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IFNγ producing CD8$^+$ T cells modified to resist major immune checkpoints induce regression of MHC class I-deficient melanomas

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Abbreviations: eTC, effector T cell; LN, lymph node; MFI, mean fluorescence intensity; STAT5, Signal transducer and activator of transcription 5; TA, tumor antigen; TILs, tumor infiltrating leukocytes.

Tumors with reduced expression of MHC class I (MHC-I) molecules may be unrecognized by tumor antigen-specific CD8$^+$ T cells and thus constitute a challenge for cancer immunotherapy. Here we monitored development of autochthonous melanomas in TiRP mice that develop tumors expressing a known tumor antigen as well as a red fluorescent protein (RFP) reporter knock in gene. The latter permits non-invasive monitoring of tumor growth by biofluorescence. One developing melanoma was deficient in cell surface expression of MHC-I, but MHC-I expression could be rescued by exposure of these cells to IFNγ. We show that CD8$^+$ T cells specific for tumor antigen/MHC-I were efficient at inducing regression of the MHC-I-deficient melanoma, provided that the T cells were endowed with properties permitting their migration into the tumor and their efficient production of IFNγ. This was the case for CD8$^+$ T cells transfected to express an active form of STAT5 (STAT5CA). The amount of IFNγ produced *ex vivo* from T cells present in tumors after adoptive transfer of the CD8$^+$ T cells was correlated with an increase in surface expression of MHC-I molecules by the tumor cells. We also show that these CD8$^+$ T cells expressed PD-1 and upregulated its ligand PDL-1 on melanoma cells within the tumor. Despite upregulation of this immunosuppressive pathway, efficient IFNγ production in the melanoma microenvironment was found associated with resistance of STAT5CA-expressing CD8$^+$ T cells to inhibition both by PD-1/PDL-1 engagement and by TGFβ1, two main immune regulatory mechanisms hampering the efficiency of immunotherapy in patients.

Cancer immunotherapy aimed at promoting tumor antigen (TA)/MHC – specific T cell responses has been hampered by two main hurdles: tumor-induced immunosuppression and tumor immune escape. Thus, while tumor infiltration by CD8$^+$ cytotoxic (granzyme B (GzmB) positive) T cells and Th1 (IFNγ producing) T cells has been associated with good clinical outcome in many different tumor types in patients (reviewed in[1]), intra-tumor or systemic Th2 polarization has been associated with poor prognosis for some human cancers (reviewed in[1]), including melanoma.[2] Tumor-recruited macrophages[3,4] and neutrophils[5] that express a tumor-induced type-2 program, as well as immature myeloid cells (or Myeloid derived suppressor cells, MDSC),[6] have all been associated with pro-angiogenic and immunosuppressive functions.

Evidence for the existence of immune escape tumor variants stems from data on spontaneous or carcinogen-induced tumor development in animal models presenting genetic defects in immune effectors, as well as from observations on tumors recurring after immunization in both patients and mice. Thus, it was observed that mice genetically deficient for components of adaptive immunity and of IFNγ signaling were significantly more prone to spontaneous epithelial tumor development than their wild type counterparts. In addition, the tumors developing in the immune-deficient mice appeared more immunogenic than those arising in immune-competent mice, an observation interpreted as evidence for tumor "immunoediting."[7] Interestingly, the molecular phenotype of the less-immunogenic tumor variants was shown

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to be associated with down-regulation of TAP1, one of the antigen processing machinery components responsible for loading peptides onto MHC-I molecules and induction of T cell responses. More recently, Schreiber's group identified mutations expressed in the highly immunogenic methylcholanthrene-induced sarcomas developed in Rag-deficient mice, demonstrating that the strong immunogenicity of an unedited tumor can be ascribed to expression of highly antigenic mutant proteins. They further showed that outgrowth of tumor cells that lack these strong antigens as a consequence of a T-cell-dependent immunoselection process represents one mechanism of cancer immunoediting.[8] Evidence for outgrowth of tumors lacking TA expression following TA-directed immunization or adoptive transfer of T cells has also been documented for transplanted tumors in mice.[9-13]

In melanoma patients, loss of TA expression has been observed after adoptive transfer of MART-1-specific T cells.[14,15] Emergence of multiple MHC-I defects was reported in melanoma cells following strong T cell-mediated immune selection[16] (reviewed in[17]). In patients immunized with MART-1 and TYROSINASE-derived peptides loss of MHC-I or selectively HLA-A2 was observed in the melanoma lesions which progressed in the presence of a TA-specific T cell response.[18] Similar results were observed[19] with either loss of TA expression or loss of MHC-I expression in distinct metastases of a melanoma patient who had been immunized with three TA peptides in adjuvant.

Defects in MHC-I expression on tumor cells have been classified as either irreversible or cytokine-reversible.[20] The irreversible phenotype generally results from structural defects due to mutations/deletions in MHC-I or β2m encoding genes, whereas defects in the transcriptional regulation of those genes are generally at the basis of the reversible phenotype. The latter often involves a coordinated downregulation of MHC-I encoding genes and/or of genes encoding components of the antigen processing machinery.[21]

IFNγ has been shown to enhance the MHC-I antigen processing and presentation pathway.[22,23] However, IFNγ was also found to up-regulate expression of immunosuppressive ligands such as PDL-1 on tumor cells, including melanomas.[24] In addition, systemic administration of IFNγ in clinical trials has led to adverse reactions and negative clinical outcome.[25]

Genetically engineered mouse melanoma models that recapitulate key aspects of human tumor development are essential to address the reciprocal influences of immune components and developing tumors both in the tumor microenvironment and systemically.[26-29] We have developed such a mouse model (TiRP) in which induced melanomas express a natural cancer-germline tumor antigen, P1A,[30] that may be targeted by P1A-specific $CD8^+$ T cells (TCRP1A;[31]). Adoptively transferred TCRP1A T cells (naïve or pre-activated) failed to accumulate in TiRP melanomas and demonstrated impaired effector functions ($GzmB^-$).[26,32] However, providing increased cytokine receptor signaling through forced expression of a constitutively active STAT5 (STAT5CA) in antitumor $CD8^+$ T cells used in adoptive therapies greatly improved their tumor infiltration and increased their expression of effector molecules ($GzmB^+$/$IFN\gamma^+$), promoting regression of TiRP melanomas.[32] Mechanistically, the active STAT5CA appeared to provide (i) long-term upregulation of genes involved in the $CD8^+$ T cell effector program (Granzymes, IFNγ, TNFα, CCL3…) as well as the transcription factors T-Bet and Eomes; (ii) repression of genes encoding the IL-6R and TGFβRII subunits and (iii) reduced sensitivity to tumor-derived IL-6.[33]

We here characterize a TiRP melanoma with undetectable surface MHC-I molecules. We addressed the question as to whether adoptive therapy with tumor-specific T cells may be efficient against melanomas if the defects in MHC-I expression were reversible. We show that STAT5CA-expressing $CD8^+$ T cells stimulate upregulation of MHC-I expression and induce regression of the melanomas in a manner concomitant with their capacity to infiltrate the tumor and to produce IFNγ. We further demonstrate that STAT5CA-expressing $CD8^+$ T cells are resistant to the effects of TGFβ1 and PD-1, two major immune-suppressive mechanisms acting in the melanoma microenvironment.

