Trends in Immunology

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Review

Dynamic Post-Transcriptional Events Governing CD8$^+$ T Cell Homeostasis and Effector Function

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Effective T cell responses against infections and tumors require a swift and ample production of cytokines, chemokines, and cytotoxic molecules. The production of these effector molecules relies on rapid changes of gene expression, determined by cell-intrinsic signals and environmental cues. Here, we review our current understanding of gene-specific regulatory networks that define the magnitude and timing of cytokine production in CD8$^+$ T cells. We discuss the dynamic features of post-transcriptional control during CD8$^+$ T cell homeostasis and activation, and focus on the crosstalk between cell signaling and RNA-binding proteins. Elucidating gene-specific regulatory circuits may help in the future to rectify dysfunctional T cell responses.

Highlights

The rapid remodeling of the T cell proteome upon activation depends on the integration of transcriptional and post-transcriptional control of gene expression.

Post-transcriptional events are heterogeneous, dynamic, transcript specific, and cell type specific.

RNA-binding proteins mediate post-transcriptional events and their activity is strictly regulated by signal transduction.

Post-transcriptional control preserves the silent state of memory T cells, and tailors the magnitude and kinetics of cytokine production in effector T cells.

Upon chronic insults, CD8$^+$ T cells can fail to respond, partially because cytokine mRNA is not stabilized and translated.

Regulatory Mechanisms Driving Effective CD8$^+$ T Cell Responses

Cytotoxic CD8$^+$ T cells have a key role in fighting pathogenic insults and in immunosurveillance. This includes clearance of primary infections and killing of malignant cells, as well as long-term protection by memory T cells against secondary infections [1,2]. The effectiveness of CD8$^+$ T cells to clear target cells is defined by their capacity to produce effector molecules, such as cytokines, chemokines, and cytotoxic granule contents. Whereas these effector molecules are essential for killing infected cells and for preventing pathogenic spread, they are also highly toxic. In fact, aberrant cytokine production strongly correlates with the development of autoimmune diseases and inflammatory pathologies, such as rheumatoid arthritis, multiple sclerosis, and various intestinal and skin disorders [3–5]. Stringent regulation of inflammatory gene expression is thus key for protective, yet balanced immune responses.

Several regulatory nodes define the extent of cytokine production (i.e., protein production) upon T cell activation. Protein production generally initiates with the transcription of DNA into mRNA, a process that depends on the accessibility of genes to, and the availability of, transcription factors. The transcriptional regulatory networks that control mammalian T cell effector functions are well studied and described elsewhere [6,7]. However, the amount of newly transcribed mRNA is not solely defined by transcription rates [8]. Genome-wide studies in bacteria and mammalian cells demonstrated that mRNA and protein abundance do not follow a linear correlation [9–11]. For instance, in *in vitro* activated murine CD4$^+$ T cells, the correlation coefficient is 0.49 [12]. This discordance between mRNA and protein expression has been attributed to several mechanisms of post-transcriptional regulation, including mRNA stability, translation efficiency, and protein degradation.

Transcripts encoding effector molecules and regulatory proteins are generally unstable but become stabilized upon T cell activation [13,14]. This increased mRNA stability is required to augment the numbers of transcripts available for protein production and to prolong the immune

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response [15]. In addition to mRNA stabilization, the protein output is also regulated by its availability for ribosome recruitment (defined, for example, by mRNA localization) and by the efficacy of translation initiation and elongation [13,14,16]. These post-transcriptional events are mediated by *trans*-acting factors, such as **RNA-binding proteins (RBPs)** (see Glossary) and/or **non-coding RNAs** [microRNAs (miRs) and long non-coding RNAs (lncRNAs)] that recognize specific *cis*-regulatory elements on target mRNAs [17,18]. RBPs and miR can act synergistically or in competition to destabilize mRNA targets and inhibit their translation into proteins (Box 1) [19]. The importance of activation-induced alterations in miR expression and activity in T cells has been reviewed elsewhere [20]. However, how RBPs mediate post-transcriptional regulatory events in T cells remains largely unexplored. In this review, we discuss recent findings elucidating how RBPs alter their function to govern $CD8^+$ T cell activation and differentiation. We focus on the role of the **Zinc finger binding protein 36 (ZFP36) family of RBPs**. ZFP36 proteins can block aberrant cytokine production by murine T cells in the absence of stimulation, and can modulate the effector function of T cells upon viral infection [21,22]. Based on this example, here we propose a model where RBP-mediated post-transcriptional control instructs $CD8^+$ T cells to respond to antigenic and co-stimulatory signals in a heterogeneous and dynamic fashion.

RNA-Binding Proteins Prevent Protein Production in Non-activated Memory $CD8^+$ T Cells

In mammals, memory $CD8^+$ T cells provide life-long protection against previously encountered pathogens [23]. The capacity of memory T cells to produce effector molecules within a few hours of re-infection substantially contributes to preventing pathogen spreading and disease development. Their 'ready-to-go' state is empowered by a permissive epigenetic signature, characterized by histone modifications on promoters and distal regulatory elements, which facilitate the accessibility of genes to transcription factors [24–26]. These epigenetic alterations also allow for the constitutive expression of cytokine mRNAs in both circulating and **tissue-resident memory T cells** [15,27,28]. The 'pre-arming' with mRNAs encoding effector molecules is a common determinant of immune cells and enables swift responses to pathogenic

Box 1. Cooperation and Competition of miRs and RBPs

miRs and RBPs share the ability to regulate mRNA stability and translation through direct recognition of mRNAs. miRs are important regulators of gene expression during T cell development and effector functions [126]. In mice, miRNAs generally repress T cell proliferation, differentiation, and cytokine production. Deletion of Dicer, a critical enzyme for miR maturation, impairs the response of murine $CD8^+$ and $CD4^+$ T cells upon *in vitro* stimulation and *in vivo* infection with *Listeria monocytogenes*-OVA [127,128]. Let-7 miRs are essential for maintaining the naïve phenotype of murine $CD8^+$ T cells [129], whereas miR15/16 participate in restricting memory formation [130]. miR29 suppresses murine $CD4^+$ T cell responses by targeting directly *Ifng* mRNA, or by targeting mRNA encoding the transcription factors T-bet and Eomes [131,132]. By contrast, miR155 can promote murine $CD8^+$ T cell responses by inhibiting the antiproliferative effect of type I interferon [133].

