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Programmed Death-1 Shapes Memory Phenotype CD8 T Cell Subsets in a Cell-Intrinsic Manner

Joanna J. Charlton,*†,1 Ioannis Chatzidakis,*†,1 Debbie Tsoukatou,* Dimitrios T. Boumpas,*† George A. Garinis,*† and Clio Mamalaki*

Memory phenotype T cells, found in unimmunized mice, display phenotypic and functional traits of memory cells and provide essential protection against infections, playing a role in both innate and adaptive immune responses. Mechanisms governing homeostasis of these memory phenotype T cells remain ill-defined. In this study, we reveal a crucial role of the negative costimulator programmed death-1 (PD-1) in regulating developmental fates of memory phenotype cells. Thus, in lymphoid organs and tissues of PD-1 knockout (KO) mice a marked accumulation of functional effector memory (TEM) phenotype CD8 T cells was observed. TEM phenotype cells from PD-1 KO mice exhibit decreased proliferation but increased survival potential. These cells could produce effector molecules constitutively, in response to phorbol esters or through bystander activation by innate stimuli. Similarly, in lymphopenia-induced proliferating CD8 T cells, whereby normally naive T cells acquire a memory phenotype, skewing toward a TEM phenotype was prominent in the absence of PD-1. Acquisition of the TEM phenotype was a CD8 T cell–intrinsic phenomenon as demonstrated by mixed bone marrow transfer experiments. Importantly, adoptively transferred PD-1 KO CD8 central memory T (TCM) cells converted into the TEM phenotype, indicating that PD-1 sets a major checkpoint in the TCM to TEM phenotype differentiation process. This was reflected by distinct patterns of gene expression of PD-1 KO TCM phenotype cells revealed by global transcriptional analysis. Additionally, adoptively transferred PD-1 KO TEM phenotype cells converted to a lesser degree to a TCM phenotype. Collectively, these data suggest that PD-1 shapes memory phenotype CD8 T cell subsets. The Journal of Immunology, 2013, 190: 6104–6114.

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emory phenotype (MP) T cells are found in normal, unimmunized mice and display phenotypic and functional traits of memory cells; they account for 10–20% of T cells in young mice, and their number increases with age. It is thought that they are generated as a result of lifetime exposure to various environmental Ags, self-Ags (1), or even simply by homeostatic expansion mechanisms. Signaling by IL-7 and/or other common γ-chain cytokines, such as IL-15, can induce naïve T cells to undergo homeostatic proliferation and convert into cells with a memory phenotype (2). Apart from their role in secondary adaptive immune responses, MP cells seem to display important innate immune responses that provide early protection against pathogens during a primary response mostly by producing IFN-γ (bystander activation) in response to IL-12, IL-18, and IFN-α/β produced by macrophages and dendritic cells (3, 4). MP and Ag-specific memory CD8 T cells can be broadly divided into central memory (TCM) and effector memory (TEM) cells based on differential expression of CCR7 and CD62L and different properties regarding effector functions, migration to lymphoid organs or tissues, as well as proliferation in response to Ag or cytokines (5). TCM cells are CD44hiCD62LloCCR7hi and migrate preferentially to lymph nodes, whereas TEM cells are CD44hiCD62LhiCCR7lo and are mostly located in spleen, peripheral tissues, and bone marrow. TEM cells provide immediate effector functions at the site of pathogen entry through production of lytic molecules such as perforin and granzymes as well as IFN-γ (5–8).

Several models have been proposed to explain the lineage relationship of TEM and TCM Ag-specific memory subsets. The question of memory subset interconversion has been addressed in different experimental systems, and both conversion of TEM to TCM cells (9, 10) and TCM to TEM cells have been reported (11–13). Importantly, recent studies have shown that a single naive precursor cell is able to give rise to all different memory subsets (14, 15). Although mechanisms governing subset differentiation of memory T cells is the subject of intense investigation, homeostasis of MP T cell subsets is less well studied.

Costimulation has been shown to be a critical parameter in determining the developmental fate of memory T cells (16–22). Programmed death-1 (PD-1) is an immunoreceptor that belongs to the CD28/CTLA-4 family and is expressed (among others) on activated CD4 and CD8 T cells. PD-1 negatively regulates TCR signaling upon engagement of one of its ligands PD-1 and PD-1 ligand 2 (23, 24). Apart from the established role of PD-1 in peripheral T cell tolerance, its role in immunity and infection is also well described. PD-1 is highly expressed on virus-specific
CD8 T cells in chronic infections and is correlated with an "exhausted" T cell phenotype that is reversed upon PD-1 neutralization (25, 26). The PD-1 pathway can compromise CD8 T cell responses during some acute infections and contributes to the functional impairment of "helpless" CD8 T cells (27). The role of PD-1 in generation, maintenance, and function of MP CD8 T cells is less clear. MP CD8 T cells express PD-1, especially in aged mice, but to a lesser extent compared with MP CD4 T cells (28), and most PD-1–expressing MP CD8 T cells belong to the TEM phenotype. Interestingly, PD-1 expression on MP CD8+ CD122+ T cells defines an IL-10–producing regulatory T cell population (29). In settings of lymphopenia, a short-lived PD-1– fraction has been identified among homeostatically proliferating (lymphoepnea-induced proliferating, LIP) CD8 T cells, characterized by poor functional responses (30).

