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,*.
Centre d’Immunologie de Marseille-Luminy (CIML), Université de la Méditerranée, UMR65461; INSERM UMR6312; CNRS UMR61023; 13288 Marseille, France.

Running title: Engineering efficient anti-melanoma CD8+ T cells

Keywords: mouse melanoma model; immunotherapy; cancer-germline tumor antigen; cytokines; transcription factor.

*Corresponding author: Nathalie AUPHAN-ANEZIN, CIML, Parc Scientifique de Luminy, Case 906, 13288 Marseille, cedex 09, France. Phone: +33 491269155; Fax: +33 491269130; email:auphan@ciml.univ-mrs.fr

The authors declare that they have no conflict of interest.

Nonstandard abbreviations used: TA, tumor antigen; TC, T cell; eTC, effector T cell; STAT5, Signal transducer and activator of transcription 5; MFI, mean fluorescence intensity; IL-2c, IL-2/IL-2mAb complex; LN, lymph node; TILs, tumor infiltrating leukocytes.

Word count: 5 217

6 figures (+ 6 supplemental figures)
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 TC with poor replicative potential was also sufficient to convert them into long-lived antigen-responsive eTC. In transplanted mastocytoma- or melanoma bearing hosts, STAT5CA greatly enhanced the ability of eTC 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 to untransduced TC including when the latter control cells were combined with infusion of IL-2/anti-IL-2 complexes. In tumors arising in the autochthonous TiRP transgenic model of melanoma associated with systemic chronic inflammation, endogenous CD8+ TC were non-functional. In this setting, adoptive transfer of STAT5CA-transduced TC produced superior anti-tumor effects compared to non-transduced TC. Our findings imply that STAT5CA expression can render TC 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 TC.
Introduction

The discovery of tumor antigens (TA) recognized by autologous T cells (TC) in patients with melanoma has led to clinical protocols for adoptive transfer of TA-specific TC. The efficacy of this treatment remains poor (1-3), for several reasons. In order to eliminate tumor cells, naïve CD8+ TC must differentiate into effector TC (eTC) acquiring lytic enzyme-containing granules and the capacity to secrete cytokines. However, TA-specific TC may undergo incomplete differentiation (4) or be tolerized upon encounter with TA (5). During prolonged antigen contact within tumors, CD8+ TC may become functionally impaired and subsequently deleted. Methods to enhance in vivo maintenance and function of transferred eTC are consequently required. TC therapy should aim at transferring long-lived anti-tumor eTC (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 IL-2R affect differentiation of fully competent CD8+ eTC (7). These results support use of IL-2 as adjuvant to increase reactivity of CD8+ eTC, as TA 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+ eTC.

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+ TC (10). Transcription factor manipulation succeeded in promoting macrophage cell differentiation while preserving self-renewal capacity (11). Genetic modification of TC 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\(^+\) TC capable of self-renewal are regulated by cytokine receptors sharing the \(\gamma c\) chain, including IL-2, IL-7 and IL-15. STAT5 is a transcription factor activated downstream of these cytokine 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) (12). STAT5CA was also shown to promote hematopoietic stem cell self-renewal (13).

We here investigate long-term behavior of CD8\(^+\) eTC expressing this active STAT5CA. We show that STAT5CA expression in CD8\(^+\) TC favors acquisition of a phenotype reminiscent of effector memory TC while maintaining the increased potential for antigen recall responses associated with central memory TC. We also evaluate the potential of STAT5CA-expressing CD8\(^+\) eTC for anti-tumor 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 \(\text{Ink4a/Arf}\) is associated with a gain-of function mutation of \(H-Ras\) and expression of mouse cancer-germline gene \(P1A\). We show that STAT5CA-expressing CD8\(^+\) eTC infiltrate autochthonous melanomas and remain functional in the immunosuppressive environment (15) of those tumors. Compared to unmanipulated CD8\(^+\) eTC, STAT5CA-expressing CD8\(^+\) eTC develop higher cytolytic activity against antigen-expressing tumors, associated with strong Tc1 (IFN-\(\gamma\)+) responses upon restimulation. Infusion of STAT5CA-expressing CD8\(^+\) eTC induces tumor regression more efficiently than infusion of CD8\(^+\) eTC, alone or in combination with IL-2/IL-2 mAb complexes. STAT5CA-mediated reprogramming applied at different stages of CD8\(^+\) TC differentiation was also efficient at boosting cytotoxic activity and tissue-migratory properties of antigen-experienced TC.

