Cutting Edge: Programmed Death-1 Defines CD8^+CD122^+ T Cells as Regulatory versus Memory T Cells

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Recent convincing data have shown that naturally occurring CD8+CD122+ T cells are also regulatory T cells. Paradoxically, CD8+CD122+ T cells have been well described as memory T cells. Given their critical role in tolerogenesis versus long-term immunity, it is important to reconcile this profound dichotomy. In this study, we reported that CD8+CD122+ T cells contain both programmed death-1 (PD-1)+ and PD-1− populations. It was CD8+CD122+PD-1+ T cells, but not their PD-1− counterparts, that suppressed T cell responses in vitro and in vivo. This suppression was largely dependent on their production of IL-10. Moreover, the costimulatory signaling of both CD28 and PD-1 is required for their optimal IL-10 production. In contrast, Ag-specific CD8+CD122+ PD-1− T cells were bona fide memory T cells. Thus, CD8+CD122+ T cells can be either regulatory T or memory T cells, depending on their PD-1 expression and Ag specificity. This study reconciles previously contradictory findings and has important implications for tolerance induction. The Journal of Immunology, 2010, 185: 000–000.

**Materials and Methods**

**Mice and Abs**

Wild-type (WT) BALB/c (H-2d) and C57BL/6 (H-2b) mice were purchased from National Cancer Institute (National Institutes of Health, Bethesda, MD). Rag1−/−, IL-10−/− mice, and Thy1.1+ congenic mice were all in B6 background and purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in a specific pathogen-free environment, and all animal experiments were approved by the Animal Care and Use Committee of the University of Texas Health Science Center (Tyler, TX). Anti-IL-10 Ab and rPD-L1 Fc (rPD-L1) were purchased from R&D Systems (Minneapolis, MN), whereas anti–IL-15 Ab was from eBioscience (San Diego, CA). Activating anti-CD3 and anti-CD28 Abs and blocking anti–PD-1 Ab (J43) were purchased from BD Biosciences (San Jose, CA), whereas CTLA4-Ig was bought from BioExpress (West Lebanon, NH).

**Skin transplantation**

Skin donors were 6- to 8-wk-old BALB/c (H-2b) mice, and allograft recipients were 6- to 8-wk-old Rag1−/− mice (B6 background, H-2b). Full-thickness trunk skin was transplanted to the dorsal flank area of recipient mice. Skin allograft rejection was defined as graft necrosis >90%, as described previously (23).

**Analysis of T cell proliferation in vitro by [3H]thyminidine uptakes**

Purified CD8+CD122+ or CD8−CD122− T cells derived from WT or 2C-Tg mice were cultured in 96-well plates (Corning Costar, Corning, NY) in complete RPMI 1640 medium. Irradiated BALB/c spleen cells were also added to the culture to serve as both alloantigen and APC. Twenty-four hours later, cells were harvested and analyzed by a scintillation counter. Cells were pulsed with [3H]thyminidine for last 8 h before harvesting.

**T cell phenotyping, CFSE staining, and FACS sorting or analysis**

For phenotyping, spleen cells from WT B6 mice were isolated and stained with anti-CD8 PE, anti-CD122 FITC, anti-CD44 allophycocyanin or anti-CD62L allophycocyanin, and anti–PD-1 biotin, followed by streptavidin-PerCP (BD Biosciences). For intracellular FoxP3 staining, cells were permeabilized before staining with anti-FoxP3 allophycocyanin in separate samples.
Cells were finally analyzed by a FACSCalibur (BD Biosciences). For CFSE staining, T cells from Thy1.1+ mice were first labeled with CFSE dye (Invitrogen, San Diego, CA; 5μM) at 37°C for 8 min, washed, cultured in the complete RPMI 1640 medium (10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin), stained with anti-CD90.1 PE (BD Biosciences), and analyzed by FACS. To isolate CD3+CD25+ naive T cells, splenocytes were stained with anti-CD3 PE, anti-CD25 FITC, and anti-CD44 PerCP (BD Biosciences), and CD3+CD25+CD44low T cells were then sorted out by FACSAria (BD Biosciences). CD8+CD122+PD-1+ or their PD-1− counterparts were sorted after staining with anti-CD8 PE, anti-CD122 FITC, and anti–PD-1 biotin, followed by streptavidin PerCP. The purity of sorted cells was typically 96%.

Measurement of cytokines in the supernatant
CD8+CD122+ T cells isolated from WT mice were cultured in complete RPMI 1640 medium in 96-well plates in the presence of plate-bound anti-CD3 Ab alone or together with activating anti-CD28 Ab, rPD-L1, CTLA4-Ig, anti–PD-L1 blocking Ab, or T cell-depleted syngeneic splenocytes (APC).

