Naive tumor-specific CD4+ T cells differentiated in vivo eradicate established melanoma

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In vitro differentiated CD8+ T cells have been the primary focus of immunotherapy of cancer with little focus on CD4+ T cells. Immunotherapy involving in vitro differentiated T cells given after lymphodepleting regimens significantly augments antitumor immunity in animals and human patients with cancer. However, the mechanisms by which lymphopenia augments adoptive cell therapy and the means of properly differentiating T cells in vitro are still emerging. We demonstrate that naive tumor/self-specific CD4+ T cells naturally differentiated into T helper type 1 cytotoxic T cells in vivo and caused the regression of established tumors and depigmentation in lymphopenic hosts. Therapy was independent of vaccination, exogenous cytokine support, CD8+, B, natural killer (NK), and NKT cells. Proper activation of CD4+ T cells in vivo was important for tumor clearance, as naive tumor-specific CD4+ T cells could not completely treat tumor in lymphopenic common gamma chain (γ_c)–deficient hosts. γ_c signaling in the tumor-bearing host was important for survival and proper differentiation of adoptively transferred tumor-specific CD4+ T cells. Thus, these data provide a platform for designing immunotherapies that incorporate tumor/self-reactive CD4+ T cells.

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Abbreviations used: ACT, adoptive cellular therapy; mRNA, messenger RNA; TCM, central memory T cell; TEM, effector memory T cell; Tg, transgenic; T reg cell, regulatory T cell; TRP, tyrosinase-related protein.

Adoptive cellular therapy (ACT) of cancer using in vitro differentiated CD8+ T cells is a powerful treatment against established cancer in humans and mice. In recent years, great progress has been attained in the understanding of the mechanisms involved in enhancing treatment of large established tumors (Gattinoni et al., 2006). Lymphodepletion before adoptive therapy greatly enhances ACT in humans and mice through the creation of cytokine sinks, removal of regulatory T cells (T reg cells), and the release of toll-like receptor agonists (Gattinoni et al., 2005a; Paulos et al., 2007; Dudley et al., 2008). Recent evidence suggests that irradiation also enhances the expression of ICAM and VCAM in the tumor vasculature allowing tumor-reactive T cells to enter more readily (Quezada et al., 2008). Although CD8+ T cells are potent mediators of antitumor immunity, there has been little focus on tumor-specific CD4+ T cells. CD4+ Th cells are important in immunity because in the absence of help, CD8+ T cells can be deleted or lose the capacity to develop into memory CD8+ T cells upon rechallenge (Janssen et al., 2003; Antony et al., 2005; Williams et al., 2006). Therefore, the use of tumor/self-reactive CD8+ T cells in the adoptive immunotherapy of cancer may face similar fates because T cells must remove tumor antigen in the context of persisting self-antigen, which in some cases leads to autoimmunity (Gattinoni et al., 2006; Rosenberg et al., 2008). Adoptive cell therapies that incorporate CD4+

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T cells are far superior to therapies that only use $CD8^+$ T cell clones (Dudley et al., 2002). Therefore, one theoretical means of improving immunotherapy to self may involve the provision of tumor-reactive or self-reactive $CD4^+$ T cells (Nishimura et al., 1999; Marzo et al., 2000; Antony et al., 2005), but a more direct role for $CD4^+$ T cells in tumor immunity remains unclear (Ho et al., 2002; Muranski and Restifo, 2009).

Recently, adoptive transfer of in vitro differentiated tumor-specific $CD4^+$ T cells in humans and mice has shown promise against cancer as a therapy (Nishimura et al., 1999; Perez-Diez et al., 2007; Hunder et al., 2008; Muranski et al., 2008). This has rekindled the idea of using antigen-specific $CD4^+$ Th during immunotherapy because $CD4^+$ Th cells can mediate the proper signals required in vivo to activate $CD8^+$ T cells and other cells of the innate immune system (Kahn et al., 1991; Hung et al., 1998; Nishimura et al., 1999; Antony et al., 2006; Williams et al., 2006). In fact, several preclinical and clinical trials have shown the importance of CD4 help during immunotherapy of cancer (Nishimura et al., 1999; Antony et al., 2006; Dudley et al., 2008). However, isolation of tumor-specific $CD4^+$ T cells has been difficult (Wang, 2001) and only a few MHC class II vaccines have been produced as a result of the lack of knowledge of how to generate vaccines that specifically activate Th cells instead of tumor-specific $Foxp3^+$ T reg cells (Rosenberg, 2001; Vence et al., 2007). In addition, lack of appropriate mouse models to study tumor-specific $CD4^+$ T cell responses to self-antigens has hindered progress in our understanding of the role of $CD4^+$ T cells in maintaining immunity to cancer.

Now, with a better understanding of $CD4^+$ T cell biology, the use of cytokines to differentiate and expand T cells in vitro has led to a panoply of CD4 lineages with specific in vivo functions (Weaver and Rudensky, 2009). For example, in vitro differentiated $CD4^+$ Th17 tumor-specific T cells have shown superiority over $CD4^+$ Th1 differentiated T cells in the adoptive immunotherapy of cancer in a mouse model of melanoma (Muranski et al., 2008). IL-2 and IL-7 in vitro expanded NY-ESO-1–specific $CD4^+$ T cells in humans have also shown clinical promise in one patient who had not received prior lymphodepleting conditioning or a vaccine (Hunder et al., 2008). Although these are promising studies, the mechanisms involved in the direct therapy of cancer by $CD4^+$ T cells remain elusive. Likewise, methods for enhancing adoptive immunotherapy without prior in vitro manipulation that may lead to the terminal differentiation of T cells also remain unclear (Gattinoni et al., 2005b, 2009; Klebanoff et al., 2005). Although such manipulations can lead to vaccine independence (Klebanoff et al., 2009), long-term benefits from in vivo differentiation may outweigh in vitro stimulation because the in vivo environment may provide the correct signals that cannot be attained in a culture dish.

To test a direct role for $CD4^+$ T cells in the immunotherapy of cancer, we used a gp75/tyrosinase-related protein (TRP) 1–specific $CD4^+$ TCR transgenic (Tg) mouse that produces class II–restricted T cells that recognize mouse TRP-1 in the context of $I\text{-}A^b$ (Muranski et al., 2008). TRP-1 is expressed in malignant melanoma and in the skin and eyes of mice and humans; therefore, this model mimics the human condition as closely as possible. Surprisingly, we found that adoptive transfer of naive TRP-1–specific $CD4^+$ T cells into lymphopenic animals bearing large established melanoma caused tumor regression and depigmentation independent of vaccination, cytokine administration, and $CD8^+$, B, NK, and NKT cells. This therapy was dependent on common gamma chain (γ_c) signaling in the host for survival and differentiation of $CD4^+$ T cells in vivo. These data provide a better understanding for the design of immunotherapies that incorporate tumor/self-reactive $CD4^+$ T cells.

RESULTS

Autoimmunity and cancer regression with adoptive transfer of naive tumor-specific TRP-1 $CD4^+$ T cells into lymphopenic mice TRP-1–specific $CD4^+$ TCR Tg mice were created on a C57BL/6 background that contains TRP-1 as a self-antigen (Muranski et al., 2008). These mice develop vitiligo (depigmentation) slowly with age (Fig. S1 A) but can develop vitiligo rapidly with genetic removal of Foxp3 (denoted $Foxp3^{sf}$), which is required for development of T reg cells that suppress autoimmune disease (Kim et al., 2007; Fig. 1 A and Fig. S1 B). TRP-1–specific $CD4^+$ TCR Tg mice, which express TRP-1 antigen, have activated T cells that are characterized by low expression of CD62L and high expression of CD44 on TRP-1 $CD4^+$ T cells in vivo (Fig. S1 C, top). Because we wanted to study naive $CD4^+$ T cells, $Tyrp1^{+/+}$ TRP-1 $CD4^+$ Tg mice were crossed with white-based brown mutation mice ($Tyrp1^{B\text{-}w}$) containing a radiation-induced inversion interrupting the *tyrp1* gene (Smyth et al., 2006). RT-PCR confirmed that no *Tyrp1* messenger RNA (mRNA) was present in $Tyrp1^{B\text{-}w}$ mice, and $Tyrp1^{B\text{-}w}$ mice that express the Tg TCR do not develop vitiligo (unpublished data). These mice have a light-brown coat color (Fig. S1 D) and were further crossed with $RAG^{-/-}$ mice to generate naive monoclonal TRP-1–specific $CD4^+$ T cells (Fig. S1 C, bottom). This mouse model is unique in that it develops T cells specific for TRP-1 that are both $Foxp3^-$ and $Foxp3^+$ on a $RAG^{-/-}$ background. The expression of Foxp3 is at the natural physiological ratio of 5–15% (Fig. S1 E).

Once TRP-1–specific $CD4^+$ TCR Tg mice on a $Tyrp1^{B\text{-}w}$-$RAG^{-/-}$ background were obtained (hereafter denoted as TRP-1 $CD4^+$ T cells), TRP-1 $CD4^+$ T cells were transferred into nontumor-bearing $RAG^{-/-}$ mice and observed for signs of specific immunity (e.g., depigmentation). Indeed, ~5–6 wk after transfer, the mice developed progressive depigmentation as indicated by coat color changes from black to white (Fig. 1 B). Depigmentation progressed until the mice became completely white.

To determine if adoptive transfer of TRP-1 $CD4^+$ T cells could mediate the regression of established tumors, WT, $RAG^{-/-}$, and irradiated WT mice (5 Gy) were given 2×10^5 B16 tumor cells s.c. On day 7, after tumor was visible and palpable, sorted TRP-1 $CD4^+$ T cells from 2×10^5 $Tyrp1^{B\text{-}w}$-$RAG^{-/-}$ mice were transferred i.v. by tail vein injection into

tumor-bearing mice. Adoptive transfer of TRP-1 $CD4^+$ T cells into lymphopenic mice caused infiltration of tumor-specific T cells into and regression of established tumors (Fig. 1, C and D; and Fig. S1 F). Irradiated WT mice had a transient antitumor response, indicating a temporary lymphodepletion as seen in mice and patients that are lymphodepleted (Dudley et al., 2005; Gattinoni et al., 2005a; Zhang et al., 2005).

