

cell Biology

Dynamic changes in tRNA modifications and abundance during T cell activation

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Edited by Nahum Sonenberg, McGill University, Montreal, QC, Canada, and approved July 28, 2021 (received for review April 14, 2021)

The tRNA pool determines the efficiency, throughput, and accuracy of translation. Previous studies have identified dynamic changes in the tRNA (transfer RNA) supply and mRNA (messenger RNA) demand during cancerous proliferation. Yet dynamic changes may also occur during physiologically normal proliferation, and these are less well characterized. We examined the tRNA and mRNA pools of T cells during their vigorous proliferation and differentiation upon triggering their antigen receptor. We observed a global signature of switch in demand for codons at the early proliferation phase of the response, accompanied by corresponding changes in tRNA expression levels. In the later phase, upon differentiation, the response of the tRNA pool relaxed back to the basal level, potentially restraining excessive proliferation. Sequencing of tRNAs allowed us to evaluate their diverse base-modifications. We found that two types of tRNA modifications, wybutosine and ms²t6A, are reduced dramatically during T cell activation. These modifications occur in the anticodon loops of two tRNAs that decode "slippery codons," which are prone to ribosomal frameshifting. Attenuation of these frameshiftprotective modifications is expected to increase the potential for proteome-wide frameshifting during T cell proliferation. Indeed, human cell lines deleted of a wybutosine writer showed increased ribosomal frameshifting, as detected with an HIV gag-pol frameshifting site reporter. These results may explain HIV's specific tropism toward proliferating T cells since it requires ribosomal frameshift exactly on the corresponding codon for infection. The changes in tRNA expression and modifications uncover a layer of translation regulation during T cell proliferation and expose a potential tradeoff between cellular growth and translation fidelity.

transfer RNA | T cell activation | tRNA-modifications | HIV

ew cells in the adult mammalian body can proliferate under normal conditions. One example of a fundamental programmed proliferation processes is evoked in clonal expansion of selected cells of the adaptive immune system, following the encounter with foreign antigens. Upon recognition of a cognate antigen, naïve T cells (and B cells) undergo massive proliferation and differentiation, hence changing their status from arrested naïve cells to highly proliferating effector cells (1). The integrity of the immune response is dependent on the precise regulation of proliferation rates, number of cell cycles, and cell death. In addition, a portion of the population is differentiated into memory cells, which remain in the body for extended periods of time. A major regulatory challenge and point of vulnerability is to allow such massive cellular proliferation while circumventing the risk of cancerous growth.

A crucial part of cell proliferation is the ability to massively translate new proteins. Translation control was shown to regulate gene expression in the immune system (2-4). However, the regulation of translation elongation, and especially tRNA (transfer RNA) availability, was not explored. We have previously shown that genes that are up-regulated in proliferating cancerous cells or

upon induced pluripotency have a distinct translation program from that of arrested cells (5, 6). In particular, mRNAs (messenger RNAs) corresponding to cell-autonomous functions, related to proliferating cells, are enriched with a specific set of codons, while mRNA of multicellular functions, related to nondividing cells, are enriched with a different set of preferred codons. The tRNA pool in these cells is dynamically regulated and its dynamic corresponds to the changes in codon usage, as also reflected in differential tRNA essentiality in proliferation and cell arrest (7, 8). Following these observations, it is essential to explore how the tRNA pool changes in a natural proliferation process, and what the contributions of the tRNA pool regulation to the integrity dynamics and of the immune response are.

Beyond changes in the expression level of tRNAs, these RNAs are among the most highly posttranscriptionally modified molecules in the mammalian transcriptome (9, 10). Essentially all tRNA molecules undergo diverse chemical modifications on many of their bases, and the various nucleotide modifications can affect tRNA folding and stability, amino acid loading, and codon-anticodon base pairing of tRNAs to their corresponding codons (11-15). Modifications around the anticodon loop of the tRNA are known to affect translation fidelity, speed, and tendency to frameshift (15–18). Modification levels are regulated and can change in a

Significance

The tRNA pool decodes genetic information during translation. As such, it is subject to intricate physiological regulation in all species, across different physiological conditions. Here, we show a program that governs the tRNA pool and its interaction with the transcriptome upon a physiological cellular proliferation: T cell activation. We found that upon antigenic activation of T cells, their tRNA and mRNA pools undergo coordinated and complementary changes, which are relaxed when cells reduce back their proliferation rate and differentiate into memory cells. We found a reduction in two particular tRNA modifications that have a role in governing translation fidelity and frameshift prevention. This exposes a vulnerability in activated T cells that may be utilized by HIV for its replication.

The authors declare no competing interest.

This article is a PNAS Direct Submission

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/ doi:10.1073/pnas.2106556118/-/DCSupplemental.

Published October 12, 2021.

Author contributions: R.R., M.P., O.D., N.F., and Y.P. designed research; R.R., M.P., I.E.-M., Y.M., Y.S., O.M., and S.R.-Z., performed research; R.R. contributed new reagents/analytic tools; R.R., A.N., N.S.-G., and Y.P. analyzed data; and R.R., O.D., T.S., N.F., and Y.P. wrote the paper.

¹Deceased February 19, 2021.

dynamic manner across physiological conditions (19–21), in cancer (22, 23), and during development (11). Yet the dynamics, regulation, and biological function—both at the molecular and physiological level—of most such modifications are not well-characterized.

Here we set to investigate the control of the tRNA pool in a physiologically normal and programmed proliferation process, by following T cells upon their antigen receptor activation. We examined the codon usage changes during T cell activation and the corresponding changes in tRNA abundance. We also examined in parallel the posttranscriptional chemical base modifications of the tRNAs. We found that upon activation, T cells reprogram their tRNA pool to serve the altered codon usage demand of the proliferation-related genes. Interestingly though, at the later stage of the response, when T cells differentiate, the tRNA response is relaxed back toward base level, where it is no longer adapted to the proliferation-related codon usage. Furthermore, we found that at the pick of their proliferation, T cells exhibit a sharp decline of two tRNA base modifications. Both of these modifications are known to protect the ribosome from −1 frameshifting at "slippery" frameshift-prone codons. Interestingly, the reproduction of HIV-1, which preferentially targets proliferating T cells, depends exactly on such -1 frameshifting that occurs on one of these slippery codons (24). We indeed found that the knockout of a key enzyme in one of these two tRNA modifications pathways, in human cells, induced a frameshift at the HIV frameshifting slippery codon sequence. While the biological reason for the reduction of this modification in T cells is still unknown, we hypothesize that it may represent a tendency to trade off translation accuracy for speed, a potential requirement for fast proliferation.

