Walking the wire
Post-transcriptional regulation of T cell effector functions in health and disease
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Chapter 6

Critical role of post-transcriptional regulation for IFN-γ in tumor-infiltrating T cells

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Abstract
A hallmark of effective T cell responses against tumors is the production of Interferon gamma (IFN-γ). However, tumor-infiltrating T cells (TILs) gradually lose their capacity to produce IFN-γ and therefore fail to clear malignant cells. Dissecting the underlying mechanisms that block cytokine production is thus key for improving T cell products. Here we show that even though TILs maintain substantial expression levels of Ifng mRNA, post-transcriptional mechanisms impede the production of IFN-γ protein due to loss of mRNA stability. CD28 triggering but not PD1 blocking antibodies effectively restores the stability of Ifng mRNA. Intriguingly, TILs devoid of AU-rich elements within the 3’Untranslated Region maintain stabilized Ifng mRNA and produce more IFN-γ protein than wild type TILs. This sustained IFN-γ production translates into effective suppression of tumor outgrowth, which is almost exclusively mediated by direct effects of IFN-γ on the tumor cells. We therefore conclude that post-transcriptional mechanisms could be modulated to potentiate effective T cell therapies in cancer.
**Introduction**

Cytotoxic CD8⁺ T cells can be very potent in anti-tumoral therapies. In fact, more than 50% of the patients suffering from metastatic melanoma respond to T cell therapy with *ex vivo* expanded tumor-infiltrating T cells (TILs), of which 20% experience complete remission¹,².

A critical feature of CD8⁺ T cell responses is the release of effector molecules, and the pro-inflammatory cytokine interferon gamma (IFN-γ) is key herein. Deletion of the IFN-γ gene, and loss of the IFN-γ receptor signaling pathway resulted in spontaneous tumor development in mice, and in loss of tumor suppression³,⁴. A high IFN-γ-mediated gene signature has been associated with better survival for melanoma patients⁵. In addition, genetic screens revealed that modulating IFN-γ responses in tumors leads to loss of responsiveness to immunotherapies⁶, which is further emphasized by the fact that genetic variations of the interferon signaling pathway in humans correlate with cancer risk and survival⁷.

A major limitation of effective anti-tumor responses by TILs is the loss of effector function, i.e. the failure to produce effector molecules such as IFN-γ⁸,⁹,¹⁰. This occurs upon chronic exposure to antigen and to inhibitory molecules, upon glucose restriction, and upon increased fatty acid oxidation¹¹,¹²,¹³,¹⁴,¹⁵,¹⁶. However, whether these events fully explain the loss of effector function within the tumor microenvironment is yet to be determined.

Recently, the importance of post-transcriptional mechanisms for cytokine production has become appreciated¹⁷,¹⁸,¹⁹,²⁰. AU-rich elements (AREs) within the 3'Untranslated region of mRNAs play a critical role in determining the fate of mRNA¹⁷,¹⁸,¹⁹,²⁰. This encompasses the regulation of mRNA stability, subcellular localization of the mRNA, and translation efficiency. We recently found that these regulatory mechanisms differentially dictate the cytokine production of T cells²¹. The immediate production of IFN-γ mainly depends on rapid translation of pre-formed mRNA, whereas prolonged cytokine production relies on *de novo* transcription and increased mRNA stability²¹. Furthermore, limited *de novo* transcription and the lack of *Ifng* mRNA stabilization effectively restrict the magnitude and duration of IFN-γ production²². Whether and how post-transcriptional mechanisms govern the production of IFN-γ in T cells during an acute infection, and how this compares to cytokine production during chronic antigen exposure in tumors is not well understood.

Here, we discovered a hitherto unappreciated role of post-transcriptional regulation that restricts the production of IFN-γ by T cells within the tumor environment. Importantly, removing AREs from the *Ifng* locus was sufficient for TILs to retain the production of IFN-γ, and thus their capacity to suppress the tumor outgrowth. We therefore propose that adoptive T cell therapy could be potentiated by relieving IFN-γ from post-transcriptional control mechanisms.
Results

Germ-line deletion of AREs within the Ifng 3’UTR augments and prolongs protein production in T cells upon infection

We first interrogated how the 3’UTR of Ifng controls the protein production in activated T cells. To this end, we compared OT-I TCR transgenic T cells from wild type (WT) mice with those from mice that lack the ARE region within the Ifng 3’UTR (ARE-Del)\(^{23}\). FACS-sorted naive WT and heterozygous ARE-Del OT-I T cells were activated for 1 day with OVA\(_{257-264}\) peptide-loaded bone marrow-derived dendritic cells, and the production of IFN-γ was measured upon incubation with brefeldin A for the last 3h of culture, without the addition of exogenous peptide. The antigen threshold of 0.1nM peptide to drive detectable IFN-γ and TNF-α production was equal for ARE-Del and WT T cells (Fig 1A). However, at all peptide concentrations ARE-Del T cells produced markedly higher levels of IFN-γ protein, but not of TNF-α (Fig 1A, B). This difference in IFN-γ production between WT and ARE-Del T cells was maintained at day 3 post activation (Fig 1B). Thus, germ-line loss of AREs within the Ifng 3’UTR does not result in qualitative, but in quantitative differences in IFN-γ production.

