TGF-ß superfamily enhances the antigen-induced IFN-γ production by effector/memory CD8+ T cells

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Abstract. Ag-specific effector/memory CD8+ T cells play an important role, not only in viral eradication, but also in T cell-mediated tumor rejection. Increasing evidence suggests that TGF-ß plays a critical role in the tumor escape from immune surveillance. Although it is known that TGF-ß directly suppresses the activation of naïve T cells, the direct effects of TGF-ß on effector/memory CD8+ T cells have not yet been fully investigated. The present study evaluated the effect of TGF-ß on effector/memory CD8+ T cells using Ag-specific, mouse-derived, effector/memory CD8+ T cell clones, designated as 6C2. Notably, pretreatment of TGF-ß1 caused an approximate 100% enhancement of IFN-γ production in response to peptide stimulation. TGFß-RI kinase inhibitor reduced the enhancement of peptide-induced IFN-γ secretion by TGF-ß1. In addition, either Activin-A or BMP-4 pretreatment caused an approximate 100% enhancement of IFN-γ production in the peptide effect. These results suggest a contradictory effect of the TGF-ß superfamily on effector/memory CD8+ T cells.

Introduction

Ag-specific effector/memory CD8+ T cells play an important role, not only in viral eradication, but also in T cell-mediated tumor rejection through their ability to produce various factors against certain pathogens and tumor cells (1). Tumor infiltrating lymphocytes (TILs) are found in different types of tumors, and their presence has been correlated with a more favorable outcome in patients with various types of cancer (2-5). Therefore, for over a century, a significant investment towards developing antitumor effector/memory CD8+ T cells by cancer vaccines has been ongoing. Despite much effort, vaccination approaches to date can at best induce objective cancer regression in only a small minority of patients with solid cancers (6). These difficulties in relation to cancer immunotherapy are explained, in part, owing to the induction of anergy in TILs by the tumor microenvironment (7). Among the various mechanisms that are employed by the tumor microenvironment, TGF-ß is recognized to have a central role in the induction of T cell anergy (8,9). There is accumulating evidence that TGF-ß suppresses the immune system to evade host immunity through generation of regulatory T cells, suppression of NK and NKT cells and inhibition of antigen presentation on antigen-presenting cells (8-11). In addition, TGF-ß controls T cell homeostasis by directly inhibiting both T cell proliferation and activation in naïve CD4+ and naïve CD8+ T cells (8). The mechanisms by which TGF-ß inhibits naïve T cell proliferation and activation have been well investigated (8,12,13). However, it has been reported that the majority of TILs have an effector/memory phenotype (14), and, more importantly, the effects of TGF-ß on effector/memory CD8+ T cells have not yet been fully investigated. In addition, it has been reported that TGF-ß has immunostimulatory effects through the generation of Th17 cells, a T cell subset crucial for the induction of autoimmune tissue injury (15). Thus, it would be simplistic to suggest that TGF-ß is always immunosuppressive. Recently, adoptive cell transfer (ACT), the infusion of *ex vivo* expanded tumor-reactive effector/memory CD8+ T cells to cancer patients, has been reported to be an effective therapy for cancer regression (1). In ACT, effector/memory CD8+ T cells can be expanded without any influence of the inhibitory effect for T cell proliferation by TGF-ß. This means that transferred effector/memory CD8+ T cells, generated in the absence of TGF-ß, encounter TGF-ß in the tumor microenvironment for the first time in ACT therapy. Therefore, it is necessary to clarify the direct effects of TGF-ß on effector/memory CD8+ T cells.

In this study, we used mouse-derived, Ag-specific-effector/memory CD8+ CTL clones, designated as 6C2 cells, in order to evaluated the effects of TGF-ß on effector/memory CD8+ T cells. 6C2 cells were generated as hepatitis B surface antigen (HBsAg)-specific CTL clones in 1993 (16).

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Since then, they have been maintained and used to elicit acute hepatitis in hepatitis B virus (HVB) transgenic mice (16,17). 6C2 cells readily proliferate and demonstrate effective cytotoxicity in response to Ag stimulation (16,17). Because of these characteristics, these cells are defined as effector/memory $CD8^+$ T cells. We also evaluated the effects of the TGF-ß superfamily of cytokines, Activin-A and BMP-4 (18,19) on these cells. Notably, 6C2 cells were not only resistant to the inhibitory effect of TGF-ß1 but also showed enhanced IFN-γ production upon pretreatment with TGF-ß1. TGFß-RI kinase inhibitor reduced the enhancement of peptide-induced IFN-γ secretion by TGF-ß1. These results suggest that 6C2 cells may have a functional TGF-ß receptor, and signals through the TGF-ß receptors may contribute to the enhancement of IFN-γ production. In addition, pretreatment with either Activin-A or BMP-4, members of the TGF-ß superfamily, resulted in an approximate 100% enhancement of IFN-γ production in the peptide effect. These results, in contrast to the inhibitory effect of TGF-ß in naïve cells, suggest a divergent effect of the TGF-ß superfamily on effector/memory $CD8^+$ T cells.

