Walking the wire

Post-transcriptional regulation of T cell effector functions in health and disease

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Chapter 7

General Discussion

Introduction

Protective T cell effector functions rely on the fine-tuned modulation of gene expression. Changes in gene expression generally initiate with transcription of DNA into mRNA[1]. In T cells this process is triggered by antigen recognition, co-stimulation and/or cytokine signals[2]. Activation of mRNA transcription is however not sufficient to shape the quality and the magnitude of T cell responses. In fact, the abundance of transcript levels is not only regulated by transcriptional rates, but it also depends on the mRNA turnover[3], which can dynamically change during T cell activation[4]. In addition, for efficient translation of mRNA into proteins, the localization of mRNA and its availability for ribosome recruitment is also important, as well as the efficacy of initiation and elongation of mRNA translation[5, 6]. These post-transcriptional events are all guided by *trans*-acting factors, such as RNA-binding proteins (RBPs) and/or antisense RNAs that recognize specific *cis*-regulatory elements on target mRNAs[7, 8, 9]. Furthermore, recent studies revealed the existence of epigenetic imprinting of mRNA molecules, such as N^6-methyladenosine or 5-methylcytosine modifications, which can also steer the post-transcriptional control[10, 11, 12]. Thus, not only quantitative changes, but also qualitative changes in gene expression define the fate of the mRNA and consequentially the makeup of the cellular proteome.

In this thesis, I investigated the post-transcriptional regulatory mechanisms that tune the magnitude and timing of cytokine production in T cells. My research highlights the dynamics and heterogeneity of post-transcriptional control that rapidly adapts to environmental changes and the state of T cell differentiation. I demonstrate that a cytokine mRNA can be regulated by different post-transcriptional events during the course of its life (*summarized in Figure 1*). This results in a complex and versatile regulatory network that involves mRNA stability and protein translation and that tightly balances initiation, duration and magnitude of inflammatory gene expression. In addition, the regulation of mRNA stability and protein translation occurs in a transcript-specific manner, thereby different cytokine mRNAs follow individual regulatory programs to meet their required terms of production (*summarized in Figure 2*). These novel insights in the regulation of cytokine production provide important information that may be exploited in the future to rectify dysfunctional immune responses.

Post-transcriptional regulation dictates the quiescent state of memory $CD8^+$ T cells

Memory $CD8^+$ T cells provide a lifelong protection against previously encountered pathogens. Vaccinia virus-specific memory T cells were for example found in the blood of survivors after more than 40 years of infection[13], and in 50% of volunteers after more than 50 years of vaccination[14]. Although memory T cells patrol the human body in a quiescent state, they are constantly poised to respond to re-infection and to rapidly produce cytokines. Their 'ready-to-go' state is empowered by a permissive epigenetic signature[15, 16, 17] and the constitutive expression of pre-formed cytokine mRNAs[18, 19]. Interestingly, the 'pre-arming' with effector mRNAs is a general mechanism employed by different cells of the immune system to swiftly respond to (re)infection. In fact, constitutive expression of cytokine mRNAs is not only found in memory T cells, but also in NK cells, NKT cells, innate lymphoid cells (ILCs) and myeloid cells, such as mast cells, basophils and eosinophils[20, 21, 22].

In *chapter 2* we investigated the role of post-transcriptional control in preventing aberrant cytokine production from pre-formed mRNA in resting memory T cells. This correlated well with NK cells that also do not translate IFN-γ, granzyme B and perforin mRNA unless activated with cytokines[20, 21]. However, the molecular mechanisms that maintain deployment-ready mRNA silent were not yet understood. In *chapter 2* we show that memory T cells rapidly degrade pre-formed cytokine mRNA and actively block its translation into protein to guarantee a total safeguard and maintain their silent state. mRNA decay is important to limit the levels of pre-formed cytokine mRNA, however it is not sufficient to avoid chronic protein production. The direct block of translation initiation is therefore essential. We found that the interaction of the RNA-binding protein (RBP) ZFP36L2 with AU-rich elements (AREs) within the 3'UTR of pre-formed *Ifng* and *Tnfa* mRNA obstructs the recruitment of these short-lived mRNAs to ribosomes and thus impedes their translation (Fig 1). We hypothesize that this translational block also prevents chronic IFN-γ production in NK cells and NKT cells. This regulatory mechanism may however be even more broadly employed provided that AREs are widely expressed through inflammatory genes[23], and that many immune cells, but also epithelial cells[24], contain pre-formed cytokine mRNA.