We next compared the in vivo responsiveness of ARE-Del T cells to an acute infection with that of WT T cells. We transferred 1x10\(^3\) FACS-sorted naive WT or ARE-Del OT-I T cells into C\(^{57}\)BL/6J/Ly5.1 recipient mice and infected the mice the following day with 2.5x10\(^4\) Listeria monocytogenes expressing Ovalbumin (LM-OVA;\(^{24}\)). T cell expansion during the course of infection was similar between T cells derived from both genetic backgrounds, as judged from the percentage of WT and ARE-Del OT-I T cells in the peripheral blood (Fig 1C). The ex vivo production of IFN-γ in WT T cells from peripheral blood samples peaked 9 days after infection, as judged by incubation with only brefeldin A for 3h prior to analysis (Fig 1C, D). Interestingly, ARE-Del T cells produced maximal levels of IFN-γ already at day 6 post infection, and the mean fluorescence intensity levels of IFN-γ per transferred ARE-Del T cells were substantially higher at all time points measured (Fig 1C, D).

Memory T cells become rapidly reactivated upon secondary infection and produce massive amounts of cytokines\(^{25, 26}\). Indeed, more than 80±6% and 90±2% of WT and ARE-Del memory T cells, respectively, produced IFN-γ after 6h of reinfection with high dose LM-OVA (2.5x10\(^2\); Fig 1E). At 24h after reinfection, the ex vivo production of IFN-γ by WT T cells dropped by half to 36±20%, whereas 75±10% of ARE-Del T cells retained high IFN-γ levels (Fig 1E). In conclusion, whereas the antigen threshold of ARE-Del T cells equals that of WT T cells, ARE-Del T cells outcompete WT T cells in magnitude and duration of IFN-γ production.

Sustained IFN-γ production by tumor-infiltrating ARE-Del T cells

We next questioned whether ARE-Del T cells also responded more vigorously to tumor cells. We co-cultured Ovalbumin-expressing B16F10 melanoma cells (B16-OVA;\(^{27}\)) for 4h with ARE-Del and WT OT-I T cells that were activated and expanded in vitro\(^{22}\). Again, T cells from both genetic backgrounds produced the effector cytokines TNF-α, IL-2, and IFN-γ with a similar sensitivity (Fig 2A, Fig S1A). Nevertheless, the percentage of IFN-γ-producing ARE-Del T cells was significantly higher (Fig 2A).

To determine how T cells responded to established tumors in vivo, we injected 1x10\(^6\) in vitro activated and expanded WT or ARE Del OT-I T cells into B16-OVA tumor-bearing mice that were injected with 3x10\(^4\) cells subcutaneously 7-10 days earlier. 14 days post T cell
Figure 1: Germ-line deletion of AREs within the Ifng 3' UTR induces superior IFN-γ production

(A) Naive CD44<sup>low</sup> CD62L<sup>hi</sup> WT and ARE-Del OT-I T cells were co-cultured for 24h with OVA<sub>257-264</sub> peptide-loaded bone marrow derived dendritic cells (DCs) as indicated. For the last 3h, 1μg/ml brefeldin A (Brfa) was added prior to analysis of IFN-γ (left panel) and TNF-α (right panel) production by intracellular cytokine staining. (B) Representative dot plots of IFN-γ producing WT and ARE-Del T cells at day 1 (top), and day 3 (bottom) post activation as described in (A). (C) C57BL/6J/Ly5.1 mice received 1x10<sup>3</sup> naive WT, or ARE-Del OT-I T cells, and were infected the next day with 2x10<sup>4</sup> LM-OVA. The % (left), and the IFN-γ production (right) of transferred OT-I T cells was determined in peripheral blood samples after 3h incubation with Brfa by flow cytometry. (D) Representative IFN-γ stainings of WT (filled histogram) and ARE-Del (black line) T cells in peripheral blood at indicated time points. (E) Representative dot plots of mice rechallenged with 2x10<sup>5</sup> LM-OVA 35 days post primary infection. 6h and 24h post secondary infection, the IFN-γ production of splenic T cells was measured after 3h incubation with Brfa. [Unpaired student t-test; (A) n=3 mice per group; *p<0.05. (C) n=8 mice per group; **p<0.005; ****p<0.0001].
transfer, we analysed the phenotype and the effector function of tumor-infiltrating T cells (TILs). The percentage of WT and ARE-Del T cell infiltrates and their expression levels of CD44 and CD62L was equal (Fig 2B, C), and the percentage of TILs expressing the degranulation marker CD107a was not different between WT and ARE-Del TILs (Fig 2D).

Chronically activated T cells gradually lose their capacity to produce effector molecules\(^\text{28}\). In line with that, the TNF-α and IL-2 production of WT and ARE-Del TILs was undetectable (Fig 2D). Also the production of IFN-γ was limited with a mere 19±8% of the WT TILs producing detectable levels directly ex vivo (Fig 2E). In sharp contrast, 62±10% ARE-Del T cells produced IFN-γ (Fig 2E). The superior IFN-γ production of ARE-Del T cells was also evident from the IFN-γ production per cell, as measured by mean fluorescence intensity levels of the IFN-γ+ T cells (Fig 2F). Of note, the superior IFN-γ production by ARE-Del T cells was independent of the tumor size (Fig 2F). Furthermore, whereas the addition of

![Figure 2: ARE-Del T cells retain IFN-γ production within the tumor environment](image)

