Walking the wire

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

Salerno, F.

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Chapter 3

Distinct PKC-mediated post-transcriptional events set cytokine production kinetics in CD8^+ T cells

Fiamma Salerno^a, Nahuel A. Paolini^a, Regina Stark^a, Marieke von Lindern^a, and Monika C. Wolkers^a

^a Sanquin Research, Department of Hematopoiesis and Landsteiner Laboratory, Academic Medical Centre (AMC), University of Amsterdam, Amsterdam, The Netherlands

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Abstract
Effective T cell responses against invading pathogens require the concerted production of three key cytokines: TNF-α, IFN-γ, and IL-2. The cytokines functionally synergize, but their production kinetics widely differ. How the differential timing of expression is regulated remains however poorly understood. We compared the relative contribution of transcription, mRNA stability, and translation efficiency on cytokine production in murine effector and memory CD8+ T cells. We show that the immediate and ample production of TNF-α is primarily mediated by translation of pre-formed mRNA through Protein Kinase C (PKC)-induced recruitment of mRNA to polyribosomes. Also the initial production of IFN-γ utilizes translation of pre-formed mRNA. However, the magnitude and subsequent expression of IFN-γ, and of IL-2 depends on calcium-induced de novo transcription and PKC-dependent mRNA stabilization. In conclusion, PKC signaling modulates translation efficiency and mRNA stability in a transcript-specific manner. These cytokine-specific regulatory mechanisms guarantee that T cells produce ample amounts of cytokines shortly upon activation and for a limited time.
Introduction

Effective CD8+ T cell responses require the production of three key cytokines: Tumor Necrosis Factor alpha (TNF-α), Interferon gamma (IFN-γ), and Interleukin-2 (IL-2). Intriguingly, T cells release these cytokines in a serial fashion, and not simultaneously1,2, reflecting their consequential functions. The immediate production of TNF-α induces apoptosis of infected cells, and promotes leukocyte infiltration by regulating chemokine release and vascular permeability3,4. The slightly later produced IFN-γ blocks microbial replication and potentiates innate immune responses by directly activating macrophages5 and recruiting neutrophils6. The survival and proliferation signals provided by IL-2 are required at later stages of activation. Importantly, all three cytokines have adverse effects when aberrantly produced, which results in immunopathology for TNF-α and IFN-γ7,8, and in Activation-induced Cell Death of T cells for IL-29.

Several mechanisms drive cytokine production upon T cell activation. The transcription factors NFAT, AP-1 and NFκB are engaged downstream of Ca2+ signaling, protein kinase C (PKC) and (MAP)-Kinase signaling10,11,12,13. PKC, MAPK and PI3K/Akt signaling can also control the turn-over of cytokine mRNAs14,15,16, whereas mTOR is critical to drive the translation of mRNA into protein17,18. How these regulatory networks feed into sequential cytokine production, however, is yet to be determined. We hypothesized that each cytokine requires custom-made regulatory pathways to obtain appropriate anti-microbial responses without inducing severe side effects. Here, we show that the timing of these three cytokines is determined by differential control of transcription, mRNA stability and translation. Importantly, PKC signaling orchestrates this timing in a cytokine-specific manner.

Results

Sequential production of cytokines in T cells

To determine the kinetics of cytokine release, we activated OT-I T cells for 20h with OVA257–264 peptide/CD80 expressing cells17. Activated T cells were removed from the antigen after 20 hours and rested for 3-13 days in the presence of IL-7. We then re-activated these effector T cells with OVA257–264 peptide in the presence of brefeldin A (BrefA) to block protein secretion, and measured the intracellular cytokine levels of TNF-α, IFN-γ, and IL-2.

TNF-α was detectable within 30 min of stimulation and reached its plateau within the first 2h, whereas IFN-γ and IL-2 were measured from 1h and 2h of activation onwards (Fig 1A). Protein levels of all cytokines steadily increased until 6 hours (Fig 1A). Whereas this experimental set-up displays the maximal capacity of cytokine production, it cannot distinguish whether cytokines are continuously or transiently generated. Therefore, we added BrefA to activated T cells at later time points, for a maximum of 2h (Fig 1B). More than 90% of T cells produced TNF-α during the first hours, which dropped to 20% in the last 2h of stimulation (Fig 1B). IL-2 production also declined in the last 2h, whereas IFN-γ production was robust over time (Fig 1B)1,2. Of note, the kinetics of cytokine production was independent of culture conditions with different cytokines, i.e. IL-7, IL-2 or IL-15 (Fig S1A). Furthermore, the low affinity T4 variant of OVA257–264 peptide (aa: SIITFEKL)19 only altered the magnitude of response but not the kinetics (Fig S1A).