Results

Characterization of a RFP+ autochthonous melanoma mouse model: establishment of a melanoma cell line presenting reversible defects in MHC-I surface expression

Melanoma development is induced in TiRP mice by subcutaneous injection of 4OH-tamoxifen (**Fig. S1A**) but depending on their anatomical location, follow-up of these tumors was not always easy. To measure more precisely melanoma development, TiRP mice were crossed with the ROSA-td-RFP reporter mice[34] (**Fig. S1B**): RFP^+ expression was detected upon melanocyte-specific Cre activation concurrent with the initiation of melanoma development (**Fig. S1C**). Of note, while tumor cells transfected with fluorescent reporter genes often lose expression of the tracer once injected *in vivo* in absence of selection, we never noticed extinction of the fluorescent signal encoded at the ROSA26 locus. From several TiRP-RFP mice developing melanomas *in situ*, tumor cell lines were established *in vitro* which expressed distinct levels of the P1A-restricting MHC-I molecules $H\text{-}2L^d$ (**Fig. 1A**): T-RFP-92 and -95 expressed $H\text{-}2L^d$ at the same level as the previously derived T-429,[26,32] while $H\text{-}2L^d$ was undetectable on T-RFP-69, which was also found negative for $H\text{-}2K^d$ expression (not shown). These results suggest that the T-RFP-69 tumor manifests a global defect in expression of MHC-I surface molecules. We next examined whether IFNγ treatment would affect $H\text{-}2L^d$ expression on T-RFP-69 cells (**Fig. 1B**). After overnight culture with IFNγ, $H\text{-}2L^d$ was increased on a fraction of the tumor cells: $H\text{-}2L^d$ positive and negative cells were sorted and the negative fraction was subjected to a second IFNγ treatment, which resulted in a homogenous $H\text{-}2L^d$ high expression. Those data showed that T-RFP-69 cells maintained an IFNγ-responsive MHC-I expression.

We additionally tested whether T-RFP-69 cells differed from the previously established T-429 melanoma line in terms of their expression of the TiRP transgene-encoded $HRas^{G12V}$ and P1A (Trap1a) transcripts (**Fig. 1C**). High levels of both transcripts were observed in the two melanoma lines, the expression level of the P1A transcripts being similar to that of the endogenous P1A in the P511 mastocytoma line (**Fig. 1C**).

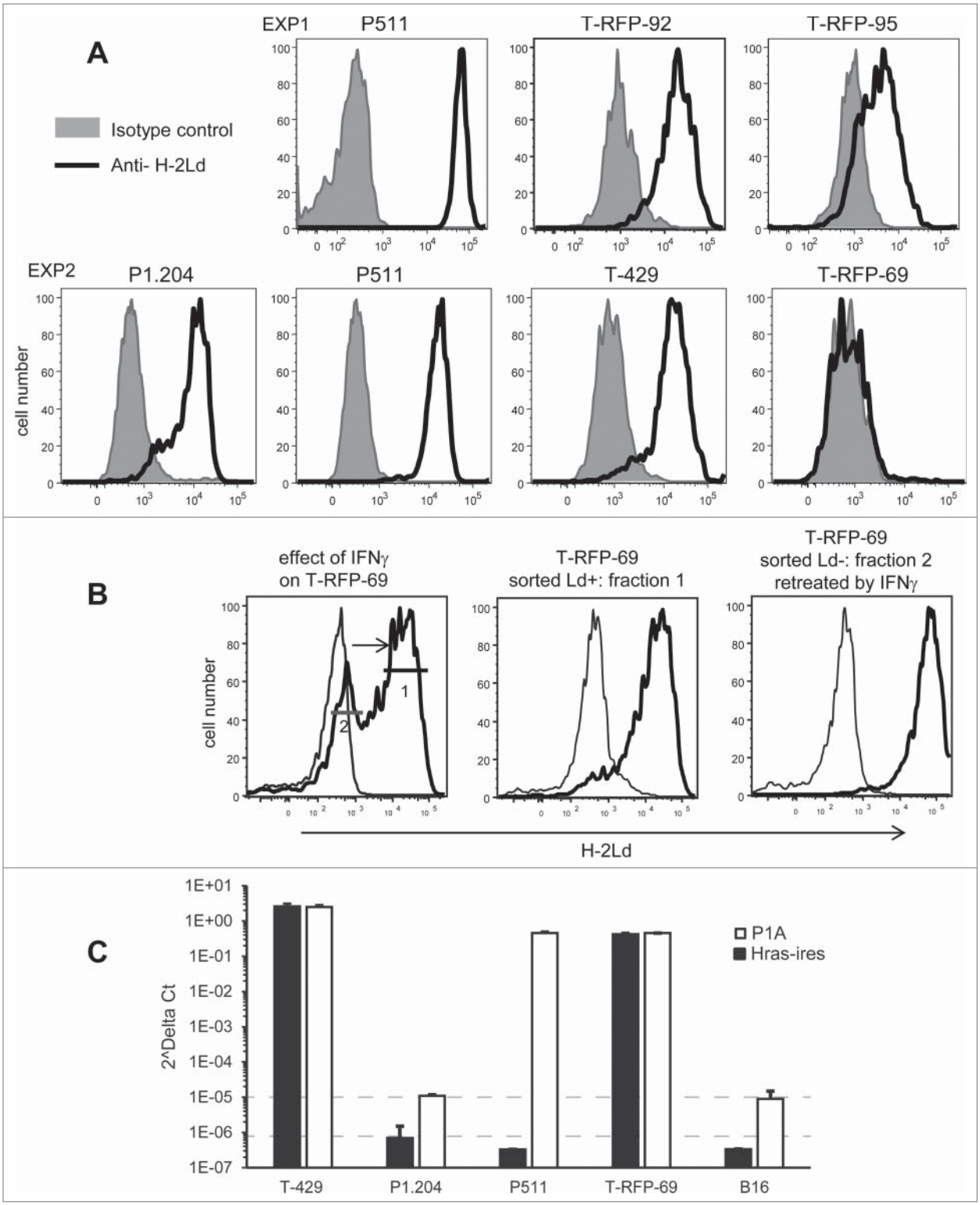

Figure 1. Characterization of different TiRP melanoma cell lines expressing distinct levels of MHC class I. (**A**) TiRP melanoma cell lines isolated from 4OH-tamoxifen treated TiRP or TiRP-RFP mice (see methods) are stained by an anti-H-2L^{d} mAb (black line) or an isotype control (gray). Mastocytoma tumors expressing (P511) or not (P1.204) the P1A Ag are included as controls.(**B**)**:** Expression of H-2L^{d} on untreated (thin line) or IFNγ-treated (bold line) T-RFP-69 tumors. Cells showing high (fraction 1) and low (fraction 2) H-2L^{d} expression are sorted. An additional IFNγ-treatment is applied to fraction 2 (left panel). (**C**)**:** mRNA was extracted from the same cell lines as in A and qRT-PCR was conducted to measure expression of transcripts encoding HRas^{G12V} and P1A. A P1A-negative melanoma (B16) was included as a control. Results are representative of two or three experiments done in duplicate.

