


Paradox Found: Global Accounting of Lymphocyte Protein Synthesis

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Abstract

Rapid lymphocyte cell division places enormous demands on the protein synthesis machinery. Flow cytometric measurement of puromycylated ribosome-associated nascent chains after treating cells or mice with translation initiation inhibitors reveals that ribosomes in resting lymphocytes *in vitro* and *in vivo* elongate at typical rates for mammalian cells. Intriguingly, elongation rates can be increased up to 30% by activation *in vivo* or fever temperature *in vitro*. Resting and activated lymphocytes possess abundant monosome populations, most of which actively translate *in vivo*, while *in vitro*, nearly all can be stalled prior to activation. Quantitating lymphocyte protein mass and ribosome count reveals a paradoxically high ratio of cellular protein to ribosomes insufficient to support their rapid *in vivo* division, suggesting that the activated lymphocyte proteome *in vivo* may be generated in an unusual manner. Our findings demonstrate the importance of a global understanding of protein synthesis in lymphocytes and other rapidly dividing immune cells.

eLife assessment

This study addresses how protein synthesis in activated lymphocytes keeps up with their rapid division, with **important** findings that are of significance to cell biologists and immunologists endeavouring to understand the 'economy' of the immune system. The work is supported by **solid** data. Because it proposes non-conventional mechanisms, the study sets the scene for further work in this area.

Introduction

Naïve lymphocytes are among the smallest nucleated cells in mammals – nearly devoid of cytoplasm, with few mitochondria – and have minimal metabolic activity, consistent with doubling times on the order of hundreds to thousands of days, respectively, for B cells ^{1,2,3} and T cells ³. Within a day of activation by cognate antigen, lymphocytes begin to divide rapidly, with reported doubling times as rapid as 6 hours ^{4,5}. Such Jekyll and Hyde behavior requires

massive induction of DNA and protein synthesis to support daughter cell production ⁶ as well as synthesizing large amounts of immune regulatory (e.g., cytokines) and effector molecules (e.g., antibody and cytokines) ^{7–9}.

Pioneering studies of protein synthesis regulation in lymphocytes utilizing radiolabeled amino acids on mitogen-activated human peripheral blood lymphocytes reported 7 to 20-fold increases in protein synthesis activity ^{10,11}. While this is an impressive increase, it was assumed that sufficient protein was synthesized to enable the generation of daughter cells with the same protein content as their progenitor. Moreover, radiolabeling, like all methods, is imperfect, and its accuracy as a measure of protein synthesis rates depends on assumptions that are nearly impossible to definitively verify ¹². Applying new methods to old problems is a tried-and-true method for generating new insights and discoveries.

Indeed, newer methods, including ribosome profiling ¹³, tRNA arrays ¹⁴, and tandem mass spectrometry ¹⁵, are revolutionizing the field of protein synthesis. This includes extending classical methods. Puromycin (PMY) is an aminonucleoside antibiotic that mimics tyrosine-tRNA, binding the ribosome A site and causing rapid chain termination by covalently attaching to the C-terminus of the nascent chain. PMY was first applied in classical protein synthesis studies ¹⁶ and remains a workhorse in understanding ribosomal catalysis of protein synthesis ^{17–20}.

We developed the ribopuromycylation method (RPM) to better localize and quantify active protein synthesis. RPM uses a brief pulse of PMY to label elongating nascent chains frozen on ribosomes by treating cells with a translation elongation inhibitor. Ribosome-bound nascent chains are then detected using a PMY-specific monoclonal antibody in fixed and permeabilized cells via standard immunofluorescence ²¹ or flow cytometry ²².

Here, we use RPM, and the ribosome transit assay (RTA), an extension of RPM that measures elongation rates, in conjunction with classic techniques to quantify the number and protein synthesis activity of ribosomes in resting and activated human and mouse lymphocytes. Our findings reveal novel features of lymphocyte translation as well as a discrepancy in the protein synthesis capacity of T cells with respect to their rapid *in vivo* division rates, emphasizing the importance of quantitative accounting as a reality check for our limited understanding of fundamental aspects of cell biology and immunology.

Results

Characterizing protein synthesis in human lymphocytes *ex vivo* with flow RPM implicates widespread ribosome stalling in non-activated cells

We first used flow RPM to compare translation in non-activated vs. PMA/ionomycin/IL-2-activated human lymphocyte subsets after 2 and 5 days in culture (**Figure 1A**). We devolved the total flow RPM signals into T cell (CD4⁺, CD8⁺) and B cell (CD19⁺) subsets to follow distinct patterns of protein synthesis in each population (Supplemental Figure 1A). Comparing lymphocytes from 3 donors revealed considerable donor heterogeneity in RPM staining of day 2 activated cells and proliferation of lymphocyte subpopulations.

We performed RPM on peripheral blood mononuclear cells labeled with CFSE to track cell division by dye dilution (Supplemental Figure 1B). On day 2, activated CD8⁺ T cells demonstrated a wide range of RPM staining, with nearly all divided cells at day 5 CFSE^{low} and RPM^{high}. Some divided

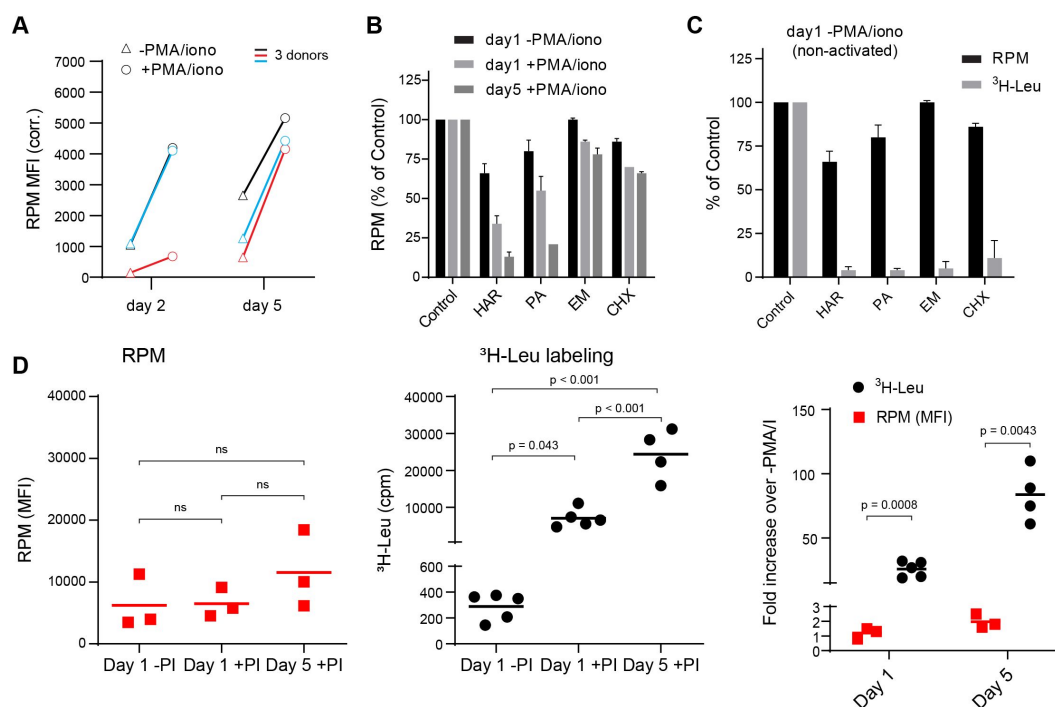


Figure 1

Stalled ribosomes in resting *ex vivo* human lymphocytes

(A) Primary human lymphocytes from three independent donors were cultured in PMA/ionomycin and IL-2 (+PMA/iono) or IL-2 only (-PMA/iono) for up to 5 days. CD45⁺ cells were processed for flow RPM. (B) Primary human lymphocytes were cultured *ex vivo* as indicated, followed by a 15 minute treatment with vehicle, harringtonine (HAR, 5μg/mL), pactamycin (PA, 10μM), emetine (EME, 25μg/mL), or cycloheximide (CHX, 200μg/mL), and all cultures were then treated with puromycin (PMY, 50μg/mL) for 5 minutes. Cells were harvested, and RPM staining was performed. Gated on CD45⁺ cells. Error bars represent standard deviation of two independent experiments. (C) Radioactive amino acid incorporation (0.2 mCi/mL [³H]-Leu for 5 min) or RPM (as in B) in day 1 non-activated human lymphocytes. Error bars represent standard deviation of two independent experiments. (D) Radioactive amino acid incorporation and RPM in rested and activated human lymphocytes. RPM MFI values (gated on CD45⁺ cells) on the left, [³H]-Leu incorporation (cpm) in the middle, and ratios of the activated to the resting cells on the right. Each point represents a single donor; bars indicate the mean from 3-5 independent donors. Left and middle panels: one-way ANOVA pairwise p-values; right panel: unpaired t-test p-values with Welch's correction.

cells exhibited near baseline RPM signals, however, consistent with their return to a resting state. Interestingly, although non-activated cells did not divide, ~50% demonstrated increased RPM staining.

We noted that the RPM signal in PMA/ionomycin-activated CD8⁺ T cells was only 2-to 5-fold higher than in non-activated cells. This increase is modest compared to the ~15-fold activation-induced increase in protein synthesis in original studies ^{10,11}. To examine this discrepancy, we first incubated cells for 15 min with initiation inhibitors (harringtonine, HAR; pactamycin, PA) or elongation inhibitors (emetine, EME; cycloheximide, CHX), followed by RPM staining. Elongation inhibitors had minor effects on RPM of activated or resting cells (**Figure 1B**), as expected due to ribosome retention of nascent chains ²¹. Initiation inhibitors, however, clearly discriminated between resting and activated cells. RPM signal was diminished by up to 80-90% on day 5 post-activation. Note that at the standard translation rate of 6 amino acids/sec, 15 min is sufficient time to complete translation of all but the very longest transcripts.

We repeated this experiment using day 1 resting lymphocytes to directly compare flow RPM with classical metabolic radiolabeling with [³H]-Leucine ([³H]-Leu) (**Figure 1C**). All inhibitors nearly completely blocked incorporation of [³H]-Leu into proteins, suggesting that there were actively translating ribosomes in resting cells and that the inhibitors were active, even though RPM labeling was only weakly impacted. We also performed a time course examining [³H]-Leu incorporation compared to flow RPM signal in day 1 resting, day 1 activated, and day 5 activated human lymphocytes. Plotting the ratios between activated and non-activated cells from RPM flow vs. [³H]-Leu incorporation revealed a substantial difference between the two methods (**Figure 1D**).

Thus, we cannot attribute the persistence of flow RPM staining in translation initiation inhibitor-treated resting lymphocytes to incomplete inhibition of protein synthesis. Instead, these data are consistent with a significant fraction of “stalled” ribosomes in cultured resting cells, *i.e.*, ribosomes with nascent chains that are not actively translating. Stalled ribosomes would be labeled with PMY, as originally described in neurons ²³, but would not incorporate [³H]-Leu, just as we observe with resting lymphocytes.

Flow RPM measures ribosome elongation rates in live cells

To extend these findings, we developed a variation of approaches that use initiation inhibitors to measure ribosome transit times, for example by conversion of polysomes to monosomes ²⁴ or ribosome profiling ¹³. To derive a relative ribosome transit rate, we incubate cells with the initiation inhibitor HAR for increasing times before shifting cells to 4°C to halt ribosome elongation and process for RPM staining (**Figure 2A**).

We validated this approach in HeLa cells whose ribosome transit times are well characterized ²⁵. This revealed a curve that follows one phase exponential decay (**Figure 2B**; gating strategy in Supplemental Figure 1C), with a calculated half-life to decay of 70-150 seconds. Including EME with HAR prevented decay of the RPM signal, as predicted, since EME blocks elongation while enabling (even enhancing) puromycylation ^{21,26}.

Immunoblotting of puromycylated nascent chains validated the approach by showing a time-dependent decrease in puromycin signal and increased Mr of nascent chains after blocking initiation (**Figure 2C**). This is expected since nascent chains present at later time points after blocking initiation will be longer. Incubation of EME with HAR greatly retarded the loss of signal and the shift to longer puromycylated nascent chains.

We applied this RPM-based “ribosome transit assay” (RTA) to investigate translational control in human lymphocytes. Day 5 activated lymphocytes behaved similarly to HeLa cells in their RTA half-life and EME sensitivity (**Figure 2D**). By contrast, in day 1 resting lymphocytes, there was a

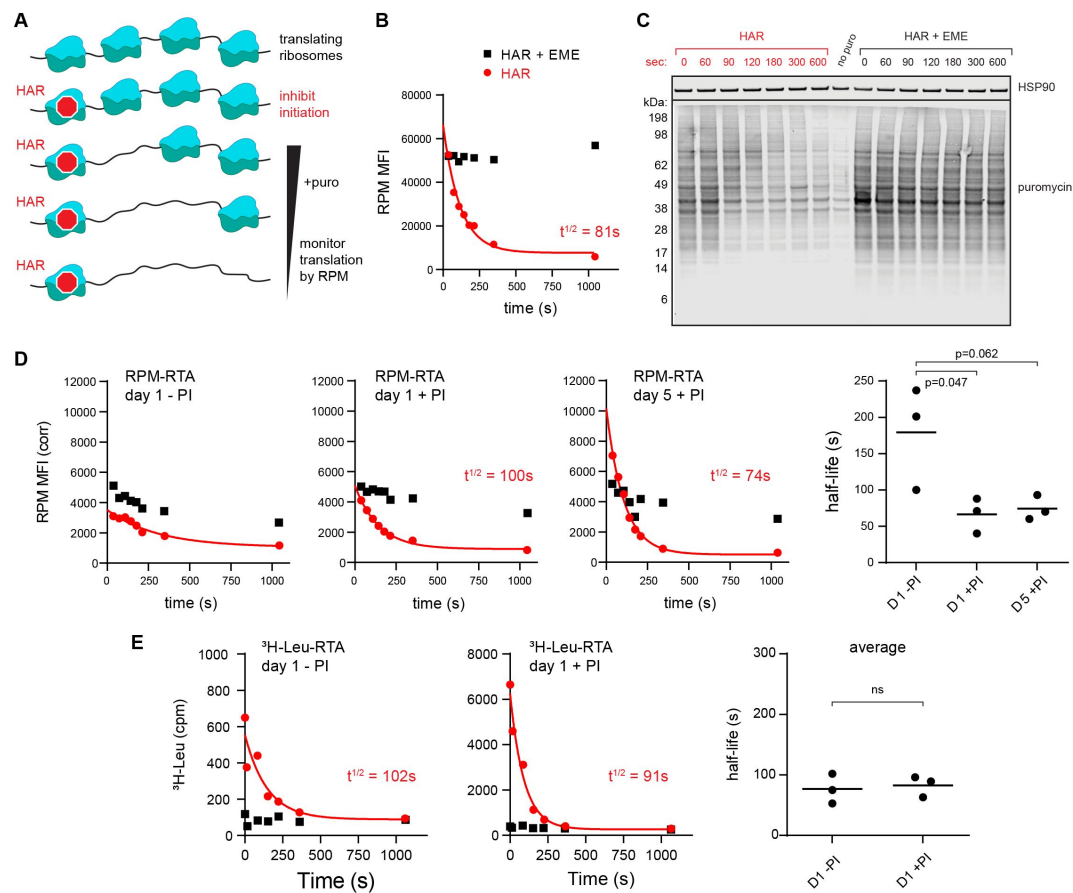


Figure 2

RPM measures ribosome transit times in HeLa and human lymphocytes

(A) Schematic representation of the ribopuromycylation (RPM) Ribosome Transit Analysis (RTA) method. Translation initiation is blocked and the decrease in RPM is monitored as the elongating ribosomes run off mRNA. (B) RPM-RTA in HeLa cells. Harringtonine (HAR, 5 µg/mL) is used to inhibit new ribosome initiation; emetine (EME, 25 µg/mL) is used to freeze ribosomes on mRNA; puromycin (PMY, 50 µg/mL) generates RPM signal. Curve is fitted using one phase exponential decay, and ribosome transit times are expressed as RPM half-time to decay. (C) Same as B, but cells are instead lysed in the presence of MG-132 and subjected to anti-puromycin western blot analysis. (D) Representative plots of the RPM-RTA signal in resting and activated human lymphocytes (left three panels). Gated on CD45+ cells. Far right, ribosome transit times determined from 3 independent donors. Each dot represents data from one individual donor; the horizontal bars indicate the mean. P-values indicate one-way ANOVA pairwise comparisons. (E) Ribosome transit times as in A but determined by [³H]-Leu incorporation instead of RPM. After treatment with HAR or HAR plus EME, cells were labeled for 5 minutes in 0.25 mCi/mL [³H]-Leu. Right panel, ribosome transit times determined by [³H]-Leu incorporation from three independent donors. Each dot represents data from one individual donor; the horizontal bars indicate the mean. Unpaired t-test.

limited decay in the signal. Further, the decay was similar in EME-treated cells, consistent with the idea that the flow RPM signal in day 1 resting lymphocytes predominantly represents stalled ribosomes with bound nascent chains.