In this study we demonstrate a crucial role of PD-1 in differentiation of MP CD8 T cells. Our data reveal that PD-1 impedes accumulation of TEM phenotype CD8 T cells through promoting their apoptotic death and by inhibiting conversion of TCM to TEM phenotype.

Materials and Methods

Mice

PD-1 knockout (KO) (31), GFP-transgenic (32), and DsRed-transgenic mice (33) have been previously described. All mice were backcrossed to the C57BL/10 background for 10 generations. C57BL/10 (referred to as wild-type, WT) and C57BL/10.PD-1–deficient mice (PD-1 KO) were used in the current study. Mice were maintained in the Institute of Molecular Biology and Biotechnology colony. All experiments were approved by the General Directorate of Veterinary Services, Region Crete.

Flow cytometry

Cells from spleen, thymus, lymph nodes, and blood were prepared for flow cytometry as previously described (34). The following Abs, as well as PE–Cy5, anti–CD8a-allophycocyanin, anti–CD8b-allophycocyanin, anti–CD69–PE, anti–CD44–PE, anti–CD28–PE, anti–CD45–PE, anti–Gr1–PE, and anti–CD127–PE, were from BD Biosciences (BD Biosciences) plus complement (Cedarlane Laboratories, catalog no. CL5031) was used according to the manufacturer’s instructions, and subsequently stained for intracellular cytokines and analyzed by flow cytometry.

Transfer of sorted CD8+ T cell subsets

CD8+ T cells were purified from spleen with the negative selection MACS magnetic beads separation system (Miltenyi Biotec) according to the manufacturer’s instructions. Purified CD8+GFP+ T cells were stained with anti–CD44–PerCP-Cy5.5, anti–CD8–allophycocyanin, and anti–CD62L–PE for the purification of TEM (CD8+CD44hiCD62Lhi), TCM (CD8+CD44hiCD62Llo), or naive cells (CD8+CD44lo) and sorted with a Dako MoFlo T high-performance cell sorter. Cells (1.5 × 106) were then adoptively transferred into WT and PD-1 KO mice. Mice were killed on day 42, and CD62L and CD44 expression on donor-derived GFP+CD8+ T cells. In the case of naive cells, recipients were sublethally irradiated (450 rads).

For SNARF-1 (seminaphorhodafuor-1-carboxylic acid acetate succinimidyl ester; Molecular Probes) labeling, purified cells (10–20 × 106/ml) were labeled with 25 μM SNARF-1 in PBS, for 30 min at 37°C, as described (34).

Microarray hybridizations and analysis

Spleen cells from 7-mo-old WT and PD-1 KO mice were sorted for CD8 TCM cells as described above. RNA was then extracted by standard procedures according to manufacturer’s instructions (Qiagen). For genome-wide expression analysis of these cell populations, synthesis of double-stranded cDNA and biotin-labeled cRNA was performed according to the instructions of the manufacturer (Affymetrix). Fragmented cRNA preparations were hybridized to full mouse genome oligonucleotide arrays (15,000; 430G 2.0 array; Affymetrix). Initial data extraction and normalization within each array were performed by means of GeneChip operating software (Affymetrix). Microarrays complied with the Minimum Information About a Microarray Experiment and are available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress, accession number E-MTAB-1509). Expression intensities from the PD-1 KO TCM phenotype genome-wide data and corresponding controls were log transformed and normalized within and between arrays with the quantile normalization method using the R open statistical package (http://www.r-project.org/). Two-tailed, paired t-test analysis or a two-way ANOVA was used to extract the statistically significant data from each group of mice by means of the Spotfire Decision Site software package 7.2 version 10.0 (TIBCO Spotfire, Somerville, MA). The criteria for significance were set at p ≤ 0.05 and a ± 1.5-fold or more change in gene expression. The Affymetrix 430 2.0 arrays include several internal controls to ensure accurate and reproducible measurement of gene expression changes. For each probe set, signals were considered to be valid when they were marked as "present" (for more information, see http://www.affymetrix.com) and exhibited a signal >40 in at least one microarray hybridization. All probe sets with a signal <40 and ± 1.5-fold or more change in gene expression were considered for further analysis. Significant overrepresentation of fifth-level gene ontology terms describing "biological process" annotation (GOTERM_BP_5) was identified with the National Institute of Allergy and Infectious Diseases Database for Annotation, Visualization and Integrated Discovery Web site (http://www.david.abcc.ncifcrf.gov). Generation of mixed bone marrow chimeras

Bone marrow was obtained from femurs of GFP-transgenic and PD-1 KO mice. Mature T cells were first depleted by the use of anti-CD90.2 (BD Biosciences) plus complement (Cedarlane Laboratories), according to manufacturers’ instructions. Contamination of bone marrow cells with mature T cells was <0.1%. A mixture of 105 WT and PD-1 KO bone marrow cells
at a 1:1 ratio was injected i.v. into DsRed mice lethally irradiated with 950 rads. Cells from these chimeras were analyzed after 8 wk.