Results

A Global Codon Usage Shift, toward AT-Ending Codon, Followed by a Relaxation During T Cell Activation and Differentiation. An important aspect of translation regulation is the dynamic change of supply (tRNA and anticodons) and demand (mRNA and codons) (7). To study translation regulatory dynamics during a central process of mammalian cell proliferation and differentiation, we followed T cells as they are triggered to switch from a naïve to an activated state. Naïve CD4⁺ T cells were isolated from mice spleen and activated using anti-CD3/anti-CD28 activation beads (Materials and Methods). Samples were taken at the naïve stage ("time point zero"), and after 20, 48, and 72 h. At the later time points (i.e., 48 and 72 h) cells were sorted according to the expression of the CD62L and CD44 markers. High CD62L and CD44 cells (CD62L⁺ CD44⁺) correspond with differentiation toward precursor central memory (25), while CD44⁺, a marker of T cell activation, was high in all activated cells (Fig. 1A and SI Appendix, Fig. S1-1). The experiment was repeated twice. We sequenced both mRNAs and tRNAs from each sample. Unsorted samples from one biological repeat were analyzed also by ribosomal protection sequencing (Ribo-Seq) and liquid chromatography/mass spectrometry (LC/ MS) shotgun analyses (Materials and Methods and Fig. 1A). By examining the expression pattern of cell cycle genes, we found that 20 h after activation cells are at the peak of S-phase gene expression, while the peak of M-phase gene expression is at 48 h after activation (Fig. 1B). Those results are in line with the known dynamic of T cell activation in vitro (25).

We characterized changes in mRNA codon usage by examination of the change in the representation of each of the 61-amino acid coding codons in the mRNA pool at each time point. We first examined and verified that changes in mRNA expression were in a good correlation between the two biological repeats (*SI Appendix*, Fig. S1-2). The "demand" for tRNA by each codon type in the transcriptome is computed as the sum, over all mRNAs, of the product of mRNA expression level and the number of appearances of each codon in each gene (5) (*Materials and Methods*). We found that relative to the naïve cells, activated proliferating cells show an increase in representation of the codons, defined before by Gingold et al. (5) as the "proliferation codons" signature (Fig. 1*C* and *SI Appendix*, Fig. S1-3). There is a statistically significant difference between the fold-change in representation in the transcriptome of AT vs. GC ending codons at each time point relative to the naïve cell (*SI Appendix*, Fig. S1-4). The proliferation codons tend to end with A or T nucleotides at the third codon position, in contrast to the differentiation codons, which tend to have G or C at their third positions. Although the enrichment in proliferation codons is seen in all samples, when compared to naïve cells, the fold-change in codon usage is maximal at the 20-h sample, and it is then reduced 72 h after activation, as 72 h CD62L⁺ cells show the least difference in codon usage from naïve cells (Fig. 1*C* and *SI Appendix*, Fig. S1-3*A*).

We characterized each sample based on the average codon usage of the genes expressed in it, normalized by their expression level, using principal component analysis (PCA) (Fig. 1D and SI Appendix, Fig. S1-3B). The analysis shows a marked change in codon usage at 20 h, relative to the naïve population, and a gradual return toward base levels with 72 h CD62L⁺ cells located closest to the naïve samples. We then turned to examine which genes derive the codon usage trend. We first checked the genes that belong to the gene ontology (GO) categories associated with the different stages of the cell cycle (G0-early G1, G1, S, G2M, M). We found that the first PC of the codon usage PCA is in high correlation with the expression of S-phase genes (R = 0.95)(Fig. 1E). The correlation to G1, G2/M, and M-phase GO categories was also high, but lower than S-phase (R = 0.67, R = 0.74, R = 0.55, respectively) (Fig. 1F and SI Appendix, Fig. S1-5), while developmental process-related genes are negatively correlated with the first PC (SI Appendix, Fig. S1-5). Indeed, the changes in codon usage at 20 h and 48 h, but not 72 h compared to naïve samples is in high correlation with the codon usage of S- and M-phase genes (Fig. 1G and SI Appendix, Fig. S1-6). Apart from cell cycle-related GO categories, the most correlative GO categories are: "negative regulation of oxidative stress-induced cell death," "regulation of DNA metabolic process," "cellular respiration," and "purine ribonucleoside monophosphate metabolic process" (Dataset S1). We conclude that the induction of genes belonging to these functional categories drives the proliferationcodon usage signature that we observe in activated T cells.

Induction of a Proliferation tRNA Signature Is Followed by Relaxation at the Differentiation Phase. We next set to examine what the changes are in the tRNA pool and if they correspond to the changes in demand as reflected by the codon usage, adapting a recently developed tRNA sequencing protocol (26) [see Materials and Methods for further details and Aharon-Hefetz et al. (8) for additional quality assessment]. In the downstream analysis, we grouped together read counts of all tRNAs of the same anticodon that have at least a minimal tRNAScan prediction score [tRNAscan-SE (27)] above 50. We calculated the expression of each such isoacceptor group as the sum of read counts mapped to each of its corresponding genes. The experiment was done in two biologically independent experiments, with three technical repeats for each time point, showing a high correlation between the two biological experiments (SI Appendix, Fig. S2-1). Sequencing results were validated by real-time qPCR, showing similar trends for three tested tRNA types (SI Appendix, Fig. S2-2). We found a significant positive correlation between tRNA expression levels and expressed codon usage in the naïve cells, with high levels of both CG ending codons and matched tRNA (Fig. 2A) (Pearson $R = 0.66, P < 10^{-8}$). Twenty hours after activation of the cells, the tRNAs that decode the AT ending codons are up-regulated compared to naïve T cells (Fig. 2B) (rank-sum test P < 0.05). Beyond this time point, we did not observe such significant difference in expression fold-change between tRNAs that code for AT ending codons and CG ending codons.