FIGURE 1. Phenotypes of naturally occurring CD8+CD122+ T cells. A. Spleen cells isolated from WT naive B6 mice were stained for CD44, CD62L, PD-1, CD4, CD25, and CD127, as well as intracellular FoxP3. Filled purple histograms exhibit CD127 expression after gating on PD-1+ or PD-1− population of CD8+CD122+ cells. One representative of three separate FACS data is shown. B. FACS-sorted CD8+CD122+PD-1+ and CD8+CD122+PD-1− T cells were cultured in the presence of plate-bound anti-CD3 Ab and T cell-depleted syngeneic splenocytes (APC). Seventy-two hours later, the amount of IL-10 produced per 10⁵ cells was measured and calculated. *p < 0.05; #p < 0.05.

FIGURE 2. CD8+CD122+PD-1+ cells are Treg cells, whereas their PD-1− counterparts can be memory T cells. A. One day before skin grafting from BALB/c donors, Rag1−/− mice received 1×10⁶ CD3+CD25−CD44low responder T cells alone (control, □, n = 6), 2.5×10⁵ CD8+CD122+PD-1− cells alone (○, n = 8), both control and CD8+CD122+PD-1− cells lacking IL-10 (▲, n = 8), or CD8+CD122+PD-1− cells alone (●, n = 8), both control and CD8+CD122+PD-1− cells treated with neutralizing anti–IL-10 Ab (0.1 μg; days 0, 2, 4, 6, and 10) (◆, n = 7), both control and CD8+CD122+PD-1− cells treated with neutralizing anti–CD28 Ab (2.5 μg/ml) (□, n = 8), both control and CD8+CD122+PD-1− cells treated with neutralizing anti–CD28 Ab (2.5 μg/ml), rPD-L1 (5 μg/ml), or APC. In some groups, CTLA4-Ig and anti–PD-1 blocking Ab (J43) (5 μg/ml) were added to the culture. Seventy-two hours later, the amount of IL-10 produced per 10⁵ cells was measured and calculated. *p < 0.05; #p < 0.05.

CUTTING EDGE: CD8+CD122+ CELLS ARE EITHER MEMORY OR Treg CELLS

FIGURE 1.

A

B

C

FIGURE 2.

A

B

C

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Thirty-two hours later, IL-10 levels in the supernatant were detected by ELISA, according to manufacturer’s instructions (Invitrogen).

Results and Discussion

Because memory-like T cells undergoing homeostatic proliferation contained both PD-1- and PD-1+ fractions (21), we examined whether naturally occurring CD8\(^+\)CD122+ T cells also express PD-1. As shown in Fig. 1A, a substantial subset of these cells (30–40%) is PD-1+. Moreover, CD8\(^+\)CD122+ T cells were also CD44\(^{low}\)/CD62L\(^{high}\), resembling a central memory phenotype. However, they did not express FoxP3, suggesting that they are not conventional FoxP3+ Treg cells. Interestingly, CD8\(^+\)CD122+PD-1+ cells were largely CD127 (IL-7R\(^{a}\)) negative, whereas 50% of CD8\(^+\)CD122+PD-1+ T cells were CD127+. Given that expression of CD25 (IL-2R\(^{a}\)) and CD127 on CD4+ T cells discriminates between Treg cells and conventional T cells with CD25+CD127+ being CD4+ Treg cells (24), expression of CD122 (IL-2R\(^{b}\)) and CD127 on CD8\(^+\) cells may define Treg and memory T cells. Hence, CD8\(^+\)CD122+PD-1+ cells could be Treg cells because they are CD127+. Naturally occurring CD8\(^+\)CD122+PD-1+ cells could also include both CD127+ memory T cells and homeostatically proliferated T cells that are largely CD127+ (21). In contrast, we examined IL-10 production by naturally occurring CD8\(^+\)CD122+ T cells 72 h after cell cultures with anti-CD3 Ab and APC. As shown in Fig. 1B, activating CD8\(^+\)CD122+ T cells via anti-CD3 plus APC induced a much higher level of IL-10 production by their PD-1+ than PD-1- components, implying that PD-1 signaling favors their production of IL-10. This is consistent with a previous study that PD-1 signaling preferentially stimulated IL-10 production (25). We then determined the role of T cell costimulation in IL-10 production by CD8\(^+\)CD122+PD-1+ cells. As shown in Fig. 1C, either activating anti-CD28 Ab or rPD-L1 treatment increased IL-10 levels compared with anti-CD3 alone (anti-CD28: 1.7 ± 0.4 versus 0.9 ± 0.2 or rPD-L1: 1.9 ± 0.3 versus 0.9 ± 0.2, both p < 0.05), whereas the presence of APC or treatments with both anti-CD28 Ab and rPD-L1 further increased IL-10 production compared with a single costimulation via either anti-CD28 or rPD-L1. Blocking CD28 by CTLA4-Ig or PD-1 signaling by anti–PD-1 Ab (J43) suppressed IL-10 production (1.8 ± 0.3 versus 3.9 ± 0.7 and 1.6 ± 0.2 versus 3.9 ± 0.7, both p < 0.05), whereas blocking ICOS signaling did not (data not shown). Isotype control Abs did not alter IL-10 levels. These findings suggest that simultaneous signaling via both CD28 and PD-1 is required for their optimal IL-10 production.