Results

**Increased numbers of TEM phenotype CD8 T cells in lymphoid organs and tissues of PD-1 KO mice**

We analyzed splenocytes from young (2- to 4-mo-old) and middle-aged (7- to 14-mo-old) C57BL/10 (WT) and C57BL/10.PD-1 KO (PD-1 KO) mice for the presence of CD8^+CD44^hi (MP CD8) cells. As expected (1), middle-aged WT mice had accumulated more CD8^+CD44^hi T cells than did young WT ones (9.6 versus 3.8 × 10^6; Fig. 1A). Splenocytes from either young or middle-aged PD-1 KO mice contained slightly, but significantly, higher numbers of MP CD8 T cells compared with WT mice of respective age (Fig. 1A). When we further categorized these cells to T CM phenotype (CD44^hiCD62L^hi) or TEM phenotype (CD44^hiCD62L^lo) we found that young and middle-aged PD-1 KO mice contained ~3- and ~5.5-fold, respectively, more TEM phenotype CD8 cells in spleen than did their WT counterparts (Fig. 1B, 1C). As expected, TEM phenotype cells expressed low levels of CCR7, as shown by co-regulation of CD62L and CCR7 expression on WT and PD-1 KO CD8 T cells (Fig. 1D). Naive and T CM phenotype CD8 T cell numbers were not significantly different between WT and PD-1 KO mice in any age group (Fig. 1C).

Because TEM cells migrate preferentially to tissues, we analyzed CD8^+ T cells isolated from liver, lung, peritoneal cavity, and bone marrow. In all tissues the percentage of TEM phenotype cells among CD8^+ T cells was significantly higher in PD-1 KO mice. Similar results were obtained in blood (Fig. 2A). When we consider that recovered CD8^+ T cells were more numerous in all PD-1 KO tissues examined, TEM phenotype cells were from ~5-fold (in bone marrow) to ~9-fold (in lung) more abundant when compared with tissues from WT animals (Fig. 2B). It is possible that the observed differences were due to increased preferrence of PD-1 KO TEM phenotype CD8 cells to migrate from lymph nodes to tissues. However, when lymph nodes from WT and PD-1 KO mice were examined, the same trend was observed; that is, TEM phenotype CD8 T cells were significantly more numerous in lymph nodes from PD-1 KO mice (Fig. 2).

**Phenotypic and functional analysis of PD-1 KO TEM phenotype CD8 T cells**

CD44^hiCD62L^loCCR7^lo TEM phenotype cells have been reported to express CD127 (IL-7Rα) and CD122 (IL-2Rβ-chain), whereas they lack CD25 (IL-2Rα). We investigated expression of several activation/memory markers on the surface of accumulated PD-1 KO TEM phenotype CD8 T cells (Fig. 3A; we found that TEM phenotype cells from both PD-1 KO and WT mice were CD25^−, consistent with a memory and not a recently activated effector phenotype. CD122 was found to be expressed on a larger fraction of PD-1 KO TEM phenotype cells compared with WT (93 versus 65%), suggesting a possible role of IL-15 in the homeostasis of the accumulated cells (1). Although CD122 was expressed on a slightly lower percentage of TEM cells from PD-1 KO mice, the absolute number of CD127 TEM phenotype CD8^+ cells was found to be 3-fold higher compared with WT spleens as a consequence of increased numbers of TEM cells in spleen of PD-1 KO mice (Fig. 3B). Interestingly, there was a percentage of PD-1 KO and WT TEM phenotype CD8^+ cells that expressed the early activation marker CD69, and this was increased in the PD-1 KO cells (Fig. 3A). However, these cells could not be typical effectors because they were uniformly CD25^−.

GzmB is one of the most important effector molecules produced by armed cytotoxic CD8 T cells. GzmB expression was assayed ex vivo in WT and PD-1 KO CD8 T cells from middle-aged mice. In TEM phenotype CD8 T cells there was a discrete GzmB^hi population that was significantly larger when cells came from PD-1 KO mice (5 versus 23%) (Fig. 3C).

One of the cardinal features of memory CD8 T cells is the fast recall responses, for example, production of effector molecules after brief stimulation with phorbol esters. In such an assay, IFN-γ is accumulated only in CD44^hi MP cells and not in naive CD8
T cells. Because it was not possible to assess IFN-γ production by T<sub>EM</sub> and T<sub>CM</sub> subsets owing to rapid shedding of CD62L after TCR stimulation (36), we performed this assay on isolated CD8<sup>+</sup>CD44<sup>hi</sup>CD62L<sup>lo</sup> TEM phenotype cells. As shown in Fig. 3D and 3E, a higher proportion of T<sub>EM</sub> phenotype CD8<sup>+</sup> T cells from PD-1 KO spleens produced IFN-γ. Additionally, a smaller percentage of PD-1 KO T<sub>EM</sub> cells produced IL-2 compared with PD-1 KO T<sub>CM</sub> cells (Fig. 3F), in agreement with previously described subset phenotypes (8, 10).

To investigate whether accumulation of PD-1 KO T<sub>EM</sub> phenotype CD8<sup>+</sup> cells is due to increased proliferation, we analyzed cell cycle by Ki-67 expression and BrdU incorporation assays. Both of these experiments showed that PD-1 KO T<sub>EM</sub> phenotype cells cycle slower than do their WT counterparts, thus strongly suggesting that their accumulation is not due to enhanced rate of proliferation (Fig. 3G, 3H). Next, we wanted to examine whether cell survival is involved in accumulation of PD-1 KO T<sub>EM</sub> phenotype cells. Ex vivo annexin V binding assays showed that a higher percentage of WT T<sub>EM</sub> phenotype cells was annexin V<sup>+</sup> (Fig. 3I), indicating a contribution of survival in the accumulation of PD-1 KO T<sub>EM</sub> phenotype cells.