WT and ARE-Del OT-I T cells were activated for 20h with MEC.B7.SigOVA cells and subsequently cultured with rIL-2 for 4 days. T cells were then (A) co-cultured for 4h with indicated amounts of B16-OVA cells in the presence of BrfA, or (B-G) injected (1×10^6 WT or ARE-Del OT-I T cells) i.v. into C57BL/6J/Ly5.1 mice bearing B16-OVA tumors that had reached a size of ~8mm\(^3\). 14 days later, tumors were excised and analyzed for (B) percentage and (C) CD44 and CD62L expression of WT (gray histograms) and ARE-Del OT-I (black line) TILs. (D-F) Intracellular staining for CD107a, TNF-α, IL-2 (D), and IFN-γ (E-F) of WT and ARE-Del OT-I TILs was performed ex vivo after 4h incubation with BrfA monensin. Data were pooled from 3 (B-D) and 5 (F) independently performed experiments (mean ± SD), or representative of 3 (C) and 5 (E) independently performed experiments. (A) n=4; (B-E, G) n=9-12; [Unpaired student t-test; ns=non-significant; *p<0.05, ****p<0.0001]. (F) n=22 WT, 23 ARE-Del mice [Unpaired student t-test; ****p<0.0001]. (G) Spleen- and tumor-derived OT-I T cells were activated for 4h with 100nM OVA\(^{257–264}\) peptide or with PMA/ionomycin in the presence of BrfA, or were left untreated (-). Data were pooled from 3 independently performed experiments (n=10-12; mean ± SD). [Unpaired student t-test; ****p<0.0001]. For representative dot plots, see Suppl Fig 1B.
Post-transcriptional regulation in TILs

Exogenous OVA$_{257-264}$ peptide to spleen-derived T cells from tumor-bearing mice resulted in massive cytokine production, TILs were unresponsive to additional antigen (Fig 2G, Fig S1B). Only by bypassing the proximal TCR signaling with PMA/ionomycin resulted in potent IFN-γ production of WT TILs (Fig 2G, Fig S1B), implying that regulatory factors other than antigen loss caused the block of IFN-γ production in this model. In conclusion, AREs within the Ifng locus promote the loss of IFN-γ production in TILs through post-transcriptional repression.

**ARE-Del T cell therapy substantially delays the tumor outgrowth**

To determine whether ARE-Del T cells also had a higher therapeutic potential, we followed the tumor outgrowth in B16-OVA tumor bearing mice that were left untreated, or that received 1×10$^6$ WT, or ARE-Del OT-I T cells. Mice that did not receive T cell therapy reached the maximal acceptable tumor size of 1000mm$^3$ within 20 days post tumor injection (Fig 3A, B). As previously shown$^{27}$, T cell therapy with WT OT-I T cells significantly delayed the tumor outgrowth (Fig 3A), and it increased the 50% survival rate from 18 days to 25 days (p=0.0005; Fig 3B). Remarkably, T cell transfer with ARE-Del T cells substantially extended this therapeutic effect, increasing the 50% survival rate from 25 days to a striking 43 days when compared to T cell transfer with WT T cells (p=0.02; Fig 3A, B). Altogether, our data demonstrate that removal of AREs within the Ifng 3’UTR suffices to significantly potentiate the therapeutic effects of T cell therapy.

**Increased IFN-γ production by ARE-Del TILs alters the phenotype of macrophages**

IFN-γ can exert pleiotropic effects on the immune system and on the inflamed tissue$^{28, 30}$. To identify the mechanisms that ARE-Del T cells use to block the tumor outgrowth, we first analyzed the composition and functionality of lymphoid and myeloid tumor infiltrates. The absolute numbers of live CD45$^+$ cells were equal in tumors from mice treated with WT or ARE-Del T cells (Fig 4A). This was also reflected by the percentages of CD3$^+$ T cells, CD8$^+$ T cells, CD4$^+$ T cells, regulatory T cells, NK cells, and B cells found within the lymphoid infiltrates (Fig 4B). Furthermore, the endogenous CD8$^+$ T cell and NK cell infiltrates had a similar potential to produce IFN-γ or to express the degranulation marker CD107a (Fig S1C).

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Figure 3: Sustained IFN-γ production by ARE-Del TILs results in superior anti-tumor responses

(A) Tumor size and (B) survival of B16-OVA tumor-bearing mice that were treated with 2×10$^6$ WT OT-I, or 2×10$^6$ ARE-Del OT-I T cells, or left untreated (no T cells) at day 7 after tumor injection. Lack of survival was defined as death or tumor size >1000mm$^3$. Data represent 2 independently performed experiments. [n=7 mice/group; Gehan-Breslow-Wilcoxon test; p<0.0001].
To study the myeloid tumor infiltrates, we distinguished three different populations based on their forward scatter/side scatter profile and on the expression of CD11b, Ly6G/C and F4/80\(^{31}\). The percentage of tumor-infiltrating CD11b\(^{hi}\) Ly6G/C\(^{hi}\) neutrophils was equal in tumors treated with WT and ARE-Del T cells (Fig 4C), and their activation status did not change as judged from the expression levels of CD63 and ICAM-1 (CD54) (Fig S1D). Likewise, migrating CD11b\(^{hi}\) F4/80\(^{int}\) monocytes that represent the major source of macrophages in inflamed tissues\(^{31}\) did not change (Fig 4C, D). Interestingly, the percentage of fully differentiated CD11b\(^{hi}\) F4/80\(^{hi}\) macrophages consistently increased from 7±3% in mice treated with WT T cells to 12±4% in mice that received ARE-Del T cells (p=0.0057; Fig 4C, D). CD11b\(^{hi}\)F4/80\(^{hi}\) macrophages expressed higher MHC-I and MHC-II levels than the CD11b\(^{hi}\)F4/80\(^{int}\) monocytic fraction (Fig 4E). Treatment with ARE-Del T cells further enhanced the levels of MHC-II (Fig 4E). T cell therapy with ARE-Del cells also reduced the expression levels of the mannose receptor CD206 (Fig 4F), a marker that is associated with the anti-inflammatory phenotype of macrophages\(^{32}\). These findings thus indicate that the continuous IFN-\(\gamma\) production in tumors by ARE-Del T cells augments the numbers of tumor-associated macrophages with a pro-inflammatory phenotype.

