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 naïve 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

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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*,

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2012). Intra-tumor T cells expressed high levels of inhibitory receptors such as PD-1 and had poor capacity to produce IFN γ 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, *Nr4a2*, 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 *Nr4a2* 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 TGF β 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 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 IFN γ , 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 IFN γ 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 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 (*Klrc1*), and CTLA-4 (*Ctla4*) 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 *Ifng* 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 *Cd69*, *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

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 γ , 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, T⁺).

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.

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 (*Tnfrifp3*) encoding an inhibitor of the NF- κ B 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 2. Tumor-induced exhaustion of CD8 T cells in the TiRP model shows many similarities to virus-induced exhaustion.

After the analysis of the microarray data, fold changes between activated (Act) and naïve (N) CD8 T cells and between exhausted (Exh) and naïve CD8 T cells were calculated. Genes with a P -value lower than 0.05 and a $\log_2$ fold change > 1 (upregulated) or lower than -1 (downregulated) were selected.

- A** These lists of genes upregulated (top panel) or downregulated (bottom panel) were compared using a Venn diagram. Numbers indicate the number of genes in each category.
- B, C** Fold changes between exhausted and naïve CD8 T cells and between exhausted and activated CD8 T cells were calculated. Genes with a P -value lower than 0.05 in both conditions and a $\log_2$ fold change > 1 or lower than -1 in both conditions were selected. **(B)** The list of genes upregulated in exhausted CD8 T cells was used in GOrilla to determine the enrichment of GO terms in exhausted CD8 T cells (Supplementary Table S3) and in Revigo (see Materials and Methods) to represent the enriched GO categories. **(C)** The lists of genes upregulated or downregulated differentially in the tumor-exhausted as compared to acutely activated CD8 T cells were compared using GSEA to the transcriptomic data obtained in exhausted CD8 T cells in chronic infection (ratio Day 30; Doering *et al*, 2012).

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 naïve 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 naïve 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 autochthonous tumor-induced exhaustion.