Because we wanted to study the role of $CD4^+$ T cells in tumor immunity, we focused on $RAG^{-/-}$ mice because these mice contain no $CD8^+$ T cells, B cells, or NKT cells, all of which have been described in tumor immunity (Dougan and Dranoff, 2009). Complete regression of B16 melanoma was observed in $RAG^{-/-}$ mice (Fig. 1 D). $RAG^{-/-}$ mice that received 5 Gy irradiation to deplete cytokine sinks (Gattinoni et al., 2005a) and adoptive cell transfer of TRP-1

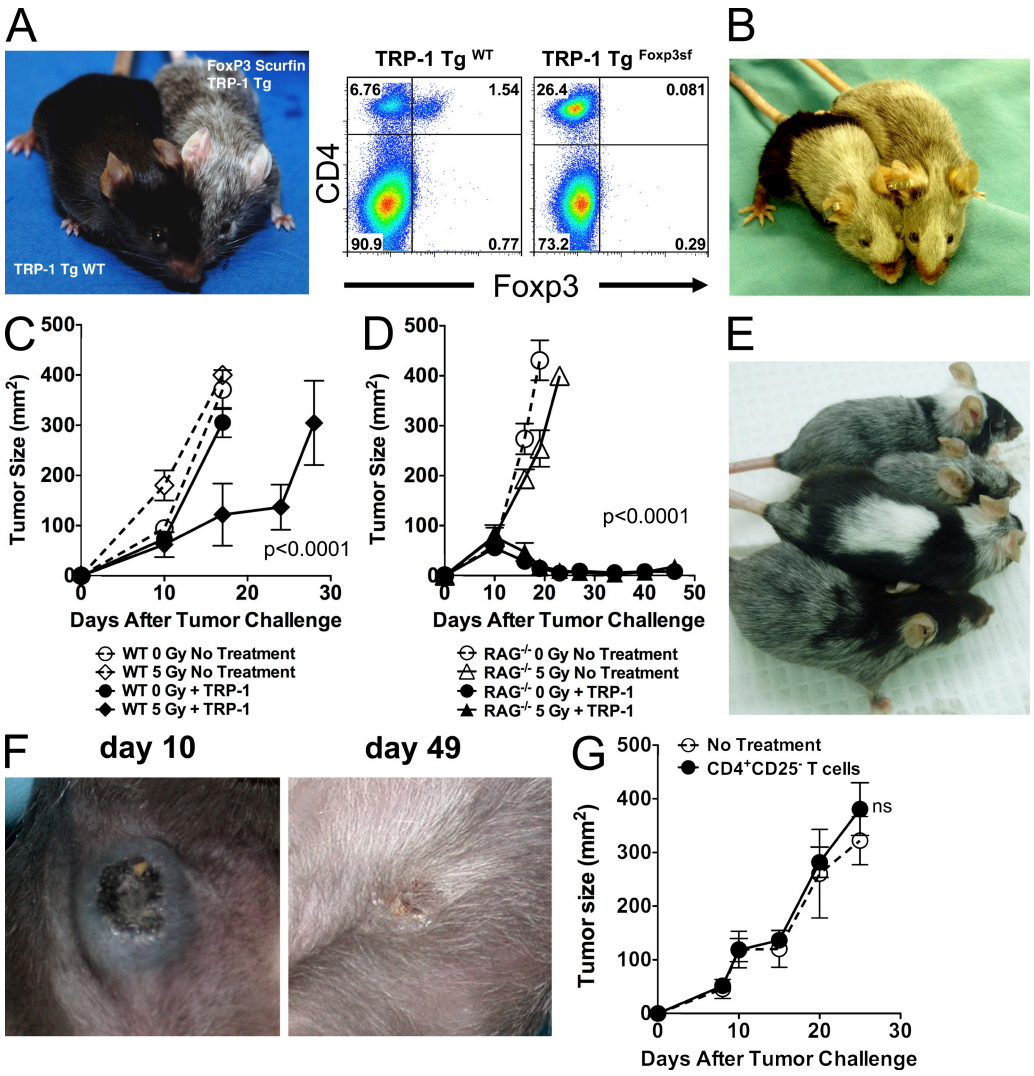

Figure 1. Treatment of established melanoma with adoptive transfer of TRP-1 $CD4^+$ T cells into lymphopenic mice is specific. (A) Enhanced specific autoimmune disease in TRP-1 $CD4^+$ $Foxp3^{sf}$ (Foxp3 negative) mice. TRP-1 $CD4^+$ WT Tg mice (left; $n = 13$) and TRP-1 $CD4^+$ $Foxp3^{sf}$ mice (right; $n = 7$) were compared for incidence of depigmentation over time. 1-mo-old littermates are shown. TRP-1 $CD4^+$ $Foxp3^{sf}$ mice have no $Foxp3^+$ T cells as shown by flow cytometry. (B) Depigmentation (vitiligo) can be adoptively transferred to lymphopenic hosts through TRP-1–specific $CD4^+$ T cells. 2×10^5 TRP-1 $CD4^+$ T cells from *Tyrp1*$^{B\text{-}w}$$RAG^{-/-}$ mice were transferred to nontumor-bearing $RAG^{-/-}$ hosts. Mice ($n = 20$) developed depigmentation after 35–45 d. A representative picture is shown. (C) Tumor-bearing WT mice were irradiated with 500 rads (5 Gy) or not irradiated (0 Gy) on day 7 after tumor challenge, and 2×10^5 naive TRP-1 $CD4^+$ T cells were adoptively transferred by i.v. tail vein injection. Experiments were repeated two times. $P < 0.0001$ for WT mice receiving 5 Gy and TRP-1 $CD4^+$ T cells versus no treatment. (D) Tumor-bearing $RAG^{-/-}$ mice were irradiated with 500 rads (5 Gy) or not irradiated (0 Gy) on day 7 after tumor challenge, and 2×10^5 naive TRP-1 $CD4^+$ T cells were adoptively transferred by i.v. tail vein injection. Experiments were repeated nine times. $P < 0.0001$ for $RAG^{-/-}$ no treatment versus $RAG^{-/-}$ + TRP-1 $CD4^+$ T cells (0 Gy or 5 Gy). (E) Whole body vitiligo in tumor-bearing mice treated with TRP-1 $CD4^+$ T cells ($n = 40$ mice). (F) Tumor regression in lymphopenic mice treated with TRP-1 $CD4^+$ T cells at days 10 and 49. The picture is representative of 40 mice over eight different experiments. (G) Adoptive transfer of 10^6 open repertoire $CD4^+CD25^-$ T cells into lymphopenic tumor-bearing $RAG^{-/-}$ mice on day 7 after tumor challenge does not affect tumor growth. Data represent three independent experiments. $n = 5$ mice/group; P = NS. Error bars indicate SEM.

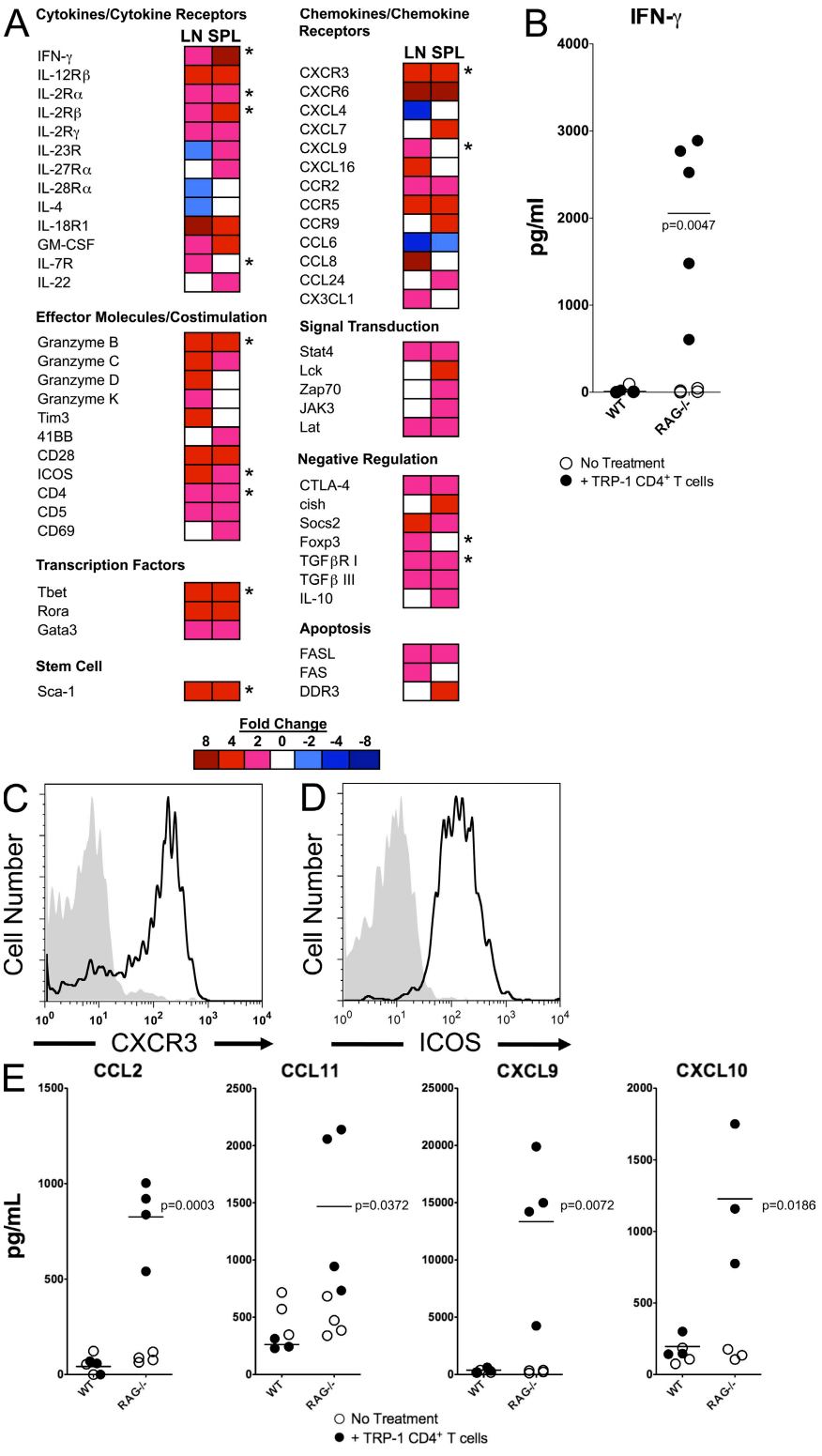

Figure 2. TRP-1 CD4⁺ T cells treating established tumors differentiate into Th1 CD4⁺ cytotoxic T cells in lymphopenic mice. (A) Gene expression analysis of TRP-1 CD4⁺ T cells from spleen and LNs of lymphopenic mice undergoing tumor regression. Heat maps represent fold changes in mRNA expression between naive TRP-1 CD4⁺ T cells and TRP-1 CD4⁺ T cells differentiated in vivo for 1 wk. The gene array is representative of one experiment. * indicates data confirmed by flow cytometry or multiplex assay. (B) IFN-γ levels in the serum of tumor-bearing WT and RAG$^{-/-}$ mice with and without adoptive transfer of 2 × 10^5 TRP-1 CD4⁺ T cells 1 wk after transfer. Open circles represent individual mice receiving no treatment and closed circles represent individual mice receiving TRP-1 CD4⁺ T cells. Horizontal bars indicate means for treated groups only (n = 3–5 mice/group). Data are representative of

$CD4^+$ T cells also had complete regression. As seen in non-tumor-bearing $RAG^{-/-}$ mice, depigmentation was present in tumor-bearing $RAG^{-/-}$ mice at ~5–wk after treatment (Fig. 1 E). Tumor regression was dramatic, with large established tumors regressing to a small scar (Fig. 1 F). $RAG^{-/-}$ mice do not have endogenous $CD8^+$ T, B, or NKT cells; therefore, this therapy was completely independent from these cell types. Because TRP-1 $CD4^+$ T cells came from *Tyrp1*$^{B\text{-}w}$$RAG^{-/-}$ mice, there was no concern for contamination from other cells that may contribute to tumor therapy.

