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Inhibition of TGF-β1 Signaling Promotes Central Memory T Cell Differentiation

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This study affirmed that isolated CD8+ T cells express mRNA and produce TGF-β following cognate peptide recognition. Blockage of endogenous TGF-β with either a TGF-β–blocking Ab or a small molecule inhibitor of TGF-βRII enhances the generation of CD62L<sup>high</sup>/CD44<sup>high</sup> central memory CD8<sup>+</sup> T cells accompanied with a robust recall response. Interestingly, the augmentation within the central memory T cell pool occurs in lieu of cellular proliferation or activation, but with the expected increase in the ratio of the Eomesoderm/T-bet transcriptional factors. Yet, the signal transduction pathway(s) seems to be noncanonical, independent of SMAD or mammalian target of rapamycin signaling. Enhancement of central memory generation by TGF-β blockade is also confirmed in human PBMCs. The findings underscore the role(s) that autocrine TGF-β plays in T cell homeostasis and, in particular, the balance of effector/memory and central/memory T cells. These results may provide a rationale to targeting TGF-β signaling to enhance Ag-specific CD8<sup>+</sup> T cell memory against a lethal infection or cancer. The Journal of Immunology, 2013, 191: 2299–2307.

Recent progress in immunology has emphasized the importance of generating better quality memory T cells. Central to those efforts has been an expectation that the development of a robust long-term T cell memory would fortify vaccines and enhance host protection against infectious diseases and cancer immunotherapy. Indeed, it has been reported that, in both mice and nonhuman primates, central memory CD8<sup>+</sup> T cells are superior to effector memory CD8<sup>+</sup> T cells as mediators of host immune-based protection against viruses and cancer (1–3). In mice, central and effector memory CD8<sup>+</sup> T cells can be separated into two distinct populations according to their respective CD44 and CD62L expression levels (1, 3–6). A CD44<sup>high</sup>/CD62L<sup>low</sup> CD8<sup>+</sup> T cell population that is found mainly in peripheral tissues and rapidly acquires effector functions constitutes the effector memory, whereas CD8<sup>+</sup> T cells expressing a CD44<sup>low</sup>/CD62L<sup>high</sup> population, which typically reside in secondary lymph nodes where they acquire profound proliferative capacities upon Ag recognition, represent the central memory T cells. Investigators are beginning to unravel the molecular pathways that regulated the differentiation of long-lived central memory T cells. Along with those phenotypic markers, certain cytokines, such as IL-2 and IL-15, and selective intracellular signal transduction molecules, such as AMPK-α and mammalian target of rapamycin (mTOR), have been implicated in the differentiation of effector to central memory CD8<sup>+</sup> T cells (6–10).

TGF-β is a well-known immune-suppressive cytokine that affects multiple cell types within the immune system. For example, TGF-β controls T cell homeostasis by directly inhibiting both proliferation and activation of naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells (11).

Disruption of TGF signaling in naive T cells results in the emergence of autoimmune diseases in mice (11, 12). The inhibitory effects of TGF-β are not limited to the activation of naive T cells. CD8<sup>+</sup> T cells activated in the presence of exogenous TGF-β do not acquire CTL function (13), and CD4<sup>+</sup> T cells fail to become Th1 or Th2 cells (14, 15). However, the fact that CD8<sup>+</sup> T cells produce endogenous TGF-β upon activation (16) and despite the presence of the cytokine, naïve T cells still differentiate into effector cells in a vaccine setting. These observations underscore the intriguing differential effects of endogenous and exogenous levels of TGF-β on T cell activation and differentiation. Because exogenous TGF-β effects on T cells present a seemingly different set of parameters, such as the complexities of the activation mechanism of latent TGF-β (17), the current study focused on the physiological changes associated with CD8<sup>+</sup> T cell generation and differentiation and autocrine TGF-β.

Splenocytes from H-2Db–restricted NP68-specific CD8<sup>+</sup> TCR transgenic (Tg) mice (F5 mice) offer a potential in vitro model to directly examine the role(s) that TGF-β and small molecule TGF-β receptor inhibitors play in CD8<sup>+</sup> T cell differentiation (18). Upon stimulation with cognate peptide, the CD8<sup>+</sup> F5 T cells acquire both phenotypic changes and immune effector functions that are reminiscent of those described during the generation of an in vivo Ag-specific T cell response, that is, priming, expansion, contraction, and memory. The present study affirmed that isolated CD8<sup>+</sup> T cells express mRNA and produce TGF-β following cognate peptide recognition. In addition, blockage of endogenous TGF-β with either a TGF-β–blocking Ab or a small molecule inhibitor of TGF-βRII enhances the generation of central memory T cells. Interestingly, the augmentation within the central memory T cell pool occurs in lieu of cellular activation and seems to be mediated via a pathway independent of SMAD. The findings underscore the role(s) that autocrine TGF-β plays in T cell homeostasis and, in particular, the balance between effector and central memory T cells.

Materials and Methods

Animals

Female C57BL/6 mice (8–12 wk old) were obtained from the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD).
F5 mice that are Tg for nucleoprotein of influenza virus A/NT/60/68 (5′66ASSENMDAM74°; NP68)-specific, H-2Db-restricted TCR (19, 20) were obtained from Taconic Farms (Hudson, NY). All mice were housed and maintained in microisolator cages under specific pathogen-free conditions and in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. All experimental studies were carried out under the approval of the Intramural Animal Care and Use Committee. Splenocytes from SMAD2 conditional knockout mouse were provided by J. Kang of the University of Massachusetts Medical School, Worcester, MA (21).

