Induction of a distinct CD8 Tnc17 subset by transforming growth factor-β and interleukin-6

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Abstract: Cross-talk between TGF-β and IL-6 has been shown to direct the differentiation of $CD4^+$ cells into special IL-17-secreting cells, which are termed Th17 cells. In this study, we demonstrated that TGF-β and IL-6 could stimulate $CD8^+$ cells to differentiate into noncytotoxic, IL-17-producing cells in MLC. These IL-17-producing $CD8^+$ cells exhibit a unique granzyme $B^-IFN\text{-}\gamma^-IL\text{-}10^-$ phenotype. The mRNA level of Th2/T cytotoxic 2 (Tc2) transcription factors GATA3 and Th1/Tc1 transcription factors T-box expressed in T cell (T-bet) as well as its target H2·O-like homeobox (Hlx) is decreased in $CD8^+$ cells from TGF-β- and IL-6-treated MLC. In addition, these $CD8^+$ cells display a marked up-regulation of retinoic acid-related orphan receptor-γt, a key IL-17 transcription factor. These results demonstrate that the existence of an IL-17-producing $CD8^+$ subset belongs to neither the Tc1 nor the Tc2 subset and can be categorized as a T noncytotoxic 17 (Tnc17) subset. *J. Leukoc. Biol.* 82: 354–360; 2007.

Key Words: *IL-17 · mixed lymphocyte culture · cytotoxic T lymphocyte*

INTRODUCTION

Cytokines play key roles in regulating the development of immune effector cells and possess direct effector functions in fighting diseases. TGF-β has been found at the site of most tumors [1], and it inhibits the proliferation and functional differentiation of T lymphocytes [2, 3] and other immune cells [4, 5]. Further, TGF-β production by tumor cells prevented activation of CTL function [3]. This effect was presumably a result of the inhibition by TGF-β of the expression and function of IL-2 and IL-2Rs [6, 7] and cytolytic gene products [8], or it is the result of inducing T regulatory cells (Tregs) [9–13]. It has also been demonstrated that TGF-β plays a synergistic role with IL-10 to polarize tumor-infiltrating lymphocytes to predominantly Th2/T cytotoxic 2 (Tc2) phenotypes [14]. As CTL and Th1-associated cytokine production are important for achieving effective, immune-mediated tumor eradication, suppression of these functions by TGF-β would effectively subvert a proper immune response.

IL-6 may also play a pivotal role in cancer development. It is known that IL-6 can be a differentiation inducer in lung adenocarcinoma cells [5, 6] and other tumors [7, 15]. It is not clear whether IL-6 is secreted by cancer cells or by the immune system in response to the tumor or both. However, several recent reports have highlighted the nature of cross-talk between IL-6 and TGF-β [16, 17]. IL-6 was shown to be able to antagonize tumor-derived TGF-β and restore lymphokine-activated killing activity [9] and plays a key role in T cell activation by overcoming the suppressive effect of $CD4^+CD25^+$ Tregs [17], indicating that IL-6 may interfere with TGF-β in initiating the induction of $CD4^+CD25^+$ Tregs. During the past year, a new subset of $CD4^+$ cells, Th17, has been identified and is characterized by production of IL-17. Th17 cell differentiation is initiated by TGF-β and IL-6 [18–20]. It has shown recently that the retinoic acid-related orphan receptor (ROR)γt is the key transcription factor that orchestrates the differentiation of Th17 [21]. However, the role and effects of TGF-β and IL-6 on $CD8^+$ cell differentiation remain relatively undefined. We have found recently that high levels of TGF-β and IL-6 are present in malignant effusion of cancer patients [22]. We hypothesize that the cross-talk between TGF-β and IL-6 may modulate the $CD8^+$ CTL to kill tumor cells.

In this study, we showed that IL-6 acted cooperatively with TGF-β to elicit a high frequency of IL-17-secreting $CD8^+$ cells with a noncytotoxic phenotype. IFN-γ as well as IL-10 was not expressed in these IL-17-secreting $CD8^+$ cells. It leads to the fact that IL-17-producing $CD8^+$ cells may represent a distinct subset of $CD8^+$ cells, T noncytotoxic 17 (Tnc17).

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MATERIALS AND METHODS

Mice and cell lines

Female, 6- to 8-week-old BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan, ROC). All mice were housed at the Laboratory Animal Center of the National Health Research Institutes (NHRI; Taiwan, ROC). All of the animal studies were approved by the Animal Committee of the NHRI and performed according to their guidelines. The cell lines used in the study include the thymoma EL-4 of H-2^b and plasmacytoma P815 of H-2^d.