MHC-I deficiency prevents activation of adoptively transferred naïve P1A-specific CD8$^+$ T cells

We performed adoptive transfers using naïve P1A-specific CD8$^+$ T cells that additionally expressed the luciferase reporter gene (TCRP1A Luc$^+$ cells) in mice transplanted with T-RFP-69 tumors. Non-invasive fluorescence and bioluminescence (**Figs. 2A, B**) were used to monitor *in vivo* tumor growth and intra-tumor T cell accumulation, respectively. Naïve TCRP1A cells did not accumulate in large numbers inside the tumor and did not control tumor growth, while those cells successfully colonized MHC-I sufficient T-429 melanomas and even more efficiently the immunogenic mastocytoma P1A$^+$ (P511) tumor. In this last condition, we have previously shown that naïve TCRP1A T cells became activated in the LN draining the tumor and then migrated to the tumor site where they were restimulated, presumably by the tumor itself.[11] We here evaluated the efficiency of the melanoma cell lines to trigger proliferation of CFSE-labeled naïve TCRP1A T cells in the tumor draining LNs (**Figs. 2C–D**). Rag-1$^{-/-}$ mice either tumor-free or bearing a P1A$^-$ tumor (P1.204) were included as controls and allowed evaluation of the homeostatic proliferation (gray histograms). While both P1A$^+$ mastocytoma (P511) and T-429 melanoma induced high proliferation of naïve TCRP1A cells, T-RFP-69 melanoma was significantly (**Fig. 2D**) less efficient.

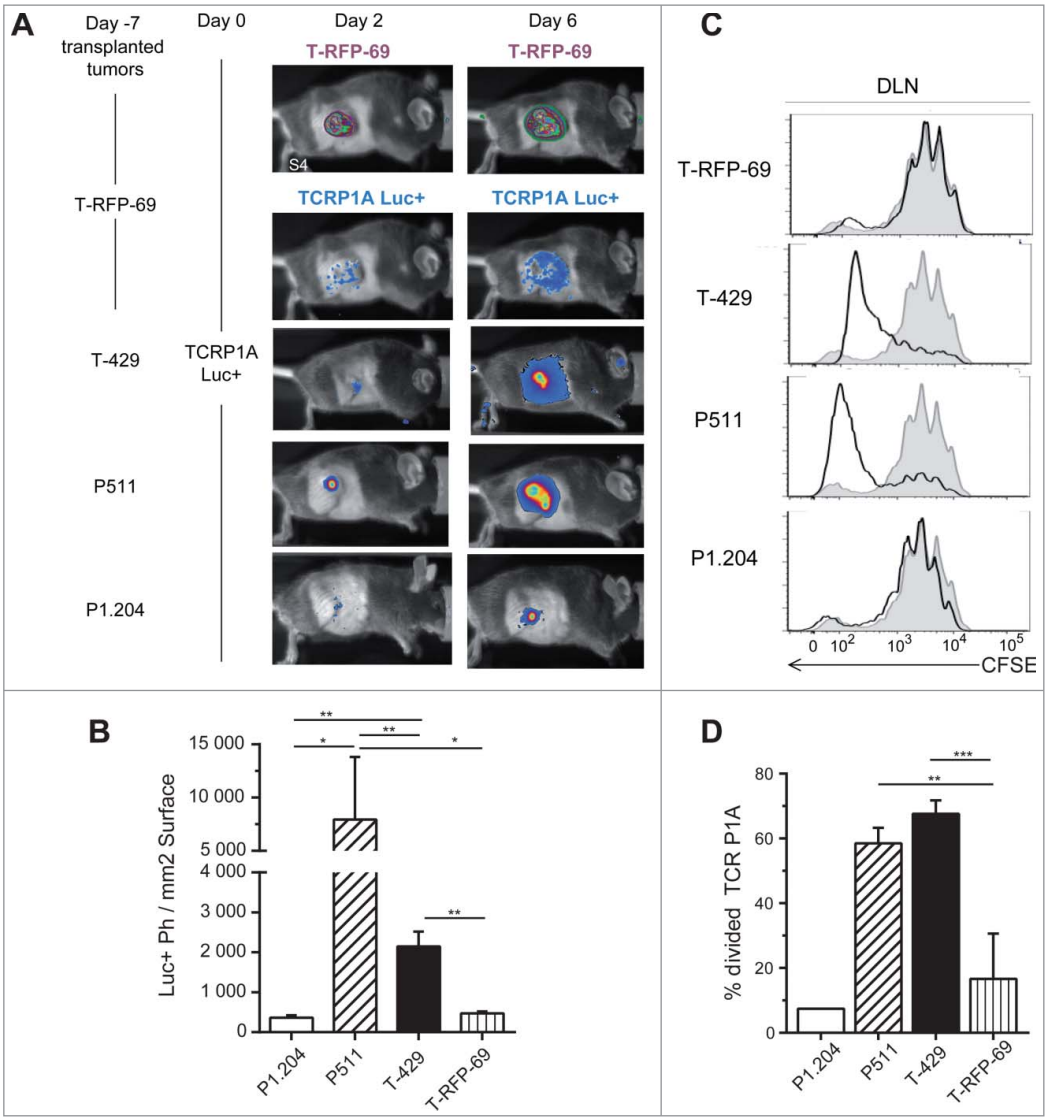

Figure 2. Naive TCRP1A CD8$^+$ TCs fail to infiltrate transplanted T-RFP-69 tumors. (**A–D**) Rag-1$^{-/-}$B10.D2 mice were inoculated s.c. with 10^6 tumor cells. Fifteen (TiRP melanomas) or 7 (mastocytomas) d later, mice received either PBS or 10^6 TCRP1A Luc+ naïve T cells (**A and B**) or CFSE labeled-TCRP1A naïve T cells (**C and D**). (**A and B**) Fluorescence signal relative to T-RFP-69 development and luminescent signal relative to TCRP1A Luc$^+$ T cells were recorded at the indicated time after T cell transfer. (**C and D**) 3 d after adoptive transfers, T cell proliferation was analyzed in tumor draining (DLN). T cells were also injected in tumor-free mice: this gives the level of homeostatic proliferation which is used as a basal control (gray histograms) in the overlays.

STAT5CA-expressing TCRP1A cells efficiently infiltrate and induce regression of melanoma tumors presenting reversible defects in MHC-I expression

We recently showed that forced expression of a constitutively active STAT5 in tumor-specific CD8$^+$ TCs (TCRP1A eTC-STAT5CA) greatly improved their tumor infiltration after adoptive transfer into tumor-bearing mice and promoted regression of TiRP melanomas.[32] We here analyzed whether those manipulated CD8$^+$ T cells could induce regression of MHC-I deficient T-RFP-69 melanomas. TCRP1A Luc$^+$ T cells were activated *in vitro* by their cognate P1A peptide and transduced or not by retroviral particles encoding STAT5CA. At the end of a 3 d culture, TCRP1A Luc$^+$ cells were injected in Rag-1$^{-/-}$ mice bearing a transplanted T-RFP-69 tumor. Bioluminescence recordings (**Fig. 3A**) revealed a higher intratumor accumulation of TCRP1A Luc$^+$ eTC-STAT5CA than TCRP1A Luc$^+$ eTC (see **Fig. 3B** for statistics). While the former T cells induced tumor regression, the latter failed to do so (**Fig. 3A, C**). For two individual mice injected with TCRP1A Luc$^+$ eTC-STAT5CA, **Fig. 3D**

