Naturally Occurring PD-1+ Memory Phenotype CD8 T Cells Belong to Nonconventional CD8 T Cells and Are Cyclophosphamide-Sensitive Regulatory T Cells

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Naturally Occurring PD-1+ Memory Phenotype CD8 T Cells Belong to Nonconventional CD8 T Cells and Are Cyclophosphamide-Sensitive Regulatory T Cells

Koji Sakuraba,*† Kensuke Shibata,* Yukihide Iwamoto,† Yasunobu Yoshikai,* and Hisakata Yamada*

CD8 T cells expressing memory markers exist in naive mice and are thought to be of heterogeneous origin. It was recently reported that among such memory-phenotype (MP) CD8 T cells in naive mice, those expressing programmed death-1 (PD-1) have immune regulatory activity, but their origin and relationship with other regulatory CD8 T cell subsets remain unclear. In the current study, we examined detailed characteristics and functions of PD-1+ MP CD8 T cells in naive mice. Their expression pattern of surface molecules resembled that of exhausted CD8 T cells seen in chronic viral infection. However, PD-1+ MP CD8 T cells were detected from neonatal periods, even in the thymus; thus, they are naturally occurring. By analyzing bone marrow chimera mice in which conventional CD8 T cells and represent the CP-sensitive suppressor CD8 T cells.

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emoery CD8 T cells, which are responsible for the secondary immune responses, arise from naive CD8 T cells after an exposure to foreign Ags and are phenotypically distinct from naive CD8 T cells. However, in addition to bona fide memory CD8 T cells, there are subsets of T cells expressing memory markers, such as CD44. An example of such memory phenotype (MP) CD8 T cells is those that have been proliferated under lymphopenic conditions (1, 2). Although relatively high affinity for self-peptide/MHC complex is required for the proliferation and phenotypic conversion, they are basically originated from conventional naive CD8 T cells specific for foreign Ags. Another subset of MP CD8 T cells are distinct from conventional CD8 T cells and are also called innate CD8 T cells. These nonconventional CD8 T cells are positively selected by hematopoietic cells, rather than thymic epithelium, and develop independent of Tec family kinase signaling (3). Nonconventional CD8 T cells include CD8 T cells restricted with nonclassical MHC class I b molecules, as well as those restricted with classical MHC class I a molecules. As a part of innate immune system, nonconventional CD8 T cells are equipped with effector functions in situ and contribute to host defense by producing IFN-γ in response to cytokine stimulation (e.g., IL-12 and IL-18) in an Ag-independent manner (4). Thus, MP CD8 T cells that are known to exist in naive mice are likely of heterogeneous origin.

One of the characteristics of MP CD8 T cells is the expression of CD122 (IL-2/15Rβ-chain), which is responsible for their IL-15 dependency. However, interestingly, it was reported that CD122+CD8 T cells in naive mice represented CD8 regulatory T cells (Tregs), which were found in search of cell population(s) responsible for the development of autoimmunity in CD122-deficient mice (5). CD122+ CD8 T cells inhibited autoimmunity caused by CD122+ CD8 T cells in vivo and suppressed proliferation and cytokine production by CD122+ CD8 T cells in vitro. Lately, Dai et al. (6) clarified that, among CD122+CD8 T cells in naive mice, only programmed death-1 (PD-1)+ population has the regulatory activity. PD-1 is a negative costimulatory molecule transiently induced on T cells after TCR stimulation, but is continuously expressed on virus-specific CD8 T cells during chronic viral infection, which is involved in their exhausted state (7). However, it was reported that PD-1 was also expressed on a part of MP CD8 T cells arisen from lymphopenia-induced proliferation (8). In addition, it is unknown whether nonconventional MP CD8 T cells express PD-1 or not. Thus, the origin of PD-1+ MP CD8 T cells in naive mice remains unclear.

The presence of CD8 T cells with immune regulatory activity has long been recognized. In addition to CD122+CD8 T cells, various subsets of CD8 T cells were reported to have regulatory activity (9–11). In earlier studies, it was shown that injecting
cyclophosphamide (CP) in mice enhanced immune responses that were suppressed by complementing T cells from nontreated mice (10). Thus, these suppressor T cells were CP sensitive and were often shown to be CD8\(^+\) (Lyt2\(^+\)) T cells (12). However, identification of such suppressor CD8 T cell population is yet to be done, although CP sensitivity of Foxp3\(^+\) CD4 Tregs was reported instead (13).