Ribosomal engagement and translational initiation generally commences at the 5'UTR of an mRNA[25]. It is therefore counterintuitive that the 3'UTR-bound ZFP36L2 can repress the translational initiation process, and suggests that other trans-acting factors must cooperate in this mechanism of regulation. Already in 1969, Spirin introduced the concept of 'informosomes'. To strictly regulate both their biogenesis and function, mRNA molecules can bind different RBP complexes during different stages of their lifetime: '*Omnia mea mecum porto*' ('All that is mine I carry with me')[26, 27]. We therefore propose that ZFP36L2 acts in the context of a larger RBP-RNA complex that maintains the equilibrium between synthesis, degradation and a translational stall of pre-formed cytokine mRNA in memory T cells. In macrophages another member of the ZFP36 RBP family, tristetraprolin (TTP), links mRNA decay to block of translation. TTP recruits the DCP2 decapping complex and the CCR4-Not deadenylase complex at the 3' end of target mRNAs[28], and interacts with the cap-binding translation repression 4EHP-GYF2 complex at their 5'-end[29, 30]. Also ZFP36L2 is possibly engaged in the bridge between the 3'- and the 5'-ends of *Ifng* and *Tnfa* mRNA. However, because ZFP36L2 lacks the conserved tetraproline motifs required for the binding of TTP to the 4EHP-GYF2 complex, the binding partners of ZFP36L2 in memory T cells are likely to be different from the binding partners of TTP in macrophages. Of note, in contrast with the direct link between mRNA decay and translational block in macrophages, we found that degradation of pre-formed *Ifng* and *Tnfa* mRNA is independent from ARE-ZFP36L2 interaction in memory T cells. Thus, future investigations, applying for example RNA interactome capture approaches[31], are required to fully unravel the RBP-complex that keeps memory T cells post-transcriptionally silent.

RBP-RNA complexes also determine the localization of the mRNA within cytosolic compartments. In the intestinal epithelium, mRNA localization does not generally overlap with the sites where ribosomes reside[32]. Upon stimulation by nutrients, mRNA molecules are mobilized towards the ribosomes and mRNA translation occurs directly at the site where protein production is required[32]. Because only a few mRNA molecules need to be transported to give rise to high amounts of proteins, asymmetrical distribution of the mRNA appears to be more efficient than transporting already translated proteins[33]. In neurons,