Materials and methods

Materials. Mouse IFN-γ, TNF-α and IL-2 ELISA kits, and recombinant TGF-ß1, Activin-A and BMP-4 were purchased from R&D Systems, Inc. (Minneapolis, MN). The mouse granzyme B ELISA kit, anti-mouse CD27-FITC mAb, Armenian hamster IgG-FITC mAb, CD28-PE mAb and Golden Syrian hamster IgG-PE mAb, were purchased from e-Bioscience (San Diego, CA). Anti-mouse CD8-FITC mAb and CD8-PE mAb were purchased from BD Pharmingen (San Diego, CA). TGFß-RI kinase inhibitor was purchased from Calbiochem (La Jolla, CA). The Cytotoxicity Detection Kit^{Plus} (LDH) was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

Cell culture. HBsAg-specific, mouse-derived, H-2d-restricted, $CD8^+$ CTL clone cells designated 6C2, which recognized an epitope (IPQSLDSWWTSL) located between residues 28-39 of HBsAg, P815 cells (H-2d) and cell lines designated P815-env, which were transfected with the HBV large envelope, were generously provided by Dr Kiminori Kimura (Tokyo University, Tokyo, Japan). 6C2 cells ($1.5x10^5$/well) were cultured with mitomycin C-treated P815-env transfectants ($1.5x10^5$/well) in complete RPMI-1640 culture medium containing streptomycin (100 mg/ml), penicillin (100 U/ml), 20% FCS, 2-mercaptoethanol ($5x10^{-5}$ M), and 5.0% EL-4 supernatant, in 24-well plates at 37˚C in a humidified atmosphere of 5% CO_2/95% air. Thereafter, the 6C2 cells were restimulated and maintained weekly.

Synthetic peptides. HBsAg 28-39 peptide (IPQSLDSWWTSL) (HBs peptide) was synthesized and purchased from Peptide-Institute Inc. (Minoh, Japan). The peptides were dissolved in 100% DMSO at a concentration of 1 mg/ml and stored at -20˚C until used. The stored peptide was diluted with RPMI-1640 and used for the experiments. The maximum concentration of DMSO was 0.01%, which did not affect the assay for either ELISA or the FACS analysis.

ELISA assay. The levels of various cytokines in the medium were measured by means of the respective ELISA kits according to the manufacturers' instructions. The assay kits are able to detect mouse IFN-γ, TNF-α, IL-2 and granzyme B in the range between 9.4-600, 23.4-1500, 15.6-1000 and 19.5-5000 pg/ml, respectively. When the samples generated values higher than the maximum detection limits, they were adequately diluted with calibrator diluent provided with the kit and re-assayed.

Cytotoxicity assay. The cytolytic activity of 6C2 cells was measured by means of the Cytotoxicity Detection Kit^{Plus} (LDH) according to the manufacturer's instructions. Based on the preliminary experiment, 6C2 cells and target cells (P815; $1.0x10^5$ cells/well) were coincubated at an E:T ratio of 10:1 in the presence of diminishing concentrations of HBsAg peptide (0.1-300 ng/ml) for 4 h (96-well U-bottom plates), and LDH activity released from damaged cells was measured using a microplate reader at 492 nm. All experiments were performed in triplicate.

Flow cytometry. Cultured cells were collected at the indicated time points and washed twice by PBS. The cells were incubated for 15 min at room temperature with the following fluorescent-labeled conjugated mAbs: anti-CD8-FITC, anti-CD8-PE, anti-CD27-FITC or isotype control (Armenian hamster IgG)-FITC, and anti-CD28-PE or isotype control (Golden Syrian hamster IgG)-PE. The samples were acquired using a FACScalibur™ Flow Cytometer (BD Immunocytometry Systems, San Jose, CA), and the data were analyzed using the CellQuest™ software.

Statistical analysis. Significant differences between the groups were statistically evaluated by means of the non-parametric Mann-Whitney U test using Statview 4.1 (Abacus Concepts). A value of $p<0.05$ was considered to be statistically significant. All values in the figures were expressed as the mean ± SE.

