Molecular profiling of CD8 T cells in autochthonous melanoma identifies Maf as driver of exhaustion

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Abstract

T cells infiltrating neoplasms express surface molecules typical of chronically virus-stimulated T cells, often termed “exhausted” T cells. We compared the transcriptome of “exhausted” CD8 T cells infiltrating autochthonous melanomas to those of naive and acutely stimulated CD8 T cells. Despite strong similarities between transcriptional signatures of tumor- and virus-induced exhausted CD8 T cells, notable differences appeared. Among transcriptional regulators, Nr4a2 and Maf were highly overexpressed in tumor-exhausted T cells and significantly upregulated in CD8 T cells from human melanoma metastases. Transduction of murine tumor-specific CD8 T cells to express Maf partially reproduced the transcriptional program associated with tumor-induced exhaustion. Upon adoptive transfer, the transduced cells showed normal homeostasis but failed to accumulate in tumor-bearing hosts and developed defective anti-tumor effector responses. We further identified TGFβ and IL-6 as main inducers of Maf expression in CD8 T cells and showed that Maf-deleted tumor-specific CD8 T cells were much more potent to restrain tumor growth in vivo. Therefore, the melanoma microenvironment contributes to skewing of CD8 T cell differentiation programs, in part by TGFβ/IL-6-mediated induction of Maf.

Keywords Maf; melanoma; T-cell exhaustion; TGFβ

Subject Categories Immunology; Molecular Biology of Disease

DOI 10.15252/embj.201490786 | Received 12 December 2014 | Revised 2 June 2015 | Accepted 8 June 2015

Introduction

The adaptive immune system plays a central, yet complex role in controlling tumor growth. It can suppress tumors by destroying cancer cells or inhibiting their growth. It can also promote tumor progression either by selecting for tumor cells that are more fit to survive an immune attack (Schreiber et al., 2011) or by establishing conditions within the tumor microenvironment that facilitate tumor outgrowth (DeNardo et al., 2009). Nevertheless, the presence of CD8 T cells associated with the expression of effector molecules, such as granzyme B (GZMB) or perforin, inside primary tumors has been correlated with a better prognosis for patients affected with a wide variety of cancers (Fridman et al., 2012). In spite of this, T cells infiltrating tumors often share features with exhausted T cells in chronic infection (Zajac et al., 1998). The molecular bases characterizing this state have been thoroughly detailed in previous studies using LCMV and other viral chronic infections (Day et al., 2006; Wherry et al., 2007). In tumors and chronic infections, CD8 T cells express inhibitory receptors including PD-1, Lag-3, and CTLA-4 (Ahmadzadeh et al., 2009; Baitsch et al., 2011). It is now well recognized that these receptors inhibit T-cell-mediated protection from chronic infections and tumors. In particular, blockade of PD-1 has been shown to restore function in exhausted CD8 T cells during chronic viral infection (Barber et al., 2006). Accordingly, the therapeutic use of “blocking” antibodies specific for CTLA-4, PD-1, and PD-L1 is increasingly useful for cancer patients (Topalian et al., 2012; Wolchok et al., 2013). This approach represents a breakthrough for the treatment of patients with various solid tumors (Drake et al., 2014). However, except for inhibitory receptors, mechanisms that regulate tumor-induced T-cell exhaustion are poorly documented (Baitsch et al., 2011). While several transcription factors (T-bet, Blimp-1, and Batf) have been shown to play a role in chronic infection (Shin et al., 2009; Angelosanto & Wherry, 2010), little is known concerning the establishment of T-cell exhaustion in cancer.

We developed a mouse model of induced melanoma based on conditional deletion of tumor suppressor genes with concomitant expression of a natural mouse tumor antigen (TIRP mice) (Huijbers et al., 2006). In this model, tumor-intrinsic factors control the development of aggressive tumors and their expression of an inflammatory/immunosuppressive program (Soudja et al., 2010; Wehbe et al.,...
Intra-tumor T cells expressed high levels of inhibitory receptors such as PD-1 and had poor capacity to produce IFNγ upon restimulation, suggesting that they were exhausted (Soudja et al., 2010; Auphan-Anezin et al., 2013). Taking advantage of this model, we here establish the gene expression signature associated with CD8 T-cell exhaustion during autochthonous melanoma development. We show that tumor- and virus-induced exhaustion share many features, with the expression of genes encoding molecules such as inhibitory receptors or particular transcription factors. Among the latter, Nrg4a2, encoding an orphan nuclear receptor, is highly expressed in both virus- and tumor-induced exhaustion, whereas Maf is highly overexpressed in tumor-exhausted CD8 T cells and only very weakly during chronic viral infection (Crawford et al., 2014). We confirm the overexpression for both genes in Melan-A-/MART-1-specific CD8 T cells isolated from tumor-infiltrated lymph nodes (TILN) from melanoma patients. Overexpression of Maf by retroviral transduction of CD8 T cells dampens their intra-tumor accumulation and anti-tumor activity, while overexpression of Nrg4a2 does not affect CD8 T-cell properties. Importantly, we show that Maf expression in anti-tumor CD8 T cells contributes to their polarization toward an exhausted phenotype. Finally, we show that TGFβ and IL-6 are capable of inducing Maf expression in CD8 T cells in vitro and that Maf-depleted tumor-specific CD8 T cells have heightened capacity to eliminate melanoma cells in vivo.

Results

T cells infiltrating autochthonous melanomas in TiRP mice are functionally exhausted and express characteristic phenotypic markers of exhaustion

We have previously shown that “aggressive” inflammatory melanomas that developed in TiRP mice were infiltrated by T cells lacking effector molecules and expressing high levels of PD-1, indicating an exhausted phenotype (Soudja et al., 2010; Auphan-Anezin et al., 2013). We further characterized phenotypically and functionally these tumor-infiltrated lymphocytes (TILs). CD8 T cells were isolated from tumors, tumor-draining lymph nodes (TDLN), or spleens of TiRP mice with induced melanomas. TILs expressed high levels of various inhibitory receptors (PD-1, LAG-3, GP49b) at their surface (Fig 1A). They expressed CD69, suggesting that these cells were still stimulated within the tumor microenvironment. CD44 was expressed at an intermediate level between the low level on naïve CD8 T cells and the high level on the fraction of splenic antigen-experienced CD8 T cells (Fig 1A). Ex vivo, both CD8 T cells from TDLN and TILs showed a weak level of GZMB compared to TILs from a tumor rejected after transfer of specific CD8 T cells (P511 mastocytoma, Fig 1B) (Shanker et al., 2007). After restimulation, a very low percentage of CD8 T cells from TILs produced IL-2 or IFNγ, while splenic CD8 T cells from the same melanoma-bearing mice showed efficient cytokine production (Fig 1B and C). The percentage of CD8 T cells producing IL-2 or IFNγ was similar whether they were taken from TDLN of melanoma-bearing or from LN of control mice. Altogether, these data confirm that CD8 TILs from the induced melanomas are exhausted.