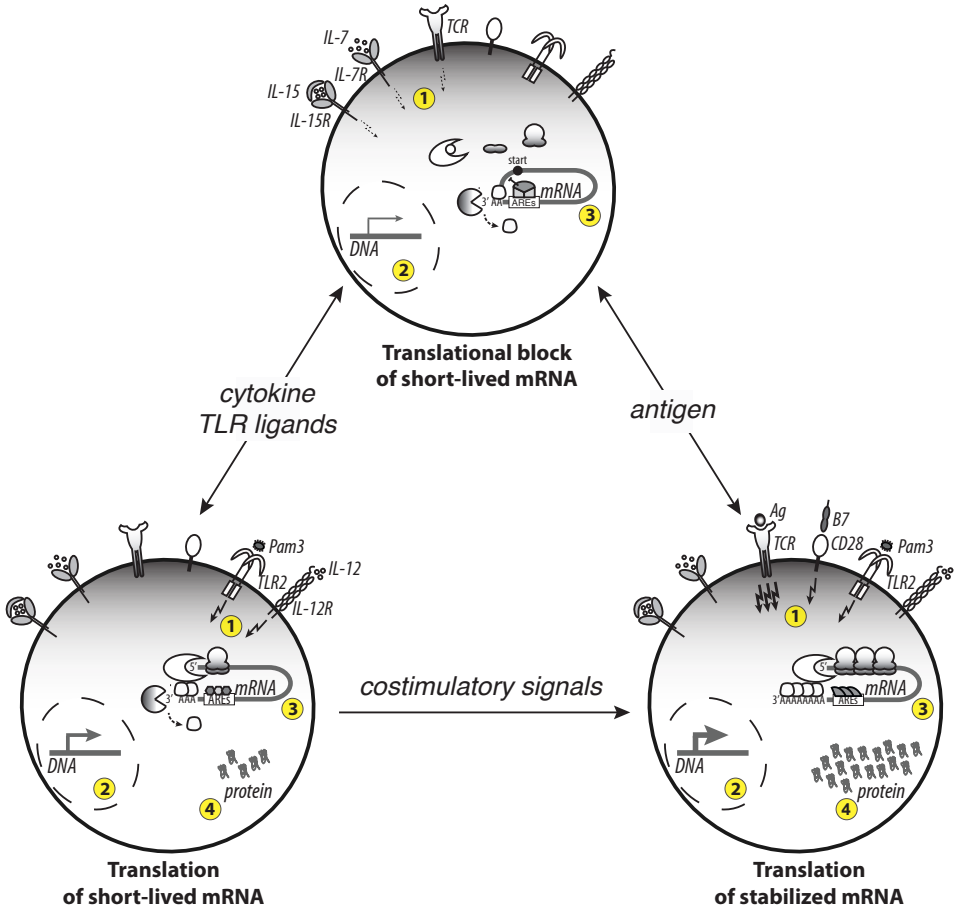

Figure 1: Rapid switch of post-transcriptional regulatory events upon (re)activation of memory T cells

Steady state (top): (1) Tonic signaling through the TCR and IL-7- and IL-15-mediated stimulation support homeostatic functions of memory T cells in the absence of infection. (2) Constitutive mRNA transcription ensures basal levels of pre-formed cytokine mRNAs. (3) mRNA decay and ARE-dependent block of ribosome recruitment impede the translation of pre-formed mRNA into protein.
Antigen-independent activation (left): (1) Memory T cells can respond to non-related infections through cytokine- (as in example IL-12) and TLR-mediated triggering. (2) *De novo* mRNA transcription and (3) mRNA translation are engaged. (4) Rapid ARE-dependent degradation of newly synthesized mRNA contains the levels of protein production.
Antigen-dependent activation (right): (1) When memory T cells encounter their cognate antigen, the signal strenght and duration of stimulation determine the amont of cytokine produced. Costimulatory molecules, such as CD28 and TLR2, can enhance T cell activation. (2) Increased de novo mRNA transcription and (3) ARE-dependent mRNA stabilization promote (4) high levels of protein production.

β-actin mRNA is pre-positioned all along the dendrites[34], yet its translation is repressed in distal dendrites, and active in proximal dendrites[35]. Whether pre-formed cytokine mRNA is asymmetrically distributed in memory T cells is still to be determined. However, ZFP36L2-RNA complexes could make pre-formed mRNA unavailable for translation by keeping it away from the ribosomes. Because ZFP36L2 dissociates from *Ifng* and *Tnfa* mRNA within the first 2h of T cell activation (*chapter 2*), other RBPs competing for the same ARE-binding site could therefore interact with pre-formed cytokine mRNA and mobilize it towards the site where protein production is required. Interestingly, T helper cells use two directionally distinct pathways to secrete IFN-γ and TNF-α upon stimulation. IFN-γ is secreted towards the immunological synapse, whereas TNF-α is released in a multidirectional fashion[36]. It is therefore possible that different ARE-BPs recruit pre-formed *Ifng* and *Tnfa* mRNA upon T cell activation, thereby promoting their specific translocation and local mRNA translation. Pull down experiments in *chapter 2* identified ZFP36L1 as a second RBP that selectively binds the ARE-containing region of the *Ifng* 3'UTR. Interestingly, ZFP36L1 appears to have an opposite expression profile compared to ZFP36L2. Whereas ZFP36L1 expression is hardly detectable in resting memory T cells, its mRNA and protein levels significantly increase upon 2h of T cell stimulation (*chapter 2* and data not shown). Thus, ZFP36L1 and ZFP36L2 might have a *yin-yang* behavior, and their functional switch could be dictated by post-translational modifications acquired upon T cell activation. Different intracellular signaling pathways can in fact induce post-translational modifications of RBPs, among which phosphorylation is the best studied[37, 38, 39]. Phosphorylation can modify the composition of the RBP-RNA complex by causing structural changes and altering the affinity of RBP to the target mRNAs. For example, in macrophages TNF-α protein production occurs upon p38 MAPK/MK2-dependent phosphorylation of the RBP TTP[40, 41]. Phosphorylated TTP decreases its affinity to the ARE, inhibits its ability to replace the competing RBP HuR, and permits HuR to bind and enhance the translation of *Tnfa* mRNA[42]. Because ribosome recruitment of pre-formed *Tnfa* and *Ifng* mRNA requires the activation of the PKC signaling (*chapter 3*), it is tempting to speculate that PKC can directly or indirectly modify ZFP36L2 and inactivate its inhibitory function.