Results

Functional and phenotypic characteristics of HBsAg-specific CTL. To evaluate the functional characteristics of 6C2 cells, the cytotoxicity assay was performed to confirm the functional avidity of the CTL clone. 6C2 cells exhibited half-maximal lysis when the dose of the peptide ranged between 3 and 10 ng/ml (2.1 and 7.0 nM; Fig. 1A). These results are consistent with the findings of a previous report by Ando *et al* (16). To confirm the further functional characteristics of the 6C2 cells, the cytokine production (IFN-γ, TNF-α, IL-2 and granzyme B) in response to Ag stimulation by the peptide (HBsAg 28-39) was evaluated. Ag stimulation elicited IFN-γ, TNF-α and granzyme B secretion in a dose-dependent manner in the range between 0.1 and 300 ng/ml (Fig. 1B-D). In contrast, Ag stimulation did not elicit any IL-2 secretion in the 6C2 cells (data not shown).

Since expression of CD28 and CD27 is particularly useful for distinguishing between subsets of differentiated $CD8^+$ T cells (21,22), the cell surface expression of CD27 and CD28 was examined by flow cytometry at 2, 4 and 7 days after the

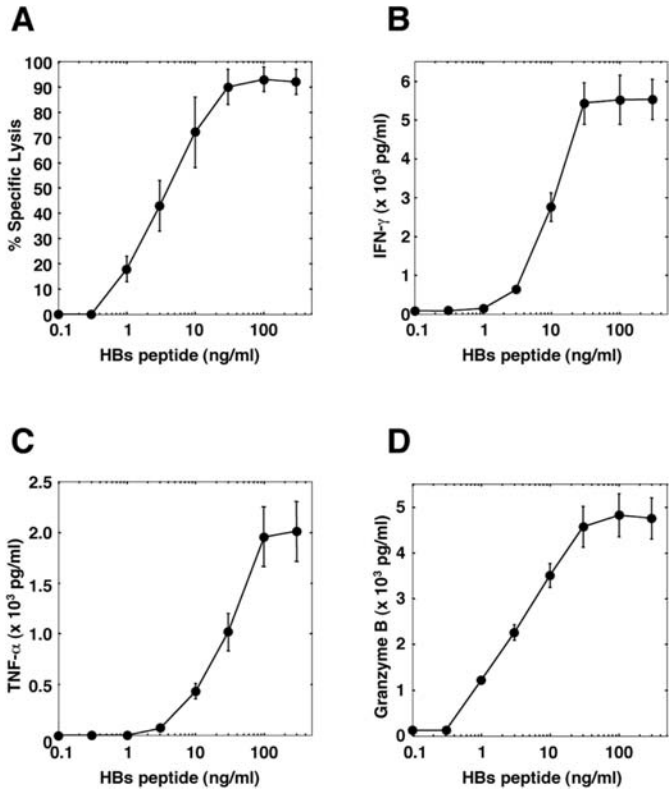

Figure 1. Functional characteristics of HBsAg-specific CTL clone, 6C2. The cultured cells were collected 4 days after restimulation and then washed with RPMI containing 0.3% FCS and used for subsequent experiments. (A) Cytolytic activity of 6C2 cell was measured by means of the Cytotoxicity Detection Kit^{Plus} (LDH). 6C2 and target cells (P815; 1.0x10^5 cells/well) were coincubated at an E:T ratio of 10:1 in the presence of diminishing concentrations of HBs peptide (0.1-300 ng/ml) for 4 h (96-well U-bottom plates), and LDH activity released from damaged cells was measured using a microplate reader at 492 nm. (B, C and D) The cells were stimulated by the various doses of HBs peptide (0.1-300 ng/ml) for 4 h in 24-well plates. The levels of (B) IFN-γ, (C) TNF-α and (D) granzyme B in the medium were measured by means of the respective ELISA kits. All experiments were performed in triplicate.