**Continuous IFN-\(\gamma\) production by ARE-Del T cells directly affects the tumor outgrowth**

IFN-\(\gamma\) can also directly act on tumor cells\(^{30}\). Indeed, treating B16-OVA melanoma cells with recombinant IFN-\(\gamma\) (rIFN-\(\gamma\)) induced the expression of PD-L1, MHC-I and MHC-II, which was completely lost when the IFN-\(\gamma\) receptor 1 was deleted by CRISPR/Cas9 (IFN-\(\gamma\)-R\(^{-/-}\)) (Fig S1E, A).

Figure 4: ARE-Del TIL therapy promotes the pro-inflammatory profile of tumor-associated macrophages

(A) Absolute numbers of live CD45\(^{+}\) infiltrates in tumors from mice that had received WT OT-I (grey) or ARE-Del OT-I (white) T cells. (B) Percentage of CD3\(^{+}\) cells, CD3\(^{+}\)CD8\(^{+}\) OT-I T cells (CD8\(^{+}\)), CD3\(^{+}\)CD4\(^{+}\)FoxP3\(^{-}\) T cells (CD4\(^{+}\)), CD3\(^{+}\)FoxP3\(^{+}\) regulatory T cells (Treg), CD3 NK1.1\(^{+}\) cells (NK), and CD3 CD19\(^{+}\) B cells from CD45\(^{+}\) tumor-infiltrating population. (C-D) Percentage of CD11b\(^{hi}\) Ly6G/C\(^{hi}\), CD11b\(^{hi}\) F4/80\(^{int}\) and CD11b\(^{hi}\) F4/80\(^{hi}\) cells of CD45\(^{+}\) population, and (D) representative dot plot of CD11b\(^{hi}\) F4/80\(^{int}\) and CD11b\(^{hi}\) F4/80\(^{hi}\) cells. (E) MHC-I (left) and MHC-II (right) expression levels on tumor-infiltrating CD11b\(^{hi}\) monocytes and CD11b\(^{hi}\)F4/80\(^{hi}\) macrophages. (F) CD206 expression on CD11b\(^{hi}\) F4/80\(^{hi}\) tumor-infiltrating macrophages. Data were pooled from 3 independently performed experiments ± SD. [(A-E) n=12; (F) n=6-9 mice/group. [(C, F) Unpaired Student \(t\)-test; (E) One-way ANOVA with Tukey’s multiple comparison; ns=non-significant; *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001].
Similarly, high expression levels of PD-L1, MHC-I and MHC-II were found on tumor cells ex vivo after treatment with WT T cells, and these markers were further enhanced upon ARE-Del T cell therapy (Fig 5A). Moreover, B16-OVA cells cultured with rIFN-γ lost their capacity to expand (Fig 5B). This was at least in part due to a block in proliferation as determined with the cell trace dye CFSE (Fig 5C). As expected, IFN-γR-/- B16-OVA cells were refractory to rIFN-γ (Fig 5B, C).

We next determined the effect of IFN-γ in vivo on B16-OVA IFN-γR-/- and Cas9 control tumors in mice treated with WT or ARE-Del T cells. Again, the percentage of OT-I T cell infiltrates was equal, and ARE-Del T cells maintained their superior production of IFN-γ, whether the tumors expressed IFN-γR or not (Fig 5D, E). However, the therapeutic advantage of ARE-Del T cells over WT T cells was completely lost on IFN-γR-/- tumors (Fig 5F). In conclusion, relieving IFN-γ from post-transcriptional regulation boosts the therapeutic potential of T cell therapy predominately through direct effects of IFN-γ on the tumor cells.
IFN-γ production by ARE-Del T cells correlates with increased mRNA stability

Our data thus far demonstrate that AREs are instrumental for the loss of cytokine production in TILs. Of note, the critical role of post-transcriptional regulation is further emphasized by the discrepancy of Ifng mRNA levels and protein levels. Despite the loss of IFN-γ production, WT TILs expressed significantly higher levels of Ifng mRNA than spleen-derived OT-I T cells (Fig 6A). ARE-Del TILs expressed another 2-fold more Ifng mRNA than WT TILs (Fig 6A). Also human melanoma-specific TILs completely fail to produce IFN-γ protein upon activation⁹, ³³. Yet, gene expression data reveal that melanoma-specific TILs express 6.7 fold more IFNG mRNA than their peripheral blood-derived counterparts⁹.