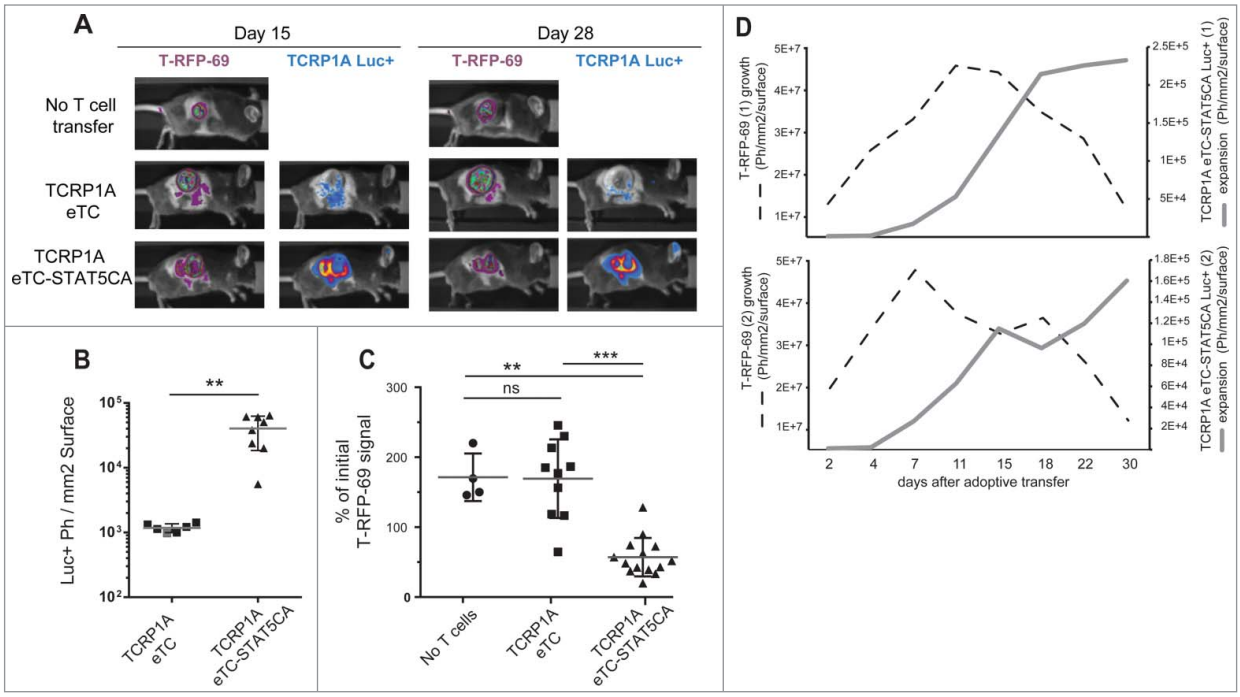

Figure 3. Pre-activated TCRP1A eTCs do not infiltrate transplanted T-RFP-69 tumors unless manipulated to express an active form of STAT5 (eTCs-STAT5CA). (**A–D**) Rag-1$^{-/-}$B10.D2 mice were inoculated s.c. with 10^6 T-RFP-69 tumors. Fifteen days later, mice received either PBS or pre-activated TCRP1A Luc$^+$ eTC or pre-activated TCRP1A Luc$^+$ eTC-STAT5CA (10^6 cells each). Fluorescence signals measuring tumor development and luminescent signals measuring TCRP1A Luc$^+$ T cells were recorded at the indicated times after T cell transfer. Images representative of six independent experiments are shown in (**A**). At day 15 (peak of TCRP1A Luc$^+$ eTC accumulation), the luciferase signals (photon emission/mm^2 on a defined area) are reported for transferred TCRP1A Luc$^+$ eTC or TCRP1A Luc$^+$ eTC-STAT5CA T cells (**B**). At day 10, peak of tumor growth recorded in mice receiving TCRP1A eTC-STAT5CA) the tumor-related fluorescence signals measured 10 d later are reported as a % of the initial tumor signal (**C**). The same measurements were conducted for both untreated mice and mice that received TCRP1A eTCs. In **D**, two examples of simultaneously recorded tumor growth (left axis) and TCRP1A Luc$^+$ eTC-STAT5CA accumulation (right axis) are reported (as photons/mm^2 on a defined area).

shows that tumor growth continued for 7–10 d, followed by tumor regression after T cells started to accumulate within the tumor (from day 7 to day 15–18).

STAT5CA-expressing TCRP1A cells are stimulated more efficiently by TiRP melanomas than unmanipulated TCRP1A eTCs even when the latter are inoculated in contact with the tumors

We next wondered whether the inefficiency of unmanipulated TCRP1A eTCs to induce melanoma regression was the consequence of their poor capacity to infiltrate and accumulate within the tumor mass. If so, this limitation should be bypassed by their inoculation in direct contact with tumor cells. For this purpose, air-pouches were raised on the dorsum by subcutaneous air injection. CTV-labeled TCRP1A eTCs or eTCs-STAT5CA were co-injected with tumor cells into the pouches and recovered from the pouches 3 d later for analysis of their proliferation (**Fig. S2**). It should be noted that in these experiments, we could not use TCRP1A eTCs or eTCs-STAT5CA directly after their *in vitro* stimulation and transduction as cells are still proliferating as a result of the recent TCR stimulation. Instead, activated T cells were adoptively transferred into congenic Rag-1$^{-/-}$ hosts where they differentiate into memory cells, as previously described.[32,33] A month later, TCRP1A eTCs or eTCs-STAT5CA recovered from recipients' LNs and spleens were included in restimulation assays in the pouches. While eTCs-STAT5CA showed a limited dilution of the CTV staining when inoculated with the P1A-negative (P1.204) control tumor, those cells divided up to 3 or 4 times when injected with the T-RFP-69 or T-429 melanomas, respectively (**see Figs. S2A and S2C** for related statistical analyses). Interestingly, TCRP1A eTCs also showed an efficient proliferation triggered by co-injection with T-429 melanomas, while their response to T-RFP-69 tumors was less effective (**Fig. S2B–C**). However, cell yields showed an advantage for TCRP1A eTCs-STAT5CA as compared to eTCs for all samples, suggesting a better survival of divided cells when STAT5CA is expressed.

Upregulation of MHC-I on deficient TiRP tumor cells and efficient IFNγ production in melanomas infiltrated by STAT5CA-expressing TCRP1A cells

We next evaluated the consequences within the T-RFP-69 tumors of the T cell adoptive transfers. Twenty days after adoptive therapies with TCRP1A T cells, T-RFP-69 tumors were recovered and separated into three parts for (i) TILs extraction and their analyses by flow cytometry; (ii) overnight culture to measure cytokine production in tumor supernatant; (iii) immunohistology conducted on tumor slices. CD8$^+$ staining on CD45$^+$ cells demonstrates massive tumor infiltration by eTCs-