In the current study, we characterized PD-1\(^+\) MP CD8 T cells in naive mice to clarify their origin and relationship with other regulatory CD8 T cell subsets. We found that PD-1\(^+\) MP CD8 T cells in normal mice are naturally occurring and are a unique subset of nonconventional CD8 T cells in that they were positively selected by hematopoietic cells, but were IL-15 independent. It was also revealed that PD-1\(^+\) MP CD8 T cells were highly sensitive to CP treatment among T cell subsets in naive mice and were able to suppress delayed-type hypersensitivity (DTH) responses in mice pretreated with CP.

**Materials and Methods**

**Mice**

C57BL/6 mice were purchased from Charles River Japan (Yokohama, Japan). C57BL/6-background IL-15 knockout (KO) mice and \(\beta_2\)-microglobulin (\(\beta_2\)m) KO mice were purchased from Taconic (Germantown, NY). IL-15 transgenic (Tg) mice were reported previously (14). The mice were bred under specific pathogen-free condition in our institute, and 2-d to 12-wk-old mice were used for the experiments. The study design was approved by the Committee of Ethics on Animal Experiment at the Faculty of Medicine, Kyushu University. Experiments were conducted under the control of the Guidelines for Animal Experimentation.

**Abs and flow cytometric analysis**

FITC-conjugated anti-CD44 (IM-7) and anti-Ly6C (AL-21) mAbs, Alexa Flour 488-conjugated anti–IFN-\(\gamma\) (XMG1.2) mAb, allophycocyanin-conjugated anti-CD8a (53-6.7), anti-CD4 (RM4-5) and anti-BrdU mAbs, and PE-conjugated streptavidin were purchased from Becton-Dickinson Biosciences (San Jose, CA). FITC-conjugated anti-CD69 (H1.2F3), anti-CD127 (A7R34), anti-CD122 (TM-b1), anti-Foxp3 (FJK-16s), and anti–IL-10 (JES5-16E3) mAbs, PE-conjugated CD25 (PC61.5) and anti-CD122 (TM-b1), PerCP-Cy5.5–conjugated anti-CD44 (IM-7) and anti-CD45.1 (A20) mAbs; and biotin-conjugated anti-CD279 (PD-1, RMPI-30) mAbs were purchased from eBioscience (San Diego, CA). FITC-conjugated anti-CD62L (MEL-14) mAbs were purchased from Caltag Laboratories (Carlsbad, CA). Alexa Fluor 647-conjugate anti–Tim-3 (B8.2C12), allophycocyanin-conjugated anti-CD223 (LAG-3, C7B7W), and anti-CD152 (CTLA-4, 1C10-4B9) mAbs were purchased from BioLegend (San Diego, CA). For cell surface staining, a single-cell suspension was incubated with the optimal concentration of fluorescent mAbs in PBS for 20 min at 4°C.

**Intracellular staining**

For intracellular cytokine staining, cells were stimulated with 25 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 1 \(\mu\)g/ml brefeldin A (Sigma-Aldrich) was added for the last 3 h of incubation. Stained cells were run on a FACS-Flow Kit.

**BrdU incorporation assay**

Mice received i.p. injection of 1.5 mg BrdU 1 d before sacrifice. After cell surface staining, intracellular BrdU staining was performed according to the manufacturer’s instruction (Becton-Dickinson Biosciences; BrdU Flow Kit).

**CT treatment**

Mice were administrated i.p. with 200 mg/kg CP (Sigma-Aldrich). Spleen cells were harvested on days 2, 4, and 8, and the number of T cell subsets was calculated after flow cytometric analysis. Alternatively, mice were treated with 200 mg/kg CP 4 d before immunization for DTH experiments.