When T cells produce two or more cytokines, they represent the most potent
effector cells against pathogens\textsuperscript{20, 21}. We found that the overall percentage of cytokine-producing T cells was constant, but the production profile evolved over time (Fig 1C, Fig S1B). TNF-α dominated the first 2h (91±4%, of which 54±5% produced TNF-α alone, and 37±6% in combination with IFN-γ and/or IL-2). At 4h most T cells (90±2%) produced at least 2 cytokines. At 6h, the T cell response shifted towards IFN-γ production (90±5%, of which 48±6% produced IFN-γ alone, and 51±5% together with TNF-α and/or IL-2; Fig 1C, Fig S1B). Also memory T cells rapidly produce cytokines upon reinfections\textsuperscript{22}. Indeed, naturally occurring CD44\textsuperscript{hi} memory-like OT-I T cells that can protect from reinfections\textsuperscript{23} showed similar production kinetics as in vitro generated effector T cells, irrespective of antigen affinity (Fig 1D). Importantly, effector memory (EM) and central memory (CM) T cells from Lymphocoriomeningitis virus (LCMV)-infected mice (d60) also rapidly produced TNF-α and IFN-γ upon stimulation with the cognate GP33 and NP396 peptides, and IL-2 production was delayed and of short nature (Fig 1E). Different cytokine production kinetics is thus a general feature of effector and of memory T cells.

Figure 1: TNF-α, IFN-γ and IL-2 follow individual production kinetics
(A, B) Cytokine production of resting CD8\textsuperscript{+} OT-I T cells stimulated with 100nM OVA peptide. Brefeldin A (BrfA) was present throughout the stimulation (A) or added for the last 0.5, 1, or 2h of activation (B). (C) Cytokine profile analysis of T cells activated for 2, 4 or 6h from (B). Top panel: percentage of T cells that produce at least one cytokine. Bottom panel: cytokine production of responding T cells. Pooled data from 3 independently performed experiments (mean±SD; n=7 mice). (D) CD44\textsuperscript{hi} memory-like OT-I T cells activated with peptide-loaded bone marrow derived dendritic cells. N4: SIINFEKL, T4: SIITFEKL (n=5 mice). (E) Effector memory (EM, left) and central memory (CM, right) T cells from LCMV infected mice (d60) were reactivated with GP33 and NP396 peptide (n=5 mice).
Pre-formed Tnfa and Ifng mRNA drives rapid protein production

We next interrogated what determines the differential onset of cytokine production. Rapid protein synthesis can be initiated from pre-formed mRNA. Interestingly, at all culture conditions tested resting CD8⁺ T cells contained more Tnfa mRNA than Ifng mRNA, and Il2 mRNA was almost undetectable (Fig S1C, Fig S2A). To examine whether pre-formed mRNA can drive the rapid TNF-α production, we pre-treated T cells with actinomycin D (ActD) to block de novo transcription, or with cycloheximide (CHX) to block de novo translation prior to activation. CHX significantly blocked the production of TNF-α, but ActD failed to do so.

Figure 2: Pre-formed cytokine mRNA promotes early T cell responsiveness

(A) Heatmap of the relative expression (Z-scores) of Tnfa, Ifng and Il2 mRNA (ref 24: GSE70813). (B) Tnfa, Ifng and Il2 mRNA levels of CD8⁺ CD44low naive T cells and CD8⁺ CD44hi memory-like T cells (n=10 mice) [Paired student t-test; **p<0.005; ***p=0.0001]. (C) Relative percentage of TNF-α, IFN-γ, and IL-2 producing CD8⁺ CD44hi T cells pre-treated with Actinomycin D (ActD) or cycloheximide (CHX) for 30 min prior to 2h stimulation with OVA peptide (n=6 mice). (D) Cytokine production of EM and CM T cells from LCMV-infected mice pre-treated with ActD prior to 2h GP33 and NP396 peptide stimulation. Representative of 5 mice from 2 independently performed experiments.
The percentage of TNF-α producing T cells remained 76±15% of the control at 30min of stimulation (Fig S2B). Thus, CD8\(^+\) T cells primarily employ translation of pre-formed mRNA to produce TNF-α. Interestingly, pre-formed *Ifng* mRNA also allows for rapid protein production. After 1h of activation, 42±4% of IFN-γ producing T cells remained in the presence of ActD (Fig S2C). IL-2, however, depended almost exclusively on *de novo* transcription. Only 13±4% T cells remained IL-2\(^+\) with ActD (Fig S2D).