Molecular characterization of TILs from autochthonous melanomas

To explore the molecular bases of tumor-induced exhaustion, we performed a transcriptomic analysis of CD8 T cells sorted by flow cytometry from autochthonous melanomas of TiRP mice. We compared their profile with the transcriptomic profile of sorted naïve CD44 low CD8 T cells and of activated TCR transgenic CD8 T cells (TCRP1A) obtained 4 days after infection with an adenovirus expressing P1A, the antigen recognized by the TCRP1A (see Materials and Methods). Using a cutoff of a two-fold change in comparison to naïve CD8 T cells with a statistically significant P-value ($P < 0.05$), 2,192 known genes were found upregulated in activated CD8 T cells versus 629 in CD8 TILs (hereafter also called exhausted T cells) and 1,630 were downregulated in activated CD8 T cells versus 656 in exhausted CD8 T cells (Fig 2A). More than half of the genes upregulated in activated CD8 TILs (330) were also upregulated at similar levels in activated CD8 T cells, while about a third of the genes downregulated in CD8 TILs were also downregulated in activated CD8 T cells (Fig 2A).

Exhaustion has been associated with high-level expression of inhibitory receptors that are normally transiently upregulated on effector T cells but are rapidly downregulated when the pathogen is cleared (Wherry et al., 2007). In our setting, we confirmed that exhausted CD8 T cells inside the melanomas expressed high levels of PD-1 (Pdcd1), LAG-3 (Lag3), Tim-3 (Havcr2), GP49b (Lilrb4), NKG2A (Kirc1), and CTLA-4 (Cila4) transcripts compared to naïve CD8 T cells (Supplementary Table S1). These transcripts were expressed at a higher (CTLA-4, GP49b), a similar (PD-1, NKG2A), or a lower (TIM-3, LAG-3) level in exhausted compared to activated CD8 T cells (Table 1, Supplementary Tables S1 and S2).

We also looked for key genes involved in CD8 T-cell differentiation. The transcription factor Eomesodermin (Eomes) transcript was upregulated to similar levels in exhausted and in activated CD8 T cells compared to naïve CD8 T cells (Supplementary Table S1). Transcripts of genes involved in cytolytic functions such as Gzma, Gzmb, and Gzmc were upregulated in both exhausted and activated conditions compared to the naïve condition, but with a higher level in activated CD8 T cells (Supplementary Table S1). For genes encoding cytokines, whereas the expression of Il10 transcripts was higher in exhausted compared to activated T cells (Table 1), both exhausted and activated CD8 T cells expressed similar levels of Il11 transcripts (Supplementary Table S1). Expression of Cd44 transcripts was much higher in activated compared to exhausted CD8 T cells (Supplementary Table S1). Compared to activated CD8 T cells, TILs did not upregulate Cd25 (Il2ra) expression, associated with strong TCR stimulation and IL-2 production (Verdeil et al., 2006), but still expressed high levels of Ccl9, Ccl3, or Ccl5 transcripts, whose expression is usually measured at early time points following TCR stimulation. This suggests that some pathways of stimulation persist in the TILs within the melanomas.

We then looked at genes specifically up- or downregulated in exhausted CD8 T cells compared to both naïve and activated CD8 T cells (Table 1, Supplementary Table S3). We studied the enrichment of GO terms associated with the genes from these two lists (Supplementary Table S4). The most represented group of genes with an upregulated expression consisted in “negative regulation of biological/cellular processes”, followed by “homeostatic process
and regulation of gene expression” (Fig 2B, Supplementary Table S4). Among the genes falling into the category of negative regulation, we found genes involved in the regulation of T-cell migration like Rgs1 and Rgs16 whose products negatively regulate chemokine receptor activation (Gibbons et al, 2011), genes encoding phosphatases like Ptpre and Dusp1, whose products regulate MAPK phosphorylation (Hammer et al, 2006; Zhang et al, 2009), and a gene (Tnfaip3) encoding an inhibitor of the NF-κB pathway (Giordano et al, 2014), as well as genes encoding transcription factors (Nr4a2, Nr4a3, Maf, Stat3, Bcl6; see Table 1, Supplementary Table S4). CD101, a surface molecule shown to inhibit TCR/CD3 phosphorylation in humans (Soares et al, 1998) and expressed on highly suppressive Tregs in mice (Fernandez et al, 2007), was also specifically expressed on exhausted CD8 T cells (Table 1, Fig 1A).

Figure 1. CD8 T cells infiltrated in autochthonous melanomas in TiRP mice are phenotypically and functionally exhausted.

A. TILs and splenocytes from TiRP mice with melanoma were analyzed by flow cytometry for the expression of the indicated molecules at the surface of TILs (blue line) or splenocytes from tumor-bearing (black line) or non-bearing (solid gray) mice. Mean of the percentage of positive TILs (blue bars, \( n = 6-10 \)) or CD8+ splenocytes from control mice (white bars, \( n = 6 \)) for the indicated molecules are shown.

B, C. TILs and splenocytes were restimulated using PMA/ionomycin (IFN\( \gamma \), IL-2) or left un-stimulated (GzmB), and labeled for the indicated molecules at the surface of TILs (blue line) or splenocytes from tumor-bearing (black line) or non-bearing (solid gray) mice. For GzmB labeling, TILs from P511 mastocytoma were used as a positive control (dotted line). (C) Mean of the percentage of CD8 T cells from lymph node (LN), spleen (S), or TILs (T) positive for the indicated molecules are shown in blue bars (\( n = 6-10 \)). White bars represent the mean of CD8 T cells from lymph nodes positive for the indicated markers from six control mice (ctLN). For GzmB labeling, TILs from P511 mastocytoma were used as a positive control (gray bar, *). Data information: Means were compared using Student’s t-test (two tailed comparison). *P < 0.05, **P < 0.01, ****P < 0.0001. Error bars display standard deviation.
Many genes that were downregulated during exhaustion are involved in metabolic processes, suggesting a metabolic arrest of T cells inside the tumor (Supplementary Table S4). In summary, the transcriptional signature of exhausted CD8 T cells showed high levels of inhibitory receptors, altered expression of transcription factors, and of numerous genes involved in negative regulation of biological processes including strong downregulation of genes involved in metabolic processes.