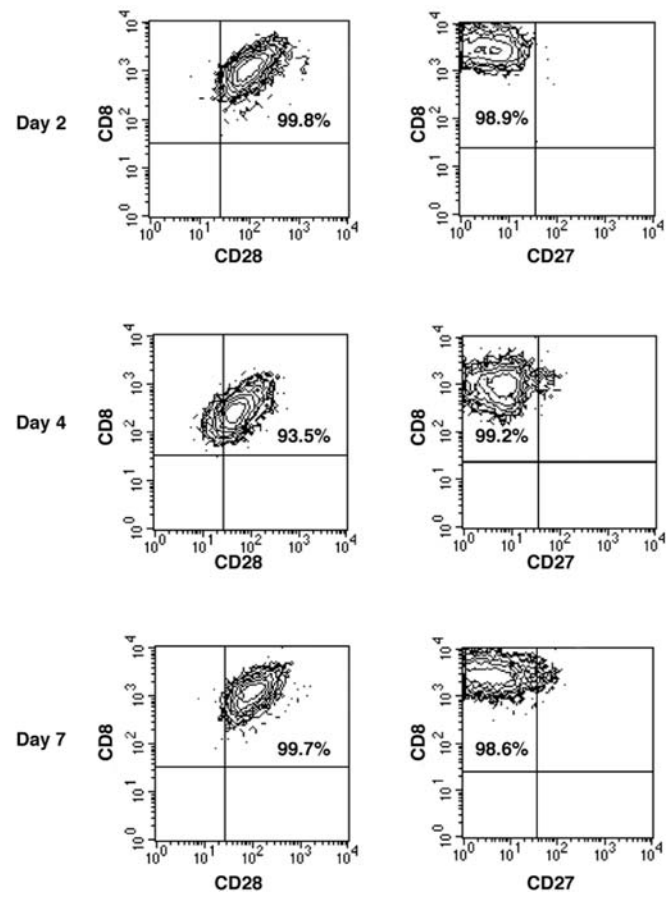

Figure 2. Phenotypic characteristics of HBsAg-specific CTL clone, 6C2. The cultured cells were collected each day after restimulation, and then washed with RPMI containing 0.3% FCS and used for subsequent experiments. 6C2 cells were stained simultaneously with either anti-CD8-FITC mAb and anti-CD28-PE mAb, or anti-CD28-FITC mAb and anti-CD8-PE mAb. The inset number indicates the percentage of CD8$^+$/CD28$^+$ cells or CD8$^+$/CD-27$^-$ cells of 6C2 cells.

restimulation (Fig. 2) to evaluate the phenotypic characteristics of the 6C2 cells. This showed that 6C2 cells constitutively expressed CD8 and CD28, though the expression levels of these markers decreased slightly at day 4. In contrast, 6C2 cells were constitutively CD27$^-$ cells. Taken together, these results suggest that 6C2 cells are CD8$^+$ T cells with the CD27$^-$ CD28$^+$ phenotype of effector/memory cells.

Effect of TGF-ß1 on the Ag-induced cytokine production by 6C2 cells. To clarify the effect of TGF-ß1 on the cytokine production by 6C2 cells in response to Ag stimulation, the effect of TGF-ß1 on Ag-induced IFN-γ secretion was evaluated. The results showed that TGF-ß1 (10 ng/ml), which alone failed to affect the IFN-γ secretion (data not shown), significantly enhanced the Ag-induced IFN-γ secretion when the dose of the peptide was between 3 and 10 ng/ml (Fig. 3A). In addition, TGF-ß1 amplified Ag-induced (3 ng/ml) IFN-γ secretion in a dose-dependent manner in the range between 1 and 10 ng/ml (Fig. 3B). TGF-ß1 at 10 ng/ml resulted in ~100% enhancement of IFN-γ secretion in response to the Ag stimulation (Fig. 3A and B). In contrast, TGF-ß1 (10 ng/ml) failed to affect Ag-induced IFN-γ secretion when the peptide dose was >30 ng/ml, at which dose the effect of Ag-induced IFN-γ secretion reached a plateau (Fig. 3A). Similarly, TGF-ß1 (10 ng/ml) enhanced Ag-induced TNF-α secretion when the dose of the peptide was between 3 and 10 ng/ml, but not >30 ng/ml (Fig. 4A). TGF-ß1 at 10 ng/ml caused ~100% enhancement of TNF-α secretion in response to the Ag stimulation (Fig. 4A). Notably, TGF-ß1 (10 ng/ml) failed to affect Ag-induced granzyme B secretion at any doses of the peptide (Fig. 4B). In addition, TGF-ß1 (10 ng/ml) had no effect on the cytotoxic activity of 6C2 cells for target cells (P815 cells) pulsed with 3 and 10 ng/ml of peptide (HBsAg 28-39; data not shown). To confirm whether TGF-ß1 enhanced Ag-induced IFN-γ and TNF-α production by 6C2 cells, intracellular IFN-γ and TNF-α staining was performed. This demonstrated that TGF-ß1 truly enhanced Ag-induced IFN-γ production (5.18±1.4% for 3 ng/ml peptide alone vs. 8.88±2.5% for 2 h pretreatment of TGF-ß1 with 3 ng/ml peptide, as measured during the 4-h stimulation; Fig. 5A). Similarly, TGF-ß1 truly up-regulated Ag-induced TNF-α production (3.54±1.1% for 3 ng/ml peptide alone vs. 6.39±2.3% for 2-h pretreatment of TGF-ß1 with 3 ng/ml peptide, as measured during the 4-h stimulation; Fig. 5B).

