediated activation of CTLs was inhibited by the addition of IFN-γ, IL-4, or IL-10 to the IL-2 culture. For further expansion of the CTLs, dendritic cells (DCs) induced from PBMCs with IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) were required as APCs. These results indicate that IFN-γ and IL-2 are important in the activation of APCs and CTLs, respectively, while GM-CSF and IL-4 are needed for the induction of DCs, which in turn are required for further expansion of mature CTLs. These results are important in allowing for a better understanding of the cellular and molecular basis of tumor-specific immunity, and also for the development of peptide-based specific immunotherapy.

Key words: SART1 antigen — Cytokine — Cytotoxic T lymphocyte — Cancer vaccine

Many different peptide antigens recognized by histocompatibility leukocyte antigen (HLA)-class I-restricted cytotoxic T lymphocytes (CTLs) against melanoma cells have been identified in the past 6 years.[1–8] Some of these antigens are under clinical trials as cancer vaccines, and major tumor regression has been reported in several HLA-A1$^+$ melanoma patients who have received vaccine therapy with MAGE3 peptide.[9] Further, peptide-based therapy has resulted in tumor regression in HLA-A2$^+$ melanoma patients.[10, 11] We have reported that nonapeptides of the SART1 antigen are available for specific immunotherapy for HLA-A2601$^+$ or A2402$^+$ epithelial cancer patients.[12, 13] Some other peptides are also able to induce CTLs against epithelial cancer cells.[14, 15] Therefore, these peptides could be a new tool in specific immunotherapy for cancer patients. Although there have been a large number of studies in this field, the differences in the mechanisms by which peptides induce CTLs appear to be significant.[13, 16–26] Identification of the cytokine combinations required for optimal CTL induction by these peptides will be important in developing a better understanding of the cellular and molecular basis of tumor-specific immunity, and for peptide-based specific immunotherapy. Therefore, we have investigated in this study the cytokine combinations required for the induction of HLA-class I-restricted and tumor-specific CTLs by a SART1-derived peptide. We report that interferon (IFN)-γ and interleukin (IL)-2 are important for the activation of antigen-presenting cells (APCs) and CTLs, respectively, while granulocyte macrophage-colony-stimulating factor (GM-CSF) and IL-4 are needed for dendritic cells (DCs), which in turn are critical to the expansion of mature CTLs.

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MATERIALS AND METHODS

Cytokines and APCs The cytokines used in this study were IL-2 (kindly provided by Shionogi Research Lab., Osaka), IFN-γ, GM-CSF, IL-4 and IFN-α (Pepro Tech.,

Rocky Hill, NJ), tumor necrosis factor (TNF)-α (Funakoshi, Tokyo), IL-12 (kindly provided by the Genetics Institute, Cambridge, MA), and IL-10 (Genzyme Corp., Cambridge, MA). Unseparated peripheral blood mononuclear cells (PBMCs) from healthy donors were incubated with various doses of cytokines for 20 h, pulsed with a peptide (10 μM) for 2 h, irradiated (30 gray), washed, and then used as APCs. DCs were also used as APCs. For the preparation of DCs, PBMCs were incubated with 10 units (u) /ml of IL-4 and 10 u /ml of GM-CSF for 7 days by the methods reported previously.[27, 28] More than 70% of the adherent cells after that culture were DCs, as determined by the staining of these cells with anti-CD11c, -DR, -CD80, and -CD83 monoclonal antibody (mAb) (IMMUNOTECH., Marseille, France) followed by an analysis with FACScan (Becton Dickinson, Tokyo) as reported.[29] The cells were then irradiated (30 gray), pulsed with a peptide (10 μM) for 2 h, washed, and used as APCs. Untreated or pre-treated PBMCs were also tested for the expression of the HLA-A24 molecules after staining with anti-HLA-A24 (A11.1M) mAb reactive to the HLA-A24 molecule (ATCC, Rockville, MD).