To dissect the mechanisms that drive the continuous IFN-γ production in ARE-Del T cells we set up an in vitro co-culture system with tumor cells. When antigen-experienced T cells were exposed to B16-OVA tumor cells for 1 day, both WT and ARE-Del OT-I T cells potently produced IFN-γ (Fig 6B). However, re-exposure to freshly seeded B16-OVA cells for a second time showed a substantial reduction of the IFN-γ production of WT T cells (from 75±13% to 51±6%), and this response was almost completely lost after a third exposure to B16-OVA cells (9±6%; Fig 6B). In contrast, ARE-Del T cells maintained their reactivity for an extended period, and 57±15% of the T cells retained their IFN-γ production at day 3 (Fig 6B). Intriguingly, irrespective of the loss of cytokine production in WT T cells, the Ifng mRNA levels of WT and ARE-DEL T cells were indistinguishable at day 1 and 2 of co-culture with

Figure 6: Superior IFN-γ production by ARE-Del TILs correlates with increased mRNA stability

(A) Ifng mRNA expression of FACS-sorted spleen- and tumor-derived OT-I T cells (pooled from 2-4 mice), 14 days after i.v. T cell transfer in B16-OVA tumor bearing mice. Data were pooled from 4 independently performed experiments (mean ± SD; n=9 mice/group). [One-way ANOVA with Tukey's multiple comparison; *p<0.05; ****p<0.0001]. (B-C) WT and ARE-Del OT-I T cells were co-cultured with B16-OVA cells at a 6:1 effector:target (E:T) ratio for indicated time. B16-OVA were refreshed daily. (B) Intracellular IFN-γ and TNF-α staining was performed at day 1 to 3 after adding BrfA during the last hour of culture. (C) Ifng mRNA expression was analyzed by RT-PCR. Representative dot plot (B) and pooled data ± SD (C) from 4 independently performed experiments. [Unpaired Student t-test; *p<0.05] (D-E) Ifng mRNA decay of resting T cells, and of T cells co-cultured in vitro with B16-OVA cells for 1 or 3 days (D), or Ifng mRNA decay of in vivo tumor derived WT and ARE-Del TILs (E) measured by adding 1μg/ml ActD for indicated time points. Presented data are pooled from 4 (n=5; D) and 2 (n=5; E) independently performed experiments (mean ± SD). [Unpaired Student t-test; *p<0.05; **p<0.005; ***p<0.0005]
B16-OVA cells (Fig 6C). At day 3, however, ARE-Del T cells maintained significantly higher levels of Ifng mRNA compared to WT T cells (Fig 6C).

We next determined whether the increased mRNA levels were due to stabilization of Ifng mRNA in ARE-Del T cells. Resting T cells have a $t_{1/2} = \sim 30$ min prior to exposure to tumor cells, as determined by blocking de novo transcription with Actinomycin D (Fig 6D). When cultured with tumor cells for 1 day, T cells substantially increased the stability of Ifng mRNA in both ARE-Del and WT T cells to $t_{1/2} > 2$ h (Fig 6D). However, whereas WT T cells lost mRNA stability by day 3 of co-culture with tumor cells ($t_{1/2} = \sim 1$ h), ARE-Del T cells maintained stable Ifng mRNA throughout the entire co-culture ($t_{1/2} > 2$ h; Fig 6D). Importantly, this disparity of Ifng mRNA turn-over rates was also found in FACS-sorted TILs from B16-OVA tumor bearing mice that displayed a $t_{1/2} = \sim 30$ min for WT TILs as opposed to $t_{1/2} > 1$ h for ARE-Del TILs (Fig 6E). Thus, stabilized mRNA and consequently elevated Ifng mRNA levels potentially promote the superior and prolonged cytokine production by ARE-Del TILs within the tumor environment.

### CD28 costimulation but not PD-1 blockade restores IFN-γ production through mRNA stabilization

We next sought to identify signals that support the stabilization of Ifng mRNA in T cells. Programmed death 1 (PD-1) and Lymphocyte-activation gene 3 (Lag-3) are two exhaustion markers that are highly expressed on T cells upon chronic antigen exposure (Fig 7A, Fig S2A, B). Blocking PD-1 can effectively reinvigorate T cell responses against tumors34, 35. Indeed, co-culturing T cells for 3 days with B16-OVA tumors in combination with αPD-1 significantly increased the production of IFN-γ of WT T cells (from 9±5% to 24±13%; p=0.03), and of ARE-Del T cells (from 66±7% to 79±8%; p=0.04. Fig 7B; Fig S2C).

Recent studies showed that PD-1 blocks T cell function by interfering with CD28 signaling36, 37. In line with that, CD80/CD86 blockade annihilated the increased IFN-γ production of PD-1 blockade, and reduced the levels of IFN-γ back to 8±4% WT and 66±13% ARE-Del T cells (Fig 7B; Fig S2C). Interestingly, providing CD28 costimulation during T cell culture with tumor cells did not change the IFN-γ production of ARE-Del T cells when compared to non-treated T cells (p=0.1). In sharp contrast, CD28 costimulation significantly restored the responsiveness of WT T cells (from 9±5% to 20±11%; p=0.04; Fig 7B; Fig S2C) to levels that were similar to the one’s reached with PD-1 blockade (24±13%). Moreover, combining PD-1 blockade with CD28 costimulation further augmented the production of IFN-γ by WT T cells when compared to single treatments (34±15%; p=0.03 compared to aPD-1; p=0.006 compared to αCD28; Fig 7B; Fig S2C).