What also remains unknown are the signals that enable the formation of RBP-RNA complexes to ensure post-transcriptional silencing in resting memory T cells. Answers to this question may be found in the signaling pathways that are required to maintain functionally competent memory T cells during steady state. The quiescent state of memory T cells is in fact not a static 'off' state, but rather results from a dynamic equilibrium between TCR-dependent and cytokine-dependent signaling (Fig 1). Resting T cells passively receive tonic signals through their TCR by constitutively active kinases like lymphocyte-specific protein tyrosine kinase (Lck)[43, 44], zeta-chain-associated protein kinase (ZAP)-70[45], and signal-transducing adaptor proteins like SLP-76[46]. This is in part achieved by the recognition of self-peptide/MHC complexes on dendritic cells that T cells encounter during their scanning for foreign antigens [47]. In addition, a fraction of TCR clusters occasionally oscillate between an inactive conformation and a primed form[48]. This could increase the accessibility of the CD3ε cytoplasmic chain to phosphorylation[48, 49], and allow the transmission of TCR-dependent signals in spite of the absence of stimulation. Because the adaptor protein SLP-76 is required to activate TCR-dependent calcium flux and MAPK signaling, and to induce cytokine production upon reactivation[46], TCR-dependent tonic signaling could possibly support the constitutive transcription of cytokine mRNA in memory T cells (Fig 1). Interestingly,

tissue-resident memory CD8+ T cells express even higher levels of pre-formed mRNAs than circulating memory T cells[18], thus suggesting a role for epithelial cells in the maintenance of basal mRNA levels, possibly through the expression of Notch ligands[50].

In addition to TCR-dependent signaling, common-γ chain cytokines such as IL-7 and IL-15 are crucial for survival and homeostatic proliferation of memory T cells[51, 52, 53], and they may support ATP production through fatty-acid oxidation[54, 55]. Cytokine-dependent signaling also propagates the permissive epigenetic signature of memory T cells to the daughter cells, thus preserving their 'ready-to-go' state[56]. Whether these signaling pathways also regulate post-transcriptional silencing of pre-formed cytokine mRNA in memory T cells is yet to be determined. Both IL-7 and IL-15 receptors act through the Jak/STAT and PI3K/Akt intracellular pathways[57, 58, 59], which support the micro-RNA mediated repression of mRNA translation in T cells[60, 61]. We therefore hypothesize that common-γ chain cytokines can also control the activity of RBPs and govern the formation of RBP-RNA inhibitory complexes.

Activated CD8+ T cells tweak cytokine production through customized regulation of gene expression

When quiescent memory T cells encounter their cognate antigen, they are rapidly activated into highly proliferative cytotoxic effector T cells[2]. However, not only antigens can activate memory T cells, but they can also be triggered in an antigen-independent manner by the inflammatory environment (Fig 1)[62, 63, 64, 65]. This dual function makes memory T cells an active component of the first line of defense that prevents pathogen spreading early during infection.

- ***Antigen-dependent activation***

Memory CD8+ T cells respond to their cognate antigen by releasing two or more cytokines, and this polyfunctional activity is considered the most protective form of memory responses. Whereas most essays do not distinguish the time frame when these cytokines are produced, we and others found that cytokine production occurs in a serial fashion rather than simultaneously[66, 67, 68]. In *chapter 3* we show that the gene-specific interplay between *de novo* transcription, mRNA stability and translation efficiency defines the production kinetics of TNF-α, IFN-γ and IL-2 (Fig 2).

TNF-α is the first cytokine to be produced upon TCR triggering, with TNF-α protein already detectable within the first 30 min of stimulation. Its rapid release is not dependent on *de novo* transcription, but almost completely relies on translation of pre-formed mRNA. TCR-mediated PKC signaling regulates the rapid recruitment of pre-formed *Tnfa* mRNA into ribosomes. This process may occur through AREs. In particular, PKC may modify ZFP36L2 and decrease its affinity for *Tnfa* mRNA, thus releasing it from the translational stalling. Interestingly, TCR stimulation does not modulate the turnover of *Tnfa* mRNA. The constitutive decay of *Tnfa* mRNA could hence be intrinsically 'encoded' in its transcript and depend on the presence of constitutive decay elements (CDE) within the *Tnfa* 3'UTR[69]. CDEs are in fact binding sites for the RBP Roquin[70], and they interact through stem-loop mRNA structures in an ARE-independent manner[71]. Of note, whereas TNF-α production in T cells almost exclusively relies on translation from pre-formed mRNA, its production strongly depends on transcription and mRNA stabilization in macrophages[69], thus revealing a cell-