Materials and Methods

Mice: Mice heterozygous for the H-2L\(^d\)/P1A\(_{35-43}\)-specific TCR-transgene (TCRP1A) (16) and TiRP mice (\(\text{Tyr-iRas-P1A-transgenic Ink4a/Arf}^{\text{floxed/floxed}}\)) (15) were kept on the Rag-1\(^-/-\)B10.D2 background.
Melanomas were induced in TiRP mice as described (14). To generate (β-actin-LucXTCRP1A) mice with ubiquitous luciferase expression, we crossed LucRep-transgenic (17) with Cre-deleter (18) mice, and crossed offspring with TCRP1A Rag-1−/−B10.D2. Lys-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:** TC were prepared from lymph nodes (LN) or spleen using standard procedures. For analysis of tissue infiltrating TC, donor mice were anesthetized and perfused with PBS. Livers were dispersed and passed over Ficoll-Paque™ (Amersham Biosciences AB, Uppsala, Sweden). For solid tumor infiltrating leukocytes (TILs), tissues were cut in small pieces with the GentleMacs Dissociator (Mylenyi Biotech), incubated 40min in medium containing Collagenase I (200u/ml) and DNase 1 (16µg/ml) before loading over Ficoll-Paque™.

**TC activation and retroviral infections:** TCRP1A CD8+ TC were stimulated with 10⁻⁷M P1A35-43 (LPYLGWLVF) peptide and retrovirally transduced 20h later, as reported (12). Cultures were continued for another 48h. Average transduction efficiency was 58% for TCRP1A eTC-STAT5CA, 31% for TCRP1A eTC-GFP (see Fig.1A). Untransduced and transduced CD8+ eTC were either analyzed directly or adoptively transferred into congenic mice or sorted based on GFP expression.

**Flow cytometry:** Antibodies were from BD Biosciences, except anti-GzmB mAb (Invitrogen). Cells (10⁶) were analyzed on a FACSCalibur or a LSR2 561 cytometer (BD Biosciences). Data were analyzed using FlowJo (Treestar Inc., CA) or Diva (BD Biosciences) software. For Intracellular cytokine staining and degranulation assays, CD8+ TC were stimulated ex vivo for 4h 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 (4µM). In all cases, CD8+ TC 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 P1A transcripts and deleted for gene Ink4a/Arf genes (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.

P1A-positive mastocytoma P815 and its P1A-negative variant P1.204, obtained from Dr. B. Van den Eynde (Ludwig Institute for Cancer Research, Brussels) (21), were transfected to express luciferase and used as described (16). Tumor cells (10^6) were inoculated s.c. and TCRP1A eTC were adoptively transferred i.v. 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, 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 4µg IL-2 + 20 µg MAB602 (referred to as IL-2c).

**Results**

**STAT5CA-expressing CD8^+ eTC are more efficient than control CD8^+ eTC for host colonization upon adoptive transfer and show increased accumulation in non-lymphoid tissues.**

CD8^+ TC expressing a transgenic-TCR (TCRP1A) specific for peptide P1A_{35-43} encoded by mouse P1A cancer-germline gene were activated in vitro. They were transduced 20h later with retroviral particles encoding a constitutively active form of STAT5 (referred to as STAT5CA hereafter)-IRES-eGFP (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^+ TC were either left untransduced (TCRP1A eTC) 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 to GFP^+ cells among control TCRP1A eTC-GFP (Fig.1A), suggesting a natural enrichment of the former cells. These TCRP1A eTC were adoptively transferred into either
immune-sufficient or -compromised congenic hosts. While untransduced TCRP1A eTC (Fig.1B) or TCRP1A eTC-GFP (Fig.S1A) showed poor survival upon transfer, TCRP1A eTC-STAT5CA efficiently colonized the hosts. Injection of as many as 8x10^6 untransduced TCRP1A eTC was less efficient than transfer of 3x10^5 or 10^4 TCRP1A eTC-STAT5CA in establishing a pool of long-lived CD8^+ eTC (Fig.1B). This greater efficiency resulted from both increased proliferation during the first week in vivo and a reduced contraction phase (Grange et al, in preparation (24)).