Reagents

TGF-β mAb (clone 1D11) was purchased from R&D Systems (Minneapolis, MN). SD208 was purchased from Sigma-Aldrich, dissolved in DMSO, and diluted in culture media; maximum concentration was 0.1%. Human rTGF (rhTGF)-β1 was purchased from PeproTech. H-2Db–restricted influenza virus A/NT/60/68 peptide (5′66ASSENMDAM74°; NP68 peptide) was synthesized by CPC Scientific.

Poxvirus constructs

Recombinant fowlpox viruses containing murine B7-1, ICAM-1, and LFA-3 genes in combination with nucleoprotein of influenza virus A/NT/60/68 (5′66ASSENMDAM74°;NP68) (rVF-NP68-TRICOM) have been described previously (22). The recombinant fowlpox virus containing the gene for murine GM-CSF has also been described previously (23).

In vitro T cell assay

Primary splenocytes were dispersed into single-cell suspensions, the RBCs were removed by lysis, and the remaining cells were seeded into 6-well plates at 6 × 105 cells/ml in complete RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin medium. Splenocytes from C57Bl6 mice were seeded into 6-well plates at 3 × 106 cells/ml in complete RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin medium) (4352339E). Mean cycle threshold (Ct) = Ct(GAPDH) − Ct(target gene)]]. The ratio of mRNA expression of target gene versus GAPDH was defined as 2(−ΔCt).

Cytokine assays

Mouse IFN-γ, IL-2, and TGF-β ELISAs were performed using Quantikine ELISA kits (R&D Systems, Minneapolis, MN). The TGF-β3 ELISA was performed using a kit from MyBioSource.com. Each ELISA protocol was carried out according to the manufacturers’ instructions. Human IFN-γ ELISA was performed using human IFN-γ ELISA kit (Invitrogen), according to the manufacturer’s protocol.

Flow cytometry analysis

Mouse splenocytes or human PBMCs were stained with Abs to the following cell surface markers: mouse CD8a, CD19, CD44, CD62L, and human CD8, CD4, CD45RA, and CD45RO, which were purchased from BD Biosciences (Mountain View, CA). Abs that recognize mouse IL-7R (CD127), T-bet, and Eomesoderm (Eomes) were purchased from eBioscience, and the human-CCR7 Ab was purchased from R&D Systems. Annexin V staining was performed using annexin V staining kit (BD Biosciences). Cells were also stained with appropriate isotype-matched controls. To identify influenza A NP68-specific cells, splenocytes were stained with NP68 dextramer (ASSENMDAM) (Immudex) or lymphocytic choriomeningitis virus dextramer (5′66FQPQNGQFI74°) (Immudex). Intracellular protein staining was performed using Foxp3 staining buffer set (eBioscience) and protocol. Stained cells were acquired using a FACScan or LSII flow cytometer (BD Biosciences). Dead cells were excluded from the analysis based on scatter profile.

Western blot analysis

Western blot was conducted, as previously described (18), using phospho-specific Abs against SMAD2 (Ser465/467) (3101), SMAD3 (Ser423/425) (9520), p44/2 MAPK (Thr202/Tyr204) (4370), p38MAPK (Thr180/Tyr182) (4511), SAPK/JNK (Thr183/Tyr185) (4671), p70S6 kinase (Ser371) (9208), p70S6 kinase (Thr389) (9234), and 86 ribosomal protein (Ser240/244) (5364). All of the phospho-specific Abs were purchased from Cell Signaling Technology (Danvers, MA). Detection was performed with the Odyssey Infrared imaging system (LI-COR Biotechnology).

Adaptive transfer

Splenocytes from F5 mice were precultured for 1 h with 3 μm SD208 or vehicle, and then stimulated with 10−5 μg/ml cognate peptide for 96 h. Splenic CD8+ T cells were purified with magnetic beads, and 1 × 107 cells/ml were labeled with 1 μm CFSE, incubated for 10 min at 37°C, and washed twice with PBS. Purified CD8+ T cells (1 × 105) were adoptively transferred into naive C57Bl6 mice on day 0, and the mice were vaccinated with rVF-NP68-TRICOM on day 3 to induce a recall response.

Statistical analysis

Statistical significance was calculated using GraphPad Prism statistical software (version 5.0c; GraphPad Software). Where not specified, results of tests of significance are reported as p values, derived from either the two-tailed unpaired Student t test or using one-way ANOVA, followed by Tukey’s multiple comparison test to compare between the groups. In the graphic representations of data, y-axis error bars indicate the SEM for each point on the graph.