To determine whether tumor treatment in lymphopenic hosts was specific, we sorted "open-repertoire" $CD4^+CD25^-$ T cells from WT mice and transferred 10^6 $CD4^+$ T cells into $RAG^{-/-}$ mice on day 7 after tumor inoculation (Fig. 1 G). $CD4^+CD25^-$ T cells have virtually no $Foxp3^+$ T cells and have been shown to help tumor-specific $CD8^+$ T cells maintain treatment of established tumors without the addition of exogenous cytokines (Antony et al., 2006). However, by themselves, $CD4^+CD25^-$ open-repertoire T cells did not affect tumor growth in lymphopenic mice. Therefore, we conclude that TRP-1 $CD4^+$ T cells are specific for their tumor/self-antigen in vivo and that the antitumor effect is independent of $CD8^+$, B, and NKT cells.

TRP-1 $CD4^+$ T cells differentiate into Th1 cytotoxic T cells in lymphopenic mice

Because it has been shown that in vitro differentiated Th17 tumor-specific T cells were superior to other $CD4^+$ T cell lineages when treating established tumors (Muranski et al., 2008), we wanted to determine the in vivo default differentiation program that leads to strong tumor regression by TRP-1 $CD4^+$ T cells. After 1 wk in vivo, adoptively transferred $CD4^+$ T cells were FACS sorted from tumor-draining LNs and the spleens of mice undergoing tumor regression and analyzed by gene microarray. These cells were compared with FACS-sorted naive TRP-1 $CD4^+$ T cells. Only genes with a twofold or greater difference were displayed. As shown in Fig. 2 A, TRP-1 $CD4^+$ T cells transferred into a lymphopenic environment defaulted to a $CD4^+$ Th1 program, as indicated by high expression of *Tbet*, *Ifng*, *Il18r*, *Il2r*, *Il12r* (Th1 priming), *Il23r* (Th1 memory; Robinson and O'Garra, 2002), *Il27r* (early Th1 priming), *Tim3*, *Cxcr3*, *Ccr2*, and *Ccr5* mRNA, with evidence of some Th17-associated genes (*Il22*, *Il23r*, and *Rora*) and Th2 genes (*Il10* and *gata3*) but not *Ccr6*, *Il4*, *Il5*, *Il17a*, *Il17f*, *or Rorc*. Genes for TCR signaling were up-regulated in the spleen (*Lck* and *Zap70*) but not in the LN, probably indicating recently activated trafficking T cells, suggested by increased *Cd69*, *Cd5* (strong TCR signals), and *Lat* expression. *Jak3* was up-regulated in TRP-1 T cells, indicating that γ_c signaling was active. *Stat4* was highly expressed, demonstrating that IL-12, and possibly IL-23, signals were potentiating IFN-γ production. Co-activation genes, including 41BB and ICOS, were highly up-regulated. Both are involved in enhancing the activation of T cells (Tamada and Chen, 2006; Stephan et al., 2007; McNamara et al., 2008). Genes related to CTL effector functions were increased; *Gzmb*, *Gzmc*, *Gzmd*, and *Gzmk* were highly up-regulated, with only *Gzmb* and *Gzmc* staying at high levels in the spleen. *Fasl* was also up-regulated.

Genes that regulate the expansion of T cells by controlling T cell activation in a negative manner—*TgfβrI*, *Ctla-4*, *Cish*, *Il10*, and *Socs2*—were also up-regulated (Fig. 2 A). High IFN-γ levels were confirmed in the serum of lymphopenic mice undergoing tumor treatment but not in WT mice or untreated lymphopenic $RAG^{-/-}$ mice (Fig. 2 B). CXCR3 and ICOS, both Th1-associated molecules, were also expressed by TRP-1 $CD4^+$ T cells (Fig. 2, C and D).

Next, we looked at effector molecules in TRP-1 $CD4^+$ T cells and found perforin, granzyme B, and LAMP-1 (CD107a), all of which are involved in degranulating cytotoxic T cells (Fig. 3). Because TRP-1 $CD4^+$ T cells expressed granzyme B on gene array (Fig. 2) and in the spleen (Fig. 3), these data suggest that the antitumor $CD4^+$ T cells are cytotoxic. Thus, naive TRP-1 $CD4^+$ T cells differentiated into a Th1 phenotype in vivo and expressed cytotoxic T cell–associated genes and molecules that may assist in antitumor immunity.

Adoptive transfer of naive TRP-1 $CD4^+$ T cells into lymphopenic mice changes chemokine expression patterns

CXCR3, CCR2, and CCR5 are chemokine receptors that help draw Th1 $CD4^+$ T cells into inflamed tissues. Because we saw high chemokine receptor expression on TRP-1 $CD4^+$ T cells (Fig. 2, A and C), we checked the serum of lymphopenic and nonlymphopenic mice for the specific ligands and found high expression of IFN-γ–inducible CXCL9 and CXCL10 (the ligands for CXCR3), as well as CCL2 (the ligand for CCR2) and CCL11 (the ligand for CCR2 and CCR5), in lymphopenic mice during tumor regression (Fig. 2 E). CCL3 (the ligand for CCR5) was also moderately high in the serum of lymphopenic mice during tumor regression (unpublished results). CCL2 is known to draw inflammatory monocytes from the blood into LN and activate Th1 $CD4^+$ T cells (Nakano et al., 2009). We saw accumulation of inflammatory monocytes ($CD11b^{high}GR\text{-}1^+$) in the spleen, LN, and tumors of treated groups (Fig. S2). These cells are class II^+ and are known to secrete IFN-γ that may help aid Th1 differentiation (Nakano et al., 2009). Other chemokine receptors were up-regulated on TRP-1 $CD4^+$ T cells, including CXCR6, which is involved in trafficking to the spleen, and CCR9 (Fig. 2 A). Lastly, a multitude of inflammatory

three experiments (no treatment vs. treatment; P = 0.0047). (C) CXCR3 expression on naive TRP-1 $CD4^+$ T cells before transfer (gray histogram) and 1 wk after in vivo differentiation (solid line). (D) ICOS expression on naive and 1wk in vivo differentiated TRP-1 $CD4^+$ T cells. (E) Serum chemokines levels in WT and $RAG^{-/-}$ mice treated or not (n = 3–5 mice/group). Open circles represent individual mice receiving no treatment and closed circles represent individual mice receiving TRP-1 $CD4^+$ T cells. Horizontal bars indicate means for treated groups only. Data are representative of three experiments.

chemokines were differentially expressed by TRP-1 CD4$^+$ T cells in the LN and spleen (Fig. 2 A). Therefore, TRP-1 CD4$^+$ T cells activated by lymphopenia-induced proliferation may help trigger Th1 chemokines, which could enhance immunotherapy by recruiting inflammatory monocytes and CXCR3$^+$CCR2$^+$CCR5$^+$ TRP-1 CD4$^+$ T cells to appropriate sites for activation and tumor infiltration.

Mechanisms of activation and effector function of TRP-1 CD4$^+$ T cells during lymphopenia

It is known that MHC class I is up-regulated on tumor cells in vivo in the presence of IFN-γ$^+$ tumor-specific CD8$^+$ T cells (Palmer et al., 2008). However, it is not known whether IFN-γ enhances MHC class II expression on established tumors in vivo. Because we saw high levels of IFN-γ expression in the serum and on gene microarray, we evaluated the expression of MHC class II in the tumor microenvironment. 1 wk after adoptive transfer with TRP-1 CD4$^+$ T cells, lymphopenic mice were sacrificed and tumors were imaged for MHC class II expression by confocal microscopy. Lymphopenic mice that had received no TRP-1 CD4$^+$ T cells had no evident expression of MHC class II on tumor cells in vivo. However, in mice that had received TRP-1 CD4$^+$ T cells, MHC class II expression was highly expressed on tumor cells in vivo (Fig. 4 A). To our knowledge, this is the first time class II up-regulation in vivo by B16 melanoma has been reported. This may be similar to the mechanism of induction of MHC class II reported in certain autoimmune diseases (Belfiore et al., 1991).

To determine if host MHC class II was important in activating TRP-1 CD4$^+$ T cells in mice with MHC class II$^{+/+}$ tumors, tumor-bearing irradiated and nonirradiated MHC class II$^{-/-}$ mice were treated with naive TRP-1 CD4$^+$ T cells. Mice were irradiated to mimic the lymphopenic environment. Tumor treatment in both groups was comparable to the no-treatment groups (Fig. 4 B), suggesting, as previously shown, that MHC class II is needed to prime naive CD4$^+$ T cells (Beutner and MacDonald, 1998). This implies that MHC class II expression on the tumor was not sufficient to activate naive CD4$^+$ T cells. However, in vitro activated TRP-1 CD4$^+$ T cells can treat MHC class II$^+$ tumors in MHC class II$^{-/-}$ mice, and anti-class II antibodies can block this effect (see Quezada et al. in this issue), showing that the function of activated CD4$^+$ T cells is independent of host class II.

Next, to determine if tumor antigen alone is enough to activate TRP-1 CD4$^+$ T cells, tumor-bearing female *Tyrp1*$^{B\text{-}w}$-RAG$^{-/-}$ mice, which express no TRP-1 antigen, were treated with TRP-1 CD4$^+$ T cells. In the absence of self-antigen in the periphery, tumor treatment was complete and irradiation did not affect the antitumor response (Fig. 4 C). Even though the tumor may be able to express class II, these experiments demonstrate that T cell activation by host MHC class II$^+$ cells may be essential for the success of treatment in the lymphopenic setting. These experiments also show that the tumor/self-antigen, TRP-1, expressed only by the tumor, is adequate to activate naive TRP-1 CD4$^+$ T cells transferred into *Tyrp1*$^{B\text{-}w}$RAG$^{-/-}$ mice.

IFN-γ has been attributed to the mechanism of action by which CD4$^+$ T cells eradicate tumors (Mumberg et al., 1999; Nishimura et al., 1999; Corthay et al., 2005; Muranski et al., 2008). We administered anti–IFN-γ antibodies (500 µg/mouse) once, at the time of adoptive cell transfer, and found that anti–IFN-γ antibodies were able to attenuate the antitumor response in lymphopenic mice (Fig. 4 D).