Bona fide memory T cells specific for *Herpes Simplex Virus* (HSV) and LCMV-specific memory T cells\(^24\) also contain pre-formed *Tnfa* and *Ifng* mRNA, but not of *Il2*, in almost all memory T cell subsets (Fig 2A). Similarly, CD44\(^{hi}\) memory-like T cells express significant *Tnfa* and *Ifng* levels (Fig 2B). Importantly, both CD44\(^{hi}\) T cells and LCMV-specific EM and CM T cells use pre-formed *Tnfa* and *Ifng* mRNA for cytokine production, shown by the negligible effect of ActD on their protein levels (Fig 2C, D). Of note, the levels of pre-formed *Ifng* mRNA were higher in memory than in effector T cells (compare Fig 2A, B with Fig S2A), which corresponds with higher usage of pre-formed mRNA (Fig 2C, D) and a more similar onset of production with TNF-α (Fig 1D, E). Again, IL-2 production primarily depended on *de novo* transcription (Fig 2C, D). Combined, our data demonstrate that TNF-α, IFN-γ, and IL-2 follow different production kinetics in effector and in memory CD8\(^+\) T cells, which is at least in part determined by the levels of pre-formed mRNA available for rapid translation.

**PKC signaling contributes to translation initiation**

Translation of pre-formed *Tnfa* and *Ifng* mRNA into protein suggests a swift engagement of cytokine mRNA into polyribosomes, a process that is determined by the availability of the eukaryote initiation factor 4E (eIF4E)\(^{25, 26}\). eIF4E is captured by hypo-phosphorylated eIF4E-binding proteins (4E-BPs). Phosphorylation of 4E-BP by mTOR releases eIF4E to bind mRNA 5'-cap structures to facilitate the formation and scanning of the pre-initiation scanning complex, which allows ribosomes to assemble at start codons\(^26\). Indeed, resting T cells primarily expressed the unphosphorylated α-isoform and the partially phosphorylated β-isoform of 4E-BP1 that both repress translation (Fig 3A)\(^{25}\). Stimulation with OVA peptide rapidly decreased the unphosphorylated α-isoform and induced the fully phosphorylated, inactive γ-isoform of 4E-BP1 (Fig 3A).

We and others showed that mTOR activity drives the translation of cytokines\(^{17, 27}\). In line with that, pre-incubating T cells with rapamycin to inhibit mTOR blocks 4E-BP1 phosphorylation (Fig 3B). TCR-mediated PI3K/Akt signaling phosphorylates mTOR and its downstream target, the ribosomal protein S6 (Fig 3C, Fig S3A). This was effectively blocked when T cells were pre-treated with the pan-PI3K inhibitor ZTSDK474 (Fig 3C, Fig S3A). TCR triggering, however, also engages other crucial signaling pathways, such as the Ca\(^{2+}\) flux that activates calcineurin\(^{28}\), and Protein Kinase C (PKC)\(^{29}\). We therefore questioned whether Ca\(^{2+}\) flux and/or PKC signaling also mediate mTOR activity and thus translation. Inhibiting calcineurin with cyclosporine A (CsA) did not affect mTOR and S6 phosphorylation (Fig 3C, Fig S3A). In contrast, blocking PKC with LY-333531 (specific inhibitor of PKC-β isoform) or sotrastaurin (pan-PKC inhibitor) prior to activation significantly impaired mTOR and S6 phosphorylation (Fig 3C, Fig S3A). Thus, TCR-dependent activation of PKC can enhance mTOR activity.

PKC promoted mTOR and S6 phosphorylation independently from PI3K signaling. Stimulating T cells with PMA alone to directly engage PKC sufficed for mTOR and S6
phosphorylation, whereas promoting the intracellular Ca\textsuperscript{2+} influx with ionomycin had no effect (Fig S3B). Furthermore, pre-treating cells with PKC inhibitors LY-333531 and sotrastaurin effectively blocked mTOR and S6 phosphorylation upon PMA/ionomycin stimulation (Fig 3D, Fig S3C). Importantly, the PI3K inhibitor ZTSK474 and the calcineurin inhibitor CsA failed to do so, demonstrating that PKC-mediated activation of mTOR was independent from PI3K and Ca\textsuperscript{2+} influx (Fig 3D). T cell activation with PMA alone was also sufficient to induce the hyperphosphorylated inactive γ-isoform of 4E-BP1 (Fig S3D). Again, this shift was blocked by rapamycin, and by LY-333531 or sotrastaurin, but not by CsA (Fig 3E). In accordance with a more diverse signal upon TCR triggering and a possible redundancy between the PI3K and the PKC pathway, inhibiting PKC upon OVA peptide stimulation did not reduce the migration of 4E-BP1 towards its γ-isoform (Fig S3E). Altogether, our data show that PKC signaling converges with the PI3K pathway to regulate mTOR activity and to initiate mRNA translation of activated T cells.