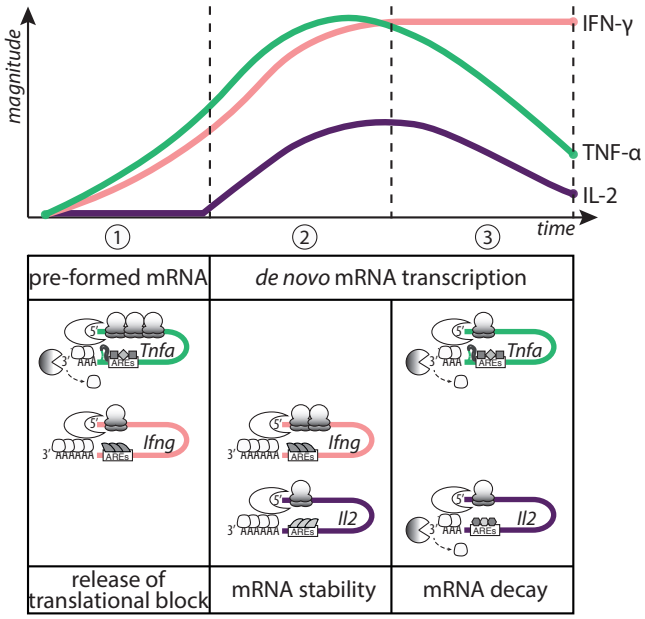

Figure 2: Cytokine-specific post-transcriptional regulatory programs during T cell activation

(Top) TNF-α, IFN-γ and IL-2 follow individual kinetics of production upon T cell activation.
(1) The immediate production of TNF-α and IFN-γ is promoted by the costitutive expression of pre-formed mRNA, which is released from its translational block and rapidly engaged by ribosomes. (2) Initiation of IL-2 production and increased magnitude of IFN-γ response require *de novo* mRNA transcription and mRNA stabilization. (3) Timely shut-down of TNF-α and IL-2 production is guaranteed by mRNA decay.
All cytokine-specific post-transcriptional events are possibly regulated through dynamic changes of ARE-RBP complexes.

type specific program of regulation.

In contrast to TNF-α, IL-2 protein levels are only detectable from 2h of T cell activation onwards. Its production entirely relies on *de novo* transcription and mRNA stabilization. Whereas mRNA stability contributes to reach optimal responses during the first 4h of activation, rapid *Il2* mRNA decay shuts down the protein expression in due time. Thus, a dynamic regulation of *Il2* mRNA turnover is crucial to shape the IL-2 production kinetics. This is again regulated through PKC, and it appears to depend on the presence of AREs within its 3'UTR[72, 73, 74].

Interestingly, IFN-γ production employs all three mechanisms studied, i.e. *de novo* transcription, mRNA stabilization and translation efficiency. However, their relative contribution shifts in time: whereas the instantaneous production of IFN-γ requires rapid translation of pre-formed mRNA, the magnitude and length of the response is determined by *de novo* transcription and mRNA stabilization (Fig 2). The delayed contribution of *de novo* transcription is also supported by the fact that mRNA transcription in T cells mainly relies on *de novo* recruitment of RNA polymerase II to gene loci, and not on the usage of stalled RNA Pol[75]. Of note, also for IFN-γ, post-transcriptional processes are governed by the PKC signaling and possibly modulated through dynamic changes of ARE-RBP complexes.