To independently measure ribosome transit times in day 1 resting vs. activated lymphocytes, we treated cells for increasing times with HAR and then pulse-labeled with [^3H]-Leu (**Figure 2E** [↗](#)). This showed that both resting and activated cells demonstrated a decay half-life of ~90-100 seconds, similar to the RTA values for activated lymphocytes and HeLa cells.

Based on these findings, we conclude that:

1. A large fraction of ribosomes stalled in resting cultured lymphocytes.
2. Elongation occurs at similar rates for HeLa cells and lymphocytes, with the active ribosomes in resting lymphocytes translating at a similar rate as fully activated lymphocytes.
3. RTA provides a simple flow cytometric measure of ribosome transit rates, confirming and extending the findings of Arguello *et al.* [27](#) [↗](#) who reported a highly similar method.

Resting human lymphocytes have a dominant monosome population

Protein synthesis is generally believed to occur predominantly in polysome structures, consisting of multiple ribosomes transiting a single mRNA [28](#) [↗](#). Classic [29](#) [↗](#), [30](#) [↗](#) and more recent studies [31](#) [↗](#) have established, however, that resting lymphocytes have few polysomes and provided evidence for active monosome translation by their stability in high salt, which dissociates non-translating ribosomes [32](#) [↗](#).

Confirming these reports, we found that a large fraction of assembled ribosomes in resting human lymphocytes fractionate as monosomes in sucrose gradients (Supplemental Figure 2A). Polysome abundance increases over two days post-activation. Treating freshly isolated human lymphocytes with CHX to freeze ribosomes [33](#) [↗](#), [34](#) [↗](#) did not increase polysome recovery (Supplemental Figure 2B). These findings, coupled with our RPM/RTA measurements, indicate that stalled ribosomes are likely monosomes.

Protein synthesis in mouse lymphocytes *ex vivo*

Working with human lymphocytes is problematic – preparations between individuals vary considerably, and the manipulations required to isolate lymphocytes from donor blood, such as elutriation and Percoll gradient purification, increase the time cells spend outside their physiological environment.

Seeking a more reproducible system without the impact of potential artefactual stalling of the translation machinery, we turned to OT-I TCR transgenic mice [35](#) [↗](#). OT-I cells are CD8⁺ T cells specific for a defined cognate ligand (mouse K^b MHC class I molecules bound to the ovalbumin-derived SIINFEKL peptide) that can be activated *in vitro* or *in vivo*. OT-I T cells can be obtained from spleen or lymph nodes (LNs) in reasonable numbers at ~80% purity without further manipulation.

RTA analysis revealed that there was no decay in RPM signal for *ex vivo* day 1 resting OT-I T cells, consistent with near total stalling of once-translating ribosomes, as we identified in human lymphocytes (**Figure 3A** [↗](#), middle panel). By contrast, in freshly isolated OT-I cells, the RPM signal decays by 50%, consistent with active translation by 50% of the ribosomes with the rest of the ribosomes likely stalled or poised. The signal decay $t_{1/2}$ of 40 seconds is consistent with translation

of shorter than average mRNAs or stalling on partially translated mRNAs. Polysomes were a minor fraction in freshly isolated mouse lymphocytes, even when mice were pre-treated with CHX (Supplemental Figure 2C, further addressed in the next section).

By contrast, day 2 activated *ex vivo* OT-I T cells demonstrated a 20-fold increased RPM signal relative to resting cells, a near total signal decay with a $t_{1/2}$ of ~70 seconds (Figure 3A, right panel), and a preponderance of polysomes (Supplemental Figure 2D). This is consistent with the large fractional engagement of ribosomes upon activation. Notably, the decay rate is faster than observed in previous conditions and intriguingly, the rate increases by ~20% at a “fever” temperature of 39.5 C° (Figure 3B). This suggests that lymphocytes may be able to exceed the standard mammalian cell elongation rate of ~6 residues/second³⁶, particularly under fever conditions, when maximizing T cell protein synthesis is likely at a premium to support their anti-viral activity by rapid division and production of effector molecules.

Protein synthesis in mouse lymphocytes and innate immune cells *in vivo*

Mammalian cells evolved, of course, in mammals, not in plastic flasks nurtured by synthetic media in a 20% oxygen atmosphere. We therefore adapted the RTA assay to mice. To simultaneously measure resting and activated T cells, we adoptively transferred CFSE-labeled OT-I T cells into congenic B6 mice, which we infected with SIINFEKL-expressing vaccinia virus (VACV) to activate OT-I cells. We then injected mice with HAR for 0-10 min, followed by PMY injection and flow RPM processing of harvested splenocytes (Figure 4A). With each mouse providing a single data point, we could generate RTA curves for non-activated host CD4 and CD8 cells as well as transferred OT-I cells activated by VACV infection (Figure 4B). These curves show that nearly all ribosomes with nascent chains in both resting and activated lymphocytes are actively elongating proteins *in vivo*.

The elongation rate *in vivo* is surprisingly slower than the *in vitro* rate. Notably, this experiment used our original protocol of PMY treatment alone²² since EME, the inhibitor used to stabilize puromycylated polypeptides on ribosomes *in vitro*²¹ was ineffective *in vivo*. We found, however, that CHX is active *in vivo*, arresting the accumulation of puromycylated polypeptides for at least 60 min after injecting PMY (Supplemental Figure 3A). We therefore modified the RTA by simultaneously treating animals with CHX with PMY to determine the relative amount of ribosome-associated nascent chains *in vivo*. This enabled comparison of translation activity in various immune cell types using 15 min HAR pretreatment values to subtract the signal from stalled ribosomes. The number of translating ribosomes varies over a narrow range among resting splenic lymphocytes, NK cells, macrophages, and neutrophils (Supplemental Figure 3B).

Using this improved RPM protocol, one day after infecting mice with VACV we now measured a ~15-fold increase in translating ribosomes in activated OT-I T cells *in vivo* (Supplemental Figure 3C; gating strategy in Supplemental Figure 3D) as compared to the 10-fold increase we previously reported²². As cell division progressed over the next two days, the signal from translating ribosomes decreased (Supplemental Figure 3E-F). Comparing the results from Supplemental Figure 3C (OT-I cells) with Supplemental Figure 1B (polyclonal human CD8+ T cells) reveals what we had described previously²², that a transgenic T cell population has much less spread of RPM staining when compared to activated polyclonal T cells in C57/BL6J mice after VACV infection, or here when comparing to all human CD8+ T cells.

We next performed the modified RTA to measure translation rates in OT-I cells *in vivo* on day 2 and 3 post-infection with VACV-SIINFEKL (Figure 4C). Addition of CHX to the *in vivo* RTA is important because of the well-characterized “leakiness” of harringtonine³⁷; indeed, ribosome transit times in activated OT-I cells were now in line with the *in vitro* rates, and were ~20% faster than transit times in recipient (non-activated) T cells.

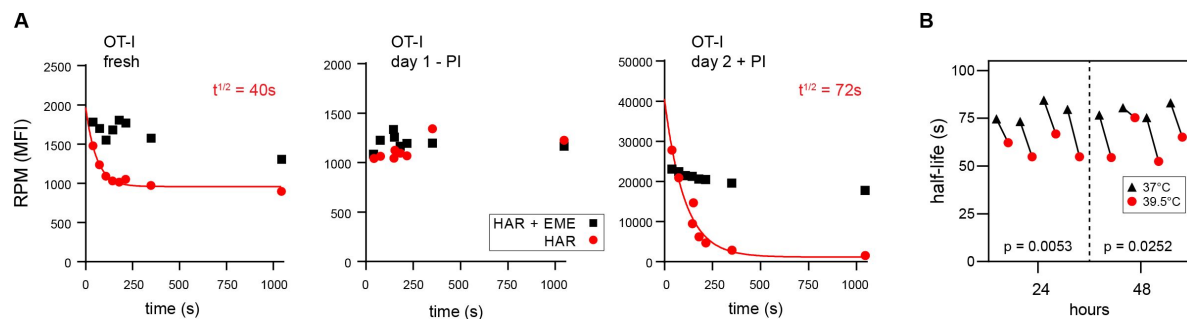


Figure 3

RPM ribosome transit analysis of OT-I T cells *in vitro*

(A) Lymphocytes from spleens and lymph nodes from transgenic OT-I mice were isolated, and either used immediately, cultured for one day in the absence of PMA/ionomycin, or cultured for 2 days in the presence of PMA/ionomycin and IL-2. RPM-RTA analysis was conducted to determine ribosome transit half-lives, both with and without EME. (B) Lymphocytes from spleens and lymph nodes from transgenic OT-I mice were isolated, labeled with CFSE, and cultured under activating conditions for either 24 or 48 hours. Cells were harvested, and RPM-RTA was performed at both 37°C and 39.5 °C. Half-life of RPM signal by RTA is plotted; p-values determined by paired t-test analysis.

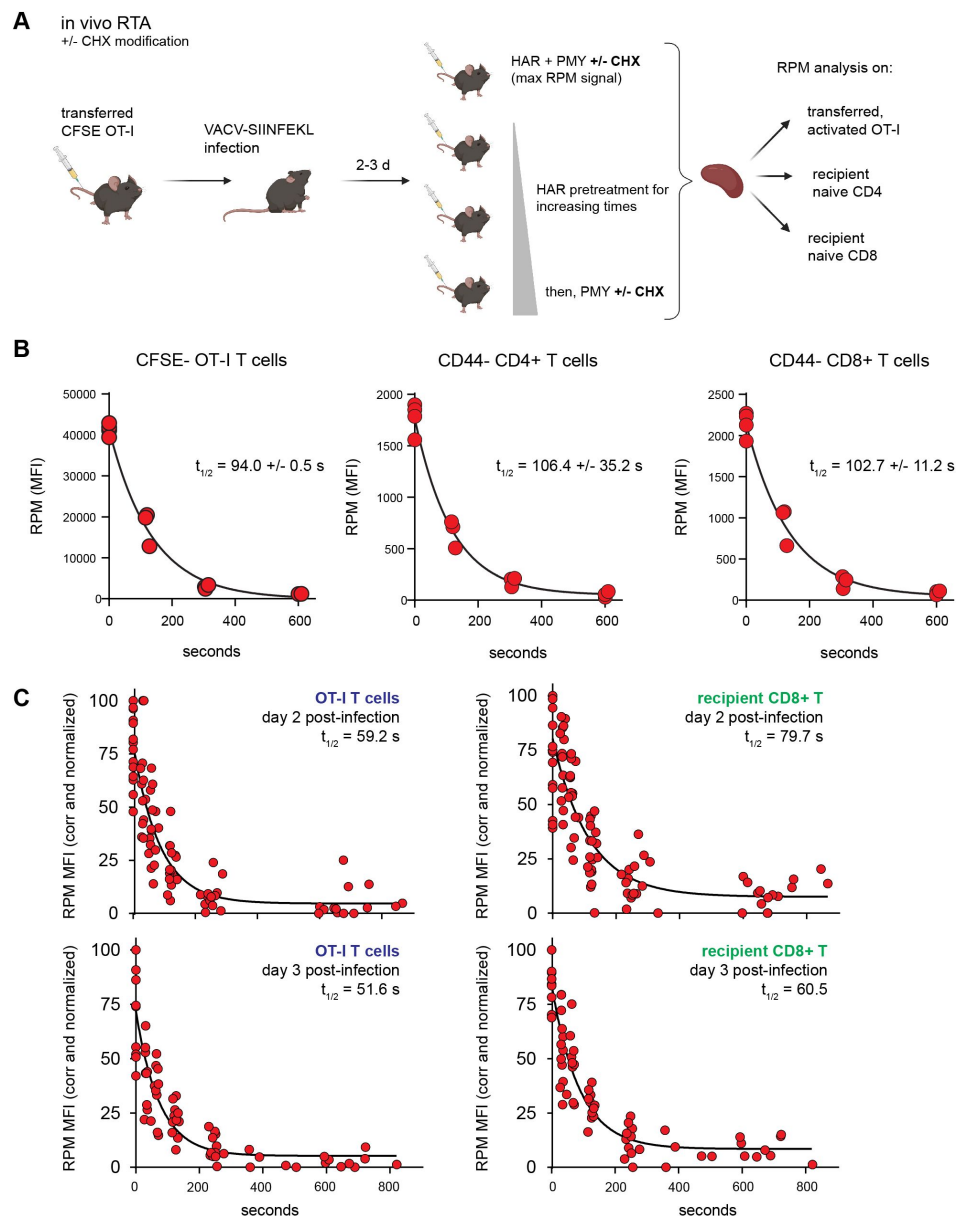


Figure 4

Translation rates of resting and activated T cells *in vivo*

(A) Depiction of the *in vivo* RPM-RTA method. Labeled OT-I T cells are first adoptively transferred, followed by VACV-SIINFEKL infection of mice. RTA analysis is performed by intravenous injection of HAR followed by PMY (+/- CHX to prevent leakiness from HAR inhibition alone). Spleens are harvested for RPM analysis on both endogenous and transferred T cells. Schematic designed with Biorender. (B) CFSE-labeled Ly5.2⁺ (CD45.2⁺CD45.1⁻) OT-I T cells were adoptively transferred into Ly5.1 (CD45.1⁺CD45.2⁻) mice, which were then infected with VACV-SIINFEKL to activate the OT-I cells. Three days after infection, mice were intravenously injected with HAR simultaneously with PMY for 5 minutes (maximum signal), or first injected with HAR for ~110, ~275, or ~575 seconds before being injected with PMY for 5 minutes. Splenocytes from mice were harvested, surface stained for gating and activation markers as indicated, fixed and permeabilized, and stained for RPM. Gates were CFSE^{low} OT-I CD8⁺ T cells to measure decay in activated cells, and CD44⁺CD8⁺ or CD44⁺CD4⁺ T cells to measure decay in resting T cells. The curve was generated by fitting to a one phase exponential decay. Representative of two independent experiments, 2-4 mice per group, with the mean and standard deviation of the calculated half-life decays as indicated. (C) RTA, with the CHX modification, of adoptively transferred OT-I T cells or un-activated host CD8⁺ T cells in mice infected for 2 or 3 days with VACV-SIINFEKL. 3-4 independent experiments combined, normalized by setting maximum background-subtracted signal to 100.