A critical aspect of post-transcriptional regulation is the crosstalk between different *trans*-acting factors and the dynamic consequences that these interactions may have on the fate of target mRNAs. miRs and RBPs can cooperate to regulate gene expression, or can exert opposite effects and regulate each other's function [19]. For instance, miR16 and ZFP36 cooperate to accelerate mRNA degradation in HeLa cells. ZFP36 does not directly bind miR16, but interacts with components of the RNA-induced silencing complex (RISC) to assist miR16 in targeting ARE-expressing mRNAs [134]. Similarly, HuR contributes to recruiting let-7- and miR19-containing RISC to *MYC* and *RHOB* target mRNAs, to repress their translation in HeLa cells and human keratinocytes, respectively [135,136]. RBPs can also directly bind miRs, or compete for overlapping binding sites. For example, Roquin simultaneously interacts with the RISC component Argonaute2, miR146a, and the *Icos* target mRNA in murine $CD4^+$ T cells, thereby facilitating the decay of both miRs and mRNAs [137]. However, Roquin can also outcompete miR17-92 to bind and regulate *Pten* mRNA, thus controlling T helper 17, T follicular helper, and T follicular regulatory cell differentiation in mice [138]. HuR is also known to compete with miRs and to protect target mRNAs from miR-mediated mRNA decay in human and mouse [139,140]. These few examples show that RBPs can regulate many target mRNAs by using different regulatory strategies. Elucidating the interplay between miRs and RBPs in T cells will contribute to shedding light on the versatility and complexity of post-transcriptional regulatory networks.

Glossary

4sU labeling: method to metabolically label newly synthesized RNA molecules by incorporation of 4-thiouridine (4sU) to distinguish *de novo* transcribed RNA (i.e., 4sU-labeled RNA) from pre-existing RNA (containing unmodified uridine; U).
Anergic self-tolerant T cells: subset of T cells that escape negative selection, are self-reactive, but become functionally unresponsive (anergic) through a process of peripheral T cell tolerance.
Antigen affinity: strength by which an antigen binds its specific TCR.
Bystander stimulation: type of T cell stimulation occurring in the absence of cognate antigen and of non-self-specific TCR triggering.
Crosslinking immunoprecipitation (CLIP): method used to study genome-wide RNA–protein interactions at a single nucleotide resolution, based on UV-crosslinking and immunoprecipitation.
Fluorescence *in situ* hybridization (FISH): method used to visualize nucleic acids by microscopy (e.g., confocal) based on complementary fluorescent probes.
Innate lymphoid cells (ILCs): group of innate immune cells belonging to the lymphoid lineage but that do not express antigen-specific receptors.
Marginal zone B cells: mature B cells residing into the marginal zone of the spleen; they mount rapid antibody responses to both T cell-dependent and T cell-independent antigens.
Non-coding RNA: group of RNA molecules that are not necessarily translated into protein, but regulate gene expression at the transcriptional and/or post-transcriptional level. This includes miRs, small interfering RNAs, small-nucleolar RNAs, lncRNAs, circular RNAs, piwi-interacting RNAs, as well as yet-to-be-discovered small regulatory RNAs.
P-body: RNA–protein complex that aggregates in the cytoplasm of yeast and mammalian cells, and contains proteins and enzymes mainly involved in RNA decapping and decay.
PIM-family kinases: serine/threonine kinases involved in cell survival and proliferation comprising three family members: PIM-1, PIM-2, and PIM-3.
Ribosome footprinting (or RIBO-seq): method used to identify actively translated RNA molecules, based on deep sequencing of ribosome-protected mRNA fragments.

insults. In fact, it is also described for natural killer (NK) cells, NKT cells, **innate lymphoid cells** (ILCs), mast cells, basophils, and eosinophils [29–31]. However, the constitutive expression of effector mRNAs also poses a health risk. Effector molecules are highly toxic, and aberrant protein production could lead to chronic activation, inflammation, tissue damage, and autoimmune diseases [3–5]. It is therefore imperative that protein production from preformed mRNAs should occur primarily upon pathogenic or aberrant cellular insults.

To preserve their silent state in the absence of infection, memory T cells tightly regulate the turnover of preformed cytokine mRNA and the initiation of its translation into protein (Figure 1A) [21]. In resting murine $CD8^+$ T cells, the mRNA of effector molecules is generally unstable, and this limits its accumulation [15]. However, rapid mRNA turnover is not sufficient to avoid aberrant protein production. It was recently shown that the translation of preformed mRNA in murine $CD44^{hi}$-memory-like $CD8^+$ and $CD4^+$ T cells was actively blocked by the RBP ZFP36L2 [21]. This process depended on the presence of AU-rich elements (AREs) within the 3′ untranslated region (3′UTR) of *Ifng* and *Tnfa* mRNA, and ZFP36L2 directly bound to these AREs in human and mouse T cells [21]. The interaction between AREs and ZFP36L2 hampered the recruitment of preformed mRNA to ribosomes, blocked its translation into protein, and prevented aberrant cytokine production in non-activated $CD44^{hi}$-memory-like $CD8^+$ and $CD4^+$ T cells in mice [21]. Moreover, germ-line deletion of the *Ifng* ARE region [32] has also resulted in chronic IFN-γ production in resting murine NK cells and NKT cells [33], indicating that the ARE-dependent translational block of cytokine production from preformed mRNA also occurs in other immune cells. Of note, a recent study in mice showed that skin-resident *Staphylococcus epidermidis*-specific $CD4^+$ and $CD8^+$ T cells constitutively expressed a **type 2 immunity** transcriptome; however, protein production of type 2 cytokines (i.e., IL-5 and IL-13) only occurred upon intradermal injection of chitin or exposure to insect bites [34]. Given that most transcripts encoding inflammatory mediators, including *Il5* and *Il13*, contain AREs in their 3′UTR [35], it is conceivable that the translational block mediated by ZFP36L2 (or by another ARE-BP) is more broadly applied to block the undesirable release of effector molecules under homeostatic conditions.