Results

TGF-β production by isolated CD8+ T cells following cognate peptide stimulation

Two to 4 h after recognition of the cognate peptide-bound MHC in the presence of anti-CD28, CD8+ T cells isolated from F5 TCR.Tg mice produced a spike in TGF-β mRNA. The CD8+ T cells also acquired an effector memory phenotype (i.e., CD44 high/CD62Llow) following cognate peptide stimulation has been tied to the acquisition of effector functions and proliferation, respectively. Indeed, beginning at 96 h post–peptide stimulation, the proliferating T cells endogenously produce substan- tial amounts of IFN-γ, IL-2, and TGF-β, which are secreted into the culture supernatant (Fig. 1). This time interval has been referred to as the T cell priming phase (18). From 24 to 72 h post–cognate peptide stimulation, the proliferating T cells endogenously produce substantial amounts of IFN-γ, IL-2, and TGF-β, which are secreted into the culture supernatant (Fig. 1). The per- centage of CD8+ T cells expressing surface markers indicating a central memory phenotype, CD62LhiCD244high, increased from 11% at 24 h to >65% at 72–96 h, respectively (Supplemental Fig. 1B). T cell production of IFN-γ and IL-2 following cognate peptide stimulation has been tied to the acquisition of effector functions and proliferation, respectively. Indeed, beginning at 96 h post–peptide stimulation, if those CD8+ T cells were rested for an additional 48 h in the presence of a low dose of IL-2, two distinct cell populations emerged that were isolated by flow cytometry based on their CD62L expression levels (Supplemental Fig. 1B, day 6 panel). When each CD8+ T cell population was restimulated with the cognate peptide, the CD62LhiCD244high CD8+ T cells produced higher levels of IL-2 (597 versus 224 pg IL-2/ml; p < 0.0001) (Supplemental Fig. 1C), and had a higher proliferative response (Supplemental Fig. 1F) than the corre-
The corresponding CD62L low/CD44 high CD8+ T cells. Those functional differences are consistent with identifying those cells as central and effector memory T cells (1, 3–6), respectively. Of interest was the T cell function(s), particularly those of T cell differentiation, which may be associated with endogenous TGF-β production, which became the focus of subsequent study.

**FIGURE 1.** Temporal-dependent in vitro production of TGF-β, IFN-γ, and IL-2 by isolated CD8+ T cells following cognate peptide stimulation. Splenic CD8+ T cells from TCR Tg mice for the nucleoprotein of influenza virus NP68 (F5 Tg-mice) were isolated by magnetic beads and stimulated with 10^{-4} μg/ml cognate peptide (NP68 peptide), 1.0 μg/ml H2Db-dimer X, and 2.0 μg/ml anti-CD28. (A) TGF-β1 mRNA as measured by quantitative PCR at the indicated time points. (B) TGF-β1, (C) IFN-γ, and (D) IL-2 production in the T cell culture supernatant as measured using appropriate cytokine ELISAs. Data represent the mean ± SE of triplicate samples from three independent experiments.

**FIGURE 2.** Blockade of TGF-β signaling increased central memory T cell phenotype. Splenic CD8+ T cells isolated from F5 mice were pretreated with either the anti–TGF-β mAb (0.1–10 μg/ml) or TGF-βRI kinase inhibitor, SD208 (0.3–3.0 μM), for 1 h prior to stimulation with 10^{-4} μg/ml cognate peptide (NP68 peptide), 1.0 μg/ml H2Db-dimer X, and 2.0 μg/ml anti-CD28, and all analyses were performed 72 h later. (A) Flow cytometric analyses of F5-CD8+ T cells, as determined by anti-CD62L and either anti-CD44 (upper panel) or anti-CD127 (lower panel) are shown. (B) Total number of CD62L high/CD44 high central memory (solid bars) versus CD62L low/CD44 high effector memory (open bars) CD8+ T cells recovered and assessed by trypan blue exclusion. *p < 0.05 (0.1 μg/ml versus 1.0 and 10 μg/ml anti–TGF-β mAb), **p < 0.01 (untreated versus 0.1 μg/ml versus 1.0 and 10 μg/ml anti–TGF-β mAb). (C) IFN-γ production as measured by ELISA. **p < 0.01 untreated versus 0.1 μg/ml anti–TGF-β mAb), ***p < 0.001 (untreated versus 1.0 and 10 μg/ml anti–TGF-β mAb). (D) SD208 effects on the phenotypic changes of F5-CD8+ T cells, as determined by anti-CD62L and anti-CD44. (E) Total number of CD62L high/CD44 high central memory (solid bars) versus CD62L low/CD44 high effector memory (open bars) CD8+ T cells recovered and assessed by trypan blue exclusion. *p < 0.05 (untreated versus 0.3 μM SD208), **p < 0.05 (untreated versus 1 μM SD208), ***p < 0.001 (untreated versus 3 μM SD208). (F–H) Splenocytes from C57BL/6 mice were preincubated with various doses of SD208 (0.3–3.0 μM) for 1 h, and stimulated with 2.5 μg/ml anti-CD3 and 1.25 μg/ml anti-CD28, and all analyses were done 72 h later. (F) Flow cytometric analyses of CD8+ and CD4+ T cells stained with anti-CD62L and anti-CD44 Abs. Numbers in each upper right quadrant denote percentage of cells. (G and H) Total number of (G) CD8+ or (H) CD4+ CD62L high/CD44 high central memory (solid bars) versus CD62L low/CD44 high effector memory (open bars) T cells recovered following stimulation with cognate peptide. Cell numbers were determined by trypsin blue exclusion, and the total number of CD8+, CD4+, CD62L high/CD44 high, CD62L low/CD44 high cells was determined based on flow cytometry data. (G) CD8+: *p < 0.05 (0.3 versus 1.0 μM and 1.0 versus 3.0 μM SD208), ***p < 0.001 (untreated versus 0.3, 1.0, and 3.0 μM; 0.3 versus 3.0 μM SD208). (H) CD4+: ***p < 0.001 (untreated versus 0.3, 1.0, and 3.0 μM SD208). Data are the mean ± SE of triplicate samples of at least three independent experiments. Statistical significance was measured by one-way ANOVA, followed by Tukey's multiple comparison test.
Blockade of TGF-β signaling increases central memory phenotype in isolated T cells from F5 TCR.Tg and wild-type mice