Exhausted T cells from autochthonous melanomas or from chronically LCMV-infected mice show strong similarities at the molecular level

Exhaustion of CD8 T cells has been thoroughly detailed in chronic virus infection (Shin et al., 2009; Wherry, 2011; Doering et al., 2012). Differences between the tumor environment and the environment created during chronic infection could have consequences on the transcriptional program of T cells present in these contexts. To answer this question, we performed a “Gene Set Enrichment Analysis” (Subramanian et al., 2005) using the set of genes characterizing the melanoma-exhausted T cells (e.g. genes with an increased or decreased expression in exhausted T cells from tumors compared to naive and to activated T cells) in comparison with the set of genes previously identified in virus-induced exhaustion [e.g. data obtained 30 days after initiation of a chronic infection using LCMV clone 13 compared to naive CD8 T cells (Doering et al., 2012)].

The analysis shows statistically significant (nominal P-value < 0.0001), concordant differences shared by the two CD8 T-cell states of exhaustion for both the overexpressed (Fig 2C, left) and under-expressed (Fig 2C, right) set of genes from the tumor-exhausted CD8 T cells. Principal component analysis (PCA) performed on those same samples confirmed these results (Supplementary Fig S1). Our data thus show that the molecular pattern seen in T-cell exhaustion is very similar whether it is induced by a chronic infection or by the melanoma microenvironment with the expression of transcripts coding for common inhibitory receptors, transcription factors, and molecules involved in signaling (genes indicated by an “a” in Table 1).
### Table 1. Genes overexpressed during autophagous tumor-induced exhaustion.

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After the analysis of the microarrays, fold change between exhausted (exh) and naive (naive) CD8 T cells and between exhausted and activated (act) CD8 T cells were calculated. Genes with a p-value lower than 0.05 (bold values) in both conditions and a log2 fold change > 1 in both conditions are shown (see Materials and Methods for p-value determination).

*Indicates genes commonly upregulated according to the GSEA analysis (Supplementary Table S3) in our study and the study from Doering et al (2012).
**Nr4a2 and Maf are overexpressed in both murine and human CD8 TILs**

One aim of our study was to determine potential transcriptional regulators favoring exhaustion establishment in TILs. We chose to focus our studies on the two transcriptional regulators with the highest fold increase in exhausted CD8 T cells compared to naive CD8 T cells, Nr4a2 and Maf (Table 1). While the former transcription factor was highly expressed in both virus- and tumor-induced exhaustion, Maf was highly overexpressed in tumor-exhausted CD8 T cells (Table 1) and only very weakly during chronic viral infection (Crawford et al., 2014). We validated our microarray data by measuring the level of expression of those two transcripts in CD8 T cells compared to naive T cells, with an average of a 25- and a four-fold increase compared to the median value obtained in naive T cells, for MAF and NR4A2, respectively. Blood-derived activated Melan-A-/MART-1-specific CD8 T cells showed an intermediate level of expression for MAF (seven-fold increase compared to naive T cells) and a level of NR4A2 expression that was similar to that of naive T cells. These results validate our transcriptomic data for CD8 in the spleen (17%) and was further raised in CD4 TILs (35.6%). MAF+ CD8 T cells were only found among TILs, ranging from 6 to 42% of the CD8 TILs (average of 22%).

We then determined whether our findings in a melanoma mouse model were applicable to humans. Therefore, we used RNA from sorted naive T cells (CD8⁺, CD45RA⁺, CCR7⁺, CD27⁺, CD28⁻) from healthy donors and from Melan-A-/MART-1-specific CD8 T cells isolated from the blood or from metastasized LNs of melanoma patients as previously described (Baitsch et al., 2011). We measured by real-time quantitative reverse transcription PCR (qRT-PCR) the relative levels of MAF and NR4A2 in those samples. For both genes, we found a significant increase in tumor-infiltrated CD8 T cells compared to naive T cells, with an average of a 25- and a four-fold increase compared to the median value obtained in naive T cells, for MAF and NR4A2, respectively. Blood-derived activated Melan-A-/MART-1-specific CD8 T cells showed an intermediate level of expression for MAF (seven-fold increase compared to naive T cells) and a level of NR4A2 expression that was similar to that of naive T cells. These results validate our transcriptomic data for CD8

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**Figure 3.** Maf and Nr4a2 are overexpressed in CD4 and CD8 TILs.

A-D (A) CD4 and CD8 T cells were sorted from tumors of TiRP mice (three independent samples). RNA levels for Maf and Nr4a2 from these cells (Exh) were compared to those from naive CD4 and CD8 T cells by qRT-PCR. CD8⁺ (C) or CD4⁺ (D) T cells from spleens of tumor-free mice (solid gray), and from spleens of tumor-bearing TiRP mice (black) and TILs (blue) were analyzed by flow cytometry for the expression of Maf. Data from several experiments (each dot represents one mouse) are recapitulated on the right panel, also indicating the percentage of positive cells after labeling with an isotype-matched mAb on TILs (Tiso). (B) Comparison by qRT-PCR of the levels of MAF and NR4A2 in human naive T cells isolated from PBMC and activated Melan-A-/MART-1-specific CD8 T cells from PBMC (PBMC) or tumor-infiltrated lymph nodes (TILN) of melanoma patients. Data were compared using Student’s t-test (two tailed comparison). *P < 0.05, **P < 0.01, ***P < 0.001. Error bars display standard deviation.
T cells and suggest that there is convergence of gene expression in both CD4 and CD8 TILs in mice and between human and murine CD8 TILs, at least for the expression of Nr4a2 and Maf.