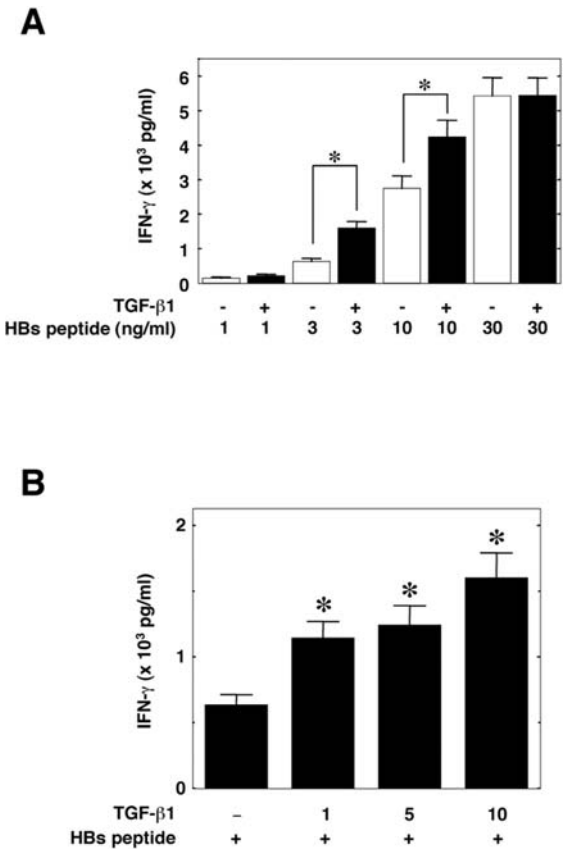

Figure 3. Effect of TGF-ß1 on the Ag-induced IFN-γ secretion by 6C2 cells. The cultured cells were collected 4 days after restimulation, and then washed with RPMI containing 0.3% FCS and used for subsequent experiments. (A) The cells were pretreated with 10 ng/ml of TGF-ß1 or vehicle for 2 h, and then stimulated by the various doses of HBs peptide for 4 h in 24-well plates. (B) The cells were pretreated with various doses of TGF-ß1 or vehicle for 2 h, and then stimulated by 3 ng/ml of HBs peptide for 4 h in 24-well plates. The levels of IFN-γ in the medium were measured by means of the ELISA kits. All experiments were performed in triplicate. *p<0.05 as compared to the value of HBs peptide alone.

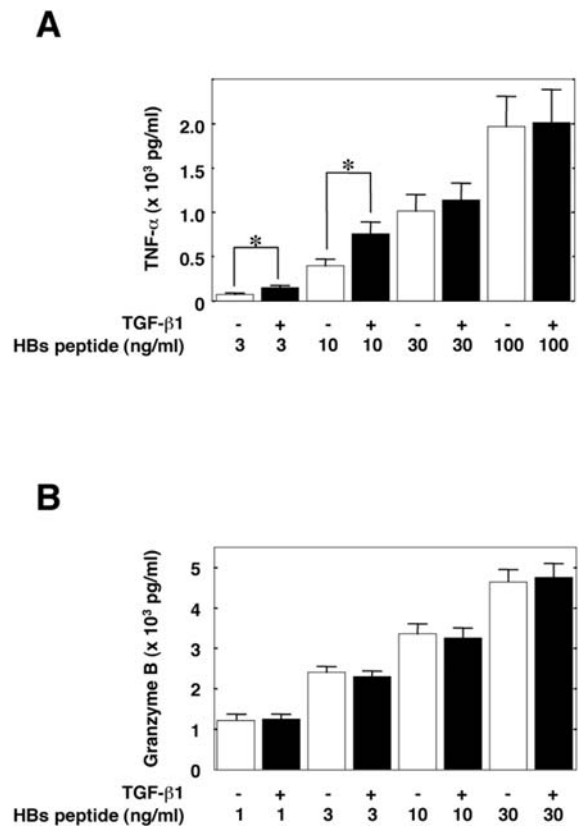

Figure 4. Effect of TGF-ß1 on the Ag-induced TNF-α and granzyme B secretion by 6C2 cells. The cultured cells were collected 4 days after restimulation, and then washed with RPMI containing 0.3% FCS and used for subsequent experiments. The cells were pretreated with 10 ng/ml of TGF-ß1 or vehicle for 2 h, and then stimulated by the various doses of HBs peptide for 4 h in 24-well plates. The levels of (A) TNF-α and (B) granzyme B in the medium were measured by means of the respective ELISA kits. All experiments were performed in triplicate. *p<0.05.

Effect of TGFβ-RI kinase inhibitor on the enhancement of Ag-induced IFN-γ secretion by TGF-β1 in 6C2 cells. The TGF-ß ligand initiates signaling by binding to and bringing together TGF-ß receptor type I (TGFß-RI) and type II (TGFß-RII), receptor serine/threonine kinases, on the cell surface (18,19). This allows TGFß-RII to phosphorylate the TGFß-RI kinase domain, which then propagates the signal through phosphorylation of the Smad proteins (18,19). To evaluate whether the up-regulatory effect of TGF-ß1 on the Ag-induced IFN-γ secretion is mediated by the TGF-ß receptors in 6C2 cells, the effect of TGFß-RI kinase inhibitor on the enhancement of Ag-induced IFN-γ secretion by TGF-ß1 was analyzed. This showed that TGFß-RI kinase inhibitor reduced the enhancement of Ag-induced IFN-γ secretion by TGF-ß1 (Fig. 6). These results suggest that TGF-ß1 enhances the Ag-induced IFN-γ secretion through its TGF-ß receptors.