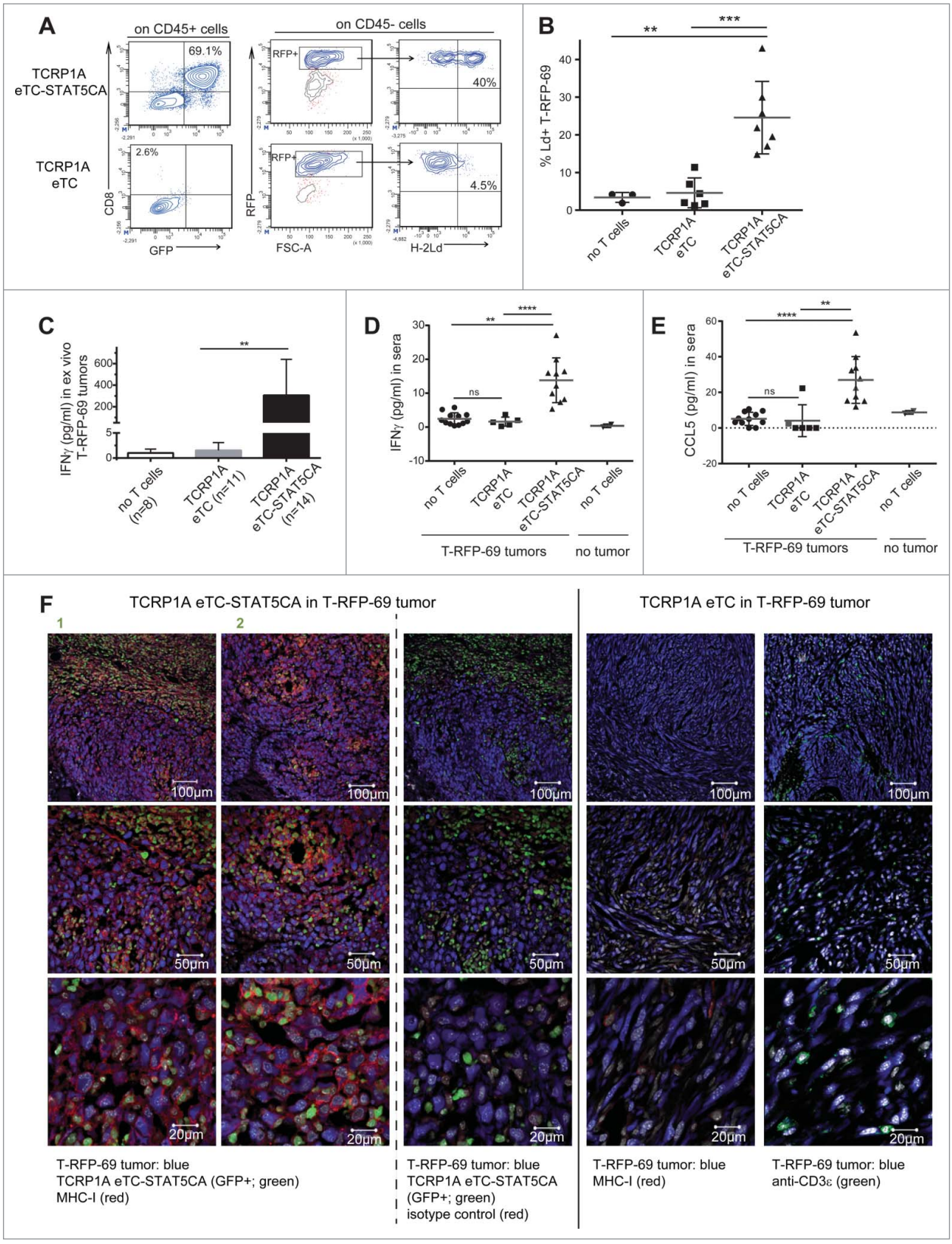

Figure 4. For figure legend, see page 8.

STAT5CA as compared to eTCs (**Fig. 4A**, left panel and **Fig. 4B**). Importantly, analysis of $CD45^-$ RFP^+ tumor cells recovered from mice injected with eTCs-STAT5CA revealed an important increase of $H\text{-}2L^d$ expression at the surface of a fraction of tumor cells, while this characteristic was not found in tumors from untreated mice or mice receiving eTCs (**Fig. 4A** right panel and **Fig. 4B**).

Multiplex immunoassays were conducted to quantify cytokines produced by the whole tumors after a 20 h culture (**Fig. 4C** and **Table S1**). Sera of tumor-bearing mice were also recovered and included in these multiplex analyses (**Figs. 4D–E** and **Table S1**). IFNγ was among the few cytokines that were detected in higher amounts both in *ex vivo* cultures (**Fig. 4C**) and sera (**Fig. 4D**) from mice that received eTCs-STAT5CA as compared to eTCs or from untreated animals. Higher levels of CCL5 (RANTES) were detected only in sera of tumor-bearing mice injected with eTCs-STAT5CA (**Fig. 4E** and **Table S1**). Comparison of sera from individual tumor-bearing mice before and after T cell inoculation (**Fig. S3B**) demonstrated a clear correlation between the presence of IFNγ and the inoculation of STAT5CA-expressing T cells. These results are in agreement with the increased capacity of eTCs-STAT5CA for IFNγ secretion triggered by TCR stimulation *in vitro* (**Fig. S3A**) in correlation with their enhanced transcript expression.[33]

Multiplex analyses of tumor supernatants also showed an increase in IL-18 when tumor-bearing mice received TCRP1A eTCs-STAT5CA as compared to eTCs or to no treatment (**Table S1, Fig. S3C**). Memory $CD8^+$ T cells have been shown to produce high levels of IFNγ in response to different inflammatory cytokines, in particular to combinations of IL-12 and IL-18.[35] Therefore, we measured the response of purified (NK cell depleted) TCRP1A eTCs-STAT5CA and eTCs after 40 h of stimulation with IL-12 or/and IL-18. While each cytokine alone was inefficient at inducing IFNγ secretion (not shown), their combination was quite effective, with an advantage again for the STAT5CA-expressing eTCs (**Fig. S3E**) over control eTCs and naïve $CD8^+$ T cells. Altogether, since very low levels of IL-12 were detectable in *ex vivo* tumors (**Table S1, Fig. S3D**) and IL-18 alone was not efficient at inducing IFNγ secretion by the T cells, it appears most likely that intra-tumor IFNγ production by STAT5CA-expressing T cells required cognate recognition and was not solely cytokine-driven.

Immunostaining on tumor slices confirmed the high tumor infiltration by green GFP^+ STAT5CA-expressing T cells (**Fig. 4F**; left panels) together with the increased MHC-I expression (intense red) at the surface of T-RFP-69 tumors. In tumors from mice that received control eTCs (**Fig. 4F**; right panels), fewer T cells were found and the rare cells detected as MHC-I-positive were not RFP^+, thus non-tumor cells.

We next analyzed whether the observation made using TiRP melanomas expressing P1A could be reproduced with a different melanoma. Using the B16F10 melanoma model expressing the LCMV gp33 epitope (B16-gp33), it was reported that gp33/$H\text{-}2D^b$-specific $CD8^+$ T cells from P14 mice were inefficient at preventing the growth of B16-gp33 melanoma cells.[36] In this setting, we tested whether transduction of P14 T cells with STAT5CA improved their capacity to control B16-gp33 tumor growth. Indeed, P14 eTC-STAT5CA were more efficient than P14 eTCs at inducing tumor shrinkage (**Fig. S4A**) in correlation with their higher capacity to infiltrate the tumor (**Fig. S4B**) and with higher production of IFNγ (**Fig. S4C**).

Efficient IFNγ production in the melanoma microenvironment is associated with resistance of STAT5CA-expressing $CD8^+$ T cells to inhibition by TGFβ1 and by PD-1/PDL-1 engagement

We previously provided evidence for the constitutive activation of the TGFβ pathway in the amelanotic inflammatory TiRP melanomas ([37] and **Fig. S5A** for TGFβ1 secretion by T-RFP-69 melanomas). Massague and co-workers have shown that TGFβ1 induced the repression of transcripts encoding effector molecules (IFNγ and GzmB) during naïve to effector $CD8^+$ T cell differentiation.[38] However, the behavior of antigen-experienced T cells exposed to TGFβ1 has not been reported. We therefore explored the sensitivity of TCRP1A eTCs-STAT5CA and eTCs to TGFβ1. The cells were stimulated for 48h by anti-CD3 +/− TGFβ1. qRT-PCR analyses showed that eTCs-STAT5CA maintained a high level of IFNγ (**Fig. 5A**) and GzmB (**Fig. S5B**) transcripts in both conditions, while those transcripts were expressed at a lower level in eTCs and were significantly inhibited in presence of TGFβ1. IFNγ secretion in culture supernatants was assessed by Elisa in the same conditions. eTCs-STAT5CA produced larger amounts of IFNγ and were insensitive to TGFβ1, while the lower production of IFNγ by eTCs was significantly inhibited in the presence of TGFβ1 (**Fig. 5B**).