In conclusion, T<sub>EM</sub> phenotype CD8<sup>+</sup> T cells are substantially accumulated in lymphoid organs and tissues of PD-1 KO mice where they display significantly enhanced characteristics of TEM cells, and decreased potential to apoptosis may contribute to their accumulation.

**PD-1 pathway prevents differentiation of LIP memory CD8 T cells to T<sub>EM</sub> phenotype**

Naive T cells undergoing lymphopenia-induced homeostatic proliferation acquire a MP similar to central memory cells without passing through an effector phase (37, 38), and they become capable of mediating protective immunity against pathogens (39). To examine whether PD-1 mutation perturbs normal development of LIP memory T cells we transferred purified naive (CD44<sup>lo</sup>) GFP, WT or GFP.PD-1 KO CD8<sup>+</sup> T cells to sublethally irradiated WT hosts. CD8<sup>+</sup> T cell subset analysis showed that by day 20, a significant population of T<sub>EM</sub> phenotype PD-1 KO cells arose and...
became by far the predominant one in host spleens (Fig. 4A). Higher numbers of PD-1 KO T EM phenotype cells were recovered when compared with WT, with a parallel decrease in the number of PD-1 KO TCM phenotype cells (Fig. 4B). These results might suggest that in the absence of PD-1, T EM cells accumulate at the expense of the other CD8 subsets. Importantly, a much larger fraction of PD-1 KO-derived T EM cells were GzmB hi when assayed directly ex vivo (Fig. 4C). Additionally, analysis of transferred cells at earlier time points in the host’s blood (day 5) revealed that initially both WT and PD-1 KO naïve donor cells gave rise mostly to T CM phenotype cells (Fig. 4D); at later time points T EM phenotype cells progressively emerged and formed the largest subpopulation by day 20, when PD-1 KO cells were transferred. This suggests that PD-1 regulates T CM to T EM subset differentiation in lymphopenic conditions. The fact that we transferred purified naïve WT or PD-1 KO CD8+ T cells and hosts were always WT is suggestive of a CD8 cell-intrinsic mechanism.

In conclusion, our results show that PD-1 signaling in CD8 T cells can modulate the homeostasis of the MP pool by impeding differentiation toward a functional T EM phenotype, most probably from a T CM phenotype intermediate.

Accumulation of T EM phenotype CD8 T cells depends on cell-intrinsic mechanisms

To examine further whether the effect of PD-1 was indeed intrinsic to the CD8 T cells, we performed mixed bone marrow chimera experiments transferring mixtures consisting of equal numbers of PD-1 KO and GFP.WT bone marrow cells to lethally irradiated DsRed.WT hosts. In these settings, PD-1 KO and WT CD8 T cells mature and respond to the same environmental cues, and any observed differences should be attributed to intrinsic factors. Eight weeks after transfer we analyzed thymi, spleens, and lymph nodes from hosts and the ratios of donor-derived WT and PD-1 KO T cells were evaluated. Analysis of thymi showed equal contribution of WT- and PD-1 KO-derived cells in thymocytes and similar percentages of CD8 single-positive (SP) cells (Fig. 5A). The mean PD-1 KO/WT ratio for these chimeric mice was 1.0 for CD8+ SP thymocytes (Fig. 5A, right), suggesting that PD-1 KO bone
marrow cells had no general thymic developmental advantage over WT counterparts. In contrast, the majority of donor-derived CD8+ cells in spleens were of PD-1 KO origin (Fig. 5B), suggesting that postthymic events are the cause of increased PD-1 KO-derived peripheral CD8+ T cells. Further subtype analysis in spleens and mesenteric lymph nodes showed that there was a significantly higher proportion of T EM phenotype cells in PD-1 KO mice, whereas a smaller proportion of recovered PD-1 KO TCM phenotype donor-derived cells. This was also true but to a lesser degree for PD-1 KO TCM phenotype donor-derived cells. Similar degrees of abnormal conversion and high recoveries were also obtained when PD-1 KO TCM cells were transferred to WT hosts but not when WT TCM cells were transferred to PD-1 KO mice (Supplemental Fig. 1), indicating that the above-described phenomenon was a result of the lack of PD-1 in donor TCM cells.

It was possible that accumulating PD-1 KO T EM phenotype cells might have arisen from overt proliferation of residual T EM cells in the purified TCM cell “preparation.” To exclude this, we analyzed Ki-67 expression in GFP+ PD-1 KO TCM and T EM phenotype cells on days 21 and 42 after transfer of GFP+ TCM phenotype cells. Ki-67 expression was lower in the T EM phenotype subset compared with TCM phenotype when analyzed in the same host (Fig. 6C), thus showing that GFP+ T EM phenotype cells in PD-1 KO hosts were not outnumbered by vast proliferation of contaminant T EM phenotype cells. For the same purpose we transferred purified SNARF-1–labeled GFP+PD-1 KO TCM phenotype cells to PD-1 KO hosts and compared dye intensity dilution in GFP+ TCM and T EM phenotype cells. No consistent difference was observed when profiles for these subsets were overlaid (Fig. 6D). These data indicate that accumulated T EM phenotype cells, after PD-1 KO TCM cell transfers, do not originate from overt expansion of residual cotransferred T EM cells.