MLC

Splenocytes were obtained from BALB/c (H-2^d) and C57BL/6 (H-2^b) mice, and single cell suspensions were made. BALB/c splenocytes were used as responders (R), and 2000 rads X-irradiated C57BL/6 splenocytes were used as stimulators (S) at a R:S ratio of 3:1. Cells were resuspended at 3.3×10^6 cells/ml in RPMI-1640 medium containing 5% FBS, penicillin and streptomycin, gentamicin, HEPES, and 2-ME. Cells were seeded in 24-well tissue-culture plates at 2 mL per well. Recombinant TGF-β (1 ng/mL) and/or IL-6 (100 ng/mL; R&D Systems, Minneapolis, MN, USA) were added in parallel cultures as indicated. After 4–5 days of culture, proliferation responses, cell-mediated cytotoxicity, and cytokine profiles were determined.

Determination of proliferation responses

The cell density of BALB/c (H-2^d) splenocytes was adjusted to 3×10^7/mL and labeled with 10 uM CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Eugene, OR, USA) at 37°C for 10 min. Cells were washed with culture medium once and used as responders. The MLC was set as described above. Recombinant TGF-β (1 ng/mL) and/or IL-6 (100 ng/mL) were added as indicated. After 4 days of culture, the cells were harvested for staining. Nonspecific binding was blocked by incubation with rat anti-mouse CD16/CD32 antibody (BD Bioscience, San Jose, CA, USA) in PBS for 10 min at 4°C. Cells were stained with anti-CD8 antibodies conjugated with PE (BD Biosciences). After washing, cells were acquired and analyzed on a FACSCalibur flow cytometer with CellQuest software. Proliferative $CD8^+$ cells were determined by fluorescence intensity of CMFDA dilution.

Determination of cytolytic activity

EL-4 (H-2^b) or P815 (H-2^d) was labeled with 0.5 μM CMFDA at 37°C for 20 min. Cells were washed with culture medium once and used as target cells. Effector cells were prepared from H-2^d (BALB/c) against H-2^b (C57/BL6) MLC on Day 5. Target cells were cocultured with effector cells at the indicated E:T ratios. After 1.5 h incubation at 37°C, cells were stained with Annexin V conjugated with PE (R&D Systems) according to the manufacturer's instructions. CMFDA-positive cells (10,000) were harvested and analyzed on a FACSCalibur flow cytometer with CellQuest software. Cytolytic activities were determined by the percentages of Annexin V-positive cells within the CMFDA-positive gate. Less than 5% of target cells were Annexin V-positive when cultured with medium only in each experiment.

Intracellular staining

The MLC was established as described above. On Day 5, cells were restimulated with anti-CD3 antibody (2C11) and Brefeldin A (eBiosciences, San Diego, CA, USA) for 4 h. Cells were first stained with PE-Cy5-conjugated anti-CD8 antibody (eBiosciences) and then treated with fixation and permeabilization buffer (eBiosciences) according to the manufacturer's directions. Intracellular staining was performed using FITC- or PE-conjugated antibodies to IFN-γ, IL-10, and Granzyme B (eBiosciences). The PE-conjugated anti-IL-17 antibody was purchased from BD Biosciences. Isotype-matched antibodies conjugated with FITC or PE were used as negative controls. $CD8^+$ cells were further gated for evaluation of cytokine expression profiles.

Real-time quantitative PCR (qPCR) analysis of gene expression in $CD8^+$ cells

The MLC was set as described above and supplemented with or without recombinant TGF-β (1 ng/mL) and IL-6 (100 ng/mL). After 4 days of culture, $CD8^+$ cells were purified using anti-CD8 magnetic microbeads and MACS columns (Miltenyi Biotec, Bergishc Galdbach, Germany) according to the manufacturer's instruction (purity was >95%). Total RNA of isolated cells was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. RNA (0.5–1 μg) was reverse-transcribed to cDNA with an oligo-dT primer in a 20-μl vol by using SuperScript III RT (Invitrogen, Carlsbad, CA, USA). The mouse Universal Probe Library (UPL) set (Roche, Mannheim, Germany) was used to perform the real-time qPCR assay for gene expression in isolated cell populations. The specific primers and UPL number were as follows: hypoxanthine guanine phosphoribosyl transferase (HPRT), 5′-ggagcggtagcacctcct-3′ (forward) and 5′-ctggttcatcatcgctaatcac-3′ (reverse) with UPL#69; T-box expressed in T cell (T-bet), 5′-tcaaccagcaccagacagag-3′ (forward) and 5′-aaacatcctgtaatggcttgtg-3′ (reverse) with UPL#19; H2·O-like homeobox (Hlx), 5′-aagccagaccgaaagcag-3′ (forward) and 5′-tgcgcctccttagagtgc-3′ (reverse) with UPL#88; GATA3, 5′-cttatcaagcccaagcgaag-3′ (forward) and 5′-cccattagcgttcctcctc-3′ (reverse) with UPL#77; RORγt, 5′-ttcaccccacctccactg-3′ (forward) and 5′-caagggatcacttcaatttgtg-3′ (reverse) with UPL#56. The reaction mixture contained 5 ng cDNA, 0.2 μM primers, and LightCycler 480 Probe Master (Roche) and was performed in a LightCycler 480 system (Roche). All qPCRs were carried out with an initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 2 s. Target gene expression was calculated using the comparative method for relative quantity upon normalization to HPRT gene expression.