Combined, our data demonstrate that the mere presence of common regulatory elements within cytokine mRNAs, such as AREs, poorly predict the fate of their encoding transcripts. Rather, their fate is determined by several signaling pathways that are activated upon T cell stimulation (Fig 1). In *chapter 3* and *4*, we dissected the role of three major TCR-dependent pathways in the cytokine-specific regulation of gene expression. We corroborated previously published data showing that Ca^{2+} flux and PI3K/Akt signaling are required for *de novo* transcription of cytokine mRNAs[76, 77, 78, 79], whereas mTOR activity controls the initiation of mRNA translation[5]. Although PKC signaling enhances Ca^{2+}-dependent mRNA transcription, we found that PKC mainly customizes post-transcriptional regulatory events that control the production kinetics of TNF-α, IFN-γ and IL-2. The concerted action of these intracellular signaling pathways is in turn regulated by the quality, strength and duration of the TCR engagement. In *chapter 3* we investigated how the antigen load regulates cytokine production. Interestingly, ribosomal recruitment of pre-formed *Tnfa* mRNA is activated in a switch-like manner and, only when a certain concentration of antigen is present, substantial production of TNF-α protein is allowed. Similarly, antigen concentration appears to control *Ifng* mRNA turnover (*chapter 5*). About 20 years ago, Viola and Lanzavecchia proposed that T cells produce IFN-γ only when ≥8000 TCRs were triggered, independently from the nature of the ligand[80]. This activation threshold is lowered to about 1500 TCRs when costimulatory molecules are also engaged[80]. In fact, TCR and costimulatory pathways synergize to strengthen the signal transduction cascade (Fig 1). Because the sum of multiple stimuli, such as TCR triggering, co-stimulation, and cytokines allows to calculate the number of division that T cells undertake during proliferation[81], we aim to use a similar approach to calculate the requirements for optimal cytokine production. Unraveling the relative contribution of different stimuli to cytokine production may in the future allow predicting the efficacy of immunomodulatory therapies.

- ***Antigen-independent activation***

Memory T cells can be activated not only through antigen recognition by the TCR, but also by inflammation-dependent bystander activation[62, 63, 64]. Independently from their cognate antigen, memory T cells can sense the inflammatory environment, and promote the activation and recruitment of other immune cell types like monocytes/macrophages and neutrophils to the site of infection[82, 83]. Thus, antigen-non-specific memory T cells can actively contribute to the initiation of inflammatory responses.

Upon bystander stimulation, memory T cells undergo a robust program of activation. They induce the expression of CD69, CD25 and CD11a surface molecules, increase the expression levels of transcription factors like T-bet and Eomesodermin, and release cytotoxic molecules such as IFN-γ, granzyme B and perforin, but not TNF-α[84]. In *chapter 4* we demonstrate that in vitro generated resting $CD8^+$ T cells can directly recognize microbial-derived danger signals by responding to Toll-like receptors (TLR)-2 and 7 ligands. TLRs can be engaged in an antigen-dependent (*chapter 5*) and in an antigen-independent manner (*chapter 4*). Interestingly, when TLRs are engaged in the absence of the antigen, T cells exclusively promote the production of IFN-γ, and none of the other cytokines measured.

Antigen-independent activation of memory T cells can also be achieved by multiple combinations of pro-inflammatory cytokines. IL-12 and IL-18 strongly induce IFN-γ production[63, 64], whereas IL-15 and type I interferons enhance the cytolytic activities[85, 86]. In

addition, memory CD8$^+$ T cells increase the expression of NK-cell-specific markers such as NKG2D upon prolonged exposure to high levels of IL-15[87]. Memory T cells that express the NKG2D receptor support early pathogen clearance during *Listeria monocytogenes* infection in a TCR-independent manner[88]. Bystander-activated memory T cells share multiple phenotypic and functional features with NK cells and ILC1, which also produce IFN-γ in response to, for example, IL-12 and IL-15 stimulation[88, 89, 90]. This functional redundancy may represent an advantage for the host, since NK cells, ILC1 and memory T cells appear to be differently located within the epithelium of barrier tissues[90]. However, because most of the studies on bystander-activated memory T cells were performed *in vitro* or in relatively 'clean systems' like transgenic or knock out mice, it is still unclear whether activation with pro-inflammatory cytokines occurs in the complete absence of TCR signaling during *in vivo* infections. It was recently described that, despite of the absence of antigen, cytokine-mediated bystander activation of CD8$^+$ T cells is TCR signaling dependent and may require self-pMHC-T cell interaction[91]. In addition, TCRs can also be cross-reactive[92]. Molecular mimicry among pathogens results in non-specific activation of memory T cells and production of granzyme B[85]. For example, mice latently infected with *Murine gamma-herpesvirus 68* (MHC-68) or *Murine cytomegalovirus* (CMV) acquire resistance against *Listeria monocytogenes* and *Yersinia pestis* bacterial infections[93]. In addition, TCRs specific for *Lymphocytic choriomeningitis virus* (LCMV)-derived antigens appear to be highly cross-reactive for *Pichinde virus* or *Vaccinia virus* epitopes[94].