**Cell purification and transfer**

CD8 T cells were negatively selected by staining with anti-CD4 (OKT3) and anti–I-A/E (M5/114.12.5) mAbs, followed by incubation with Dynabeads sheep anti-rat IgG. The enriched CD8 T cells were stained with FITC-conjugated anti-CD44 mAb, allophycocyanin-conjugated anti-CD8a mAb, and biotin-conjugated anti–PD-1 mAb, followed by PE-conjugated streptavidin, and PD1-positive or -negative CD44\(^{hi}\)CD8 T cells were sorted by using a FACS Aria II (Becton-Dickinson Biosciences). The purity of PD1-positive CD44\(^{hi}\)CD8 T cells was >85%, and PD1-negative CD44\(^{lo}\)CD8 T cells was >95%. CD25\(^{+}\)CD4 T cells were FACS sorted after staining with PE-conjugated anti-CD25 and allophycocyanin-conjugated anti-CD4 mAbs. The purity of CD25\(^{+}\)CD4 T cells was >95%. Cells were harvested, washed with HBSS, and injected i.v. into recipient mice (1 \(\times\) 10\(^7\)/each), which had been treated with CP 4 d before. The mice were subsequently immunized with methylated BSA (mBSA) to induce DTH response.

**Induction and assessment of DTH**

Mice were immunized s.c. with 100 \(\mu\)g mBSA (Sigma-Aldrich) emulsified in CFA containing 60 \(\mu\)g Mycobacterium tuberculosis H37RA (Difco, Detroit, MI). Eleven days after the immunization, the mice were challenged by an injection of 100 \(\mu\)g mBSA into one rear footpad, and the other rear footpad received PBS injection as a control. Footpad swelling was measured using a dial caliper (PEACOCK model G; Ozaki Manufacturing) 24, 48, and 72 h after the challenge. The magnitude of the DTH response was determined as the difference in footpad thickness between mBSA- and PBS-injected footpads.

**Statistics**

Statistical significance was calculated using the Student t test using Prism software Version 4.0a (GraphPad Software). The p value <0.05 was considered to be statistically significant.

**Results**

**PD-1\(^+\) MP CD8 T cells are phenotypically distinct population of MP CD8 T cells in naive mice**

Dai et al. (6) showed PD-1\(^+\) CD122\(^{hi}\)CD8 T cells in naive mice exhibited regulatory activity. Because there is a heterogeneity in the expression levels of CD122 in CD44\(^{hi}\) mp CD8 T cells (Fig. 1A, upper left), we first analyzed the relationship between PD-1\(^+\) CD122\(^{hi}\)CD8 T cells and CD44\(^{hi}\)CD8 T cells in the spleen of naive mice. PD-1\(^+\) cells were found in CD8 T cells expressing intermediate level of CD122 (Fig. 1A, upper right). When expression of PD-1 was plotted against CD44, a marker of MP CD8 T cells, virtually all PD-1\(^+\) cells were detected in CD44\(^{hi}\) T cells (Fig. 1A, lower left). Thus, PD-1 is expressed on a part of CD44\(^{hi}\)CD8 T cells that express intermediate levels of CD122 (Fig. 1A, lower right). Because expression of CD44 discriminates MP CD8 T cells more clearly than CD122, we used CD44 as the marker of MP CD8 T cells in the following experiments.

In contrast to the majority of CD44\(^{hi}\)CD8 T cells, which were CD62L\(^{lo}\), Ly6C\(^{hi}\), CD69\(^{lo}\), and CD127\(^{lo}\), most of PD-1\(^+\) CD44\(^{hi}\)CD8 T cells were CD62L\(^{hi}\), Ly6C\(^{lo}\), CD69\(^{hi}\), and CD127\(^{lo}\) (Fig. 1B). These phenotypes of the PD-1\(^+\) MP CD8 cells in naive mice resemble those reported for the virus-specific exhausted CD8 T cells (15). In addition, the frequency of cells capable of producing IFN-\(\gamma\) was lower in PD-1\(^+\) than PD-1\(^-\) cells, although the expression levels of IFN-\(\gamma\) were similar (Fig. 1C). They did not produce significant level of IL-10. Foxp3 was virtually absent in either PD-1\(^+\) or PD-1\(^-\) MP CD8 T cells (Fig. 1D). PD-1\(^+\) MP CD8 T cells were also negative for Tim-3, LAG-3, and CTLA-4, and were positive for CD28 (data not shown).
PD-1+ MP CD8 T cells naturally occur in naive mice