Effect of the TGF-β superfamily on the Ag-induced IFN-γ secretion by 6C2 cells. The TGF-ß superfamily of cytokines contains two subfamilies, the TGF-ß/Activin/Nodal subfamily and the BMP (bone morphogenetic protein)/GDF (growth and differentiation factor)/MIS (Müllerian inhibiting substance) subfamily, defined by the sequence similarity and the specific signaling pathways that they activate (18,19). To clarify whether other TGF-ß subfamilies can enhance the Ag-induced IFN-γ secretion by 6C2 cells, the effect of Activin-A or BMP-4 on the Ag-induced IFN-γ secretion was investigated. The results showed that Activin-A, which alone failed to affect IFN-γ secretion (data not shown), significantly enhanced Ag-induced IFN-γ secretion by 6C2 cells, Activin-A at 30 ng/ml caused ~100% enhancement of IFN-γ secretion in response to the Ag stimulation (Fig. 7A). The enhancement level of Activin-A in the Ag-induced IFN-γ secretion was similar to the TGF-ß1 effect. In addition, BMP-4, which alone failed to affect IFN-γ secretion (data not shown), significantly amplified Ag-induced IFN-γ secretion; BMP-4 at 100 ng/ml resulted in ~100% enhancement of IFN-γ secretion in response to the Ag stimulation (Fig. 7B). These results suggest that the TGF-ß superfamily of cytokines have similar effects on the enhancement of Ag-induced IFN-γ secretion through respective receptors by effector/memory CTL.

Discussion

Analogous to memory B lymphocytes (20), memory $CD8^+$ T cells are heterogeneous with respect to phenotypic markers,

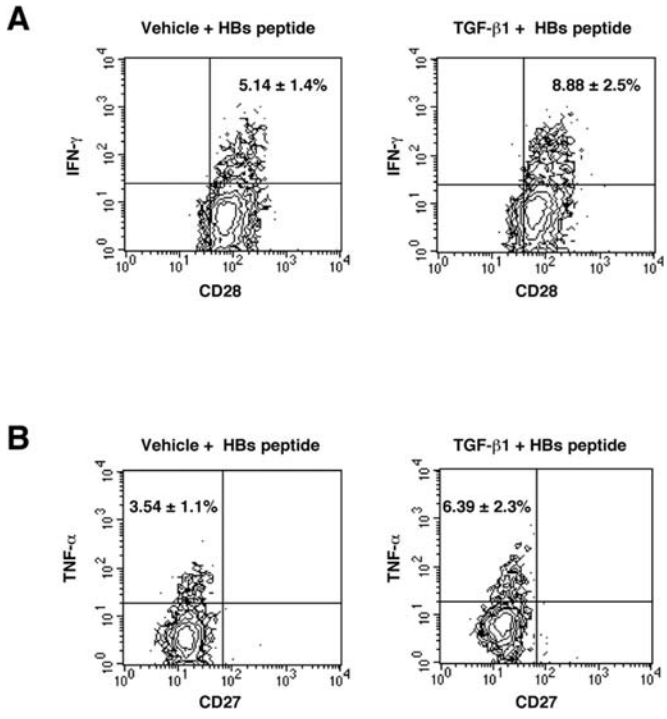

Figure 5. Effect of TGF-ß1 on the Ag-induced IFN-γ and TNF-α production by 6C2 cells. The cultured cells were collected 4 days after restimulation, and then washed with RPMI containing 0.3% FCS and used for subsequent experiments. The cells were pretreated with 10 ng/ml of TGF-ß1 or vehicle for 2 h, and then stimulated by 3 ng/ml of HBs peptide for 4 h in 24-well plates. The cells were stained either with the (A) anti-CD28-PE mAb and anti-IFN-γ-APC mAb, or (B) anti-CD27-FITC mAb and anti-TNF-α-PE mAb. The inset number indicates the percentage of (A) $CD28^+/IFN\text{-}\gamma^+$ cells, or (B) $CD27^-/TNF\text{-}\alpha^+$ cells of 6C2 cells. All experiments were performed in triplicate.