**Differential role of PKC and Ca\textsuperscript{2+} in cytokine production**

We next questioned how PKC and Ca\textsuperscript{2+} signaling contribute to cytokine production. As antigen stimulation alike, PMA/ionomycin activation induced cytokine production with identical production kinetics (Fig 4A; Fig S4A, B). Intriguingly, however, PKC and Ca\textsuperscript{2+} signaling differentially contributed to the individual cytokine production. Ionomycin only marginally supported TNF-α, while IFN-γ largely depended on Ca\textsuperscript{2+} signaling. In sharp contrast, PMA stimulation alone had limited effects on IFN-γ, but greatly promoted TNF-α production (Fig 4A; Fig S4A). This finding was confirmed with PKC and calcineurin inhibitors in PMA/ionomycin- and in antigen-stimulated T cells (Fig 4B, C). This indicates that PKC and

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**Figure 3: PKC signaling promotes translation initiation**

(A, B) 4E-BP1 expression in resting and OVA peptide-stimulated T cells (A), and in OVA-stimulated T cells pretreated for 30min with rapamycin (B). (C, D) pmTOR and pS6 levels 30min after T cell activation with OVA peptide (C), or with PMA/ionomycin (D). Indicated inhibitors were added 30min before T cell activation. (E) 4E-BP1 expression of PMA/ionomycin activated T cells that were pretreated or not with indicated inhibitors. Representative of 2 independently performed experiments (A, B, E), and of 10 mice from 3 experiments (C, D).
**Figure 4: Role of PKC and Ca$^{2+}$-dependent signaling in cytokine production**

(A) Resting T cells were stimulated or not for 4h with PMA, ionomycin, or with both (PMA/iono). Numbers in dot plots indicate percentage of TNF-α+ and/or IFN-γ+ T cells. Histogram depicts IL-2 production of the same sample. (B, C) T cells were pre-treated or not with indicated inhibitor prior to activation with PMA/ionomycin (B), or with OVA peptide (C). Representative of 3 independently performed experiments. (D-F) TNF-α (D), IFN-γ (E) and IL-2 (F) protein levels (left panels) and mRNA levels (right panels) of CD44hi memory-like T cells were determined after 4h and 2h of indicated stimulation, respectively (n=4 mice). [One-way ANOVA with Tukey’s comparison; *p<0.05; **p<0.005; ***p=0.0005; ****p<0.0001]. (G-I) Polysome fractionation of T cells activated for 2h with indicated stimuli. Tnfa (G), Ifng (H), and Il2 (I) mRNA distribution through all 20 collected fractions. Representative of two independently performed experiments.

Ca$^{2+}$ signaling have divergent roles for cytokine production also when all TCR-mediated signaling nodes are present (Fig 4C, Fig 5A-D).

Strikingly, cytokine production did not necessarily correlate with mRNA levels. PMA alone induced substantial production of TNF-α protein in effector and CD44hi memory-like T cells (Fig 4A, D; Fig 5A), but this did not correspond with significant changes in Tnfa mRNA (Fig 4D; Fig 5A). Increases of transcript levels became apparent only upon combined PMA/ionomycin activation (Fig 4D). In contrast, Ifng mRNA already increased upon single stimulation with ionomycin, and was further boosted by the combination of PMA/ionomycin (Fig 4E; Fig 5A). Thus, Ifng but not Tnfa transcript levels match with the protein levels.
We next questioned how PKC and Ca\(^{2+}\) signaling influenced the translation efficiency of \(Tnfa\) and \(Iffg\) mRNA. We fractionated polyribosomes from cytoplasmic extracts of activated T cells by sucrose gradient centrifugation, and determined the distribution of \(Tnfa\) and \(Iffg\) transcripts between free and polyribosome-bound fractions of increasing density (Fig 4G, H; Fig S4F). A large fraction of \(Tnfa\) mRNA remained as free-mRNA upon T cell activation with ionomycin alone (Fig 4G). PMA activation, however, shifted \(Tnfa\) from free-mRNA towards polyribosome-bound fractions. These findings are fully compatible with the capacity of PKC to phosphorylating 4E-BP1 (Fig 3E) and to produce TNF-α protein (Fig 4 A-D). Combined PMA/ionomycin activation resulted in further shifts of \(Tnfa\) mRNA from light to heavy-polyribosomes (Fig 4G), indicating that Ca\(^{2+}\) signaling - although not sufficient to initiate \(Tnfa\) translation - can contribute to protein production by increasing \(Tnfa\) mRNA levels, and by enhancing polysomal recruitment.