Another common mechanism of immunosuppression in the tumor microenvironment involves the T cell expressed PD-1 receptor engagement by its PDL-1 ligand. PD-1 is found transiently expressed following stimulation of naïve T cells by their cognate Ag and is thought to contribute to the dampening of T cell proliferation. Additionally, PD-1 is found stably expressed at a high level on chronically stimulated T cells including virus- and tumor-specific T cells.[39] In this latter case, PD-1 expression is

Figure 4 (See previous page). TCRP1A eTCs-STAT5CA accumulating inside transplanted T-RFP-69 tumors induced an upregulation of MHC class I molecules on the tumor cells coincident with their secretion of IFNγ. (**A–E**) Rag-$1^{-/-}$B10.D2 mice were inoculated s.c. with 10^6 T-RFP-69 tumors. Fifteen days later, mice received either PBS or pre-activated TCRP1A eTC or pre-activated TCRP1A eTC-STAT5CA (10^6 cells each). (**A–C**) Day 20 post-transfer, tumor mass were recovered and cells analyzed both for tumor-infiltrating $CD8^+$ T cells (left panel, (**A**); gating on $CD45^+$ cells) and $H\text{-}2L^d$ expression on RFP+ tumor cells (right panel, (**A**); gating on $CD45^-$ cells). At the same time, pieces of the recovered tumor mass were cultured *ex vivo* for 24 h and IFNγ was measured in the supernatant (**C**): IFNγ units per mg of cultured tumors are reported. IFNγ (**D**) and CCL5 (**E**) were also quantified in recipient mice' sera. Immuno-stainings on tumor slices were performed. (**F**) Anti-MHC-I or its isotype control is shown in red; RFP from tumors are shown in blue; GFP from TCRP1A eTC-STAT5CA is shown in green while pre-activated TCRP1A eTC were revealed by an anti-CD3ε also in green.

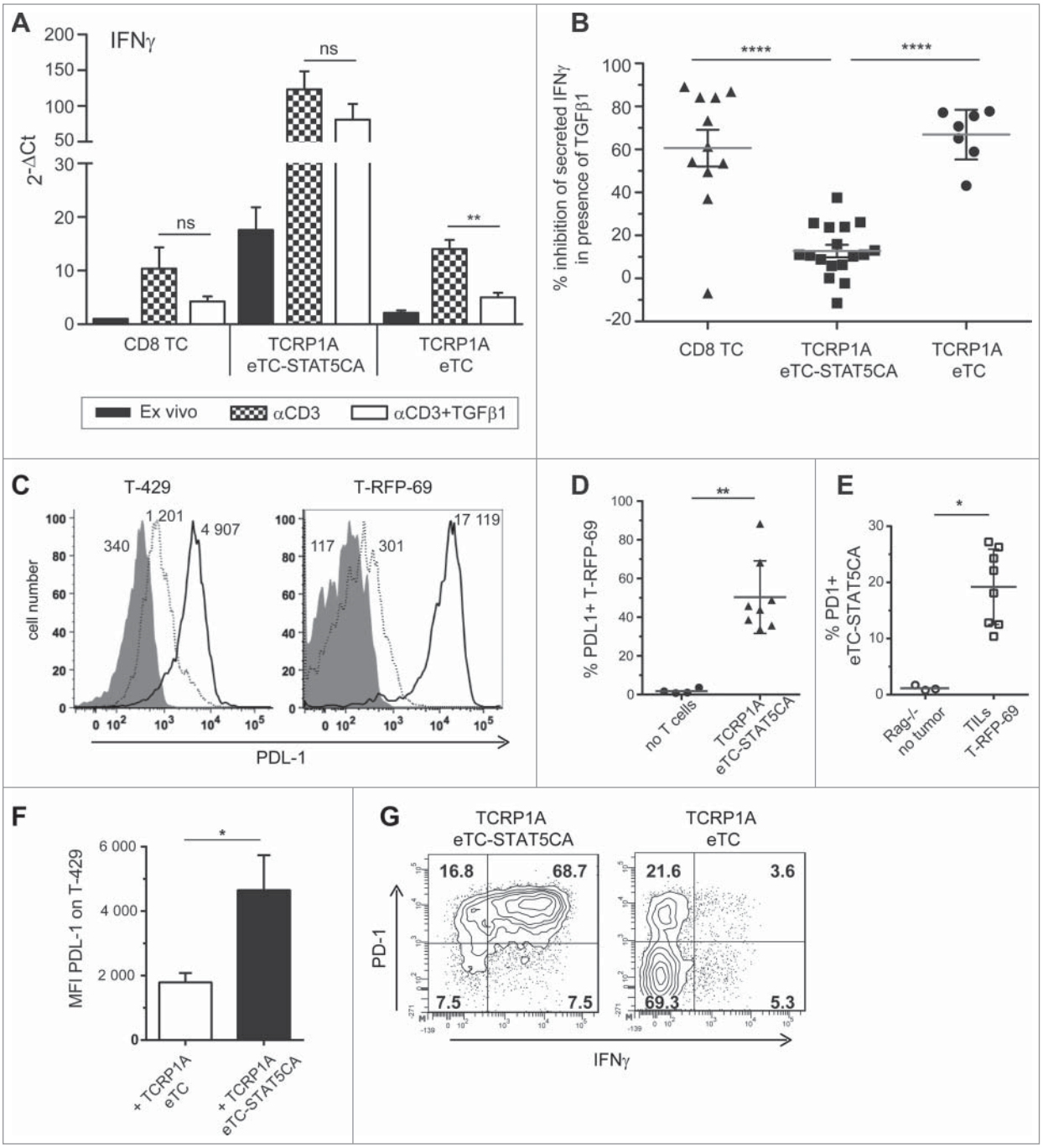

Figure 5. Activation of IFNγ production in TCRP1A eTCs-STAT5CA is insensitive to TGFβ1 and to PD-1 mediated immunosuppression. (**A and B**) As in Fig. 5, TCRP1A eTCs-STAT5CA or eTCs recovered from long-term injected Rag-1−/−B10.D2 mice (pooled lymph nodes and spleens) were stimulated 48 h with anti-CD3 in the absence or presence of TGFβ1. Naïve $CD8^+$ T cells stimulated by anti-CD3 mAb and soluble anti-CD28 mAb are included as control. IFNγ was measured at the mRNA (**A**) and protein (**B**) levels by qRT-PCR and Elisa, respectively. Data are representative of four independent experiments with 2 to 3 mice per group. (**C):** Expression of PDL-1 on untreated (thin line) or IFNγ-treated (bold line) T-429 or T-RFP-69 tumors. Staining with an isotype control (gray) is included and MFI is reported. (**D and E**) Rag-$1^{-/-}$B10.D2 mice inoculated s.c. with 10^6 T-RFP-69 tumor cells received 15 d later pre-activated TCRP1A eTC-STAT5CA (10^6). Day 20 post-transfer recovered tumors were analyzed for PDL-1 expression on RFP+ tumor cells (**D**; gating on $CD45^-$ cells) and for PD-1 expression on tumor-infiltrating $CD8^+$ T cells (**E**; gating on $CD45^+$ cells). (**F–G**) Rag-$1^{-/-}$B10.D2 mice inoculated s.c. with 10^6 T-429 tumor cells received 15 d later either pre-activated TCRP1A eTC or pre-activated TCRP1A eTC-STAT5CA (10^6 each). Day 12 post-transfer, recovered tumors were analyzed for PDL-1 expression on tumor cells (**F**; gating on $CD45^-$ cells). Tumor-infiltrating $CD8^+$ T cells (**G**; gating on $CD45^+$ cells) were restimulated 4 h with anti-CD3 bound to a PDL-1+ tumor and stained both for PD-1 and IFNγ.