Fig. 2. tRNA avilability and TE change during T cell activation. (A) Expressed codon usage (as in Fig. 1C, not normalized to amino acid, x axis) and tRNA expression (for W value for codons, see *Materials and Methods*) in naïve cells are correlated (Pearson R = 0.66, $P = 9 \times 10^{-9}$). Red indicates AT ending codons, blue indicates CG ending codons. (B) Dynamics of tRNA expression during T cell activation. Shown here is a heatmap representation of changes in tRNA expression at each time point, normalized by expression in naïve T cells. Rows are sorted based on the fold-change (FC) between naïve and 20 h after activation. Marked in red are tRNAs that decodes AT ending codons, marked in blue are tRNA that decodes CG ending codons, and in black tRNAs that decodes both (by wobble interaction), iMet and Sec tRNAs. (C) A-site codon occupancy, calculated by ribosome footprint, normalized to the frequency of each codon in the genes sequence. Each plot is a histogram of the changes in codon occupancy in the indicated sample compared to the naïve T cells (red, AT ending codons; blue, CG ending codons; Wilcoxon rank-sum test P < 0.05 for 48 and 72 h). (D) Pearson correlation of mRNA expression, tAI, and TE averaged for gene groups derived from GO categories (P value for all correlations <10⁻¹⁰, except for tAI-TE at 72 h [n.s.]). (E) A PCA projection of the mouse codon usage of gene sets derived from GO categories. Each point represents one gene set. Gene sets corresponding to tissue-specific GO terms are to the left side, and those corresponding to proliferation-related GO terms are to the right. The color code in the Top panel represent changes at the mRNA level, averaged over all the genes in each gene set. In the Middle panel, each gene category is color coded according to the relative change in availability of the tRNAs that correspond to the codon usage of its constituent genes, averaged over all genes in the gene set. A red color for a given gene set indicates that on average the genes in that set have codons that mainly correspond to the tRNAs that are induced in the condition, whereas a blue color indicates that the codon usage in the set is biased toward the tRNAs that were repressed in that given condition. In the Bottom panel the color code indicates TE calculated based on Ribo-seq by ratio of reads of the ribosome protected fragment (reads per kilobase and million mapped reads, RPKM) divided by read count of mRNA-seq (RPKM). (F) Correlation of mRNA expression, tAI, and TE to the first PC, calculated based on average codon usage of each GO category (shown in E). P value for all correlations <10⁻¹⁰.

We specifically inspected two unique tRNAs: for Selenocysteine and the initiator-methionine tRNA (tRNA^{iMet}). We found that both these tRNAs were down-regulated at all time points following the T cell activation (Fig. 2B and SI Appendix, Fig. S2-3 A and B). The decline of the Seleno-cysteine tRNA in proliferation is in line with our previous observations (5). However, the decline in expression of the tRNA^{iMet} is contrasted with the induction of this tRNA in other proliferating cells (5, 28). We analyzed the expression of translation initiation factors and found two clusters of expression, one that is elevated upon T cell activation and the other that is reduced, similarly to tRNA^{iMet} (*SI Appendix*, Fig. S2-3*C*). This inconsistency warrants further investigation in the future.

Besides changes at the anticodon levels, which are based on averaging entire families of tRNAs that share the same anticodon, we also examined individual genes from each tRNA family. We found pronounced changes in expression in several specific tRNA genes within tRNA families (*SI Appendix*, Fig. S2-4 and S2-5). This could be taken to indicate regulation on individual tRNA

gene copies, which can differ in their function either in translation or in other functions, such as a source of tRNA fragments (29, 30). In three tRNA families-Ala-AGC, Ala-TGC, and Val-AAC-the increase in the anticodon level in activated cells results from up-regulation of one or two tRNA genes that are lowly expressed in the naïve cells. In other cases, we found changes in the tRNA gene levels, without changing the total anticodon level.

We next aimed to reveal how coordinated are the various levels at which translation is regulated as the cells complete a cycle of proliferation and differentiation. To that end, we examined mRNA expression, tRNA expression, and translation efficiency (TE) as assessed by ribosome profiling sequencing (Ribo-seq) from the same cultures. While mRNA- and tRNA-sequencing (mRNA-/ tRNA-seq) reveal, respectively, the demand and supply for the translation process, Ribo-seq measurement provides a snap-shot of actively translating ribosomes across the body of each translated mRNA. When analyzing the mRNA-seq and Ribo-seq libraries across coding regions, the expected distinct profiles of ribosome accumulation at the start and end of the ORF in the Ribo-seq library, as well as alignment of reads to the correct ribosome reading frame, confirm the overall quality of the libraries (SI Appendix, Fig. S2-6). For each codon, we calculated A-site occupancy, normalized to codon representation, inferred from ribosome footprint (31). We found that the A-site occupancy in CG ending codons is increased at 48 and 72 h after activation compared to the naïve sample (Fig. 2C). However, we did not find a correlation between tRNA expression changes (fold-change to naïve) and A-site occupancy (SI Appendix, Fig. S2-7)

To assess the adaptiveness of each gene's sequence to the tRNA supply we used a modification of the tRNA adaptation index (tAI) score (32), which calculates codon adaptiveness based on the tRNA availability. Whereas in the original tAI measure tRNA availability is given as a static tRNA gene copy number in the genome, here we determined the tRNA availability based on the tRNA-seq read count for each anticodon (Materials and Methods). This allows us to determine TE for each codon in a dynamic fashion, across the process of T cell activation. We next computed the ratio of this tAI score for each gene given the tRNA pool at a given time point, normalized to the score computed based on the naïve tRNA pool. The ratio is higher than one at a given time point during T cell activation for genes with a codon bias that corresponds to tRNAs that are induced at that time point. tAI and TE are distinct dynamic measures of genes' translation and the correlation between them has so far not been assessed. While TE captures both initiation rate and translation speed, tAI evaluates the availability of tRNAs needed to translate a gene.

Overall, at the single-gene level, we found low yet significant correlations between changes in mRNA expression, tAI, and TE (SI Appendix, Fig. S2-8 and Dataset S4). When examining the correlation between mRNA levels and each of these measures of translation, we found a modest correlation (Pearson's correlation = 0.11, but highly significant P values due to involvement of 4,474 genes in the calculation) between tAI and mRNA levels at 20 h and 48 h, and a slightly lower correlation between mRNA and TE at these times points. Interestingly, these correlations become (significantly) negative at the 72-h time point, perhaps suggesting the action of means to restrain mRNA's transcription response (SI Appendix, Fig. S2-8). Yet, when analyzed at a higher level of granularity of GO functional gene sets, we found a significant correlation between the changes in tAI and TE at 20 h and 48 h after activation compared to the naïve cells (Fig. 2D and SI Appendix, Fig. S2-9). The existence of such a correlation between entire functional categories but not at individual gene level suggests that different genes in shared functional categories use different regulatory means (e.g., tRNA availability or engagement with ribosome) to respond in the same directions. At 72 h after activation, there is a negative correlation between

changes in mRNA expression compared to naïve and changes in TE and tAI compared to naïve.

