Activated STAT5 Promotes Long-Lived Cytotoxic CD8⁺ T Cells That Induce Regression of Autochthonous Melanoma

Magali Grange¹,²,³, Michel Buferne¹,²,³, Grégory Verdeil¹,²,³, Lee Leserman¹,²,³, Anne-Marie Schmitt-Verhulst¹,²,³, and Nathalie Auphan-Anezin¹,²,³

Abstract

Immunotherapy based on adoptive transfer of tumor antigen-specific CD8⁺ T cell (TC) is generally limited by poor in vivo expansion and tumor infiltration. In this study, we report that activated STAT5 transcription factors (STAT5CA) confer high efficiency on CD8⁺ effector T cells (eTC) for host colonization after adoptive transfer. Engineered expression of STAT5CA in antigen-experienced TCs with poor replicative potential was also sufficient to convert them into long-lived antigen-responsive eTCs. In transplanted mastocytoma- or melanoma-bearing hosts, STAT5CA greatly enhanced the ability of eTCs to accumulate in tumors, become activated by tumor antigens, and to express the cytolytic factor granzyme B. Taken together, these properties contributed to an increase in tumor regression by STAT5CA-transduced, as compared with untransduced, TCs including when the latter control cells were combined with infusion of interleukin (IL)-2/anti–IL-2 complexes. In tumors arising in the autochthonous TiRP transgenic model of melanoma associated with systemic chronic inflammation, endogenous CD8⁺ TCs were nonfunctional. In this setting, adoptive transfer of STAT5CA-transduced TCs produced superior antitumor effects compared with nontransduced TCs. Our findings imply that STAT5CA expression can render TCs resistant to the immunosuppressive environment of melanoma tumors, enhancing their ability to home to tumors and to maintain high granzyme B expression, as well as their capacity to stimulate granzyme B expression in endogenous TCs. Cancer Res; 72(1); 76–87. ©2011 AACR.

Introduction

The discovery of tumor antigens recognized by autologous T cells (TC) in patients with melanoma has led to clinical protocols for adoptive transfer of tumor antigen-specific TCs. The efficacy of this treatment remains poor (1–3), for several reasons. To eliminate tumor cells, naive CD8⁺ TCs must differentiate into effector TCs (eTC) acquiring lytic enzyme-containing granules and the capacity to secrete cytokines. However, tumor antigen-specific TCs may undergo incomplete differentiation (4) or be tolerized upon encounter with tumor antigen (5). During prolonged antigen contact within tumors, CD8⁺ TCs may become functionally impaired and subsequently deleted. Methods to enhance in vivo maintenance and function of transferred eTCs are consequently required. TC therapy should aim at transferring long-lived antitumor eTCs (6) with (i) decreased thresholds for TCR signaling and enhanced ability to proliferate in response to antigen alone, thus bypassing costimulation requirement for activation, (ii) increased cytolytic activity, (iii) adequate expression of adhesion molecules or chemokine receptors to allow migration to peripheral tumor-invaded tissues, and (iv) increased resistance to tumor-derived immunosuppressive molecules. Both avidity of TCR stimulation and signals from the interleukin (IL)-2R affect differentiation of fully competent CD8⁺ eTCs (7). These results support use of IL-2 as adjuvant to increase reactivity of CD8⁺ eTCs, as tumor antigen are generally poorly immunogenic. However, IL-2 contributes to expansion/function of CD4⁺CD25⁺ T regulatory cells with immunosuppressive properties (8), so alternative approaches may improve in vivo expansion and function of CD8⁺ eTCs.

Cell programming by manipulation of transcription factors is under investigation in a wide variety of biological areas. Terminally differentiated cells are usually limited in their proliferative capacity (9), a characteristic also applying to CD8⁺ TCs (10). Transcription factor manipulation succeeded in promoting macrophage cell differentiation while preserving self-renewal capacity (11). Genetic modification of TCs for use in adoptive transfer has been limited to a small number of genes. Therefore, modifying ‘master switch’ genes such as transcription factor—rather than genes encoding effector molecules—might globally enhance TC functions.