Because both CD28 stimulation and PD-1 blockade effectively increased the IFN-γ production in WT T cells, but only PD-1 blockade acted on ARE-Del T cells, it suggested to us that these two pathways may employ different mechanisms. We therefore determined the effect of PD-1 blockade and/or CD28 costimulation on the stability of Ifng mRNA. As expected, irrespective of the antibody treatment, the Ifng mRNA in ARE-Del T cells was always stabilized (Fig S2D). However, despite significant increases in the IFN-γ protein production, PD-1 blockade in WT T cells did not increase the Ifng mRNA levels, or its stability (Fig S2E, Fig 7C). mRNA levels and stability were identical between PD-1 blockade alone or in combination with αCD80/86 (Fig S2E, Fig 7C). In sharp contrast, however, CD28 costimulation effectively stabilized Ifng mRNA in WT T cells (Fig 7C). PD-1 blockade could
further potentiate the \textit{Ifng} mRNA stabilization when combined with CD28 costimulation, which was also concomitant with increased \textit{Ifng} mRNA levels and IFN-\( \gamma \) protein production (Fig 7C, Fig S2C, E). Combined, whereas CD28 signaling and PD-1 blockade primarily govern IFN-\( \gamma \) production through different pathways, they collaborate in restoring the production of IFN-\( \gamma \) in tumor-exposed T cells.

Altogether, our study reveals that post-transcriptional regulation blocks the production of IFN-\( \gamma \) in TILs, and that loss of this regulatory mechanism can greatly improve the therapeutic effect of T cell therapy.

\textbf{Figure 7: CD28 triggering restores \textit{Ifng} mRNA stability of WT TILs}

(A) Representative dot plot of PD-1 and LAG-3 expression of endogenous CD8\(^+\) TILs, WT and ARE-Del OT-I TILs analyzed directly \textit{ex vivo} 14 days after T cell transfer in B16-OVA tumor bearing mice. For pooled data see Suppl Figure 2A. (B-C) WT and ARE-Del OT-I T cells were co-cultured \textit{in vitro} with B16-OVA cells as described above for 3 days. When indicated, T cells were daily treated with 10\( \mu \)g/ml anti-PD1 blocking antibody, with 10\( \mu \)g/ml anti-CD28 antibody, with 10\( \mu \)g/ml anti-CD80 and 10\( \mu \)g/ml anti-CD86 blocking antibodies, or a combination thereof. (B) IFN-\( \gamma \) protein production was assessed by flow cytometry upon addition of BrfA during the last hour of culture. For pooled data see Suppl Figure 2C. (C) \textit{Ifng} mRNA decay was measured by adding 1\( \mu \)g/ml ActD for indicated time points (n=5). Representative dot plots (B) or data pooled ± SD (C) from 4 independently performed experiments.
Discussion

The production of IFN-γ by TILs is critical for effective anti-tumoral responses. Chronic antigen exposure and immunosuppressive signals within the tumor environment, however, impede the effector function of TILs. Here we show that the loss of IFN-γ production is linked to rapid *Ifng* mRNA decay that is mediated by AREs located within its 3'UTR.

Despite the epigenetic changes that arise early during T cell activation, tumor-specific TILs maintain higher levels of *Ifng* mRNA. As human melanoma-specific TILs alike, TILs isolated from B16-OVA tumors expressed higher levels of *Ifng* mRNA than blood- or spleen-derived T cells of the same antigen specificity (Fig 6). Yet, TILs fail to translate the mRNA into protein. Even though nutritional and metabolic restrictions within the tumor environment can block protein production, we show here that the mere removal of ARE sequences from the *Ifng* 3'UTR is sufficient to bypass this inhibitory state. Thus, ARE-dependent mRNA stabilization is one of the mechanisms that ensures effective IFN-γ production by TILs.

Which signals interfere with the stability of *Ifng* mRNA in TILs is yet to be determined. We previously showed that PKC signaling is critical for the stabilization of *Ifng* mRNA in activated T cells. CD28 costimulation that can stabilize cytokine mRNA also amplifies PKCθ signaling in T cells. In line with that, CD28 costimulatory signals in tumor-exposed T cells effectively stabilized *Ifng* mRNA and restored IFN-γ protein production. The recovery of protein production upon PD-1 blockade was similar to that of CD28 costimulation. However, in line with recent studies that found no changes in *Ifng* mRNA levels and/or epigenetic signature upon PD-1 blockade, this treatment also failed to stabilize *Ifng* mRNA in dysfunctional T cells. These findings were unexpected because PD-1 signaling recruits SHP-1/2 and dephosphorylates Zap70, which in turn inactivates PKC signaling. Interestingly, PD-1 signaling recruits SHP2 also to CD28 and thus blocks T cell function by inactivating CD28 signaling. The efficacy of anti PD-1 therapy may therefore depend on the levels of actual CD28 signaling induced. That CD28 signaling stabilizes *Ifng* mRNA while PD-1 blockade fails to do so could therefore result from different signal strengths from these two antibody treatments. Alternatively, PD-1 blockade may also act on signaling pathways other than those involved in mRNA stabilization, which may or may not become engaged by CD28 costimulation. Because mRNA levels and stability do not change upon PD-1 blockade but the IFN-γ protein production resembles that of CD28 costimulation, it is tempting to speculate that the observed IFN-γ protein production upon PD-1 blockade is mediated through increased translation efficiency.