In parallel, we examined the response of each gene and genes' functional categories in light of their codon usage programs. For that, we projected all mouse genes' GO categories on a PCA plane, based on the average codon usage of all the genes that belong to each category (Fig. 2E). The location (but not the coloring) of the GO categories is identical in all three panels of Fig. 2E, as it reflects static codon usage only. In similarity to the human genes analysis (5), on the right side are cell-autonomous functionality, such as the cell cycle genes, and functions related to gene expression, such as the ribosome, DNA replication machinery genes, while on the left side are multicellularity related functions (SI Appendix, Fig. S2-10A). While the coefficient of the first PC separate codons by their AT or CG, the second PC separate codons by their amino acid hydrophobicity (SI Appendix, Fig. S2-10 B and C). This analysis clearly shows the marked difference in codon usage of genes that fulfill proliferation and differentiation functions, captured by the first PCA component. To examine the correspondence between changes in tRNA supply, TE, and mRNA level, we colored the GO categories based on averaged (z-transformed) change in mRNA expression (Fig. 2 E, Top), based on changes in tAI calculated for averaged codon usage of the GO categories genes (Fig. 2 E, Middle), and based on averaged (z-transformed) changes in TE (Fig. 2 E, Bottom), between activated T cells at 20 h after activation and naïve cells. We found that the proliferation-related GO categories on the right side of the PCA projection are strongly induced at the mRNA level at 20 h after activation compared to naïve cells. The signature is stronger at 48 h and reduced at 72 h after activation, reflecting a reduction in proliferation gene expression at 72 h.

The dynamics of changes in TE of GO categories, in correlation with the first PC component, recapitulates the changes in mRNA expression. The correlation of changes in tAI with the first PC calculated for GO categories is highest at 20 h after activation compared to naïve cells, and becomes negative at 72 h after activation (Fig. 2F and SI Appendix, Fig. S2-11). Hence, at 72 h after activation, the proliferation-related genes are still induced at the mRNA level, but to a lesser extent compared to the 20 h, and the tRNA pool supply is no longer biased toward the codon usage of the genes that belong to these functionalities. This is accompanied by a negative correlation between changes in mRNA expression to TE and tAI (r = -0.1, r = -0.2, respectively) (Fig. 2D). The relationships between the tRNA and mRNA pool at 20 h, but not in 72 h, in which genes that serve cell-autonomous functions are induced at the mRNA level, and tRNAs whose anticodons match their codon usage are induced as well, resemble the situation seen in diverse cancer types (5). It is possible to speculate that the lack of correlation between supply and demand at the 72-h time-point might ensure that the proproliferation mRNAs that may still exist in the cell at this stage will not be translated efficiently, restraining potential undesired excessive proliferation. In summary, this analysis reveals some level of coordination between mRNA and tRNA supply and demand during the dynamic process of T cell activation, yet this response relaxes, and with it this coordination, and it also shows that functionally related genes might use different regulatory means to respond in similar directions.

tRNA Modifications Involved in Translation Frame Maintenance Are Reduced in Activated T Cells. tRNA molecules are extremely rich with posttranscriptional RNA modifications, of which some can govern the stability, TE, decoding rate, and fidelity (11, 12, 14, 33). Chemical modifications of RNAs are reliably detected and quantified by means such as MS (34); in particular, MS has been used to analyze tRNAs (9, 22, 35). An interesting question is: Can modifications on RNAs be detected using RNA-seq methods, given that many modifications can often cause failure

in the reverse-transcription (RT) reaction (13)? In modified positions, the reverse transcriptase can either transcribe the original nucleotide, abort the reaction (resulting in truncated fragments), or introduce typical nucleotide mismatches compared to the genomic sequence. Indeed, in several protocols for sequencing of RNA, and tRNAs in particular, certain modifications are enzymatically removed from the transcription prior to sequencing (26, 36). Yet, in agreement with recent works (13, 37), we realized that beyond our ability to uniquely detect and quantify a modified tRNA, the disadvantage due to RT disruption can actually turn into an opportunity: the ability to quantify the modification level.

To calculate the percentage of modified tRNA, as these may be dynamically regulated, we relay the fact that the fraction of RNA copies that carry the modification can be calculated as the sum of mismatches and RT-abortion truncated reads at the position, normalized to the total number of reads that map, either perfectly or not, to the gene sequence. Modifications that do not interfere with the RT process are not detectable through this method. We note that the data that emerge from sequencing do not allow us to fully detect the actual chemical nature of the modified nucleotide, only relative changes in the level of editing, but crossing the read data with databases of annotated modifications may allow us to infer the nature of modification and to detect changes in the fraction of edited transcripts. We note that our estimation of modification levels, which is based here on the percentage of truncated reads and reads with mismatched nucleotides, provides only a lower bound on the modification level since some of the modified RNA positions may be reversedtranscribed with no errors or abortions.

To determine which modifications can be detected using tRNA-seq we crossed annotated modification positions from the MODOMICS database (9), with tRNA-sequencing data collected in this study and human (8) and Escherichia coli (38) data from previous studies. We found that out of 46 modification types annotated in MODOMICS for the indicated species, 13 modifications were detected based on mismatches or RT abortions in our protocol (SI Appendix, Fig. S3-1 and Table S1). Undetectable annotated modifications are either not causing mismatches or RT abortions, or they may have not been sufficiently modified in the sample measured. For mice, only 12 tRNA species were annotated with their modifications in the MODOMICS database, and in total they represent 18 chemical modification types, appearing in 196 modification positions in total. Through changes in tRNA reads, we have been able to detect 5 of 18 annotated modifications (m1A, m1G, m2,2G, I, and wybutosine) in 24 known positions. In addition, 615 unannotated sites appear to be at least minimally modified (at least 10% of the reads; see detailed modification list in Dataset S2).