**Overexpression of Maf dampens CD8 T-cell anti-tumor response**

We further focused our study on the effects of Nr4a2 and Maf expression in CD8 T cells. To test whether forced expression of these transcription factors in CD8 T cells would dampen their anti-tumor efficiency, we used a tumor model in which untransduced CD8 T cells were able to induce tumor regression. This was not the case in the TIRP mouse model (Grange et al., 2012). We therefore took advantage of a model using the P511 mastocytoma naturally expressing the P1A-encoded antigen. In this model, as few as 10⁴ naïve tumor-specific CD8 T cells were capable of inducing a strong but transient regression of the tumor burden (Shanker et al., 2007). We hypothesized that if our gene of interest played a role in the establishment/maintenance of a dysfunctional state in tumor-infiltrating CD8 T cells, their enforced expression would dampen the anti-tumor response. Rag⁻/⁻ B10.D2 mice were injected subcutaneously with P511 cells. One week later, pre-activated T cells expressing a transgenic TCR recognizing the P1A-encoded Ag (TCRP1A T cells) were adoptively transferred into the animals. Tumors reach their maximal size 7 days after T-cell transfer and generally shrink and disappear 15 days after transfer (Fig 4D; described in Shanker et al., 2007). We used TCRP1A T cells that are also transgenic for the luciferase encoding gene (TCRP1A-luc) to follow the accumulation of T cells inside the tumor. TCRP1A-luc cells were activated in vitro and transduced with retroviruses encoding Maf and gfp (maf), Nr4a2 and gfp (nr4a2), or gfp alone (mock) (Supplementary Fig S2A). After 3 days, T cells were FACS sorted according to their expression of GFP. GFP⁺ TCRP1A-luc T cells were transferred into tumor-bearing mice. We monitored by bioluminescence the infiltration of the tumors by the TCRP1A-luc T cells. Photon emission measured in the tumors showed no difference when TCRP1A T cells were transduced with nr4a2 compared to mock (Fig 4A, Supplementary Fig S2B). However, we detected a much lower signal for maf-transduced as compared to mock-transduced TCRP1A-luc T cells (Fig 4B, Supplementary Fig S2B). This suggests a strong decrease in intra-tumor accumulation of Maf-overexpressing CD8 T cells. This was confirmed by determining the proportion of GFP⁺ maf-transduced TCRP1A T cells at day 7, which was much lower in LN, spleen, and tumor compared to their mock-transduced counterparts (Fig 4C). The overall tumor regression induced by adoptively transferred T cells was also less pronounced for maf-transduced as compared to mock-transduced TCRP1A T cells, whereas nr4a2-transduced TCRP1A T cells were as effective as their control counterparts (Fig 4D). Tumor shrinking was delayed for 5 days when maf-transduced as compared to mock-transduced TCRP1A T cells were used in the adoptive transfer and the tumor never totally disappeared from the animal (Fig 4D).

To determine whether this negative effect was linked to an early defect in T-cell survival after transfer or to a defect occurring after tumor-induced reactivation, we mixed mock-transduced GFP⁺ CD45.1 TCRP1A with maf-transduced GFP⁺ CD45.2 TCRP1A T cells at a 1:1 ratio. We transferred these cells into Rag⁻/⁻ B10.D2 mice that were injected or not with P511 cells 7 days earlier. In tumor-free mice, the CD45.1/CD45.2 T-cell ratio was unchanged 7 days post-transfer (Fig 4E). This ruled out any difference in survival during homeostasis after adoptive transfer. In tumor-bearing mice, a four-fold ratio in favor of the mock-transduced TCRP1A T cells was observed both in the LN and inside the tumor (Fig 4E). In vitro, 2 days post-transduction of TCRP1A T cells with mock- or maf-retroviral constructs, we only detected a small decrease in cell proliferation and no effect on cell apoptosis determined by annexin V labeling (Fig 4F). These results differ from previous work reporting increased sensitivity to apoptosis in vitro for CD8 T cells from mice expressing maf as a transgene in both CD4 and CD8 T cells (Peng et al., 2009). This apparent discrepancy may stem from differences in the level of maf expression or the state of differentiation of the CD8 T cells at which maf was expressed. Altogether, the results presented here suggest that maf-expressing CD8 T cells developed a defective tumor-induced effector response as compared to control T cells and failed to accumulate in tumor-bearing hosts. Although maf overexpression may affect CD8 T-cell survival at later time points, no effect was observed up to 7 days after transfer in non-tumor-bearing mice.

**Overexpression of maf polarizes CD8 T-cell differentiation toward an exhausted phenotype**

To determine how Maf expression influences the T-cell anti-tumor response, we analyzed by flow cytometry TCRP1A T cells ex vivo (from LN, spleen, or TILs), 7 days after transfer into P511-bearing mice. Maf-transduced GFP⁺ CD8 T cells expressed a higher level of PD-1 and a lower level of GzmB compared to mock-transduced T cells (Fig 5A). To test if the functional capacities of these cells were further affected, we stimulated the cells from the tumor-draining LN with PMA/ionomycin ex vivo. Clearly, fewer maf-transduced than mock-transduced CD8 T cells produced IFNγ (9.7 compared to 23.7%) and IL-2 (0.83 compared to 4.1%) (Fig 5B). These data were confirmed in the experiment where we transferred mixed mock-transduced GFP⁺ CD45.1 with maf-transduced GFP⁺ CD45.2 TCRP1A T cells as described above (Fig 4E). Upon restimulation, the proportion of IL-2 and of IFNγ-producing T cells from tumor-draining LN was, respectively, three- and four-fold higher for CD45.1 mock-transduced as compared to CD45.2 maf-transduced TCRP1A T cells (Fig 5C). We failed to detect any GFP⁺ CD8 T cells among the maf-transduced CD45.2⁺ population at day 10 after transfer (Supplementary Fig S2D). This would suggest that the tumor elimination observed after transfer of these cells was driven by tumor-specific CD8 T cells that do not overexpress MAF.

To analyze the contribution of MAF to the expression of genes associated with T-cell exhaustion in our previous settings, we measured the level of transcripts from a panel of genes found overexpressed in exhausted CD8 T cells. It includes genes encoding molecules involved in transcriptional regulation (Nr4a2, Tnfαip3, Stat3, and Bcl6), inhibitory receptors (Pdcd1 (PD-1), Lilrb4 (GP49b)), Il10, and small G protein inhibitors (Rgs1, Rgs16). Mock- or maf-transduced TCRP1A T cells were sorted either 2 days after in vitro transduction or 7 days post-transfer ex vivo into tumor-bearing mice as previously described. In in vitro-sorted cells, we already detected higher levels of Il10, Rgs1, and Bcl6 transcripts in maf-transduced as compared to mock-transduced TCRP1A T cells (Fig 5D). On ex vivo-sorted TCRP1A T cells, the whole panel of genes was overexpressed with a 2- to 3-fold increase for Bcl6, Rgs1, Stat3, Pdcd1, and Lilrb4 and a higher increase for Nr4a2, Rgs16, and Il10 (Fig 5E). Together, these data show that high levels of MAF in CD8 T cells during an
anti-tumor response drive the development of CD8 T cells with an exhaustion-like transcriptional program.