Induction of CTLs by peptides The methods by which CTLs were induced by the SART1$^{690-698}$ (EYRGFTQDF) peptide possessing the ability to induce HLA-A24-restricted and tumor-specific CTLs have been described previously.[13] Briefly, in the presence of the peptide-pulsed autologous PBMCs acting as APCs (2×10^5 cells), PBMCs (2×10^6 cells) from HLA-A2402 homozygous or heterozygous healthy volunteers were incubated in one well of a 24-well plate containing 2 ml of the culture medium (45% RPMI-1640 medium, 45% AIM-V medium [GIBCO BRL, Walkersville, MA], 10% FCS [EQUITECH BIO, Ingram, TX] with 100 u/ml of IL-2, and 0.1 mM MEM nonessential amino acids solution [GIBCO BRL]) (termed the culture medium). At days 7 and 14 of culture, the PBMCs were collected, washed, and stimulated with the irradiated autologous APCs that had been pre-incubated with the same peptide at the same dose for 2 h. The cells were harvested at day 21 of the culture and were tested for their activity in producing IFN-γ in response to various target cells in triplicate assays by ELISA. The deviations in the triplicate determinants were usually <10% of the mean values. Some of the cells were further incubated in the wells of a round-bottomed 96-well micro-culture plate with the culture medium in the presence of irradiated autologous PBMCs acting as feeder cells. These cells from the micro-culture were re-checked for activity to produce IFN-γ in response to tumor cells at around days 10–18 of culture, and were tested for their cytotoxicity at different effector-to-target (E/T) cell ratios in a 6-h ^{51}Cr-release assay at around days 14–20 as reported.[13] A two-tailed Student's t test was used for the statistical analysis.

RESULTS

Cytokines for APCs We investigated whether the pretreatment of APCs with various cytokines could augment the following peptide-mediated CTL induction. Unseparated PBMCs of HLA-A2402 heterozygotes or homozygotes were incubated for 20 h with no cytokine, or with IFN-γ (100 u/ml), IFN-α (100 u/ml), IL-12 (100 u/ml), TNF-α (100 u/ml), IL-4 (10 u/ml), IL-10 (2 pg/ml), or GM-CSF (2 u/ml). These cells were pulsed for 2 h with the SART1$^{690-698}$ peptide (10 μM), irradiated, washed, and then used as APCs to induce CTLs in the autologous PBMCs in culture with IL-2 (100 u/ml) alone. The same stimulation was repeated at days 7 and 14, followed by the harvesting of effector cells at day 21 for measurement of their CTL activity. PBMCs from HLA-A24 heterozygous donors produced low but significant (P=0.03) levels of IFN-γ at an E/T ratio of only 8 by recognition of the KE4 esophageal squamous cell carcinoma (SCC) cells (HLA-A2402$^+$, SART1$_{259}^+$), but not of the QG56 lung SCC cells (HLA-A2402$^-$, SART1$_{259}^+$) or VA13 fibroblast cells (HLA-A2402$^-$, SART1$_{259}^-$) when the untreated APCs were used (Fig. 1). Pretreatment of the APCs with IFN-γ significantly (P=0.024 or 0.009 at an E/T ratio of 4 or 8, respectively) increased the levels of IFN-γ production by the CTLs in an HLA-A24-restricted manner as compared to those by the CTLs and APCs with no cytokine pretreatment. Pretreatment of the APCs with IFN-α also significantly (P=0.032 at an E/T ratio of 8) increased the levels of IFN-γ production by the CTLs in an HLA-A24-restricted manner. In contrast, the pretreatment with IL-4, IL-10, or GM-CSF did not increase but rather significantly decreased these levels ($P<$ at least 0.01), resulting in no CTL induction. Pretreatment with IL-12 or TNF-α increased the levels of IFN-γ production by recognition of KE4 and, to some extent, by recognition of the other two target cells, suggesting that the pretreatment primarily increased the lymphokine-activated killer (LAK) cell activity. Since the results suggest that the pretreatment of APCs with IFN-γ significantly augments the subsequent CTL induction by the peptide, 10 and 1000 u/ml of IFN-γ were used for the study. However, 10 u/ml of IFN-γ did not significantly augment the peptide-induced CTL activity, and 1000 u/ml rather inhibited it in association with an increase in LAK cell activity (data not shown).

To confirm the IFN-γ-mediated augmentation of CTL activity, the PBMCs of day 21 that had been induced by the peptide loaded on the IFN-γ-pretreated APCs were further expanded in the presence of autologous feeder cells for an additional 20 days. This expansion was followed by a test of the CTL activity of the PBMCs by both IFN-γ release and 6-h ^{51}Cr-release assays. These PBMCs produced very high levels of IFN-γ and also showed significant levels of cytotoxicity against KE4 tumor cells in an