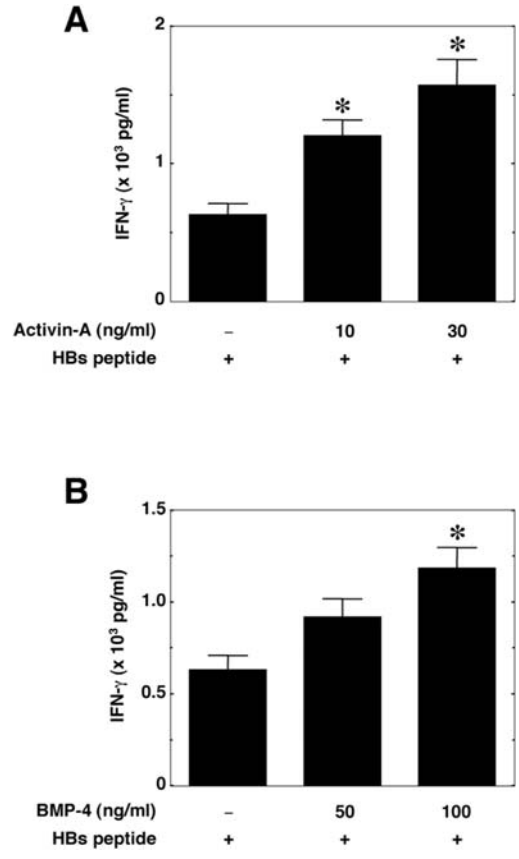

Figure 7. Effects of Activin-A or BMP-4 on the Ag-induced IFN-γ secretion by 6C2 cells. The cultured cells were collected 4 days after restimulation, and then washed with RPMI containing 0.3% FCS and used for subsequent experiments. The cells were pretreated with various doses of (A) Activin-A, (B) BMP-4, or vehicle for 2 h, and then stimulated by 3 ng/ml of HBs peptide for 4 h in 24-well plates. The levels of IFN-γ in the medium were measured by means of the ELISA kits. All experiments were performed in triplicate. *p<0.05 as compared to the value of HBs peptide alone.

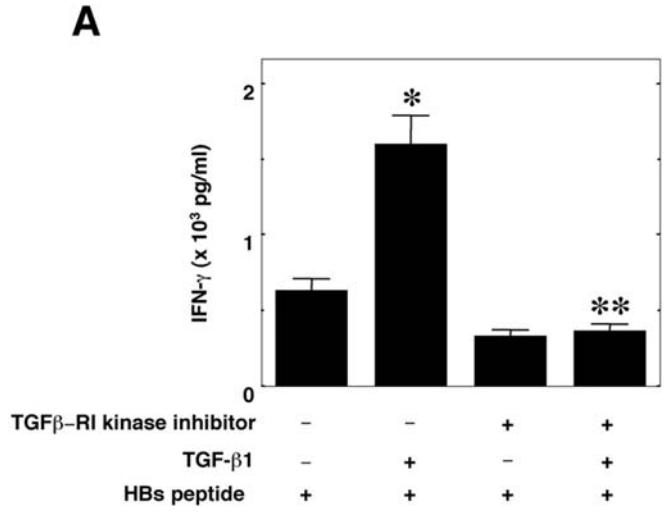

Figure 6. Effect of TGFß-RI kinase inhibitor on the enhancement of Ag-induced IFN-γ secretion by TGF-ß1 in 6C2 cells. The cultured cells were collected 4 days after restimulation, and then washed with RPMI containing 0.3% FCS and used for subsequent experiments. The cells were pretreated with various doses of TGF-ß1 or vehicle for 2 h, and then stimulated by 3 ng/ml of HBs peptide for 4 h in 24-well plates. The levels of IFN-γ in the medium were measured by means of the ELISA kits. All experiments were performed in triplicate. *p<0.05 as compared to the value of HBs peptide alone. **p<0.05 as compared to the value of TGF-ß1 with HBs peptide (without TGFß-RI kinase inhibitor).

effector functions and tissue-homing capabilities. One model to classify memory T cells is to divide the populations by the expression of CD27 and CD28. A linear differentiation model has been proposed for Ag-experienced T cells with the $CD27^+$ $CD28^+$ 'early' and the $CD27^+$ $CD28^-$ 'intermediate' stages expressing low or intermediate levels of perforin, respectively; a $CD27^-$ $CD28^-$ 'late' stage is thought to represent fully differentiated effector T cells (21,22) because of their high perforin content, ability to produce a large amount of IFN-γ after stimulation, and direct *ex vivo* mediation of cytotoxicity (21-23). In contrast, the characteristics of $CD27^-$ $CD28^+$ memory CD8 T cells are still poorly understood. The present study showed that 6C2 cells, HBsAg-specific CTL clones, are $CD8^+$ T cells with the $CD27^-$ $CD28^+$ phenotype. Virus-specific $CD8^+$ T cells with a $CD27^-$ $CD28^+$ phenotype, known to have higher proliferative ability (21,24), are a rare subset especially in both the acute and chronic phases of persistent human viral infections (22). Virus-specific $CD27^-$ $CD28^+$ $CD8^+$ T cells also play a key protective role in ensuring stable control of viral replication (25). According to these findings, 6C2 cells might be a rare subset of effector/memory $CD8^+$ T cells. In order to evaluate whether these phenotypical characteristics can explain the enhancing effect of IFN-γ

production by the TGF superfamily in these cells, further study in relation to the phenotypical characteristics of the several types of effector/memory CD8+ T cell clones should be carried out in the future.