CD8⁺ eTC function and maintenance of memory CD8⁺ TCs capable of self-renewal are regulated by cytokine receptors sharing the γc chain, including IL-2, IL-7, and IL-15. STAT5 is a transcription factor activated downstream of these cytokine receptors.
receptors upon JAK3-mediated phosphorylation and dimerization. The effect of IL-2 on expression of genes involved in CD8+ eTC functions could be mimicked by expressing a constitutively active form of STAT5 (STAT5CA; ref. 12). STAT5CA was also shown to promote hematopoietic stem cell self-renewal (13).

We here investigate long-term behavior of CD8+ eTCs expressing this active STAT5CA. We show that STAT5CA expression in CD8+ eTCs favors acquisition of a phenotype reminiscent of effector memory TCs while maintaining the increased potential for antigen recall responses associated with central memory TCs. We also evaluate the potential of STAT5CA-expressing CD8+ eTCs for antitumor responses upon adoptive transfer in tumor-bearing hosts. We used genetically engineered TiRP mice (14, 15) in which inducible tumor development recapitulates key aspects of human melanoma. In these mice, melanocyte-specific deletion of Ink4a/Arf is associated with a gain-of-function mutation of H-Ras and expression of mouse cancer germline gene PIA. We show that STAT5CA-expressing CD8+ eTCs infiltrate autochthonous melanomas and remain functional in the immunosuppressive environment (15) of those tumors. Compared with unmanipulated CD8+ eTCs, STAT5CA-expressing CD8+ eTCs develop higher cytolytic activity against antigen-expressing tumors, associated with strong Tc1 (IFN-γ+) responses upon restimulation. Infusion of STAT5CA-expressing CD8+ eTCs induces tumor regression more efficiently than infusion of CD8+ eTCs alone or in combination with IL-2/IL-2 monoclonal antibody (mAb) complexes. STAT5CA-mediated reprogramming applied at different stages of CD8+ TC differentiation was also efficient at boosting cytolytic activity and tissue-migratory properties of antigen-experienced TCs.

Materials and Methods

Mice

Mice heterozygous for the H-2Ld/P1A35–43-specific TCR transgene (TCRP1A; ref. 16) and TiRP mice (Tyr-iRas-PIA-transgenic Ink4a/Arflox/lox; ref. 15) were kept on the Rag-1−/−B10.D2 background. Melanomas were induced in TiRP mice as described (14). To generate (β-actin-Luc/TCRP1A) mice with ubiquitous luciferase expression, we crossed LucRep-transgenic (17) with Cre-deleter (18) mice and crossed offspring with TCRP1A Rag-1−/−B10.D2. Ly5-eGFP mice (19) given by Dr. T. Graf were crossed on the B10.D2 background. All these mice were bred in the CIML animal facility. Animal experiments respected French and European directives.

Cell preparation

TCs were prepared from lymph nodes or spleen using standard procedures. For analysis of tissue-infiltrating TCs, donor mice were anesthetized and perfused with PBS. Livers were dispersed and passed over Ficol-Paque (Amersham Biosciences AB). For solid tumor-infiltrating leukocytes (TIL), tissues were cut in small pieces with the GentleMacs Dissociator (Mytenyi Biotec), incubated for 40 minutes in medium containing Collagenase I (200 μg/mL) and DNase I (16 μg/mL) before loading over Ficol-Paque.

TC activation and retroviral infections

TCRP1A CD8+ TCs were stimulated with 10−7M P1A35–43 (LPYLGWLVF) peptide and retrovirally transduced 20 hours later, as reported (12). Cultures were continued for another 48 hours. Average transduction efficiency was 58% for TCRP1A eTC-STAT5CA, 31% for TCRP1A eTC-GFP (Fig. 1A). Untransduced and transduced CD8+ eTCs were either analyzed directly or adoptively transferred into congenic mice or sorted on the basis of green fluorescent protein (GFP) expression.

Flow cytometry

Antibodies were from BD Biosciences, except anti-GzmB mAb (Invitrogen). Cells (106) were analyzed on a FACSCalibur or a LSRII 561 cytometer (BD Biosciences). Data were analyzed using FlowJo (Treestar Inc.) or Diva (BD Biosciences) software. For Intracellular cytokine staining and degranulation assays, CD8+ TCs were stimulated ex vivo for 4 hours with FcR-bearing (P1.204) tumor cells (see hereafter), in the absence or presence of anti-CD3, together with anti-CD107a mAb (20) and monensin (4 μmol/L). In all cases, CD8+ TCs were stained for CD8 and IFN-γ using the Cytofix/Cytoperm Kit (BD Biosciences).