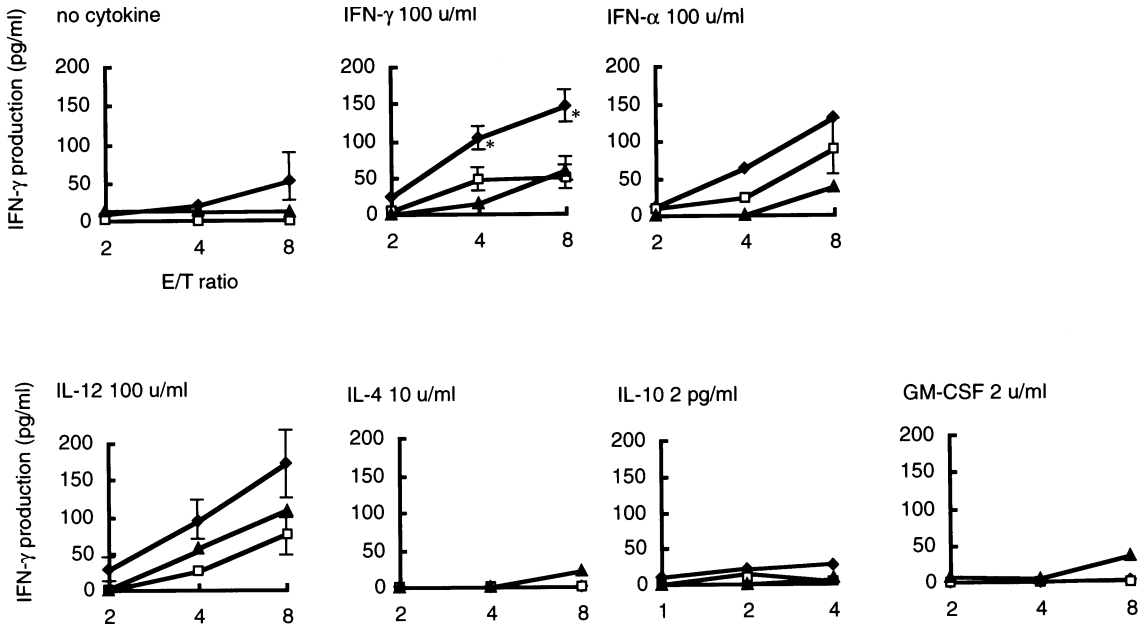

Fig. 1. Pretreatment of APCs with cytokines. Unseparated PBMCs of HLA-A2402 heterozygotes were incubated for 20 h with no cytokine or with IFN-γ (100 u/ml), IFN-α (100 u/ml), IL-12 (100 u/ml), TNF-α (100 u/ml), IL-4 (10 u/ml), IL-10 (2 pg/ml), or GM-CSF (2 u/ml). These cells were pulsed for 2 h with the $SART1^{690-698}$ peptide (10 μM), irradiated, washed, and then used as APCs (2×10^5 cells/2ml/well) to induce CTLs in the autologous PBMCs (2×10^6 cells/2ml/well) in culture with IL-2 alone (100 u/ml). The same stimulation was repeated at days 7 and 14 followed by the harvest of effector cells at day 21 for the measurement of their activity in producing IFN-γ by recognition of the KE4 esophageal SCC (HLA-A2402$^+$, $SART1_{259}^+$), the QG56 lung SCC (HLA-A2402$^-$, $SART1_{259}^+$), or VA13 fibroblast cells (HLA-A2402$^-$, $SART1_{259}^-$) in triplicate assays at three different effector-to-target (E/T) ratios by ELISA (the limit of sensitivity: 5 pg/ml). The deviations of the triplicate determinants were usually <10 % of the mean values. Representative results for the PBMCs from an HLA-A24 heterozygous donor are shown in the figure. ◆ KE4, □ QG56, ▲ VA13. A two-tailed Student's *t* test was used for the statistical analysis of the values of IFN-γ production measured by the triplicate assays, and ∗ indicates *P* values of less than 0.05 between the level of IFN-γ produced by the CTLs that were stimulated by the peptide-pulsed APCs pretreated with no cytokine and that by the CTLs stimulated by APCs pretreated with cytokine. The *P* values are given in the "Results" section. In addition, the levels of IFN-γ produced by the CTLs that were stimulated by the peptide-pulsed APCs pretreated with IFN-γ were significantly higher ($P<0.01$) than those by the CTLs that were stimulated by the peptide-pulsed APCs pretreated with IL-4, IL-10, or GM-CSF both at E/T ratios of 4 and 8.

HLA-24 restricted manner (Fig. 2). In contrast to the heterozygotes, the pretreatment of APCs with IFN-γ or IL-12 of PBMCs from HLA-A24 homozygotes failed to augment the peptide-induced CTL activity (data not shown).