AREs are not only present in inflammatory genes. They are found in ~16% of the human transcriptome [36]. New techniques that map RNA–protein interactions genome-wide to a single nucleotide of resolution (Box 2) have revealed that one RBP can interact with a subset of mRNAs sharing specific *cis*-regulatory elements, such as AREs [37]. In fact, ZFP36L2 does not only bind cytokine mRNAs [21]; together with its paralog ZFP36L1, it can also regulate the expression of transcription factors, kinases, and cell cycle genes in mice [37–40]. For example, CD2-Cre recombinase-mediated conditional deletion of ZFP36L1 and ZFP36L2 in mice resulted in increased expression of the oncogenic transcription factor Notch-1 in differentiating lymphocytes, which contributed to the development of T cell leukemia, relative to wild-type (WT) control mice [38,39]. Crosslinking immunoprecipitation (iCLIP) experiments in LPS-stimulated murine B cells revealed that ZFP36L1 targeted mRNAs encoding **PIM-family kinases** and cell cycle genes to enforce quiescence on late pre-B cells [37]. ZFP36L1 can also bind mRNAs encoding the transcription factors IRF8 and KLF2 to regulate the localization and survival of **marginal zone B cells** [40]. The mode of action of ZFP36L1 and ZFP36L2 is not yet fully elucidated. Nevertheless, these examples clearly demonstrate the critical role of RBPs in modulating gene expression in immune cells and in preserving their quiescent state in the absence of infection.

How Does ZFP36L2 Convey a Translational Block?

Given that ribosomal engagement and translation initiation occur at the 5′UTR of the mRNA, it appears counterintuitive that an RBP binding to the 3′UTR, as ZFP36L2 does, represses translation. However, the 5′UTR and the 3′UTR form a so-called mRNA closed-loop structure, which is

RNA-binding proteins (RBPs): proteins that contain domains known to directly interact with RNA molecules, or that reside within RBP complexes even if they do not directly contact RNA in some structurally characterized conformations.

Stress granule: RNA–protein aggregate containing translation initiation factors, poly(A) binding proteins, and ribosomal subunits; forms in the cytoplasm of eukaryotic cells undergoing stress.

Tissue-resident memory T cells: subset of non-circulating memory T cells that reside in barrier tissues, including skin, intestine, female reproductive tract, and lungs.

Toll-like receptor (TLR): class of transmembrane receptors recognizing structurally conserved molecules derived from pathogens or endogenous damage signals.

Type 2 immunity: adaptive immune response characterized by the production of IL-4, IL-5, IL-9, and IL-13, typically associated with allergic inflammation, tissue repair and fibrosis.

Zinc finger binding protein 36 (ZFP36) family of RNA-binding proteins: known to interact with ARE-containing mRNAs to regulate their stability and/or translation. It has three members: ZFP36 (also known as tristetraprolin; TTP or TIS11), ZFP36L1 (TIS11B or BRF1) and ZFP36L2 (TIS11D or BRF2). In rodents, a fourth family member has been reported, although ZFP36L3 is absent in humans.