Log ₂ FC	Exh/N	Exh/Act	Act/N	Log ₂ FC	Exh/N	Exh/Act	Act/N	Log ₂ FC	Exh/N	Exh/Act	Act/N
Cytoskeleton/Cell adhesion				Signaling				Miscellaneous			
Spp1	3.5	4.6	−1.0	Rgs1 ^a	10.2	6.9	3.3	Samd3 ^a	6.3	5.1	1.3
Ctnnd2	1.5	2.3	−0.8	Camk2n1 ^a	5.8	6.0	−0.2	Tmem50b	5.5	2.8	2.7
Krt77	1.5	1.8	−0.3	Ptpr ^a	5.3	5.1	0.2	F2r ^a	5.0	2.0	3.0
Ahnak	1.3	2.2	−0.9	Dusp1 ^a	5.0	5.7	−0.7	Ctss	5.1	3.9	1.2
Jam2	1.2	1.9	−0.7	Lphn1	4.7	5.5	−0.8	Ssty2	4.5	4.0	0.5
Krt80	1.2	1.8	−0.6	Rgs16 ^a	4.2	3.7	0.5	Ifit3 ^a	4.4	5.7	−1.3
Tpm3	1.1	1.4	−0.4	Il10	3.9	1.8	2.1	Dennd4a ^a	4.4	4.1	0.2
				Sh2d2a ^a	3.6	3.0	0.6	Gch1	4.3	5.7	−1.4
Chromatin/DNA Repair				Sfn	3.2	4.2	−1.0	Hba−a1 ^a	4.1	3.7	0.4
Hspa1a ^a	6.4	7.1	−0.7	Rab22a	1.9	1.9	−0.1	Tmem88	4.0	3.7	0.2
Pot1b ^a	3.6	3.2	0.4	Ptger1	1.7	2.1	−0.4	Padi2	3.9	3.0	0.9
Zfyve27	3.0	4.5	−1.5	Gkap1	1.7	2.1	−0.4	Prl8a8	3.8	5.0	−1.1
Phf21a	2.2	2.8	−0.6	Peg12	1.5	2.7	−1.1	Ern1 ^a	3.6	3.7	−0.2
Meg3	2.2	2.8	−0.7	Klk1b3	1.5	1.9	−0.4	Ier2 ^a	3.4	2.0	1.4
Mum1	1.6	3.4	−1.9	Klk13	1.1	2.1	−1.0	Phf20 l1	3.4	1.9	1.5
Suv420 h1	1.5	2.6	−1.1	Plekhh3 ^a	1.0	2.0	−1.0	C1qc ^a	3.3	4.0	−1.5
								Zfyve27	3.0	4.5	−0.7
Inhibitory receptors				Regulation of transcription				Spata20	2.9	4.6	−1.7
Lilrb4 ^a	10.4	5.4	5.0	Nr4a2 ^a	7.2	6.4	0.8	Eif1	2.6	3.7	−1.1
Ctla4 ^a	4.5	1.7	2.8	Maf	6.2	4.0	2.2	Mndal	2.5	1.3	1.2
Cd101	2.3	3.0	−0.8	Nr4a3 ^a	5.9	5.7	0.2	Nprl3	2.4	3.4	−1.0
				Tnfrsf3 ^a	4.2	6.8	−2.6	Fam172a	2.4	5.3	−2.9
Metabolism				Stat3 ^a	4.0	3.1	0.9	Mapre3	2.2	2.1	0.2
Trf	6.7	7.4	−0.7	Mxd4	3.9	3.2	0.6	Adm2	2.2	3.2	−1.0
Abcb1a ^a	3.5	3.6	−0.1	Zmiz2	3.9	2.7	1.2	Gvin1	2.0	1.4	0.6
Dnaja4 ^a	3.4	4.5	−1.0	Supt5 h	3.1	1.1	2.0	Chd2	1.9	2.1	−0.1
Ch25 h ^a	3.2	2.9	0.3	Fosb ^a	2.9	5.5	−2.6	Rnf212	1.9	3.0	−1.1
Adssl1	2.9	4.7	−1.8	Bcl6	2.5	2.2	0.4	Spink3	1.9	2.6	−0.7
Apoe	2.5	2.5	−0.1	Hipk2	2.3	2.5	−0.2	Tmem214	1.7	1.3	0.4
Dct	2.4	2.6	−0.2	Lefty1	2.2	2.8	−0.6	Asb11	1.7	2.7	−1.0
Mmp1a	2.3	2.9	−0.6	Skil ^a	1.8	2.5	−0.7	Cyp2a4	1.6	2.2	−0.6
Dhrs7c	2.2	2.4	−0.2	Atf4	1.7	1.8	−0.1	Kcna5	1.5	2.9	−1.4
Ccbl1	1.9	2.4	−0.5	Jun	1.6	4.1	−2.5	Best2	1.5	3.0	−1.5
Pde10a	1.8	1.6	0.2	Sp100	1.4	1.3	0.0	Mettl11b	1.5	2.5	−1.0
Fdft1	1.7	1.4	0.3	Mitf	1.2	1.6	−0.4	Sh3 bp4	1.5	1.9	−0.5
Gk2	1.6	2.0	−0.4					Slc29a4	1.5	2.8	−1.4
				Ubiquitination				NEAT1	1.4	2.2	−0.8
Surface receptor/ligand				Fbxo40	4.6	4.4	0.1	Adig	1.4	2.1	−0.7
Gp49a ^a	8.0	4.6	3.5	Otud7b	2.8	1.8	1.0	Muc15	1.4	3.2	−1.8
Cyslr2	3.8	4.1	−0.3					Birc7	1.3	2.5	−1.2
Adrb2 ^a	2.5	3.6	−1.1	Migration				Fut4	1.3	1.6	−0.3
Ifngr1 ^a	2.1	2.7	−0.6	Cxcl10 ^a	5.5	4.5	1.0	Crygs	1.1	1.8	−0.6
Cd69 ^a	2.1	3.5	−1.4	Ccl3 ^a	5.5	4.9	0.6	Ripply2	1.0	1.4	−0.4
Itgav ^a	2.0	2.0	0.0	Ccl5 ^a	3.9	5.1	−1.3				
Cd7	1.8	2.7	−0.8								
Clec1b	1.2	1.4	−0.3								

After the analysis of the microarrays, fold change between exhausted (exh) and naïve (naïve) 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).