Tumor transplantation and bioluminescence imaging

Luciferase-expressing (Luc−) melanoma cell line T429-Luc− was derived from melanoma line T429 (expressing H-RasG12V and PIA transcripts and deleted for Ink4a/Arf genes; ref. 15) established in culture from an induced amelanotic melanoma (15) after transfection with vector pEGFP-Luc (BD Biosciences/BD Clontech) by Lipofectamine 2000 (Invitrogen Life Technologies), followed by G418 selection. PIA-positive mastocytoma P815 and its PIA-negative variant P1.204, obtained from Dr. B. Van den Eynde (Ludwig Institute for Cancer Research, Brussels, Belgium; ref. 21), were transfected to express luciferase and used as described (16). Tumor cells (106) were inoculated subcutaneously and TCRP1A eTCs were adoptively transferred intravenously in mice bearing a solid tumor mass.

Preparation and administration of cytokine complexes

Recombinant human IL-2 (TECIN, Roche, received from Linda Sherman, Scripps Research Institute, La Jolla, CA) and MAB602 mouse anti-Human IL-2 (R&D system) were incubated at 1-to-1 molar ratio. Mice received intravenous injections (daily for the first 3 days and every other day until the end of experiments) of complexes containing 4 μg IL-2 + 20 μg MAB602 (referred to as IL-2c).

Results

STAT5CA-expressing CD8+ eTCs are more efficient than control CD8+ eTCs for host colonization upon adoptive transfer and show increased accumulation in nonlymphoid tissues

CD8+ TCs expressing a transgenic-TCR (TCRP1A) specific for peptide P1A35–43 encoded by mouse P1A cancer germline gene were activated in vitro. They were transduced 20 hours later with retroviral particles encoding a constitutively active form of STAT5 (referred to as STAT5CA hereafter)-IRES-eGFP.
(ref. 12; TCRP1A eTC-STAT5CA). STAT5CA bears (i) substitution S710F that reduces its sensitivity to phosphatases; and (ii) substitution H299R conferring dependency on wild-type endogenous STAT5 for DNA binding (22, 23). As negative controls, TCRP1A CD8+ TCs were either left untransduced (TCRP1A eTCs) or transduced to express eGFP alone (TCRP1A eTC-GFP). We observed that the fraction of GFP+ cells among TCRP1A eTC-STAT5CA increased with time during in vitro culture as compared with GFP+ cells among control TCRP1A eTC-GFP (Fig. 1A), suggesting a natural enrichment of the former cells. These TCRP1A eTCs were adoptively transferred into either immune-sufficient or immune-compromised congenic hosts. Whereas untransduced TCRP1A eTCs (Fig. 1B) or TCRP1A eTC-GFP (Supplementary Fig. S1A) showed poor survival upon transfer, TCRP1A eTC-STAT5CA efficiently colonized the hosts. Injection of as many as 8 × 10^6 untransduced TCRP1A eTCs was less efficient than transfer of 3 × 10^4 or 10^5 TCRP1A eTC-STAT5CA in establishing a pool of long-lived CD8+ eTCs (Fig. 1B). This greater efficiency resulted from both increased proliferation during the first week in vivo and a reduced contraction phase (Grange and colleagues, in preparation).

Comparative analysis also showed increased migration of TCRP1A eTC-STAT5CA in nonlymphoid tissues (liver, lung

![Image](image_url)