To determine why adoptive transfer of T cells resulted in activation of CD4$^+$ T cells in RAG$^{-/-}$ mice, we looked at CD11c$^+$ DC activation status because these cells express high levels of MHC class II in vivo. We found, after adoptive cell transfer with TRP-1–specific CD4$^+$ T cells, that CD11c^{high} DC expressed increased levels of MHC class II and CD86

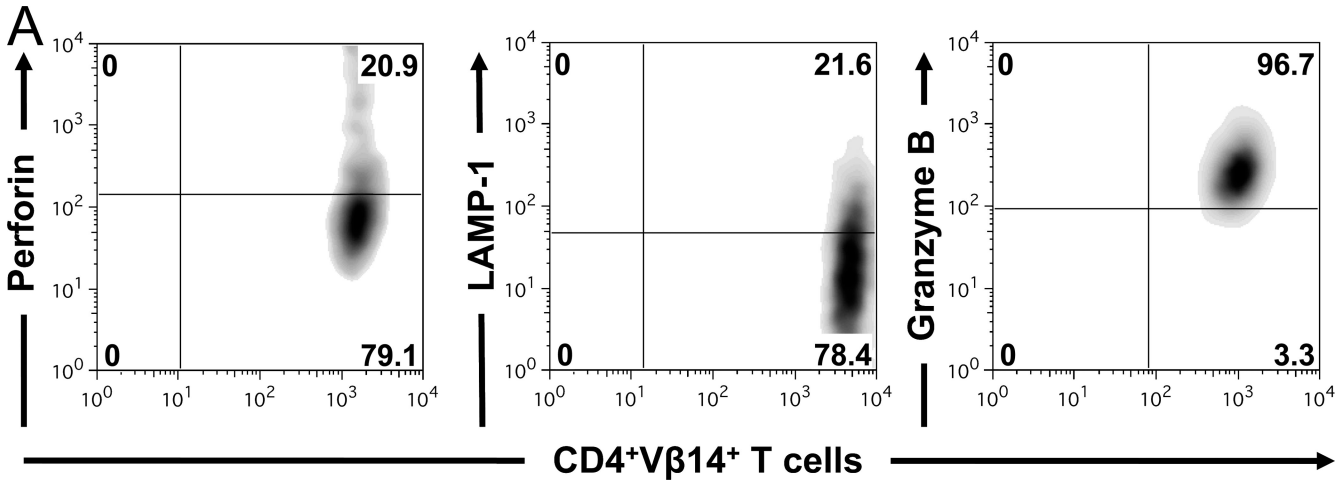

Figure 3. TRP-1 CD4$^+$ T cells become cytotoxic T cells in vivo. (A) Spleens from tumor-bearing RAG$^{-/-}$ mice treated with TRP-1 CD4$^+$ T cells on day 7 after tumor challenge were stained ex vivo with antibodies to CD4, Vβ14, perforin, LAMP-1 (CD107a), and granzyme B 1 wk after adoptive cell transfer. Intracellular staining was performed as indicated in Materials and methods. Flow cytometry shows gated TRP-1 CD4$^+$Vβ14$^+$ cells. Data represent two independent experiments (n = 5 mice/group).

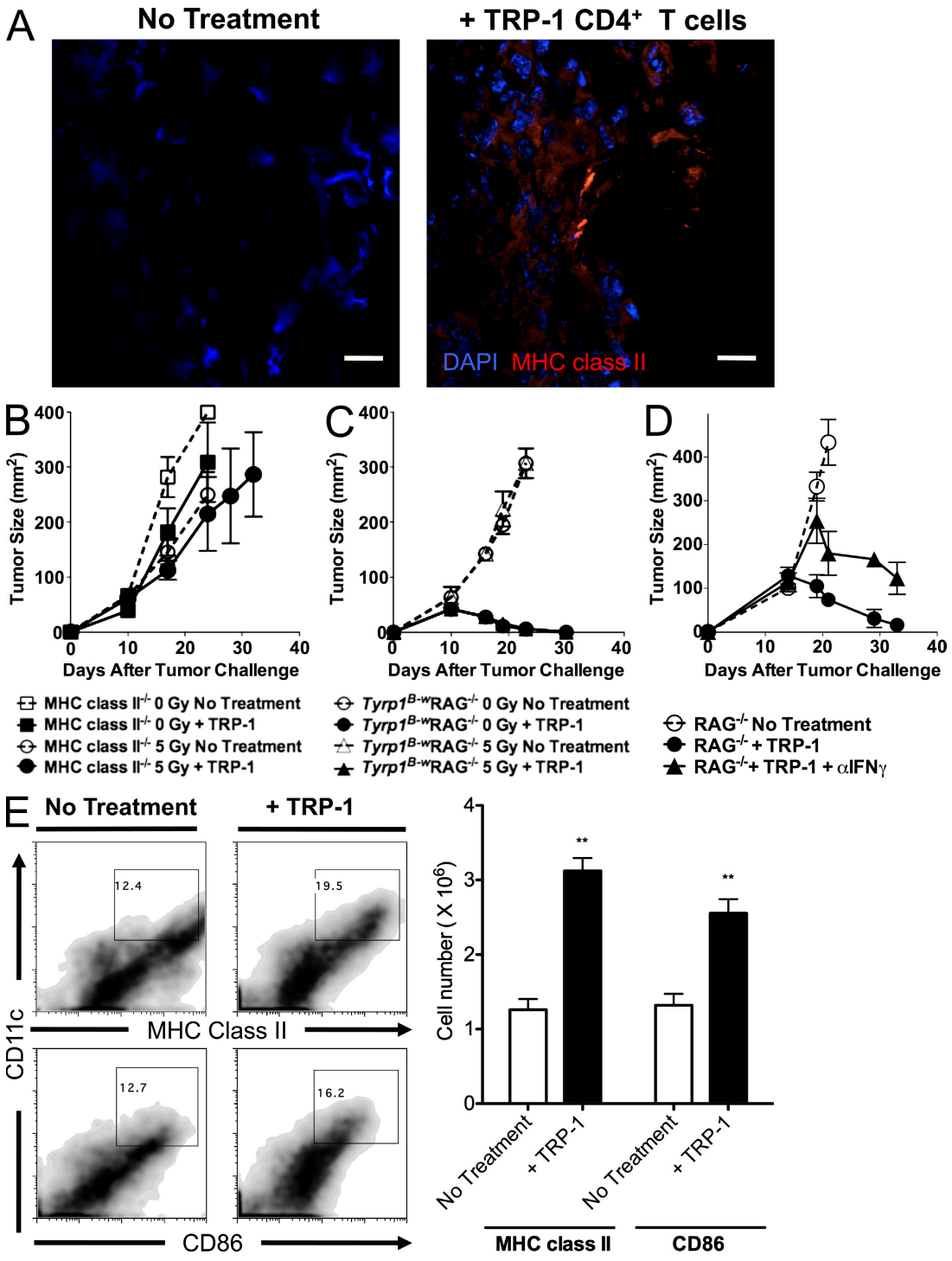

Figure 4. Mechanism of treatment by TRP-1 CD4+ T cells. (A) Confocal microscopy of day-14 tumors, 1 wk after adoptive cell transfer with 2×10^5 naive TRP-1 CD4+ T cells. Tumors were frozen in OCT, cut, and stained with DAPI (blue) and MHC class II (red). Samples were analyzed by confocal microscopy (Olympus) with a 20× oil immersion objective. Bars, 50 µm. (B) 7-d tumor-bearing MHC class $II^{-/-}$ mice were irradiated with 5 Gy or not irradiated and treated with 2×10^5 naive TRP-1 CD4+ T cells. (C) 7-d tumor-bearing non-TCR Tg *Tyrp1*$^{B\text{-}w}$RAG$^{-/-}$ mice were irradiated with 5 Gy or not irradiated and treated with 2×10^5 naive TRP-1 CD4+ T cells. (D) 11-d tumor-bearing $RAG^{-/-}$ mice were treated with naive TRP-1 CD4+ T cells or not and, in addition, some treated mice received one injection of 500 µg of neutralizing anti–IFN-γ antibodies on day 7 of ACT. Data are representative of four independent experiments with five to eight mice per group. (E) DC activation in lymphopenic mice after ACT with TRP-1 CD4+ T cells. Flow cytometry of $CD11c^{high}$MHC class II^{high} and $CD86^{high}$ DCs from tumor-bearing $RAG^{-/-}$ mice undergoing treatment or not. Bar graphs indicate absolute numbers of $CD11c^{high}$ MHC class II^+ $CD86^{high}$ cells. Values represent SEM (n = 3 mice/group; **, $P < 0.05$). Data are representative of four experiments.

when compared with nontreated groups (Fig. 4 E). Collectively, these experiments suggest that adoptively transferred TRP-1 CD4+ T cells become activated on host MHC class II+ cells and may eradicate tumors through an IFN-γ–dependent pathway that may be linked to MHC class II expression on the tumor.

Activation, persistence, and memory formation of TRP-1 CD4+ T cells

To determine how lymphopenia affects long-term maintenance and memory formation of Th1 CD4+ T cells, we analyzed TRP-1 CD4+ T cells from mice undergoing long-term tumor therapy (~270 d). It may be possible that in vivo differentiation might bestow upon T cells a signal that allows them to persist and become superior antitumor CD4+ T cells. Therefore, we looked at long-term maintenance and found that adoptively transferred TRP-1 CD4+ T cells can maintain tumor regression and persist at relatively high levels 270 d after transfer (Fig. 5, A and B). We also looked at memory formation and activation markers that differentiate between effector memory T cells (TEMs) and central memory T cells (TCMs). CD62L and CD44 were high and low, respectively, as expected before transfer of CD4+ T cells (Fig. 5 C). Naive T cells were also IL-7Rα^{high} (Fig. 5 D). After 1 wk, CD4+ T cells became activated as shown by the phenotype CD62L^{low}, CD44^{high}, and IL-7Rα^{low} (Fig. 5, C and D). Mice were analyzed for adoptively transferred T cells again 270 d later. CD4+ T cells remained at high levels and were mostly TEM (CD62L^{low}