Comparative analysis also showed increased migration of TCRP1A eTC-STAT5CA in non-lymphoid tissues (liver, lung (not shown)) as compared to untransduced (Fig.1B) or GFP-transduced (Fig.S1A) eTC. Host colonization by TCRP1A eTC-STAT5CA was less efficient in immune-competent recipients, although preferential homing into peripheral tissues was maintained (Fig.1C) for prolonged periods (97 days, Fig.1D), demonstrating that mutated STAT5CA was not immunogenic per se. Tissue homing correlated with acquisition of an effector memory phenotype: CD44^hi, CD122^hi, CD62L^{med} (spleen)/^{low} (lung), CD127^o (Fig.1E). In contrast, untransduced TCRP1A eTC developed a CD44^hi, CD62L^hi, CD122^{med}, CD127^hi phenotype (Fig.1E) reminiscent of central memory TC (Fig.S1B for TCRP1A eTC-GFP). Importantly, natural enrichment was also observed with polyclonal CD8^+ eTC-STAT5CA as compared to CD8^+ eTC-GFP (Fig.S2A) and phenotypic characteristics also distinguished STAT5CA-transduced from either GFP-transduced (Fig.S2B) or untransduced (Fig.S2C) polyclonal CD8^+ eTC. These results exclude any bias conferred by TCR specificity for differentiation into long-lived CD8^+ eTC and modulation of activation and migration marker expression.

To evaluate their functional potential, TCRP1A CD8^+ eTC-STAT5CA 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.1F for lung). In comparison, TCRP1A eTC-GFP were only weakly active (Fig.1F). TCRP1A eTC-STAT5CA expressed high amounts of GzmB protein, whereas untransduced and GFP-transduced TCRP1A eTC were weakly positive for GzmB ex vivo (Fig.1E, Fig.S1B) and required reactivation to up-regulate its expression (not shown). Additionally, as compared to untransduced TCRP1A eTC, TCRP1A eTC-STAT5CA were highly efficient IFN-γ
producers following brief in vitro restimulation (Fig.1G). Importantly, no constitutive IFN-γ production was detected in those cells, indicating regulated cytokine production.

**Differentiated antigen-experienced CD8⁺ TC can be reprogrammed by STAT5CA.**

In experiments presented in Fig.1B, naïve CD8⁺ TC were transduced 20h after antigen-stimulation. We asked whether STAT5CA expression could reprogram antigen-experienced CD8⁺ TC ex vivo. These cells are more representative of CD8⁺ TC present in tumor-bearing hosts. We adoptively transferred untransduced TCRP1A eTC 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). While the efficiency of retroviral infection of Ag-experienced TCRP1A eTC 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 up-regulation 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). Additionally, TCRP1A eTC-STAT5CA after secondary transfer were as efficient as primary TCRP1A eTC-STAT5CA at eliminating P1A-peptide pulsed targets in 5h in vivo cytolytic assays (not shown). Altogether, STAT5CA expression in antigen-experienced CD8⁺ TC appeared to reprogram their migration and functional potential and to enhance their survival upon transfer into congenic hosts.

**STAT5CA-expressing CD8⁺ eTC promote antigen-specific regression of transplanted mastocytomas.**

The specificity of the anti-tumor response was addressed through a simple experimental model in which P1A⁺ (P815) or P1A⁻ (P1.204) mastocytomas expressing luciferase (Luc⁺) are injected in Rag-1⁻⁻B10.D2 mice (Fig.S3) as previously reported (16). Adoptive transfers were performed 7 days after tumor cell inoculation (defined as day 0), time at which all Rag-1⁻⁻B10.D2 recipients had developed solid tumors. These tumors continued to grow in the untreated (PBS) group (Fig.S3A). In tumor-
bearing mice infused with naïve CD8+ TCRP1A TC, P1A+ tumors grew until day 6 but disappeared between day 9 and 13. However, a relapse occurred in all mice, due to emergence of P1A TA loss variants (Fig.S3B). Similar relapses were also observed in mice receiving untransduced TCRP1A eTC or TCRP1A eTC-GFP (Fig.S3A-B). In the group receiving TCRP1A eTC-STAT5CA, no relapses were observed in 7 out of 8 recipients that received 10^6 cells, and in 3 out of 6 recipients injected with 5x10^5 cells (Fig.S3A-B). Occurrence of TA-negative variants was previously reported for mastocytoma P815 (16, 21, 25), in which X chromosome encoded P1A gene is monoallelic. Addition of peptide P1A35-43 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 eTC (Fig.S3D) may be a contributing factor, in addition to efficient activation of their effector functions (Fig.1F-G). However, mice infused with TCRP1A eTC-STAT5CA failed to reject P1A- (P1.204) tumors (Fig.S3C), according to the specificity of their cytolytic activity. This was also correlated with their differential expansion in P1A+ versus P1A- tumor-bearing mice (Fig.S3E and next section).