Statistical analysis

The statistical significance of differential findings between experimental groups was determined by an unpaired Student's *t*-test. Data were considered statistically significant if $P \leq 0.05$.

RESULTS

IL-6 abrogated the inhibition by TGF-β of $CD8^+$ cell proliferation in MLC

A murine MLC model was used in this study to investigate the role of TGF-β and IL-6 in regulating the CD8-mediated immune responses. Cell proliferation was analyzed by determining the CMFDA intensity. Results are shown in **Figure 1**. Approximately 42% of total $CD8^+$ cells showed diluted CMFDA in unmodified MLC (control-MLC). However, in the presence of TGF-β (TGF-β-MLC), the percentages of divided $CD8^+$ cells in total $CD8^+$ cells were reduced significantly to 32% ($P<0.0005$, compared with control-MLC). In contrast, when the MLC was supplemented with IL-6 (IL-6-MLC), the percentage of proliferating cells was elevated significantly to 49% ($P<0.016$, compared with control-MLC) of the whole $CD8^+$ population. It is most interesting that the presence of IL-6 plus TGF-β (TI-MLC) strongly stimulated $CD8^+$ cell proliferation. Close to 55% of $CD8^+$ cells showed diluted CMFDA ($P<0.009$, compared with control-MLC).

IL-6 was unable to restore the cytotoxic response inhibited by TGF-β

The above result demonstrated that IL-6 was able to abrogate the suppressive effect of TGF-β on $CD8^+$ cell proliferation. Here, we investigated whether the cytotoxic response was modulated by TGF-β and/or IL-6. The effect of TGF-β and IL-6 on allo-specific CTL activity is shown in **Figure 2**. Specific CTL activity in MLC was obtained against the haplotype-matched, allogeneic target cells (EL4 of H-2^b) and not for the syngeneic target cells (P815 of H-2^d). In TGF-β-MLC, the cytotoxic activity was abolished; in contrast, the killing activity

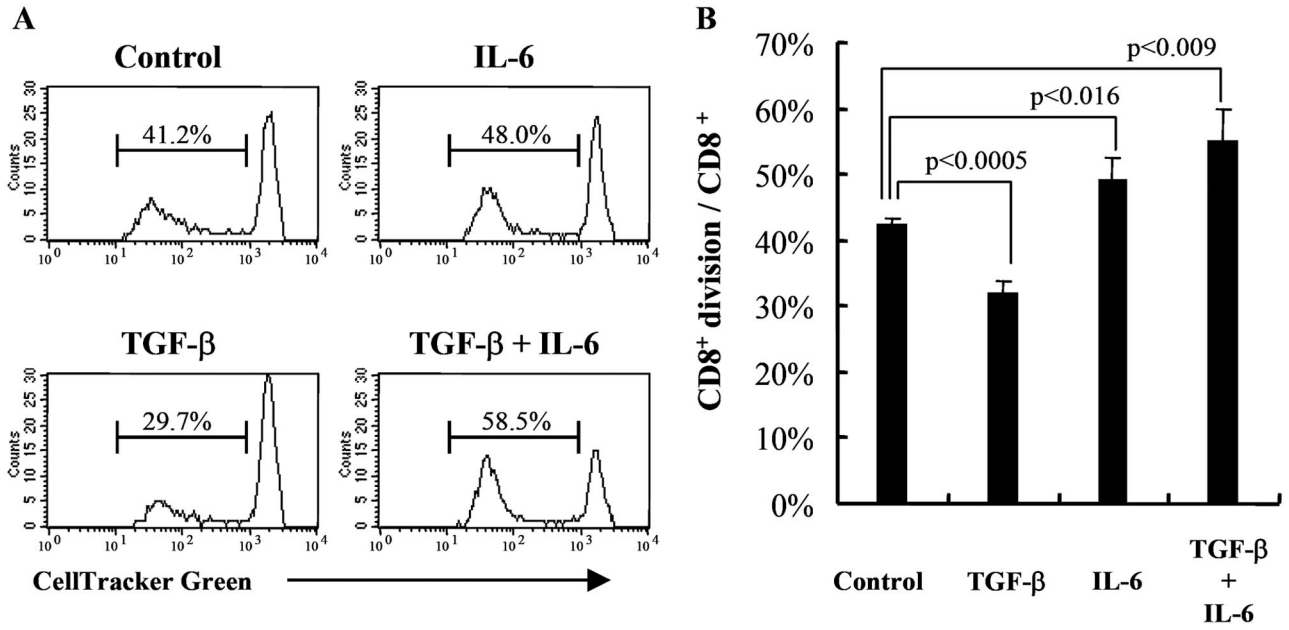

Fig. 1. IL-6 restores the inhibitory effect of TGF-β on $CD8^+$ cell proliferation successfully. MLC, consisting of 2000 rads X-irradiated C57BL/6 splenocytes and BALB/c splenocytes, prelabeled with CMFDA, were established with the addition of TGF-β (1 ng/mL) and/or IL-6 (100 ng/mL) as indicated. (A) $CD8^+$ cell proliferation was assessed by determining CMFDA dilution in cells positively stained for CD8 on Day 4. A representative experiment is shown. (B) The percentages of $CD8^+$ division over total $CD8^+$ cells were plotted. The means with SD from three independent experiments are shown.

was enhanced in IL-6-MLC. However, IL-6 was unable to restore the allo-specific cytolytic activity fully in the TI-MLC. The experiments have been repeated three times, and the results were reproducible. These results were quite different from the proliferation data.