In conclusion, post-transcriptional regulatory mechanisms impede the production of IFN-γ by TILs. Of note, removing AREs in only one *Ifng* allele in the transferred T cells as we did in this study is sufficient to restore the production of IFN-γ to levels that effectively delay the tumor outgrowth. Interfering with the instability of *Ifng* mRNA could thus significantly potentiate the efficacy of T cell therapy against tumors.
Materials and Methods

Mice and cell culture
C57BL/6J/Ly5.1 mice, C57BL/6J.OT-I (OT-I) and C57BL/6J.OT-I ARE-Del transgenic mice (ARE-Del) were bred and housed in the animal department of the Netherlands Cancer Institute (NKI). All animal experiments were performed in accordance with institutional and national guidelines and approved by the Experimental Animal Committee of the NKI.

Cells were cultured in IMDM (GIBCO-BRL) supplemented with 8% FCS, 15μM 2-mercaptoethanol, 2mM L-Glutamine, 20 U/mL penicillin G sodium, and 20μg/mL streptomycin sulfate.

In vitro T cell activation and Listeria monocytogenes-OVA infection
FACS-sorted naive CD8+ CD44lo CD62Lhi T cells from WT or ARE-Del OT-I splenocytes were co-cultured for 1 or 3 days with bone marrow derived DCs loaded with indicated amounts of OVA257-264 peptide as previously described21. For infections, 1×10^3 naive WT or ARE-Del OT-I (Ly5.2) T cells were adoptively transferred into C57BL/6J/Ly5.1 recipient mice. The next day, mice were infected i.v. with 2.5×10^8 CFU Listeria monocytogenes strain expressing Ovalbumin (LM-OVA). For reinfections, mice received 2.5×10^5 CFU of LM-OVA 35 days upon a first infection. 6h and 24h later, peripheral blood and spleens were collected, and single cell suspensions were incubated with 1μg/ml brefeldin A for 3h before proceeding with FACS staining.

For T cell transfer into tumor bearing mice, CD8+ T cells were purified from spleens and lymph nodes of WT and ARE-Del OT-I mice by MACS selection (Miltenyi CD8 isolation kit; 95-99% purity). T cells were activated as previously described22. T cells were harvested, removed from the stimuli and put to rest for 4 days at a density of 0.5x10^6/ml with 120IU/ml recombinant interleukin 2 (rIL-2; Proleukin). Prior to T cell transfer dead cells were removed with Lympholyte M gradient (Cedarlane).

Generation and analysis of B16-OVA IFN-γR−/− cells
IFN-γR−/− B16-OVA cells were generated using CRISPR-Cas9 and guide RNA targeting the first exon of Ifngr1: forward 5’TGGAGCTTTGACGAGCACTG3’, reverse 5’CAGTGCTCGTCAAAGCTCCA3’, as predicted with the CRISPR design tool (http://crispr.mit.edu/). Guide RNA was subcloned into the PX458 vector (Addgene #48138; kind gift from F. Zang, MIT, Boston), and the Ifngr1-targeting gRNA, or the empty Cas9 vector alone was transfected into B16-OVA cells by CaPO4 transfection. GFP-expressing cells were single-cell sorted (BD FACSaria III Cell Sorter), and the Ifngr1 knockout clones was identified by Sanger sequencing (forward: 5’CTTGCGGACTTGGCGACTAGTCTG3’, reverse: 5’CTGCCGTGGAAACTAACTGTAAAA3’). The loss of Ifngr1 was validated by the loss of upregulation of the IFN-γ responsive genes PDL-1, MHC-I and MHC-II upon overnight exposure with 50 IU/ml recombinant murine IFN-γ (rIFN-γ, PeproTech). The proliferative capacity of IFN-γR−/− B16-OVA, and Cas9 control cells was determined by cell count and by 12.5nM CSFE labeling over 3 days of culture.

B16 melanoma tumor model
For in vivo studies, mice were injected subcutaneously with 3×10^5 B16-OVA cells 27, or with 3×10^6 Cas9 control or IFN-γR−/− B16-OVA cells. When the tumors reached ~8mm^3, mice
received 1-2×10⁶ WT or ARE-Del CD8⁺ OT-I/Ly5.2 T cells i.v.

Tumor infiltrates were analyzed 14 days after T cells transfer. For tumor growth studies, mice were sacrificed when the tumor reached a size of ~1000mm³. Excised tumors were cut into small pieces and digested with 100μg/ml DNase I (Roche) and 200U/ml Collagenase (Worthington) at 37°C for 30 min. Cells were counted, and when possible lymphocytes were enriched on a Lympholyte M gradient (Cedarlane). T cells were incubated with 1μg/ml brefeldin A alone for 4h or, when indicated, they were stimulated for 4h with 100nM OVA257-264 peptide or with 10ng/ml PMA and 1µM ionomycin (both Sigma-Aldrich).

For in vitro studies, WT and ARE-Del OT-I T cells were activated for 20h with MEC.B7.SigOVA cells, cultured for 4 days with rIL-2 in the absence of antigen, and then reactivated by coculture with pre-seeded B16-OVA cells for 1 to 3 days. B16-OVA cells were refreshed daily at a 6:1 effector:target ratio. When indicated, T cells were daily treated with 10mg/ml of the following purified antibodies: anti-mouse CD28 (PV-1; Bioceros), anti-mouse CD279 (PD-1; 29F.1A12), anti-mouse CD80 (16-10A1), and anti-mouse CD86 (GL-1; all eBioscience).