These studies used two TGF-β blocking reagents, an anti–TGF-β mAb and a TGF-βR1 kinase inhibitor, SD208, to examine what consequences might occur in the transition of peptide-stimulated T cells through the memory differentiation program with the interruption of biological contributions of TGF-β. As previously described, 72 h after post-cognate peptide stimulation, 65–70% of the CD8+ T cells from F5 TCR.Tg mice expressed high levels of CD62L and CD44 (i.e., CD62Lhigh and CD44high), indicative of a central memory phenotype. The addition of the anti–TGF-β-specific mAb to the F5 CD8+ T cell culture media resulted in a dose-dependent increase in the percentage of cells expressing that phenotype (i.e., 85%: 10 μg/ml anti–TGF-β mAb, Fig. 2A). Other studies have used the expression of the homeostatic cytokine receptor, CD127, as another phenotypic marker for central memory T cells. Flow cytometric analysis confirmed that TGF-β blockade also increased CD62Lhigh/CD127high central memory phenotype from 40 to 58% (10 μg/ml TGF-β mAb, Fig. 2A, lower panel). The acquisition of the phenotypic markers indicative of memory T cell differentiation occurred in the absence of any measurable changes in T cell proliferation, as determined by CFSE (data not shown). As a result, there was an overall increase in the absolute number of central memory T cells (Fig. 2B, solid bars) with a commensurate reduction in the number of CD62Llow/CD44high effector memory T cells (Fig. 2B, open bars). Blockade of endogenous TGF-β slightly reduced IFN-γ production, an indicator of CD8+ T cell activation (Fig. 2C). In addition, no TGF-β3 was found in these culture supernatants (data not shown). To examine whether the observed increase in memory T cells coincided with the interruption of TGF-β signaling, isolated CD8+ T cells were stimulated with peptide-bound MHC and anti-CD28 and simultaneously treated with SD208, a TGF-βR1 kinase inhibitor. That also increased the percentage of T cells with the CD62Lhigh/CD44high central memory phenotype in a dose-dependent manner (i.e., 66% vehicle versus 89% at 3 μM, Fig. 2D). The interruption of TGF-β signaling by SD208 treatment also increased the total number of central memory T cells (Fig. 2E, solid bars) while reducing CD62Llow/CD44high effector memory T cells (Fig. 2E, open bars).

When CD4+ or CD8+ T cells isolated from naive, wild-type B6 mice were stimulated in vitro with anti-CD3 and anti-CD28, they proceeded through the same priming, expansion, and contraction phases as previously described for the F5 CD8+ cells. In vitro treatment of either CD8+ or CD4+ T cells isolated from naive B6 mice with SD208, followed by anti-CD3 and anti-CD28 stimulation, resulted in a significant dose-dependent increase in the percentage of cells expressing the CD62Lhigh/CD44high central memory phenotype (Fig. 2F). For example, the percentage of CD8+ T cells that expressed high CD62L and CD44 levels increased from 27% in untreated cells to 41, 45, and 57% following treatment with 0.3, 1.0, and 3.0 μM SD208, respectively. SD208 treatment up to 1 μM had no detrimental effect on the growth of either CD8+ or CD4+ T cells (data not shown), thereby increasing the total number of CD62Lhigh/CD44high central memory T cells.

**FIGURE 3.** Exogenous TGF-β inhibits central memory CD8+ T cell differentiation. Splenocytes from F5 mice were preincubated with 0.1–5.0 ng/ml rhTGF-β1 for 1 h and then stimulated with 10-4 μg/ml cognate peptide for 72 h. (A) Flow cytometric analysis of the phenotypic changes 72 h after peptide stimulation. Cells were stained with anti-CD8a, anti-CD62L, and anti-CD44 (upper panel in [A]) or anti-CD127 (lower panel in [A]). Numbers in each quadrant denote percentage of cells. (B) IFN-γ production in the culture supernatant measured by ELISA. ***p < 0.001 (untreated versus 0.1, 0.5, 1.0, and 5.0 ng/ml rhTGF-β1). (C) Number of CD62Lhigh/CD44high central memory (solid bars) versus CD62Llow/CD44high effector memory (open bars) CD8+ T cells recovered following stimulation with cognate peptide. Total viable cells were determined by trypan blue exclusion, and the total number of CD8+, CD62Llow/CD44high, CD8+, CD62Llow/CD44high, CD8+, CD62Llow/CD44high, CD8+ cells was determined based on flow cytometry data. Data are the mean ± SEM of triplicate samples from three independent experiments. (D) Apoptotic cells, as determined by staining with anti-CD8a and annexin V Abs, after exogenous TGF-β preincubation and peptide stimulation. ***p < 0.01 (untreated versus 1.0 ng/ml rhTGF-β). ***p < 0.001 (untreated versus 0.1, 0.5, and 5.0 ng/ml rhTGF-β). Data are the mean ± SE of triplicate samples from three independent experiments. Statistical significance was measured by one-way ANOVA, followed by Tukey’s multiple comparison test.
memory cells in both T cell compartments (Fig. 2G, 2H, solid bars). In the culture supernatants containing splenic T cells in the presence of 3 μM SD208, there was an increase of IL-2 levels (data not shown), which might account for the reduction of CD4+ T cells via activation-induced cell death (Fig. 2H). In any case, the findings argue that the differentiation of central memory T cells by either CD8+ cells from F5 TCR.Tg mice or CD8+ and CD4+ T cells from wild-type B6 mice can be controlled, in part, through TGF-β receptor signaling.