Given that CD8\(^+\)CD122+ T cells contained both PD-1- and PD-1+ populations, they could exhibit a differential function. We then examined their in vivo function to either reject a graft or suppress graft rejection. FACS-sorted CD8\(^+\)CD122+PD-1-, CD8\(^+\)CD122+PD-1+, and/or responder T cells (CD3+CD25-CD44\(^{low}\)) from WT B6 mice were transferred to Rag1-/- mice that were transplanted with a skin graft from BALB/c mice 1 d later. As shown in Fig. 2A, neither CD8\(^+\)CD122+PD-1- nor CD8\(^+\)CD122+PD-1+ cells alone rejected a skin allograft, whereas naive CD3+CD25+ T cells rejected all grafts within 25 d. CD8\(^+\)CD122+PD-1+ cell transfer significantly suppressed allograft rejection mediated by CD3+CD25+ T cells (median survival time [MST] = 110 versus 17 d, p < 0.05) with 57% of grafts surviving for >100 d, whereas CD8\(^+\)CD122+PD-1- cell transfer did not delay allograft rejection (MST = 19 versus 17 d, p > 0.05). Interestingly, transfer of CD8\(^+\)CD122+ T cells, which contained both PD-1- and PD-1+ cells, also suppressed allograft rejection (MST = 41 versus 17 d, p < 0.05), albeit at a much smaller magnitude compared with PD-1+ fraction (MST = 41 versus 110 d, p < 0.05). Blocking IL-10 using neutralizing Abs or IL-10-/- CD8+ T cells significantly, but not totally reversed CD8\(^+\)CD122+PD-1+ cell-mediated suppression (anti–IL-10 Ab: 35 versus 110 or 17 d, and IL-10-/-: 29 versus 110 or 17 d, all p < 0.05). Isotype control Ab did not alter graft rejection (data not shown). These data suggest that PD-1-, but not PD-1+, subset of CD8\(^+\)CD122+ T cells is Treg and that in vivo suppression mediated by CD8\(^+\)CD122+PD-1+ T cells is mostly, but not totally, dependent on their production of IL-10, strongly supporting previous studies on the role of IL-10 in the suppression mediated by CD8\(^+\)CD122+ T cells (1–3). Interestingly, their suppression of graft rejection is

**FIGURE 3.** CD8\(^+\)CD122+PD-1+ T cells, but not their PD-1- counterparts, suppress T cell responses in vitro. A, CFSE-labeled CD3+CD25+CD44\(^{low}\)T cells (4 × 10^5/well) from naive Thy1.1 congenic mice were cultured alone, together with 1 × 10^5 per well of CD8\(^+\)CD122+PD1- cells or CD8\(^+\)CD122+PD1+ cells from WT B6 mice or with CD8\(^+\)CD122+PD1- cells from IL-10-/- mice in the presence of plate-bound anti-CD3 Ab and T cell-depleted syngeneic splenocytes (APC). The ratio of suppressor versus responder is 1:4. In some groups, anti–IL-10 Ab (10 μg/ml) was added to the culture. Seventy-two hours after the cultures, cells were stained for Thy1.1 marker and analyzed by FACS. B, Intracellular staining for IFN-γ after similar cell cultures without CFSE labeling. Histograms (A, B) are shown after gating on Thy1.1+ population. Numbers are shown as mean ± SD from three separate experiments.
also partially dependent on IL-15 (Supplemental Fig. 1), implying that IL-15 is important, but not absolutely required for their suppression.