TGF-β and IL-6 induce IL-17-producing $CD8^+$ cells

The $CD8^+$ cells in TI-MLC showed the highest level of proliferative ability but had a low level of cytotoxicity (Figs. 1 and 2). However, $CD8^+$ cells in MLC were shown to play the major role of performing cytotoxic activity (data not shown). These results indicate that the effect of TGF-β and IL-6 on proliferation of $CD8^+$ cells is different from that on the cytotoxicity of $CD8^+$ cells. To address this issue, the cytokine production profile of $CD8^+$ cells was evaluated by analyzing the intracellular staining. As shown in **Figure 3**, ~44% and 32% of $CD8^+$ cells contributed to IFN-γ production in control- and IL-6-MLC, respectively. TGF-β induced profound suppression of IFN-γ-secreting $CD8^+$ cells. Less than 3% of $CD8^+$ cells expressed IFN-γ in TGF-β- and TI-MLC. Approximately 10% of $CD8^+$ T cells were able to secrete IL-10 in IL-6-MLC. The frequency of IL-10-secreting $CD8^+$ T cells decreased slightly to 6% in TI-MLC. No significant numbers of IL-10-producing

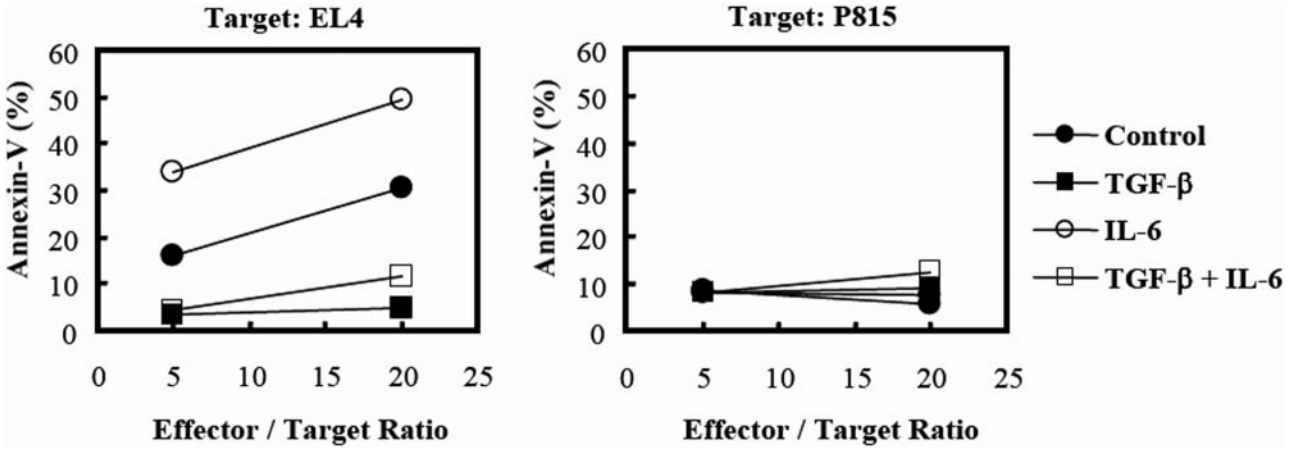

Fig. 2. Modulating effects of IL-6 and TGF-β on allocytotoxicity. BALB/c ($H\text{-}2^d$) against C57BL/6 ($H\text{-}2^b$) MLC was established with the addition of TGF-β (1 ng/mL) and/or IL-6 (100 ng/mL) as mentioned. Cultured cells were harvested on Day 5 as effector cells and incubated with CMFDA-labeled target cells EL4 ($H\text{-}2^b$) or P815 ($H\text{-}2^d$) for 1.5 h at E:T ratios at 20:1 or 5:1. The cytotoxicity was then determined by labeling cells with PE-conjugated Annexin V, and the CMFDA-positive cell population was analyzed by flow cytometry. Data are representative of three independent experiments.

