Quantitative analysis of T cell proteomes and environmental sensors during T cell differentiation

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Quantitative mass spectrometry reveals how CD4+ and CD8+ T cells restructure proteomes in response to antigen and mammalian target of rapamycin complex 1 (mTORC1). Analysis of copy numbers per cell of 9,400 proteins provides new understanding of T cell phenotypes, exposing the metabolic and protein synthesis machinery and environmental sensors that shape T cell fate. We reveal that lymphocyte environment sensing is controlled by immune activation, and that CD4+ and CD8+ T cells differ in their intrinsic nutrient transport and biosynthetic capacity. Our data also reveal shared and divergent outcomes of mTORC1 inhibition in naïve versus effector T cells: mTORC1 inhibition impaired cell cycle progression in activated naïve cells, but not effector cells, whereas metabolism was consistently impacted in both populations. This study provides a comprehensive map of naïve and effector T cell proteomes, and a resource for exploring and understanding T cell phenotypes and cell context effects of mTORC1.
Fig. 1 | Proteome remodeling during T cell differentiation. a, Total protein content of naïve, 24 h TCR-activated (TCR) and effector (Eff) populations. b, Mean fluorescence intensity (MFI) of forward and side scatter for naïve CD4+ and CD8+ T cells. c, Heat maps of CD4+ and CD8+ proteomes during differentiation. The full list of proteins within the heat map is provided in Supplementary Data 1. BR, biological replicate. Relative protein abundance is graded from low (blue) to high (red) to allow comparisons between different T cell differentiation stages. d, Numbers of proteins changing in abundance during T cell differentiation: naïve to TCR activated, and naïve to effector (P < 0.05; fold change >1.5 (two-tailed t-test with unequal variance) or found in one population but not detected in another population). e, Proportion of the cell mass corresponding to proteins increasing, decreasing or not changing in naïve cells in response to TCR triggering. The numbers of proteins in each category are also provided. Proteins were categorized as changing as described for d. f, Protein copy number comparisons between naïve and effector populations for CD4+ and CD8+ T cells. Proteins highlighted in red were significantly different between naïve and effector cells (P < 0.05; fold change >2 s.d. from the mean fold change; two-tailed t-test with unequal variance) or were found in one population but not detected in another population. The dashed line represents the mean fold change between naïve and effector cells. GZMB, granzyme B; KLF2/3, Kruppel-like factor 2/3. g, Abundance of effector molecules in CTLs and Th1 cells. PRF1, perforin-1. h, Abundance of GZMB and PRF1.

In a and d–h, n = 6 biologically independent samples for CD8+ naïve cells and n = 3 biologically independent samples for each of the other T cell populations. In b, n = 4 biologically independent samples. In c, n = 3 biologically independent samples for each T cell population. Histogram bars represent means ± s.d.
To establish whether cell mass increases in activated T cells reflect scaled increases in the expression of existing proteins or the expression of new proteins, we used nearest-neighbor analysis and Pearson correlation to align the expression profiles of >8,000 proteins in T cell populations. This analysis showed that immune activation does not scale up all proteins, but dynamically reshapes proteome landscapes (Fig. 1c and Supplementary Data 1). There were increases in the abundance of >6,000 proteins in activated CD8+ cells and almost 5,500 proteins in activated CD4+ cells compared with naïve cells (Fig. 1d). However, 1,300–1,800 proteins did not change abundance, and a substantial proportion of the naïve cell proteome (7–9%) was downregulated as T cells responded to antigen (Fig. 1c–e). Proteins whose expression decreased after immune activation had diverse functions (Supplementary Data 2) and included translational repressors, cell cycle inhibitors and transcription factors.

Protein copy number comparisons determined the proteins that are exclusively found at a particular stage of T cell differentiation and the proteins that are expressed in all populations at either equal, enriched or depleted levels (Fig. 1f and Supplementary Data 2 and 3). We detected >800 proteins in effector CD4+ and CD8+ cells that were not found in naïve T cells (Fig. 1f), including effector molecules such as interferon-γ (IFN-γ), granzymes and perforin (Fig. 1f and Supplementary Data 2). Interestingly, effector Tc1 cells expressed high levels of granzymes and perforin comparable to those of CTLs (Fig. 1g,h). The pattern of expression of transcription factors and chromatin regulators was also remodeled as T cells differentiated (Fig. 2a,b). For example, immune activation caused T cells to downregulate their expression of Kruppel-like family transcription factors, which maintain pluripotency and cell quiescence (Fig. 2a). The complexity and extent of the transcription factor and proteome remodeling driven by immune activation was remarkable. However, it was striking that there were more similarities than differences between CD4+ and CD8+ T cells, reflecting that the bulk of cellular proteins comprise core machinery essential to every cell (Supplementary Fig. 1).