The results of phenotypic analysis suggest PD-1+ MP CD8 T cells in naive mice represent exhausted CD8 T cells that have been induced in the periphery. To test this possibility, we performed longitudinal study examining the presence of PD-1+ MP CD8 T cells in the spleen and the thymus of naive mice from the birth. PD-1+ MP CD8 T cells were detected as early as day 2 after birth not only in the spleen, but also in the thymus (Fig. 2A). Although absolute number of PD-1+ MP CD8 T cells increased with age (Fig. 2B), the percentage of PD-1+ cells among CD44high CD8 T cells was even higher in the neonatal period than the later time periods (Fig. 2C). In addition, in adult mice, CD44high CD8 T cells in the thymus had higher percentage of PD-1+ cells than those in the spleen. These suggest that PD-1+ MP CD8 T cells naturally occur in the thymus, similar to Foxp3+ CD4 Tregs as well as nonconventional CD8 T cells, although possible involvement of phenotypic conversion of naive conventional CD8 T cells induced by the lymphopenia in neonatal period cannot be excluded.
PD-1+ MP CD8 T cells are positively selected by hematopoietic cells

To verify whether the naturally occurring PD-1+ MP CD8 T cells belong to nonconventional CD8 T cell lineage, we examined bone marrow chimera mice in which β2m KO mice were used as the recipients. Although the development of CD44low naive CD8 T cells was severely impaired in the β2m KO recipients, there was a comparable number of MP CD8 T cells with normal frequency of PD-1+ cells (Fig. 3A, 3B). There was also no difference in the absolute number of PD-1+ MP CD8 T cells between WT and β2m KO recipients (Fig. 3C). The phenotypes of PD-1+ MP CD8 T cells developed in β2m KO recipients were similar to those in WT mice (data not shown). Therefore, PD-1+ MP CD8 T cells in naive mice are most likely selected by hematopoietic cells and therefore belong to nonconventional CD8 T cells. We detected few, if any, CD8 T cells expressing CD44 and PD-1 in unmanipulated β2m KO mice (data not shown), suggesting that CD8 T cells that developed totally independent of β2m did not significantly affect the analysis.

PD-1+ MP CD8 T cells are IL-15 independent

Although IL-15 dependency is known as a feature of MP CD8 T cells including nonconventional CD8 T cells, PD-1+ MP CD8 T cells expressed relatively low level of CD122, as shown in Fig. 1. Actually, the presence of IL-15–independent population of MP CD8 T cells in naive mice was also reported (16). So, we examined the number of PD-1+ MP CD8 T cells in mice deficient for or overexpressing IL-15 (Fig. 4). Although CD44highCD8 T cells were reduced in IL-15 KO mice (Fig. 4B, 4D), the percentage of PD-1+ cells in CD44high cells was increased (Fig. 4A, 4C). Conversely, CD44highCD8 T cells were increased in mice overexpressing IL-15, whereas the percentage of PD-1+ cells in CD44high cells was decreased. By calculating the absolute number, it was revealed that the number of PD-1+ CD44highCD8 T cells was not affected by the lack or overexpression of IL-15 (Fig. 4E). PD-1+ cells in IL-15 KO and Tg mice were phenotypically similar to those in WT mice (data not shown). Thus, PD-1+ cells are a unique population of nonconventional CD8 T cells that are IL-15 independent.

PD-1+ MP CD8 T cells are rapidly cycling and CP sensitive

It seems likely that PD-1+ MP CD8 T cells represent the IL-15–independent population of MP CD8 cells in naive mice reported by Boyman et al. (16). Expression pattern of surface markers, as shown in Fig. 1, was also compatible. Because rapid turnover was reported to be another characteristic of the IL-15–independent MP CD8 T cells, we compared steady state BrdU incorporation of PD-1+ MP CD8 T cells with that of other T cell populations, including PD-1− MP CD8 T cells as well as CD25+ Foxp3+ CD4 Tregs. In fact, PD-1+ MP CD8 T cells were the highest in the percentage of cells uptaking BrdU among CD8 T cell subsets in naive mice (Fig. 5). Thus, PD-1+ MP CD8 T cells showed rapid turnover at a steady state. We also found that the percentage of BrdU+ cells in CD25+
CD4 T cells, most of which were Foxp3+ (data not shown), was much lower than that of PD-1+ MP CD8 T cells and was comparable to that of CD4 non-Tregs as well as naive CD8 T cells. These data raised a possibility that PD-1+ MP CD8 T cells are highly sensitive to CP treatment, which preferentially deplete cycling cells and were also used to deplete suppressor T cells in earlier studies (10). So, we measured the number of various T cell populations in the spleen of naive mice daily after CP administration. Although CP treatment decreased overall cell number of CD8 and CD4 T cells (Fig. 6A, 6D), MP CD8 T cells, especially those expressing PD-1, were highly sensitive to CP treatment, as shown by the decreased percentages among CD8 T cells (Fig. 6B, 6C). The phenotype of the remaining small number of PD-1+ cells was similar to those in nontreated mice (data not shown). In contrast, CP treatment decreased only the absolute number, but not the percentage of CD25+ Foxp3+ CD4 T cells among CD4 T cells (Fig. 6E, data not shown), indicating that they have the same CP sensitivity as other CD4 T cells. Thus, PD-1+ MP CD8 T cell population was the most sensitive to CP treatment among T cell subsets.