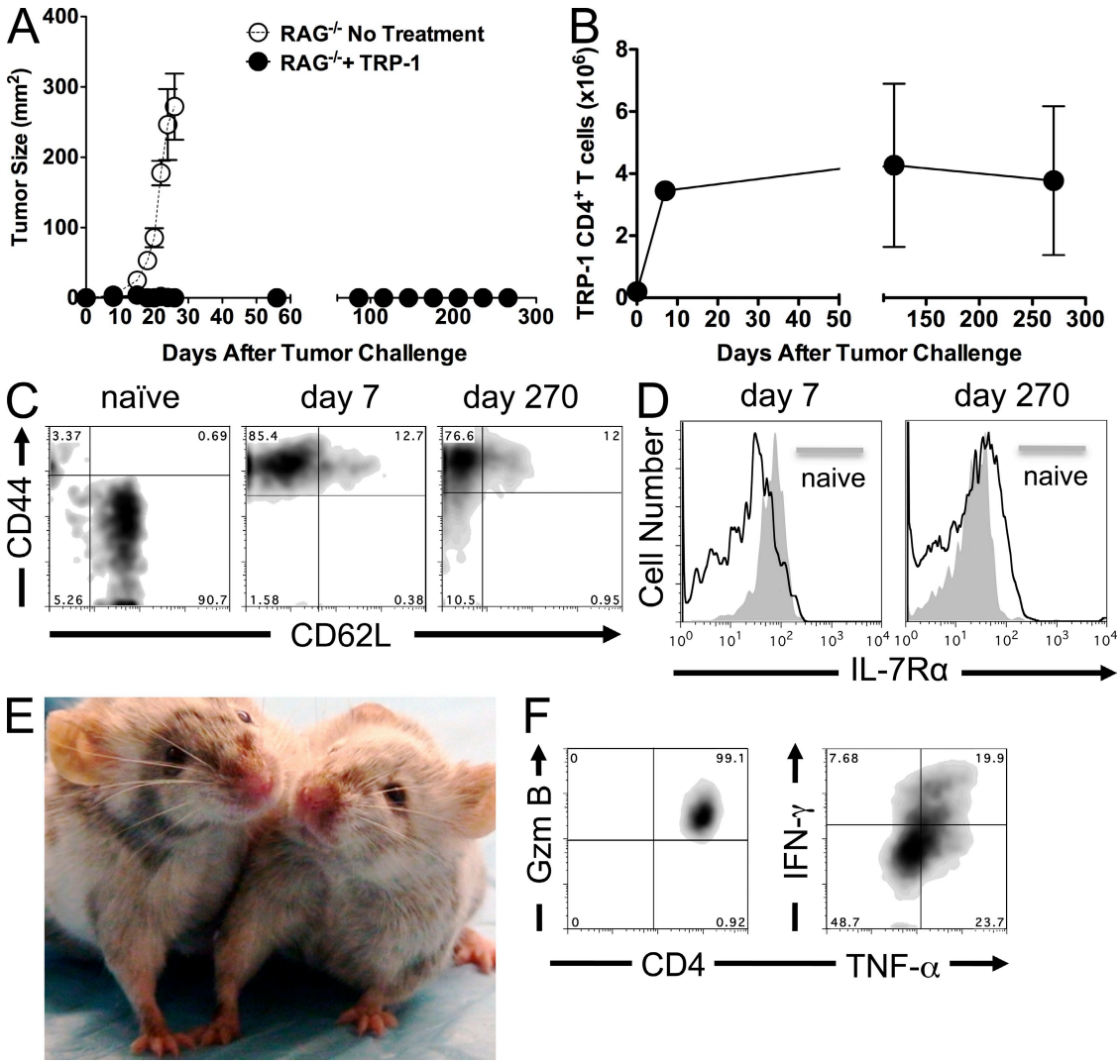

Figure 5. Activation, persistence, and memory formation of TRP-1 CD4+ T cells. (A) Long-term tumor regression. Lymphopenic mice were treated on day 7 after tumor challenge with adoptive transfer of naive TRP-1 CD4+ T cells and followed for 270 d. (B) TRP-1 CD4+ T cells persist for long periods in lymphopenic mice. Error bars indicate SEM. (C) Flow cytometry of CD44 and CD62L expression on naive TRP-1 CD4+ T cells before transfer, after 1 wk of in vivo differentiation, and at 270 d. (D) IL-7Rα expression on naive (shaded) and 1-wk in vivo differentiated and 270-d persisting TRP-1 CD4+ T cells (solid line). (E) Phenotype of 270-d-old treated RAG$^{-/-}$ mice. (F) Granzyme B, IFN-γ, and TNF expression in TRP-1 CD4+ T cells isolated on day 120 after treatment from tumor-free mice. Data are representative of two experiments (n = 5 mice/group).

and CD44^{high}), but ~12% of CD4$^+$ T cells were TCM (CD62L^{high} and CD44^{high}; Fig. 5 C).

To assess whether TRP-1 self-reactive T cells converted to T reg cells over this time span, we looked at Foxp3 expression in adoptively transferred cells and found that in long-term responder mice, Foxp3 expression remained stable at 5–15% (Fig. S3). Mice also became completely depigmented (Fig. 5 E). Lastly, granzyme B, IFN-γ, and TNF were evident in TRP-1 CD4$^+$ T cells 120 d after transfer, indicating that they were effector T cells (Fig. 5 F). Therefore, tumor-specific CD4$^+$ T cells differentiated in vivo can persist for long periods of time and appear to continue to clear antigen based on the progressive depigmentation, granzyme B expression, and TEM phenotype. TRP-1 CD4$^+$ TCM cells may also renew the TRP-1 CD4$^+$ TEM pool over time.

NK cells are not required for tumor therapy with TRP-1 CD4$^+$ T cells

To determine host mechanisms that affect the activation and persistence of adoptively transferred CD4$^+$ T cells, we transferred TRP-1 CD4$^+$ T cells into RAG$^{-/-}\gamma_c^{-/-}$ hosts, which lack NK cells in addition to T, B, and NKT cells (Cao et al., 1995). Previously, it was shown that treatment of a 1-d tumor was hindered in RAG$^{-/-}\gamma_c^{-/-}$ mice (Perez-Diez et al., 2007), and this was attributed to a lack of NK cells. In agreement with the previous study, we found that tumor therapy was hindered even when supplemented with irradiation (Fig. 6 A). To examine this further, we depleted NK cells in tumor-bearing RAG$^{-/-}$ mice starting on days 0 and 7 after tumor challenge, 1 d after ACT, and then weekly. TRP-1 CD4$^+$ T cells were transferred into tumor-bearing RAG$^{-/-}$ mice on day 8. We found that NK1.1 depletion (1 mg of anti-NK1.1/mouse) did not affect tumor treatment in a negative manner (Fig. 6 B). We also irradiated RAG$^{-/-}$ mice with 5 Gy. The results were similar to those associated with NK cell depletion (Fig. 6 A). Next, we transferred 5 × 10^6 sorted NK cells into RAG$^{-/-}\gamma_c^{-/-}$ mice along with TRP-1 CD4$^+$ T cells. Treatment was similar to RAG$^{-/-}\gamma_c^{-/-}$ mice with TRP-1 CD4$^+$ T cells alone (Fig. 6 C).

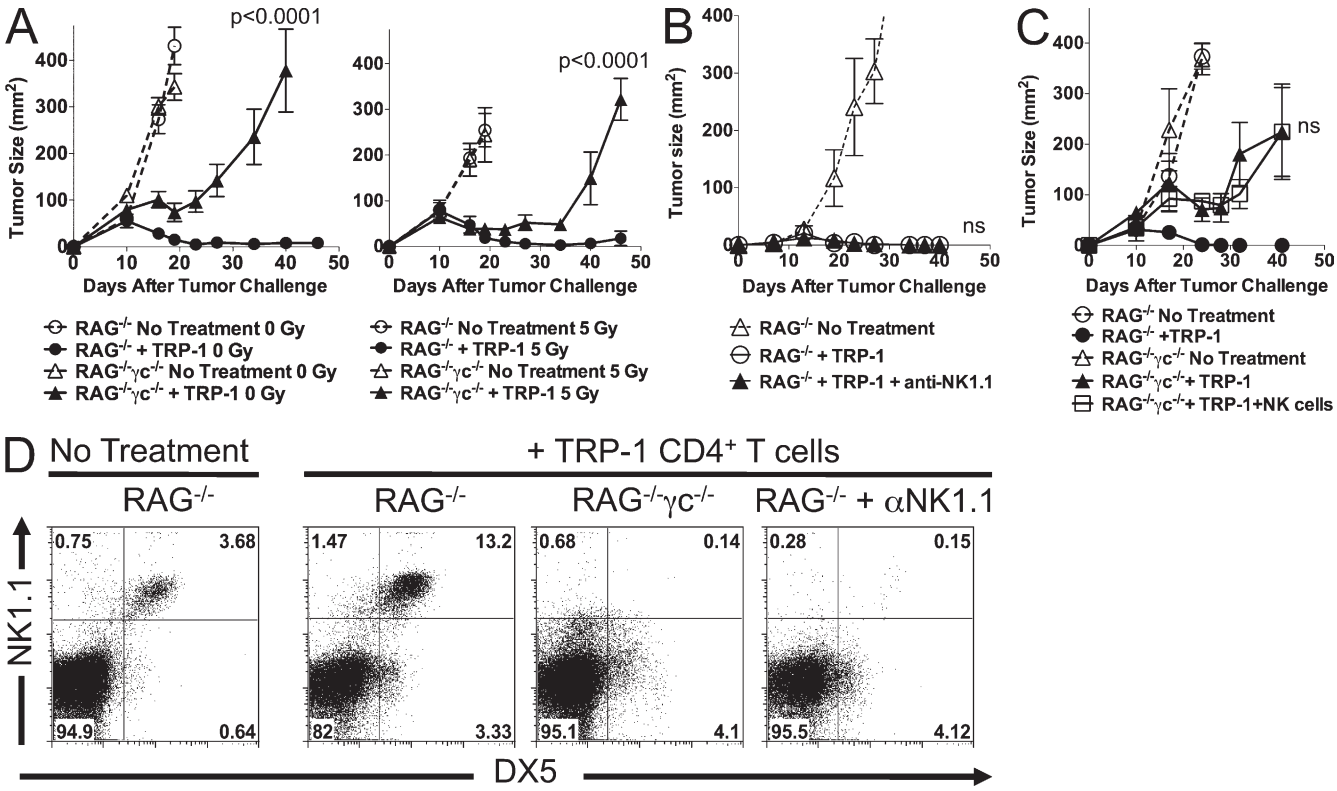

Figure 6. NK cells are not required for tumor therapy with TRP-1 CD4$^+$ T cells. (A) Tumor-bearing RAG$^{-/-}$ and RAG$^{-/-}\gamma_c^{-/-}$ mice were treated on day 7 without or with irradiation (5 Gy) and with and without ACT with 2 × 10^5 naive TRP-1 CD4$^+$ T cells. Data are representative of three experiments ($P < 0.0001$). The p-value indicated is for RAG$^{-/-}$ + TRP-1 versus RAG$^{-/-}\gamma_c^{-/-}$ + TRP-1 CD4$^+$ T cells for both 0 and 5 Gy. (B) Tumor-bearing RAG$^{-/-}$ mice received 1 mg of anti-NK1.1 antibodies weekly starting 1 d before ACT and were compared with tumor-bearing RAG$^{-/-}$ mice receiving no treatment or 2 × 10^5 naive TRP-1 CD4$^+$ T cells. Data represent three experiments with five to eight mice per group. P = NS for RAG$^{-/-}$ + TRP-1 versus RAG$^{-/-}$ + TRP-1 + anti-NK1.1. (C) 7-d tumor-bearing RAG$^{-/-}$ and RAG$^{-/-}\gamma_c^{-/-}$ mice received naive TRP-1 CD4$^+$ T cells plus sorted NK cells from WT mice where indicated (P = NS for addition of NK cells). Data represent two independent experiments. Error bars indicate SEM. (D) Flow cytometry of NK cells (NK1.1$^+$DX5$^+$ cells) in the LNs of RAG$^{-/-}$, RAG$^{-/-}$ + anti-NK1.1 antibodies, and RAG$^{-/-}\gamma_c^{-/-}$ mice as indicated 3–4 wk after adoptive T cell transfer. Data are representative of three independent experiments (n = 2–3 mice/group).

NK cells are required for efficient priming and activation of CD4+ T cells in vivo (Martín-Fontecha et al., 2004). We found that NK cells entered the LN readily in tumor-bearing RAG$^{-/-}$ mice receiving CD4+ T cells but not in RAG$^{-/-}$-$\gamma_c^{-/-}$ mice, untreated RAG$^{-/-}$ mice, or NK-depleted RAG$^{-/-}$ mice (Fig. 6 D). These data suggest that NK cells are not required for activation of naive tumor-specific CD4+ T cells in vivo.