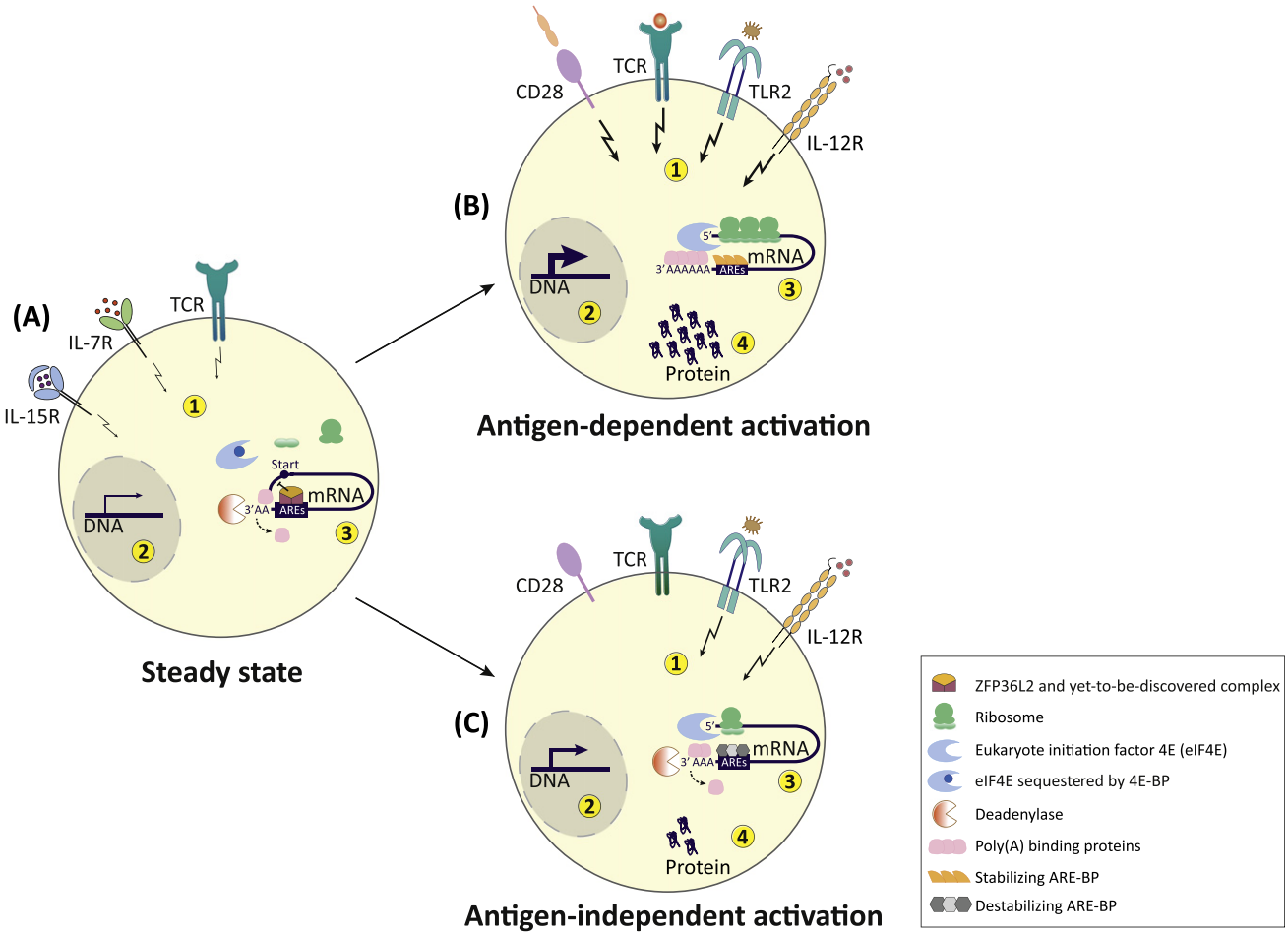

Figure 1. Rapid Switch of Post-Transcriptional Regulatory Events upon (Re)activation of Murine Memory CD8$^+$ T Cells. (A) Steady state: (1) Tonic signaling through the T cell receptor (TCR) and interleukin (IL)-7- and IL-15-mediated stimulation support homeostatic functions of memory T cells in the absence of infection. (2) Constitutive mRNA transcription ensures basal levels of preformed cytokine mRNAs. (3) mRNA decay and AU-rich element (ARE)-dependent block of ribosome recruitment impede the translation of preformed mRNA into protein. (B) Antigen-dependent activation: (1) When memory T cells encounter their cognate antigen, the signal strength and duration of stimulation determine the amount of cytokine produced. Co-stimulatory molecules [such as cluster of differentiation (CD)28 and Toll-like receptor 2 (TLR2)] and cytokines (such as IL-12) can enhance T cell activation. (2) Increased *de novo* mRNA transcription and (3) ARE-dependent mRNA stabilization promote (4) high amounts of protein production. (C) Antigen-independent activation: (1) Memory T cells can respond to the inflammatory environment in an antigen-independent manner. Stimulation can occur through cytokine receptors (e.g., IL-12R) and TLRs. (2) *De novo* mRNA transcription and (3) mRNA translation are engaged. (4) Rapid ARE-dependent degradation of newly synthesized mRNA limits the amounts of protein production. Abbreviations: BP, binding protein; ZFP36L2, Zinc finger binding protein 36 like 2.

mediated by a large RBP complex (Figure 1A) [41]. This structure allows RBPs interacting with the 3′ end to modulate processes at the 5′ end, such as mRNA decapping, translation repression, or translation initiation [42–44]. For example, in HEK 293T cells, ZFP36 (also known as tristetraprolin) recruits the DCP2 decapping complex and the CCR4-Not deadenylase complex at the 3′ end of target mRNAs [45]. ZFP36 also interacts with the cap-binding translation repression 4EHP-GIGYF2 complex at the 5′ end of target mRNAs in HEK 293T cells, in RAW264.7 macrophage-like cells, and in murine bone marrow-derived macrophages [46–48]. This combined interaction with the 3′ end and the 5′ end of an mRNA may allow ZFP36 to link mRNA decay to the block of translation. It is conceivable that ZFP36L2 also acts in a larger RBP–RNA complex that brings the 3′UTR and the 5′UTR in close vicinity and, therefore, stalls preformed

Box 2. Methods for Studying RBP–RNA Complexes

RNA is bound at all times by RBPs. In 2012, two studies provided the first comprehensive human mRNA-binding proteome [141,142]. This was achieved by ultraviolet (UV) crosslinking of RBPs to RNA followed by oligo(dT) capture and mass spectrometry (referred to as RNA interactome capture, a method that was recently further improved [143]). Overall, 1542 RBPs were identified. Of those, ~700 bind to mRNA and regulate mRNA-related processes [144]. Computational approaches can further help predict the RNA-binding capacity of proteins [145]. In addition, by extracting UV-crosslinked RNA–protein complexes with organic phase separation methods, the analysis of the genome-wide prevalence of RBPs has been extended to other RNA biotypes beyond mRNA [146–149]. Thus, all these methods can now be applied to different cell types and RNA species.

To identify mRNA targets of a specific RBP, RNA immunoprecipitation (RIP) followed by RT-PCR or deep-sequencing analysis (RIP-Seq) can be used [21,150,151]. However, native RIP (i.e., without crosslinking) only identifies abundant mRNAs or strong RBP–RNA interactions that are resistant to stringent washing steps. Therefore, UV-mediated crosslinking and RNA fragmentation were introduced and used for **crosslinking immunoprecipitation** (CLIP) [152]. Many CLIP approaches have been developed to map the binding of individual RBPs at a single-nucleotide resolution (reviewed in [153]).

RBPs can regulate the fate of mRNA by determining its stability, translation efficiency, and subcellular location. To monitor changes in mRNA stability, chemical inhibitors of *de novo* transcription, such as actinomycin D, α-amanitin, or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), have been widely used. Alternatively, metabolic labeling of RNA molecules with 4-thiouridine (4sU) or 4-thiouracil (4tU) can measure *de novo* transcriptional rates and mRNA turnover rates in pulse-chase experiments [154–156]. Recently, **4sU labeling** was applied to single-cell analysis [157]. To study global changes of translational efficiency, tRNA analogs (i.e., puromycin or *O*-propargyl-puromycin; OPP) or an amino acid analog of methionine [i.e., L-homopropargylglycine (HPG) or L-azidohomoalanine (AHA)] can be incorporated into nascent polypeptide chains and label *de novo* synthesized proteins *in vitro* and *in vivo* [65,158,159]. Polysome fractionation and **ribosome footprinting** (or RIBO-seq) instead measure translation efficiency in a gene-specific manner [15,22,160]. Furthermore, confocal microscopy with **fluorescence *in situ* hybridization** (FISH) probes can be used to determine the subcellular localization of RNA [161–163]. However, this method remains challenging in primary lymphocytes due to their small cell size and low cytoplasmic content.

Many novel tools are now available to unravel the dynamic behavior of RBPs in different cellular contexts. The challenge for immunologists is to apply these technologies to limited numbers of primary cells.

cytokine mRNA in memory T cells. However, because ZFP36L2 lacks the conserved tetra-proline motifs that ZFP36 uses to interact with the 4EHP-GIGYF2 complex, the binding partners of ZFP36L2 may be different. Thus, identifying the interacting partners of ZFP36L2 is key to better understanding how the translational block might be conferred in memory T cells.