Additionally, we purified T EM phenotype CD8+ T cells from GFP+ WT or GFP+PD-1 KO spleens and transferred them separately to WT or PD-1 KO mice, respectively. Fig. 6E (upper panel) shows the purity of transferred cells. When analyzing mice that received T EM phenotype cells, T EM to TCM conversion was moderate for WT donor cells, whereas a smaller proportion of recovered PD-1 KO donor cells bore the TCM phenotype, consistent with less T EM to TCM conversion (Fig. 6E, lower panel). A significantly higher recovery of T EM phenotype PD-1 KO donor-derived cells was observed (Fig. 6F), which may be partly attributed to their enhanced survival.

In conclusion, these results provide strong evidence that PD-1 regulates differentiation of TCM to T EM phenotype CD8+ cells in nonimmunized, naive mice both by inhibiting TCM to T EM conversion and by promoting T EM to TCM conversion.

Absence of PD-1 exerts genome-wide gene expression changes in TCM phenotype CD8+ cells

We have shown that transferred TCM phenotype CD8+ cells from PD-1 KO mice, but not WT, can give rise predominantly to a T EM phenotype population (Fig. 6A, 6B). Analysis of TCM phenotype CD8+ cells for CD69, Ly6C, CD25, CD127, and CD122 surface expression revealed indistinguishable patterns between PD-1 KO

To investigate whether aberrant conversion between MP subsets contributes to accumulation of T EM phenotype CD8+ T cells in PD-1 KO mice, we purified both T CM and T EM phenotype CD8+ T cells from GFP+WT or GFP+PD-1 KO spleens and transferred them separately to WT or PD-1 KO mice, respectively. Fig. 6A (upper panel) shows the purity of TCM phenotype CD8+ T cells. When analyzing host mice that received T CM phenotype cells, little conversion of T CM to T EM cells was found in WT mice after 42 d (Fig. 6A, lower panel, left). In PD-1 KO mice, however, a striking conversion of T CM to T EM phenotype was observed (Fig. 6A, lower panel, right) (~80% of donor-derived cells from PD-1 KO hosts that received TCM CD8+ T cells were of a T EM phenotype). This was accompanied by a substantially higher recovery of PD-1 KO T EM phenotype donor-derived cells (Fig. 6B). This was also true but to a lesser degree for PD-1 KO TCM phenotype donor-derived cells. Numbers indicate percentages in each region. Plots are representative of one experiment with three mice per group.

FIGURE 4. Fate of naive WT and PD-1 KO CD8+ cells transferred to sublethally irradiated WT hosts. GFP+CD8+CD44lo cells from spleens of 2- to 4-mo-old PD-1 KO and WT mice were isolated by FACS sorting. Purified cells were then adoptively transferred into sublethally irradiated WT mice. On day 20, mice were sacrificed and spleens were analyzed. (A) Spleenocytes were analyzed for CD8+, CD44, and CD62L expression. Numbers indicate percentages in each region. Plots are representative of three individual experiments (WT, n = 7; PD-1 KO, n = 9). (B) Total numbers of GFP+ MP CD8+ T cell subsets found in spleen. Error bars indicate SD. (C) Ex vivo GzmB expression on day 20, gated on T EM phenotype CD8+ cells (shaded region, isotype control; thin line, GFP+WT; thick line, GFP+PD-1 KO). (D) GFP+CD8+ cells in hosts’ blood were examined, as in (A), on days 5, 10, and 20. Numbers indicate percentages in each region. Data are representative of one experiment with three mice per group.

demonstrate that the absence of PD-1 results in accumulation of CD8 T EM phenotype cells in a cell-intrinsic manner.

PD-1 regulates interconversion of TCM and T EM phenotype CD8+ T cells

Absence of PD-1 exerts genome-wide gene expression changes in TCM phenotype CD8+ cells

We have shown that transferred TCM phenotype CD8+ cells from PD-1 KO mice, but not WT, can give rise predominantly to a T EM phenotype population (Fig. 6A, 6B). Analysis of TCM phenotype CD8+ cells for CD69, Ly6C, CD25, CD127, and CD122 surface expression revealed indistinguishable patterns between PD-1 KO
and PD-1 KO (GFP +DsRed 2) CD8+ T cells from thymi, spleens, and lymph nodes were analyzed by flow cytometry 8 wk after bone marrow reconstitution in irradiated DsRed hosts. (A) Representative dot plots with donor-derived WT (GFP +DsRed 2) and PD-1 KO (GFP +DsRed 2) thymocytes. The expression of CD4 and CD8 was analyzed in gated populations. Numbers indicate percentages in each region (upper left, CD8 SP; lower right, CD4 SP; upper right, double-positive; lower left, double-negative). Column represents the average value of PD-1 KO/WT CD8 SP thymocyte ratios with error bar indicating SD. Data are representative of two individual experiments (n = 6). (B) Total numbers of CD8+ WT and PD-1 KO cells in spleens with error bars indicating SD. (C) Donor-derived WT (GFP +DsRed 2) and PD-1 KO (GFP +DsRed 2) CD8+ T cells from spleens and mesenteric lymph nodes (LN) were further analyzed for expression of CD44 and CD62L. Numbers indicate percentages in each region. Data are representative of three individual experiments with three to four mice per group. (D) Total numbers of WT and PD-1 KO CD8+ cell subsets in spleens with error bars indicating SD. (E) Similar analysis of donor-derived WT (GFP +DsRed 2) and PD-1 KO (GFP +DsRed 2) T cells from spleens after bone marrow reconstitution in irradiated DsRed host as in (C). Data are representative of one individual experiment with three mice per group. (F) Total numbers of CD8+ WT and PD-1 KO cells in spleens with error bars indicating SD, as in (E).