These results indicate that:

1. Monitoring accurate translation and rates *in vivo* is possible and avoids artifacts associated with *ex vivo* lymphocyte cultures.
2. Activation increases elongation rates in lymphocytes by ~20%.

Contribution of monosomes vs. polysomes to T cell translation

We next biochemically characterized translation in resting OT-I cells *in vivo* or OT-I cells activated *in vitro* by PMA/ionomycin/IL-2. We treated animals/cells with CHX/PMY, isolated ribosomes from cell lysates on sucrose gradients in monosome and polysome fractions, blotted fractions onto nitrocellulose and stained with antibodies against RPL7 or PMY. The robust PMY signal shows that, contrary to recent claims^{38,39}, PMY does not completely release nascent chains when ribosomes are previously exposed to CHX (**Figure 5A**).

After setting the puromycylation:RPL7 ratio in polysomes to 100% (assuming that all ribosomes in the polysome fraction are actively translating), we found that 33% of monosomes in resting *in vivo* OT-I T cells and 52% of monosomes in day 2 activated OT-I T cells were puromycylated. Since *in vivo* RTA indicates that there is essentially no stalling of puromycylated ribosomes (**Figure 4C**), these data demonstrate robust translation in T cell monosomes. Assuming equal elongation rates, ~38% and ~32% of overall translation would occur in monosomes of resting *in vivo* and activated *in vitro* cells, respectively. We note, however, that since PMY reduces the number of polysomes recovered from CHX-treated cells by 5-10%, a small fraction of translating monosomes probably derive from the polysome population.

The high fraction of monosome-based translation is surprising in activated cells. We noted that the activation protocol for OT-I T cells we used is far less effective than that published by Tan *et al.*³¹ which includes SIINFEKL antigenic stimulation along with PHA and ionomycin. Bulk peptide-antigen stimulation directly *ex vivo* is not possible with human cells, but it is with transgenic murine T cells, and the methodological adaptation enhances activation. Direct comparison of the protocols confirmed the superiority of Tan *et al.*, as shown by activation markers (CD69, CD25, CD44), cell size (measured by side scatter, which correlates well with automated diameter measurements), and cell division (Supplemental Figure 4A-B). OT-I cells activated by this protocol yielded large increases in the observed polysome fractions of activated splenocytes, or lymph-node-derived lymphocytes (**Figure 5B**). This was also evident during high salt fractionation conditions, where we found that 500mM was necessary to fully dissociate non-translating ribosomes compared to the often used 300mM (Supplemental Figure 5A-B). T cell ribosomes had quantifiable but low levels of monosomes under these high salt conditions (Supplemental Figure 5C-D; note that we could not obtain enough activated OT-I cells *in vivo* for these experiments).

These findings indicate that monosomes make a major contribution to translation in resting T cells but are likely to make a minor contribution in fully activated cells. These results might also complicate the conclusion reached by Geraschchenko *et al.*⁴⁰ that harringtonine may only be useful until 45 seconds after the start of treatment, as the assumption was made that polysomes were the only ribosome subset actively translating mRNA.

Accounting for translation in lymphocytes: measuring the protein-to-ribosome ratio

Cells need to synthesize sufficient proteins to regenerate a complete proteome each division cycle. This number will depend on the division rate, cell size, protein concentration, and protein loss due to degradation and export (secretion, release of exosomes, loss of other cellular material). To understand how the protein synthesis apparatus enables such rapid T cell division times, we

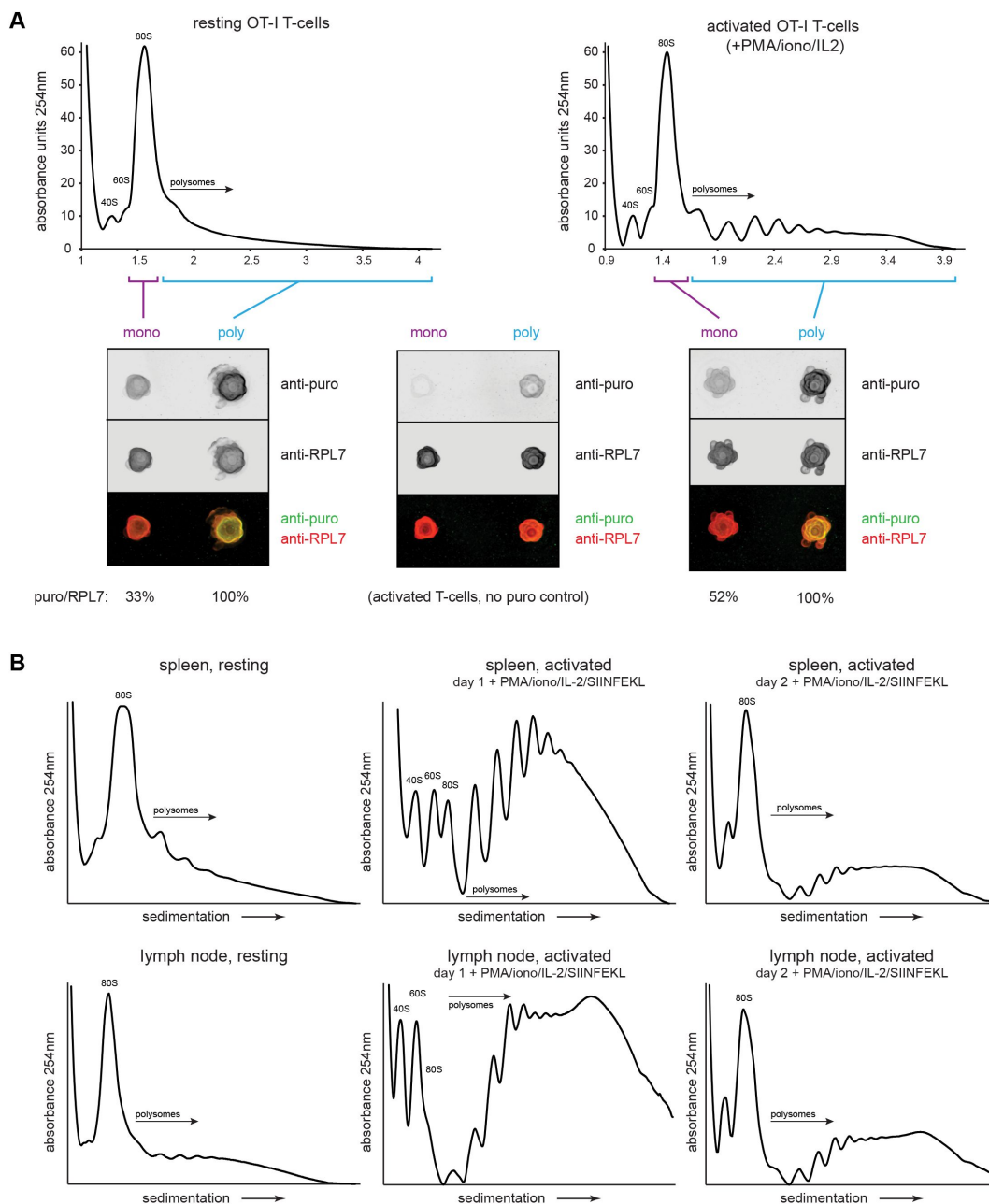


Figure 5

Puromycylation reveals percentage of actively translating monosomes in resting and activated T cells

(A) OT-I mice were treated intravenously with CHX and PMY, and lymphocytes from the spleens and lymph nodes were isolated and subjected to polysome profiling by ultracentrifugation through 15-45% sucrose gradients (resting OT-I T cells). OT-I T cells activated *in vitro* for 2 days with PMA/ionomycin and IL-2 (without cognate SIINFEKL peptide) were treated either with CHX alone (no PMY control) or CHX with PMY and subjected to polysome profiling. The indicated fractions were collected, pooled, and their ribosomes were re-isolated and dotted onto a nitrocellulose membrane for blotting with antibodies against PMY and RPL7. After subtraction of background signal from the anti-puro antibody (middle panel), the PMY/RPL7 ratio of monosomes was expressed relative to that of polysomes, which was defined as 100% translating. Representative of two independent experiments. (B) For resting T cells, OT-I mice were treated intravenously with CHX, and lymphocytes from the spleens or lymph nodes were isolated and lysed. For activated T cells, lymph node or splenic OT-I T cells were stimulated *in vitro* for 2 days with PMA/ionomycin, IL-2, and exogenous SIINFEKL, followed by treatment with CHX for 5 minutes. For both resting and activated cells, ribosome-containing lysates were fractionated via ultracentrifugation on 15-45% sucrose gradients.

quantitated a number of critical protein synthesis parameters in resting and activated OT-I T cells (**Figure 6A** [↗](#)). For these experiments, we used the optimized protocol for *in vitro* OT-I T cell activation [31](#) [↗](#) that greatly increased the fraction of ribosomes in polysomes on day 1 post activation.

Automated microscope measurements revealed that OT-I T cells increase in diameter from the resting state to the day 1 and day 2 activated states, with a corresponding calculated volume increase (based on spherical geometry) of ~2.9 fold (**Figure 6B** [↗](#)). To quantify protein content, we determined total tryptophan (Trp) autofluorescence of fully denatured proteins in a total cell lysate [41](#) [↗](#). Protein content per T cell increases ~5-fold following activation (**Figure 6C** [↗](#)), from 421 million proteins per cell (assuming an average length of 472 aa and a proteome Trp content of 0.69% [41](#) [↗](#)) to 2.15 billion proteins per cell in day 2 activated cells, resulting in a net 1.7-fold increase in protein concentration (**Figure 6D** [↗](#)).

We determined the number of ribosomes per cell using a Bioanalyzer electrophoresis device to measure the amount of 18S and 28S rRNA in purified total RNA based on staining with a RNA-binding dye and utilizing a spike-in standard mRNA to control for yield loss during RNA purification (**Figure 6E** [↗](#)). The maximal number of translating ribosomes is limited by the less abundant subunit, which in all cases is the 60S subunit (typically 75-90% of the 40S subunit). 60S subunits increased both in absolute terms and per unit cell volume as T cells became activated, reaching a maximum of ~3.6 million copies per T cell following 2d *in vitro* activation from 264,000 copies in resting T cells (**Figure 6F-G** [↗](#)). These numbers are similar to those reported by Wolf *et al.* [42](#) [↗](#), but should be more accurate since Wolf *et al.* used total cellular RNA content to estimate ribosomes.

Could there be a significant pool of non-functional ribosomes in the nucleus, where initial assembly occurs, and which occupies nearly 50% of the volume of resting T lymphocytes [43](#) [↗](#) and 34% of activated T cells [44](#) [↗](#)? Immunoblotting of fractionated nuclei shows that the distribution of ribosomes in lymphocytes is similar in resting and activated OT-I T cells and HeLa cells, with only a small fraction of total ribosomal subunits detected in nuclear lysates (Supplemental Figure 6).

The ratio of proteins to ribosomes is critical since it dictates the minimal time to replicate the proteome during cell division. This dropped up to 3-fold as T cells became activated (**Figure 6H** [↗](#)). Since mammalian ribosomes elongate at ~6 residues per second [13](#) [↗](#), [36](#) [↗](#), we calculated the minimal time for a ribosome to recreate the proteome based on the protein/maximally assembled ribosome ratios, not accounting for protein degradation or secretion.

For HeLa cells, it would take a ribosome 19.9 h to synthesize 910 “average” proteins of 472 amino acids, reasonably close to the reported doubling time of ~24 hours. For OT-I T cells, with an *in vitro* doubling time of ~9.7 h, the calculated minimal proteome duplication time is also within shouting distance – 10.1 h by day 2. Therefore, the division rates of *in vitro* activated OT-I T cells, and HeLa cells can be approximated from the number of proteins and functional ribosomes translating a full capacity.