Figure 1. TCRP1A eTC-STAT5CA colonize hosts and express effector functions more efficiently than control TCRP1A eTC-GFP. A, TCRP1A CD8+ TCs were transduced to express eGFP alone or in combination with STAT5CA. Percentages of GFP+ among CD8+ TCs are reported, with autofluorescence (gray), 72-hour and 96-hour poststimulation in vitro. B, TCRP1A eTCs (8 × 10^5) or TCRP1A eTC-STAT5CA (3 × 10^6, middle or 10^5, right) were adoptively transferred into Rag-1−/− B10.D2 mice. Dot plots of CD8 expression versus GFP of transferred CD8+ eTCs are shown in spleen and liver days 42 and 48 posttransfer. C and D, TCRP1A eTC-STAT5CA (10^6, C; 5 × 10^5, D) were adoptively transferred in Ly5-eGFP B10.D2 mice for 21 (C) or 97 (D) days. Ly5-eGFP mice expressing eGFP in myeloid cells only were used as GFP tolerant hosts. Percentages of GFP+ eTCs among total gated CD8+ TCs (corresponding exclusively to transferred eTCs) are reported in lymph node and spleen (C and D), lung and liver (C). E, activation or migration markers (as indicated) are reported for splenocytes from Rag-1−/− B10.D2 mice 42 days after transfer of 10^5; TCRP1A eTC-STAT5CA (blue line; gated CD8+ GFP+) or untransduced TCRP1A eTCs (green line; gated CD8+); TCRP1A naive CD8+ TCs are shown in gray. For GzmB, the MFI ratio between anti-GzmB and isotype control (not shown) staining is reported on gated CD8+ eTC splenocytes. Representative of 5 experiments. LN, lymph node.
Engineering Efficient Antimelanoma CD8+ T Cells

To evaluate their functional potential, TCRP1A CD8+ eTCs were recovered from lung or spleen 30 days after infusion in Rag-1-/- B10.D2 mice. They exhibited high cytolytic activity against P1A+ but not P1A- targets (Fig. IF for lung). In comparison, TCRP1A eTC-GFP were only weakly active (Fig. IF). TCRP1A eTC-STAT5CA expressed high amounts of GzmB protein, whereas untransduced and GFP-transduced TCRP1A eTCS were weakly positive for GzmB ex vivo (Fig. IE, Supplementary Fig. S1B) and required reactivation to upregulate its expression (not shown). In addition, as compared with untransduced TCRP1A eTCS, TCRP1A eTC-STAT5CA were highly efficient IFN-γ producers following brief in vitro restimulation (Fig. IG). Importantly, no constitutive IFN-γ production was detected in those cells, indicating regulated cytokine production.

Differentiated antigen-experienced CD8+ TCs can be reprogrammed by STAT5CA

In experiments presented in Fig. 1B, naive CD8+ TCs were transduced 20 hours after antigen stimulation. We asked whether STAT5CA expression could reprogram antigen-experienced CD8+ TCs ex vivo. These cells are more representative of CD8+ TCS present in tumor-bearing hosts. We adoptively transferred untransduced TCRP1A eTCS into primary hosts, recovered them at late time points (Fig. 2A) and transduced them to express STAT5CA. We next transferred them into secondary hosts (Fig. 2A). Although the efficiency of retroviral infection of Ag-experienced TCRP1A eTCS was low (about 10%; not shown), TCRP1A eTC-STAT5CA were strongly enriched 35 days after the second transfer, suggesting that STAT5CA also conferred a survival advantage at this stage. These cells expressed an effector memory TC phenotype, including GzmB upregulation and migration toward tissues (Fig. 2B). Ex vivo restimulation with P1A+ tumor cells triggered efficient IFN-γ secretion by reprogrammed TCRP1A eTC-STAT5CA (Fig. 2C). In addition, TCRP1A eTC-STAT5CA after secondary transfer were as efficient as primary TCRP1A eTC-STAT5CA at eliminating P1A-peptide pulsed targets in 5-hour in vivo cytotoxicity assays (not shown). Altogether, STAT5CA expression in antigen-experienced CD8+ TCS seemed to reprogram their migration and functional potential and to enhance their survival upon transfer into congenic hosts.

STAT5CA-expressing CD8+ eTCS promote antigen-specific regression of transplanted mastocytomas