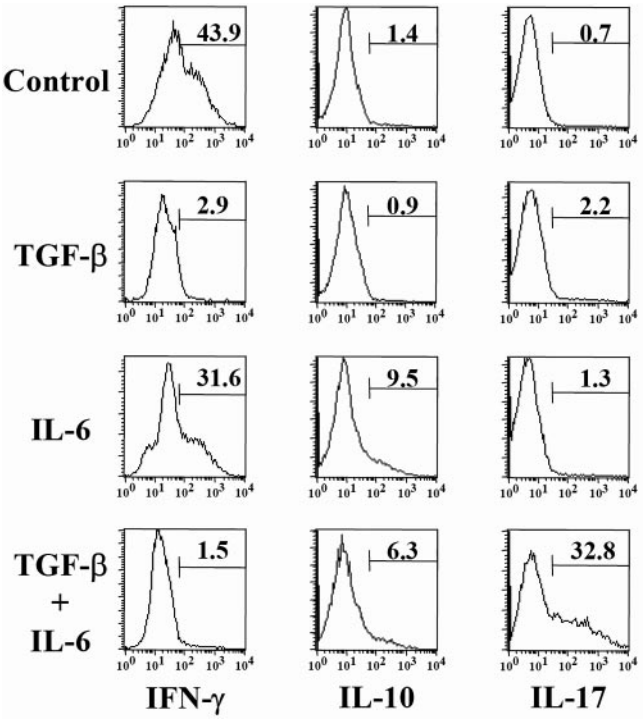

Fig. 3. IFN-γ, IL-10, and IL-17 expression profiles of $CD8^+$ T cells in IL-6- and/or TGF-β-treated MLC, which when consisting of C57BL/6 and BALB/c splenocytes, were established with the addition of TGF-β (1 ng/mL) and/or IL-6 (100 ng/mL) as mentioned. The cultures were harvested after 5 days and stained with anti-CD8 followed by intracellular staining for IFN-γ, IL-10, or IL-17. $CD8^+$ cells were gated, and expression of cytokine profiles was plotted. The numbers in the histograms indicate the percentage of positive cells. Data shown are representative of three independent experiments.

$CD8^+$ cells were detected in control- and TGF-β-MLC. It is striking that more than 30% of $CD8^+$ T cells expressed IL-17 in TI-MLC, and low percentages of the $CD8^+$ cell population expressed IL-17 in control-, TGF-β-, and IL-6-MLC. All experiments have been repeated three times, and the results were reproducible.

Distinct IL-17^+granzyme B^- and IL-10^+granzyme B^- $CD8^+$ T cells were induced by TGF-β and IL-6

Granzyme B is known to play a pivotal role in CTL function to eliminate virus-infected cells or tumor cells. Next, we have further assessed whether the expression of granzyme B in these IL-17-secreting CD8 T cells was shown to bear good proliferative but poor cytotoxic ability. From **Figure 4**, we found that in control- and IL-6-MLC, 28% and 37% of $CD8^+$ cells were IFN-γ^+granzyme B^+ phenotypes, respectively. However, less than 2% of $CD8^+$ cells had an IFN-γ^+granzyme B^+ phenotype in TGF-β- and TI-MLC. Regarding $CD8^+$ cells with the IL-10^+granzyme B^+ phenotype, only in IL-6- and TI-MLC, there were significant numbers of $CD8^+$ cells to produce IL-10. However, the IL-6-MLC produced a higher frequency of the IL-10^+granzyme B^+ phenotype in $CD8^+$ cells than the TI-MLC (9.1% vs. 2.9%). Few IL-17^+granzyme B^+CD8^+ cells could be found in control-, TGF-β-, and IL-6-MLC. Less than 2% of $CD8^+$ cells were the IL-17^+granzyme B^+ phenotype in TI-MLC. Therefore, it appears that the majority of IL-17-producing $CD8^+$ cells does not express granzyme B. The experiments were performed three times, and the results were reproducible. In fact, granzyme B-expressing $CD8^+$ cells are diminished by the addition of TGF-β. In contrast, IL-6 enhances the expression of granzyme B in CD8 cells. However, IL-6 was unable to reverse the suppressive effect of TGF-β on granzyme B production. It can be concluded that the profile of granzyme B expression in $CD8^+$ cells correlated with the results of their cytotoxic activity as shown in Figure 2.

To verify whether IL-17-expressing $CD8^+$ cells in TI-MLC expressed IFN-γ or IL-10, several experiments were set up, and the results are shown in **Figure 5**. More than 26% of IL-17-expressing $CD8^+$ cells do not produce IFN-γ or IL-10. There were merely 6% IL-17^-IFN-γ^+ and 3% IL-17^-IL-10^+ phenotype $CD8^+$ cells. Less than 2% of $CD8^+$ cells were the IL-17^+IFN-γ^+ or IL-17^+IL-10^+ phenotype. The experiments have been repeated twice, and the results were reproducible. These results demonstrate that most of IL-17-secreting $CD8^+$ cells were the IFN-γ^-IL-10^- phenotype.