Paradoxical discrepancy in OT-I cell division rate and protein synthesis capacity

We extended these findings to OT-I T cells *in vivo*, determining first that adoptively transferred OT-I T cells divide most rapidly between day 1 and 2 of activation during acute viral infection, with an average doubling time of 6.8 h, slowing to approximately 7.7 h by day 2 post-infection (via CFSE labeling; Supplemental Figure 3C-E). We sorted for transferred OT-I T cells on day 2 post-infection and measured cell size, protein, and ribosome numbers (“*ex vivo* day 2” measurements in **Figure**

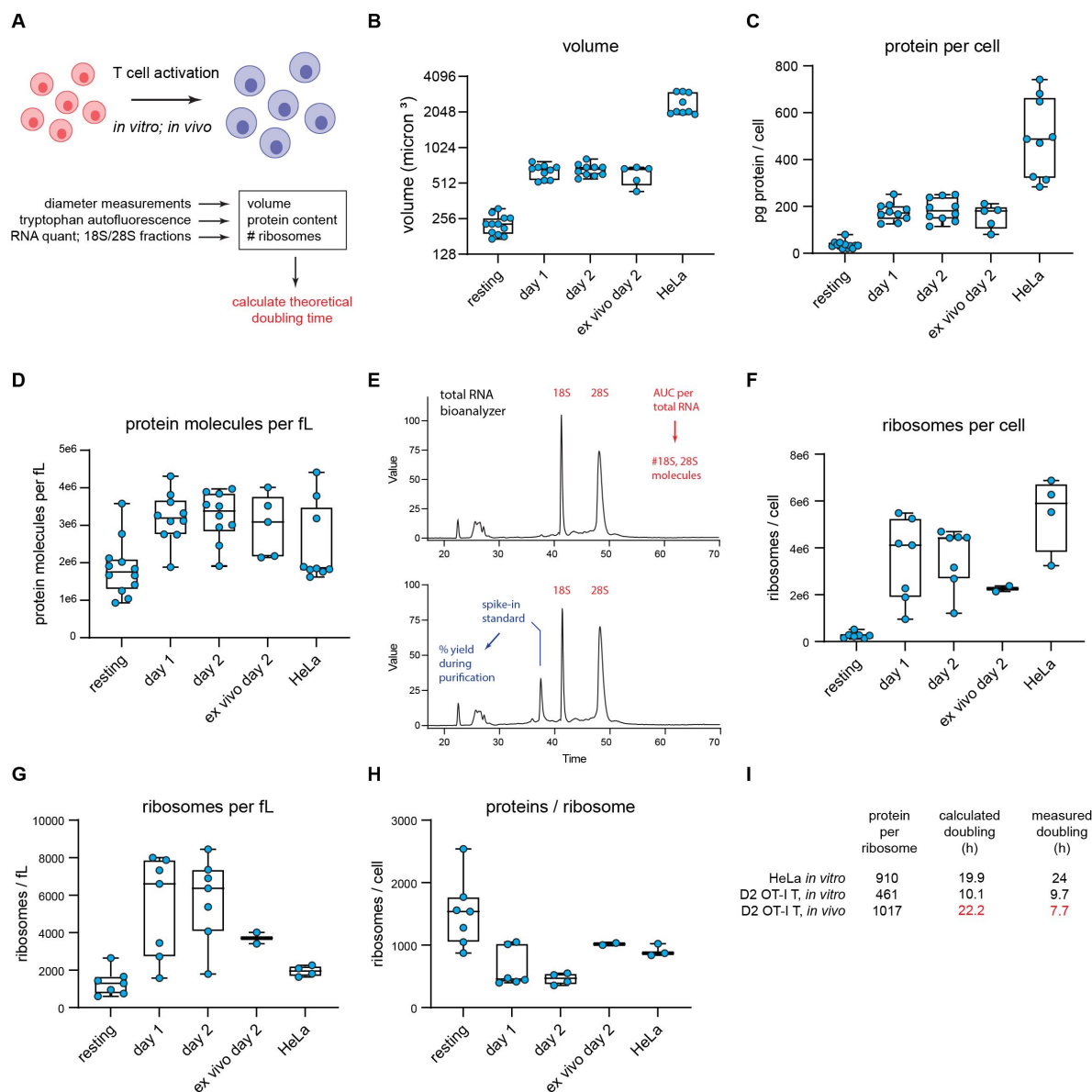


Figure 6

T cell accounting reveals discrepancy in proteome duplication rate for activated T cells *in vivo*

(A) Measurements made to calculate *in vitro* and *in vivo* rates of T cell division. (B) Volume calculations based on diameter measurements made by automated cell counter for the indicated cell types. Day 1 and day 2 represent *in vitro* activated OT-I T cells. *Ex vivo* day 2 represent cells activated *in vivo* for 2 days, followed by isolation and processing. (C) Protein content per cell as measured by tryptophan fluorescence of denatured lysates. (D) Protein molecules per fL, assuming an average protein length of 472 aa and average amino acid mass of 110 Da. (E) Example output from custom bioanalyzer method to determine number of ribosomes per cell. Total RNA is quantified and the bioanalyzer is used to determine area under the curve for 18S and 28S percentage of total RNA. Additionally, an exogenous mRNA standard is spiked into the sample prior to RNA isolation to determine the percent loss in yield during the purification procedure. Combined, this method allows for the accurate determination of total number of 18S and 28S molecules per cell. (F) Number of ribosomes per cell for the indicated cells. (G) Ribosome per fL for the indicated cells. (H) The protein/ribosome ratio, a representation of how many proteins a single ribosome would need to create to duplicate the proteome. (I) Discrepancy between measured and calculated rates of division for OT-I T cells activated and dividing *in vivo*.

6 [graphs](#)). Cells activated *in vivo* were similar in size and protein content to *in vitro* activated cells, but the protein-to-ribosome ratio was significantly higher than *in vitro* activated T cells due to the presence of 2.3 million vs. 3.6 million 60S subunits in maximally *in vitro* activated T cells.

Remarkably, the ratio of proteins to ribosomes (1017) at this juncture dictates a minimal proteome duplication time of 22.2 h, nearly 3x the measured doubling time of 7.7 h. While our RTA measurements support a higher elongation rate *in vivo* ($t_{1/2}$ =55 sec vs. 70 sec in HeLa cells), the 27% increase (7.6 residues per second) does not come close to accounting for the discrepancy. Thus, a paradox: protein synthesis activity or capacity of *in vivo* activated T cells does not support their doubling times.

Discussion

Lymphocytes protect jawed vertebrates against viral and cellular microbes and tumors. To counter the essentially infinite diversity of antigens expressed by pathogens, lymphocytes evolved to generate an enormous repertoire of specific antigen receptors. Hosts hope to never need the repertoire, and until a cognate antigen appears, metabolic processes, including protein synthesis, are minimized (this might also extend the life span of naive cells since the generation of defective ribosomal products (DRiPs) and other damaging chemical byproducts will also be minimized). Upon activation, lymphocytes divide rapidly to achieve numbers capable of exerting effective immunity. Here, we studied aspects of protein synthesis in lymphocytes, a field fairly dormant since the pioneering studies by the Kay and Cooper laboratories in the 70's but now experiencing a renaissance [31](#), [42](#), [43](#), [45](#)–[47](#).

To measure translation elongation rates *in vivo* and *in vitro*, we developed flow RTA, which is far simpler and cheaper than original [36](#) and recent [13](#), [24](#) methods and provides information at the level of individual cells simultaneously phenotyped by standard flow cytometry markers. While this work was in progress, the Pierre lab described “SunRiSE”, a nearly identical approach, to measure elongation rates *in vitro*, observing similar puromycylation decay rates following HAR treatment [27](#). While our findings are similar regarding the elongation rates of fibroblasts and lymphocytes, the addition of elongation inhibitors to the protocol (CHX or EME) greatly reduces the leakiness of harringtonine, thereby improving the calculated elongation rate accuracy.

RTA revealed exciting facets of lymphocyte translation. We find that a significant fraction of *ex vivo* lymphocytes possess stalled/primed ribosomes that puromycylate nascent chains but do not transit mRNA. Graber *et al.* [23](#) used the original RPM protocol to show that primary neurons possess substantial numbers of stalled ribosomes, apparently to facilitate rapid translation upon synaptic signaling. Otherwise, to our knowledge, such prolonged ribosomal stalling has not been described in mammalian cells. These experiments may also be useful in examining the phenomenon of “poised mRNA”, originally described in lymphocytes for cytokine mRNAs and more recently expanded on with advanced sequencing techniques [48](#), [49](#).

Our improved RTA protocol reveals the dramatic upregulation of protein synthesis by OT-I CD8⁺ T cells activated *in vivo*, with a 15-fold increase in translation in day 1 activated OT-I T cells vs. resting OT-I T cells. Activated T cells divide every 6.8 h from day 1 to day 2 post-VACV-SIINFEKL infection. This is consistent with a prior OT-I study in mice infected with a different SIINFEKL expressing VACV [50](#). Importantly, by i.v. delivery of translation inhibitors, we show that RTA can be used to measure elongation rates *in vivo*. Though we focus on lymphocytes, *in vivo* RTA can be used to study any cell type in animals that can be analyzed *ex vivo* by flow cytometry.

Contrary to observations *in vitro*, ribosomes are not stalled in naïve mouse T cells *in vivo*, as we show via RTA analysis of non-activated T cells. Importantly, ribosome transit times were up to ~30% faster in activated cells, consistent with the idea that lymphocytes can accelerate translation

to support activation and the rapid cell division that ensues. Similarly, OT-I T cells increased elongation rates *in vitro* when incubated at fever temperature (39.5 °C). While such accelerated translation may decrease translational fidelity, the impact may be lessened by the terminal nature of lymphocyte division, since the vast majority of activated cells apoptose within weeks of activation.

We additionally provide initial measurements of numbers of ribosomes and their protein synthesis activity, key values in accounting for the macroeconomics of T cell protein synthesis. Of particular importance is the ratio of cellular proteins to ribosomes; in conjunction with the elongation rate, this value dictates the minimal time (*i.e.*, no protein degradation or secretion) for duplicating the proteome⁵¹. Using the mammalian cell “speed limit” of 6 residues per second^{13,36} mouse T cells do not appear to possess sufficient ribosomes to support a 6-8 h division time. Even if ribosomes in *in vivo* activated T cells are translating at 7.8 residues per second, the time required to synthesize the proteome is 2.3x greater than the observed replication time (15.5 h vs. 6.8 h).

The discrepancy is further exacerbated when accounting for protein secretion and degradation of DRiPs (30% of nascent proteins)^{52–55} and retirees ($t_{1/2}$ of ~32 h over the entire proteome^{56,57}) and the presence of stalled and resting ribosomes. Together, this likely doubles the time required to synthesize the proteome.

Something is obviously wrong. T cell doubling times of 6.8 h are very likely to be accurate, as they are simple to measure and are routinely reported in mouse T cell studies. Quantitating proteins, however, is more challenging than it might seem. Where are the potential gremlins?

1. *Quantitating cellular protein.* We initially used the various dye binding assays for quantitating cellular protein content. While these assays vary notoriously for quantitating different proteins, they provide similar values for the cellular proteome. Moreover, they were in good agreement with a completely independent method based on Trp fluorescence, which we consider the gold standard for protein quantitation⁴¹. We use Wisniewski and Gaugaz’s value for Trp abundance in the proteome (0.69%) and note that this value is nearly identical to values obtained using the abundance of Trp totaled up from proteomic analysis of 11 different human tumor cell lines⁵⁸ as well as a transgenic T cell⁵⁹. One possible source of error is the free metabolic pool of tryptophan, but this is likely to be less than 5% of protein tryptophan⁶⁰. Another is the presence of serum proteins in cellular lysates. These are unlikely, however, to significantly contribute to our values since we fail to see FBS-derived-BSA in SDS-PAGE of total cell lysates.
2. *Quantitating Ribosomes.* We originally quantitated ribosomes using antibodies specific for ribosomal proteins in immunoblots of total cell lysates with purified ribosomes as a standard. We eventually recognized, however, that this approach is limited by heterogeneity in ribosome composition^{61,62}, as well as the presence of free pools of any given ribosomal subunit. It dawned on us that ribosomes are, in principle, simple to quantitate based on their RNA species, which account for >80% of total cellular RNA. Though we initially quantitated rRNA species on agarose gels with purified ribosomes as a standard, we believe quantitation is more accurate using a Bioanalyzer with doped-in highly purified RNA as an absolute staining standard and a control for yield loss during purification of samples.

3. *Ribosome Elongation Rates*. The classical value of ~6 or fewer residues per second for radiolabeling studies of cultured cells seems likely to be accurate based on ribosome profiling ¹³[13](#),⁶³[63](#). A recent ribosome profiling study extends these findings to mice, with *in vivo* elongation rates of 6.8, 5.2, and 4.4 amino acids per second for liver, kidney, and skeletal muscle, respectively ⁶⁴[64](#). Our *in vivo* RTA data demonstrate that translation elongation in *in vivo* activated T cells is 30% faster than in cultured cells and thus is likely to be up to ~7.8 residues/sec. We further note that it is likely that puromycylation detects only a subset of nascent chains. Indeed, in dozens of studies (including our **Figure 2C** ⁴²[42](#)), immunoblots of puromycylated proteins detect discreet bands in SDS-PAGE gel rather than the expected smear if all chains are randomly puromycylated at all lengths. This may be due to non-random incorporation of puromycin, non-random antibody detection of puromycylated nascent chains, or a combination of both. Though it seems improbable, it is possible that this bias influences RTA inferred elongation rates.

While one or more of these values may yet be inaccurate, we note that Wolf *et al.*'s ⁴²[42](#) mass spectrometric-based measurements of *in vitro* T cell protein synthetic capacity supports and even exacerbates the paradox. We must therefore consider the possibility that that lymphocytes are in such a hurry to divide that they resort to the extraordinary measure of acquiring proteins from resting lymphocytes or other cell types.

There are reports that neurons acquire ribosomes from Schwann cells ⁶⁵[65](#),⁶⁶[66](#) and that cancer cells acquire mitochondria from immune cells ⁶⁷[67](#). Further, through trogocytosis, lymphocytes acquire cell surface molecules from other cells ⁶⁸[68](#). We are, however, proposing that lymphocytes acquire a significant fraction of their proteome, perhaps via something akin to emperipolesis or entosis ⁶⁹[69](#), where cells actively enter homotypic cells and can even divide while residing inside ⁷⁰[70](#).

In any event, our findings clearly indicate how much remains to be learned about basic lymphocyte cell biology and the importance of simple accounting in squaring our models of cell biology with reality.

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Experimental Procedures

Mice

Specific-pathogen-free C57BL/6 mice were purchased from the Jackson Laboratory or from Taconic. OT-I TCR transgenic mice were acquired from the NIAID Intramural Research Repository. All mice were housed under specific pathogen-free conditions (including murine norovirus, mouse parvovirus, and mouse hepatitis virus) and maintained on standard rodent chow and water supplied *ad libitum*. All animal studies were approved by and performed in accordance with the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases. For acute infections, and to generate memory T cells, CFSE-labeled Ly5.2⁺ (CD45.2⁺CD45.1⁻) OT-I T cells were adoptively transferred into Ly5.1 (CD45.1⁺CD45.2⁻) mice. A subset of these mice was infected with VACV-SIINFEKL for indicated times to activate OT-I T cells, with some mice left uninfected where specified. For experiments done directly on memory OT-I T cells, assays were done 8-9 weeks after infection.

***In vivo* RPM, *in vivo* RPM-RTA, and relative protein synthesis determination**

For the standard and CHX-improved *in vivo* RPM assays, mice were intravenously injected with 100µl of a 10mg/ml solution of PMY in PBS that was warmed to 37°C, or PMY, as just described, along with 0.34mg per mouse of CHX. After indicated times, mice were sacrificed, and organs were collected into complete RPMI on ice (Gibco RPMI supplemented with 7.5% fetal calf serum). For the *in vivo* RTA, or the CHX-improved *in vivo* RTA, mice were intravenously injected with 100µg of HAR simultaneously with 1mg of PMY, or 1mg of PMY and 0.34mg of CHX for 5 minutes (for the maximum signal) or first intravenously injected with 100µg of HAR for the times indicated before being intravenously injected with 1mg of PMY, or PMY and 0.34mg of CHX for 5 minutes. To determine relative levels of active protein synthesis, two sets of mice were required. In the first set, mice were intravenously injected with 100µg of HAR for 15 minutes, and then intravenously injected with 1mg of PMY and 0.34mg of CHX for 5 minutes before spleens were harvested. In the second set, mice were intravenously injected simultaneously with 0.34mg of cycloheximide and 1mg of PMY for 5 minutes before spleens were harvested.