It is well known that IL-4, as well as IL-10, counteracts the activity of IFN-γ.[16, 17] Therefore, PBMCs of HLA-24 heterozygotes were pretreated with no cytokine, with IFN-γ alone, with IFN-γ plus IL-4, or with IFN-γ plus IL-10, and were used as APCs for the induction of CTLs by the peptide. Addition of IL-4 significantly neutralized the IFN-γ-mediated augmentation of CTL induction, although the HLA-A24-restricted CTL activity was still detectable (Fig. 3). On the other hand, the HLA-A24-restricted CTL activity became undetectable when IL-10 was added. This could be due in part to IL-4-mediated inhibition of LAK activity,[30] whereas IL-10 mainly inhibited IFN-γ-mediated CTL induction, but not LAK activity.

HLA-A24 antigen expression of the pretreated APCs

To understand the mechanisms of IFN-γ-mediated augmentation, the expression levels of the HLA-A24 antigens for PBMCs pretreated with various cytokines were measured. The expression levels of the HLA-A24 antigens in the IFN-γ-pretreated PBMCs of heterozygotes were higher than those of the untreated PBMCs, as determined by a comparison of the mean channels of HLA-A24 antigen expression after staining of PBMCs with anti-HLA-A24 mAb (Fig. 4). IL-12 or IFN-α slightly increased the HLA-A24 antigen expression, whereas IL-4, IL-10, and GM-CSF failed to increase the expression. A portion of the results is shown in Fig. 4.

The expression levels of the HLA-A24 antigens for PBMCs pretreated with no cytokine, with IFN-γ alone, with IFN-γ plus IL-4, or with IFN-γ plus IL-10 were also measured to better understand the counteraction mecha-

nisms of IL-4 or IL-10 against the IFN-γ-mediated augmentation of CTL activity through APCs. IFN-γ-induced augmentation of HLA-A24 antigen expression on the surface of PBMCs was abrogated by the addition of either IL-4 or IL-10 to the culture (data not shown).

Cytokines for CTLs We investigated cytokines required for the differentiation and proliferation of CTLs in the presence of the peptide-pulsed irradiated PBMCs acting as APCs by incubation of PBMCs with IL-2 (100 u/ml) alone, IL-2 plus IFN-γ (100 u/ml), IL-2 plus IL-10 (2 pg/ml), or IL-2 plus IL-4 (10 u/ml). Both the *n*-fold increase as compared to the original number of applied cells and the level of IFN-γ production in response to the tumor cells at an E/T ratio of 10 were measured at day 21 (Fig. 5). The PBMCs cultured with IL-2 alone produced significant levels of IFN-γ in an HLA-24-restricted manner