The mechanisms and signaling pathways that promote the binding of ZFP36L2 to cytokine mRNA in non-activated T cells are also not well understood. mRNA molecules are constantly decorated with RBPs, yet the components of RBP–RNA complexes undergo dynamic signal-dependent alterations [49]. Noteworthy, the quiescent state of T cells is not a static 'off' state. When naive $CD4^+$ T cells from uninfected mice were immediately fixed upon isolation, low-level phosphorylation of S6 protein was observed, pointing to mTORC-1 activity and, thus, basal translation of mTOR targets during homeostasis [50]. In memory T cells, the constitutively active kinases lymphocyte-specific protein tyrosine kinase (Lck), zeta-chain-associated protein kinase (ZAP)-70, and the adapter protein signal-transducing adaptor protein SLP-76 confer tonic signals through the T cell receptor (TCR) [51–54]. These signals are required to maintain functionally competent naïve and memory T cells [52–54], and to reduce the threshold for T cell activation. In addition, the common γ-chain binding cytokines IL-7 and IL-15 ensures memory T cell survival and homeostatic proliferation [55,56], and propagates the permissive epigenetic signature of human memory T cells to daughter cells [57]. Thus, TCR and cytokine receptor-mediated tonic signals preserve the 'ready-to-go' state and, therefore, may also control the amount of preformed cytokine mRNA in memory T cells (Figure 1A). Furthermore, tonic signals may also regulate the stability and activity of RBPs [49,58]. Phosphoproteomic analysis of *in vitro* expanded murine $CD8^+$ T cells revealed that IL-2-dependent JAK1/3 signaling dynamically remodeled the

phosphorylation profile of the ZFP36 family of RBPs [59,60]. Given that the JAK/STAT and PI3K/AKT pathways are also activated downstream of IL-7 and IL-15 receptors [61], tonic signals received through these cytokine receptors might regulate the activity of ZFP36L2 to block translation of cytokine mRNA in memory T cells, thus preventing aberrant cytokine production from preformed mRNA in the absence of pathogenic insults.

T Cells Tune Cytokine Production by Context-Dependent Regulation of Gene Expression

Activated $CD8^+$ T cells rapidly divide and acquire effector functions. This process is initiated by antigen recognition, co-stimulation, and/or cytokine signals (Figure 1B) [62]. T cell activation involves extensive chromatin remodeling, increased transcription rates, and global alterations in translation [6,7,63–65]. Suboptimal TCR signaling results in limited induction of protein translation, concomitant with impaired proliferation [66].

Memory $CD8^+$ T cells rapidly differentiate into effector T cells, which produce substantial amounts of effector molecules within a few hours. This includes cytokines, such as TNF-α and IFN-γ, granzymes, perforins, and chemokines, such as CCL3, CCL4, and CCL5. Protective T cell responses are defined by the combined production of several effector molecules. For instance, the production of the three key cytokines TNF-α, IFN-γ, and IL-2 identifies effective $CD8^+$ T cell responses against viruses and tumors [67,68]. Even though TCR triggering drives the production of all three cytokines, their onset, duration, and magnitude of production in murine and human T cells is gene specific [15,69,70]. In fact, the interplay of *de novo* transcription with mRNA stability and translation efficiency is specific to each cytokine and dictates the temporal order of protein production (Figure 2) [15].

The imminent production of TNF-α upon antigen-specific activation of murine $CD8^+$ T cells is enabled by the dissociation of ZFP36L2 from preformed *Tnfa* mRNA and by its rapid recruitment to ribosomes ('Initiation' in Figure 2) [15,21]. Given that *Tnfa* mRNA does not become stabilized upon TCR triggering, TNF-α protein is only transiently produced during an early phase of activation and almost completely relies on the translation of preformed mRNA ('Resolution' in Figure 2) [15]. Similar to TNF-α, the early production of IFN-γ upon peptide stimulation of murine TCR transgenic OT-I T cells depends on preformed mRNA ('Initiation' in Figure 2) [15]. However, *de novo* transcription and mRNA stabilization of *Ifng* mRNA are also sequentially used to match the magnitude and the duration of IFN-γ production to the antigen load ('Peak' and 'Resolution' in Figure 2) [71]. Because T cells do not express preformed *Il2* mRNA, IL-2 production exclusively relies on *de novo* transcription. This is reflected by the later onset of protein production in human and mouse $CD8^+$ T cells [15,69,70]. As for IFN-γ, the kinetics and magnitude of IL-2 production are further shaped by the dynamic regulation of mRNA stability ('Peak' and 'Resolution' in Figure 2) [15]. These cytokine-specific regulatory networks and kinetics of production are determined by the role of different signaling pathways (discussed later). The engagement of signaling pathways is regulated by the quality (i.e., **antigen affinity** and type of co-stimulatory molecules engaged), strength (i.e., antigen load or stimulation doses), and duration of T cell stimulation [71].

Notably, *Tnfa*, *Ifng*, and *Il2* mRNAs all contain AREs within their 3′UTR. However, the mere presence of AREs does not predict whether mRNA stability or regulation of translation is the prime modulator for protein output. Studies performed in Jurkat T cells, primary murine $CD8^+$ T cells, and macrophages revealed that *Tnfa*, *Ifng*, and *Il2* mRNAs are putative targets of several ARE-BPs, such as the three ZFP36 family members, as well as the RBPs HuR, AUF-1, TIA-1, and others [21,22,72–75]. The mechanistic relationship between these ARE-BPs remains however