found on T cells infiltrating the tumor but not on those present at distant lymphoid sites ([40] and **Fig. S6A–B**), suggesting that stable PD-1 expression requires constant TCR engagement. Cytokines that activate STAT5 can rescue PD-1 inhibition on recently activated T cells.[41] Given the high and stable PD-1 expression on TILs (Fig. **S6A–B**), it was of interest to evaluate whether STAT5 activation could also rescue TILs activation. Indeed, eTCs-STAT5CA did not differ from endogenous $CD8^+$ T cells for the expression level of PD-1 in MHC-I-positive T-429 tumors (**Fig. S6A**). However, a lower PD-1 expression was measured on eTCs-STAT5CA TCRP1A cells infiltrating MHC-I^{low} T-RFP-69 melanomas (**Fig. S6B**; see legend).

We further evaluated the level of PDL-1 expression on TiRP melanoma cells exposed or not to IFNγ. While both T-429 and T-RFP-69 cell lines expressed low basal levels of PDL-1, incubation with IFNγ triggered in a dose-dependent fashion (**Fig. S6C**), a mild (× 4.1) and high (× 56.8) PDL-1 upregulation on those cells, respectively, when compared to basal levels (**Fig. 5C**). Consistent with the production of IFNγ by eTCs-STAT5CA TCRP1A cells infiltrating T-RFP-69 melanomas (**Fig. 4C**), an upregulation of PDL-1 expression was also observed on T-RFP-69 cells from tumors infiltrated by those T cells (**Fig. 5D**). Furthermore, analysis of tumor-infiltrating eTCs-STAT5CA revealed an up-regulated cell surface expression of PD-1 in the T-RFP-69 tumor microenvironment (**Fig. 5E**). Those results suggested that eTCs-STAT5CA were resistant to PD-1 mediated inhibition. To evaluate the resistance of eTCs-STAT5CA as compared to control eTCs, we used TILs recovered from MHC-I sufficient T-429 tumors as those expressed high level of PD-1 (**Fig. S6A**). In this model the tumor cells express PDL-1 (**Fig. 5F**) and the TCRP1A TILs (either eTCs-STAT5CA or eTCs) express similar levels of PD-1 (**Fig. 5G**). After an *in vitro* restimulation by anti-CD3 bound to PDL-1^+ tumor cells, PD-1^+ eTCs-STAT5CA were indeed capable of producing IFNγ, while eTCs failed to do so (**Fig. 5G**).

Discussion

It was recently reported that regression of melanoma metastases was associated with the upregulation of genes involved in antigen presentation and in interferon-mediated responses. This was not observed in progressing melanoma metastases in the same patients.[42] Therefore, reversion of MHC-I expression defects in tumor cells can restore immunogenicity and contribute to T cell mediated rejection.

Using the TiRP melanoma mouse model, we here characterized a melanoma line, T-RFP-69 that is deficient for surface expression of MHC-I as detected by flow cytometry. We show that when infiltrated by IFNγ-secreting T cells this MHC-I deficient TiRP melanoma recovered surface MHC-I expression on some of the tumor cells. These results suggest that the T-RFP-69 melanoma might express levels of MHC-I sufficient to trigger initial activation of P1A-specific eTC-STAT5CA, the reactivity of these T cells being further amplified by the IFNγ-mediated increase in MHC-I expression. The requirement for TA-specific restimulation of eTC-STAT5CA was previously demonstrated by their inability to accumulate within a P1A-negative tumor and to induce its regression.[32] This was further confirmed here, even when these T cells were coinjected with a TA-negative tumor into a pouch. In the latter setting, we additionally provided evidence that TCRP1A eTCs could be stimulated by the T-RFP-69 tumor cells to perform a few cycles of division, suggesting that by bypassing the tumor stroma/architecture a low but significant anti-TA reactivity of the eTCs can be revealed even to MHC-I^{low} tumors. Of note, eTC-STAT5CA showed higher levels of surface TCR expression as compared to unmanipulated eTC (data not shown), which may enhance their reactivity toward tumors expressing low levels of TA/MHC-I complexes.

IFNγ was also shown to increase CCR5 expression on T cells[43] and we observed that eTC-STAT5CA have a $CCR5^{hi}$ phenotype (expression of CCR5 transcripts has been reported in[33] and validated at the protein level (data not shown)). Additionally, CCL5 was found to be produced at higher levels in tumor-bearing mice injected with eTC-STAT5CA. CCR5/CCL5 chemo-attraction might further amplify the accumulation of eTC-STAT5CA at inflammatory sites.

Intra-tumoral accumulation of Th-1 / Tc-1 TILs with preserved cytokine secretion is an important parameter in the reversion of the tumor escape variants to an immunogenic phenotype. In this respect, TGFβ1, a cytokine produced by a number of cancer cells, has been shown to exert its immunosuppressive activity in part by its capacity to inhibit both Th-1 / Tc-1 cytokine production and Tc-1 / NK cytolytic effector programs.[44,45] We here report that STAT5CA-expressing T cells are very efficient IFNγ producers in a TGFβ1 insensitive manner. The latter property may be linked to reduced expression of the TGFβRII by STAT5CA-expressing T cells[33] as well as to positive effects of IL-2R/STAT5 on IFNγ transcription counteracting the repressive effects of TGFβ1/Smad2-3 signaling.[38] This observation is reminiscent of the abrogation of TGFβ1-mediated signaling by IL-2[38] or by IL-15,[46] two STAT5 activating cytokines.

Antitumor responses are associated with chronic stimulation of tumor-specific T cells resulting in their acquisition of an exhausted phenotype, akin to the one found in chronic viral infections. This phenotype includes expression of a large panel of inhibitory receptors, among which PD-1. Antibodies blocking the PD-1 inhibitory receptor or its ligand PDL-1 are effective in therapy in patients with non–small-cell lung cancer, melanoma and renal-cell cancer.[47] Importantly, patients bearing PDL-1–negative tumors were unresponsive to this therapy[48] and a host response based on IFNγ production was detected in PDL-1–expressing tumors. Altogether, those results suggest a negative feedback loop whereby IFNγ secretion by tumor-infiltrating T cells up-regulates PDL-1, which in turn negatively regulates activation of PD-1^+ T cells. It should be noted that PDL-1 can be expressed by tumor cells or by tumor-infiltrating monocytes/myeloid suppressor cells. Consequently, the efficacy of treatments with anti-PD-1 or PDL-1 mAbs will be restrained by the tumor cell/tumor microenvironment PDL-1 status,[24] a constraint applying to both endogenous and adoptively transferred T cell responses. Nevertheless, we demonstrate here that expression of

STAT5CA in tumor-specific $CD8^+$ T cells renders them resistant to PD-1 mediated signaling.