and WT cells (Supplemental Fig. 2). To examine whether TCM phenotype CD8 cells from PD-1 KO mice had already adopted a different transcriptional profile at the time of transfer, we performed transcriptome analysis on TCM phenotype CD8 cell subpopulations derived from PD-1 KO and WT spleens. First, all significantly differentially expressed genes from the PD-1 KO and WT TCM phenotype CD8 cells were classified as having increased or decreased expression. Two-tailed, pairwise ANOVA of Affymetrix complete mouse genome arrays revealed 237 annotated genes with significantly changed expression patterns between WT and PD-1 KO TCM CD8 cells (p ≤ 0.05, 1.5-fold change up- or downregulated) (Supplemental Table I), a number that significantly exceeds the number of genes that are expected to occur by chance under these selection criteria. Using this dataset, we then identified those biological processes with a significantly disproportionate number of responsive genes in the TCM phenotype CD8 cell subset relative to those contained in the Affymetrix arrays as shown in Fig. 7A. Selected genes and the magnitude of over- or underexpression are graphically depicted in Fig. 7B. Among these, there are genes involved in T cell costimulation (CD24, Icos, ICAM1, Tnfsf11b [TNFR2]), apoptosis/survival (Bcl2a1, Bcl13, TNF, Xiap), signal transduction (Jak1, Map3k8 [Tpl-2], Gadd45b, Socs3), as well as T cell migration/adhesion/inflammation (Ccl3, Cxcl9, Nrp1, [neuropilin-1], Lgals3 [galec tin-3]). Differentially expressed transcription factors included Rel, STAT1, Irf4, Irf8, and the less characterized Atf3, Ahr, and Bhlhe40 (Dec1). Ahr is able to modulate CD62L expression in primary responses (40) and under certain conditions it diminishes memory CD8 pool but not CD8 cell responses (41). Bhlhe40 transcription factor, which has recently been shown to be important in generation of regulatory T cells (42), is one of the most upregulated genes in PD-1 KO TCM CD8 cells (3.8-fold). Interestingly, upregulation of IL12Rb1 was accompanied by increased expression of genes previously characterized as positively regulated by IL-12 and/or IFN-α/β, such as Gadd45b, Bcl13, TNF, Lgals3, Cxcl13, Bhlhe40, Cdk11a, and Aft3 and IL12Rb1 itself (43–45). Importantly, when these cytokines are used as signal 3 on CD8 T cells they downregulate CD62L and CCR7 more efficiently than signal 1 and 2 alone (43).

Because many of the annotated genes functions were related to cell death, we compared ex vivo annexin V binding between WT and PD-1 KO TCM phenotype CD8 T cells. A higher percentage of PD-1 KO TCM phenotype cells were annexin V+ , indicating an increased propensity to apoptosis (Fig. 7C). When gating on CD62L hi , CD62L int , and CD62L lo MP PD-1 KO CD8 T cells, we observed a correlation of CD62L downregulation with increased annexin V binding (Fig. 7D). This may suggest that the increased annexin V binding of PD-1 KO TCM phenotype cells reflects their predisposition to become (CD62L lo ) T EM cells.

Overall, our results show that PD-1 KO TCM phenotype CD8 cells bear a distinct gene expression profile, and ablation of the PD-1 pathway had exerted an impact before the acquisition of the T EM phenotype. This may indicate that in transfer experiments PD-1 KO TCM phenotype cells are already preprogrammed, at least at the transcriptional level, to differentiate to T EM phenotype cells where further reprogramming takes place. Moreover, their profile indicates that TCM phenotype CD8 T cells may respond differently to IL-12 and IFN-α/β cytokines.

**Superior bystander production of IFN-γ by T CM phenotype PD-1 KO CD8 cells after innate stimulus**

MP CD8 T cells have been shown to produce IFN-γ driven by IL-12, as well as IFN-α/β produced by macrophage/dendritic cells, in
FIGURE 6. Fates of memory CD8 T cell subsets in adoptive transfer experiments. Purified GFP+CD8+ TCM phenotype cells from 5- to 7-mo-old GFP.WT and PD-1 KO were adoptively transferred into WT and PD-1 KO mice. (A) Representative dot plots with CD62L and CD44 expression on purified TCM phenotype cells before adoptive transfer (upper panel) and on day 42 on donor-derived GFP+CD8+ cells (lower panel). Numbers indicate percentages in each region. Data are representative of four individual experiments (WT, n = 10; PD-1 KO, n = 12). (B) Total numbers of recovered GFP+CD8+ TEM and TCM phenotype cells from WT and PD-1 KO host spleens as in (A). For comparison, the numbers of transferred cells per host (input) are indicated. (C) Mean percentages of Ki-67+ cells among donor-derived GFP+PD-1 KO CD8+ subsets on days 21 and 42 after transfer with error bars indicating SD. Data are representative of two individual experiments with three mice per group. (D) SNARF-1 profiles of donor-derived CD8+PD-1 KO TCM and TCM phenotype cells in host spleens on day 13 (thick line, PD-1 KO TCM; shaded area, PD-1 KO TCM). Data are representative of one experiment with four mice per group. (E) Purified GFP+CD8+ TEM phenotype cells from 5- to 7-mo-old GFP.WT and GFP.PD-1 KO mice were adoptively transferred into WT and PD-1 KO mice. Input and output of TCM-transferred cells, as in (A). (F) Total numbers of recovered GFP+CD8+ cell subsets from WT and PD-1 KO host as in (B) (WT, n = 6; PD-1 KO, n = 10).