Single cell preparation from organs

Isolated organs were crushed between two frosted microscope slides, and the resultant single cell suspension was filtered through a 70µm mesh screen. The filtered single cell suspension was then centrifuged, resuspended in ACK lysing buffer (Lonza) to lyse red blood cells, centrifuged again, and resuspended in complete RPMI for counting on a Nexcelom Cellometer Vision using Trypan Blue (Lonza BioWhittaker) for live/dead cell discrimination and cell diameter measurements.

CFSE labeling

Spleens and inguinal, mediastinal, cervical, mesenteric, and popliteal lymph nodes from OT-I TCR transgenic or C57BL/6 mice were processed into a single cell suspension, red blood cells were lysed in ACK lysing buffer, and the resultant cells filtered through a 70 µm mesh screen. After two washes in PBS, cells were counted on a Nexcelom Cellometer Vision using Trypan Blue for dead cell exclusion, and cells were labeled in 5 µM CFSE (Invitrogen) in PBS at 1×10^7 cells per ml for 18 minutes in a 37° water bath with mixing every 6 minutes. Cells were washed three times in PBS, recounted, and adoptively transferred into the indicated mice or cultured as specified.

Human lymphocyte purification and culture conditions for human and mouse lymphocytes

Elutriated human lymphocytes were from healthy anonymous donors at the NIH Clinical Center Department of Transfusion Medicine. After collection, elutriated lymphocytes were purified on a discontinuous 35-70% Percoll (Amersham Biosciences) gradient and washed once with ACK lysing buffer (Life Technologies) to remove contaminating red blood cells. For time-course experiments, purified lymphocytes were resuspended in PBS and labeled with CFSE where indicated (as described above) to enable tracking of cell division over time. Lymphocytes were plated at $1-2 \times 10^6$ cells/mL in RPMI: RPMI 1640 (Gibco) supplemented with 15% FCS, 25mM HEPES (Corning Cellgro), 1mM sodium pyruvate (Gibco), and 55uM BME (Gibco). Depending on the experiment, media was also supplemented with recombinant human IL-2 (BRB NCI Frederick, 25 U/mL), PMA (Sigma, 1ng/mL), and ionomycin (Sigma, 100ng/mL). For OT-I T cell cultures, PMA was added at 100ng/ml instead, and, where noted, SIINFEKL was added as well (100nM) for optimal activation. Lymphocytes were cultured in 6% CO₂ at 37°C and allowed to sit overnight prior to any experiments unless noted (noted as “freshly isolated”). For time-course experiments, lymphocytes were cultured for up to 5 days and resuspended in fresh media every 2 days. Cell counts, diameters, and viabilities (through Trypan blue exclusion) were made on a Nexcelom Cellometer Vision cell counter. Cell volumes were calculated assuming spherical geometry.

In vitro RPM and RPM staining

For each sample, cells were resuspended at 2×10^7 cells per mL and 100 μ L transferred into 96-well round-bottom plates. When indicated, the media contained protein synthesis inhibitors at the following concentrations: 5 μ g/mL HAR (Santa Cruz Biotechnology), 25 μ g/mL EME (Calbiochem), 200 μ g/mL CHX (Sigma), 50 μ g/mL anisomycin (Sigma), or 10 μ M pactamycin (Sigma). After a 15-minute incubation at 37°C, 50 μ L of 3X PMY (Calbiochem) media was added (150 μ g/mL, for a final concentration of 50 μ g/mL) and the cells were incubated for an additional 5 minutes before shifting to ice and adding 100 μ L of cold PBS. Cells were then stained with ethidium monoazide (10 μ g/mL in PBS, Molecular Probes) for live/dead cell discrimination. After thorough washing, and a 10-minute incubation with heat-inactivated sera, or 2.4G2 to block Fc receptors, cell surface antigens were labeled for 30 minutes at 4°C with the following antibodies: For human lymphocyte stains, antibodies against: CD3 ϵ PerCP-eFluor 710 (clone OKT3, eBioscience), CD19 PE (clone HIB19, eBioscience), CD45 APC-eFluor 780 (clone 2D1, eBioscience), CD4 PE-CF594 (clone RPA-T4, BD) or CD4 PE-Cy7 (clone RPA-T4, eBioscience), and CD8 α BV421 (clone RPA-T8, BD). For mouse lymphocyte stains, antibodies were: CD3 ϵ BV786 (clone 145-2C11, BD), CD4BV510 (clone RM4-5, BD), CD5 APC-R700 (clone 53-7.3, BD), CD8 α PE-CF594 (clone 53-6.7, BD), CD11b PE-Cy7 (clone M1/70, eBioscience), CD19 APC-Cy7 (eBio1D3, eBioscience), CD25 BV650 (PC61, BD), CD44 BV605 (IM7, BD), CD44 eFl450 (clone IM7, eBioscience), CD45.1 APC (clone A20, eBioscience), CD45.1 eFl450 (clone A20, eBioscience), CD45.2 eFluor450 (clone 104, eBioscience), CD45.2 PE-Cy7 (clone 104, eBioscience), CD69 PerCP-Cy5.5 (clone H1.2F3, Invitrogen), γ δ TCR PE (eBioGL3, GL3, eBioscience), Gr1 PE (clone RB6-8C5, BD), NK1.1 FITC (clone PK136, eBioscience), TCR β 711 (H57-597, BD), and V β 5.1/V β 5.2 PE (clone MR9-4, BD). All antibodies were used at 1:150 dilution in buffered saline supplemented with 0.1% BSA. Next, cells were simultaneously fixed and permeabilized in fix/perm buffer (1% PFA, 0.0075% digitonin in PBS) for 20 minutes at 4°C. Intracellular PMY was labeled with an anti-PMY antibody (clone 2A4) directly conjugated with Alex Fluor 647 (conjugated using the Life Technologies Protein Labeling Kit per the manufacturer's instructions) for 1 hour. Cells were thoroughly washed and resuspended in buffered saline supplemented with 0.1% BSA, flow cytometry performed on a BD LSRII or BD LSRFortessa X-20, and resulting data analyzed with FlowJo software. To gate on OT-I T CD8 $^+$ T cells, setup was: singlets by FSCa and FSCw, lymphocytes by SSCa and FSCa, EMA $^-$ (live/dead cell marker), CD3 $^+$ CD19 $^-$, CD8 $^+$ CD4 $^-$, CD45.2 $^+$ CD45.1 $^-$, and V β 5 $^+$, and activation markers as indicated. For thymocyte subsets, gating setup was singlets by FSCa and FSCw, lymphocytes by SSCa and FSCa, EMA $^-$, and then subsets on combinations of CD3 ϵ , CD4, CD8 α , CD19, CD25, CD44, CD69, γ δ TCR, and TCR β . For human lymphocytes, gating setup was singlets by FSCa and FSCw, lymphocytes by SSCa and FSCa, EMA $^-$, and on subsets as indicated.

Amino acid radiolabeling

The following reagents were used for radioactive amino acid labeling: DMEM (for labeling HeLa cells) or RPMI minus leucine (RPMI without L-leucine, L-glutamine, and sodium pyruvate from MP Biomedicals, supplemented with Glutamax and 1mM sodium pyruvate) for labeling human lymphocytes with or without inhibitors and for the [3 H]-Leu (Perkin Elmer) ribosome transit analysis. Cells were kept at 37°C throughout the experiment and labeling. Cells were resuspended in complete RPMI at 1×10^7 cells/ml and 1ml was aliquoted into fresh Eppendorf tubes. Next, cells were spun at 300g for 4 minutes and pre-treated with protein synthesis inhibitors (concentrations as in RPM Staining above) in complete RPMI for 15 minutes. Pre-treated cells were spun at 300g for 4 minutes, resuspended in 200 μ L of labeling media (RPMI-Leu) containing 0.2mCi/mL [3 H]-Leu in the absence or presence of protein synthesis inhibitors as indicated. After a 5-minute labeling period, protein synthesis was stopped by adding 1mL of ice-cold PBS containing 200 μ g/mL of CHX. For all labeling experiments, after washing cells thoroughly in ice cold PBS, cells were lysed in 100 μ L or 200 μ L of 2% SDS lysis buffer (2% SDS, 50mM Tris-HCl pH 7.5, 5mM EDTA, 15U/mL DnaseI (Roche), cOMplete mini EDTA-free protease inhibitor tablet (Roche) in water) and boiled for 30-60 minutes to ensure complete lysis. Protein amounts were quantified by the DC Protein Assay (BioRad) or by tryptophan fluorescence measurements⁴¹. To quantify the amount of [3 H]-Leu

incorporated into proteins, equal amounts of lysate (six replicates per condition) were spotted onto a 96-well DEAE filter mat (PerkinElmer) and the mat was dried at 60°C. The mat was then soaked in a 10% trichloroacetic (TCA) (Calbiochem) solution for 30 minutes at room temperature, washed twice in 70% ethanol, dried at 60°C, placed in a MicroBeta sample bag (PerkinElmer) with ~6mL of BetaPlate Scint (PerkinElmer), and heat sealed. Radioactivity was quantified in a 1450 MicroBeta TriLux scintillation counter. To determine the total amount of amino acid incorporated into proteins, dilutions of the [³H]-Leu stock were counted and used as standards.

***In vitro* RPM Ribosome Transit Analysis (RTA)**

RPM-RTA: For each time point, 1×10^6 lymphocytes were transferred to a fresh conical tube and resuspended in 250μL of the appropriate media. Cells were kept at 37°C (or 39.5°C when indicated) throughout the experiment. An equal volume of 2X inhibitor media was added to each tube at the indicated time and the tube was vortexed briefly to mix. Depending on the time course, the 2X inhibitor media contained HAR (Santa Cruz Biotechnology) at 10μg/mL (final concentration 5μg/mL), or HAR at 10μg/mL and EME (Calbiochem) at 50μg/mL (final concentrations of 5μg/mL and 25μg/mL, respectively). At the end of the time course, an equal volume (250μL) of 3X puromycin (PMY) (Calbiochem) media (150μg/mL, for a final concentration of 50μg/mL) was added to each tube and the tube was vortexed briefly to mix. Cells were incubated for 5 minutes with PMY before adding an excess of ice-cold PBS to quench the ribopuromycylation reaction. The cells were then stained for analysis by flow cytometry as described above.

[³H]-Leu RTA: Cells were kept at 37°C throughout the experiment. For each time point, 30×10^6 lymphocytes were transferred to fresh Eppendorf tubes and resuspended in 50μL of the RPMI-Leu. 50μL of 2X inhibitor media was added to each tube and the tubes were vortexed briefly to mix. The inhibitor concentrations are as noted above. At the indicated times, an equal volume (100μL) of [³H]-Leu labeling media (RPMI-Leu media and [³H]-Leu in a 1:1 ratio, 0.5mCi/mL) was added and cells were labeled for 5 minutes. To stop [³H]-Leu incorporation, an excess of ice-cold PBS containing 200μg/mL CHX and 1mg/mL leucine was added before placing the cells on ice. Cells were lysed and [³H]-Leu incorporation quantified as described under “Amino acid radiolabeling” subsection.

Polysome profiling

A 15-45% continuous sucrose gradient was made in Thinwall polyallomer tubes (Beckman Coulter) from 15% and 45% sucrose (MP Biomedicals) solutions in gradient buffer (20mM Tris-HCl pH 7.4, 5mM MgCl₂, 100mM KCl, supplemented with 100μg/mL CHX (Sigma) and 10U/mL RNaseOUT (Invitrogen)). Briefly, 5mL of the 15% sucrose solution was carefully layered onto 5mL of the 45% sucrose solution, and the tube was placed horizontally at 4°C, typically overnight or for at least 2.5 hours before the experiment. For each gradient, cells were harvested and washed in cold PBS as described above. For cell lysis, cells were first swelled by adding 950μL of a cold hypotonic buffer (20mM Tris-HCl pH 7.4, 5mM MgCl₂, 10mM KCl, 40U/mL RNaseOUT, 0.1U/μL SupraseIn, supplemented with Complete EDTA-free protease inhibitors (Roche)). After 10 min of cell swelling, NP-40 was added to a final concentration of 0.5%, the resultant lysate mixed, incubated on ice for 3 min, and spun at 7000 rpm for 2 min to remove nuclei. Post-nuclear lysates were then brought to 100mM KCl (or 300mM or 500mM NaCl where indicated), layered onto 15-45% continuous sucrose gradients, and spun for 100 min at 38,000rpm at 4°C in a SW41Ti rotor (Beckman Ultracentrifuge). Gradients were syringe-fractionated mechanically from the bottom up and monitored for absorbance at 254nm (Teledyne Isco) to obtain polysome profiles. When indicated, area under the curve measurements were calculated by a trapezoidal method from the resulting curves. When required by the experiment, we concentrated monosome and polysome fractions for immunoblotting. To pellet the ribosomes, we spun the pooled monosome or polysome fractions for 1 hour or O/N at 39,000 rpm at 4°C in the SW41Ti rotor on a 34% sucrose cushion. The resultant ribosome pellet was resuspended in 2% SDS extraction buffer.

Quantitating cellular proteins

We quantitated total protein in cell lysates based on Trp fluorescence ⁴¹[\[link\]](#). Briefly, cells (1-2 million lymphocytes per 100µl) were lysed for 10-30 minutes at 95°C in a solution of 2% SDS, 0.1 M Tris-HCl, 50 mM DTT (pH 7.8) with 15U/mL DnaseI (Roche) and a cOmplete mini EDTA-free protease inhibitor tablet (Roche) added fresh. An 8M urea, 10mM Tris-HCl with 0.5mM DTT solution was freshly prepared, and 200µl added to wells of a flat-bottomed black polystyrene plate, and 2-4 µl of either cell lysates or a Trp standard solution was added to individual wells in triplicate. Fluorescence emitted at 350nm after excitation at 295nm was measured. We also compared this assay with the commercially available DC protein assay (Bio-Rad, performed according to the manufacturer's instructions), and found that the assays generated similar values.

Quantitating cellular ribosomes

After lymphocyte isolation, the PBS-washed cell pellet was dissolved in TRIzol; a spike-in mRNA standard was added at this step (CleanCap EGFP mRNA from TriLink, L-7601) to account for RNA loss during processing. RNA purification was conducted as described in the manufacturer's TRIzol protocol, with 5µg of glycogen used as carrier and the isopropanol precipitation step conducted overnight at -20°C. The final RNA pellet was dissolved in 50µL of ultra-pure water and roughly quantified to determine appropriate range for the Agilent bioanalyzer chip. Samples, including fresh spike-in mRNA alone, were loaded and run on RNA Nano Bioanalyzer chips (Agilent RNA Nano 6000), with a 70°C heating step and run on a 2100 Agilent Bioanalyzer. Bioanalyzer 2100 Expert software was used to determine total RNA concentration of each sample and percent area under curve of each peak (mRNA spike-in at approximately 1000 bp, 18S rRNA at around 1800 bp, 28S rRNA at around 4000 bp). The yield of the RNA prep was calculated as follows:

(mRNA spike-in standard peak from an RNA-purified sample) / (average of 2-3 standard peaks from 75ng/µL standard wells) = (fraction of RNA that remains after the purification process). We next converted the 18S and 28S ng/µL values to 'number molecules per cell' using the number of cells that originally went into the RNA purification, the RNA yield described above, and the following values: mouse 18S = 6.40E+05 g/mol; mouse 28S = 1.60E+06 g/mol; human 18S = 6.40E+05 g/mol; human 28S = 1.70E+06 g/mol.