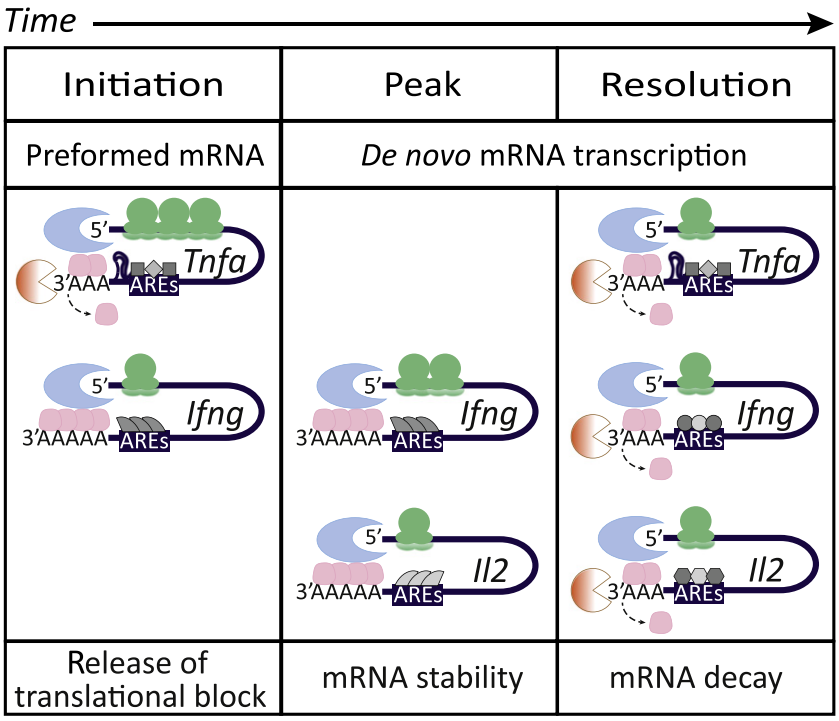

Figure 2. Examples of Cytokine-Specific Regulatory Networks during CD8$^+$ T Cell Activation. Tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-2 follow individual kinetics of production upon activation of human and mouse CD8$^+$ T cells. Initiation: the immediate production of TNF-α and IFN-γ is promoted by the constitutive expression of preformed mRNA, which is released from its translational block and rapidly engaged by ribosomes. Peak: initiation of IL-2 production and increased magnitude of IFN-γ response require *de novo* mRNA transcription and mRNA stabilization. Resolution: timely shutdown 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 AU-rich element–RNA-binding protein (ARE–RBP) complexes.

unresolved. We speculate that co-regulation through different RBPs and/or other *trans*-factors, such as non-coding RNA, defines a dynamic and flexible regulation. We anticipate that these regulatory mechanisms and the activity of *trans*-acting factors are cell type and transcript specific, depend on the subcellular localization of the target mRNA, and are instructed by context and signal strength.

The transcript specificity of RBPs can be influenced by their affinity to target mRNAs. For example, the RBPs HuR and TIA-1 recognize generic U-rich elements [76,77], whereas the three ZFP36 family members specifically interact with AU-rich elements. However, ZFP36, ZFP36L1, and ZFP36L2 may bind AREs with different affinity, given that structural studies have suggested limited homology (60%) within the RNA-binding tandem zinc finger domain between ZFP36 and ZFP36L1/ZFP36L2 [78,79].

Another important determinant of RBP activity is the subcellular localization of mRNA. ARE-mediated mRNA decay generally takes place in processing bodies (**p-bodies**), whereas translationally stalled mRNAs are associated with **stress granules** (reviewed in [13,80]). P-bodies and stress granules are dynamic structures comprising a conglomeration of RNA molecules and RBPs. RBPs can operate within different intracellular compartments. Studies in HeLa and NIH 3T3 cell lines revealed that the RBPs Roquin and Regnase-1 recognize and degrade

the same mRNA targets but, while Roquin acts in p-body and/or stress granules, Regnase-1 interacts with ribosomes within the endoplasmic reticulum [81]. Likewise, ZFP36L1 can interact with target mRNAs in HeLa cells in membrane-free structures associated with the endoplasmic reticulum [82]. Whether similar mechanisms also occur in T cells remains unknown and is challenging to study due to the small cell size and low cytoplasmic content of primary T cells.

RBPs can also be expressed, and thus be active, during different stages of T cell activation and differentiation. For example, whereas ZFP36L2 is already expressed by quiescent $CD8^+$ T cells, ZFP36 and ZFP36L1 expression increases upon activation [21,22]. Of note, ZFP36 can autoregulate its own abundance by binding to the AREs within the *Zfp36* 3′UTR in mouse RAW264.7 macrophage-like and human THP-1 myelomonocytic cells [83,84]. Given that *Zfp36l1* and *Zfp36l2* mRNAs also contain AREs, cross-regulation of these three family members is feasible. In conclusion, whether ARE-BPs cooperate in post-transcriptional regulatory events, or whether they act independently in a spatially and temporally compartmentalized manner is still under debate and requires evaluation at the level of individual transcripts.

Is Post-Transcriptional Regulation Involved in Antigen-Independent $CD8^+$ T Cell Responses?

Memory T cells can also be activated in an antigen-independent manner. They respond to cytokines, such as IL-12, IL-18, IL-15 and type I interferons (IFN-α and IFN-β), and to **Toll-like receptors** (TLR2 and TLR7) [85–88]. After receiving this so-called **bystander stimulation**, murine memory T cells undergo a robust program of activation that includes increased expression of the transcription factors T-bet and Eomesodermin, the activation markers CD69, CD25 and CD11a, and the cytotoxic molecules IFN-γ, granzyme B, and perforin [89]. Sensing inflammation-derived molecules renders memory T cells an active component of the first line of defense that prevents pathogen spreading early during infection [90,91].

Notably, antigen-dependent and antigen-independent stimulation of T cells display different cytokine production profiles [71]. Even though memory T cells contain preformed mRNAs encoding IFN-γ and TNF-α [15], only IFN-γ is produced upon TLR-dependent activation of murine $CD8^+$ T cells [88]. However, TLR triggering induces IFN-γ production exclusively by *de novo* transcription without inducing mRNA stabilization. Furthermore, preformed *Ifng* mRNA is not used (Figure 1B,C) [88], indicating that TLR engagement is not sufficient to release the translational block of preformed *Ifng* mRNA. Although bystander activation probably defines the most rapid memory T cell response, the inability to translate preformed mRNA and the requirement for *de novo* transcription without mRNA stabilization may provide a safeguard to limit T cell responses to non-cognate infections. Deregulating bystander activation could in fact cause the exacerbation of cytokine production, which in turn could lead to tissue damage and immunopathology [92].

Signaling Pathways Driving Cytokine Production and RBP Activity in $CD8^+$ T Cells

Diverse stimuli regulate cytokine production by activating specific signaling cascades. Pioneering studies in human and mouse T cells demonstrated that Ca^{2+} flux, Protein Kinase C (PKC), PI3K/AKT, and MAPK signaling promote the *de novo* transcription of cytokine mRNAs, whereas mTOR signaling steers mRNA translation [16,93,94]. These signaling pathways are central to driving T cell responses upon antigen recognition, engagement of co-stimulatory receptors, and bystander activation (Figure 3).

Transcription of effector molecules encoding mRNAs relies on crosstalk between different signaling cascades, mediated through TCR engagement and costimulation (reviewed in [93,95,96]).