As for various inhibitory receptors bearing ITIM/ITSIM motives in their cytoplasmic domains, ligation of PD-1 is thought to induce its phosphorylation and to increase its association with the SHP-1/2 phosphatases that in turn dampen TCR signaling by abrogation of the PI3K and ERK pathways. Reduced ERK activation upon PD-1 ligation can be overcome through cytokine receptor signaling, particularly cytokines that activate STAT5.[49] SHP-2 can also dampen phospho-STAT5 signaling, a mechanism suggested to occur in regulatory T cells from patients chronically infected with HCV.[50] Therefore the expression of the active form of STAT5CA, previously shown to present increased resistance to dephosphorylation,[51] may confer increased resistance to the action of phosphatases recruited to various inhibitory receptors. Attempts to conduct biochemical analyses on TILs failed due to the paucity of T cells and to the surrounding dying tumors, both precluding purification of high T cell numbers.

This study showed that two major immune checkpoints hampering the efficiencies of immunotherapies, namely immunosuppression by tumor-produced TGFβ1 and by immune-induced PD-1/PDL-1 engagement could be bypassed by adoptive therapy with $CD8^+$ T cells expressing STAT5CA. This was the case even for tumors presenting a reversible MHC-I deficiency, as production of IFNγ was correlated with increased expression of MHC-I within the tumor.

Further beneficial antitumor effects may result from the production of IFNγ such as (i) sensitization of tumor stroma cells to cytolytic T cells and their subsequent destruction[13,52]; and (ii) M1 polarization of the M2-type tumor-associated myeloid cells. The TGFβ1-resistant IFNγ production by the eTC-STAT5CA may be particularly relevant to provide the latter effect. Such reprogramming of macrophages[53] and myeloid derived cells[54] within mouse tumors was recently obtained with adoptively transferred $CD8^+$ T cells engineered to express IL-12, which occurred partly through IL-12-induced IFNγ production.

Materials and Methods

Mice

TiRP mice (Tyr-iRas-P1A-transgenic Ink4a/$Arf^{flox/flox}$)[30] were kept on the B10.D2 background. To generate TiRP mice expressing a Cre-inducible Red Fluorescence Protein (RFP), *ROSA-tdRFP* reporter KI mice,[34] $RFP^{flox/flox}$ mice, were first backcrossed to $Ink4a/Arf^{flox/flox}$ B10.D2 mice. Next, TiRP mice were crossed with $Ink4a/Arf^{flox/flox}$ $RFP^{flox/flox}$ mice and screened for presence of one allele of the TiRP transgene and one allele of the RFP^{flox} KI (designated as TiRP-RFP mice hereafter). Melanomas were induced in TiRP or TiRP-RFP mice as described.[30] Mice heterozygous for the $H\text{-}2L^d$/$P1A_{35\text{-}43}$-specific TCR-transgene (*TCRP1A*)[11] were kept on the Rag-$1^{-/-}$B10.D2 background. TCRP1A mice with ubiquitous luciferase expression (βactin-Luc × TCRP1A) were obtained as described.[32] Rag-$1^{-/-}$B10.D2 mice were also used. P14 TCR mice on the C57BL/6 (CD45.2) background[55] were also used. All these mice were bred in the CIML animal facility. Animal experiments respected French and European directives.

Cell preparation

T cells were prepared from lymph nodes (LN) or spleen using standard procedures. For solid tumor infiltrating leukocytes (TILs), tissues were cut in small pieces with the GentleMacs Dissociator (Miltenyi Biotech), incubated 40 min in medium containing Collagenase I (200 μg/mL) and DNAse 1 (16 μg/mL) before loading over Ficoll-Paque™.

T cell activation and retroviral infection was performed as described.[32] *In vitro* restimulation was conducted on the following cells: eTCs-STAT5CA or eTCs adoptively transferred in syngeneic Rag deficient mice recovered from recipients' pooled LNs and spleens; $CD8^+$ T cells from B10.D2 mice as control. Cells (10^6) were stimulated by coated anti-CD3 mAb (and soluble anti-CD28 mAb for naïve $CD8^+$ T cells). Cultures were conducted in the absence or presence of TGFβ1 (R&D Systems; 5 ng/mL) for 48 h. IFNγ in supernatants was measured by Elisa (AN18 and biotinylated R46A2 Abs and streptavidin-HRP (R&D Systems)).

Flow cytometry

Antibodies were from BD Biosciences, except anti-$H\text{-}2L^d$ reactive mAb (28.14.8) and its isotype control (both from eBioscience). Cells (10^6) were analyzed on a LSR2 cytometer (BD Biosciences). Data were analyzed using FlowJo (Treestar Inc., CA) or Diva (BD Biosciences) software. For Intracellular cytokine staining, $CD8^+$ T cells were stimulated *ex vivo* for 4 h in the presence of monensin (4μM) and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences).

Quantitative RT-PCR was performed as previously described[33]

Tumor transplantation

Melanoma cell lines T-429[26] and T-RFP-69 were established in culture from induced amelanotic melanomas. P1A-positive (P815 subline) mastocytoma P511 and its P1A-negative variant P1.204 obtained from Dr. B. Van den Eynde (Ludwig Institute for Cancer Research, Brussels) have been described in[32]. Tumor cells (10^6) were inoculated s.c. and TCRP1A T cells were adoptively transferred i.v. in mice bearing a solid tumor mass. B16F10 melanoma cells expressing the glycoprotein epitope amino acid 33–41 (B16-gp33), a kind gift from Dr. H. Pircher (University of Freiburg, Germany), were inoculated in C57BL/6 (CD45.1) mice that were adoptively transferred with $CD8^+$ T cells from P14 TCR mice as described.[56]

Biofluorescence and bioluminescence monitoring

The Berthold "Nightowl" instrumentation was used to monitor sequentially RFP fluorescence emission from tumor cells (550 nm excitation and 600 nm emission) and luminescence emission from transferred TCRP1A Luc^+ T cells as previously described.[11,32]

Immunohistology

Tumors were harvested and fixed in antigenfix (Diapath) for 3 h, then washed in phosphate buffer and dehydrated in 30% sucrose in phosphate buffer overnight. Cut 8 μm frozen sections were stained with the indicated antibodies: biotin- anti-H-2^d (34-1-2S) or control Ig, both from Biolegend, followed by streptavidin-Alexa-647; anti-CD3 mAb (17A2) + anti-rat IgG-Alexa-488).

Immunofluorescence confocal microscopy was performed with a Leica SP5 confocal microscope. Separate images were collected for each fluorochrome and overlaid to obtain multicolor images.

Multiplex analyses

Mice sera and tumor supernatants were assessed using ProcartaPlex mouse Cytokine & Chemokine Panel 26plex, mouse G-CSF/CSF-3 Simplex and mouse VEGF-A simplex (all from eBioscience; see supplemental Table 1 for complete analyte overview) according to the manufacturer's protocols. Assays were read on a MAGPIX® instrument (Millipore).

Statistical analyses

They were done with GraphPad Prism 6 software (La Jolla, CA, USA), unpaired t test, Two-tailed p: *, $p < 0.05$; **,$p < 0.01$; ***, $p < 0.001$. ****, $p < 0.0001$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the pub lisher's website.

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