Scaling versus enrichment of core proteins and processes in differentiating T cells. Many proteins change copy numbers in immune-activated versus naïve T cells, but as immune activation increases the total T cell mass, only proteins whose abundance drops below or exceeds the total cell mass will change concentration. Moreover, proteins with unchanged copy numbers in naïve versus activated cells decrease concentration. Hence, >6,500 proteins increased in copy number in CTLs versus CD8+ naïve cells, but only 3,300 proteins increased in concentration (Fig. 3a). One way to assess the scaling versus enrichment or depletion of core subcellular compartments is to consider what percentage a protein group contributes to total protein mass in different populations. For example, nuclear envelope protein copy numbers increase in activated versus naïve T cells, but the percentage of cell mass that comprises nuclear envelope proteins decreases (Fig. 3b,c). Immune-activated T cells thus do not equally scale up their nuclear envelope proteins compared with other proteins, which is consistent with images showing increases in cytoplasm volumes of effector versus naïve cells. What about metabolic compartments? There is enrichment of total glycolytic pathway and mitochondrial proteins in activated versus naïve T cells (Fig. 3b,c and Supplementary Fig. 2), although it should be highlighted that these protein groups are already abundant in naïve T cells. Glycolytic enzymes represent 2–3% of naïve T cell proteomes versus 4–5% in effectors, while mitochondrial proteins are 12–13% of naïve T cells versus 15–16% of effectors (Fig. 3c). An examination of mitochondrial proteins revealed that some increased in scale with the overall increase in cell mass that occurred during T cell differentiation, while others increased beyond scaling. For example, mitochondrial ribosomal proteins are tenfold more abundant in effector cells versus naïve cells (Fig. 3d). Another interesting example of scaling versus enrichment is that of hexokinase 1 and 2 (HK1 and HK2). HK2—one of the hexokinases that phosphorylate glucose to direct glucose metabolic pathways—showed a >1,000-fold increase in effector versus naïve T cells (Fig. 3e). Interestingly, despite this huge increase in HK2 expression, it proved non-essential for T cell activation19,20. The current data explain why HK2 is redundant, as they show that HK1 concentrations in naïve and activated T cells are very high and sufficient to compensate for HK2 loss (Fig. 3e and Supplementary Fig. 2).

As activated T cells increase volume, they increase cell surface area and may change the membrane densities of plasma membrane proteins. Simple modeling, which did not model alterations in membrane ruffling, predicted a threefold increase in membrane surface area in CTLs versus naïve T cells (Fig. 3f). Molecules such as Thy-1 cell surface antigen and CD45, had higher copy numbers in activated versus naïve CD8+ T cells, but did not increase membrane density after immune activation (Fig. 3f). In contrast, the amino acid transporters solute carrier family 1 member 5 (SLC1A5) and solute carrier family 7 member 5 (SLC7A5) increased 40-fold in copy number in CTLs versus naïve CD8+ cells—a tenfold increase in membrane density (Fig. 3f). Further analysis showed that naïve T cells had low expression of all amino acid and glucose transporters versus high levels of these transporters in activated T lymphocytes (Fig. 4a). In comparison, mitochondrial transporters were highly abundant in naïve T cells. Previous studies have shown increased amino acid and glucose transport in activated T cells compared with naïve T cells, and noted lower nutrient transport in effector CD4+ versus CD8+ populations21,22. The present data explain this result: activated CD4+ T cells have a similar nutrient transporter repertoire but consistently express lower copies of key amino acid and glucose transporters than activated CD8+ T cells (Fig. 4a).

In this respect, although nutrient transporter expression in naïve T cells is very low compared with activated T cells, transporters are not completely absent. For example, the System L amino acid transporter SLC7A5 was found at approximately 3,000 copies in naïve CD4+ T cells and 8,000 copies in naïve CD8+ T cells (Fig. 4b). These data predict that there is some System L transport activity in naïve T cells, and that CD8+ T cells would have higher basal levels than CD4+ cells. To challenge this prediction, we used a sensitive flow cytometry assay to assess System L amino acid transport activity in naïve T cells23. The data show that naïve CD4+ and CD8+ T cells have a detectable basal System L transport activity mediated by SLC7A5. Crucially, the data show that naïve CD8+ cells have a higher System L transport capacity than naïve CD4+ cells (Fig. 4c,d). These results highlight the predictive value of the proteomic ruler methodology.