Because the swift cytokine release by memory CD8$^+$ T cells is promoted by rapid translation of pre-formed mRNA upon TCR triggering (*chapter 2* and *3*), it would be interesting to assess whether antigenic stimulation is required to engage this 'ready-to-be-translated' template. One would argue that bystander activation is the most rapid memory T cell response, hence TCR-independent signaling could also release pre-formed *Ifng* mRNA from its translational block. However, preliminary *in vitro* data presented in *chapter 5* indicate that bystander activation induces IFN-γ production later than TCR triggering. In line with these data, TLR-mediated IFN-γ production relies exclusively on *de novo* transcription and does not promote mRNA stabilization (*chapter 4*). The different onset of IFN-γ production between antigen-dependent and antigen-independent stimulation thus suggests an exclusive usage of pre-formed cytokine mRNA in response to the cognate antigen. This hypothesis is also supported by two other observations: 1) although IFN-γ and TNF-α are both encoded by pre-formed mRNA in memory T cells, only IFN-γ, and not TNF-α, is produced upon bystander activation; 2) TLRs, as well as cytokine receptors, primarily activate the PI3K/Akt and MAPK pathways without engaging PKC[95]. We show in *chapter 3* that PKC is sufficient and required to recruit pre-formed mRNA to ribosomes and to stabilize cytokine mRNA. The incapacity to translate pre-formed mRNA, and the requirement for *de novo* transcription without mRNA stabilization, could thus represent a safeguard to limit memory T cell responses to generic infections. Deregulation of bystander activation could in fact cause the exacerbation of cytokine production, which could potentially degenerate in cytokine storms, tissue damage and immunopathology.

Rapid mRNA turnover impairs cytokine production of exhausted T cells

Persistent exposure to antigen during tumor formation or chronic infection causes a hyporesponsive state of $CD8^+$ T cells that is referred to as exhaustion[96]. Exhausted $CD8^+$ T cells are characterized by increased expression of inhibitory receptors, such as programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein (CTLA)-4 or lymphocyte-activation gene (Lag)-3, and by the gradual loss of proliferative capacity and cytokine production[97, 98, 99]. Thus, exhausted $CD8^+$ T cells fail to execute their effector functions and gradually lose their protective role. Blocking PD-1 and/or CTLA-4 signaling with antibodies restores the effector functions of tumor-infiltrating lymphocytes (TILs) and chronically infected T cells, thereby enhancing both the control of tumor growth and viral infections[100, 101, 102, 103, 104, 105, 106].

Although many studies have reported an inverse correlation between the loss of cytokine production and the increased recruitment of inhibitory receptors on the surface of exhausted T cells, it is still under debate how and if these two events directly correlate with each other. PD-1 engagement attenuates TCR activation by inhibiting TCR-mediated phosphorylation of ZAP70 and its association with CD3ζ[107]. PD-1 triggering also interferes with the flux of intracellular Ca^{2+} [108], which activates the transcription factor NFAT. NFAT strongly promotes the transcription of cytokine mRNA when associated to AP-1[109]. However, during chronic activation of T cells, NFAT mainly works independently from AP-1 and enhances the expression of inhibitory receptors, thus promoting T cell exhaustion[110]. PD-1-mediated signaling can also inhibit PKCθ[107] and PI3K/Akt intracellular pathways, thus blocking IL-2 production and T cell proliferation[111]. PKCθ and PI3K/Akt signaling pathways can be activated downstream of the TCR, and of the co-stimulatory molecule CD28. CD28 was recently identified as a novel direct target of PD-1[112], and it was found essential for the positive outcome of anti-PD-L1 treatment in tumor-bearing mice[113]. This thus suggests that exhausted T cells have a cell-intrinsic requirement for CD28-mediated signaling to mount effective immune responses.

Whereas the transcriptome and epigenetic landscape of exhausted T cells has been widely studied in the past years[114, 115, 116, 117, 118, 119, 120], the possible role of other regulatory pathways in controlling T cell exhaustion are much less investigated. In *chapter 6* we reveal that post-transcriptional regulatory events are crucial in dampening IFN-γ production of TILs. We show that the progressive loss of IFN-γ production in TILs directly correlates with the acquired loss of *Ifng* mRNA stability. The rapid turnover of *Ifng* mRNA offers only a limited window for mRNA translation, thus resulting in impaired protein production. *Ifng* mRNA stability of TILs is regulated through AREs, and deletion of the ARE region within one allele of the Ifng gene is sufficient to restore T cell effector functions and to delay tumor outgrowth.