Based on modification calling by mapping mismatches and RT abortion at each position, we could quantify changes in modification levels throughout the T cell activation process. We examined separately the modifications around the anticodon (and one nucleotide upstream and downstream), which are known to have a higher effect on tRNA decoding role (39) (Fig. 3A), and the modifications that occur elsewhere in the tRNA molecule, which are involved in addition in many other aspects of tRNA biology (40) (SI Appendix, Fig. S3-2). We found that the level of most modifications remained constant during the T cell activation process. However, two types of modifications located on $tRNA^{Lys}(UUU)$ and $tRNA^{Phe}(GAA)$ show a marked reduction in modification level in activated cells at 20 h compared to the naïve cells. The two modifications are within the anticodon loop, on position 37 (i.e., one nucleotide upstream from the anticodon) (Fig. 3A). We also detected changes in modification levels of tRNA^{Arg}(UCU) isodecoders at a position more distant from the anticodon. According to the annotation in MODOMICS, this modification can be assumed to be m³C 32 (13) (SI Appendix, Fig. S3-2). We focus below on the modifications in $tRNA^{Lys}(UUU)$ and $tRNA^{Phe}(GAA)$.

Interestingly, both of the anticodon modifications that changed in level during T cell activation are involved in decoding of "slipperv codons," which are known to be involved in ribosomal frameshift (17, 41-43). Position 37 of tRNA^{Phe}(GAA) is annotated in MODOMICS to have the wybutosine (yW) modification. yW and its derivatives, peroxywybutosine (o2yW) and hydroxywybutosine (OHyW), are tricyclic nucleosides with a large side chain found at position 37 of mammalian tRNAPhe. yW derivatives play a critical role in efficient codon recognition, decoding efficiency, and reading frame maintenance (17, 43-45). We found that the RT-abortions fraction at position 37 of tRNAPhe(GAA), assumed to result from the yW or derivates presence, changed dynamically with T cell activation. Its levels were high at naïve T cells, low at 20 and 48 h after activation, and elevated back at 72 h after activation yet to a lower level compared to the initial level (Fig. 3B). While RT abortion due to the modification decreased during T cell activation, mismatch fractions increased in the activated cells (Fig. 3C), showing a distinct mismatch pattern (SI Appendix, Fig. S3-3A) that is similar to the pattern observed in annotated positions of m1G modifications (SI Appendix, Fig. S3-1). This indicates that in the activated cells, m1G modification is not processed to yW. However, we did not observe any significant reduction in mRNA expression of yW biosynthesis enzymes (trmt-5, tyw-1, tyw-3, lcmt-2, tyw-5) (Dataset S3). For tRNA^{Lys}(UUU), we found RT abortions in a similar pattern to tRNA^{Phe(GAA)}, without the appearance of mismatches in the activated cells (Fig. 3D and SI Appendix, Fig. S3-3B). The intermediate modification t⁶A, which might occur in the activated cells, cannot be detected using our tRNA-seq protocol, since it does not result in a detectable mismatch pattern (SI Appendix, Fig. S3-1). Our data support the presence of the bulky modification 2-methylthio-6-threonylcarbamoyl-A (ms²t⁶A), as shown before in mouse (18) and human (9, 46), as this modification was shown to cause RT abortion in human (13). While the two above-mentioned modifications [i.e., t yW on tRNA^{Phe}(GAA) and ms²t⁶A on tRNA-^{Lys}(UUU)] show a dynamic change in levels during T cell activation, the downstream modification m1A at position 58 remained constant for both tRNAs (SI Appendix, Fig. S3-3C). The retainment of this modification on both tRNAs indicates that these tRNAs maturate and are processed properly.

We were looking for a sequencing independent means to identify and quantify these modifications, and turned to the direct analysis of tRNA modifications by MS. For that, class I tRNA fractions were obtained from the naïve CD4⁺ T cells and activated cells at 20, 48, and 72 h. Each tRNA fraction was digested by RNase T₁ and subjected to the shotgun analyses by capillary LC/MS (47, 48). As shown in Fig. 3E, we successfully detected the anticodoncontaining fragments of cytoplasmic tRNA^{Lys}(UUU). Judging from the exact molecular mass, we found four kinds of fragments with different modification status. The fully modified fragment containing 5-methoxycarbonylmethyl-2-thiouridine [mcm $(5)s^2U$] at position 34 and ms²t⁶A at position 37 was detected as a major species in all four time points during the activation process (Fig. 3E). This fragment was further probed by collision-induced dissociation (CID) to confirm the position of each modification (SI Appendix, Fig. S3-4). In addition, we detected three hypomodified fragments bearing mcm (5)s²U34 and t⁶A37, mcm⁵U34 and ms²t⁶A37, and mcm⁵U34 and t⁶A37 (Fig. 3*E*). Over time after activation, these hypomodified fragments increased significantly, suggesting that the 2-methylthio group of ms²t⁶A37 and 2-thio group of mcm (5)s²U34 are down-regulated at 20 and 48 h after activation. Since the 2-methylthio group of ms²t⁶A37 hinders RT and can be detected by an RT stop signature, the LC/MS data clearly explain the unique behavior in the tRNA-seq data upon T cell activation.



Fig. 3. Changes in tRNA modifications during T cell activation. (*A*) Average anticodon loop modification levels based on tRNA-seq from the naïve and activated (20 h) T cells ($n = 3 \pm SD$). Threshold for modification calling is: minimum coverage of modified position = 20 reads, modification fraction above 10% in at least one sample. (*B*) RT-abortion fraction at position 37 of reads aligned to tRNA^{Phe}(GAA). Triangles and circles are the first and second experimental sets correspondingly (ANOVA *P* value <10⁻⁵ for all samples compare to naïve, <0.01 for all samples except for naïve compared to 72 h CD62⁺). (*C*) Mismatches fraction at position 37 of reads aligned to tRNA^{Phe}(GAA). Triangles and circles are the first and second experimental sets correspondingly (ANOVA *P* value <10⁻⁵ for all samples compare to naïve, <0.01 for all samples except for 72 h CD62⁺ compared to naïve, naïve vs. 72 h CD62⁺ *P* < 0.05, second set, n.s). (*D*) RT-abortion fraction at position 37 of reads aligned to tRNA-LysUUU (ANOVA *P* < 10⁻⁷ for all samples except for 72 h CD62⁺). At this position, there are no detected changes in mismatch levels (*SI Appendix*, Fig. S3-3). (*E*) MS shotgun analysis of cytoplasmic tRNA modifications during T cell activation. Shotgun analysis of class I tRNA fraction from mouse CD4⁺ T cells collected at 0 (naïve), 20, 48, and 72 h after activation. The tRNA fraction was digested by RNase T1 and subjected to capillary LC/MS. Top to bottom panels represent XICs for negative ions of anticodon-containing fragments of cytoplasmic tRNA^{Lys} bearing mcm (5)s²U34 and t⁶A37, mcm⁵U34 and ms²t⁶A37, and mcm⁵U34 and t⁶A37, respectively. The percentage of each peak represents relative abundance of the corresponding fragment.