In contrast to \(Tnfa\), we found \(Iffg\) already in polyribosomes in ionomycin activated cells (Fig 4H). PMA stimulation, however, was more effective at evoking high-density ribosome loading of \(Iffg\) mRNA (Fig 4H). Because ionomycin also induced de novo transcription of \(Iffg\) (Fig 4E; Fig S4E), we postulate that newly synthesized \(Iffg\) mRNA is immediately engaged by ribosomes, resulting in the observed protein production.

IL-2 production required both PMA and ionomycin stimulation for measurable increases of mRNA levels and cytokine production (Fig 4A, F). As \(Iffg\) mRNA alike, \(Il2\) mRNA always associated with polyribosomes, which was further enhanced by combined PMA/ionomycin stimulation (Fig 4I). Nevertheless, \(Il2\) mRNA levels at ionomycin activation alone are possibly too low to allow for detectable protein production (Fig 4A, F). Thus, IL-2 requires both PKC and Ca\(^{2+}\) signaling for detectable protein levels.

**Signal strength dictates protein production in a cytokine-specific manner**
The levels of cytokines produced by T cells depends on costimulatory signals, on the affinity and the amount of antigen\(^{30,31}\). We stimulated T cells with low (0.1nM), intermediate (1nM) and high (100nM) OVA peptide levels to determine the effect on cytokine mRNA expression and protein levels. OVA\(^{low}\) did not drive protein production of any of the three cytokines. Upon activation with OVA\(^{int}\), a substantial fraction of T cells produced TNF-α (48±14%, Fig 5A), and a minor but significant fraction produced IFN-γ (21±3%; Fig 5B). IL-2 was almost undetectable (2±1%; Fig 5C), and T cells only produced significant IL-2 levels when activated with OVA\(^{hi}\).

Strikingly, again only activation with OVA\(^{hi}\) resulted in elevated \(Tnfa\) transcript levels, but OVA\(^{int}\) failed to induce Tnfa mRNA despite the substantial TNF-α protein production (Fig 5D). We therefore interrogated how antigen load affected the recruitment of mRNA to polysomes. In T cells activated with OVA\(^{low}\), \(Tnfa\) was primarily found as free-RNA (Fig 5D). Importantly, activation with OVA\(^{int}\) totally shifted \(Tnfa\) towards polyribosome-bound fractions. This was not further amplified with OVA\(^{hi}\), suggesting that the translation efficiency could not increase anymore (Fig 5D). Thus, TNF-α production depends on recruitment of pre-formed \(Tnfa\) mRNA to polyribosomes, which can be further amplified by de novo transcription at high antigen levels.

In sharp contrast, \(Iffg\) and \(Il2\) transcript levels directly correlated with protein levels: OVA\(^{int}\) and OVA\(^{hi}\) significantly increased \(Iffg\), as did OVA\(^{hi}\) for \(Il2\) (Fig 5E, F). Furthermore, \(Iffg\) and \(Il2\) almost completely associated with polyribosomes at any condition tested (Fig 5E, F),
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Again indicating that cytoplasmic Ifng and Il2 mRNA immediately engages with ribosomes. Altogether, we show that mRNA transcription and mRNA translation differentially contribute to the production of TNF-α, IFN-γ and IL-2.

PKC-signaling stabilizes Ifng and Il2 transcripts for optimal protein production

In resting T cells Tnfa mRNA levels are higher than those of Ifng and Il2 (Fig 2A). However, after 2h activation with OVA hi, Ifng and Il2 mRNA levels steeply increase and outnumber those of Tnfa mRNA (Fig 6A), independently of the culture conditions or the antigen affinity (Fig S5A). Rapid accumulation of transcripts can be promoted by de novo transcription and mRNA stabilization. mRNA stability was associated with increased translation efficiency, including transcripts that contain the regulatory AU-rich elements within their 3’UTR, like Tnfa, Ifng, and Il2.16, 32 We thus questioned whether optimal cytokine production correlated with mRNA stability. T cells activated for 2h with OVA hi or PMA/ionomycin were treated with ActD over the course of the next 2h to measure the mRNA decay rate. The half-life of Tnfa was unaffected by T cell stimulation irrespective of the stimulus used (t1/2 = ~60min; Fig 6B; Fig S5B), which was in line with its moderate increase in mRNA levels (Fig 6A). In sharp contrast, Ifng and Il2 mRNA transcripts were substantially stabilized upon T cell activation to t1/2 > 2h (Fig 6C, D, Fig S5B). CsA also did not alter the stability of Ifng, and only slightly reduced the half-