γ_c signaling on host cells is required for the proper activation, differentiation, and survival of naive tumor-specific TRP-1 CD4+ T cells in vivo

We were perplexed as to why RAG$^{-/-}\gamma_c^{-/-}$ mice failed to control tumor immunity even when WT NK cells were present. TRP-1 CD4+ T cells were analyzed 1 wk after transfer from RAG$^{-/-}$ and RAG$^{-/-}\gamma_c^{-/-}$ hosts. Expression of CD62L was low on both populations, indicating activation in vivo (Fig. 7 A). However, CD122, ICOS, and CD25 were expressed at lower levels on TRP-1 CD4+ T cells from RAG$^{-/-}\gamma_c^{-/-}$ hosts when compared with TRP-1 CD4+ T cells from RAG$^{-/-}$ mice (Fig. 7 A). Consistent with these findings, IFN-γ and the CXCR3 ligand CXCL9, but not CXCL10, were lower in the serum of RAG$^{-/-}\gamma_c^{-/-}$ hosts (Fig. 7 B and not depicted). Surprisingly, 1 wk after therapy, there were more TRP-1 CD4+ T cells in RAG$^{-/-}\gamma_c^{-/-}$ hosts than in RAG$^{-/-}$ hosts (Fig. 7 C), which is in agreement with recent findings by others (Guimond et al., 2009). We therefore looked at other time points in vivo and found that TRP-1 CD4+ T cells failed to persist after 4 wk in RAG$^{-/-}\gamma_c^{-/-}$ lymphopenic hosts (Fig. 7 C). Looking at other parameters, we found that TRP-1 CD4+ T cells from RAG$^{-/-}$ mice produced IFN-γ, but not IL-17, when stimulated ex vivo. However, TRP-1 CD4+ T cells from RAG$^{-/-}\gamma_c^{-/-}$ mice failed to produce IFN-γ or IL-17 (Fig. 7 D). Because failure to produce IFN-γ may be related to their failure to differentiate, we stained for the Th1 transcription factor Tbet and found that expression was considerably lower in TRP-1 CD4+ T cells from RAG$^{-/-}\gamma_c^{-/-}$ hosts (Fig. 7 E). Further exploration showed that CD11c^{high} DCs expressed less MHC class II and CD80 but similar levels of CD40 in RAG$^{-/-}\gamma_c^{-/-}$ hosts when compared with RAG$^{-/-}$ hosts (Fig. 7 F). Lastly, it was possible that transferred TRP-1 CD4+ T cells could have converted to T reg cells, which lead to tumor progression, but that was not evident beyond 4 wk after therapy (Fig. S4).

Collectively, the data suggest that γ_c signaling on host cells may be required for proper activation of naive CD4+ T cells. This may occur through IL-12 signals that increase class II and CD80 expression on host DCs (Ohteki et al., 2001, 2006; Terme et al., 2008). Consistent with this, MHC class II and CD80 were decreased in RAG$^{-/-}\gamma_c^{-/-}$ hosts receiving TRP-1 CD4+ T cells. These experiments suggest that lack of γ_c signaling, possibly on host DC, in RAG$^{-/-}\gamma_c^{-/-}$ mice might hinder proper activation of adoptively transferred naive TRP-1 CD4+ T cells, which eventually leads to their failure to survive and maintain treatment of established tumors.

DISCUSSION

ACT using a patient's own immune cells to treat established cancer has been highly successful therapy against melanoma (Rosenberg et al., 2008) and is increasingly being used against other cancers as well (Stephan et al., 2007; Pule et al., 2008). ACT using CD4+ T cells was recently attempted in a patient with metastatic melanoma using NY-ESO-1–specific CD4+ T cells expanded in vitro in IL-7 and IL-2 (Hunder et al., 2008). Surprisingly, adoptive transfer therapy with CD4+ T cells led to objective cancer regression without prior vaccination or lymphoconditioning. Although this was reported in only one patient, the findings support the possibility of treating established tumors without vaccination or radiation therapies that may cause extended morbidity in patients with prolonged and severe sickness. In the prior study, epitope spreading was reported as the likely cause of the enhanced treatment, indicating that CD4+ T cells helped a CD8+ T cell response to the established tumor as shown in preclinical models (Marzo et al., 2000; Antony et al., 2006). In this paper, we describe a CD8+ T cell– and vaccine-independent therapy in which in vivo differentiated Th1 CD4+ T cells recognize self and eradicate established tumors.

Although we focused on a naive T cell– and a vaccine-independent therapy, we do not advocate that vaccines are ineffective or that in vitro differentiation of T cells should not be attempted. Recently, it was shown that a tumor vaccine was required to enhance the immunotherapy of cancer when using in vitro–activated antitumor CD8+ T cells and CD4+ Th cells (Overwijk et al., 2003; Antony et al., 2005). Vaccine independence was seen only after extreme radiation therapy (9 Gy) before ACT or in vitro programming of TEM cells (Wrzesinski et al., 2007; Klebanoff et al., 2009). The importance of activating CD4+ T cell help with a tumor vaccine has also been strengthened of late. A study using a modified TRP-1 vaccine could induce either tumor immunity or autoimmunity depending on the modification in the vaccine (Engelhorn et al., 2006). When the modified vaccine activated CD4+ T cell help through enhanced processing of MHC II peptides, tumor immunity was induced (Engelhorn et al., 2006). This new strategy to stimulate CD4+ T cells against self emphasizes the need to understand how CD4+ T cells become activated in vivo.

In this study, we demonstrate that naive CD4+ T cells transferred into lymphopenic hosts differentiated into Th1 cytotoxic T cells expressing the hallmark genes associated with effector T cells—Tbet, IFN-γ, CXCR3, granzyme B, perforin, and LAMP-1—and caused the regression of established tumors without the aid of CD8+ T, B, NK, or NKT cells. CD4+ T cells have been shown to play a role in cancer immunotherapy through a multitude of mechanisms; CD4+ T cells can help cytotoxic CD8+ T cells eradicate tumors (Hung et al., 1998; Nishimura et al., 1999; Toes et al., 1999; Sutmuller et al., 2001; Behrens et al., 2004a,b; Antony et al., 2005; Engelhorn et al., 2006; Brandmaier et al., 2009), and CD4+ T cells can condition a DC through CD40L to enhance CD8+ CTL function (Bennett et al., 1998; Kalams and

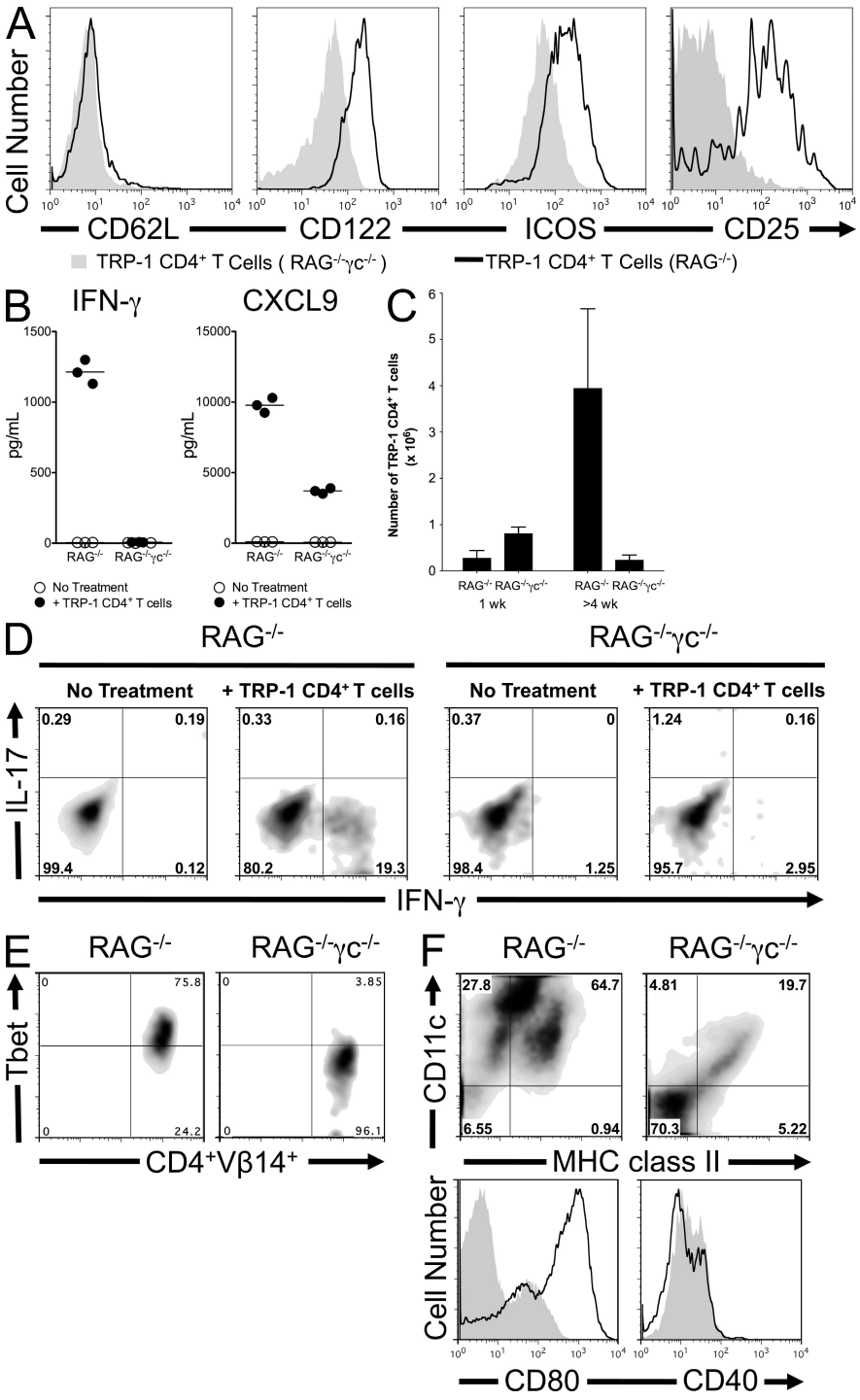

Figure 7. γ_c signaling on host DC is required for survival and differentiation of TRP-1 T cells in vivo. (A) Differential activation of TRP-1 CD4+ T cells in RAG$^{-/-}$ and RAG$^{-/-}\gamma_c^{-/-}$ hosts. Spleens from RAG$^{-/-}$ or RAG$^{-/-}\gamma_c^{-/-}$ mice were isolated and stained for TRP-1 CD4+ T cells. Shown are CD62L, CD122, ICOS, and CD25 expression on gated TRP-1 CD4+ T cells from indicated host. (B) IFN-γ and CXCL9 are differentially expressed in the serum at 1 wk in RAG$^{-/-}$ and RAG$^{-/-}\gamma_c^{-/-}$ hosts after TRP-1 CD4+ T cell transfer. Horizontal bars represent mean. (C) TRP-1 CD4+ T cells expand in RAG$^{-/-}\gamma_c^{-/-}$ hosts initially but fail to survive after 4 wk. Error bars indicate SEM. (D) Flow cytometry of IFN-γ and IL-17 expression in TRP-1 CD4+ T cells isolated from tumor bearing RAG$^{-/-}$ and RAG$^{-/-}\gamma_c^{-/-}$ mice 4 wk after transfer. T cells were activated with PMA and ionomycin for 4 h and then fixed and permeabilized and stained with anti–IFN-γ and IL-17 antibodies. (E) Tbet expression in TRP-1 CD4+ T cells 4 wk after transfer. (F) MHC class II, CD80, and CD40 expression in RAG$^{-/-}\gamma_c^{-/-}$ hosts. Top flow diagram indicates CD11c^{high} MHC class II+ cells; bottom flow histograms show CD80 and CD40 expression on gated CD11c^{high} MHC class II+ cells. Solid line represents RAG$^{-/-}$ mice treated with TRP-1 CD4+ T cells. Shaded histogram represents RAG$^{-/-}\gamma_c^{-/-}$ mice treated with TRP-1 CD4+ T cells. Data are representative of three independent experiments (n = 5 mice/group).