In the shotgun analysis of CD4⁺ T cells, we clearly detected the anticodon-containing fragments of tRNA^{Phe}. OHyW is a major modification and o2yW is a minor modification in naïve cells (*SI Appendix*, Fig. S3-5). Upon activation, both yW derivatives get plunged drastically (*SI Appendix*, Fig. S3-5), indicating that biogenesis of yW derivatives is impaired after T cell activation. This result nicely explains the deep-sequencing data for tRNA^{Phe}(GAA) upon T cell activation. In addition to the two modifications we found to decrease during T cell activation using tRNA-seq, we further explored the MS generated for additional potential changes in modification levels. We could identify and quantify t6A-containing fragments from four other tRNAs (tRNA^{iMet}, tRNA^{Met}, tRNA^{Thr}, tRNA^{Asn}). We found that the level of this modification remains consistently high throughout T cell activation (*SI Appendix*, Fig. S3-6). This modification was not detected by the tRNA-seq, since t6 does not

interfere with reverse-transcription in our protocol (*SI Appendix*, Fig. S3-1). Thus, it seems that while some modifications can be identified by both methods, some are detected only by the former, while others only by the latter, establishing the complementary nature of these two technologies in detecting and quantifying RNA modifications.

Reduced yW Modification Level Increases Ribosomal Frameshift Level.

Since we could not detect intermediate modifications of yW derivatives from tRNA^{Phe} by MS, and to estimate the effect of the modification on ribosomal frameshift, we first approached this modification by a combined tRNA-seq and a gene-deletion approach. Specifically, we used CRISPR/cas9 to knock out a yW modification enzyme, coded by the gene *TYW1*. This enzyme catalyzes the second reaction in the biogenesis of yW formation at position 37 of tRNA^{Phe}. We generated clones of HeLa *tyw1* knockout (KO) cells using two alternative gRNAs (guide RNAs) that target the second or third exon of *twy1* the gene. Indeed, the tRNA-seq–based signal for detection and quantification of yW modification level was reduced on tRNA^{Phe}(GAA) by 20 to 30%, from an initial level higher than >90% (Fig. 44). Interestingly, the total level of tRNA^{Phe}(GAA) is elevated upon this KO (Fig. 4B),

suggesting a feedback loop that could sense the reduction in the modified tRNA level and respond by elevating transcription of the tRNA molecules. Alternatively, it is possible that the modification destabilizes the tRNA.

Here too we turned into LC/MS-based chemical confirmation of the modification. We performed a shotgun analysis of tRNA fraction obtained from HeLa cells, and detected anticodoncontaining fragments bearing o2yW and OHyW from cytoplasmic tRNA^{Phe} (SI Appendix, Fig. S4-1). The peak intensities estimate that 90% of tRNA^{Phe} has OHyW, and the rest 10% has o2yW. Any other intermediates during yW biogenesis are not detected (SI Appendix, Fig. S4-1). When tywl was knocked out, both yW derivatives disappeared (SI Appendix, Fig. S4-1). Because Tyw1 is responsible for conversion from m¹G to imG-14 in yW biogenesis (44), we expected to detect an m¹G-containing fragment (ACmUGmAAm¹GAUCUAAAGp). However, we failed to detect it, unexpectedly (SI Appendix, Fig. S4-1). As m¹G can be digested by RNase T₁, we next searched for the corresponding fragments ACmUGmAAm¹Gp and AUCUAAAGp, and only found AUCUAAAGp, whose sequence was confirmed by CID (SI Appendix, Fig. S4-2), indicating that biogenesis of yW



Fig. 4. Reduction of yW modification induces translation frameshift. (A) Changes in modification levels, measured by tRNA-seq. Shown are fraction of RT abortion at position 37 of reads aligned to tRNA^{Phe}(GAA) in control cells (nontargeting gRNA) as well as levels measured for two clones of KO for *TYW1* gene. y-axis is the modification level, calculated as fraction of reads with MM (mismatch) or abortion at the indicated position. Asterisks mark the statistical significance between control levels and KO, Student *t* test **P* < 0.05, ***P* < 0.005 (*n* = 3 biological repeats). (*B*) Changes in tRNA abundance. A volcano plot showing the log2 fold-change of abundance levels of each tRNA type measured in WT vs. *TYW1*-KO. tRNA abundance is measured by the number of reads aligned to each tRNA, grouped by anticodon. Mark in red is tRNA^{Phe}(GAA). (C) A scheme for creating HeLa cells knocked out for *TYW1* and measuring frameshift using an HIV reporter. (*D*) Frameshift levels as measured by GFP expression normalized to mCherry. Shown are frameshift levels in control cells (nontargeting gRNA) as well as levels measured for two clones knocked out for the *TYW1*-KO, Student *t* test **P* < 0.05, ***P* < 0.005 (*n* = 3 biological repeats).

derivatives is impaired at the second step catalyzed by Tyw1 in the *TYW1*-KO cell.

To test the influence of the yW modification on a frameshift level, we used a fluorescent protein-based frameshift reporter (*Materials and Methods*, Fig. 4C, and *SI Appendix*, Fig. S4-3). We have recently created a library of such dual fluorescent proteins connected with diverse candidate linkers (49). To select from this library one biologically meaningful linker sequence, we returned to the biology of activated T cells. We recall that infectivity of HIV-1 is primarily focused on activated T cells, and is heavily dependent on the UUU (-1) frameshift for translation of its polymerase enzyme (24, 50). We have thus chosen the HIV gagpol frameshift sequence as the linker for our reporter (Fig. 4C).