**TGFβ and IL-6 induce Maf expression in CD8 T cells**

In CD4 T cells, Maf expression has been associated with Th2 (Ho et al., 1996), Th17 (Xu et al., 2009), TTr1 (Apetoh et al., 2010), and follicular helper T-cell (Tfh) (Crotty, 2011) differentiation in response to TCR signaling plus various stimuli including the presence of cytokines such as IL-4 (Kurata et al., 1999), IL-6 (Yang et al., 2005), TGFβ (Xu et al., 2009), or IL-27 (Apetoh et al., 2010). However, scant information exists on the control of Maf expression in CD8 T cells (Xu et al., 2009). In the TiRIP model, the tumor itself produces high levels of TGFβ, IL-6, and IL-10 (Soudja et al., 2010; Welbe et al., 2012). We tested if one of these cytokines could induce Maf expression during CD8 T-cell in vitro activation. We never
detected MAF by flow cytometry or qRT-PCR after antigenic stimulation of TCRp1A T cells (Supplementary Fig S3) or P14 T cells (Fig 6A and B) without additional cytokines. At day 3 post-activation, we observed a strong increase in Maf transcripts when TGFβ or IL-6 was present but no increase in the presence of IL-10 (Fig 6A and B; Supplementary Fig S3A). We confirmed those results by flow cytometry with the differentiation of a MAF+ population in the presence of TGFβ, IL-6, or a combination of both (from 5 to 20% of the CD8 T cells depending on the cytokine, see Fig 6A and B; Supplementary Fig S3B). We measured the level of a set of transcripts associated with exhaustion in our previous data in response to increasing amounts of TGFβ. TGFβ increased the expression of Maf, Il10, and Bcl6 and dampened the expression of Gzmb and Ifng (Supplementary Fig S3B and C). However, in contrast to the observations in the tumor-exhausted CD8 T cells, it also dampened Nr4a2 and Rgs1 expression. Expression of PD-1 was slightly increased both at the transcripts and at the protein level (Supplementary Fig S3B and C). Altogether, these data suggest that TGFβ and IL-6 in the tumor microenvironment contribute to the induction of Maf expression in exhausted CD8 T cells.

**Maf deletion in CD8 T cells diminishes the effects of TGFβ and IL-6 on their activation program**

To further characterize the importance of MAF expression during the response of CD8 T cells to antigenic stimulation, we generated...
TCR transgenic P14 mice (TCR specific for the LCMV gp33 peptide and H-2D\textsuperscript{b}) with selective deletion of Maf in mature T cells by crossing maf floxed/floxed mice (Wende et al., 2012) with P14 maT-Cre mice (Giordano et al., 2014) (P14 maT-Cre-maf fl/fl mice, Fig 6A and B). We evaluated the capacity of Maf-deficient CD8 T cells to respond to stimulation with GP33 peptide in vitro in the presence of TGF\textbeta\ or/and IL-6. When stimulated with peptide alone, we did not detect noticeable differences between WT and Maf-deficient CD8 T cells 3 days post-stimulation with regard to CD25 expression, cell number, number of cell divisions, or annexin V labeling (Fig 6C–F). Maf-deficient T cells were, however, producing more IL-2 (Fig 6G) and Ifng (Fig 6H) than their WT counterparts. Presence of 10 ng/ml TGF\textbeta\ in the culture medium dampened CD8 T-cell stimulation, affecting proliferation of the cells but not their survival (Fig 6E and F). All of the effector functions were decreased in this condition: production of IL-2, 

Figure 6. Presence of TGF\textbeta\ or/and IL-6 during CD8 T-cell activation induces maf expression.

A–H (A) CTV-labeled P14 T cells were activated in vitro with GP33 peptide (10\textsuperscript{-6} M) in the presence of TGF\textbeta\ (10 ng/ml) or/and IL-6 (10 ng/ml) for 72 h. CD8 T cells were labeled with the indicated Ab and analyzed by flow cytometry. Levels of MAF in P14 T cells from maT-Cre\textsuperscript{+} maf\textsuperscript{fl/fl} mice (gray) or from Cre\textsuperscript{−} littermates (black) (A) and their CTV profile from one representative experiment (C) or pooled percentages for the indicated molecule in P14 T cells from three independent experiments (B, D, F) are shown. P14 T-cell numbers were determined 72 h post-stimulation and normalized according to the peptide-only condition in three independent experiments (E). Supernatants harvested at the same time point were analyzed by ELISA for the presence of IL-2 (G). In (H), RNAs from the two types of P14 T cells were analyzed by qRT-PCR. Mean of relative expressions compared to beta-2-microglobulin (ΔC\textsubscript{T}) for the indicated genes and from three independent experiments is shown. Data were compared using Student’s t-test (two-tailed comparison). *P < 0.05; **P < 0.01. Error bars display standard deviation.
expression of Ifng and Gzmb (Fig 6G and H). On the opposite, we detected a significant increase in Il10 and Bcl6 expression (Fig 6H). In the Maf-deficient CD8 T cells, the proliferation was partially rescued as well as IL-2 production and CD25 expression at the cell surface (Fig 6C–E). Interestingly, the increased expression of Bcl6 and Il10 in WT P14 T cells in the presence of either TGFβ or IL-6 was not detectable in the Maf-deleted CD8 T cells (Fig 6H). The presence of 10 ng/ml IL-6 did not affect cell proliferation in either WT or Maf-deficient CD8 T cells. The number of recovered cells was, however, increased by two-fold for the Maf-deficient CD8 T cells (Fig 6E). This correlates with a decreased labeling with annexin V, suggesting a better survival of the Maf-deficient cells in the presence of IL-6 (Fig 6F). IL-6 induced the expression of Bcl6, but not Il10, abrogated the expression of Ifng, and partially decreased the expression of Gzmb in WT cells (Fig 6H). In Maf-deficient CD8 T cells, IL-6 failed to induce the expression of Bcl6, and Gzmb expression was partially restored. When IL-6 and TGFβ were combined, their effects on WT T cells were more dramatic with decreased proliferation, increased apoptosis, high levels of Bcl6 and Il10, and decreased levels of Ifng and IL-2 production (Fig 6C–H). Maf deletion strongly restored IL-2 production, cell survival, and proliferation (Fig 6C–G) and led to decreased Bcl6 and Il10 expression, but failed to restore significant levels of Ifng or Gzmb. Altogether these data show that the effects of TGFβ and IL-6 on CD8 T-cell activation are partially regulated by their induction of MAF in CD8 T cells.