**Exogenous TGF-β inhibits central memory CD8+ T cell differentiation**

The complexities of TGF-β interactions with T cells were underscored when exogenous TGF-β was added in vitro to F5 CD8+ T cells prior to stimulation with the cognate peptide. Despite endogenous TGF-β production, preincubation of those CD8+ T cells with 0.1–5 ng/ml exogenous TGF-β significantly reduced the percentage of CD8+ T cells expressing the CD62Lhigh/C44high central memory phenotype from 72 to 30% (5 ng/ml rhTGF-β) (Fig. 3A, upper panel) as well as CD62Lhigh/C127high central memory phenotype (55 versus 8%) (Fig. 3A, lower panel). Commensurate with the phenotypic changes was a substantial reduction in the amount of IFN-γ by the peptide-stimulated CD8+ T cells, which has been reported in previous studies (Fig. 3B) (11). Interestingly, the effect of exogenous TGF-β on total cell numbers was not dose dependent (Fig. 3C, bell-shaped curve). Whereas the lowest dose of exogenous TGF-β (0.1 ng/ml) slightly reduced the number of CD62Lhigh/C44high central memory T cells (Fig. 3C, solid bars), the number of T cells expressing the CD62Llow/C44high effector phenotype rose (Fig. 3C, open bars), which was consistent with reduced apoptosis at the lowest dose of exogenous TGF-β (Fig. 3D, U-shaped curve). The lowest dose of exogenous TGF-β did not affect cell proliferation in the CFSE assay (data not shown). In contrast, higher doses of exogenous TGF-β preferentially reduced the number of CD62Lhigh/C44high central memory T cells (Fig. 3D, solid bars) with an accompanying increase in annexin V staining (Fig. 3D).

**TGF-β blockade of central memory T cell differentiation is not mediated through SMAD or MAPK superfamily signaling pathways**

SMAD2, not SMAD3, was phosphorylated 15–60 min after the in vitro addition of 5 ng/ml rhTGF-β to isolated nonactivated CD8+ T cells (Supplemental Fig. 2). SD208 addition suppressed the TGF-β-induced SMAD2 phosphorylation in a dose-dependent manner, starting at 0.3 μM (Fig. 4A). Other well-known SMAD-independent pathways, such as Erk, p38MAPK, and JNK (24), were not activated by exogenous TGF-β stimulation in nonactivated CD8+ T cells, and SD208 addition was inconsequential (Fig. 4A). These results implicated SMAD2 signaling in central memory CD8+ T cell differentiation. Next, splenocytes from SMAD2 conditional knockout mice were stimulated with anti-CD3 and anti-CD28 in the presence of various doses of the anti–TGF-β mAb. Contrary to our expectations, TGF-β blockade still increased the percentage of T cells expressing the CD62Lhigh/C44high central memory phenotype in both CD4+ and CD8+ T cells (Fig. 4B). These observations suggested that, even though SMAD2 is the signaling pathway for TGF-β, it does not seem to be involved in the differentiation pathway for central memory T cells. The possibility of crosstalk between TGF-β signal and mTORC1 pathway, which is known to be involved in central memory differentiation, was subsequently evaluated. TGF-β blockade had no effect on mTORC1 pathway (phosphorylation of p70S6 kinase and S6 ribosome protein) of peptide-activated CD8+ F5 T cells (Fig. 4C), suggesting no crosstalk between TGF-β signaling and mTORC1 pathways. These findings suggest that the TGF-β signaling interruption that facilitates central memory T cell differentiation does not occur via the SMAD2, MAPK superfamily, or mTOR signal transduction pathways, thus suggesting the involvement of a yet-to-be-defined non-traditional pathway(s) that mediates those changes.

**Change of transcriptional factors, Eomes, and T-bet, following TGF-β blockade**

Investigators have reported that both Eomes and T-bet (Th21) are two T-box–containing transcriptional factors that are regulated by mTOR activity and consequently control effector and memory functional decisions in CD8+ T cells (25, 26). Even though the present data do not implicate mTOR signaling during TGF-β blockade, contributing to central memory T cell differentiation, the fate of those transcriptional factors during this treatment was of interest. Blocking of TGF-β signaling with the addition of the anti–TGF-β mAb to CD8+ F5 T cells had no demonstrable effect on T-bet mRNA levels (Fig. 5A), whereas Eomes mRNA expression was significantly upregulated at 72 h following peptide stimulation (0.0042 vehicle versus 0.0115 anti–TGF-β mAb; p < 0.001) (Fig. 5B). These changes led to an overall increase in the Eomes/T-bet mRNA ratio at 72 h (1.05 versus 3.52; p < 0.01) (Fig. 5C). mRNA expression levels of other transcriptional factors, such as Tcf-7, Bmi-1, Bcl-6, and Blimp-1 (Prdm1) (25, 27), were
unchanged following the addition of the anti–TGF-β mAb (Supplemental Fig. 3). When Eomes and T-bet protein levels were measured by intracellular FACS-based staining, the addition of the anti–TGF-β Ab to F5 CD8+ T cells had a dose-dependent increase in Eomes (38.1 versus 48.9% at 10 μg/ml TGF-β mAb), whereas T-bet levels were decreased (43.1 versus 30.4% at 10 μg/ml TGF-β mAb) (Fig. 5D). Those changes resulted in an increased Eomes/T-bet protein ratio (Fig. 5E), which is consistent with differentiation of memory T cells (25).