We transfected the *TYW1*-KO cells, as well as control cells, with the fluorescent frameshift reporter and measured the frameshift level. We found that the frameshift level is significantly elevated by 6% in the mutant compared to the wild-type (Fig. 4D). This indicates that down-regulation of yW modification can indeed exert an effect on frameshift in general, and that it might affect the production of HIV polymerase, which depends on ribosomal frameshift.

A Tradeoff between Translation Fidelity and Speed. Why do activated T cells reduce the level of the two tRNA modifications that have a role in reducing ribosomal frameshift? We hypothesize that the T cell system may have evolved to trade off translation fidelity and speed, as discussed in other contexts (38, 51). One possibility is that this reduction in modifications allows cells to translate proteins more rapidly, a necessity under conditions that require rapid growth in biomass production, toward cellular proliferation. Under such conditions, we reason, the system evolved to compromise translation fidelity (i.e., to feature enhanced frameshift). This hypothesis raises the prediction that under such a tradeoff scenario, genes expressed in T cell activation would avoid the slippery codons (UUU) in favor of synonymous codons (UUC) that are not prone to frameshifting, thus reducing translation error rate. We examined each mouse gene for its tendency to prefer either UUU or UUC for encoding phenylalanine and ranked all genes according to this codon bias. Strikingly, we found that the genes with the highest UUC/UUU ratio are enriched with several functionalities involved in T cell and other immune functions (Fig. 5A). As a negative control, we examined the other "2-box" codon duplets in the genetic code (i.e., all pairs of synonymous codons of the type XXU XXC). For each such pair, we computed across all genes the XXU/XXC preference score, and ranked the genes according to the extent of XXU avoidance. None of the other eight codon pairs showed enrichment for any functionality related to T cells or immunity (SI Appendix, Table S2). Avoidance of frameshift-prone codons in the genes expressed in proliferating T cells may reduce undesired frameshifting events that can occur due to a reduction in the tRNA modification.

Discussion

In this work, we studied the tRNA pool and its interaction with the mRNA pool in a natural physiological context of massive cellular proliferation: that of activated T cells. The interplay between the tRNA and mRNA pools in cells was shown to govern proteomewide translation (7, 52, 53). Previous studies have shown that the mammalian proliferation state affects the cellular tRNA pool, but most studies were focused on cancerous proliferation (15, 54). Noncancerous, physiologically normal proliferation may obey different dynamics. Normal cellular proliferation must be a restrained process, one that actually must have evolved to avoid undesired cancerous transformation. Normal proliferation evolved over long organismal evolutionary time scales, as opposed to cancer, which operates selection over short time scales of cellular somatic evolution. The motivation to inspect the tRNA pool under conditions of normal proliferation is thus clear.

Indeed, we found that early on upon stimulation, proliferating T cells feature transcription activation of the proliferation translation program. Induced mRNA coding genes that fulfill the proliferation needs of cells represent a proliferation codon usage program and they are rather adequately served by the proliferation-oriented tRNAs that are largely induced at this stage (see summary in Fig. 5B). Yet, after the pick of proliferation (72 h in this experiment), while many of the proliferating mRNA transcripts are still present, the tRNA pool appears to have relaxed back and might no longer translate as efficiently as the proproliferation transcripts. At this time point, the difference between the CD62L⁺ and CD62L⁻ T cells is interesting: while the tRNA pool of CD62L⁺ cells appears almost fully relaxed, the CD62L⁻ still shows some residual induction of the proliferation tRNA pool signature. This is in line with the reduced proliferation rate that was observed for memory precursors compared with effector T cells during influenza infection (55). Thus, the faster decline in the expansion of memory T cells might be regulated in part by dynamics of tRNA expression that result in an imbalance between codon supply and demand.

Curiously, the correlation between changes in supply and demand is often not high. A first potential explanation for the sometimes modest increase in tRNA levels is that relationships of supply and demand in tRNA and mRNA are known to be highly nonlinear, and often feature strong criticality in which a small change in a tRNA's level can elicit a significant effect on translation [e.g., see our work on "ramping" and codon usage (56)]. Second, the analysis was based on averaging entire families of tRNAs that share the same anticodon. It is the nature of such averages that they may dampen signals of change. Indeed, when we examine individual genes from each tRNA family, we often see more pronounced fold-changes (SI Appendix, Fig. S2-4 and S2-5). This could be taken to indicate regulation on individual tRNA gene copies, consistent with our recent publication on this topic (29). Finally, perhaps the most prominent argument in the context of this report is that in addition to a change in tRNA level, posttranscriptional changes-particularly RNA modifications-might enhance and modulate the signal, affecting function and availability of tRNAs.

This study goes beyond changes in tRNA levels, as it inspects dynamics in tRNA modifications during the T cell activation process. While most modifications on most tRNAs do not change, we found two modifications that are one nucleotide downstream of the anticodon of two tRNAs, which drop significantly during the peak of proliferation, and are relaxed back toward base level when cells differentiate into memory cells. Changes in those modifications levels are known to alter the decoding rate of the corresponding codons, the codon preference, and the support of high levels of translation required by rapidly dividing cells (44, 45, 57). Interestingly, these two tRNAs decode slippery codons that are prone for ribosomal frameshifting (17, 41, 43). It thus appears that during the peak of proliferation, cells trade off translation adequacy, and maybe even run into the risk of ribosomal frameshifting.

Why do activated T cells reduce the level of the two tRNA modifications that have a role in reducing ribosomal frameshift? One possibility is a putative beneficial effect of frameshifting on these cells at the proliferation stage. It is possible to speculate that ribosomal frameshifting might enhance the phenotypic variability of immune cells. Yet, our examination of codon usage in immunerelated genes (Fig. 5A) does not provide further support to this notion. A more conservative alternative is that the T cell system may have evolved to balance the tradeoff between translation fidelity and speed, as has been discussed in other cellular contexts (38, 51). According to this possibility, reduction in modification allows cells to translate proteins more rapidly (i.e., the presence of the modifications stabilizes the codon-anticodon pairing and increases fidelity) (16, 18, 58, 59). This is a necessity under conditions that require rapid growth in biomass, toward extensive cellular proliferation. In support of this possibility, we indeed found that