Walker, 1998; Ridge et al., 1998; Diehl et al., 1999), but the mechanisms by which $CD4^+$ T cells cause eradication of established tumors by themselves are still unknown.

Previously it was shown that $CD4^+$ T cells could prevent the growth of tumors by antiangiogenesis through the activation of IFN-γR on nonhematopoietic cells. However, this action was not shown for large vascularized tumors in a treatment and adoptive cell transfer model and it was not clear whether $CD8^+$ T cells were involved (Qin and Blankenstein, 2000). Rejection of small tumors was also shown to be an indirect process through IFN-γ–dependent activation of macrophages or by eosinophils and NK cells (Hung et al., 1998; Corthay et al., 2005). We noted high levels of CCL11 in the serum during treatment and CCL24 expression by TRP-1 $CD4^+$ T cells, which are known to recruit eosinophils, so their role cannot be excluded. Indirect effects of $CD4^+$ T cells have also been noted in a study using Tg Marylyn $CD4^+$ T cells, which recognize the male antigen H-Y. MHC class II^- tumors were amenable to treatment and this was attributed to NK cells (Perez-Diez et al., 2007). In this paper, we do not find a role for NK cells in this model.

Not surprisingly, with adoptive cell transfer therapies using in vitro differentiated $CD4^+$ T cells to treat large vascularized tumors, IFN-$\gamma R^{-/-}$ mice were shown not to respond to treatment (Muranski et al., 2008), and anti–IFN-γ diminished the antitumor effect (Nishimura et al., 1999; Muranski et al., 2008). However, IFN-$\gamma^{-/-}$ mice were able to respond to treatment with in vitro differentiated $CD4^+$ T cells, indicating that the innate source of IFN-γ (NK cells) was not needed (Qin and Blankenstein, 2000; Muranski et al., 2008). Because we saw increased MHC class II expression in the tumor microenvironment, it is possible that IFN-γ from $CD4^+$ T cells may be needed to increase MHC class II expression on tumor cells, which allows a direct interaction between T cells and cancerous cells to occur (Quezada et al., 2010). Direct killing of tumors is possible, as indicated by high granzyme B expression by TRP-1 $CD4^+$ T cells on gene microarray and expression of perforin, granzyme B, and LAMP-1 in TRP-1 $CD4^+$ T cells isolated from the spleen. IFN-γ may be working indirectly at a local level through macrophages expressing NO, TNF, ROS, and NKG2D receptors (Kahn et al., 1991; Raulet and Guerra, 2009). IFN-γ released by differentiated $CD4^+$ T cells may be so extremely high in the tumor microenvironment that the tumor vasculature expressing IFN-γ receptors undergoes anti-angiogenesis. The presence of specific disease, indicated by depigmentation and uveitis (Muranski et al., 2008), supports the theory of a direct killing mechanism against MHC class II^+ cells. Inflammatory monocytes in the tumor microenvironment may also play an indirect role in the process. Another unappreciated role of IFN-γ may be to induce the chemokines CXCL9 and CXCL10 (Luster et al., 1985), which may be required to attract $CXCR3^+$ antitumor $CD4^+$ T cells into the tumor site. Most importantly, all of these mechanisms may not be mutually exclusive.

Why then does tumor therapy fail in $RAG^{-/-}\gamma_c^{-/-}$ mice? $CD4^+$ T cells need to become properly activated cytotoxic T cells, which is not dependent on NK cells. $RAG^{-/-}\gamma_c^{-/-}$ mice express low levels of MHC class II and CD80 on $CD11c^{high}$ cells, both of which may be needed to properly activate $CD4^+$ T cells in vivo. The availability of MHC class II on APC has already been shown to be critical in regulating the homeostatic proliferation of $CD4^+$ T cells (Kassiotis et al., 2003). Therefore, removing DC cells by high-intensity lymphodepleting regimens may harm ACT with $CD4^+$ T cells. As seen in other studies, adding these cells back with lymphodepleting regimens may enhance immunotherapy (Dubsky et al., 2007; Palucka et al., 2007; Banchereau et al., 2009).

γ_c signaling is part of the IL-2 cytokine family, which includes IL-4, IL-7, IL-9, IL-15, and IL-21. Increased IL-7 and IL-15 levels in the host have been shown in the clinic and in animal models to be the mechanisms by which lymphodepleting regimens enhance adoptive immunotherapy (Gattinoni et al., 2005a; Dudley et al., 2008; Guimond et al., 2009). However, it has not been shown in a reciprocal manner that the host needs γ_c signaling for efficient activation of tumor-specific T cells. We observed that with loss of γ_c, $CD4^+$ T cell differentiation and survival were diminished by wk 4 after transfer. In $RAG^{-/-}$ γ_c^+ hosts, we saw expansion and survival of $CD4^+$ T cells past 4 wk. We also found higher expression of MHC class II on $CD11c^{high}CD86^{high}$ DC and the Th1 transcription factor Tbet in TRP-1 $CD4^+$ T cells isolated from $RAG^{-/-}$ mice when compared with TRP-1 $CD4^+$ T cells from $RAG^{-/-}\gamma_c^{-/-}$ mice. Our results differ from those in a recent study showing that γ_c signaling on DC reduces $CD4^+$ T cell homeostatic proliferation in vivo (Guimond et al., 2009). We found that TRP-1 $CD4^+$ T cells expand initially at 1 wk after adoptive cell transfer but fail to survive in $RAG^{-/-}\gamma_c^{-/-}$ hosts once tumor progresses, which is usually 4 wk or later. Surprisingly, even though there were more tumor-specific T cells in $RAG^{-/-}\gamma_c^{-/-}$ hosts, it was not enough to clear tumor. It may be possible that strong (self-reactive) TCR activation is a prerequisite for survival for self-reactive TRP-1 $CD4^+$ T cells because the previous study used polyclonal $CD4^+$ T cells or antigen-specific $CD4^+$ T cells that do not recognize an antigen in the host (Guimond et al., 2009). This strong activation (indicated by CD5 expression on TRP-1 $CD4^+$ T cells) may come from increased expression of CD80 and MHC class II on DC that requires γ_c for their activation to become sensitive to and produce IL-12, which is paramount for Th1 priming in vivo (Ohteki et al., 2001, 2006; Terme et al., 2008). During in vivo differentiation, TRP-1 $CD4^+$ T cells expressed high levels of IL12Rβ1 and IL-27R during in vivo differentiation, which are required for priming, and IL-23R, which may be important for Th1 memory formation. The strong upregulation of *Stat4* in TRP-1 T cells in vivo shows that IL-12 and/or IL-23 signals are driving their activation, as indicated by high IFN-γ production and IL-22, respectively. This is further supported by lack of IFN-γ and Tbet expression in TRP-1 $CD4^+$ T cells isolated from $RAG^{-/-}\gamma_c^{-/-}$ hosts 4 wk after transfer in addition to lack of IFN-γ and CXCL9, but not CXCL10, in the serum at 1 wk in $RAG^{-/-}\gamma_c^{-/-}$ hosts. Therefore, it appears that lack of proper DC activation leads to low

expression of MHC class II and CD80, which subsequently leads to inappropriate activation of naive TRP-1 $CD4^+$ T cells in $RAG^{-/-}\gamma_c^{-/-}$ hosts. Loss of IFN-γ expression by $CD4^+$ T cells possibly leads to loss of CXCL9 expression in the tissues, and this may lead to failure of recruitment of antitumor $CXCR3^+$ $CD4^+$ T cells to the tumor site. This pattern of recruitment may be similar to the process that occurs during nephritis or disease of the eye during herpes simplex virus type 1 infection, which requires CXCL9, but not CXCL10, for recruitment of $CXCR3^+$ T cells into the tissues (Wuest et al., 2006; Menke et al., 2008).

Why seek to understand how TRP-1 $CD4^+$ T cells are activated in vivo? More intense ablation may remove DCs that provide the secondary signals (such as IL-12 or possibly IL-23) that are needed to properly differentiate $CD4^+$ T cells in vivo into long-lived memory cells, which is similar to IL-2 for $CD8^+$ T cells (Williams et al., 2006). Less space may be the Achilles' heel of intense lymphodepletion when using $CD4^+$ T cells. This may indicate that $CD4^+$ T cells are not receiving the proper signals initially for long-lived survival. In mice irradiated with 9 Gy and humans receiving 12 Gy lymphodepleting regimens, IL-2 administration was needed to augment ACT. Transfer of naive T cells has not yet been accomplished in these settings. These cells may require administration of DC for support (Steinman and Banchereau, 2007).

How can this environment be obtained without lymphodepleting chemotherapy or irradiation? Cytokines (such as IL-7 and IL-15) found in patients receiving high-dose radiation therapy and irradiated WT and $RAG^{-/-}$ mice may be used to mimic lymphopenia in a WT host (Gattinoni et al., 2005a; Zhang et al., 2005; Guimond et al., 2009; Pellegrini et al., 2009). In humans, IL-7 has shown strong promise in recent years, and long administration has been shown to release the inhibitory networks that suppress antitumor immunity (Pellegrini et al., 2009). Cytokine/antibody immune complexes to IL-2, IL-7, or IL-15 may offer another way to mimic lymphopenia, as a much lower amount of cytokines is needed to enhance proliferation of naive and memory phenotype T cells in vivo (Boyman et al., 2006, 2008; Rubinstein et al., 2006; Cho et al., 2007). Lymphopenia-induced activation of $CD4^+$ T cells itself may program these cells to become less prone to tolerance induction seen in other systems using $CD4^+$ T cells (Sotomayor et al., 1999), and we are currently exploring this.

Administration of antibodies against costimulatory and inhibitory molecules is another method to enhance antitumor immunity. We saw high expression of CTLA-4 and 41BB on TRP-1 $CD4^+$ T cells. Anti–CTLA-4 therapy is being used in clinical trials and has been shown to enhance adoptive cell therapies already with $CD8^+$ T cells and donor lymphocyte infusions by inhibiting T reg cells and enhancing activation of non–T reg cells (Quezada et al., 2006, 2008; Peggs et al., 2009). With tumor-specific TRP-1 $CD4^+$ T cells, this enhances therapy considerably in WT and lymphopenic hosts (Quezada et al., 2010). Stimulation of 41BB also enhances immunotherapy using T cells coexpressing 41BBL (Stephan et al., 2007). It can also break tolerance to a poorly immunogenic tumor (Wilcox et al., 2002). ICOS is being considered as another immunotherapy candidate (Paulos, C. and June, C., personal communication). These therapies could be combined with ACT in the future.