Immunoblotting

To fractionate cells into nuclear and non-nuclear lysates, cells were either dissolved directly in 2% SDS extraction buffer at 95°C ("all" in Supplemental Figure 6) or subjected to a hypotonic lysis procedure. Cells were swelled with a buffer containing 20mM Tris-HCl pH 7.4, 2.5mM MgCl₂, and 10mM KCl supplemented with protease inhibitors for 10 min on ice. NP-40 was added to a final concentration of 0.5%, and the resultant lysate was mixed, incubated on ice for 3 minutes, and spun at 7000 rpm for 1 min. Non-nuclear lysates were removed and immediately dissolved in gel loading sample buffer (Life Technologies) to prevent sample degradation. Nuclei were washed gently 2x with PBS buffer containing NP-40 and protease inhibitors. Finally, nuclear proteins were extracted by dissolving pelleted nuclei in 2% SDS extraction buffer at 95°C. Equal amounts of each fraction were prepared for SDS-PAGE.

Samples were electrophoresed in 4-12% NuPAGE Bis-Tris gels (Invitrogen). Proteins were then transferred to nitrocellulose membranes (iBlot system, Novex) and membranes stained with Ponceau S and washed with PBS to confirm transfer uniformity. Next, membranes were incubated with either StartingBlock buffer (ThermoScientific) or Odyssey Blocking Buffer (Licor), followed by primary antibody prepared in StartingBlock buffer or Odyssey Blocking Buffer with 0.1% Tween-20 (Sigma). Depending on the experiment, we used the following primary antibodies: mouse anti-PMY (clone 2A4) at 6.66µg/mL; human anti-ribosomal P antigen at 1:2000 (Immunovision); rabbit anti-RPL7 at 1:1000, rabbit anti-RPL26 at 1:2000 (Bethyl); mouse anti-beta actin at 1:4000 (Licor); rabbit anti-HSP90 at 1:500 (Santa Cruz); rat anti-GRP94 at 1:1500 (Novus); rabbit anti-RPL28 at 1:500, rabbit anti-RPL6 at 1:1000, mouse anti-PDI at 1:2000 (Abcam); mouse anti-lamin A/C at

1:2000, rabbit anti-fibrillarin at 1:1000, mouse anti-RPS6 at 1:1000, rabbit anti-histone H3 at 1:2000, rabbit anti-RPL5 at 1:1000, and rabbit anti-RPS3 at 1:1000 (Cell Signaling Technology). The number of ribosomes per cell in earlier experiments was quantified by generating a standard curve using highly purified HeLa cell or canine rough microsome ribosomes (a kind gift of Chris Nicchitta, Duke University).

Membranes were washed three times in PBS + 0.1% Tween-20 (PBS-T) followed by incubation with secondary antibodies (all from Licor; used at 1:10,000) prepared in StartingBlock buffer or Odyssey Blocking Buffer. Membranes were washed 3x in PBS-T, 1x in PBS, and scanned via a Licor Odyssey CLX scanner.

Supplemental Figure 1 – RPM tracks translation in distinct cell populations over time

(A) Population frequency and RPM of resting day 2 or day 5 human lymphocytes or PMA/ionomycin/IL-2 activated day 2 or day 5 human lymphocytes. Left panel is the percent of CD45⁺ cells in the indicated population, the right panel is the RPM signal in each population. (B) Representative RPM flow cytometry plot gated on polyclonal CD8⁺ T cells. Similar data was obtained from all donors. (C) Gating strategy to quantify HeLa cell RTA assays as described in [Figure 2](#).

Supplemental Figure 2 – Dominant populations of monosomes in resting human and mouse lymphocytes

(A) Primary human lymphocytes were cultured for one day in the absence of PMA/ionomycin or for up to 2 days in the presence of PMA/ionomycin/IL-2, followed by polysome profiling. Representative of two independent experiments. (B) Freshly isolated human lymphocytes were treated with 0.1 µg/mL CHX for 30 minutes prior to cell lysis and sucrose gradient centrifugation. Representative of two independent experiments. (C) C57BL/6 mice were treated IV with vehicle or CHX. After 10 min, spleens and lymph nodes were harvested, and the resulting cells subjected to polysome profiling via ultracentrifugation through 15-45% sucrose gradients. (D) Lymphocytes or hepatocytes were harvested from OT-I mice treated IV with CHX, lysed, and processed for polysome profiling. For activated cells, OT-I T cells were treated with PMA/ionomycin and IL-2 for 2 days prior to CHX treatment and polysome profiling. Bottom right; quantification of the areas under the curve of free subunits, monosomes, and polysomes. Representative of two independent experiments.

Supplemental Figure 3 – RPM cell phenotyping and *in vivo* T cell division

(A) C57BL/6 mice were treated intravenously with CHX and PMY or only PMY. After the indicated times, splenocytes were harvested, surface stained, fixed/permeabilized, and RPM staining was performed. Representative of three independent experiments, 2-3 mice per group. (B) In one set of C57BL/6 mice, HAR was intravenously injected for 15 minutes before intravenously injecting mice with PMY for 5 min. In a second set of mice, CHX and PMY were IV injected for 5 min. Splenocytes from each set of mice were harvested, surface stained, fixed, and permeabilized, and RPM staining was performed for various immune cell subsets. To determine relative amounts of ribosomes, the

'HAR then PMY' RPM signal was subtracted from the CHX+PMY RPM signal for each cell subset after flow cytometry. Representative of two independent experiments, 2-4 mice per group. (C) CFSE-labeled Ly5.2⁺ (CD45.2⁺CD45.1⁻) OT-I cells were adoptively transferred into Ly5.1⁺ (CD45.1⁺CD45.2⁻) mice, which were then infected with VAC-SIINFEKL. 1-3 days after infection, mice were intravenously injected with CHX simultaneously with PMY for 5 min. Splenocytes from the mice were harvested, surface stained, fixed and permeabilized, and RPM staining was performed. Representative flow cytometry plots gated on OT-I T cells. (D) Gating strategy used to display and quantify *in vivo* T cell data as described in panel C and elsewhere. (E) Number of divisions (by CFSE dilution) of OT-I T cells 1-3 days after infection of mice with VACV-SIINFEKL. (F) Amount of translation as measured by RPM signal (with "no PMY" signal subtracted) in uninfected, or one-, two-, or three-day VACV-SIINFEKL-infected mice. Representative of four independent experiments, 2-3 mice per time point.

Supplemental Figure 4 – Exogenous SIINFEKL significantly enhances OT-I T cell activation *in vitro*

(A) Maximum-normalized MFI of CD69, CD25, CD44, and side scatter (SSCa), as well as CFSE expansion index, of OT-I T cells after 1 or 2 days with PMA/ionomycin or PMA/ionomycin with exogenous SIINFEKL. IL-2 was included in all conditions. (B) Side scatter is a good proxy for cell size. SSCa MFI plotted vs cell diameter as determined by automated cell counter measurements.

Supplemental Figure 5 – Salt stability of T cell ribosomes and monosome quantification

(A) Lymph node and splenic OT-I T cells were mixed and stimulated *in vitro* for 2 days with SIINFEKL, PMA/ionomycin, and IL-2. Cells were treated with CHX for 5 minutes, lysed, and brought to either 300 or 500mM NaCl final concentration. Ribosome-containing lysates were subjected to polysome profiling via ultracentrifugation through 15-45% sucrose gradients containing either 300 or 500mM NaCl. (B) Quantification of the areas under the curve of free subunits, monosomes, and polysomes for each sample. (C) For resting T cells, OT-I mice were treated IV with CHX and lymphocytes from the spleens or lymph nodes were isolated and lysed. For activated T cells, lymph node and splenic OT-I T cells were mixed and stimulated *in vitro* for 2 days with SIINFEKL, PMA/ionomycin, and IL-2, treated with CHX for 5 minutes, and lysed. Lysates were subjected to polysome profiling via ultracentrifugation on 15-45% sucrose gradients after bringing both lysate and sucrose gradients to a final concentration of 500mM NaCl to dissociate non-translating ribosomes. (D) Quantification of the areas under the curve of free subunits, monosomes, and polysomes in each sample. Representative of two independent experiments.

Supplemental Figure 6 – Fractionation of HeLa or T cells reveals few ribosomal components in nuclear lysates

HeLa cells, freshly isolated resting OT-I T cells, or OT-I T cells stimulated with PMA/ionomycin and IL-2 *in vitro* for 2 days were either lysed directly in SDS extraction buffer ("all") or subjected to a hypotonic lysis procedure to isolate non-nuclear lysates and nuclear lysates. Equal amounts of each fraction were subjected to immunoblotting for markers typical of the cytosol, ER, and nucleus. Antibodies against ribosomal proteins were used to determine where the majority of

ribosomal proteins (and therefore ribosomes) fractionated. Controls with antibodies specific for nucleolar located fibrillarin, histone H3, and lamin A/C establish lack of nuclear contamination in non-nuclear fractions. ER and cytoplasmic proteins HSP90, GRP94, PDI, and actin indicate lack of contamination in the nuclear fraction.

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Editors

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Reviewer #1 (Public Review):

The authors examine the fascinating question of how T lymphocytes regulate proteome expression during the dramatic cell state change that accompanies the transition from the resting quiescent state to the activated, dividing state. Orthogonal, complementary assays for translation (RPM/RTA, metabolic labeling) are combined with polyribosome profiling and quantitative, biochemical determinations of protein and ribosome content to explore this question, primarily in the OT-I T lymphocyte model system. The authors conclude that the ratio of protein levels to ribosomes/protein synthesis capacity is insufficient to support activation-coupled T cell division and cell size expansion. The authors hint at cellular

mechanisms to explain this apparent paradox, focusing on protein acquisition strategies, including emperipolesis and entosis, though these remain topic areas for future study.

The strengths of the paper include the focus on a fundamental biological question - the transcriptional/translational control mechanisms that support the rapid, dramatic cell state change that accompanies lymphocyte activation from the quiescent to activated state, the use of orthogonal approaches to validate the primary findings, and the creative proposal for how this state change is achieved.

The weakness of the work is that several cellular regulatory processes that could explain the apparent paradox are not explored, though they are accessible to experimental analysis. In the accounting narrative that the authors highlight, a thorough accounting of the cellular process inventory that could support the cell state change should be further explored before committing to the proposal, provocative as it is, that protein acquisition provides a principal mechanism for supporting lymphocyte activation cell state change.

Appraisal and Discussion:

1. Relating to the points raised above, two recent review articles explore this topic area and highlight important areas of study in RNA biology and translational control that likely contribute to the paradox noted by the authors: Choi et al. 2022, doi.org/10.4110/in.2022.22.e39 ("RNA metabolism in T lymphocytes") and Turner 2023, DOI: 10.1002/bies.202200236 ("Regulation and function of poised mRNAs in lymphocytes"). These should be cited, and the broader areas of RNA biology discussed by these authors integrated into the current manuscript.
2. The authors cite the Wolf et al. study from the Geiger lab (doi.org/10.1038/s41590-020-0714-5, ref. 41) though largely to compare determined values for ribosome number. Many other elements of the Wolf paper seem quite relevant, for example, the very high abundance of glycolytic enzymes (and whose mRNAs are quite abundant as well), where (and as others have reported) there is a dramatic activation of glycolytic flux upon T cell activation that is largely independent of transcription and translation, the evidence for "pre-existing, idle ribosomes", the changes in mRNA copy number and protein synthesis rate Spearman correlation that accompanies activation, and that the efficiencies of mRNA translation are heterogeneous. These data suggest that more accounting needs to be done to establish that there is a paradox.

As one example, what if glycolytic enzyme protein levels in the resting cell are in substantial excess of what's need to support glycolysis (likely true) and so translational upregulation can be directed to other mRNAs whose products are necessary for function of the activated cell? In this scenario the dilution of glycolytic enzyme concentration that would come with cell division would not necessarily have a functional consequence. And the idle ribosomes could be recruited to key subsets of mRNAs (transcriptionally or post-transcriptionally upregulated) and with that a substantial remodeling of the proteome (authors ref. 44). The study of Ricciardi et al. 2018 (The translational machinery of human CD4⁺ T cells is poised for activation and controls the switch from quiescence to metabolic remodeling (doi.org/10.1016/j.cmet.2018.08.009) is consistent with this possibility. That study, and the short reviews noted above, are useful in highlighting the contributions of selective translational remodeling and the signaling pathways that contribute to the cell state change of T cell activation. From this perspective an alternative view can be posited, where the quiescent state is biologically poised to support activation, where subsets of proteins and mRNAs are present in far higher levels than that necessary to support basal function of the quiescent lymphocyte. In such a model, the early stages of lymphocyte activation and cell division are supported by this surplus inventory, with transcriptional activation, including ribosomal genes, primarily contributing at later stages of the activation process. An obvious

analogy is the developing *Drosophila* embryo where maternal inheritance supports early-stage development and zygotic transcriptional contributions subsequently assuming primary control (e.g. DOI 10.1002/1873-3468.13183 , DOI: 10.1126/science.abq4835). To pursue that biological logic would require quantifying individual mRNAs and their ribosome loading states, mRNA-specific elongation rates, existing individual protein levels, turnover rates of both mRNAs and proteins, ribosome levels, mean ribosome occupancy state, and how each of these parameters are altered in response to activation. Such accounting could go far to unveil the paradox. This is a considerable undertaking, though, and outside the scope of the current paper.

Regarding the revised manuscript:

I am largely satisfied with the authors responses to the review and have but a few remaining thoughts, some mirrored in the comments from the other reviewers and some that came to mind upon reading the revision.

1. In the Introduction, it would be (have been) helpful if in paragraph two, it was stated that the current study was designed to test that assumption made in prior reports that the fold-increase in protein synthesis in response to mitogen activation was sufficient to endow the daughter cells with "the same protein content as their progenitor".
2. The primary conclusion, that "...protein synthesis activity or capacity of in vivo activated T cells does not support their doubling times" remains, to my eye, insufficiently supported by the data, though I agree it is a rational interpretation. My concern is that the devil is deeper in the details and without knowing the mRNA transcriptome composition pre- and post-activation, mean CDS length, 5' UTR structural features, perhaps codon optimality, etc., etc., the broader conclusion could be premature. As a first check, it would be useful to determine poly(A) mRNA and ribosome concentrations/cell, pre- and post-activation, and subsequently to compare mRNA transcriptome compositions in greater detail. Do mRNA:ribosome levels and ratios diverge as a consequence of activation? Poly(A) mRNA compositions? Does protein half-life change pre- and post-activation? mRNA half-life? My view is that additional molecular accounting is likely necessary to be confident in the primary conclusion.
3. I did not provide a clear description of the alternative interpretation I was imagining, which is that in the resting, unstimulated state, mRNA:ribosome and/or protein levels may be much higher than that necessary for lymphocyte viability. As in early development, this could be a mechanism to then provide sufficient protein synthesis capacity and/or proteins to daughter cells following activation of cell division and cell growth. In other words, it's a dynamic range question; the daughter cells exploit "unused" protein synthesis capacity to sustain their growth and division. Quantification and analysis of the additional variables noted in point 2) could reconcile the different interpretations.