The specificity of the antitumor response was addressed through a simple experimental model in which P1A+ (P815) or...
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P1A<sup>−</sup> (P1.204) mastocytomas expressing luciferase (Luc<sup>+</sup>) are injected in Rag-1<sup>−/−</sup> B10.D2 mice (Supplementary Fig. S3) as previously reported (16). Adoptive transfers were done 7 days after tumor cell inoculation (defined as day 0), time at which all Rag-1<sup>−/−</sup> B10.D2 recipients had developed solid tumors. These tumors continued to grow in the untreated (PBS) group (Supplementary Fig. S3A). In tumor-bearing mice infused with naive CD8<sup>+</sup> TCRP1A TCs, P1A<sup>−</sup> tumors grew until day 6 but disappeared between day 9 and 13. However, a relapse occurred in all mice because of emergence of PIA tumor antigen loss variants (Supplementary Fig. S3B). Similar relapses were also observed in mice receiving untransduced TCRP1A eTCs or TCRP1A eTC-GFP (Supplementary Fig. S3A and B). In the group receiving TCRP1A eTC-STAT5CA, no relapses were observed in 7 of 9 recipients that received 10<sup>6</sup> cells and in 3 of 6 recipients injected with 5 x 10<sup>5</sup> cells (Supplementary Fig. S3A and B). Occurrence of tumor antigen-negative variants was previously reported for mastocytoma P815 (16, 21, 24), in which X chromosome encoded PIA gene is monoallelic. Addition of peptide P1A<sub>35–43</sub> restores killing of such variants by TCRP1A cytolytic effectors (16). Elucidation of mechanisms by which TCRP1A eTC-STAT5CA may prevent development of such variants requires further analysis. Differential accumulation of TCRP1A eTC-STAT5CA versus TCRP1A eTC-GFP or untransduced eTCs (Supplementary Fig. S3D) may be a contributing factor, in addition to efficient activation of their effector functions (Fig. 1F and G). However, mice infused with TCRP1A eTC-STAT5CA failed to reject PIA<sup>−</sup> (P1.204) tumors (Supplementary Fig. S3C) according to the specificity of their cytolytic activity. This was also correlated with their differential expansion in PIA<sup>−</sup> versus PIA<sup>+</sup> tumor-bearing mice (Supplementary Fig. S3E and next section).

**Tumor antigen-specific intratumor accumulation and reactivation of STAT5CA-expressing CD8<sup>+</sup> eTCs**

To evaluate intratumor accumulation of TCRP1A eTCs, we used (β–actin-Luc×TCRP1A) double transgenic mice as donors of TCRP1A CD8<sup>+</sup> eTCs. After in vitro activation and transduction to express or not STAT5CA, 10<sup>6</sup> Luc<sup>+</sup> TCRP1A eTCs were injected in mastocytomas bearing hosts, and their...
in vivo localization was followed by bioluminescence monitoring (Fig. 3). Luc+ TCRP1A eTC-STAT5CA expanded in P1A+ tumor-bearing hosts to a greater extent than Luc+ TCRP1A eTCs and accumulated to a 10-fold higher extent at the tumor site (central abdomen) by day 8 (Fig. 3A and B). Presence of Luc+ TCRP1A eTCs in tumor-draining lymph node was also observed at day 8. Although accumulation of Luc+ TCRP1A eTC-STAT5CA was limited in P1A- tumor-bearing hosts, these TCs were nevertheless present in areas surrounding tumors, without affecting their growth (Supplementary Fig. S3A and C). In tumor-free hosts, in contrast, Luc+ TCRP1A eTC-STAT5CA homed preferentially to lung and liver (Fig. 3A and not shown).

Antigen-specific local reactivation of TCRP1A eTC-STAT5CA was evaluated by injecting them in hosts bearing P1A+ and P1A- mastocytomas on opposite flanks (Supplementary Fig. S4). TCRP1A eTC-STAT5CA have a GzmB(effector memory TC-like phenotype when transferred in tumor-free hosts (Fig. 1E). Upon transfer in tumor-bearing hosts (Supplementary Fig. S4), TCRP1A eTC-STAT5CA maintained a GzmB expression in spleens of the recipients. In addition, an efficient antigen-specific response was triggered in TCRP1A eTC-STAT5CA by P1A+ tumors, as shown by further upregulation of GzmB and CD25, both inside P1A+ tumors and draining lymph node (Supplementary Fig. S4).

Altogether, TCRP1A eTC-STAT5CA were specifically stimulated by P1A+ tumor cells, accumulated to a greater extent than control TCRP1A eTCs, and exhibited a higher cytolytic potential. All these parameters may contribute to efficient control of P1A+ tumor growth.