^aIndicates 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 naïve 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 and CD4 TILs obtained from TiRP melanomas (Fig 3A). We evaluated by flow cytometry the level of MAF in CD4 and CD8 T cells in tumor-free or TiRP mice. In the spleens of tumor-free animals, we found very few MAF⁺ CD8 T cells (1.9%) and some MAF⁺ CD4 T cells (around 10%; Fig 3C and D). In TiRP mice that had developed a tumor, the percentage of MAF⁺ CD4 T cells was slightly increased

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 naïve 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 naïve T cells, with an average of a 25- and a four-fold increase compared to the median value obtained in naïve 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 naïve T cells) and a level of *NR4A2* expression that was similar to that of naïve T cells. These results validate our transcriptomic data for CD8

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 naïve 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 naïve 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 needed to use 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 10^4 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^{high} 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 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-to-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 IFN γ (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 IFN γ -producing TCRP1A 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*, *Tnfrsf3*, *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, those data show that high levels of MAF in CD8 T cells during an

Figure 4. Overexpression of *Maf*, but not *Nr4a2*, dampens the anti-tumor response by CD8 T cells.

TCR1A-luc CD8 T cells were activated and transduced *in vitro* with a retrovirus encoding either *Maf* (maf) or *Nr4a2* (nr4a2) coupled to GFP or with a construct coding for GFP only (mock). Two days after transduction, GFP-expressing CD8 T cells were FACS sorted and 5×10^4 of GFP⁺ TCR1A-luc CD8 T cells were injected in Rag^{-/-} B10.D2 mice, previously injected with 10^6 P511 mastocytoma (7 days before)

- A, B** P511 infiltration by TCR1A-luc T cells transduced with the indicated construct was monitored every 2–3 days by bioluminescence. Data were compared using multiple t-test using the Sidak-Bonferroni method. Three independent experiments with 3–4 mice per experiment.
- C** At day 7 post-transfer, percentages of TCR1A CD8 T cells among CD45⁺ cells (mock- or maf-transduced) from draining LN (DLN), contralateral LN (CTLN), spleen, and TILs from two pooled experiments were determined. Data were compared using Student's t-test (two-tailed comparison).
- D** Tumor growth was monitored every 2–3 days using a caliper. Data were compared using multiple t-test using the Sidak-Bonferroni method.
- E** After sorting, CD45.1 mock-transduced and CD45.2 maf-transduced TCR1A T cells were mixed at a 1/1 ratio and transferred in Rag^{-/-} B10.D2 mice (white or purple hatched bars) or Rag^{-/-} B10.D2 mice injected with 10^6 P511 mastocytoma 7 days earlier (black or purple bars). Mean percentage of CD45.1⁺ or CD45.2⁺ among CD8 T cells at day 7 post-transfer in the LN or in TILs (two experiments with five mice per condition) are shown. Data were compared using Student's t-test (two-tailed comparison).
- F** Cell trace violet (CTV)-labeled TCR1A CD8 T cells were activated and mock- or maf transduced. Two days after transduction, cells were labeled with annexin V. CTV profile (left panel) and level of expression of annexin V (right panel) of non-transduced (solid gray), mock-transduced (black), or maf-transduced (purple) GFP⁺ CD8 T cells are shown. Representative histograms out of 4 independent experiments.

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

anti-tumor response drive the development of CD8 T cells with an exhaustion-like transcriptional program.

TGF β 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), Tr1 (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), TGF β (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 TiRP model, the tumor itself produces high levels of TGF β , IL-6, and IL-10 (Soudja *et al*, 2010; Wehbe *et al*, 2012). We tested if one of these cytokines could induce *Maf* expression during CD8 T-cell *in vitro* activation. We never

Figure 5. Overexpression of *Maf* during an anti-tumor response polarizes CD8 T cells toward an exhausted phenotype.