response to infection or a defined innate stimulus (3, 4). Given our microarray results that imply an increased response of PD-1 KO TCM phenotype CD8 cells to these cytokines, we injected WT and PD-1 KO mice with LPS and analyzed CD8 T cells for IFN-γ production shortly after injection. A higher fraction of PD-1 KO CD8 T cells is considerably higher in PD-1 KO spleens (Fig. 3B), whereas enhancement of TCR signals by OX-40 (21) and ICOS (16) promote accumulation of PD-1 KO TCM phenotype cells produced IFN-γ ex vivo (Fig. 8, middle panel). No difference in IFN-γ production was observed between TCM phenotype cells from WT and PD-1 KO mice (Fig. 8, lower panel). These results show increased indirect response of PD-1 KO TCM phenotype CD8 cells to LPS, probably through IL-12 and/or IFN-α/β, and they imply a greater bystander innate response of PD-1 KO MP CD8 T cells to various pathogens. However, incubation of PD-1 KO splenocytes with various concentrations of IL-12 or type I IFN together with IL-18 did not result in superior production of IFN-γ by PD-1 KO MP CD8 subsets in vitro (not shown). Nevertheless, this does not exclude a role of these cytokines in the increased production of IFN-γ by PD-1 KO TCM CD8 cells in vivo in the context of an inflammatory milieu induced by LPS.

Discussion

In this study we describe a previously unrecognized role of PD-1 in MP CD8 T cell formation and particularly in shaping MP subset development. We have identified a substantial increase in CD44hi CD62LloCCR7hi CD8 T cells, categorized as TCM phenotype (5), in spleen and tissues and even lymph nodes of PD-1 KO mice (Figs. 1, 2). This phenomenon was more prominent with advancing age (Fig. 1C). Despite some quantitative differences in expression of memory markers on TCM cells from PD-1 KO and WT mice, the number of CD127hi and CD122hi TCM phenotype CD8 T cells is considerably higher in PD-1 KO spleens (Fig. 3B), consistent with an MP (46, 47). Although a proportion of PD-1 KO TCM phenotype cells express CD69 (Fig. 3A), most should not be recently activated cells because no CD25hi subpopulation was identified (Fig. 3A). Moreover, recently activated, typical effector cells would decay fast in a 42-d period, something not observed in our experiments (Fig. 6F). These accumulated TCM cells, in the absence of PD-1, seem to have enhanced effector memory characteristics, as shown by higher expression of Gzmb directly ex vivo (Fig. 3C) and IFN-γ after short activation with phorbol esters (Fig. 3D, 3E). The differences in expression of cytokine receptors found (Fig. 3A) could reflect an altered responsiveness to homeostatic cytokines in the PD-1 KO mice. Further studies will determine the contribution of these cytokines in the altered homeostasis of MP cells found in the PD-1 KO mice.

Costimulatory and coinhibitory molecules have been shown to regulate memory CD8 T cell development, with a consensus that costimulation promotes formation of Ag-specific or MP cells whereas coinhibition impedes it. However, to date, variable data exist on correlation between TCR signal strength modulated by positive and negative costimulators and developmental fate toward TCM and TCM subsets. For example, whereas enhancement of TCR signals by OX-40 (21) and ICOS (16) promote accumulation of TCM cells, stronger TCR signals in the absence of BTLA lead to...
accumulation of T_CM cells (19). Our results, which show that ablation of the PD-1 pathway drives MP CD8 T cells preferentially to a T_EM phenotype, are in agreement with the notion that increased signal strength (5) and duration (9) favor skewing toward a T_EM cell subset. Homeostatic proliferation of adoptively transferred naive PD-1 KO CD8 T cells gave rise to large numbers of T_EM phenotype cells (Fig. 4A, 4B, 4D), as MP CD8 T cells closely resemble memory cells generated under lymphopenic conditions (LIP memory cells) (1). Interestingly, T_CM cells appear first (day 5, Fig. 4D), followed by substantial accumulation of T_EM cells in blood of PD-1 KO (day 20, Fig. 4D) and in spleen (day 20, Fig. 4A), which does not take place in WT donor cells to the same extent. This implies that increased duration of signal, in the absence of PD-1, favors TEM differentiation through a T_CM intermediate. This observation correlates well with the massive T_CM to TEM conversion of transferred purified PD-1 KO T_CM phenotype CD8 T cells in lymphosufficient mice (Fig. 6A, 6B) where we provided “extra time” to the transferred cells, inside the host, to differentiate to T_EM cells.