**TA-specific intra-tumor accumulation and reactivation of STAT5CA-expressing CD8+ eTC.**

To evaluate intra-tumor accumulation of TCRP1A eTC, we used (βactin-LucXTCRP1A) double transgenic mice as donors of TCRP1A CD8+ TC. After in vitro activation and transduction to express or not STAT5CA, 10^6 Luc+TCRP1A eTC 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 eTC, and accumulated to a ten-fold higher extent at the tumor site (central abdomen) by day 8 (Fig.3A-B). Presence of Luc+TCRP1A eTC in tumor-draining LN was also observed at day 8. Although accumulation of Luc+TCRP1A eTC-STAT5CA was limited in P1A- tumor-bearing hosts, these TC were nevertheless present in areas surrounding tumors, without affecting their growth (Fig.S3A, 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 (Fig.5). TCRP1A eTC-STAT5CA have a GzmB^hi effector memory TC-like phenotype when transferred in tumor free hosts (Fig.1E). Upon transfer in tumor-bearing hosts (Fig.5), TCRP1A eTC-STAT5CA maintained a GzmB^hi expression in recipients' spleens. Additionally, an efficient antigen-specific response was triggered in TCRP1A eTC-STAT5CA by P1A^+ tumors, as shown by further up-regulation of GzmB and CD25, both inside P1A^+ tumors and draining LN (Fig.5).

Altogether, TCRP1A eTC-STAT5CA were specifically stimulated by P1A^+ tumor cells, accumulated to a greater extent than control TCRP1A eTC 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^+ eTC, even when associated with infusions of IL-2 complexes.

We next evaluated anti-tumor responses in immunocompetent hosts (Fig.4) injected with a P1A-expressing luciferase- and melanoma cell line (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 eTC (10^6 cells). As poor accumulation/survival of adoptively transferred control TCRP1A eTC (Fig.1, Fig.3) may limit their anti-tumor potential, we additionally provided infusions of IL-2/IL-2mAb complexes (IL-2c) (26) that were shown to enhance expansion and activity of some anti-tumor CD8^+ TC in vivo (27).

Marked tumor regression was observed in mice receiving TCRP1A eTC-STAT5CA (Fig.4A-B). Analysis of TILs at day 11 revealed the presence of CD8^+ TC mostly composed of transferred TCRP1A eTC-STAT5CA (Fig.4C-E). In contrast, in mice receiving control TCRP1A eTC, tumors did not regress (Fig.4A-B) and these eTC hardly invaded the tumor mass (Fig.4C-E), whereas endogenous CD8^+ TC were present among TILs. IL-2c treatment for 10 days did not increase tumor-
infiltration by transferred TCRP1A eTC (Fig.4C-E). However, as previously described (27), IL-2c treatment increased numbers of both NK cells and endogenous CD8⁺ TC in the spleen (not shown), as well as among TILs (Fig.4E).

STAT5CA-expressing TCRP1A TILs expressed high levels of GzmB in comparison to endogenous CD8⁺ TILs or to 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; 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⁺ TC and slightly increased their expression of GzmB in TILs and in splenic CD8⁺ TC (Fig.S5B), but did not induce intra-tumor accumulation of infused TCRP1A eTC. Accordingly, although tumor regression in recipients infused with TCRP1A eTC and IL-2c was more pronounced than in mice receiving only TCRP1A eTC (Fig.4A-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, 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-L melanoma, we detected TCRP1A eTC-STAT5CA in the peripheral blood of mice killed for analysis at day 70. No pathological signs and no evidence for tumor escape variants were observed in these mice (data not shown).

**STAT5CA-expressing CD8⁺ eTC induced regression of autochthonous mouse melanoma.**

The stroma of naturally occurring tumors may both impede access of TC to tumors (28) and promote immuno-suppression through complex cytokine/chemokine secretion and recruitment of suppressive...
cells (29, 30). This situation is not fully reproduced in transplanted tumors. In the inducible TiRP model of melanoma expressing the P1A-encoded TA, we recently reported (15) that endogenous CD8+ TILs expressed a GzmBlow phenotype and demonstrated suppressed functions. TiRP mice developing aggressive induced melanoma tumors in a Rag-1−/−B10.D2 background were injected with either 10^6 untransduced TCRP1A eTC or TCRP1A eTC-STAT5CA. In the 9 mice receiving TCRP1A eTC-STAT5CA, we observed a very rapid and extensive tumor necrosis (Fig.6A-B). None of the mice (n=7) treated with TCRP1A eTC demonstrated 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 eTC was very limited (Fig.6D). In this latter case, injection of increased cell numbers (7x10^6) led to higher tumor infiltration but those TILs maintained a low GzmB expression (Fig.6E) and failed to produce IFN-γ (Fig.S6).