CD28-mediated signaling can promote cytokine mRNA stabilization (*chapter 6*) [121, 122]. Although CD28 is a direct target of PD-1[112], we found that blocking PD-1 signaling *per se* did not restore *Ifng* mRNA stability or the levels of *Ifng* mRNA, despite the significant increase of IFN-γ protein production. Our findings are supported by previous data showing that PD-1 blockade enhances protein production without affecting the amounts of *Ifng* mRNA and/or its epigenetic signature[118]. Combined, these data indicate that PD-1 primarily blocks the production of IFN-γ by repressing the translation of *Ifng* mRNA into protein. The block of *Ifng* mRNA translation observed in TILs resembles the already described phenotype of

anergic self-tolerant T cells[123], and quiescent memory T cells (*chapter 2*). However, whether the binding of ZFP36L2 to AREs causes the block of IFN-γ protein production in TILs is yet to be determined. Of note, the single treatment with anti-CD28 antibodies also enhanced IFN-γ production, and it did so by promoting *Ifng* mRNA stabilization. Although anti-PD-1 and anti-CD28 treatments appeared to interfere with mRNA translation and mRNA stabilization, respectively, when combined they exert cooperative effects (*chapter 6*). Thus, the increase of protein production and mRNA stabilization may depend on a threshold of CD28 signaling.

Exploiting post-transcriptional regulation for therapeutic treatments

In addition to further our understanding of post-transcriptional regulatory mechanisms, our study may also provide tools to improve tumor therapies. PD-1 checkpoint blockade is one of the most promising cancer treatment currently applied in the clinic, however it is not sufficient to re-program exhausted T cells into durable effector/memory T cells[118]. If the antigen is not cleared and remains in the system at a high concentration, T cells can become exhausted again despite of the anti-PD-1 or anti-PD-L1 treatment[118, 124]. Additional therapies must therefore be provided to improve and prolong the effect of checkpoint blockade. We propose that post-transcriptional regulation can be exploited for this purpose, and may broaden the window of opportunity of tumor therapies. Our findings could possibly be translated to clinical setting by engineering *in vitro* expanded TILs for T cell therapy. Because constitutively stable *Ifng* mRNA retains IFN-γ production of TILs and delays the tumor outgrowth, one could remove the ARE-containg region using CRISPR/Cas9 technology in the IFNG gene of human T cells before reinjecting them into the patient. Alternatively, the stability of *IFNG* mRNA and the production of IFN-γ could be prolonged by using chimeric antigen receptors (CARs) containing the intracytoplasmic domain of CD28. Because we observed a synergistic effect between CD28 and PD-1, CD28 CAR T cells could be used in combination with PD-1 blockade to not only restore T cell proliferation[125], but also IFN-γ production.

It is however important to mention that prolonged type I and type II IFN signaling was also associated to tumor resistance to checkpoint blockade[126]. Moreover, genetic mutations of IFN-receptor associated genes were found in melanoma patients that were unresponsive to anti-PD-1 or anti-CTLA-4 treatment[127, 128]. Thus, increased IFN-γ levels could also result in tumor escape. Despite its direct effect on tumor cells, IFN-γ can also promote the pro-inflammatory phenotype of tumor infiltrates, as for example macrophages[129], and act on endothelial cells to promote regression of tumor vasculature[130]. Therefore, we propose that the manipulation of Ifng gene expression, and in particular the editing of its ARE-dependent post-transcriptional regulation, might in the future be applied in the clinic and significantly enhance the treatment of solid tumors.

Remarkably, AREs are not only found in the gene encoding the pro-inflammatory cytokines we describe here, but they are found in about 80% of the transcripts encoding for cytokines and growth factors[131], and in about 16% of the total human transcriptome[132]. AREs are involved in the regulation of several biological processes that require a rapid on/off switch, as for example cell cycle, apoptosis, cellular communication, motility, metabolism, signal transduction, transcriptional and post-transcriptional control. They are not restricted to the immune system, but they can virtually regulate protein production in each cell of

our body. Because dysregulation of ARE-mediated processes has been implicated in the development of severe inflammatory diseases, neurodegenerative disorders and oncogenesis[133, 134, 135, 136, 137], their manipulation represent an appealing therapeutic approach for a various range of targets and human diseases. Thus, deepening our understanding of post-transcriptional regulatory networks is imperative to identify intelligent new targets for optimal therapeutic benefit.

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