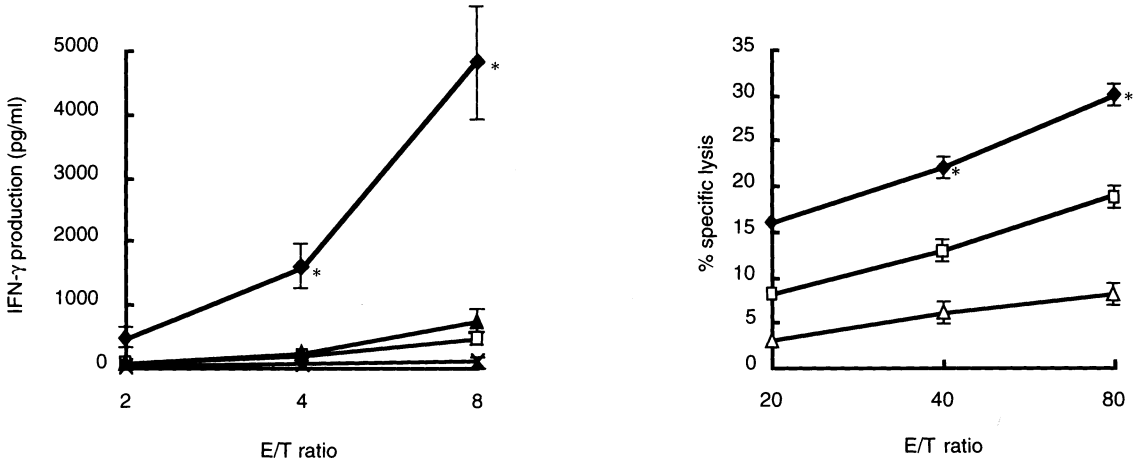

Fig. 2. CTLs induced by the IFN-γ-pretreated APCs. PBMCs at day 21 that had been induced by the peptide loaded on the IFN-γ-pretreated APCs were further expanded in the presence of autologous feeder cells for an additional 20 days followed by testing of their CTL activity both by IFN-γ release and 6-h ^{51}Cr-release assays. Detailed methods for the IFN-γ release assay are described in the legend to Fig. 1. Ef. alone in the figure indicates the IFN-γ production by effector cells alone. Detailed methods for the standard 6-h ^{51}Cr-release have been previously described.[12] Values represent the mean % specific lysis of triplicate assays, and the deviations for the triplicate assays were <10% of the mean values. The phytohemagglutinin (PHA)-blasts used for target cells indicate the autologous PHA-activated T cells. ◆ KE4, □ QG56, ▲ VA13, △ PHA-blasts, × Ef. alone. A two-tailed Student's *t* test was used for the statistical analysis of the IFN-γ production or % specific lysis measured by triplicate assays, and ∗ indicates *P* values of less than 0.05.

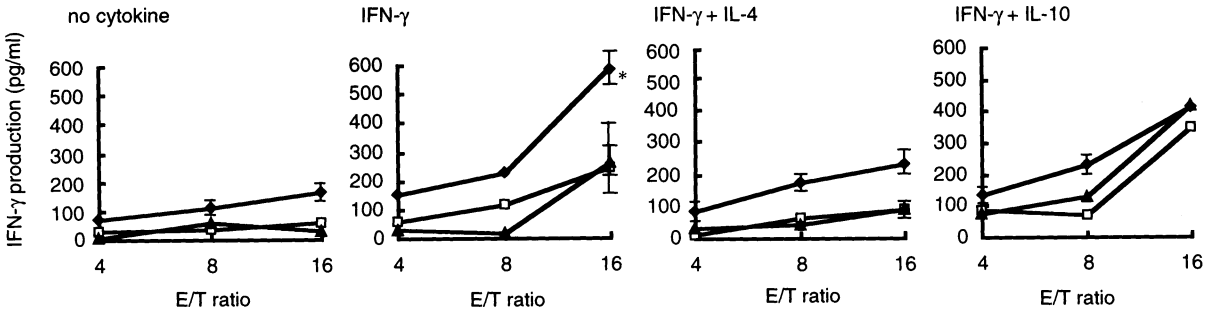

Fig. 3. Inhibition of IFN-γ-mediated augmentation by IL-4 or IL-10. PBMCs of HLA-24 heterozygotes were pretreated with no cytokine or with IFN-γ (100 u/ml) alone, IFN-γ plus IL-4 (10 u/ml), or IFN-γ plus IL-10 (2 pg/ml), and were used as APCs for the induction of CTLs by the peptide using the methods described in the legend to Fig. 1. ◆ KE4, □ QG56, ▲ VA13. A two-tailed Student's *t* test was used for the statistical analysis of the values of IFN-γ production measured by triplicate assays, and ∗ indicates *P* values of less than 0.05 between the level of IFN-γ produced by the CTLs that were stimulated by the peptide-pulsed APCs pretreated with IFN-γ and that by the CTLs stimulated by the APCs pretreated with no cytokine, IFN-γ and IL-4, or IFN-γ and IL-10.