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

Discussion

We here report that STAT5CA-transduced CD8+ eTC present many properties described to positively impact efficacy of adoptive TC therapy for solid tumors (31). In particular, they show high specific cytolytic potential, strong Tc1 recall responses and migration in tissues in a manner similar to effector memory TC (CD44hi CD62Llo). Additionally, STAT5CA-expressing CD8+ eTC demonstrated central memory characteristics of long-term survival and capacity to self-renew. At the molecular level, the combination of these properties appears to be associated with concomitant high expression of transcription factor T-Bet, characterizing effector TC, and Eomes, as in central memory TC (Grange et al., in preparation (24)).
While not constitutively activated, STAT5CA-expressing eTC were capable of enhanced secondary responses. This may be due to their increased expression of transcripts encoding effector molecules (Grange et al, in preparation (24)) allowing rapid recall responses. Moreover, expression of STAT5CA in antigen-experienced CD8+ TC endowed with poor replicative potential converted them into long-lived eTC. These reprogrammed eTC 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 (32). These mice also presented defects in LN and splenic T cell zone stroma associated with impaired recruitment of naïve TC (33), stressing the importance of tissue-migratory properties for TC in adoptive immunotherapy. Our ultimate goal was to evaluate the ability of STAT5CA-expressing CD8+ TC to infiltrate these autochthonous melanomas and to resist their immunosuppressive effects. 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 TA-specificity of the eTC-STAT5CA anti-tumor 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+ eTC 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 since 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-C) in P1A- as compared to 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 anti-tumor CD8+ eTC has been combined with IL-2 infusions to support survival and proliferation of eTC in cancer patients (6, 34, 35). Nevertheless, in vivo maintenance of CD8+ eTC was hardly increased (6), and IL-2R driven signals appeared to generate terminally differentiated eTC that rapidly became senescent (10, 36, 37). Additionally, as IL-2 contributes to expansion/function of CD4+CD25+ regulatory TC with immunosuppressive properties (38), substitutes such as use of IL-2c have been developed (26). In mice inoculated with a P1A+ melanoma, IL-2c treatment improved CD8+ eTC survival. However, IL-2c did not promote migration of infused anti-tumor CD8+ eTC inside tumors and had limited effects on GzmB expression. In contrast, STAT5CA expression induced stable phenotypic and migratory properties and survival of CD8+ eTC. The difference between STAT5CA and IL-2c treatment for modulating homing properties and survival of the transferred eTC may depend on the capacity of STAT5CA to concomitantly mimic signals elicited by IL-7 and IL-15, as well as IL-2 (39). Additionally, although they expressed PD-1 inhibitory receptors to the same extent as endogenous TC or untransduced TCRP1A eTC, 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 (40). 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 TC 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 (32). Consistent with detection of cytokines capable of activating STAT3 (41), nuclear phospho-STAT3 in tumor cells and in some infiltrating CD45 leukocytes was observed in the TiRP model (15). In CD8+ TC, STAT3 deletion improved their tumor-induced
proliferation and infiltration within tumors (42). STAT3 and STAT5 have been reported to compete for binding on a similar DNA consensus sequence on the IL-17 promoter (43). Whether this competition is a mechanism for resistance to immunosuppression of STAT5CA-expressing CD8+ eTC will be further investigated.

In conclusion, STAT5CA expression contributed to optimize anti-tumor activity of CD8+ eTC, with increased intra-tumor accumulation and strong specific Tc1 recall responses. This result suggests consideration of use of STAT5CA-transduced CD8+ TC for adoptive immunotherapy. This approach here required the use of retroviruses. Recent clinical data with retrovirus-engineered TC for cancer and HIV patients indicate that retroviral gene transfer in mature TC is safe (44). This conclusion is supported by studies showing resistance of mature TC to oncogene transformation (45). Additionally, no transformation of STAT5CA-transduced CD8+ TC was observed in this and in a previous study (46). 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+ TC specific for some poorly immunogenic TA might allow their enrichment in vitro and render them efficient for adoptive immunotherapy. The fact that antigen-experienced and polyclonal CD8+ TC can be reprogrammed by STAT5CA-expression could thus enhance the number of TA that may be targeted in adoptive therapy.