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Fig. 5. Tradeoff between translation speed and accuracy. (*A*) T cell activation and immune-related genes are avoiding the use of UUU codons. Shown here are GO categories enriched with genes that avoid the use of UUU codon normalized to phenylalanine usage. Analysis of other "U" ending codons did not show enrichment for T cell-related GO categories (*SI Appendix*, Table S2). (*B*) A schematic illustration of changes in codon representation (mRNA), changes in tRNA expression, and tRNA modification, during T cell activation. Red represents proliferation codons/tRNAs and blue represents differentiation codons/tRNAs.

genes involved in immune response—such as TCF7, CD5, Lyl1, Pdcd1, and Rhoh—tend to avoid the use of the slippery codon UUU (Fig. 5A). Notwithstanding, despite the apparent avoidance of slippery codons in the immunity-related genes, other genes expressed in T cells might still be subject to elevated frameshifting upon reduction in the modification. Thus, the second prediction of the speed–fidelity tradeoff model is that if indeed T cells experience a general, perhaps nonspecific, increase in frameshifting, they could suffer from proteotoxic stress. Interestingly we found that the proteasome—both the catalytic and regulatory subunits—is induced around 20 h upon activation, followed by relaxation at later time points, suggesting that proteotoxic stress is experienced by the proliferating cells (*SI Appendix*, Fig. S1-5, *Bottom*) (60, 61). The model that thus emerges is that T cells reduce the level of two tRNA modifications that when present, prevent frameshifting at slippery codons. In doing so, they might trade off translation fidelity and speed. They protect their most crucial and cell-specific genes from containing the slippery codons, but they may suffer from otherwise nongenespecific frameshifting activity.

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Most observations in this study were done in mouse T cells. Yet, assuming the T cell proliferation in human cells features similar reduction of the same tRNA modifications would lead to the hypothesis that the increase in the potential for ribosomal frameshifting may also occur in proliferating human T cells. Interestingly, activated human T cells are the infection targets of HIV, which necessitates calibrated frameshifting rates on the UUU slippery codon between its gag and pol ORFs (24). Thus, while T cells must be reducing tRNA modifications for their own needs and interest, it is tempting to speculate that HIV takes advantage of this dynamics that is utilized by the virus as a vulnerability.

Materials and Methods

T Cell Isolation and FACS Sorting. Two independent biological repeats were done as follows. Naïve cells were extracted from 6 and 21 spleens of B6 mice using the StemCell CD62L⁺CD44⁺ kit (female, 7 wk, 6 to 9% naïve). Each 2 (of the 6) or 7 (of the 21) spleens were pooled to a total of three samples extracted separately. Naïve cells were activated by the addition of anti-CD3/anti-CD28 activation beads (1:1 bead:cell ratio; ThermoFisher Scientific Cat#11131D). Cells were grown at 0.25×10^6 cell/ml in 24 wells, and were collected at 20, 48, and 72 h after activation.

For the first biological repeat, a portion from the naïve state was taken to FACS sorting for live cells followed by RNA extraction. At 20 h only live cells were sorted. At 48 and 72 h, cells were sorted into CD62L⁺CD44⁺ and CD62L⁻CD44⁺, as described in *SI Appendix, Extended Material and Methods*.

mRNA-seq. mRNA-seq for the first biological repeat was preform using the MARS-Seq protocol, as described previously (62), and analyzed as described in *SI Appendix, Extended Material and Methods*. Libraries were sequenced using a 75-bp single-read output run on the Illumina NEXTseq platform.

mRNA-seq for the second biological repeat was done in parallel to ribosome footprint sequencing by a tailored mRNA-seq protocol, as previously described (63) (see *SI Appendix, Extended Material and Methods* for further details)

tRNA-Seq and Read Analysis. tRNA sequencing protocol was adapted from Zheng et al. (26) with minor modifications, described in *SI Appendix, Extended Material and Methods*.

Codon Usage Calculation. Codon usage and codon bias were calculated based on mRNA expression. We summed the usage of each codon in each gene multiplied by the expression of the gene:

{Codon Usage} = $\sum_{i=1}^{n}$ codon Frequecy in gene i × mRNA expression of gene i.

Estimating tAI Based on tRNA Expression. We generated a measure of modified tAI (tRNA adaptation index, implemented in Fig. 2). Our measure is

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calculated similarly to the tAI (32), with one major change: we determined tRNA availability based on the read aligned to the indicated tRNA, as done in our previous work (5). As such, this measure can be computed for every condition. We defined the tRNA availability of each tRNA type (anticodon) by the sum reads of its tRNAs. Then, we determined the tAI of an individual codon by the extent of reads of tRNA state serve in translating it, incorporating both the fully matched tRNA as well as tRNAs that contribute to translation through wobble rules (W-value) (64, 65).

Since there are biases in the tRNA-seq methods, we employed this method as a comparative approach: we report fold-changes between conditions and not the actual value.

Frameshift Assay. Control and *TYW1*-KO HeLa cells were seeded onto six-well plates such that cell confluence will be ~70% the next day (~150,000 cells). Cells were transiently transfected with frameshift reporter plasmids. The plasmids express mCherry, followed by a HIV gag-pol frameshift signal, followed by GFP out of frame (-1). As a control, we used in-frame GFP and out of frame (+1) GFP in the same plasmid context (Fig. 4*C* and *SI Appendix*, Fig. S4-3*A*). The plasmids were generous gift from Martin Mikl, Weizmann institute, Rehovot, Israel (49). The cells were analyzed using ATTUNE FACS (ThermoFisher) 48 h after transfection. Frameshift rate was calculated as GFP/mCherry ratio, in the linear range of fluorescent slop (*SI Appendix*, Fig. S4-3).

SI Appendix, Extended Material and Methods include further details of the study materials and methods.

Data Availability. The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database (accession no. GSE165622) (66). Data sources are as follows: The coding sequences of *Mus musculus* were downloaded from the Consensus CDS (CCDS) project (https://www.ncbi.nlm. nih.gov/projects/CCDS/CcdsBrowse.cgi). For classification of gene categories, defined gene categories by biological process were downloaded from the GO project (http://geneontology.org/); to avoid too-small gene sets, we considered only those with at least 40 genes. Pathway lists were downloaded from the MODOMICS (9) database (http:// genesilico.pl/modomics/).

ACKNOWLEDGMENTS. We thank the Y.P. and N.F. laboratory members Noa Hefetz-Aharon, Martin Mikl, Schraga Schwartz, and Daniel Douek for stimulating discussions. We also thank the European Research Council, the Israel Science Foundation, and the Israel Cancer Research Fund for grant support.

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