**Increased CD8+ central memory phenotype alters in vivo proliferation upon recall response**

The functional characteristics of SD208-treated CD8+ T cells that had an increased central memory phenotype were evaluated using an adoptive T cell transfer protocol. As before, splenic CD8+ T cells from F5 mice were stimulated in vitro with the cognate peptide alone or combined with 3 μM SD208. After 96 h, consistent with previous results (Fig. 2A), 62% of the untreated CD8+ T cells expressed CD62Lhigh/CD44high central memory markers, whereas a higher percentage (89%) of SD208-treated T cells expressed the central memory phenotype (Fig. 6A). At that time, T cells were CFSE labeled and adoptively transferred into naive B6 mice. Three days posttransfer, the distribution of the transferred cells in the peripheral blood and spleen was similar (Fig. 6B, left and center graph), whereas a higher percentage of SD208-treated T cells was found in the lymph nodes (3.4 versus 2.6%; p < 0.0016) (Fig. 6B, right graph). Three days after adoptive transfer, the transferred cells had also maintained their respective CD62Lhigh/CD44high central memory phenotype (60 versus 84%, untreated versus SD208 treatment) (Fig. 6C) without any sign of proliferation (Fig. 6D). At that time, the mice were vaccinated with rF-NP68-TRICOM, the cognate peptide engineered in a recombinant avipox vector, to evaluate the ability of the transferred T cells to mount a peptide-specific recall response. Three days postvaccination, mice that were adoptively transferred with the SD208-treated T cells had higher numbers of NP68-specific CD8+ T cells (1.73%, untreated versus 6.31%, SD208 treatment, p < 0.05) (Fig. 6E, 6F). The NP68 tetramer+ SD208-treated adoptively transferred CD8+ T cells had a higher proliferative index, as determined by changes in the CSFE dye dilution assay, than the untreated cells (Fig. 6G, p < 0.01), a characteristic of central memory T cells. A low frequency of NP68-specific CD8+ T cells (0.28%) was found after rF-NP68-TRICOM vaccination of naive, nonadoptively transplanted mice (Fig. 6E). These data collectively suggest that SD208-treated cells showed increased central memory T cells phenotypically were capable of eliciting a strong recall response following encounter in vivo with the cognate peptide.

**Increased central memory T cells in human PBMCs following TGF-β blockade**

As previously stated, like their murine counterparts, isolated human T cells also produce TGF-β following in vitro stimulation (16). Therefore, it was of interest whether TGF-β blockade resulted in similar changes in cellular differentiation in human CD8+ T cells. To address that question, CD45RO+/CD8+ T cells that expressed CD45RA (98%), CCR7 (85%), and CD62L (70%) were isolated from human PBMCs (Supplemental Fig. 4). CD45RO was chosen because its expression profile on activated or memory human T cells is similar to that of CD44 on mouse T cells. Six days after in vitro stimulation in the presence of anti-CD3, ~90% of the isolated CD8+ T cells had CD45RO expression, suggestive memory differentiation (Supplemental Fig. 4B). Furthermore, 45% of the CD45RO+ cells expressed high levels of CD62L (CD62Lhigh), indicative of central memory cells. SD208 addition to the PBMC culture media resulted in a dose-dependent increase in the percentage of cells expressing that phenotype in CD8+ T cells (i.e., 61%: 3 μM SD208, Fig. 7A) as well as an increase in the mean fluorescence intensity (i.e., 9,440 with vehicle versus 15,800 with 3 μM SD208). The acquisition of the phenotypic markers indicative of memory T cell differentiation occurred in the absence of any measurable changes in viable cell number, as...
FIGURE 6. Increased central memory phenotype alters in vivo proliferation upon recall response. Splenocytes from F5 mice were preincubated with 3 μM SD208 or vehicle for 1 h, stimulated with 10^{-4} μg/ml cognate peptide for 96 h, and then stained with anti-CD8, anti-CD62L, and anti-CD44. (A) CD62L and CD44 expression on CD8 T cells 96 h after in vitro stimulation. (B) In vivo distribution of adoptively transferred F5 memory CD8 T cells after 3 d and prior to rFN68-TRICOM vaccination. Mice (3/group) were euthanized, and the peripheral blood, spleen, and inguinal lymph nodes were analyzed for NP68 dextramer staining. Each dot represents a single mouse, and the horizontal line indicates the mean. NS, no statistical significance. (C) A representative FACS plot for each group. The numbers in the top right quadrant denote the percentage of CD62L^{high}/CD44^{high} cells among CD8^{dextramer+} T cells from splenocyte. (D) A representative CFSE dye dilution for each group of CD8^{dextramer+} T cells from splenocyte. (E-G) Recall response 3 d after rFN68-TRICOM challenge. Peripheral blood from rFN68-TRICOM–vaccinated mice (n = 3/group) (no adoptive transfer, adoptive transfer with vehicle-treated cells, and adoptive transfer with SD208-treated cells) was collected and analyzed for NP68 dextramer staining. (E) A representative FACS plot for each group. The numbers in the top right quadrant denote the percentage of dextramer+ cells among CD8^{T} T cells. (F) Each dot represents a single mouse, and the horizontal line indicates the mean. (G) A representative CFSE dye dilution for each group of CD8^{dextramer+} T cells from a single experiment. Three separate experiments were carried out, and the increased proliferation in the SD208-treated T cells was statistically significant (p < 0.01), as measured using the one-way ANOVA, followed by Tukey’s multiple comparison test.

determined by trypan blue (Fig. 7B), and was CD8 T cell specific (i.e., no commensurate change in CD4 T cells; data not shown). As a result, there was an overall increase in the absolute number of central memory T cells (Fig. 7B, solid bar) in CD8 T cells and, similar to the murine cells, a dose-dependent decrease in the number of effector memory T cells (Fig. 7B, open bars). Blockade of endogenous TGF-β slightly increased IFN-γ production, an indicator of CD8 T cell activation (Fig. 7C). These findings indicate that increased central memory cell generation by TGF-β blockade is a characteristic shared by both murine and human CD8 T cells. Differential changes in IFN-γ production in the murine and human T cells may be explained by the use of different modalities to inhibit TGF-β signaling: the anti–TGF-β Ab for mouse T cells, and SD208, a TGF-βRI kinase inhibitor, for human T cells.