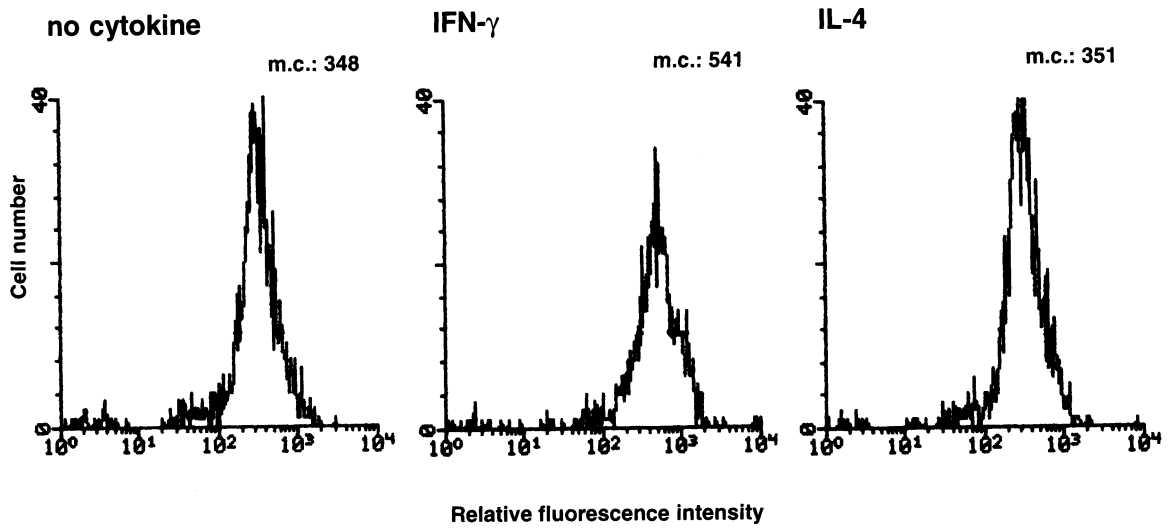

Fig. 4. Expression of HLA-A24 antigens on PBMCs. PBMCs from HLA-A24 heterozygotes were pretreated for 20 h with no cytokine, IFN-γ (100 u/ml), IL-4 (10 u/ml), or the other cytokines, washed three times, and stained with anti-HLA-A24 mAb followed by measurement of their expression on the surface by FACScan. Histograms of representative results are shown. Horizontal or vertical lines indicate the relative number of positive cells or the fluorescence intensity, respectively. m.c. indicates mean channel.

through the recognition of KE4 tumor cells. The addition of IFN-γ or IL-10, however, severely inhibited the IL-2-mediated activation of CTLs. The addition of IL-4 also inhibited the CTL activity. PBMCs in culture with IL-2 alone increased their numbers by 8-fold, whereas the addition of IFN-γ inhibited the IL-2-mediated proliferation of PBMCs, and that of IL-4 or IL-10 also decreased this proliferation.

DCs as APCs The above results suggest that IL-2 was sufficient for the activation of the peptide-induced CTLs until day 21. However, a kinetic study showed that none of the culture conditions tested, including the use of IL-2 alone, supported the further expansion of PBMCs after day 21. The PBMCs after the fourth stimulation at day 21 ceased to proliferate and then rapidly died off before day 28 of culture in the majority of donors tested, including the HLA-A24 homozygotes or heterozygotes of healthy donors as well as of cancer patients (data not shown). We then investigated whether DCs were required as APCs for the further expansion of the peptide-induced mature CTLs. PBMCs from HLA-A24 homozygous or heterozygous healthy donors were cultured with IL-2 in the presence of the peptide-pulsed autologous DCs acting as APCs, followed by measurement of both the CTL proliferation and activity every 7 days until day 35. Whole PBMCs acting as APCs were also tested as a control. Representative results from a homozygote are shown in Fig. 6. The CTL activity became significant as early as day 14 and reached its maximal level at day 21, with both DCs acting as APCs and whole PBMCs. Thereafter, the CTL activity rapidly

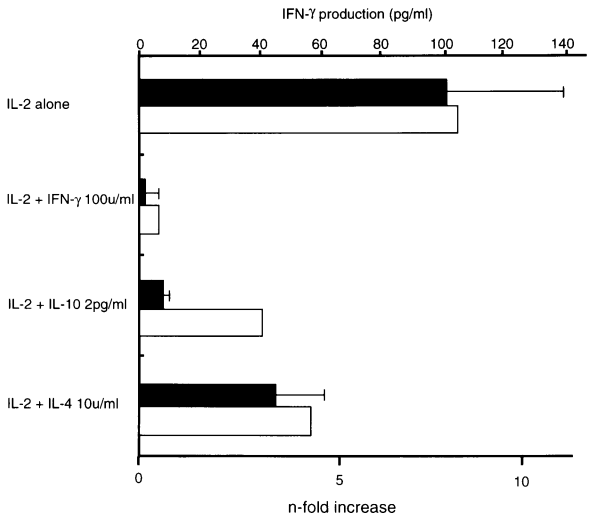

Fig. 5. Cytokines for CTL induction. PBMCs from HLA-A24 homozygotes or heterozygotes were incubated with IL-2 (100 u/ml) alone, IL-2 plus IFN-γ (100 u/ml), IL-2 plus IL-10 (2 pg/ml), or IL-2 plus IL-4 (10 u/ml) in the presence of the peptide-pulsed autologous APCs (2×10^5 cells). Methods for the CTL induction are described in the legend to Fig. 1. Both the *n*-fold increase as compared to the original number of applied cells and the levels of IFN-γ were measured at day 21. Representative results from a heterozygous donor are shown in the figure. The values shown in the figure are the levels of IFN-γ production in response to KE4 tumor cells at an E/T ratio of 10:1 after subtraction of the production in response to either QG56 or VA13 (higher values, between 50 and 100 pg/ml). □ *n*-fold increase, ■ IFN-γ production.