A–E TCRP1A CD8 T cells were activated and transduced *in vitro* either with a retrovirus encoding *Maf* (*Maf*, purple) coupled to GFP or with a construct encoding GFP only (Mock, black). Two days after transduction, GFP-expressing CD8 T cells were FACS sorted (A, B, D, E) and injected (5×10^4) in *Rag*^{−/−} B10.D2 mice, previously inoculated with 10^6 P511 mastocytoma (7 days earlier) (A, B, E). At day 7 post-transfer, mice were sacrificed. Cells from tumor-draining LN (DLN), spleen, or TILs were directly labeled (A) or restimulated and labeled (B) for the indicated molecules and analyzed by flow cytometry. (B) Numbers indicate the average of positive cells from eight mice per condition from two independent experiments. (C) CD45.1 TCRP1A T cells were mock transduced (black), and CD45.2 TCRP1A T cells were *maf* transduced (gray). Transduced T cells were sorted for GFP expression 2 days later, mixed at a 1/1 ratio, and transferred in *Rag*^{−/−} B10.D2 mice injected with 10^6 P511 mastocytoma 7 days earlier. At day 7 post-transfer, cells from DLN were restimulated and labeled for flow cytometry analysis. Mean percentage of IL-2⁺ or IFN- γ ⁺ TCRP1A CD8 T cells is shown (two experiments with five mice per condition). (D, E) GFP⁺ mock- or *maf*-transduced TCRP1A cells were sorted 2 days after *in vitro* transduction (D) or 7 days post-transfer in tumor-bearing *Rag*^{−/−} B10.D2 mice (E) and used for qRT-PCR analysis. Ratio of transcript expression in *maf*-transduced versus mock-transduced GFP⁺ CD8 T cells is shown (pooled results from 3 (D) or 2 (E) independent experiments with 2 mice per condition). Data were compared using Student's *t*-test (two-tailed comparison). **P* < 0.05, ****P* < 0.001. Error bars display standard deviation.

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 TGF β 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 TGF β , 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 TGF β . TGF β 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 TGF β 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 TGF β 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

Figure 6. Presence of TGFβ 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^{-6} M) in the presence of TGFβ (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⁺ *maf*^{fl/fl} mice (gray) or from Cre[−] 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 (Δ Ct) 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.

TCR transgenic P14 mice (TCR specific for the LCMV gp33 peptide and H-2D^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 TGFβ 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 TGFβ 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;

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 TGF β 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 TGF β 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 TGF β 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 *maf*^{fl/fl} 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⁺ *maf*^{fl/fl} mice (*maf*-KO P14 CD8 T cells) or P14 maT-Cre[−] *maf*^{fl/fl} 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 IFN γ , IL-2, and TNF α 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 TNF α but higher production of IFN γ 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 IFN γ 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 several 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 GZMB, 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 DGK α , 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 *Klrp1* in exhausted T cells, but no increase in transcripts encoding *Cdkn2a* (P16), *Cdkn1a* (P21), or *Trp53* (P53) that can be upregulated during

Figure 7. Inactivation of *Maf* in tumor-specific CD8 T cells increases their intra-tumoral accumulation, effector functions, and anti-tumoral efficacy *in vivo*.

A C57Bl/6 CD45.1⁺ mice were injected s.c. with 3×10^5 B16-GP33 melanoma cells. One week later, mice were adoptively transferred with 5×10^6 *in vitro* pre-activated CD45.2⁺ P14 T cells from *maT-Cre*⁺ *maf*^{fl/fl} mice (eight mice, gray) 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*⁺ *maf*^{fl/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 6 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; Utzschneider *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*, *Tnfrsf3*, *Stat3*, and *Bcl6*), inhibitory receptors [*Pdcd1* (PD-1), *Lilrb4* (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 TGF β -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*, *Lif*, *Il10*, *Ctla4*). 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 and, in particular, their high expression of PD-1 (Kroenke *et al*, 2012). For Tfh, induction of this program is controlled by antigen stimulation in synergy with co-stimulation from CD28, CD40L, and ICOS, receptors of the SLAM family and cytokines (IL-21, IL-6, IL-12). In our study, induction of *Maf* and *Bcl6* transcripts was observed when naïve CD8 T cells were activated in the presence of TGF β or IL-6, conditions in which transcripts encoding the effector molecules GZMB and IFN γ were repressed. TGF β may also contribute to the low-level expression of GZMB and IFN γ in the intra-tumor CD8 T cells, given the direct repressive effect described for TGF β -mediated SMAD signaling on the *Gzmb* and *Ifng* promoters (Thomas & Massague, 2005). MAF has also been described to have a direct repressive effect on the differentiation of CD4 T cells into Th1 IFN γ -producing cells (Ho *et al*, 1998). Thus, TGF β may exert repression on the induction of Th1/TC1 effector programs both directly via SMADs and indirectly via its induction of *Maf*. Using *Maf*-deleted CD8 T cells, our data show the partial dependence on *Maf* of the TGF β inhibitory effects on naïve CD8 T-cell proliferation, IL-2 production, and *Gzmb* expression in response to antigen. They also show the complete dependence on *Maf* for TGF β -induced expression of *Bcl6* and *Il10* in the same setting. Importantly, deletion of *Maf* in adoptively transferred tumor-specific CD8 T-cell effectors led to their increased capacity to induce melanoma regression. This was correlated with their higher intra-tumoral abundance, with a higher

frequency of IFN γ -producing cells and a lower surface expression of the PD-1 receptor.