<https://doi.org/10.7554/eLife.89015.2.sa2>

Reviewer #2 (Public Review):

This paper takes a novel look at the protein economy of primary human and mouse T-cells - in both resting and activated state. Their findings in primary human T-cells are that:

1. A large fraction of ribosomes are stalled in resting cultured primary human lymphocytes. and these stalled ribosomes are likely to be monosomes.

2. Elongation occurs at similar rates for HeLa cells and lymphocytes, with the active ribosomes in resting lymphocytes translating at a similar rate as fully activated lymphocytes.

They then turn their attention to mouse OT-1 lymphocytes, looking at translation rates both in vitro and in vivo. Day1 resting T-cells also show stalling - which curiously wasn't seen on freshly purified cells - I didn't understand these differences.

In vivo they show that it is possible to monitor accurate translation and to measure rates in vivo. Perhaps most interestingly they note a paradoxically high ratio of cellular protein to ribosomes insufficient to support their rapid in vivo division, suggesting that the activated lymphocyte proteome in vivo may be generated in an unusual manner.

This was an interesting and provocative paper. Lots of interesting techniques and throwing down challenges to the community - it manages to address a number of important issues without necessarily providing answers.

<https://doi.org/10.7554/eLife.89015.2.sa1>

Reviewer #3 (Public Review):

Perhaps not unexpectedly, the proposed revisions consist of textual revisions only. Yewdell added a touch of levity with his H.G. Wells foundation as a source of \$\$ for a time machine. The paper does not establish striking new facts, in my opinion, but will stimulate discussion.

One point to consider: the relevance of the human T cell activation experiments is now downplayed even further, by the authors themselves, no less. I would suggest leaving the actual data out altogether and conclude with a statement: "Similar experiments conducted on activated human T cells showed significantly worse activation and may therefore not allow a head-to-head comparison with the results of our experiments performed on mouse T cells. Not only might one consider the mode of activation (PMA/ionomycin) non-physiological, the activation status achieved was less than that seen for the OT-1 model." or something similar to that effect. In the present weakened form, I do not believe that the human data add anything of substance to the paper and are more of a distraction. The authors would increase the impact and readability of their paper if they omitted the human data.

<https://doi.org/10.7554/eLife.89015.2.sa0>

Author Response

The following is the authors' response to the original reviews.

eLife assessment

This study addresses how protein synthesis in activated lymphocytes keeps up with their rapid division, with important findings that are of significance to cell biologists and immunologists endeavouring to understand the 'economy' of the immune system. The work is supported by solid data but because it proposes non-conventional mechanisms, it requires additional explanation and justification to align with the current understanding in the field.

Public Reviews:

Reviewer #1 (Public Review):

The authors examine the fascinating question of how T lymphocytes regulate proteome expression during the dramatic cell state change that accompanies the transition from the resting quiescent state to the activated, dividing state. Orthogonal, complementary assays for translation (RPM/RTA, metabolic labeling) are combined with polyribosome profiling and quantitative, biochemical determinations of protein and ribosome content to explore this question, primarily in the OT-I T lymphocyte model system. The authors conclude that the ratio of protein levels to ribosomes/protein synthesis capacity is insufficient to support activation-coupled T cell division and cell size expansion. The authors hint at cellular mechanisms to explain this apparent paradox, focusing on protein acquisition strategies, including emperipolesis and entosis, though these remain topic areas for future study.

The strengths of the paper include the focus on a fundamental biological question - the transcriptional/translational control mechanisms that support the rapid, dramatic cell state change that accompanies lymphocyte activation from the quiescent to activated state, the use of orthogonal approaches to validate the primary findings, and the creative proposal for how this state change is achieved.

The weakness of the work is that several cellular regulatory processes that could explain the apparent paradox are not explored, though they are accessible for experimental analysis. In the accounting narrative that the authors highlight, a thorough accounting of the cellular process inventory that could support the cell state change should be further explored before committing to the proposal, provocative as it is, that protein acquisition provides a principal mechanism for supporting lymphocyte activation cell state change.

Appraisal and Discussion:

- 1. relating to the points raised above, two recent review articles explore this topic area and highlight important areas of study in RNA biology and translational control that likely contribute to the paradox noted by the authors: Choi et al. 2022, doi.org/10.4110/in.2022.22.e39 ("RNA metabolism in T lymphocytes") and Turner 2023, DOI: 10.1002/bies.202200236 ("Regulation and function of poised mRNAs in lymphocytes"). These should be cited, and the broader areas of RNA biology discussed by these authors integrated into the current manuscript.*

Good suggestion. We have added these references with a short discussion.

- 1. The authors cite the Wolf et al. study from the Geiger lab (doi.org/10.1038/s41590-020-07145, ref. 41) though largely to compare determined values for ribosome number. Many other elements of the Wolf paper seem quite relevant, for example, the very high abundance of glycolytic enzymes (and whose mRNAs are quite abundant as well), where (and as others have reported) there is a dramatic activation of glycolytic flux upon T cell activation that is largely independent of transcription and translation, the evidence for "pre-existing, idle ribosomes", the changes in mRNA copy number and protein synthesis rate Spearman correlation that accompanies activation, and that the efficiencies of mRNA translation are heterogeneous. These data suggest that more accounting needs to be done to establish that there is a paradox.*

As one example, what if glycolytic enzyme protein levels in the resting cell are in substantial excess of what's needed to support glycolysis (likely true) and so translational upregulation can be directed to other mRNAs whose products are necessary for function of the activated cell? In this scenario, the dilution of glycolytic enzyme concentration that would come with cell division would not necessarily have a functional consequence. And

the idle ribosomes could be recruited to key subsets of mRNAs (transcriptionally or post-transcriptionally upregulated) and with that a substantial remodeling of the proteome (authors ref. 44). The study of Ricciardi et al. 2018 (The translational machinery of human CD4+ T cells is poised for activation and controls the switch from quiescence to metabolic remodeling (doi.org/10.1016/j.cmet.2018.08.009) is consistent with this possibility. That study, and the short reviews noted above, are useful in highlighting the contributions of selective translational remodeling and the signaling pathways that contribute to the cell state change of T cell activation.

Our study focuses on the central issue of whether measured ribosome translation rates support rapid division. The abundance of glycolytic enzymes, mRNA copy numbers etc., are clearly interesting and critical to cell metabolism, but are irrelevant to measuring the overall translation rate and capacity of T cells.

From this perspective, an alternative view can be posited, where the quiescent state is biologically poised to support activation, where subsets of proteins and mRNAs are present in far higher levels than that necessary to support basal function of the quiescent lymphocyte. In such a model, the early stages of lymphocyte activation and cell division are supported by this surplus inventory, with transcriptional activation, including ribosomal genes, primarily contributing at later stages of the activation process. An obvious analogy is the developing Drosophila embryo where maternal inheritance supports early-stage development and zygotic transcriptional contributions subsequently assuming primary control (e.g. DOI 10.1002/1873-3468.13183 , DOI: 10.1126/science.abq4835). To pursue that biological logic would require quantifying individual mRNAs and their ribosome loading states, mRNA-specific elongation rates, existing individual protein levels, turnover rates of both mRNAs and proteins, ribosome levels, mean ribosome occupancy state, and how each of these parameters is altered in response to activation. Such accounting could go far to unveil the paradox. This is a considerable undertaking, though, and outside the scope of the current paper.

The reviewer is essentially proposing RiboSeq analysis of pre- and post-activation T cells, whereby individual mRNAs can be queried for ribosome occupancy, and where translation inhibitors could be used to quantify mRNA-specific transit rates. This is important information but would not provide a more accurate accounting of protein synthesis rates than our much more direct measurement. We note that other labs have begun to work on this exact topic, however – see both PMID: 36002234 and PMID: 32330465.

Reviewer #2 (Public Review):

This paper takes a novel look at the protein economy of primary human and mouse T-cells - in both resting and activated state. Their findings in primary human T-cells are that:

- 1. A large fraction of ribosomes are stalled in resting cultured primary human lymphocytes, and these stalled ribosomes are likely to be monosomes.*
- 1. Elongation occurs at similar rates for HeLa cells and lymphocytes, with the active ribosomes in resting lymphocytes translating at a similar rate as fully activated lymphocytes.*

They then turn their attention to mouse OT-1 lymphocytes, looking at translation rates both in vitro and in vivo. Day 1 resting T-cells also show stalling - which curiously wasn't seen on freshly purified cells - I didn't understand these differences.

This is clarified and discussed starting in the third paragraph of “Protein synthesis in mouse lymphocytes ex vivo” section. Cells cultured ex vivo for 1 day with no activation show signs of stalling, as we observed in isolated human cells. But cells immediately out of an animal show a measurable decay rate since they are obviously synthesizing proteins in vivo and are processed rapidly.

In vivo, they show that it is possible to monitor accurate translation and measure rates. Perhaps most interestingly they note a paradoxically high ratio of cellular protein to ribosomes insufficient to support their rapid in vivo division, suggesting that the activated lymphocyte proteome in vivo may be generated in an unusual manner.

This was an interesting and provocative paper. Lots of interesting techniques and throwing down challenges to the community - it manages to address a number of important issues without necessarily providing answers.

Reviewer #3 (Public Review):

This manuscript provides a more or less quantitative analysis of protein synthesis in lymphocytes. I have no issue with the data as presented, as I'm sure all measurements have been expertly done. I see no need for additional experimental work, although it would be helpful if the authors could comment on the possibility of measuring the rate of synthesis of a defined protein, say a histone, in cells prior to and after activation. The conclusion the authors leave us with is the idea that the rates of protein synthesis recorded here are incompatible with observed rates of T cell division in vivo. Indeed, in the final paragraph of the discussion, the authors note the mismatch between what they consider a requirement for cell division, and the observed rates of protein synthesis. They then invoke unconventional mechanisms to make up for the shortfall, without -in this reviewer's opinion- discussing in adequate detail the technical limitations of the methodology used.

Points #1-3 in the Discussion relate to potential pitfalls of our analyses; in point #3 we now add further limitations of RTA based on non-random detection of nascent chains due either to bias in either puromycylation or antibody detection of puromycylated nascent chains.

A key question is the broad interest, novelty, and extension of current knowledge, in comparison with Argüello's (reference 27) 'SunRise' method. It would be helpful for the authors to stake out a clear position as to the similarities and differences with reference 27: what have we learned that is new? The authors could cite reference 27 in the introduction of their manuscript, given the similarity in approach. That said, the findings reported here will generate further discussion.

We did cite this reference (27) in the section “Flow RPM measures ribosome elongation rate in live cells” giving credit where credit is due. We independently devised the method in 2014, and uniquely, to our knowledge, have applied it in vivo. We now further discuss the importance of our CHX modification to limit dissociation and increase the accuracy of RTA (second and third paragraphs of “Protein synthesis in mouse lymphocytes and innate immune cells in vivo”).

The manuscript would increase in impact if the authors were to clearly define why a particular measurement is important and then show the actual experiment/result. As an example, it would be helpful to explain to the non-expert why the distinction between monosomes, polysomes, and stalled versions of the same is important, and then explain the rationale of the actual experiment: how can these distinctions be made with confidence, and what are confounding variables?

We believe this is addressed in the section “Resting human lymphocytes have a dominant monosome population”.

The initial use of human cells, later abandoned in favor of the OT-1 in vitro and in vivo models, requires contextualization. If the goal is to address the relationship between rates of translation and cell division of antigen-activated T cells in vivo, then a lot of the work on the human model and the in vitro experiments becomes more of a distraction, unless properly contextualized. Is there any reason to assume that antigen-specific activation in vivo will impact translation differently than the use of the PMA/ionomycin/IL2 cocktail? The way the work is presented leaves me with the impression that everything that was done is included, regardless of whether it goes to the core of the question(s) of interest.

Donor PBMCs are clearly the more relevant model for understanding human T cell biology, which is why started our studies with this model. Had the manuscript strictly described mouse studies it is likely that we would be criticized for not studying human cells: Catch 22! However, as we state in the manuscript, the human cell model has a variety of technical downsides, including donor heterogeneity. PMA/ionomycin activation is also physiologically questionable, and while we could deliver a defined TCR to redirect their specificity, this is typically done after cells have been activated, since lentiviral delivery is poor in resting lymphocytes. A main point we try to make from this work is that cells derived from human blood donors show signs of ribosomal stalling by the time they are isolated and put into culture. This may limit the usefulness of studying them preactivation, although based on our mouse data, some level of stalled ribosomes may be a feature as well – to prime T cells to be ready for their massive expansion. The move to the OT-I system gave us complete control over the system, including in vivo delivery of translation inhibitors.

It would be helpful if the authors made explicit some of the assumptions that underlie their quantitative comparisons. Likewise, the authors should discuss the limitations of their methods and provide alternative interpretations where possible, even if they consider them less/not plausible, with justification. As they themselves note, improvements in the RPM protocols raised the increase in translating ribosomes upon activation from 10-fold to 15-fold. Who's to say that is the best achievable result? What about the reliability/optimization of the other measurements?

We expanded discussion of potential pitfalls of the RPM techniques and others in the Discussion section. Regarding RPM per se, we use it as a readout of ribosome time decay, so even if further optimizations can be made, the decay rates we have made should still be accurate. In addition, for our cell accounting measurements in Figure 6, we do not use RPM data and rather calculate based on the assumption that every ribosome is used for protein synthesis at a “maximal” rate of mRNA transit.

The composition of the set of proteins produced upon activation will differ from cell to cell (CD4, CD8, B, resting vs. dividing). Even if analyses are performed on fixed cells, the ability of the monoclonal anti-puromycin antibody to penetrate the matrix of the various fixed cell types may not be equal for all of them, depending on protein composition, susceptibility to fixation etc. Is it possible for puromycin to occupy the ribosome's A site and terminate translation without forming a covalent bond with the nascent chain? This could affect the staining with anti-puromycin antibodies and also underestimate the number of nascent chains.

Yes, the method (like every other one) is imperfect. Harringtonine run-off experiments show that RPM staining only detects nascent chains. Note that reference 47 reports that 75% of

translation in activated T cells is devoted to synthesizing ~250 housekeeping proteins, which are likely to be highly similar between lymphocyte subsets.