Flow cytometry

T cells were washed with FACS buffer (phosphate-buffered saline [PBS], containing 1% FCS and 2mM EDTA) and labeled for 20 min at 4°C with the following monoclonal antibodies (all from eBioscience): anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD3 (17A2), anti-CD8 (53-6.7), anti-CD4 (GK1.5), anti-FoxP3 (FJK-16s), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD107a (eBio1D43), anti-PD-1 (J43), anti-LAG-3 (eBioC9B7W), anti-NK1.1 (PK136), anti-CD19 (eBio1D3), anti-CD11b (M1/70), anti-LyG6 (RB6-8C5), anti-F4/80 (BM8), anti-CD54 (3E2), anti-CD63 (NVG-2), anti-MHC I (H-2Kb) (AF6-88.5.5.3), anti-MHC II (I-A/I-E) (M5/114.15.2), anti-PDL1 (MIH5), anti-IFN-γ (XMG1.2), anti-TNF-α (MP6-XT22), anti-IL2 (JES6-5H4). Near-IR (Life Technology) was added to the cells to exclude dead cells from analysis. For intracellular cytokine staining, cells were fixed and permeabilized with the cytofix/cytoperm kit (BD Biosciences). FoxP3 expression was determined upon fixation and permeabilization with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). When necessary, cells were incubated with anti-CD16/CD32 blocking antibody (2.4G2; kind gift from Louis Boon, Bioceros). Flow cytometry analysis was performed on LSR-II and LSR Fortessa (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, version 10).

Quantitative PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative Real-Time PCR was performed with SYBR green, a StepOne Plus RT-PCR system (both Applied Biosystems), and with previously described primers 22. Reactions were performed in triplicate. C_t values were normalized to L32 levels.

mRNA decay was determined upon treatment with 1μg/ml Actinomycin D (Sigma-Aldrich) for indicated time points. mRNA analysis of in vivo generated TILs was performed upon FACS-sorting of OT-I TILs based on the expression of congenic markers CD45.1 and CD45.2.

Statistical analysis

Results are expressed as mean ± SD. Statistical analysis between groups was performed with GraphPad Prism 6, using unpaired Student t-test when comparing 2 groups, or 1-way
ANOVA test with Tukey’s multiple comparison when comparing > 2 groups. Survival curve comparison was performed with a Gehan-Breslow-Wilcoxon test. P values < 0.05 were considered to be statistically significant.

**Author Contributions**

F.S. and M.C.W. designed experiments, F.S., A.G., J.J.F.H., and B.P.N. performed experiments, F.S., A.G., and M.C.W. analyzed data, H.A.Y. contributed the ARE-Del mice and provided intellectual input, F.S. and M.C.W. wrote the manuscript.

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**Disclosures**

The authors have no financial conflict of interest.
References


41. Lindstein, T., June, C.H., Ledbetter, J.A., Stella, G. & Thompson, C.B. Regulation of lymphokine mes-


Supplementary Figure 1: Functional analysis of WT and ARE-Del T cells upon tumor cell encounter

(A) TNF-α and IL-2 production of WT and ARE-Del OT-I T cells co-cultured for 4h with B16-OVA cells in the presence of brefeldin A. (B) Representative dot plots of IFN-γ and TNF-α production of spleen- and tumor-derived OT-I T cells that were activated for 4h with 100nM OVA peptide or with PMA/ionomycin in the presence of BrfA. (C, D) Tumor infiltrates were analyzed **ex vivo**, two weeks after WT or ARE-Del T cell transfer. (C) IFN-γ production (left) and CD107a expression (right) of endogenous CD8+ T cells and tumor-infiltrating NK cells (n=13). (D) Geo-MFI of CD63 expression (left; n=11-14) and CD54 expression (right; n=6-9) of CD11bhi Ly6G/Cihi infiltrating neutrophils. (A-D) Data were pooled from 3 independently performed experiments (mean ± SD). [Unpaired Student t-test; non-significant]. (E-F) WT B16-OVA (E) or IFN-γR-/- B16-OVA cells (F) were cultured overnight with or without 50IU rIFN-γ. PD-L1, MHC-I and MHC-II expression was measured by flow cytometry. Data representative of 3 independently performed experiments.
Supplementary Figure 2: Effect of PD-1 blockade and CD28 costimulation on WT and ARE-Del T cell therapy

(A) PD-1 and LAG-3 expression of endogenous CD8+ TILs, WT OT-I and ARE-Del OT-I TILs analyzed directly ex vivo 14 days after T cell transfer in tumor-bearing mice. Data pooled from 10 mice and 2 independently performed experiments (mean ± SD). [Unpaired Student t-test; *p<0.05]. (B) PD-1 expression of WT and ARE-Del T cells cultured for 1 day with or without (-) the presence of B16-OVA cells (6:1 E:T ratio).

(C, E) IFN-γ protein production (C) and Ifng mRNA levels (E) of WT and ARE-Del T cells cultured for 3 days with B16-OVA cells as in Fig 7 (n=4-5; mean ± SD). (D) Ifng mRNA stability of ARE-Del T cells co-cultured with B16-OVA cells with or without (-) the presence of indicated blocking/stimulatory antibodies.

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