The effects of TGF β /IL-6 and MAF expression appear to diverge for the expression of genes such as *Nr4a2* and *Rgs1* that were found upregulated in exhausted CD8 T cells as well as upon *Maf* expression, but downregulated when CD8 T cells were activated in the presence of TGF β (Fig 6C). Other factors in the tumor microenvironment may contribute to these differences.

Whichever its mode of induction within the tumor microenvironment (TGF β , IL-6, other cytokines or receptor ligands), *Maf* induction in CD8 T cells plays a role in the dampening of anti-tumor T-cell responses. Altogether, our results suggest that TGF β /IL-6-induced MAF may be one of the transcriptional mechanisms diverting intra-tumor CD8 T cells from their cytolytic effector 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 TCRP1A mice (TCRP1A-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 maT-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 TCRP1A 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 (Dyna, 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 HerculesII Kit (with the following primers: acgcgtcgacaagcttcagaactggcaatgaa

caattcc/cgcggatccttagatcacatgaaaaattcgggagaggaagg) and cloned into pRc/CMV-3X-Flag-GFP vector (Pogenberg *et al*, 2014). FL cDNA vector encoding *nr4a2* (IRCKp5014E0514Q) was obtained from ImaGenes GmbH. This cDNA was cloned into the retroviral vector pMX-IRES-GFP after PCR amplification using PFUUltra HF (Agilent) using the following primers: tcggaatataccaaagc/ttttcttttgcggcgctctttgacgtgctgg. Retroviral particles were produced as previously described (Verdeil *et al*, 2006). TCRP1A T cells were activated for 72 h with 10^{-7} M of P1A₃₅₋₄₃ (LPYLGLWLVF) peptide. About 20 h after initial stimulation, CD8 T cells were retrovirally transduced as previously described (Verdeil *et al*, 2006). Cultures were then continued for another 48 h. P14 CD8 T cells were stimulated with GP33 peptide (KAVYNFATC— 10^{-6} M) for 48 h before adoptive transfer experiments and up to 72 h for *in vitro* analysis.

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 LSR2561 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, FoxP3 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 anaesthetization 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⁺, CD44⁻, Topro3⁻). For AdP1A 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-dermally with the adenovirus 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⁻). Triplicates 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 Miltenyi 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 (log₂ 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 house-keeping beta-2-microglobulin gene. Mouse primer list: *beta2microglobulin* (agactgatacatagcctgcag/gcagggtc aaatgaatcttcag); *maf* (aac atattccatggccagg/ggatggcttcagaaactggca); *il-10* (ggttgccaagccttatcgga/acctgctccactgccttgct); *bcl6* (aaaggccggacaccagttt/ccggaggcgattaaggttga); *pd-1* (tcctctggcctctgacatac/aaccagaaggc cggtttcaa); *gzmB* (gacaa cactcttgacgtg/gatgatctcccctgccttg); *ifng* (caacagcaaggcgaaaaa/ggac ca ctcgatgagctc); *rgs-1* (gcagtgtgtcagttctctgga/agtctcacaagccaac cag); *rgs-16* (cctgcgaggagtt caagaag/cagttctcgggtctcgtgat); *stat3* (gac attcccaaggaggaggc/tacggggcagcactacct); *gp49b* (catcgataccagtagtggg ca/gggttccaactgttcagctct); *a20* (ggaaaggcagaagaagctca/cttcctcaggacc agg tcag); *nr4a2* (cactacgggtgttcgcacttg/catcccaacagctaggcact).

Human naïve CD8 T cells and Melan-A-/MART-1-specific T cells were sorted and processed as described in Baitsch *et al* (2011). Human primers list: *GAPDH* (aaaatcaagtggggcgatg/atggcatggactgtg gtcac); *MAF* (gctggggcttccaaatgtg/ccctttacgtctgcgtttgat); *NR4A2* (taac tcggctgaagccatgc/cttgaggcgaggaccatcac).

Statistical analysis

Sample means were compared using Student's *t*-test (two-tailed comparison) or multiple *t*-test 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>

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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; MG, 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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