**Maf deletion in tumor-specific CD8 T cells increases their capacity to eliminate melanoma cells in vivo**

We next evaluated the capacity of adoptively transferred Maf-deficient tumor-specific CD8 T cells to induce melanoma regression. Because of the C57BL/6 background of P14 maT-Cre mafrn/n mice, we had to use a different melanoma model. C57BL/6 (CD45.1) mice were injected s.c. with B16F10 melanoma cells expressing the LCMV GP33 epitope (B16-GP33) (Prevost-Blondel et al., 1998). One week later, WT or Maf-deficient in vitro pre-activated P14 T cells [on a C57BL/6 (CD45.2) background] were adoptively transferred into the mice. The presence of WT P14 T cells only moderately affected tumor growth in this model. However, Maf-deficient P14 T cells were much more effective and induced significantly stronger tumor regression (Fig 7A). One week post-transfer, we analyzed the endogenous CD45.1 CD8 T cells and the transferred CD45.2 P14 T cells from either P14 maT-Cre+ mafrn/n mice (maf-KO P14 CD8 T cells) or P14 maT-Cre+ mafrn/n littermates (WT P14 CD8 T cells). There was a slight increase in Maf-deficient P14 T cells among total CD8 T cells compared to WT P14 T cells in the tumor-draining LN. In TILs, we detected a much higher percentage of Maf-deficient P14 T cells, increasing the ratio of P14 CD8 T cells/endogenous CD8 T cells from 1:1 for WT P14 to 4:1 for Maf-deficient P14 T cells (Fig 7B and C). When tested for their capacity to produce cytokines upon gp33 peptide restimulation, maf-KO P14 T cells from the tumor-draining LN showed higher production of IFNγ, IL-2, and TNFα but contained similar amounts of granzyme B compared to WT P14 T cells (Fig 7D–F). In P14 T cells from TILs, we detected similar levels of granzyme B, IL-2, and TNFα but higher production of IFNγ in Maf-deficient cells (Fig 7D–F). At the cell surface from TILs, similar levels of CD44 but lower expression of the inhibitory receptors PD-1 and LAG-3 were detected on Maf-deficient compared to WT P14 T cells (Fig 7E and F).

In summary, the higher capacity of Maf-deficient tumor-specific CD8 T cells to accumulate inside the tumor and their higher potential for IFNγ production with lower surface expression of PD-1 correlated with increased capacity to eliminate tumor burden in vivo.

**Discussion**

We took advantage of an inducible melanoma in mice to characterize the gene expression signature associated with autochthonous tumor-induced exhaustion in CD8 T cells. We show a pattern of transcriptomic signatures common to virus- and tumor-induced exhaustion in CD8 T cells, sharing the main phenotypic features. These include the sustained expression of some inhibitory receptors (PD-1, CTLA-4, LAG-3, TIM-3, 2B4) (Blackburn et al., 2009) and of some transcription factors (EOMES) (Wherry, 2011), also found to be upregulated in melanoma-infiltrated human LNs (Baitsch et al., 2011). Despite this high level of conservation of gene expression, virus-induced exhaustion and tumor-induced exhaustion have some interesting differences. The transcript encoding BLIMP-1 (Prdm1), a transcriptional repressor previously described as important to induce exhaustion during chronic viral infection (Shin et al., 2009), was not upregulated in our setting. This is consistent with the upregulation of Bcl6 expression observed in the melanoma-exhausted, but not in virus-exhausted CD8 T cells (Table 1). Indeed, BLIMP-1 has been shown to downregulate Bcl6 expression in CD4 T cells (Johnston et al., 2012). It is also coherent with their low level expression of G2MB, as BCL6 has been shown to repress Gzmb expression (Yoshida et al., 2006). Quite surprisingly, we found that Foxp1, a gene recently described to regulate quiescence in TILs from ovarian and breast cancers in both human and mouse models (Stephen et al., 2014), was not overexpressed in TILs from our melanoma model. On the contrary, we found its expression to be downregulated compared to naïve or effector CD8 T cells, following the expression pattern observed in exhausted CD8 T cells obtained in chronic viral infection (Doering et al., 2012) as well as in metastasized lymph nodes of melanoma patients (Baitsch et al., 2011). This illustrates the potential diversity of T-cell dysfunctional states depending on the type of tumor and the necessity to fully characterize TILs in various cancer types and models.

CD8 T cells exhausted by viral infection or tumor exposure have common features with other dysfunctional T cells. Like exhaustion, anergy is characterized by the expression of the inhibitory receptors previously cited (Macian et al., 2002; Chikuma et al., 2009). However, as described for virus-induced exhaustion (Wherry et al., 2007), melanoma-exhausted T cells described here did not upregulate the expression of master transcription factors found in anergic T cells such as Egr2 and Egr3, or of molecules involved in the inhibition of TCR signaling like DGKα, CBL, or GRAIL (Macian et al., 2002). Repeated antigenic stimulation could also lead to senescence of T cells, a state characterized by the shortening of telomeres, phenotypic changes (expression of KLRG1 and PD-1), and defective functions (Crespo et al., 2013). We found higher levels of Klrk1 in exhausted T cells, but no increase in transcripts encoding Cdkn2a (P16), Cdkn1a (P21), or Tp53 (P53) that can be upregulated during
A combination of transcription factors is overexpressed in exhausted T cells and drives tumor-induced exhaustion in CD8 T cells. Marilyn Giordano et al

Figure 7. Inactivation of Maf in tumor-specific CD8 T cells increases their intra-tumoral accumulation, effector functions, and anti-tumor efficacy in vivo. (A) C57Bl/6 CD45.1+ mice were injected s.c. with 3 x 10^5 B16-GP33 melanoma cells. One week later, mice were adoptively transferred with 5 x 10^6 in vivo pre-activated CD45.2+ P14 T cells from maT-Cre+ maffl/fl mice (eight mice, grey) or from Cre-/- littermates (eight mice, black) or left untreated (four mice, dotted line). Tumor growth was assessed with a caliper every 2–3 days. Multiple t-test using the Sidak-Bonferroni method was used to compare tumor growth after transfer with Cre-/- maffl/fl mice or from Cre-/- littermates.

B–F In a similar experiment, mice were sacrificed on the 6th day after transfer of P14 T cells. Percentages of CD45.1+ and CD45.2+ CD8 T cells were determined by FACS analysis. Pooled data from four mice per condition (B) or one representative dot plot in LN or TILs (C) are shown. The same samples were restimulated for 45 h with GP33 peptide and labeled with the indicated Ab. Pooled data from four mice per group are shown (D). Surface expression of the indicated molecules is shown in (E), and pooled data showing the MFI from four mice per condition are shown in (F). Data were compared using Student's t-test (two-tailed comparison).

Data information: *P < 0.05, **P < 0.01. Error bars display standard deviation.

senescence (Crespo et al., 2013). Recent studies using chronic or acute LCMV infection showed that exhausted T cells maintained an exhausted phenotype after antigen withdrawal and re-expansion of the population (Schietinger et al., 2012; Utschneider et al., 2013). Altogether, those studies suggest that exhaustion of T cells is a stage of differentiation with a unique transcriptomic and epigenetic signature.