Because CD8⁺CD122⁺PD-1⁻ T cells derived from naive WT mice fail to either reject a graft or suppress graft rejection (Fig. 2A), the question that arises now is whether they are still memory T cells. It is possible that much higher numbers of these PD-1⁻ T cells from WT mice are needed to reject an allograft, given that Ag-specific CD8⁺CD122⁺ cells have been shown to be memory T cells. However, a technical difficulty would prevent us from isolating a large number of these cells that represent only a very small fraction of T cells, because up to 5 × 10⁵ CD8⁺CD122⁺PD-1⁻ T cells transferred to Rag1⁻/⁻ recipients do not reject an allograft, whereas the same number of naive CD3⁺CD25⁺ T cells do (H. Dai and N. Wan, unpublished observation). To address this issue, we used TCR-transgenic CD8⁺ T cells that specifically, but not exclusively, recognize the Ld allogeneic on cells from BALB/c mice (23, 26) and can be tracked by a clonotypic Ab, 1B2. As shown in Fig. 2B, 2.5 × 10⁵ CD8⁺CD122⁺PD-1⁻ T cells isolated from 2C TCR-transgenic mice, which were immunized with BALB/c spleenocytes 6 wk ago, rejected a BALB/c skin graft in Rag1⁻/⁻ recipients as fast as 1 × 10⁵ naive CD3⁺CD25⁺ T cells purified from WT mice (MST = 18 versus 17 d, p < 0.05). These data suggest that transgenic CD8⁺CD122⁺PD-1⁻ T cells from immunized 2C-Tg mice are donor specific and have a higher donor-specific precursor frequency than CD3⁺CD25⁺ T cells from naive WT mice. In contrast, transgenic CD8⁺CD122⁺PD-1⁻ T cells, though donor specific, failed to reject a skin allograft (Fig. 2B). Instead, they suppressed allograft rejection mediated by CD3⁺CD25⁺ T cells (MST = 98 versus 17 d, p < 0.05), confirming that they are true Treg cells. Similarly, neutralizing IL-10 largely abolished this suppression (MST = 37 versus 98 d, p < 0.05). Importantly, these transgenic cells derived from immunized mice rejected an allograft at a faster pace than their CD122⁻ naïve counterparts (MST = 18 versus 35 d, p < 0.05), demonstrating a functional memory feature. To further confirm whether they are bona fide memory T cells, CD8⁺CD122⁺PD-1⁻ T cells and their PD-1⁻ or CD122⁻ counterparts isolated from either naive WT or immunized 2C-Tg mice were cultured in the presence of irradiated BALB/c spleenocytes for 24 h to observe their proliferation. As shown in Fig. 2C, transgenic CD8⁺CD122⁺PD-1⁻ T cells proliferated much faster than their transgenic CD122⁻ naïve counterparts (cpm: 9.9 ± 1.4 versus 3.7 ± 0.8, p < 0.05), whereas proliferation of their nontransgenic WT counterparts or transgenic CD8⁺CD122⁺PD-1⁻ cells was minimal. Hence, Ag-specific CD8⁺CD122⁺PD-1⁻ T cells are indeed memory T cells.

To confirm whether CD8⁺CD122⁺PD-1⁻ T cells are bona fide Treg cells in vitro, they were isolated from naive WT mice and cocultured with CFSE-labeled or unlabeled CD3⁺CD25⁺ naïve T cells derived from Thy1.1⁺ mice in the presence of plate-bound anti-CD3 Ab and T cell-depleted syngeneic splenocytes (APC). As shown in Fig. 3D, CD8⁺CD122⁺PD-1⁻ T cells suppressed the proliferation of Thy1.1⁺CD3⁺ T cells (percentage of proliferated cells: 23 ± 3 versus 70 ± 8, p < 0.05), whereas their PD-1⁻ counterparts did not so (percentage of proliferated cells: 67 ± 7 versus 70 ± 8, p > 0.05). Moreover, they failed to exert suppression in either absence of IL-10 or presence of neutralizing anti-IL-10 Ab (percentage of proliferated cells: 66 ± 6 versus 70 ± 8, or 75 ± 9 versus 70 ± 8, both p > 0.05). Furthermore, they also suppressed intracellular IFN-γ expression by CD3⁺ T cells in an IL-10-dependent manner (Fig. 3B). Hence, our in vitro data also demonstrated that CD8⁺CD122⁺PD-1⁻ T cells are Treg cells and that their suppression is dependent on IL-10. Finally, their suppression is also dependent on PD-1 signaling as blocking PD-1 mostly reversed their inhibition of T cell proliferation in vitro (Supplemental Fig. 2). Studies by Shi et al. (4) have shown that PD-1 is not required for their suppression in vitro. The reason for this discrepancy is unclear. Perhaps it is due to the fact that we used total CD3⁺ T cells as responders and PD-1⁺ fraction of CD8⁺CD122⁺ cells as suppressors, whereas they used CD8⁺ T cells as responders and total CD8⁺CD122⁺ cells as suppressors.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