Moreover, we have also examined the mRNA expression levels of cytokines and transcription factors of $CD8^+$ cells in TI-MLC by real-time PCR. The expression of the IFN-γ mRNA level in TI-MLC was sevenfold less than control-MLC. By contrast, IL-10 and IL-17 mRNA expression was 80- and 507-fold, respectively, higher than control-MLC (data not shown). These profiles were in agreement with the intracellular cytokine data (Fig. 3). Finally, analysis of the expression of Th1/Th2 signature transcription factors showed a substantial down-regulation of T-bet, Hlx, and GATA3 mRNA (**Fig. 6, A–C**).

It has been demonstrated recently that RORγt is a key transcription factor directing the differentiation of Th17 [21]. It is notable that the mRNA expression level of RORγt was increased 30 fold (Fig. 6D). Taken together, our data suggest that the IL-17-producing $CD8^+$ cells represent a distinct T effector lineage, neither Tc1 nor Tc2.

DISCUSSION

Tumor growth and survival are affected by the cytokines present in the tumor microenvironment. We reported that a variety of cytokines was found at elevated levels in the malignant effusions of cancer patients [22]. The most prominent feature was the presence of high levels of TGF-β and IL-6 in all the effusion samples tested [22]. Recent studies have demonstrated that TGF-β and IL-6 promote the development of IL-17-producing $CD4^+$ cells, which have been called Th17 [20]. IL-17 has been shown to increase the IL-6 production [23–26]. Therefore, in such, the environmental condition will facilitate IL-17 production. Expression of IL-17 mRNA was detected at the tumor sites, including cervical carcinoma [27] and ovarian cancer [28]. However, the effect of IL-17 on tumor growth is paradoxical. Benchetrit et al. [29] reported that IL-17 is able to inhibit tumor growth by means of a T cell-dependent manner. By contrast, Tartour et al. [27] and others [30, 31] demonstrated that IL-17 promotes tumor growth via potentiation of tumor angiogenesis. Our results also demonstrated that

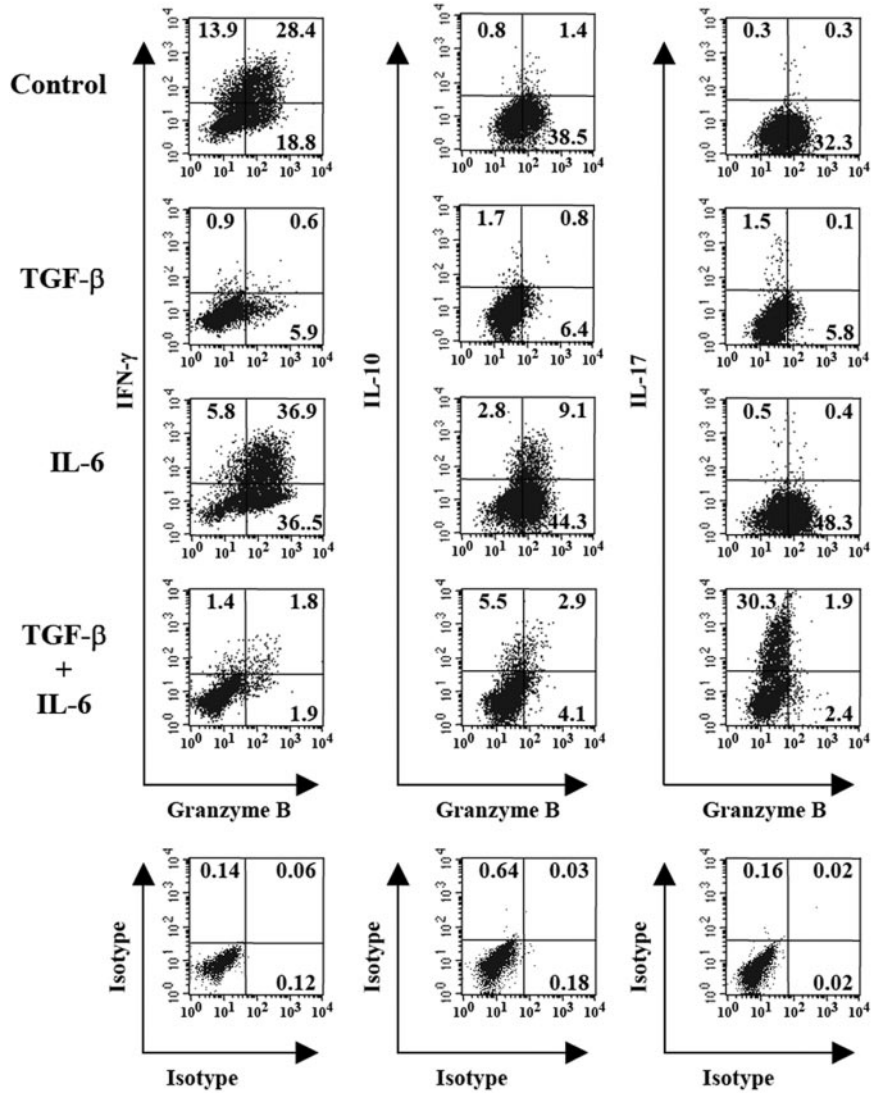

Fig. 4. Granzyme B is expressed by IFN-γ and IL-10 but not IL-17-producing CD8$^+$ T cells in TGF-β- and/or IL-6-supplemented MLC. TGF-β (1 ng/mL) and/or IL-6 (100 ng/mL) were added to MLC as described above. Cells were harvested on Day 5 and stained with anti-CD8. Intracellular staining for granzyme B and IFN-γ, IL-10, or IL-17 was performed. CD8 cells were gated and displayed as granzyme B versus IFN-γ, IL-10, or IL-17. The isotype controls were shown in the bottom panel. Numbers in quadrants indicate percent-positive cells. Data shown are representative of three independent experiments.