Regression of transplanted melanomas is more efficiently induced by STAT5CA-expressing than by control CD8+ eTCs, even when associated with infusions of IL-2 complexes

We next evaluated antitumor responses in immunocompetent hosts (Fig. 4) injected with a melanoma cell line expressing P1A and luciferase (T429-Luc+; see Materials and Methods). Mice bearing a solid tumor (see photon emission reported in Fig. 4A, day 0) were infused with either control or STAT5CA-expressing TCRP1A eTCs (106 cells). As poor accumulation/survival of adoptively transferred control TCRP1A eTCs...
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(Fig. 1, Fig. 3) may limit their antitumor potential, we additionally provided infusions of IL-2/IL-2mAb complexes (IL-2c; ref. 25) that were shown to enhance expansion and activity of some antitumor CD8\(^+\) TCs \textit{in vivo} (26).

Marked tumor regression was observed in mice receiving TCRP1A eTC-STAT5CA (Fig. 4A and B). Analysis of TILs at day 11 revealed the presence of CD8\(^+\) TILs (Fig. 4A–E, 4C–E). In contrast, in mice receiving control TCRP1A eTC, tumors did not regress (Fig. 4A and B), and these eTCs hardly invaded the tumor mass (Fig. 4C–E), whereas endogenous CD8\(^+\) TCs were present among TILs. IL-2c treatment for 10 days did not increase tumor infiltration by transferred TCRP1A eTCs (Fig. 4C–E). However, as previously described (26), IL-2c treatment increased numbers of both natural killer cells and endogenous CD8\(^+\) TCs in the spleen (not shown), as well as among TILs (Fig. 4E).

STAT5CA-expressing TCRP1A TILs expressed high levels of GzmB in comparison with endogenous CD8\(^+\) TILs or to non-STAT5CA-expressing TCRP1A eTCs alone (A–E; n = 4). A and B, tumor development was measured by counting photons emitted by luciferase-expressing tumors on a defined skin surface (mm\(^2\)S). Values between day 0 (time of adoptive transfer) and day 11 (time of analysis) are reported in A as means \pm SD for each group. For each mouse (B), photon emission is also reported as % of initial tumor photon emission, which is a ratio between day 0 (normalized to 100%) and day 11. C–E, eleven days after TC transfer, TILs were stained directly for CD8, CD45.1 (in C, host: CD45.1\(^+\); TCRP1A eTCs: CD45.2 are CD45.1-negative). D and E, ratios between transferred and endogenous CD8\(^+\) TILs (D) and absolute numbers of transferred and endogenous TILs (E) were evaluated from fluorescence-activated cell sorting analysis. Statistical analyses were done with a t test as in Fig. 3. In E, 2 by 2 comparisons of the different groups gave significant differences (*) as indicated. All other comparisons had P > 0.05 (ns).
TCRP1A eTC TILs from mice that received IL-2c infusions (Fig. 5A–C). Comparison of GzmB expression by endogenous CD8+ TILs showed marked increase in TCRP1A eTC-STAT5CA–injected and limited but significant increase in TCRP1A eTC/IL-2c–injected mice (Fig. 5A–C; Supplementary Fig. S5A for statistics). Interestingly, endogenous as well as TCRP1A eTC-STAT5CA and TCRP1A eTC/IL-2c CD8+ TILs expressed high levels of the inhibitory receptor PD-1 (Fig. 5D–F), but only TCRP1A eTC-STAT5CA CD8+ TILs responded efficiently to recall restimulation (IFN-γ secretion and CD107a exposure; Fig. 5G–I). These data suggest that IL-2c promoted proliferation of CD8+ TCs and slightly increased their expression of GzmB in TILs and in splenic CD8+ TCs (Supplementary Fig. S5B) but did not induce intratumor accumulation of infused TCRP1A eTCs. Accordingly, although tumor regression in recipients infused with TCRP1A eTCs and IL-2c was more pronounced than in mice receiving only TCRP1A eTCs (Fig. 4A and B), it was significantly weaker than in counterparts receiving TCRP1A eTC-STAT5CA. In the latter case, a significant increase in GzmB expression was observed in endogenous TILs in absence of IL-2c (Fig. 5A, Supplementary Fig. S5A), suggesting that tumor regression induced by TCRP1A eTC-STAT5CA and/or activation of TCRP1A eTC-STAT5CA also favored activation of surrounding CD8+ TILs.

When following the long-term fate of TCRP1A eTC-STAT5CA in mice that rejected a T429-Luc+ melanoma, we detected TCRP1A eTC-STAT5CA in the peripheral blood of mice killed for analysis at day 70. No pathologic signs and no evidence for tumor escape variants were observed in these mice (data not shown).