**PD-1+ MP CD8 T cells are the CP-sensitive Tregs suppressing DTH response**

To verify whether PD-1+ MP CD8 T cells represent the CP-sensitive suppressor CD8 T cells, we employed a classical DTH experiment. Mice were treated with CP 4 d before immunization with mBSA and challenged with mBSA 11 d later. As shown in Fig. 7, CP treatment enhanced DTH responses, which were significantly reversed by transferring PD-1+ MP CD8 T cells before immunization. Transferring PD-1+ CD44hiCD8 T cells showed no effect. We also examined the effect of transferring CD25+CD4 Tregs in the same system, but they failed to suppress the DTH response augmented by CP pretreatment. Thus, it was revealed that PD-1+ MP CD8 T cells in naive mice are naturally occurring CP-sensitive Tregs suppressing DTH response.

**Discussion**

Although the presence of CD8 T cells with immune regulatory activities has long been recognized, their characteristics are still not fully understood. This might be, at least in part, because of the heterogeneity and complexity of regulatory CD8 T cell subsets, each of which might be involved in different aspects of immune responses (11). Suzuki and colleagues (5) demonstrated that naturally occurring CD8 Tregs expressed CD122, although it was widely accepted that CD122 is a marker of memory CD8 T cells. This gave rise to debates on their relationship, but Dai et al. (6) finally identified PD-1 as a more selective marker of the MP CD8 Tregs.

In the current study, we examined the origin of PD-1+ MP CD8 T cells in naive mice. Because PD-1 is well known for its expression on exhausted pathogen-specific T cells in chronic infection (7), one may think that the PD-1+ MP CD8 T cells found in naive mice represent such exhausted CD8 T cells induced by chronic exposure to pathogens or environmental Ags. In this case, importantly, PD-1+ MP CD8 T cells are originated from conventional naive CD8 T cells. Similarities in the expression pattern of surface markers on PD-1+ MP CD8 T cells in naive mice and that on virus-specific exhausted CD8 T cells support this hypothesis (15). However, other findings argue against it. First, PD-1+ MP
and transferred with sorted PD-1+ (CP + PD-1+, Tregs. Alternatively, only limited subsets of PD-1+ cells have Treg regulatory activity, but their specific markers have not been unknown. It is likely that Qa-1–restricted CD8 T cells consist of nonconventional CD8 T cells (3). These suggest that the PD-1+ MP CD8 T cells in naive mice are subsets of nonconventional CD8 T cells that naturally occur in the thymus, similar to CD25+ Foxp3+ CD4 Tregs.

Perhaps the best-characterized population of CD8 Tregs are those restricted with Qa-1, a nonclassical MHC class Ib molecule (9). They target activated CD4 T cells expressing Qa-1 to suppress autoimmune responses. Interestingly, similar to PD-1+ MP CD8 T cells, MHC class Ib-restricted CD8 T cells, if not all, can undergo hematopoietic cell-induced positive selection and express memory markers (17, 18). However, it was demonstrated that the Qa-1–restricted CD8 Tregs were IL-15 dependent (19), indicating the Qa-1–restricted CD8 Tregs are not identical to the PD-1+ MP CD8 Tregs analyzed in this study. Expression of PD-1 on Qa-1–restricted CD8 T cells is also unknown. It is likely that Qa-1–restricted CD8 T cells consist of heterogeneous populations, only part of which have the regulatory activity, but their specific markers have not been reported.