Enhanced survival of PD-1 KO T_EM phenotype cells compared with WT may play an additional role in their accumulation (Fig. 3I). However, the fact that upon transfer of $1.5 \times 10^5$ purified T_CM or purified T_EM phenotype PD-1 KO cells we recovered similar numbers ($\sim 1 \times 10^5$) of PD-1 KO T_EM phenotype cells (Fig. 6B, second column versus Fig. 6E, second column) strongly implicates increased rates of T_CM to T_EM conversion as the major determinant of PD-1 KO T_EM phenotype cell accumulation, rather than enhanced survival alone.

Our results from mixed bone marrow transplantation experiments (Fig. 5), adoptive transfer of T_CM CD8 T cells (Fig. 6A, 6B, Supplemental Fig.1), and transfers of naive cells to lymphopenic hosts (Fig. 4) strongly indicate that the accumulation of PD-1 KO T_EM phenotype cells is, at least partly, a CD8 T cell–intrinsic effect. Further experiments would address the issue of whether the fate of PD-1 KO donor T_CM cells was already predetermined at the time of transfer or whether posttransfer intervention on WT T_CM cells would be sufficient to promote T_CM to T_EM differentiation. However, our microarray results, showing a discrete expression profile on PD-1 KO T_CM cells (Fig. 7A, 7B), argue in favor of the first scenario.
A recent study showed that vaccinia virus–specific PD-1 KO CD8 T cells are skewed toward T<sub>CM</sub> after acute infection (48). This does not conflict with our data because it has been shown that the type of pathogen affects memory differentiation pathways, with vaccinia virus (but not lymphocytic choriomeningitis virus) typically leading to fast emergence of T<sub>CM</sub> CD8 T cells (49). Moreover, in most acute infections, CD8 T cells rapidly stop encountering Ag (for vaccinia virus, infection is fully resolved within 2 wk) (50), and without any circumstantial or deliberate restimulation, typically most Ag-specific memory cells belong to the T<sub>CM</sub> subset. On the contrary, repetitive/continuous stimulation, either by infection or vaccination (51–53), promotes generation of cells belonging to the effector memory subset; repetitive antigenic stimulation has been shown to induce progressive decrease of CD62L surface expression (53). Importantly, in the settings of acute infection, PD-1 is shown to be expressed only transiently on CD8<sup>+</sup> T cells, whereas on chronically stimulated cells, sustained expression is observed (25, 54, 55). Therefore, with a different mode of PD-1 signaling (i.e., transient versus sustained) transition to different memory developmental pathways may take place. Thus, it is probable that settings of acute infection (48), on the one hand, and response to a plethora of Ags, with many of them repetitively encountered, on the other hand, could have a different impact on memory fate of PD-1 KO CD8 T cells. Further experiments are needed to determine whether PD-1 has the same effect on differentiation of MP phenotype cells and Ag-specific memory cells following multiple re-exposure to Ag.

Note that compared with respective MP CD8 T cells a much larger fraction of PD-1 KO LIP T<sub>EM</sub> phenotype cells produce high levels of GzmB ex vivo (Fig. 4C). Given that most of these cells recognize self-ligands, although with low affinity (56), it is reasonable to think that they could have an autoreactive potential. In line with this hypothesis, Thangavelu et al. (57), although not examining GzmB expression on T cells, have shown in a recent report that PD-1 KO recent thymic emigrants cause a lethal autoimmune-like disease in chronically lymphopenic hosts.

Overall, the emerging view is that naive WT or PD-1 KO CD8<sup>+</sup> T cells encounter Ags (commensal, environmental, or self-Ags) (2, 58) in the periphery of an immunized mouse and undergo conventional priming or homeostatic proliferation; many of these initially acquire a T<sub>CM</sub> phenotype, which in PD-1 KO cells is aberrantly transient and a large proportion of them develops stable characteristics of T<sub>EM</sub> cells. Additionally, resulting PD-1 KO T<sub>EM</sub> phenotype cells have a moderate survival advantage over the WT ones (Fig. 3I), thus further intensifying the effect of enhanced conversion.

In conclusion, our results show that PD-1 signaling in CD8<sup>+</sup> T cells can modulate the homeostasis of the MP pool through inhibiting differentiation toward a functional T<sub>EM</sub> phenotype, most probably through a T<sub>CM</sub> phenotype intermediate. These accumulated T<sub>EM</sub> phenotype cells harbor potent functional properties (Fig. 3C-E) and this could result in altered host responses against pathogens, environmental Ags, or self-Ags in the absence of an intact PD-1 pathway. Additionally, PD-1 KO MP CD8 cells may elicit superior bystander protective responses against pathogens as suggested by LPS-driven IFN-γ production, especially by T<sub>CM</sub> phenotype cells (Fig. 8). These findings can be clinically important, especially in the settings of currently developing treatments with antagonistic anti–PD-1 or anti–PD-ligand 1 Abs in cases of certain malignancies or chronic infections (26, 59). Equally important, manipulation of PD-1 pathway could enhance efficacy of certain vaccination regimens where production of T<sub>EM</sub> cells is critical (51, 60). Further studies may include a more precise analysis of accumulated Ag specificities as well as the exact time frame where PD-1 signaling on CD8 T cells is sufficient to impose a break toward T<sub>EM</sub> phenotype differentiation in naive or immunized mice.

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Disclosures

The authors have no financial conflicts of interest.

References