We think it is unlikely that only one pathway or transcription factor accounts for the entire exhaustion phenotype in the T cells, as a combination of transcription factors is overexpressed in exhausted T cells whether in our data or those from other studies. One of our objectives was to understand whether some key genes could control at least in part the stage of exhaustion. Ectopic expression of Nr4a2 has been shown to impart regulatory T-cell (Treg)-like suppressive activity on naïve CD4 T cells by inducing Foxp3 and by repressing cytokine production (Sekiya et al., 2013). However, overexpression of this molecule in tumor-specific CD8 T cells did not affect their anti-tumor response. Nr4a3, another member of the NR4A family shown to impact Treg differentiation (Sekiya et al., 2013), was also overexpressed in exhausted T cells. Perhaps the combination of high levels of these two proteins is necessary to affect CD8 T-cell responses. It is also possible that forced expression of Nr4a2 does impact CD8 T-cell differentiation, but not to the extent of affecting their anti-tumor response in our assay. In contrast, forced expression of Maf in anti-tumor CD8 T cells hampered their capacity to control tumor growth when adoptively transferred in tumor-bearing
mice. This was correlated with a defect of Maf-expressing CD8 T cells to accumulate in tumor-bearing mice as compared to control anti-tumor CD8 T cells, although their capacity to colonize the host in conditions of homeostasis was unaffected. Interestingly, forced expression of Maf in CD8 T cells partially reproduced the “exhaustion” gene expression signature, with increased expression of genes encoding transcriptional regulators (Nr4a2, Tnfaip3, Stat3, and Bcl6), inhibitory receptors ([Pdcd1 (PD-1), Llhrb4 (GP49b)], the anti-inflammatory cytokine Il10, and small G protein inhibitors (Rgs1, Rgs16), suggesting that Maf is a major driver of the exhausted phenotype.

The transcription factor MAF, which belongs to the Maf family of basic region and leucine zipper transcription factors, was first appreciated as an activator of cytokine loci, in particular IL-4, in CD4 T cells (Kim et al, 1999). It was recently found to act as a transcriptional repressor of IL-22 production in Th17 CD4 T cells, in a TGFβ-dependent manner (Rutz et al, 2011). Maf expression can also be regulated by IL-6 in CD4 T cells (Yang et al, 2005; Tsukamoto et al, 2015). Furthermore, in an exhaustive study of the regulatory network for Th17 cell specification, Maf was found to function mainly as a negative regulator, attenuating the expression of pro-inflammatory loci (e.g. Rora, Runx1, Il1r1, Ccr6, Tnf) and globally repressing genes in pathways regulated by other core transcription factors (Ciofani et al, 2012). In the same study, MAF was found to positively regulate a few loci, several linked to attenuating inflammation (e.g. Il9, Llf, Il10, Cita4). The latter two were also found upregulated in the CD8 TILs analyzed here.

It is intriguing to note that a pattern of gene expression observed in this study for tumor-exhausted CD8 T cells shares a signature of transcription factor expression characteristic of CD4 follicular helper T cells (Tfh), including the expression of Bcl6, Stat3, and Maf (Tangye et al, 2013), as well as high expression of PD-1 and low expression of Foxp1 (Wang et al, 2014). Bcl6 and Maf have also been shown to instruct the characteristic human Tfh differentiation program either directly by increasing expression of anti-inflammatory genes (Il10) or indirectly by favoring expression of transcription factors repressing effector differentiation programs (Bcl6), and potentially contributing to the increase in expression of inhibitory receptors such as PD-1. Together, our data identify novel pathways that drive the dysfunction of tumor-specific T cells, and suggest new opportunities for the development of treatments against cancer.

Materials and Methods

Mice and cell lines

TiRP-10B; Ink4a/Arf flox/flox mice, “TiRP mice,” were kept on a B10.D2 background and treated with 4OH-tamoxifen as previously described (Huijbers et al, 2006; Soudja et al, 2010). Mice heterozygous for the H-2L d/P1A35-43-specific TCR transgene (TCRP1A) (Shanker et al, 2007) were kept on the Rag-1−/− B10.D2 background. Luciferase-expressing TCRPA1A mice (TCRPA1A-luc) were generated as previously described (Grange et al, 2012). TCR transgenic P14 mice (TCR specific for the LCMV gp33 peptide and H-2D b) with selective deletion of maf in mature T cells were obtained by crossing maf floxed/floxed mice (Wende et al, 2012) with P14 mT-Cre mice (Giordano et al, 2014). Rag-1−/− B10.D2, C57BL/6 CD45.2, and C57BL/6 CD45.1 mice were also used. All these mice were bred in the CIML animal facility. Animal experiments respected French and European directives.

Cell preparation

CD8 T cells were prepared from LN or spleen of TCRPA1A Rag-1−/− B10.D2 mice or P14 C57Bl6 mice according to standard procedures. When prepared from immunocompetent B10.D2 mice, CD8 T cells were enriched using Mouse CD8 Negative Isolation Kit (Dynal, Invitrogen) according to the manufacturer’s instructions. For analysis of TILs, tumors were cut into small pieces and incubated with collagenase I for 45 min. Tumors were dispersed into a single-cell suspension and passed over Ficoll-PaqueTM solution (Amersham Biosciences AB).

CD8 T-cell activation and retroviral infections

Murine maf cDNA was amplified using the Stratagene Hercusell Kit (with the following primers: agcgcgtgacaagctctgggcaaatgga
Retroviral particles were produced (Agilent) using the following primers: tcggaaatataccaaagc/vector pMX-IRES-GFP after PCR amplification using PFUultra HF from ImaGenes GmbH. This cDNA was cloned into the retroviral cDNA vector encoding nr4a2. TCRP1A CD8 T cells were sorted by flow cytometry (CD8+, after infection, draining lymph nodes were recovered and activated Eynde (Ludwig Institute for Cancer Research, Brussels). Four days into pRc/CMV-3X-Flag-GFP vector (Pogenberg et al., 2006). FL

Flow cytometry

Antibodies were from BD Biosciences, except anti-GzmB mAb (Invitrogen) and anti-Maf mAb (eBiosciences). Cells (10^6) were analyzed on a LSR2 UV or a LSRS651 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 with ionomycin (400 ng/ml) and PMA (40 ng/ml) in the presence of Golgi stop (BD) and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences). For intracellular labeling with anti-Maf mAb, Fox3 permeabilization buffer (eBiosciences) was used according to the manufacturer’s protocol. Cells were labeled using Cell Trace Violet (life technologies) according to the manufacturer’s protocol.

Bioluminescence

The infiltration of the luciferase-expressing TCRP1A T cells was monitored by bioluminescence imaging. After i.p. luciferin (3 mg/mouse) injection, the mice were anesthetized in a chamber flushed with a mixture of isoflurane (4% in air) and placed in the NightOwl LB981 (Berthold Technologies) under continuous anaesthesia as previously described (Shanker et al., 2007).