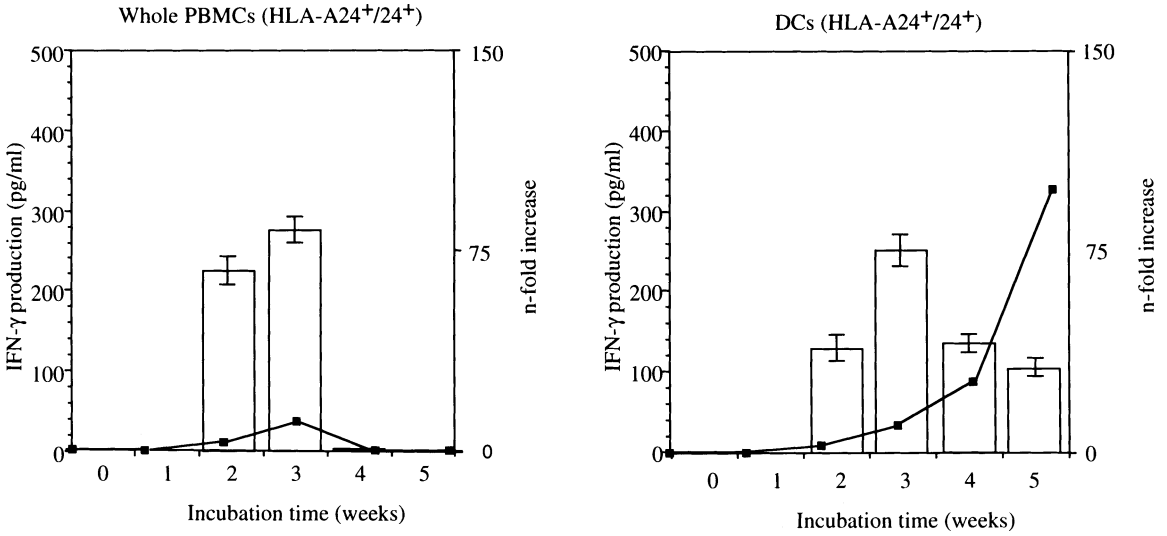

Fig. 6. Kinetic study of CTL induction. PBMCs from an HLA-A24 homozygote were incubated with either irradiated whole PBMCs (left side figure) or DCs acting as APCs (right side figure) that had been pre-incubated with the SART1$^{690-698}$ peptide followed by measurement of both the CTL proliferation and activity every 7 days. The levels of proliferation were obtained by calculating the *n*-fold increase in the originally applied number of PBMCs (2×10^6 cells). The values shown in the figure are the levels of IFN-γ production in response to KE4 tumor cells at an E/T ratio of 10:1 after subtraction of the values of IFN-γ in response to either QG56 or VA13 (higher values, between 50 and 100 pg/ml). ■ *n*-fold increase, □ IFN-γ production.

dropped to undetectable levels in the case of whole PBMCs acting as APCs, whereas it gradually decreased up to day 35 in the case of DCs acting as APCs. The levels of proliferation of effector cells were 2- and 10-fold in either case at days 14 and 21, respectively. Thereafter, the levels of proliferation dropped drastically to 0.5- and 0.2-fold at days 28 and 35, respectively, in the case of whole PBMCs acting as APCs, whereas they consistently increased up to >100-fold at day 35 in the case of DCs. These results suggest that the use of DCs as APCs is required for further expansion of mature peptide-induced CTLs.

DISCUSSION

We have shown in this study that IFN-γ plays an important role in the activation of APCs for the CTL induction by a peptide of a tumor-rejection antigen, in agreement with previously reported results.[31–33] Pretreatment of APCs with IFN-α also increased the subsequent CTL induction. IL-4 and IL-10 rather decreased it, and further inhibited the IFN-γ-mediated augmentation. The results regarding IL-10-mediated inhibition are consistent with those previously reported.[16, 17, 19–21] In contrast, conflicting results have been obtained with regard to the cytokines required for activation of CTLs *in vitro*. We have found in this study that IL-2 is sufficient for the initial culture of CTLs, and that the addition of other cytokines actually inhibits the IL-2-mediated activation of CTLs. In contrast, IL-4 or IFN-γ seems to be necessary or at least to augment the IL-2-mediated activation of CTLs in many other systems.[17, 22–24] Although the mechanisms involved in this discrepancy are unclear at present, the antigens used in the relevant experiments were different from each other. We used a peptide from a self antigen preferentially expressed in cancer cells, while others have used peptides from other tumor-rejection antigens, exogenous antigens, or tumor cells alone.