IL-17-producing CD8$^+$ cells, Tnc17, are deficient in cytolytic activity (Figs. 2 and 4). These findings provide the possible mechanism that tumor-infiltrating T cells regulate angiogenesis via elaboration of IL-17 when TGF-β and IL-6 are present at the tumor microenvironment. The consequence is tumor growth.

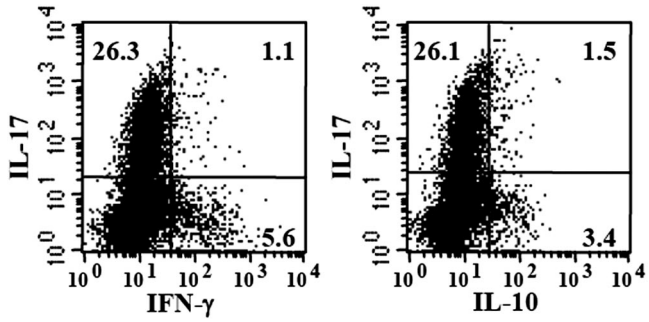

Fig. 5. IL-17-producing CD8$^+$ T cells did not express IFN-γ or IL-10 in IL-6- and TGF-β-treated MLC. BALB/c (H-2^d) against C57BL/6 (H-2^b) MLC were established in the presence of TGF-β (1 ng/mL) and IL-6 (100 ng/mL). After 5 days in culture, cells were harvested and stained with anti-CD8. Intracellular staining for IL-17 with IFN-γ or IL-10 was performed. CD8$^+$ cells were gated and plotted as IL-17 versus IFN-γ or IL-10. Numbers in quadrants indicate percent-positive cells in each quadrant. Data shown are representative of two independent experiments.

TGF-β is a pleiotropic cytokine with multiple regulatory functions in the immune system [32]. One regulatory function of TGF-β is the inhibition of naïve T cell differentiation into effector cells. Our initial experiments supported the notion that TGF-β inhibited CD8$^+$ cell proliferation (Fig. 1) and allo-specific cytotoxic activity (Fig. 2) in MLC. IL-6 was found to play an opposing role to TGF-β in several biological functions [7, 16, 33]. In contrast to the suppressive effect of TGF-β on CD8$^+$ cell proliferation and cytotoxicity (TGF-β-MLC), IL-6 enhanced CD8$^+$ cell division and allo-specific cytotoxicity, and the proliferative potential of the CD8$^+$ cell was enhanced further in TI-MLC when TGF-β and IL-6 are present. However, the generation of CD8$^+$ CTL remained to be suppressed in TI-MLC (Figs. 1 and 2). To confirm that the cytotoxic response was restricted to CD8$^+$ cells, anti-CD4, anti-CD8, and isotype control antibodies were applied in the cytotoxic assay. The cytotoxicity remained unchanged by adding anti-CD4 or isotype control antibodies but was abrogated completely by anti-CD8 antibody (data not shown). These results indicate that