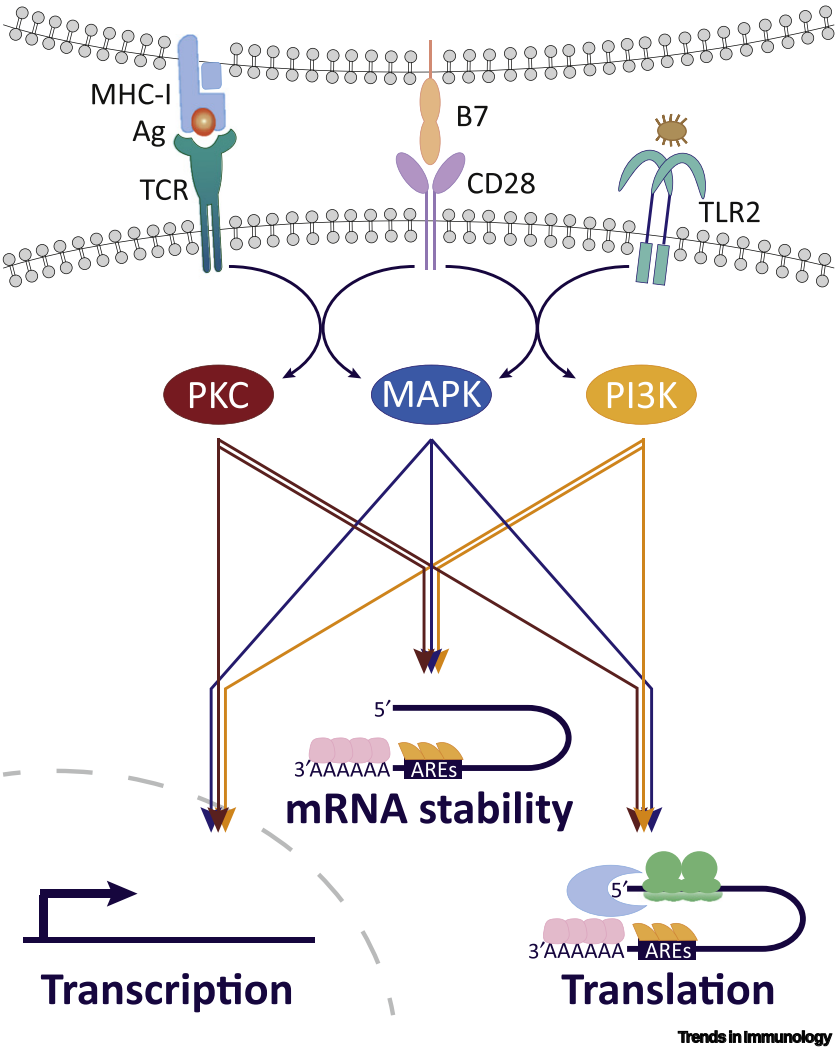

Figure 3. Protein Kinase C (PKC)-, Mitogen-Activated Protein Kinase (MAPK)-, and Phosphoinositide 3-Kinase (PI3K)-Mediated Signaling Cascades Regulate and Amplify the Transcription, Stability, and Translation of Cytokine mRNAs in $CD8^+$ T Cells. T cell activation is initiated by T cell receptor (TCR) triggering, and enhanced by co-stimulatory molecules, such as cluster of differentiation (CD)28 and Toll-like receptor 2 (TLR2). Each receptor activates three main signaling cascades dominated by the kinases PKC, MAPK, and PI3K, that can instruct both transcriptional and post-transcriptional events. We propose that the coordinated triggering of TCR, CD28, and TLR2 and the respective signaling pathways strongly enhances *de novo* transcription, as well as mRNA stability and translation initiation of cytokine mRNAs, thus enabling optimal T cell responses. Abbreviations: Ag, antigen; ARE, AU-rich element.

However, post-transcriptional regulation may primarily depend on TCR-mediated signaling [15,71,97]. Stimulating murine $CD8^+$ T cells individually with phorbol myristate acetate (PMA) or Ca^{2+} ionophore (ionomycin), and using pharmacological inhibitors showed that Ca^{2+} flux is a major driver of *de novo* transcription, whereas TCR-mediated PKC activation orchestrates cytokine production by regulating mRNA stability and translation [15]. Notably, this occurs in a transcript-specific manner: PKC signaling recruits *Tnfa* mRNA to polysomes and, thus, drives mRNA translation and protein production, a feature that is only prominent for *Tnfa* mRNA [15]. By contrast, PKC signaling stabilizes the mRNAs encoding IFN-γ and IL-2, a phenomenon that is not observed for TNF-α [15].

The differences in PKC-mediated post-transcriptional regulation may stem from different RBP-binding hubs present in the cytokine transcripts, which can comprise RNA sequences as well as secondary structures. For instance, only the 3′UTR of *Tnfa* contains a constitutive decay element (CDE) in addition to AREs [98]. Thus, it is conceivable that the CDE prevents *Tnfa* mRNA stabilization in T cells and allows for short and well-controlled cytokine production of this highly toxic cytokine. However, the inability to stabilize *Tnfa* mRNA is cell type specific. In fact, the TNF-α production in RAW264.7 macrophage-like cells also depends on *de novo* transcription and mRNA stabilization [98]. This discrepancy between macrophages and T cells in using different post-transcriptional nodes to drive TNF-α protein production may stem from different expression levels of preformed mRNA, differential RBP expression, and/or divergent signaling pathways driving their immune responses. Thus, cell specificity adds an extra challenge and layer of complexity in elucidating cytokine-specific regulatory networks.

How signaling pathways determine the function of RBPs to tailor cytokine production is only partially understood. PI3K/AKT and MAPK can phosphorylate RBPs, which defines their protein stability and function (reviewed in [99]). Using ZFP36L1 phosphorylation site mutants in mouse embryonic fibroblasts demonstrated that AKT-dependent phosphorylation protected ZFP36L1 from proteasomal degradation, yet restrained its ability to drive mRNA decay [100]. Similarly, MAP kinase signal-integrating kinases (Mnks) and p38 MAPK-activated protein-kinase-2 (MK2) can phosphorylate and inhibit the RNA-decay promoting activity of the RBPs hnRNPA1, ZFP36L1, and ZFP36, respectively, in Jurkat T cells and in HEK 293T cells [101,102]. Moreover, phosphatases are also induced upon stimulation, which further regulates the highly dynamic phosphorylation of RBPs [58]. This is exemplified by the serine-threonine protein phosphatase PP2A, which regulates p38 and MK2 as well as directly dephosphorylates ZFP36 in a mouse alveolar macrophage cell line [103].