STAT5CA-expressing CD8+ eTCs induced regression of autochthonous mouse melanoma

The stroma of naturally occurring tumors may both impede access of TCs to tumors (27) and promote immunosuppression through complex cytokine/chemokine secretion and recruitment of suppressive cells (28, 29). This situation is not fully reproduced in transplanted tumors. In the inducible TiRP model of melanoma expressing the P1A-encoded tumor antigen, we recently reported (15) that endogenous CD8+ TILs expressed a GzmBlow phenotype and showed suppressed functions. TiRP mice developing aggressive induced melanoma tumors in a Rag-1−/− B10.D2 background were injected with either 10^6 untransduced TCRP1A eTCs or TCRP1A eTC-STAT5CA. In the 9 mice receiving TCRP1A eTC-STAT5CA, we observed a very rapid and extensive tumor necrosis (Fig. 6A and B). None of the mice (n = 7) treated with TCRP1A eTCs showed tumor regression (Fig. 6B). Both analyses by cytometry (Fig. 6) and by immunostaining (not shown) showed tumor infiltration by TCRP1A eTC-STAT5CA with preserved GzmBhi expression, IFN-γ production, and CD107a exposure upon ex vivo restimulation (Fig. 6C). In comparison, tumor infiltration by
untransduced TCRP1A eTCs was very limited (Fig. 6D). In this latter case, injection of increased cell numbers (7 × 10^6) led to higher tumor infiltration, but those TILs maintained a low GzmB expression (Fig. 6E) and failed to produce IFN-γ (Supplementary Fig. S6).

Altogether, TCRP1A eTC-STAT5CA showed higher potential to infiltrate autochthonous mouse melanomas and maintained a GzmBhi expression in an immunosuppressive context.

Discussion

We here report that STAT5CA-transduced CD8^+ eTCs present many properties described to positively impact efficacy of adoptive TC therapy for solid tumors (30). In particular, they show high specific cytolytic potential, strong Tc1 recall responses, and migration in tissues in a manner similar to effector memory Tcs (CD44hi CD62Llo). In addition, STAT5CA-expressing CD8^+ eTCs showed central memory characteristic of long-term survival and capacity to self-renew. At the molecular level, the combination of these properties seems to be associated with concomitant high expression of transcription factor T-Bet, characterizing effector Tcs, and Eomes, as in central memory Tcs (Grange and colleagues; in preparation).

Although not constitutively activated, STAT5CA-expressing eTCs were capable of enhanced secondary responses. This may be due to their increased expression of transcripts encoding effector molecules (Grange and colleagues; in preparation).
allowing rapid recall responses. Moreover, expression of STAT5CA in antigen-experienced CD8+ T cells endowed with poor replicative potential converted them into long-lived eTCs. These reprogrammed eTCs also showed increased capacity for tissue infiltration and responses upon antigen recall, extending the potential application of the approach with particular relevance for tumor-bearing hosts.

In TiRP mice, developing autochthonous induced melanomas are infiltrated by PD-1+ GzmB– nonfunctional endogenous CD8+ TILs associated with systemic chronic inflammation (15), akin to that detected in a subset of melanoma patients (31). These mice also presented defects in lymph node and splenic T cell zone stroma associated with impaired recruitment of naïve TCs (32), stressing the importance of tissue-migratory properties for TCs in adoptive immunotherapy. Our ultimate goal was to evaluate the ability of STAT5CA–expressing CD8+ Tcs to infiltrate these autochthonous melanomas and to resist their immunosuppression. However, given the asynchrony of melanoma development within a cohort of treated TiRP mice and their limited availability, we first used transplanted P1A+ mastocytomas and derived P1A+ variants to establish the tumor antigen specificity of the eTC–STAT5CA antitumor effects. We also used a P1A+ melanoma line established from a TiRP-induced melanoma for the comparison of protocols involving adoptive transfers with STAT5CA–expressing or untransduced CD8+ Tcs with or without IL-2c.