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**Figure 5: Signal strength determines cytokine specific pathway for production**

(A-C) TNF-α (A), IFN-γ (B), and IL-2 (C) protein production upon 6h of stimulation with 0.1nM (low), 1nM (int) or 100nM (hi) OVA peptide. Representative of 4 independently performed experiments (n=6 mice). (D-F) Left panels: Tnfa (D), Ifng (E) and Il2 (F) mRNA levels after 2h of stimulation. mean±SD of 3 (D, F) and 4 (E) independently performed experiments (n=5 (D, F); n=6 (E) mice). [One-way ANOVA with Tukey’s comparison; *p<0.05; ***p<0.0005]. Right panels: Polysome fractionation of cytoplasmic Tnfa (D), Ifng (E) and Il2 (F) mRNA after 2h stimulation with OVA peptide. Representative of two independently performed experiments.
life of Il2 mRNA to ~115min (Fig 6 C, D; Fig S5B). However, the half-life of both Ifng and Il2 dropped back to levels of resting T cells when with PKC inhibitor LY-333531 or sotrastaurin (Fig 6C, D; Fig S5B). Furthermore, activating PKC with PMA alone was already sufficient to fully stabilize Ifng and Il2 mRNA, while ionomycin failed to do so (Fig S4C). Thus, PKC is the main driver for Ifng and Il2 stabilization.

Of note, Ifng mRNA maintained its stability also at later time points, i.e. at 4h after T cell activation (Fig 6E; Fig S5D). In contrast, the half-life of Il2 at this time point dropped back to levels of resting T cells (Fig 6F), and Tnfa maintained its low t½ of ~ 60min at any time point measured (Fig S5D). The rapid turnover of Tnfa and Il2 mRNA between 4 and 6h post-activation was in line with the profound decrease of protein production at this time point (Fig 1, Fig S4B). Combined, we here show that mRNA stability is indicative for the duration of cytokine production and that PKC signaling herein is key.

Discussion
A potent T cell response requires the coordinated production of TNF-α, IFN-γ, and IL-2. We show that effector and memory T cells employ an individual regulatory program for each cytokine to drive production with distinct kinetics. TNF-α production was fast and intense, but transient, and depended on immediate polyribosome recruitment of pre-formed mRNA. T cells also employ pre-formed mRNA for the early production of IFN-γ. In contrast, the delayed IL-2 production depended almost exclusively on de novo gene transcription. In addition, stabilization of newly synthesized mRNA defined the duration of cytokine production. Prolonged mRNA half-life supported the sustained production of IFN-γ, whereas Il2 mRNA lost its stability within 4h concomitant with a gradual loss of protein production. Although TNF-α, IFN-γ, and IL-2 employ private regulatory networks, PKC signaling is crucial.
for all three cytokines. Yet, its mode of action is cytokine-specific. PKC drives Tnfa translation without stabilizing its mRNA. Conversely, PKC mediates mRNA stability of Ifng and Il2, and enhances the shift of mRNA from light to heavy polyribosomes.

PKC activation promotes mRNA translation through mTOR, and the two mTOR targets S6 and 4E-BP1 that drive protein synthesis. Whether PKC acts directly or indirectly on these targets remains to be determined. Direct phosphorylation of S6 and 4E-BP1 by PKC has been reported, but PKC could also regulate mTOR and 4E-BP activity through i.e. ERK-dependent mechanisms. Irrespective of its mode of action, PKC-induced phosphorylation of 4E-BPs releases eIF4E, allowing the pre-initiation scanning complex to assemble at the mRNA cap. The predominant role of polysome binding and mRNA translation in regulating TNF-α, and its addiction to 4E-BP1 phosphorylation may in part explain the success of rapamycin and its analogues as immune suppressants, since those inhibitors are capable to directly block 4E-BP phosphorylation.

How PKC stabilizes cytokine mRNA is yet to be determined. In Jurkat cells, PKC phosphorylates the RNA-binding protein NF-90, which allows NF-90 to bind and stabilize Il2 mRNA. Similarly, HuR can stabilize Ifng mRNA, and in vitro data show that PKC can phosphorylate HuR. Thus, it is tempting to speculate that HuR and NF-90 promote Ifng and Il2 mRNA stability in primary CD8+ T cells in a PKC-mediated manner.