RBP phosphorylation can also modify the composition of RBP–RNA complexes by modulating the protein structure, and by altering the affinity of an RBP to the target RNA. For example, in immortalized bone marrow-derived macrophages and RAW264.7 macrophage-like cells, MK2 phosphorylated ZFP36 and reduced its ability to bind *Tnfa* mRNA [104]. Thus, phosphorylated ZFP36 failed to compete with the RBP HuR for binding and allowed HuR-mediated translation of *Tnfa* mRNA [104], a mechanism that might also apply to other cytokines [72]. In fact, PKC-mediated phosphorylation of HuR and of the RBP NF-90 allowed these two RPBs to stabilize *IFNG* and *IL2* mRNA in activated Jurkat T cells [72,75,105]. Given that ribosome recruitment of preformed *Tnfa* and *Ifng* mRNA in murine $CD8^+$ T cells depends on PKC signaling [15], we hypothesize that PKC might also directly or indirectly modify ZFP36L2 to inactivate its inhibitory function, thereby contributing to releasing the ZFP36L2-mediated translational block upon reactivation [15,21]. However, the mechanism by which PKC coordinates cytokine-specific post-transcriptional events remains to be resolved.

Post-Transcriptional Regulation Can Contribute to Impaired Cytokine Production of $CD8^+$ T Cells in Tumors

Persistent antigen exposure during a chronic infection or in the tumor microenvironment can result in the gradual loss of T cell effector function [106]. Indeed, these dysfunctional $CD8^+$ T cells express high amounts of inhibitory receptors, such as programmed cell death protein 1 (PD-1), a state also known as ‘exhaustion’ [107]. PD-1 signaling affects many pathways that drive T cell activation. For instance, PD-1 ligation counteracts TCR triggering by blocking ZAP70 phosphorylation and its association with the TCR subunit CD3ζ in human T cells [108]. In addition, PD-1 interferes with the intracellular Ca^{2+} flux [109], which activates the transcription factor NFAT. NFAT associates with AP-1 under physiological conditions and drives transcription of cytokine mRNAs upon T cell activation [110]. However, during chronic T cell activation, NFAT binds

to target genes independently from AP-1 and promotes the expression of inhibitory receptors, including PD-1 [111,112]. PD-1 also directly impairs CD28-mediated signaling [113], and blocks TCR- and CD28-mediated activation of PKCθ and PI3K/AKT, which results in loss of IL-2 production and T cell proliferation [108,114]. Given that CD28 co-stimulation supported the therapeutic benefit of anti-PD-L1 therapy in studies of CT26 colon carcinoma-bearing mice [115], it is possible that dysfunctional $CD8^+$ T cells use CD28-mediated signaling to restore effective immune responses, although this warrants further investigation.

In addition to alterations in transcriptome and epigenetic landscapes [116–119], it was recently observed that post-transcriptional events mediate $CD8^+$ T cell dysfunction in mouse and human tumor-infiltrating T cells (TILs) [120]. Specifically, TILs contain preformed *Ifng* mRNA yet fail to produce IFN-γ protein [120]. This feature resembles that of non-responsive, so-called **anergic self-tolerant T cells** [121], and of resting memory T cells [21]. The progressive loss of IFN-γ production in murine TILs directly correlated with the acquired loss of *Ifng* mRNA stability, an effect mediated by the ARE region. In fact, germ-line deletion of the ARE region was shown to prolong the stability of *Ifng* mRNA, restore IFN-γ production in TILs, and delay B16 melanoma outgrowth in mice [120]. Of note, CD28-mediated co-stimulation has been reported to stabilize *Ifng* mRNA in human $CD3^+$ T cells [122]. Engaging CD28 with stimulatory antibodies also stabilized *Ifng* mRNA in murine $CD8^+$ T cells exposed to B16 tumor cells for 3 consecutive days to mimic chronic antigen exposure [120]. Conversely, in such models, inhibiting PD-1 signaling with blocking antibodies enhanced protein production without affecting the amounts of *Ifng* mRNA, its stability, and/or the epigenetic state of the *Ifng* gene [118,120]. These data suggest that PD-1 signaling blocks the translation of *Ifng* mRNA. Whether ZFP36L2 regulates the PD-1 mediated block of IFN-γ protein production remains to be determined. Of note, expression of the ligand for PD-1, PD-L1, is also mediated by post-transcriptional events, and its mRNA stability is regulated by ZFP36 [123]. Thus, post-transcriptional regulation may be applied in various ways to block T cell function in certain tumors. Although this may be tumor and species specific, it certainly merits further attention. Given that post-transcriptional networks may boost the effectiveness of tumor therapies, identifying the RBPs and the signaling pathways that control post-transcriptional events in TILs is of paramount importance.

Concluding Remarks

In this review, we described how RBPs can mediate the fate of mRNA and, thus, define $CD8^+$ T cell effector function. We show that post-transcriptional regulatory events are versatile and context dependent, and allow for rapid and ample, yet tightly controlled, cytokine production. The dynamic interaction of RNA molecules with different RBPs was conceptually introduced in 1969 by Alexander Spirin, with the hypothesis of 'informosomes': *'omnia mea mecum porto'* ('All that is mine I carry with me') [124]. Spirin also proposed that alterations in the composition of RBP complexes defined RNA biogenesis and function [125]. Fifty years later, we are only beginning to understand which RBPs are expressed in primary T cells, how RBPs modulate T cell responses, and which signaling pathways regulate RBP activity upon T cell activation (see Outstanding Questions). Given that protein production can be regulated in a cell type-specific manner (i.e., the regulation of TNF-α production in T cells versus macrophages), one needs to be cautious in translating findings directly from one model system to the other. Thus, addressing the effects of RBPs in different T cell subsets is key and requires the implementation of recently developed methods (Box 2) to study primary T cells in depth. The combination of genome-wide RNA analysis and transcriptome-wide RBP analysis can help elucidate basic principles of RBP-mediated gene regulation. This may allow us to deepen our understanding of gene-specific regulatory networks, and inform the identification of intelligent new targets to help restore T cell effector functions for therapeutic benefit.

Outstanding Questions

Which RBPs are expressed in primary $CD8^+$ T cells and how does their landscape change upon activation?

Which mode of action is used by RBPs to modulate T cell responses? Can one RBP control the fate of different RNA targets simultaneously?

Do RBPs work alone or form dynamic complexes? How does this influence transcript-specific regulatory networks in primary T cells?

Which intracellular pathways regulate RBP activity during T cell activation and differentiation?

Which post-translation modifications affect RBP stability, the interaction of RBPs with target mRNAs, and thus RBP function in primary T cells?

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