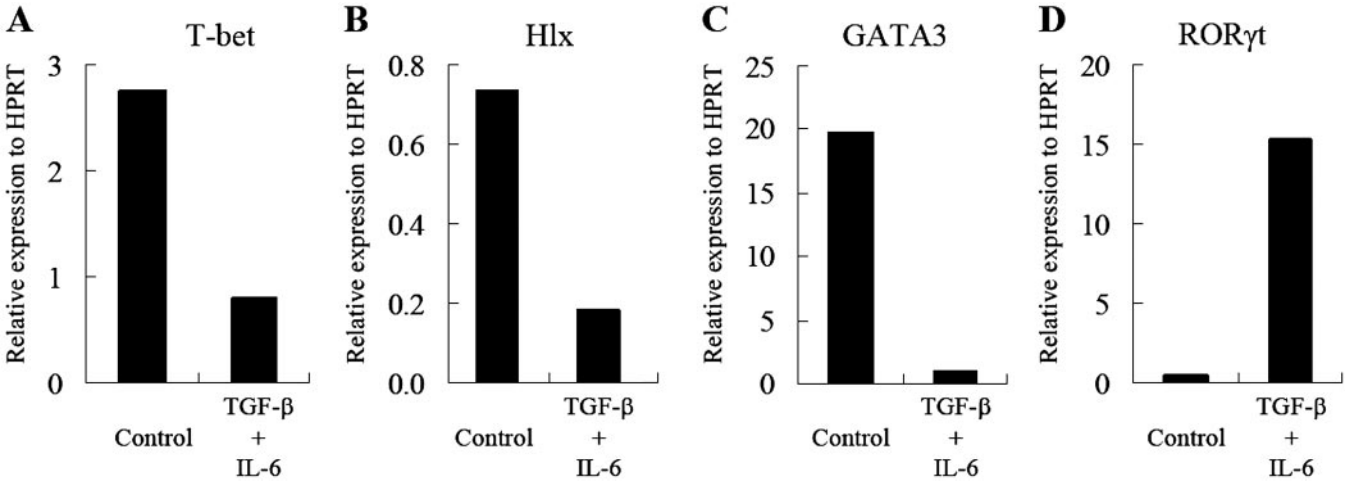

Fig. 6. IL-17-producing $CD8^+$ cells are distinct from Th1 and Th2 subsets. MLC was established in the presence or absence of TGF-β (1 ng/mL) and IL-6 (100 ng/mL). $CD8^+$ cells were purified after 4 days of culture. The mRNA levels of (A) T-bet, (B) Hlx, (C) GATA3, and (D) RORγt were determined by real-time qPCR.

cytotoxic activity was mediated mainly by the $CD8^+$ subset. Thus, there is discordance between the proliferative potential and the generation of CTL of the $CD8^+$ cells in TI-MLC. These findings uncover a novel role of the cross-regulation by TGF-β and IL-6 of the activation of $CD8^+$ cells.

Several lines of evidence have indicated that T cells modulate immune responses by producing Type 1 or 2 cytokines in vivo [34–39]. After activation, $CD4^+$ and $CD8^+$ cells differentiate into effector cells, which are specialized in terms of the cytokines that they produce. Naïve $CD8^+$ cells are able to differentiate into a Tc1 subset, which secretes IFN-γ and IL-2 predominately, and a Tc2 subset, which secretes IL-4, IL-5, and IL-10 preferentially [40–42]. We examine the functional characteristics of $CD8^+$ cells in MLC by investigating their cytokine and granzyme B expression. There were substantial numbers of IFN-γ-producing $CD8^+$ cells in control-MLC and IL-6-MLC (Fig. 3). Most of the IFN-γ^+CD8^+ cells also expressed high levels of granzyme B. In control-MLC, 67% of IFN-γ^+CD8^+ cells were granzyme B-positive cells. More than 86% of IFN-γ^+CD8^+ cells were granzyme B-positive in IL-6-MLC (Fig. 4). Naïve $CD8^+$ cells were blocked by TGF-β from differentiating into Tc1 cells. Significant numbers of IL-10-secreting $CD8^+$ cells were obtained in IL-6-MLC and TI-MLC (Fig. 3). In IL-6-MLC, ~77% of IL-10^+CD8^+ cells were the granzyme B^{high} phenotype. Less than 35% of IL-10^+CD8^+ cells expressed high levels of granzyme B when TGF-β was also present (Fig. 4). These results indicate that IL-6 skews naïve $CD8^+$ cells toward the IL-10^+ phenotype. In the presence of TGF-β and IL-6, expression of granzyme B is inhibited in the majority of IL-10^+CD8^+ cells.

It has been shown that TGF-β and IL-6 facilitate Th17 differentiation in $CD4^+$ cells [20]. We examined further whether $CD8^+$ cells would produce IL-17 in the presence of these two cytokines. Indeed, IL-17-producing $CD8^+$ cells were induced in TI-MLC but not in control-, TGF-β-, or IL-6-MLC (Fig. 3). Furthermore, up to 94% of IL-17^+CD8^+ cells were the granzyme B^{low} phenotype (Fig. 4). These observations explain clearly why proliferation of $CD8^+$ cells is enhanced in the presence of IL-6 and TGF-β, but they lack the cytolytic activity.

Like other differential processes, the naïve T cell development is mediated by lineage-specific transcription mechanisms. It was reported recently that RORγt plays the key role in Th17 differentiation [21], which resembles the roles of T-bet [43], Hlx [44], and GATA3 [45, 46] in the development of Th1 and Th2 cells. Our data indicate that $CD8^+$ cells from TI-MLC down-regulate expression of GATA3 and T-bet, as well as its downstream Hlx (Fig. 6, A–C). Conversely, expression of RORγt is elevated significantly (Fig. 6D). We also showed that most of the IL-17^+CD8^+ cells do not produce IFN-γ or IL-10 (Fig. 5). These data indicate clearly that differentiation of naïve $CD8^+$ cells into IL-17-producing cells, Tnc17, is independent of Tc1 and Tc2 cell-development programs. In this study, we provide compelling evidence to show that a previously undefined, IL-17-expressing CD8 subset is elicited in the presence of TGF-β and IL-6. The precise role of Tnc17 in tumor development will be clarified in our further studies.

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