I believe that the concept of FACS-based quantitation also requires an explanation for the nonexpert. For the FACS plots shown, the differences between the highest and lowest RPM scores for cells that divided and that have a similar CFSE score is at least 10-fold. Does that mean that divided cells can differ by that margin in terms of the number of nascent chains present? If I make the assumption that cells stimulated with PMA/ionomycin/IL2 respond more or less synchronously, why would there be a 10-fold difference in absolute fluorescence intensity (anti=puromycin) for randomly chosen cells with similar CFSE values? While the use of MFI values is standard practice in cytofluorimetry, the authors should devote some comments to such variation at the population level.

We believe that the referee is referring to Sup Fig. 1B. In this experiment the T cells are polyclonal and represent the full range of naïve to potentially exhausted differentiation states. Looking at our initial in vivo RPM study (reference 22) and comparing Figure 2 (OTI's) to Figure 3 (endogenous CD4s or CD8s), reveals more spread in the RPM values polyclonal vs. monoclonal T cells - now clarified in the third paragraph of "Protein synthesis in mouse lymphocytes and innate immune cells in vivo". Flow cytometry is by far the most accurate method for measuring fluorescence in individual cells. It is likely to be an accurate measure of the variation of nascent chains in cells in the same division cohort but likely represents the diversity of T cell activation profiles in blood of healthy donors.

It is assumed that for cells to complete division, they must have produced a full and complete copy of their proteome and only then divide. What if cells can proceed to divide even when expressing a subset of the proteome of departure (=the threshold set required for initiation of division), only to complete synthesis of the 'missing' portion once cell division is complete? Would this obviate the requirement for an unusual mechanism of protein acquisition (trogocytosis; other)?

There must be a steady state level of translation and proteome replenishment, though. If a cell can divide when it affords daughter cells with 90% of its G0 proteome (as an example), that daughter cell would either 1) be 10% smaller, or 2) require extra translation to make up for the missing proteome during its own division cycle. Though T cells do typically shrink slightly after an initial activation, cell size stabilizes over time. Requiring each daughter cell to make more and more missing proteome could be plausible, considering that initial bursts of division do take longer over time, but still, even in vitro activated T cells divide rapidly for weeks without large decreases in their division rates.

Translation is estimated to proceed at a rate of ~6 amino acids per second, but surely there is variability in this number attributable to inaccuracies of the methods used, in addition to biological variability. Were these so-called standard values determined for a range of different tissues? It stands to reason that there might be variation depending on the availability of initiation/elongation factors, NTPs, aminoacyl tRNAs etc. What is the margin of error in calculating chain elongation rates based on the results shown here?

We refer to all relevant studies we know of, including new in vivo estimates of elongation rates (reference 40).

Reviewer #1 (Recommendations For The Authors):

A "limitations of study" section would be a helpful way to detail potential contributing mechanisms that were not explored in the current study.

We have expanded the methodological limitations in the Discussion section.

Major:

1. *Broaden the scope of biological models that could explain the paradox.*

In the Discussion, we suggest that T cells acquire some fraction of their proteome through external sources and highlight some examples of this occurring.

Minor:

1. *Include Mr markers for Fig. 2C.*

Done.

1. *Though commonly used interchangeably, historically the term protein synthesis was the consequence of mRNA translation. In other words, proteins are not translated.*

Good point! We have changed the text accordingly.

1. *Include more meaningful X-axis legend in polysome gradient panels i.e., Fig. S2, e.g., fraction number.*

In most experiments, fractions were not collected. Rather, the x-axis refers to time that the sample took to be queried by the detector.

1. *Figure 3A does not report polysome profiles as described in the text, pg. 5, though this is reported in Fig S2D.*

The figure callouts were correct but confusing. We now separately refer to out each result to clarify.

1. *In Fig 5A, SDS-PAGE/anti-Puro blots would be more convincing and contain more information. The dot-blot is difficult to interpret.*

Disagree. To quantitate total anti-puromycin signal a dot blot is far better than immunoblotting, which is compromised by unequal transfer of different protein species.

1. *It's not clear why a degree of monosome translation is necessarily surprising (pg. 7).*

It's surprising since for many decades it was believed that translation by monosomes is a tiny fraction of translation. But separately, with this particular mode of activation, activated T cells displayed a preponderance of monosomes during their burst of division. When the activation method was improved, polysomes dominated. But monosome translation clearly supported T cell division during activation without cognate peptide, which was interesting.

Reviewer #2 (Recommendations For The Authors):

1. One concern is the dose of puromycin used. My understanding is that puromycin acts as a chain termination inhibitor - but is being used here predominantly as a label for nascent polypeptide chains. My concern, therefore, is the dose being used - here at 50ug/ml - which seems high and I would be concerned that at this dose it would act as a translational inhibitor rather than just labelling nascent chains, and is therefore resulting in a lower signal/background ration than expected. In human cell lines 0.1ug/ml is optimal and doses published (in cell lines) range between 1 and 10ug/ml so it will be interesting to understand why this high dose was used.

Do they have a dose-response curve - is this high dose necessary because these are primary Tcells. Can the authors show that 50 µg/mL of puromycin is optimal for studying protein translation in primary human T cells? A titration curve will help answer this question and could be included in Suppl Figure 1. This experiment is critical as the authors use a higher dose than previous studies (commonly between 1 and 10 µg/mL).

The reviewer is referencing puromycin concentrations typically used in the selection of cells – for the RPM assay, puromycin is used at saturating doses to label the maximal number of nascent chains stalled by CHX or EME pretreatment.

1. None of the figures show statistical significance.

Statistics on relevant comparisons are now indicated on figures and in legends.

1. The authors mention: "We performed RPM on cells labelled with CFSE to track cell division by dye dilution (Supplemental Figure 1B). On day 2, activated cells exhibited multiple populations, with nearly all divided cells showing a high RPM signal.". However, on day 2 it is hard to see any dividing cells in the dot plot included in the supplemental figure. Dividing cells only appear on day 5? Their statements make the subsequent paragraphs also difficult to follow.

We modified the text to clarify this data – there is likely activation-induced cell death occurring which is why there are relatively few CFSE-low cells at this timepoint, and they do exhibit a fairly wide range of RPM staining. The main point is that by day 5, nearly all divided cells exhibit high RPM.

1. "Many divided cells exhibited near baseline RPM signals, however, consistent with their return to the resting state. Interestingly, although non-activated cells did not divide, ~50% demonstrated increased RPM staining.". Again, it is hard to see the ~50% of cells with increased RPM the authors refer to in the provided supplemental figure.

This is from quantification of the flow data and is described more fully later when we discuss ribosome stalling.

1. The authors say "Thus, we cannot attribute the persistence of flow RPM staining in translation initiation inhibitor-treated cells to incomplete inhibition of protein synthesis.' - but it's unclear what this refers to as in the previous paragraph they also say: 'Initiation inhibitors, however, clearly discriminated between day 1 resting and activated cells. RPM signal was diminished by up to 8090% on day 5 post-activation.' - this is all somewhat confusing. It would be helpful to have this clarified and in the text to make more liberal use of referring to specific figures.

Figure 1B shows that RPM is maintained at fairly high levels during treatment with EME or CHX (in contrast to the initiation inhibitors HAR/PA). To rule out that the drugs were simply not active, tritiated leucine labeling was conducted to confirm that incorporation of the radiolabeled amino acid dropped to near-baseline (Figure 1C). Therefore, we can conclude that the drugs are indeed working as intended, but EME/CHX does not decrease RPM signal to the same extent that they prevent leucine incorporation.

1. Page 5 Fig 3A - I don't understand the difference between freshly isolated OT-1 cells - which don't stall and day 1 OT-1 cells which do. Why are freshly isolated cells not behaving like the naïve cells- isn't this what they would predict? Also - I accept that there is a move from monosome to polysome population between day 1 and 2 - the effect isn't huge - it would be helpful/interesting to know what has happened by day 5 - is the effect much more significant?

Freshly isolated cells are harvested from animals and immediately queried, whereas day 1 cells are cultured for 24h in the absence of any activation. Presumably, the ex vivo culture without any activation causes the mouse T cell ribosomes to stall, just as we observed in cells obtained from human donors that took hours to collect and bring to the bench. The appearance of polysomes is really related to how the activation of the cells is done... refer to Figure 5B to see how significant the polysome buildup can be!

1. Fig S3C - I don't understand how they reach the conclusion from this figure that: '~15-fold increase in translating ribosomes in activated OT-I T cells in vivo (Supplemental Figure 3C) as compared to the 10-fold increase we previously reported using the original protocol. It would very much help the reader if these calculations could be better explained.

These are simply quantifications of the RPM staining done in Supplemental Figure 3C compared to experiments done in the absence of the CHX-modified method.

1. Page 7 - They conclude that the Tan paper has superior lymphocyte activation - but presumably this depends on the signal as to whether there is more activation and how this affects the shift from monosome to polysome -ie maybe a stronger activation signal affects the distribution more - perhaps their method is the more physiological? Is their conclusion fair - that 'These findings indicate that monosomes make a major contribution to translation in resting T cells but are likely to make a minor contribution in fully activated cells.'

Yes, we believe that their published method would be more physiological with the use of the natural OT-I peptide. We conclude that although monosome translation is present (as others have published), there are relatively few monosomes in fully activated T cells. Therefore, the monosome contribution to overall translation in activated T cells appears to be minor.

1. Contrary to observations in vitro, ribosomes are not stalled in naïve mouse T cells in vivo, as we show via RTA analysis of non-activated T cells. - yes - this seems somewhat surprising - what is the explanation?

We presume this is due to the stress/non-native environment that ex vivo cultured cells are subjected to.

1. Whilst I understand the point that the authors are trying to make in Figure 1D about resting T cells having high background RPM staining due to stalled ribosomes, it is intriguing that there is almost no difference (no statistical significance provided) after 2 or 5 days of activation. Isn't this finding contrary to the one provided in Figure 1A and Suppl Figure 1B?

Figure 1A is showing the difference between no activation and activation conditions. Figure 1D is predominantly meant to show that the increase in RPM from activated cells at day 1 and day 5 are not as different as one might predict. The reason, as we describe in further experiments, is likely that cells exhibiting ribosomal stalling can incorporate puromycin, damping the “fold change” we calculate (unlike what we observe in metabolic labeling experiments in the same figure panel). Statistics have now been displayed on the graphs in Figure 1D for further clarification.

1. "Including EME with HAR prevented decay of the RPM signal, as predicted, since EME blocks elongation while enabling (even enhancing) puromycylation^{21,26}." I find this very confusing. I understand that emetine blocks protein elongation whilst enabling puromycylation, but why does it block the effect of the protein initiation inhibitor Harringtonin? Do they compete with each other?

When ribosomes are frozen with emetine, they cannot transit mRNA and “fall off”. Therefore, the inclusion of EME in these experiments is a control to ensure that we are looking at true transit and runoff of ribosomes with harringtonine treatment (explanation in the second paragraph of “Flow RPM measures ribosome elongation rates in live cells” section)

1. Can the authors explain why the RPM signal of activated OT-I cells (PMA/Iono) increases 20fold compared to resting cells, but there is only a ~2-fold increase in signal in human cells? The authors previously mentioned: "We noted that the RPM signal in activated cells was only 2- to 5fold higher than in non-activated cells. This increase is modest compared to the ~15-fold activation-induced increase in protein synthesis in original studies 10,11. To examine this discrepancy, we incubated cells for 15 min with harringtonin (HAR) or pactamycin (PA) to block translation initiation or emetine (EME) or cycloheximide (CHX) to block elongation." Would the authors have followed the same path if they had started the paper with OT-I cells?

Human cells are not as well activated as OT-I in our study. The last question is beyond the scope of our reasoning as empirical evidence-based scientists, but we have applied for funding from the HG Wells Foundation for a time machine to answer this question.

1. Authors should include representative raw data of the flow cytometries used to perform the "Ribosome Transit Assay (RTA) in Figures 2 and 3 as supplemental data.

Done; now included in Supplemental Figures 1 and 3.

1. It would be interesting to compare RPM in T cells activated with a more physiological stimulus, such as beads anti-CD3 anti-CD28 vs PMA/Iono. Particularly after showing that peptide-specific stimulation (with SIINFEKL) is more effective than PMA/Iono in activating OT-I cells and inducing polysome formation (Figures 5B and Suppl Figure 4A).

We tried plate bound anti- CD3 and anti-CD28 early in these studies, and they didn't induce as much early activation.

1. Can the authors include the gating strategy to call "activated OT-I cells" to the cells shown in Suppl Figure 3c?

A new Supplemental Figure 3D has been added showing the exact gating strategy for the OT-I cell RTA assays described in Supplemental Figure 3C and elsewhere.

1. In Figure 6B, the authors mention an increase in the volume of the cells based on the assumption of spherical morphology but then show an increase in diameter. It would be more consistent to show both parameters in the same graph.

The graph was changed to volume calculations instead of diameter for clarity. But they are linked as volume scales by radius cubed.

1. The paper's main conclusion (i.e., that the ratio of proteins to ribosomes in T cells activated in-vivo does not support their doubling time) is exciting. They conclude this after measuring cell volume, protein abundance, and ribosomes per cell. As no changes in cell volume and protein abundance between T cells activated in vitro vs in vivo were observed (Figures 6B and 6C), the difference is exclusively attributable to a reduced number of ribosomes per cell in T cells activated in vivo (Figure 6F). Critically, the measurement of ribosomes per cell in T cells activated in vivo (Figure 6F, "ex vivo day 2") includes only two data points. It is hard to understand how the authors calculated this figure's means and standard deviations as it is not described in the figure legend. From the dispersion observed for "day 1" and "day 2" in vitro-activated T cells, it seems that the variability of the assay to measure ribosome content could explain part of the phenotype. Additionally, there are several missing data points in Figure 6H. As this figure is just a transformation of Figures 6D and 6G, it isn't easy to understand why. Can I suggest that they include more data points for Figures 6F, G, and H in the ex vivo day 2' category as the two data points shown with little variability is out of keeping with the rest of the data, and may be skewing their data?

Figure 6F does not have the same number of data points as other panels because it required measurement of both protein content and ribosome number. Since the ribosome quantification method described here was developed later than some of our earlier protein measurements, not all experiments had both sets of data to properly calculate the proteins per ribosome. All data that had both values are included, though.

Reviewer #3 (Recommendations For The Authors):

Minor points:

If an increase in cell diameter is recorded upon activation, why not also provide the value for the increase in volume?

Done

Regarding the writing, the erratic punctuation/hyphenation - or lack thereof - doesn't improve readability. One example: "....consistent with the idea that the flow RPM signal in day 1 resting lymphocytes...." Perhaps better: "... consistent with the idea that the RPM signal, obtained by flow cytometry for lymphocytes analyzed on day 1 and maintained in the absence of any activating agent,..." I understand that this can make for longer sentences, but I object to the use of 'flow' as shorthand for 'flow cytometry', and to the use of day 1 as an adverb or adjective. That works as lab jargon, it's less effective in a

written text. The abbreviation 'DRiPs' is not defined. Words like 'notably', and 'surprisingly' can be eliminated.

This work would benefit from the inclusion of a section describing 'Limitations of the study'.

This is now expanded in the Discussion, as described above.