We have previously reported that the CTL activity induced by the SART1$^{690-698}$ peptide in PBMCs from HLA-A24 homozygotes is about 2 to 4 times higher than that from HLA-A24 heterozygotes in association with higher expression levels of HLA-A24 antigens in the homozygotes.[13] In this study, we showed that expression levels of HLA-A24 antigens in the IFN-γ pretreated PBMCs of heterozygotes are significantly higher than those of untreated PBMCs. In contrast to heterozygotes, the pretreatment of APCs with the IFN-γ of PBMCs from HLA-A24 homozygotes fails to augment the peptide-induced CTL activity, although the IFN-γ treatment increases the expression of HLA-A24 antigens (data not shown). These results suggest that IFN-γ increases the HLA-A24 antigen expression to an optimal level for CTL induction in the case of heterozygotes, while the levels of HLA-A24 antigens in the untreated APCs of homozygotes are sufficient for the induction of CTLs. The frequency of heterozygotes of HLA-A alleles is genetically much higher

than that of homozygotes. Therefore, the above results should be important in the development of peptide-based specific immunotherapy for cancer patients.

PBMCs died off when stimulated at day 21 by the peptide loaded onto whole PBMCs acting as APCs. DNA ladder formation was observed in these dead PBMCs (data not shown). These results suggest that re-stimulation of the mature CTLs at a higher dose of peptide leads to apoptosis of the cells, which is in agreement with previous observations.[34] Instead of whole PBMCs acting as APCs, DCs were needed for the further expansion of the mature CTLs in culture with IL-2. Although the mechanisms involved in this phenomenon have not yet been determined in detail, the use of DCs as APCs will be necessary to expand the SART1-induced CTLs on a large scale *in vitro* for adoptive cellular therapy.

In contrast to this SART1$^{690-698}$ peptide, other peptides derived from tumor-rejection antigens do not always require DCs for the expansion of the mature CTLs.[12, 35] In terms of what was tested in our laboratories, these peptides include the other SART1-derived peptides capable of inducing HLA-26-restricted CTLs at positions 736–744[12] and the MART1-derived peptides capable of inducing HLA-A2-restricted CTLs.[35] Although the mechanisms responsible for this phenomenon are also presently unclear, one reason for the discrepancy might be the cross-reactivity of the *Saccharomyces cerevisiae*-derived nonapeptide (EYRGFTPMF) to this peptide, as reported previously.[13] This fungus-derived exogenous peptide shares seven amino acids with the SART1$^{690-698}$ peptide and has activity inducing HLA-A24-restricted and tumor-specific CTLs. Human beings have a strong cell-mediated immunity against fungus.[36] *S. cerevisiae* is used in many different fermented foods and beverages, and the human body could therefore be exposed to this membrane protein on a daily base. Therefore, a relatively high number of CTL precursors recognizing this SART1$^{690-698}$ peptide may be in circulation due to the antigenic mimicry between these peptides. Indeed, the CTL precursor frequencies after the third stimulation by this SART1$^{690-698}$ peptide are >1/200, which is very high.[13] Further studies are necessary to clarify the mechanisms involved in this discrepancy.

The CTL activity expanded with DCs reached a maximal level at day 21. The decrease after day 21 could be due to the decrease in $CD3^+CD8^+$ T cells in association with the increase in $CD3^+CD4^+$ T cells. Rissoan *et al*. have recently reported the functional heterogeneity of DCs.[37] A certain type of DCs seems to induce T helper type 1 cells, while the other type of DCs induces T helper type 2 cells. Therefore, further studies of the functional heterogeneity of the DCs used in this study are needed.

We have shown in this study that IFN-γ and IL-2 are important for APC-mediated peptide presentation and for the activation of CTLs, respectively. GM-CSF and IL-4 are needed for the activation of DCs, which in turn are critical for the further expansion of the mature CTLs. These results are important in allowing for a better understanding of the cellular and molecular basis of tumor-specific immunity, and for the development of peptide-based specific immunotherapy.

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