With regard to the relationship with other reported subsets of CD8 Tregs, PD-1+ MP CD8 T cells were distinct in the expression pattern of surface molecules, including Tim-3, LAG-3, CTLA-4, and CD28. Notably, PD-1+ MP CD8 T cells were heterogeneous in the expression of surface molecules, such as CD62L and Ly6C. This suggests that there are subpopulations of PD-1+ MP CD8 Tregs. Alternatively, only limited subsets of PD-1+ cells have Treg activity. In addition, functional differences in PD-1+ MP CD8 T cells in different organs, including the thymus, remain unclear, although they showed similar phenotypes (data not shown). These are important issues to be investigated, but are currently difficult to test due to the small cell number.

Earlier studies showed that suppressor T cells were sensitive to CP treatment (10, 20). Thus, mice pretreated with CP showed exacerbated immune response, which was normalized by transferring T cells from untreated mice. As CP is an alkylating agent, it preferentially kills T cells on cell cycling. In this study, we demonstrated that PD-1+ MP CD8 T cells were the highest in the percentage of cells that had incorporated BrdU among T cell subsets in naive mice. In fact, CP treatment selectively reduced PD-1+ MP CD8 T cells. Furthermore, PD-1+ MP CD8 T cells were able to suppress DTH response that was augmented by CP treatment, suggesting that PD-1+ MP CD8 T cells represent the CP-sensitive suppressor T cells observed in the earlier studies. In contrast, CD25+ Foxp3+ CD4 Tregs, although sometimes referred to as a CP-sensitive T cell population (13), did not show higher cell cycle rate than other subset of T cells. Actually, CP treatment similarly reduced the number of CD4 Tregs and other CD4 T cells. Thus, percentage of CD4 Tregs did not change by CP treatment. In addition, we observed that purified CD25+CD4 T cells failed to suppress DTH responses. Interestingly, it was recently reported that in vivo depletion of Foxp3+ Tregs at the time of immunization rather suppressed DTH responses (21), adding support to our data.

As mentioned above, we demonstrated in this study that PD-1+ MP CD8 T cells were IL-15 independent. This is in line with the fact that IL-15–deficient mice do not spontaneously develop autoimmunity or lymphoproliferative disorder, which is in sharp contrast with the case of IL-2–deficient mice (22). Interestingly, IL-15 was shown to be dispensable for the maintenance of Ag-specific CD8 T cells in chronic viral infection, which were rather maintained by antigenic stimulation (23). Boyman et al. (16) reported that MP CD8 T cells in naive mice include IL-15–independent population, which is maintained by contact with self-peptide/MHC complex. These suggest that the PD-1+ MP CD8 T cells are maintained in a similar manner and, therefore, most likely recognize self-Ags. The expression pattern of surface molecules on PD-1+ MP CD8 T cells, which resembled that of virus-specific CD8 T cells in chronic infection, again suggests the chronic antigenic stimulation. In this regard, noteworthy is our observation that a substantial part of H-Y Ag-specific TCR Tg CD8 T cells developed in male mice expressed PD-1 (K. Sakuraba and H. Yamada, unpublished observations). However, curiously, these PD-1+ cells were CD44low, whereas PD-1+ population was uniformly CD44high. Thus, they were phenotypically distinct from the exhausted PD-1+ CD8 T cells as well as the naturally occurring PD-1+ MP CD8 T cells. Furthermore, we did not detect immune regulatory activity of these self-specific PD-1+ CD8 T cells (K. Sakuraba and H. Yamada unpublished observations), leaving the Ag specificity of PD-1+ MP CD8 Tregs unclear. Requirement of Ag recognition in exerting regulatory functions of PD-1+ MP CD8 Tregs is another important issue to be determined.

We showed in this study that naturally occurring PD-1+ MP CD8 Tregs are a novel subset of nonconventional CD8 T cells. Thus, although nonconventional CD8 T cells are well known for their effector functions in innate host defense mechanism, our results provide evidence for the presence of additional population of nonconventional CD8 T cells with immune regulatory activity. Further investigation on PD-1+ MP CD8 T cells would shed light on novel insight into the mechanism of T cell–mediated immune regulation.
Disclosures
The authors have no financial conflicts of interest.

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