In the present study, TGF-ß1 enhanced Ag-induced IFN-γ and TNF-α secretion by effector/memory CD8+ T cell clones, 6C2 cells. Although Thomas and Massague reported that TGF-ß directly suppressed CTL function (12), they analyzed the effect of TGF-ß mostly on the induction of CTL; in other words, they added TGF-ß simultaneously when naïve cells were activated by anti-CD3 or antigen. Also, in their *ex vivo* study of antigen-specific T cells, they compared CTLs generated under a normal condition and CTLs generated under neutralization of TGF-ß (12). In these experiments, they did not evaluate the effects of TGF-ß on the equally established effector/memory cells. In the present study, we evaluated the effects of TGF-ß on effector/memory CD8+ T cell clone. These differences in the experimental design may reflect the discrepancy between the former study by Thomas and Massague (12) and our present study. Since TGF-ß1 enhanced the Ag-induced IFN-γ and TNF-α secretion by 6C2 cells, these results suggest that 6C2 cells are, not only resistant to the inhibitory effect of TGF-ß1, but also showed enhanced IFN-γ production upon pretreatment with TGF-ß1. Furthermore, we showed that the TGFß-RI kinase inhibitor reduced the enhancement of peptide-induced IFN-γ secretion by TGF-ß1. Collectively, these results suggest that 6C2 might have functional TGF-ß receptors, and signals through the TGF-ß receptors may contribute to the enhancement of IFN-γ production. Since IFN-γ is known to play a central role in the induction of host defenses against tumors (26,27), and tumor cells may escape immune recognition by down-regulating components of the Ag presentation pathway (28), it is crucial that TGF-ß1 enhances IFN-γ secretion by effector/memory cells at a low Ag concentration (Figs. 3 and 4). Taken together, it is likely that the effective induction of TGF-ß-resistant effector/memory CD8+ T cells is beneficial for the induction of host defenses against tumors.

In the present study, TGF-ß1 failed to affect Ag-induced granzyme B secretion and cytotoxic activity by 6C2 cells. Activated CD8+ CTLs typically utilize two major contact-dependent pathways to kill target cells (29). One is the granule exocytosis pathway which mediates the release of membrane-pore-forming proteins such as perforin and granzyme B. The second is the secretion of soluble mediators such as IFN-γ, TNF-α and the FAS ligand to induce target cell cytotoxicity. The differential mechanisms of these two pathways may result in the differential effects of TGF-ß1 on granzyme B and IFN-γ secretion in response to Ag stimulation. Based on our findings, TGF-ß1 signaling might be involved in the production or secretion of soluble mediators such as IFN-γ and TNF-α, but not in the production or exocytosis of granule contents such as perforin and granzyme B by effector CD8+ T cells.

TGF-ß superfamily signaling involves two distinct pathways, Smad 2/Smad 3/Smad 4 and Smad 1/Smad 5/Smad 8/Smad 4 (18,19). In the present study, TGF-ß1, Activin-A and BMP-4 enhanced the Ag-induced IFN-γ secretion by 6C2 cells. These results lead us to speculate that Smad 4, a common-partner Smad, plays a crucial role in the TGF-ß superfamily-mediated enhancement of Ag-induced IFN-γ secretion in 6C2 cells. Unfortunately, the present study did not confirm the role of Smad 4 because of the inability to effectively transfect Smad 4 siRNA in 6C2 cells. Further investigation is necessary to clarify the precise mechanisms of the TGF-ß superfamily-mediated enhancement of Ag-induced IFN-γ secretion in these cells.

In conclusion, the present study evaluated the effects of TGF-ß on effector/memory CD8+ T cells using Ag-specific, mouse-derived, effector/memory CD8+ T cell clone, 6C2. Our study, in contrast to previous reports concerning the inhibitory effect of TGF-ß on naïve cells, demonstrated the immunostimulatory effects of the TGF-ß superfamily on effector/memory CD8+ T cells. These findings propose an opposing view concerning the direct effects of TGF-ß on effector/memory CD8+ T cells. In the future, further study of several types of effector/memory CD8+ T cell clones is required to evaluate whether phenotypical characteristics can explain the enhancing effect of IFN-γ production by the TGF superfamily. Also, further research regarding signaling via TGF-receptor families is required to clarify the mechanisms of the enhancing effect of IFN-γ production by the TGF superfamily in these cells.

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