**Acknowledgments**

We thank Benoit Van den Eynde for discussion and reagents, Marc Dalod, Pierre Golstein, Bernard Malissen, Jacques Nunes, Michael Sieweke and Elena Tomasello for discussions, Linda Sherman (Scripps Research Institute, San Diego, USA) for the gift of recombinant IL-2, Amandine Mas for TiRP mice treatments, and the CIML imaging and animal facilities personnel for assistance.
Grant support

This work was supported by institutional funding from INSERM and CNRS, and by grants from “Association pour la Recherche sur le Cancer (ARC)” (to NAA), “Institut National du Cancer” (INCA), the INCA PROCAN program and the European Communities “Cars Explorer project” (to AMSV), “Agence Nationale de la Recherche (ANR)” (to GV). MG received a doctoral fellowship from “ARC.”
References


Figure Legends

Figure 1: TCRP1A eTC-STAT5CA colonize hosts and express effector functions more efficiently than control TCRP1A eTC-GFP.

A: TCRP1A CD8⁺ TC were transduced to express GFP alone or in combination with STAT5CA. % GFP⁺ among CD8⁺ TC are reported, with autofluorescence (grey), 72h and 96h post-stimulation in vitro.

B: TCRP1A eTC (8x10⁶) or TCRP1A eTC-STAT5CA (3x10⁵, middle or 10⁴, right panel) were adoptively transferred into Rag-1⁻/⁻B10.D2 mice. Dot plots of CD8 expression versus GFP of transferred CD8⁺ eTC are shown in spleen and liver days 42 and 48 post-transfer.

C-D: TCRP1A eTC-STAT5CA (10⁶, C; 5x10⁵, D) were adoptively transferred in Lys-eGFP B10.D2 mice for 21 (C) or 97 (D) days. Lys-eGFP mice expressing eGFP in myeloid cells only were used as GFP tolerant hosts. % GFP⁺ eTC among total gated CD8⁺ TC (corresponding exclusively to transferred eTC) are reported in LN and spleen (C-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 10⁵: TCRP1A eTC-STAT5CA (blue line; gated CD8⁺GFP⁺) or untransduced TCRP1A eTC (green line; gated CD8⁺); TCRP1A naïve CD8⁺ TC are shown in grey. 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.

F: TCRP1A eTC-STAT5CA (blue lines) or TCRP1A eTC-GFP (green lines) were transferred into Rag-1⁻/⁻B10.D2 mice. At day 30, TC from lungs were assayed for cytotoxic activity against P1A⁺ P815 (filled symbols) or P1A⁻ P1.204 (empty symbols) tumor cells. % target lysis is shown as a function of effector/target (E/T) ratio. Results from two mice analyzed in duplicate. Representative of 3 experiments.

G: TCRP1A eTC-STAT5CA (blue) or untransduced TCRP1A eTC (green) from Rag-1⁻/⁻B10.D2 mice injected 24 days earlier were stained for CD8 and IFN-γ after a 4h culture with P1A₃₅-₄₃ peptide-pulsed
B10.D2 splenocytes (TC-depleted). % CD8⁺ TC producing IFN-γ is shown versus peptide concentration. sd are calculated from two experiments.

**Figure 2: STAT5CA-expression promotes effector functions and tissue homing independent of the activation state of TC.**

A-C: Untransduced TCRP1A eTC from primary Rag-1⁻/⁻B10.D2 hosts were recovered from LN and spleens at d95 after transfer (pooled from 2 mice (A)). Enriched CD8⁺ TC were in vitro activated with P1A₃₅-₄₃ peptide for 20h to trigger proliferation and retrovirally-transduced. After additional 48h in culture, they were injected in secondary Rag-1⁻/⁻B10.D2 hosts. 35 days later, second recipients were analyzed (spleen (A-C) and liver (A)): CD8 versus GFP dot plots are shown in A (right panel). B: Expression of markers (as indicated) and GzmB on gated CD8⁺ eTC splenocytes: the MFI ratio between GzmB and isotype control (not shown) is reported on gated CD8⁺ TC. C: A fraction of splenocytes were activated 4h with P1A⁺ P815 or P1A⁻ P1.204 tumor cells after which intracellular IFN-γ staining was performed (shown on gated CD8 TC). Representative of 3 experiments.