Discussion

In the current study, CD8 T splenic T cells from H-2Db-restricted NP68-specific TCR Tg mice (F5 mice) were used as an in vitro model to study TGF-β modulation of intrinsic T cell metabolic pathways and their role(s) in immune T cell differentiation. Upon stimulation with cognate peptide, the CD8 F5 T cells acquire both phenotypic changes and immune effector functions that are reminiscent of the in vivo phases described for an Ag-specific primary immune response, that is, expansion, contraction, and memory. During the initial 72 h after cognate peptide recognition, CD8 F5 T cells enter an expansion phase characterized by CD44 acquisition that distinguishes effector and memory T cells from their naive counterparts. This phenotypic change was immediately followed by CD62L^{high} expression, indicating a shift to central memory T cell differentiation. During that same time interval, there was an early (2- to 4-h post–peptide recognition) spike in TGF-β mRNA prior to a significant increase in TGF-β production commensurate with IL-2 and IFN-γ (Fig. 1). Endogenous TGF-β production by isolated human CD8 T cells stimulated in vitro with PHA has been reported, indicating a shared characteristic within the T cell compartments of the two species (16). The present study also showed that blockade of TGF-β actions using either a blocking Ab or a TGF-βRI kinase inhibitor increased CD62L^{high} central memory T cells independent of changes in either proliferation or cytokine secretion (Fig. 2). Whereas in prior studies the addition of IL-15 during the expansion and memory phase induced CD62L^{high} central memory T cell differentiation (8), the present findings offer a new mechanism by which T cells control their differentiation through changes in the autocrine secretion of TGF-β.

CD62L plays an essential role as a homing receptor to facilitate central memory T cells’ entry into secondary lymphoid or inflamed tissues via high endothelial venules (28). Therefore, its loss could negatively impact recruitment of memory cells to appropriate immune tissues during the generation of an immune response. CD62L expression is regulated by TACE/Adam17-mediated shedding and transcriptionally by Klf2 (6, 29, 30). In the current study, the presence of TGF-β either by endogenous production or exogenous addition reduced the number of CD62L^{high} central
memory cells. Those changes occurred with dramatic, paradoxical effects on T cell proliferation, apoptosis, and IFN-γ production. Exogenous TGF-β addition also significantly reduced CD62L expression, but with an accompanying increase in apoptosis and loss of IFN-γ production. Loss of CD62L expression on resting memory cells began ~24 h post–TGF-β addition (data not shown), suggesting that the effect might be due to changes in transcriptional regulation by Klf2, not shedding by TACE activation. Additional study is needed to address those and other possible explanations.

It was of subsequent interest to examine those intrinsic cellular pathways through which blockade of endogenously produced TGF-β enhanced immunologic memory. Recently, it was reported that inhibition of signal transduction through PI(3)K or mTOR induces CD62L^high central memory CD8^+ T cells (6, 7). Our data indicated that the signaling pathway(s) that regulates central memory differentiation by TGF-β seems to be noncanonical, independent of SMAD, MAPK superfamily, as well as the mTOR pathway (Fig. 5). It is intriguing to contrast the effects of TGF-β with those of IL-2 on memory T cell differentiation. However, the differences seem to reside in IL-2 mediating its changes via the Akt-mTOR signaling pathway, whereas TGF-β seems to signal through another pathway, not Akt-mTOR (Fig. 6). So it leads one to speculate what signals constitute whether the transition to memory T cells proceeds independent of SMAD, MAPK, and mTOR or via the Akt-mTOR pathway. Several differences, including cell type (T cell versus tumor cell) and the characteristics and context within which each cell type encounters TGF-β, might govern the selection of a particular signaling pathway. Within the first 24–36 h after recognition of its cognate peptide, endogenous TGF-β production by the CD8^+ T cells becomes measurable, which continues through the activation phase in a time-dependent fashion. Interruption of the TGF-β signaling pathway either by normal means during the T cell contraction phase or by blockade of TGF-β signaling leads to memory T cell differentiation. In contrast, when the CD8^+ T cells encounter a bolus amount of TGF-β as an external signal prior to entering the activation phase, perhaps these circumstances enact a completely different set of signaling. mTOR becomes activated when it encounters such an external signal, and, because this is such a strong signal, it requires a specific mTOR inhibitor, such as rapamycin, to inhibit the pathway leading to the transition to memory T cells. Contrary to the change in Eomes and T-Bet, other transcriptional factors that have been related to the central memory differentiation, such as Tcf7, Bmi-1, and Bcl-6 (25–27), remained unchanged following TGF-β blockade (Supplemental Fig. 3). Whether there is a common molecule in Eomes/T-bet expression that is shared by TGF-β and IL-2 or it is controlled by an independent mechanism should be a focus for future study.