Transcriptome analyses

Naïve CD8 T cells from lymph nodes of TCR transgenic TCRP1A Rag-1−/− B10.D2 mice were sorted by flow cytometry (CD8+, CD3+, CD4−, Topro3−). For AdP1At samples, naïve CD8 T cells from lymph nodes of TCR transgenic TCRP1A B10.D2 (CD45.2) were transferred into CD45.1 B10.D2 mice. One day after adoptive transfer, mice were infected intra-nodally with the adenoovirus AdP1At (coding for P1A, the antigen recognized by TCRP1A CD8 T cells) (Naslund et al., 2007), kindly provided by Benoît Van den Eynde (Ludwig Institute for Cancer Research, Brussels). Four days after infection, draining lymph nodes were recovered and activated TCRP1A CD8 T cells were sorted by flow cytometry (CD8+, CD45.2+, CD44+, Topro3+). For TILs samples, 2–3 melanoma tumors from TIRP mice were pooled. After centrifugation on Ficoll-Paque™ solution (Amersham Biosciences AB), CD8 T cells were sorted by flow cytometry (CD3+, CD8+, Topro3+). Tripletcs were used for naïve and AdP1At (activated) T cells. For TILs, T cells coming from four independent FACs sortings (with 2–3 pooled tumors each time) were used. In the TIRP model, the latency before tumor appearance is around 162 days with rapid tumor growth, once detected (Soudja et al., 2010). For our samples, latency time before tumor appearance ranged from 60 to 200 days. TILs were collected between 2 and 3 weeks after tumor detection. SuperAmp RNA amplification was performed according to Milltenyi Biotec’s undisclosed procedure, and amplified cdNA labeled with Cy3 was hybridized on Agilent Whole Mouse Genome Oligo Microarrays 8 × 60 K. Microarray data have been submitted to NCBI GEO database and are accessible with the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42824. All the data were normalized by quantiles. The statistical analysis for detecting differentially expressed genes was made using Linear Model for Microarray Data (LIMMA) based on an empirical Bayes model. Probes whose maximal gene expression value did not exceed 5 (log, scale) were removed. The remaining list contained 24,562 probes used for GSEA analysis. Genes with logFC > 1 (or < −1) and P-value < 0.05 were selected as regulated. All these selected genes were constituted as gene set for the subsequent GSEA analysis. To find GO terms that were overrepresented in exhausted T cells, we used GORilla (http://cbl-gorilla.cs.technion.ac.il/) (Eden et al., 2009). Gene Ontology terms were summarized and visualized by using REVIGO (http://revigo.irb.hr/) (Supek et al., 2011).

GSEA analysis

Data from GSE30431 were extracted from GEO. “CD8 D30 chronic” and “naïve” samples were selected. Data were normalized by RMA. 21,938 probes were taken into account. Fold changes between CD8 D30 chronic and “naïve” samples were calculated, and probes were ranked according to these ratios. All these selected genes were constituted as gene sets for the subsequent GSEA analysis (Subramanian et al., 2005).

Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated using RNeasy kit (Quiagen). RNA from sorted cell populations was reverse-transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). qRT-PCR was done by non-specific detection (Power SYBR®Green, Applied Biosystems) of cdNA. For each gene, primer pairs generated a single product and were amplified in a linear relationship with the housekeeping beta-2-microglobulin gene. Mouse primer list: beta2microglobulin (agactgatacaagcttcagc/gacggttc aataagttcttcagc); maf (aac atatcttcagccagg/ggtatgtcctacaagtcga); il-10 (ggttccagctcttgga/ acctgtccactgtctgct); bcl6 (aaatggccccacaaggagtctggaggattg/aagtctgggggcaaatggg); pd-l1 (tcctcgccctcgtcatac/accagcaagc cggttcaca); gzm-b (gacaa cactcctagtctg/gatagctctcccctccttg); ifng (caacacagaagggaaa/ggaac atctgagtcgct); rgs-1 (gcagaagctcttggtcggat/gtctcctcagcgaac); rgs-16 (ctccgtgagagg/aacgagcttctgtc); stat3 (gac atctcccaaggg/gttggggcagcacttc); gp49b (catgctcctagcatggg ca/ggttccataacggtc); a20 (ggaagagcagaagctcttctcctccgacc agg tca); nr4a2 (catcagacttgctgcatc/gtctcccaagctg).

Human naïve CD8 T cells and Melan-A-/MART-1-specific T cells were sorted and processed as described in Baitsh et al. (2011). Human primers list: GAPDH (aaatacgggctgct/ctggcactgggtgat); MAF (gtctgctcatcagcagctgctgatcgt); NR4A2 (tactggctagcctccttccagaccatc).
**Statistical analysis**

Sample means were compared using Student's t-test (two-tailed comparison) or multiple t-tests using the Sidak–Bonferroni method when multiple time points were compared. P-value is represented as following: $P < 0.05 \, (*)$, $P < 0.01 \, (**)$, $P < 0.001 \, (***)$, $P < 0.0001 \, (****)$. Error bars display standard deviation.

**Supplementary information** for this article is available online: http://emboj.embopress.org

**Acknowledgements**

We thank Thien Vu Manh for advice on data analyses, Francesco Imperatore and Pierre Perrin for technical help, Benoît Van den Eynde and Céline Powis de Tenbossche (Ludwig Institute for Cancer Research, Brussels) for reagents, Carmen Birchmeier (MDC, Berlin) and Sho Yamasaki (Kyushu University, Fukuoka), respectively, for c-Maflox/flox and for maT-Cre mice, Lee Leserman for suggestions on the manuscript, Toby Lawrence for his support, and the CIML imaging and animal facilities personnel for assistance. This work was supported by funding from INSERM and CNRS and by grants from the “Agence Nationale de la Recherche” (ANR “retour post-doctorants”: GV), the “Association pour la Recherche sur le Cancer” (ARC: AMSV), the Swiss National Science Foundation (CRSII3_141879; DS and GV) and “Institut National du Cancer” (INCa: AMSV). CH was supported by an Erasmus Fellowship from the European Commission.

**Author contributions**

NA-A, AMS-V, DS and GV contributed to the conception and design; MG, MB, LB and GV to the development of methodology; MC, CH, PB, CI, MB and GV to the acquisition of data; JM, AMS-V and GV to the analysis and interpretation of data (e.g. statistical analysis, biostatistics, computational analysis); NA-A, MS, DS, AMS-V and GV to the writing, review, and/or revision of the manuscript; MB and LV to technical or material support; and AMS-V and GV to the study supervision.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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