**Figure 3: Higher intra-tumor accumulation of TCRP1A eTC-STAT5CA than control TCRP1A eTC when transferred in hosts inoculated with P1A⁺ mastocytoma.**

A-B: Rag-1⁻/⁻B10.D2 mice were untreated or inoculated s.c. with 10⁶ P1A⁺ P815 or P1A⁻ P1.204 mastocytoma cells. Naïve CD8⁺ TC from (βactin-LucxTCRP1A) mice were used for retroviral infection. Luc⁺TCRP1A eTC-STAT5CA (blue in B-C) and Luc⁺TCRP1A eTC-GFP sorted for GFP expression (green in B-C, diamonds in C) or non-transduced (green in B-C, squares in C) Luc⁺TCRP1A eTC (10⁶ cells each) were injected in tumor-free (2 mice) or -bearing hosts. Two experiments were conducted. Bioluminescence was recorded at day 1, 3 and 8 after transfer. A: A representative image is shown for each experimental group.
B-C: CD8 TC accumulation in tumor areas is reported as number of photons/mm²S on a defined surface. Mean values +/- sd are reported at day 1 to 8 for P1A⁺ (left graph) and P1A⁻ (right graph) (B) and as values for individual mice on day 8 (C).

Statistical analyses were performed with an unpaired t test (GraphPad). Two-tailed $P$: $p<0.05$ is given as (*); $p<0.01$ as (**), $p<0.001$ as (***)

Figure 4: Higher intra-tumor accumulation and tumor regression by TCRP1A eTC-STAT5CA than control TCRP1A eTC +/- IL-2c infusions in hosts inoculated with a P1A+ melanoma.

$Lys$-eGFP CD45.1 B10.D2 mice were inoculated s.c. with $10^6$ P1A⁺Luc⁺ melanoma (T429-L) cells. Mice with solid tumors (day 0 in A) received $10^6$ TCRP1A eTC-STAT5CA (A-E; n=7), or untransduced TCRP1A eTC alone (A-C; n=7) or in combination with IL-2c infusions (A-D; n=4). A-B: Tumor development was measured by counting photons emitted by luciferase-expressing tumors on a defined skin surface (mm²S). Values between day 0 (time of adoptive transfer) and day 11 (time of analysis) are reported in A as means +/- 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: 11 days after TC transfer, TILs were stained directly for CD8, CD45.1 (in C, host: CD45.1+; TCRP1A eTC: CD45.2 are CD45.1-negative). D-E: Ratios between transferred and endogenous CD8⁺ TILs (D) and absolute numbers of transferred and endogenous TILs (E) were evaluated from FACS analysis. Statistical analyses were performed with a t-test as in Fig.3. In E, two by two comparisons of the different groups gave significant differences (**) as indicated. All other comparisons had $p>0.05$ (ns).

Figure 5: TCRP1A eTC-STAT5CA accumulating inside the tumor express high level of GzmB and produce IFNγ upon secondary stimulation despite high level of PD-1.

$Lys$-eGFP CD45.1 B10.D2 mice inoculated s.c. with $10^6$ P1A⁺Luc⁺ melanoma (T429-L) cells (as in Fig.4) received $10^6$ TCRP1A eTC-STAT5CA (A, D, G), or untransduced TCRP1A eTC alone (B, E,
H) or in combination with IL-2c infusions (C, F, I). Results are representative of 3 (TCRP1A eTC + IL-2c) to 5 (eTC-STAT5CA or TCRP1A eTC) independent experiments.

A-C: GzmB stainings of gated CD8+ TILs are shown with their respective GzmB MFI. Statistical analyses are reported in Fig.S5A.

D-F: PD-1 stainings of gated CD8+ TILs are shown with the respective percent PD-1+ TC among gated CD8+ and PD-1 MFI among CD8+PD-1+ TC. PD-1 MFI are not significantly different.

G-I: A fraction of TILs was stimulated 4h with anti-CD3 presented by FcR-bearing P1A– (P1.204) tumor and stained for CD8, CD107a and IFN-γ. Percent positive cells are reported in each quadrant.

Figure 6: TCRP1A eTC-STAT5CA infiltrate autochthonous melanomas more efficiently than control TCRP1A eTC.

TiRP Rag-1−/−B10.D2 mice developing an induced melanoma received 10⁶ TCRP1A eTC-STAT5CA (A-C), or 10⁶ (B-D) or 7×10⁶ (E) untransduced TCRP1A eTC.

A: Example of tumor regression in a TiRP mouse treated with 10⁶ TCRP1A eTC-STAT5CA. B: Mice bearing amelanotic induced melanomas were infused with 10⁶ untransduced (7 mice) or STAT5CA-transduced (9 mice) TCRP1A eTC. Tumors were measured the day of TC transfer (d0) and 7 days later. Ratios of tumor volumes, calculated as (l²xL)/2, are shown for individual mice. In the first group 2 mice died before day 7. In the second group the initial tumor size was not recorded for one mouse. Statistical analysis was performed with a t-test using GraphPad and two-tailed P. P=0.002 (**).