In this study, we focused on naive $CD4^+$ T cells, which differentiate properly in their natural state and become long-lived memory T cells. This idea was also demonstrated in another tumor model that involved activation of naive T cells via forced LIGHT expression in the tumor microenvironment (Yu et al., 2004). Although forced expression of LIGHT in human tumors may not be a clinical reality, this led to a profound tumor rejection indicating the importance of activating naive T cells in their natural state.

How do you obtain naive T cells from humans? TCR transduction of naive T cells with $CD4^+$ MHC class II tumor-specific TCRs could be attempted in humans, as previously achieved with MHC class I tumor-specific TCRs (Morgan et al., 2006; Hinrichs et al., 2009). It may be possible to transfer naive tumor-specific $CD4^+$ T cells into hosts preconditioned with noninvasive cytokine therapies that do not cause harmful side effects. Transduction of high avidity TCRs to tumor antigens into naive or cord PBL is feasible and may be augmented by high doses of IL-7 or IL-15 administration plus anti-CTLA or anti-41BB therapy.

How do you obtain nontolerant highly reactive TCRs as seen in this paper? Mouse TCRs reactive against human cancer antigens can be transfected into human T cells using gene therapy (Johnson et al., 2009). Human HLA Tg mice can be immunized with human cancer antigens to generate highly tumor-reactive TCRs, which can then be used in the immunotherapy of cancer in humans.

In summary, TRP-1 Tg mice represent a new ACT immunotherapy model for the study of ways to induce potent antitumor immunity against established melanoma without the need for cytotoxic $CD8^+$ T cells or a vaccine. Understanding the mechanisms of tumor regression in mice with lymphopenia may allow the development of new therapies that work in the absence of chemotherapy and radiation. These data suggest a new role for $CD4^+$ T cells as cytotoxic T cells.

MATERIALS AND METHODS

Mice. *Tyrp1*$^{B\text{-}w}$RAG$^{-/-}$ TRP-1-specific $CD4^+$ TCR Tg mice (B6.Cg-*Rag1*^{tm1Mom} *Tyrp1*$^{B\text{-}w}$ Tg(Tcra,Tcrb)9Rest/J; deposited at The Jackson Laboratory) and WT *Tyrp1*$^{+/+}$ TRP-1-specific $CD4^+$ TCR Tg mice were created by P.A. Antony and K. Irvine at the National Cancer Institute (Bethesda, MD; Muranski et al., 2008). Recombination-activating gene $1^{-/-}$ (*Rag1*^{tm1Mom}) mice, Foxp3^{sf}, C57BL/6 WT, and MHC class $II^{-/-}$ were purchased from The Jackson Laboratory. $RAG^{-/-}\gamma_c^{-/-}$ mice were purchased from Taconic (Cao et al., 1995). Foxp3^{sf} mice were crossed with *Tyrp1*$^{+/+}$ TRP-1-specific $CD4^+$ TCR Tg mice to generate *Tyrp1*$^{+/+}$ TRP-1-specific $CD4^+$ TCR Foxp3^{sf} Tg mice. All mice were used in accordance with guidelines from the University of Maryland Institutional Animal Care and Use Committee. All experiments were conducted with the approval of the Animal Use and Care Committees of the National Cancer Institute, Johns Hopkins University School of Medicine, and the University of Maryland School of Medicine.

Tumor lines and measurement. B16.F10 (H-2^b), hereafter called B16, is a TRP-1^+ spontaneous murine melanoma that was obtained from ATCC

and maintained in culture media as previously described (Antony et al., 2006). Tumors were injected subcutaneously at 2×10^5 cells/mouse. Tumors are measured blindly with digital calipers. The perpendicular diameters are determined and multiplied to generate the area in millimeters squared as previously described (Antony et al., 2006).

Sorting and adoptive cell transfer. TRP-1 $CD4^+$ T cells were sorted from spleens of donor *Tyrp1*$^{B\text{-}w}$RAG-1$^{-/-}$ TRP-1–specific Tg male mice. Spleens were harvested and made into single-cell suspensions. Cells were made devoid of red blood cells by ACK lysis. Subsequently, cells were counted and enriched for $CD4^+$ T cells by magnetic bead sorting using a $CD4^+$ T cell enrichment kit from Miltenyi Biotec. Enriched $CD4^+$ T cells were counted and resuspended in PBS and used in adoptive transfer studies (2×10^5 cells/mouse). NK cells were isolated from WT mice using NK cell sorting kits from Miltenyi Biotec. When indicated, 5×10^6 NK cells were transferred on the same day as TRP-1–specific T cells. Open-repertoire $CD4^+CD25^-$ T cells from WT mice were sorted as previously described (Antony et al., 2006). Mice were irradiated as previously described (Wrzesinski et al., 2007).

Depleting antibodies. Anti–IFN-γ (XMG1.2, NA/LE) and anti-NK1.1 (NA/LE, PK136) were purchased from BD.

Flow cytometry. Anti-CD4 (RM4-5), anti-CD25 (PC61), anti-CD122 (TM-β-1), anti-Vβ14 (14–2), anti-NK1.1 (PK136), anti-ICOS (7E.17G9), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD11c (HL3), anti-CD86 (GL1), anti–MHC class II (AF6-120.1), anti–IL-17 (TC11-18H10), anti–IFN-γ (XMG1.2), anti-TNF (MP6-XT22), anti-CD49b (DX5), and CD11b (M1/70) were obtained from BD. Anti–IL-7Rα (SB/199), anti-CXCR3 (CXCR3-173), anti-Foxp3 (FJK-16s), anti-Tbet (4B10), anti–MHC class II (I-A/I-E; M5/114.15.2), anti–GR-1 (RB6-8C5), anti-perforin (eBioOMAK-D), anti-NKp46 (29A1.4), anti-LAMP-1 (1D4B), anti–granzyme B (16G6), and anti-NKG2D (CX5) were obtained from eBioscience. All flow cytometry scales are log scales, if not otherwise specified. Intracellular staining for cytokines was done with the Cytofix/Cytoperm intracellular staining kit (BD). For granzyme B, Foxp3, and perforin, the Foxp3 staining buffer set was used as recommended by eBioscience. All samples were run on a FACSCalibur (BD; Department of Surgery, University of Maryland School of Medicine) and analyzed by FlowJo Software (Tree Star, Inc.).

Confocal microscopy. Confocal microscopy was done as previously described (Quezada et al., 2008) except that slides were imaged on an LSM (Olympus) with a 20× oil immersion objective. In brief, tumor was frozen in OCT solution (Sakura). 8-μm sections were cut with a microcryotome (Leica), fixed for 10 min in cold acetone, allowed to air dry, hydrated with PBS for 5 min, and then blocked with PBS with 2% FCS, 50 μg/ml 24G2 antibodies, and 5% rat, hamster, and mouse serum for 15 min. Slides were stained overnight with anti–MHC class II APC and DAPI, washed, and mounted. Samples were scanned on an inverted confocal microscope (LSM; University of Maryland Greenebaum Cancer Center) under a 20× oil immersion objective. Images were analyzed with Image J64 software (National Institutes of Health).

Gene microarrays. RNA was isolated from FACS-sorted $CD4^+$ T cells from LN and spleens of tumor-bearing RAG$^{-/-}$ mice undergoing treatment with TRP-1 $CD4^+$ T cells. RNA was isolated with RNA isolation kits (QIAGEN). Sample amplification and labeling procedures were performed using a Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies). In brief, 400 ng of total RNA was used as starting material base on available yield of RNA isolation but was kept at the same amount of input in each experiment. Total RNA was reverse transcribed into first-strand and second-strand complementary DNA by MMLV-RT using an oligo-dT primer that incorporates a T7 promoter sequence. The complementary DNA was then used as a template for in vitro transcription in the presence of T7 RNA polymerase and cyanine-labeled CTPs. The labeled complementary RNA was purified using RNeasy micro kit (QIAGEN) and followed by quantification of both concentrations of complementary RNA and dye labeled. RNA spike-in controls (Agilent Technologies) were added to RNA samples before amplification and labeling according to the manufacturer's protocol. The entire amount of each sample labeled with Cy3 or Cy5 was mixed with control targets (Agilent Technologies). Fragmentation was performed by incubating at 60°C for 30 min and stopped by adding an equal volume of 2× GE Hi-RPM hybridization buffer (Agilent Technologies). Agilent 4X44K whole mouse genome array (G4122F) with 41534 unique probes was used. Fragmented targets were added onto a microarray, assembled into a hybridization chamber (Agilent Technologies), and hybridized at 60°C for 17 h in a hybridization oven with rotation. Hybridized microarrays were washed and dried according to the Agilent microarray processing protocol. Microarrays were scanned using an Agilent G2505B Scanner controlled by Agilent Scan Control 7.0 software. Data were extracted with Agilent Feature Extraction 9.1 software. Differentially expressed targets were identified using the processed data and the log ratio generated by the software. Only values of twofold or higher were reported. Heat maps were hand generated from the gene lists. The GEO microarray data accession no. is GSE19904.

Measurement of serum cytokines and chemokines. Serum was collected via tail vein using serum collection tubes (BD). Serum was analyzed by MILLIPLEX 32-Plex assay (University of Maryland, Baltimore, Cytokine Core Laboratory, and Millipore).

Statistics. An unpaired Student's *t* test was used to compare the differences between cytokines and chemokines as indicated. Tumor curves were compared using a two-way ANOVA with nonrepeated measures. P-values of ≤0.05 were considered significant. PRISM 5.0b software was used to analyze the data (GraphPad Software, Inc.).

Online supplemental material. Fig. S1 shows the phenotype of TRP-1 Tg WT, Foxp3^{sf}, and *tyrp1*$^{B\text{-}w}$ mice. Fig. S1 also shows that TRP-1 $CD4^+$ cells are activated in WT mice but not in *tyrp1*$^{B\text{-}w}$ mice. Foxp3 expression in naive TRP-1 $CD4^+$ T cells from *tyrp1*$^{B\text{-}w}$ RAG$^{-/-}$ mice is shown. Lastly, tumor infiltration by TRP-1 $CD4^+$ T cells is shown by flow cytometry. Fig. S2 shows accumulation of inflammatory monocytes in the spleen, LN, and tumor after adoptive transfer of TRP-1 $CD4^+$ T cells into tumor-bearing lymphopenic mice. Fig. S3 shows stable expression of Foxp3 in TRP-1 $CD4^+$ T cells after tumor treatment. Fig. S4 shows that TRP-1 $CD4^+$ T cells do not convert to $Foxp3^+$ cells in RAG$^{-/-}\gamma_c^{-/-}$ tumor-bearing hosts during tumor progression. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091921/DC1.

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