Subsequent functional analyses of the F5-TCR.Tg CD62L^high memory CD8^+ cells became critical to determine whether the observed phenotypic changes truly represented central memory T cells. CD62L^high memory CD8^+ cells were generated in vitro, and, following their adoptive transfer into naive mice, were found to preferentially migrate to secondary lymph nodes (Fig. 6B). Those findings were in agreement with a previous report using lymphocytic choriomeningitis virus gp33-specific CD62L^high memory CD8^+ T cells (3). Migration to the secondary lymph nodes by the F5-TCR.Tg CD62L^high memory CD8^+ cells seemed to occur passively due to the absence of any measurable change in T cell phenotype or proliferation. Upon re-exposure to the Ag via the rF-NP34-TRICOM vaccine, there was a more robust recall response as measured by a higher number of Ag-specific CD8^+ T cells in mice that were adoptively transferred with SD208-treated F5-TCR.
Tg CD62L^high memory CD8^+ cells (Fig. 6E–G). These results also agree with previous reports that adoptively transferred CD62L^high memory CD8^+ T cells possessed superior proliferative capacity, augmented viral clearance, and were more protective against tumor challenge (1, 3). Thus, CD62L^high cells generated in vitro by TGF-β blockade of F5-TCR.Tg memory CD8^+ cells exhibit both phenotypic and functional characteristics of central memory T cells capable of fortifying the effectiveness of a vaccine. That hypothesis requires additional study because human CD8^+ T cells seem to have a similar response to blockade of TGF-β action, an increase in central memory differentiation.

Recent reports have shown that the energy-sensitive kinase mTOR responds to extrinsic factors that impact cellular metabolic states (ATP-AMP) and can alter differentiation (7, 10). The present results indicate that intrinsically the ongoing autocrine TGF-β production seems to counteract CD62L expression and differentiation of CD8^+ T cell memory. These observations raise the following question: why? As stated previously, CD62L allows central memory differentiation. A similar response to blockage of TGF-β may play a homeostatic role in establishing the size of memory T cell pools and necessary space for the deposition of future pathogen-specific memory cells. One possible explanation is that endogenous TGF-β production may have a homeostatic role in establishing the size of memory pool. If that indeed is the case, then targeting TGF-β signaling may be a viable approach when one needs to enhance Ag-specific CD8^+ T cell memory against a lethal infection or cancer.

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Disclosures

The authors have no financial conflicts of interest.

References

**Supplemental Figure 1.**

*In vitro* memory CD8\(^+\) T cell differentiation (effector memory v.s. central memory) from F5 mice. (A) Primary splenocytes (6x10\(^5\) cells/ml) from F5 mice were labeled with CFSE (final 1 \(\mu\)M) and then stimulated with 10\(^{-4}\) \(\mu\)g/ml of NP68 peptide (filled area) or vehicle (solid line) for 72 hr at which time CFSE dilution was analyzed by gating on CD8a positive cells. (B) Primary splenocytes (6x10\(^5\) cells/ml) from F5 mice were stimulated with 10\(^{-4}\) \(\mu\)g/ml of NP68 peptide and were stained with anti-CD62L, CD44 and CD8a antibodies at 24, 72, 96 hrs and day 6, respectively. At 96 hrs, cells were isolated over Ficoll and rested for 48 hrs in the presence of 10 ng/ml rmIL-2 (day 6). Numbers in the upper right quadrants denote percentage of cells. (C, D and E) On day 6, CD62L\(^{high}\)/CD44\(^{high}\) or CD62L\(^{low}\)/CD44\(^{high}\) CD8\(^+\) T cells were isolated by FACS Aria cell sorter, seeded at a density of 3x10\(^5\) cells/ml in complete media containing 10\(^{-5}\) \(\mu\)g/ml of the cognate peptide. Supernatants were collected 16 hr after restimulation and IL-2 (C) and IFN-\(\gamma\) (D) production were measured by ELISA. (E) Exogenous IL-2 (5 ng/ml) was added to the culture media for each cell type 24 hr after restimulation and total numbers of CD62L\(^{low}\) and CD62L\(^{high}\) cells recovered at day 2 and 5 were assessed by trypan-blue exclusion. Data represent the mean ± SE of triplicate samples from two separate experiments. ***, p<0.001 as determined using the one-way ANOVA followed by Tukey's multiple comparison test. NS – not significant.

**Supplemental Figure 2.**

TGF-\(\beta\) signals through the phosphorylation of SMAD-2, not SMAD-3. CD8\(^+\) T cells were incubated with 5 ng/ml of recombinant human TGF-\(\beta\)1 and harvested at the
indicated time points and examined for phosphorylation of SMAD-2 and -3 using Western immunoblots. Data are from a representative experiment that was repeated once with similar results.

**Supplemental Figure 3.**

**Changes in other transcriptional factors following TGF-β blockade.** Isolated splenic CD8\(^+\) T cells from F5 mice were pre-incubated with 1 µg/ml of TGF-β mAb for 1 hr and then stimulated with 10\(^{-5}\) µg/ml of cognate peptide (NP68 peptide), 1.0 µg/ml of H2Db-dimer X and 2.0 µg/ml of anti-CD28. (A) Tcf-7, (B) Bmi-1, (C) Bcl-6, and (D) Blimp-1 mRNA in the cells was measured by quantitative PCR at indicted time points.

**Supplemental Figure 4.**

**Phenotypic change of human PMBCs after anti-CD3 stimulation.** Human CD45RO\(^-\) cells isolated from PBMCs were stimulated with plate-coated anti-human CD3. Flow cytometric analyses were done on (A) day 0 (before stimulation) and (B) day 6 after stimulation of CD8\(^+\) T cells stained with anti-CD45RA, CD45RO, CD62L and CCR7 antibodies. Numbers in each quadrant denote percentage of cells.
Supplemental Figure 2

phospho-SMAD-3  phospho-SMAD-2  GAPDH

Time after rh-TGF-β (min)

0 15 30 60 120 240

Not detected