Accumulation of TCRP1A eTC–STAT5CA in P1A+ tumors probably resulted from antigen-induced expansion as well as a TC–intrinsic propensity to migrate to tissues/tumors because TCRP1A eTC–STAT5CA were also concentrated to some extent in P1A+ mastocytomas. This accumulation was about 3.5-fold lower (evaluation by bioluminescence in Fig. 4B and C) in P1A+ as compared with P1A+ mastocytomas, but it was almost 80-fold higher than that of control–transduced TCRP1A eTC–GFP in P1A+ mastocytomas. The presence of TCRP1A eTC–STAT5CA was not associated with regression of P1A+ tumors, however, in agreement with the TC specificity.

Targeted destruction of malignancies by adoptive transfer of antitumor CD8+ eTCs has been combined with IL-2 infusions to support survival and proliferation of eTCs in cancer patients (6, 33, 34). Nevertheless, in vivo maintenance of CD8+ eTCs was hardly increased (6), and IL-2R– driven signals seemed to generate terminally differentiated eTCs that rapidly became senescent (10, 35, 36). In addition, as IL-2 contributes to expansion/function of CD4+CD25+ regulatory Tcs with immunosuppressive properties (37), substitutes such as use of IL-2c have been developed (25). In mice inoculated with a P1A+ melanoma, IL-2c treatment improved CD8+ eTCs survival. However, IL-2c did not promote migration of infused antitumor CD8+ eTCs inside tumors and had limited effects on GzmB expression. In contrast, STAT5CA expression induced stable phenotypic and migratory properties and survival of CD8+ eTCs. The difference between STAT5CA and IL-2c treatment for modulating homing properties and survival of the transferred eTCs may depend on the capacity of STAT5CA to concomitantly mimic signals elicited by IL-7 and IL-15, as well as IL-2 (38). In addition, although they expressed PD-1 inhibitory receptors to the same extent as endogenous Tcs or untransduced TCRP1A eTCs, TCRP1A eTC–STAT5CA were found responsive to secondary stimulation. Ligation of PD-1 is thought to induce its phosphorylation and to increase its association with the SHP-2/SHP-1 phosphatases that in turn dampen TCR signaling. However, this negative PD-1–mediated signal can be overcome through cytokine receptor signaling, particularly cytokines that activate STAT5 (39). Additional analysis is required to establish the molecular bases maintaining responsiveness of TCRP1A eTC–STAT5CA.

Tumor regression induced by TCRP1A eTC–STAT5CA revealed that those Tcs remained active in the face of tumor–derived immunosuppression. In TiRP mice, a state of Th2/Th17–oriented chronic inflammation develops that resembles, in part, the Th2–dominant chronic inflammation observed in advanced melanoma patients (31). Consistent with detection of cytokines capable of activating STAT3 (40), nuclear phospho–STAT3 in tumor cells and in some infiltrating CD45 leukocytes was observed in the TiRP model (15). In CD8+ Tcs, STAT3 deletion improved their tumor–induced proliferation and infiltration within tumors (41). STAT3 and STAT5 have been reported to compete for binding on a similar DNA consensus sequence on the IL-17 promoter (42). Whether this competition is a mechanism for resistance to immunosuppression of STAT5CA–expressing CD8+ eTCs will be further investigated.

In conclusion, STAT5CA expression contributed to optimize antitumor activity of CD8+ eTCs, with increased intratumor accumulation and strong specific Tc recall responses. This result suggests consideration of use of STAT5CA–transduced CD8+ Tcs for adoptive immunotherapy. This approach here required the use of retroviruses. Recent clinical data with retrovirus–engineered Tcs for cancer and HIV patients indicate that retroviral gene transfer in mature Tcs is safe (43). This conclusion is supported by studies showing resistance of mature Tcs to oncogene transformation (44). In addition, no transformation of STAT5CA–transduced CD8+ Tcs was observed in this and in a previous study (45). Coupling of STAT5CA transduction with that of suicide genes such as thymidine kinase will allow further security for adoptive therapy. Finally, the survival advantage of STAT5CA–expressing cells over their control counterparts suggests that STAT5CA transduction of CD8+ Tcs specific for some poorly immunogenic tumor antigen might allow their enrichment in vivo and render them efficient for adoptive immunotherapy. The fact that antigen experienced and polyclonal CD8+ Tcs can be reprogrammed by STAT5CA expression could thus enhance the number of tumor antigen that may be targeted in adoptive therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Magali Grange, Michel Buferne, Grégory Verdeil, et al.


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