T cells that produce two or more cytokines are the most effective against viruses, and their capability of inducing multi-flavored responses defines the efficacy of T cell vaccines. Here, we show that the kinetics of cytokine production is a general feature of effector and memory T cells and that it is cell-intrinsic. PKC orchestrates the timing of production of each cytokine by providing a custom-made post-transcriptional regulation. Understanding the mechanisms that drive optimal cytokine production should further help to unravel how effective, multi-functional T cell responses can be generated.

Materials and Methods

Activation and expansion of murine effector and memory CD8+ T cells
Mice were housed and bred at the Netherlands Cancer Institute (NCI). Experiments are approved by the Experimental Animal Committee of the NCI. OT-I T cells were activated as previously described. Activated T cells were rested for 3-13 days in 10ng/ml recombinant murine (rm)IL-7, 120U rmIL-2 or 10ng/ml rmIL-15 (Peprotech). Splenic CD44hi OT-I T cells, and splenic CD44hiCD62L+ and CD44hiCD62L− memory T cells from LCMV-Armstrong infected mice (d60; 10^5 pfu/mouse, i.p. injection) were FACS-sorted. T cells were stimulated with 100nM OVA_257–264 peptide, or its low affinity T4 variant, with 10ng/ml PMA, 1µM ionomycin (Sigma-Aldrich), or with peptide-loaded bone-marrow derived DCs. Brefeldin A was added as indicated. DCs were incubated for 1.5h with 100nM peptide, and washed 2x. LCMV-specific memory T cells were stimulated with 5mg/ml GP33 and 5mg/ml NP396 peptide (kind gift of R. Arens, LUMC Leiden). Inhibitors were added to T cells 30 min prior to activation (Table S1).

Flow cytometry
Intracellular cytokine staining was performed with BD Cytofix/Cytoperm kit (BD Biosciences). pmTOR and pS6 was measured upon 4% PFA fixation and methanol permeabilization.
Antibodies see Table S2. Cells were acquired with LSR Fortessa (BD Biosciences), and analyzed using FlowJo software (Tree Star, version 10).

**Polysome fractionation**

40×10^6 activated T cells were washed with PBS containing 100µg/ml cycloheximide. The cytoplasmic extract was layered on a 17-50% sucrose gradient, and centrifuged for 2h at 38,000rpm at 4°C in a SW41Ti rotor (Optima L100XP ultracentrifuge; Beckman coulter). RNA was isolated by Phenol:Chloroform extraction. Total polyribosome-associated mRNA levels were quantified by RNAnano Chip assay (Agilent). Relative mRNA distribution was calculated as % of the sum of mRNA from all obtained fractions. Total mRNA levels were put at 100% for each condition.

**Quantitative PCR analysis**

cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR was performed with SYBR green on StepOne Plus RT-PCR (Applied Biosystems). Gene expression levels were calculated by the 2^ΔΔCt analysis. Cytokine mRNA half-life was determined with GraphPad Prism 6 by calculating the one phase exponential decay curve.

**Western blot**

1×10^6 cells/sample was prepared by standard procedures. Proteins were separated on a 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane by iBlot (Thermo). Anti-4E-BP1 (Cell Signaling Technology) was used, followed by goat anti-rabbit-HRP secondary antibody (Dako) was used.

**Statistical analysis**

Results are expressed as mean ± SD. Data were analyzed with GraphPad Prism6. Unpaired or paired 2-tailed Student t-test was used when comparing 2 groups, 1-way ANOVA test with Tukey’s multiple comparison or Dunnett’s multiple comparison to control group was used when comparing > 2 groups. P values of < 0.05 were considered statistically significant.

**Author contributions**

F.S., M.v.L., and M.C.W. designed research; F.S., N.A.P., and R.S. performed research; F.S., M.v.L., and M.C.W. analyzed data; F.S. and M.C.W. wrote the paper.