(C-D) 11 or (E) 7 days after adoptive transfer, splenocytes and TILs were stained directly for CD8 and GzmB. GzmB expression and MFI values are shown for gated CD8+ TC. A fraction of cells was stimulated 4h with anti-CD3 presented by FcR-bearing P1A– tumors and stained for CD8/CD107a/IFN-γ (lower panels in B and C): CD107a versus IFN-γ plots are shown for CD8+ TC with % reported in each quadrant.
Figure 1

A

TCRP1A eTC-GFP

TCRP1A eTC-STAT5CA

cell count

GFP

72h

96h

18.6

18.1

100 101 102 103 104

100 101 102 103 104

0 20 40 60 80 100

0 20 40 60 80 100

B

TCRP1A eTC

d42; 8x10^6 cells

(d42; 8x10^6 cells)

(d42; 3x10^8 cells)

CD8

Spleen

Liver

GFP

6.1

65.9

32.3

C

TCRP1A eTC-STAT5CA

d21; 2x10^5 cells

(d21; 2x10^5 cells)

(d42; 3x10^8 cells)

CD8

Spleen

Liver

GFP

80

75.4

35

D

TCRP1A eTC-STAT5CA

d97; 5x10^5 cells

(d97; 5x10^5 cells)

naive CD8+ TC

TCRP1A eTC (d42)

TCRP1A eTC-STAT5CA (d42)

CD122

CD127

CD62L

CD25

CD44

GzmB

% lysis

cell count

% CD8+ IFN+ γ

GFP

naive CD8+ TC

TCRP1A eTC (d42)

TCRP1A eTC-STAT5CA (d42)

E

F

G

Figure 1
Figure 2
Figure 3

A. 

<table>
<thead>
<tr>
<th>Day</th>
<th>Luc⁺TCRP1A eTC-STAT5CA</th>
<th>Luc⁺TCRP1A eTC-GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>P1A⁺ tumor</td>
<td>no tumor</td>
</tr>
<tr>
<td>Day 3</td>
<td>P1A⁻ tumor</td>
<td>no tumor</td>
</tr>
<tr>
<td>Day 8</td>
<td>no tumor</td>
<td>no tumor</td>
</tr>
</tbody>
</table>

B. 

- **P1A⁺ tumor**
  - Luc⁺TCRP1A eTC-STAT5CA
  - Luc⁺TCRP1A eTC-GFP or eTC

- **P1A⁻ tumor**
  - Luc⁺TCRP1A eTC-STAT5CA
  - Luc⁺TCRP1A eTC-GFP or eTC

C. 

- Day 8
  - P1A⁺
  - P1A⁻

- Luc⁺TCRP1A eTC
- Luc⁺TCRP1A eTC-STAT5CA

Ph/mm²s
Figure 4

(A) Bar graph showing the percentage of initial photon emission (transferred/endogenous) for CD8 TILs.

(B) Scatter plot comparing the percentage of endogenous and transferred CD8 TILs.

(C) Flow cytometry analysis of TILs, showing the percentage of endogenous and transferred CD8 TILs.

(D) Bar graph showing the absolute number of CD8 TILs.

(E) Bar graph comparing the absolute number of CD8 TILs in different conditions.
Figure 5

Transfer of TCRP1A eTC-STAT5CA

A. endogenous (CD45.1+) TC

B. TCRP1A (CD45.1-) eTC

C. endogenous (CD45.1+) TC

D. TCRP1A (CD45.1-) eTC

GzmB

cell count

GzmB

cell count

PD-1

PD-1

PD-1

IFNγ

IFNγ

IFNγ

CD107a

CD107a

CD107a

Figure 5

Transfer of TCRP1A eTC+ IL-2c

A. endogenous (CD45.1+) TC

B. TCRP1A (CD45.1-) eTC

C. endogenous (CD45.1+) TC

D. TCRP1A (CD45.1-) eTC

GzmB

cell count

GzmB

cell count

PD-1

PD-1

PD-1

IFNγ

IFNγ

IFNγ

CD107a

CD107a

CD107a

Figure 5

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited. Author Manuscript Published OnlineFirst on November 7, 2011; DOI: 10.1158/0008-5472.CAN-11-2187
Figure 6
Activated STAT5 promotes long-lived cytotoxic CD8+ T cells that induce regression of autochthonous melanoma.

Magali GRANGE, Michel BUFERNE, Gregory VERDEIL, et al.

_Cancer Res_ Published OnlineFirst November 7, 2011.