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**Disclosures**

The authors declare no competing financial interests.
References


Supplementary Figures

Supplementary Figure 1: Cytokine production kinetics in T cells

(A) T cells that were rested for 3 days in the presence of IL-7 (upper), IL-2 (middle), or IL-15 (lower) were activated with 100nM SIINFEKL (N4) or SIITFEKL (T4) OVA peptide for indicated time points (n=3 mice). Brefeldin A (BrfA) was added during the last 2h of culture. (B) Representative dot plots for TNF-α and IFN-γ (upper panel) and IFN-γ and IL-2 (lower panel) production of T cells that were activated for indicated time with 100nM OVA^{257–264} peptide (N4) in the presence of BrfA during the last 2h of stimulation. Numbers indicate percentage of cytokine producing T cells. (C) mRNA levels of \( Tnfa \), \( Ifng \) and \( Il2 \) of T cells that were rested for 3 days in the presence of IL-7, IL-2 or IL-15.
Supplementary Figure 2: Pre-formed cytokine mRNA promotes early T cell responsiveness

(A) Tnfa, Ifng and Il2 mRNA levels of resting T cells were measured by RT-PCR. (B-D) T cells were pre-treated for 30 min with 1µg/ml Actinomycin D (ActD) or 10µg/ml cycloheximide (CHX) and then stimulated for indicated time points with 100nM OVA peptide in the presence of BrfA. Graphs depict the relative percentage of TNF-α (B), IFN-γ (C), and IL-2 (D)-producing T cells compared to T cells activated in the absence of inhibitors. (A-D) Data are presented as mean±SD of 3 independently performed experiments. [One-way ANOVA corrected for multiple comparisons with a Tukey test; n=7 mice; *p<0.05; ***p<0.0005].

Supplementary Figure 3: mTOR and 4E-BP phosphorylation in activated T cells

(A, C) mTOR and S6 phosphorylation 30min after T cell activation with 100nM OVA, 10ng/ml PMA and 1µM ionomycin (C). Cells were also pretreated for 30min with 500ng/ml cyclosporine A (CsA), 10µM LY-333531 (LY), 5µM sotrastaurin (sotra), or 10µM ZTSK474 (ZTS). Data shown derive from 3 independently performed experiments (n=10 mice; mean ± SD). [One-way ANOVA with Dunnett’s multiple comparison. ns= non-significant; *p<0.05; ***p<0.0001].

(B) Histograms represent mTOR and S6 phosphorylation of T cells activated for 30min with indicated stimuli, or of resting cells (n=4 mice) (D, E) 4E-BP1 expression upon activation with indicated stimuli (D), and when pretreated for 30min with indicated inhibitor prior to activation (E). Representative of three (D) and two (E) independently performed experiments.
Supplementary Figure 4: Regulation of cytokine production through PKC and Ca\(^{2+}\)-dependent signaling

(A) TNF-α and IFN-γ (upper panel), and IL-2 and IFN-γ (lower panel) production of CD44\(^{hi}\) memory-like T cells activated for 4h with indicated stimuli. Unstimulated T cells were used as negative control. Representative of 4 mice from 2 independently performed experiments. (B) T cells were stimulated with PMA/ionomycin for indicated time points. The percentage of TNF-α, IFN-γ and IL-2 protein producing T cells was determined by intracellular cytokine staining (mean±SD; n=7 mice). (C-D) IL-2 production of PMA/ionomycin stimulated T cells (C), and IFN-γ and IL-2 production of OVA\(^{257–264}\) stimulated T cells (D) that were pretreated with the indicated inhibitor. Representative of 6 mice from 3 independently performed experiments. (E) Tnfa, Ifng and Il2 mRNA levels (mean ± SD) of resting T cells that were stimulated for 2h as indicated. (One-way ANOVA with Tukey’s multiple comparison; n=7 mice; ns=non-significant; *p<0.05; ***p=0.0005; ****p<0.0001). (F) Total mRNA distribution in collected sucrose fractions of activated T cells as determined by RINAnano Chip assay.
Supplementary Figure 5: Role of PKC signaling in cytokine mRNA stability

(A) Tnfa, Ifng and Il2 mRNA levels (mean±SD) 2h after activation with 100nM SIINFEKL (N4) or SIITFEKL (T4) peptide of T cells that were cultured for 3 days with IL-7, IL-2, or with IL-15 (n=3 mice). [Unpaired student t-test; n=3 mice; ns=non-significant; *p<0.05; **p=0.005].

(B) T cells were stimulated for 2h with PMA/iono were treated, and then treated with actinomycin D (ActD) alone, or in combination with 500ng/ml cyclosporine A (CsA), 10μM LY333531 or 5μM sotrastaurin for indicated time points (n=5 mice). (C) mRNA decay of T cells stimulated with indicated stimuli for 2h prior to ActD treatment (n=5 mice). (D) mRNA decay of PMA-stimulated T cells for 2h or 4h) prior to treatment with ActD (n=3 mice). (B-D) Data are presented as mean±SD of three (B, C) or two (D) independently performed experiments.
### Supplementary Table 1: Inhibitors used in T cell activation essays

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### Supplementary Table 2: Antibodies used in flow cytometry

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