Immune activation controls RNA translational machinery. T cell exit from quiescence is associated with essential increases in mRNA translation24. The current data show that immune activation results in strong upregulation of ribosomes and mRNA translational machinery. Antigen-activated T cells increased ribosome numbers almost tenfold (Fig. 5a), and strongly increased levels of eukaryotic initiation factor 4F (eIF4F) complexes that translate methyl-capped mRNAs (Fig. 5b) and eIF2 complexes, which control the transfer of tRNA (transfer RNA) to ribosomes and tRNA synthetases (Supplementary Fig. 2). Hence, more of the cell mass of activated T cells is dedicated to protein synthesis than in naïve T cells. It was also notable that CD8+ cells consistently had higher ribosome and translational complex numbers than CD4+ T cells (Fig. 5a,b). However, how cells control mRNA translation also requires understanding of translational repressor quantities in different T cell populations. T cells express the eIF4F inhibitors 4E-BP1 and 4E-BP2 (eIF4E-binding proteins 1 and 2) and the translational repressor programmed cell death 4 (PDCD4) (Fig. 5c,d)25,26. 4E-BPs bind to eIF4E and displace eIF4G to prevent active eIF4F.
Fig. 2 | Expression profile of transcription factors during T cell differentiation. Over 300 proteins annotated as DNA binding/transcription factor activity (Gene Ontology term 0003700) were identified in CD4+ and CD8+ T cell populations. Additional transcription factors without a Gene Ontology annotation were added manually. a. Transcription factor expression profiles during T cell differentiation. The full list of proteins included in heat maps is provided in Supplementary Data 1. Histograms showing protein copy numbers per cell are provided for a selection of core transcription factors essential for T cell differentiation and activity. BACH2, BTB domain and CNC homolog 2; EOMES, eomesodermin; IRF8, IFN regulatory factor 8; RUNX3, runt-related transcription factor 3.
b. Copy number comparisons for transcription factors (TFs) in different T cell populations. Scatter plots show the average copy number for TFs and allow a two-way comparison between T cell populations. Proteins that were not detected in one population are positioned on the axis. TFs significantly different between two populations (P < 0.05; fold change > 2 s.d. from the mean fold change; two-tailed t-test with unequal variance), or showing a presence/absence expression profile, are represented by a red circle, while non-significant TFs are represented by a pink circle. The dashed line represents the mean fold change between the two populations. MECP2, methyl-CpG-binding protein 2; TCF7, transcription factor 7; NFYB, nuclear transcription factor Y subunit-β; THPOK (ZBTB7B), zinc finger and BTB domain-containing 7B; TSC22D4, TSC22 domain family protein 4. For the heat maps in a, n = 3 biologically independent samples for each T cell population. For the histograms in a and plots in b, n = 6 biologically independent samples for CD8+ naïve cells and n = 3 biologically independent samples for each of the other T cell populations. Histogram bars represent means ± s.d.
Fig. 3 | Scaling versus selective enrichment of proteins and processes during T cell differentiation. **a**, Comparison of protein copy number and concentration. Volcano plots show the ratio for proteins in CTLs versus naïve CD8⁺ T cells, using copies per cell (left) or cellular protein concentration (μM; right). Horizontal dashed lines indicate P values of 0.05. Vertical dashed lines indicate fold changes of 1.5. **b**, Protein content of ribosomes (Kyoto Encyclopedia of Genes and Genomes annotation 03010), mitochondria (Gene Ontology term 0005739), the nuclear envelope (Gene Ontology term 0005635) and the glycolytic pathway. **c**, Protein content of cellular compartments relative to the total cellular protein mass (%). **d**, Expression profile of mitochondrial proteins in CTLs versus naïve CD8⁺ T cells. The vertical dashed line in the volcano plot is the mean fold change (copy number CTL/ naïve) of all proteins. **e**, Left: expression profile of mitochondrial proteins in CTLs versus naïve CD8⁺ T cells (copy number CTL/ naïve). Mitochondrial proteins are highlighted with red circles. HK1 and HK2 are highlighted with yellow circles. The vertical dashed line is the mean fold change for all proteins. Copy numbers and concentrations of HK1 and HK2 are also provided (right). **f**, Protein copy numbers relative to cell surface area. Left: the surface areas (SAs) of naïve and effector CD8⁺ T cells were estimated using the formula 4πr², assuming the radius (r) of a naïve cell to be 2.8 μm, and that of a CTL to be 5 μm. Middle: protein copy numbers per cell. Right: protein copy numbers adjusted for cell surface area (copies per μm²). SLC1A5, solute carrier family 1 member 5; SLC7A5, solute carrier family 7 member 5; THY1, Thy-1 cell surface antigen. In **a–f**, n = 6 biologically independent samples for CD8⁺ naïve cells and n = 3 biologically independent samples for each of the other T cell populations. Histogram bars represent means ± s.d. In **a, d** and **e**, P values were calculated using a two-tailed t-test with unequal variance.
translation initiation complex assembly\(^1\). PDCD4 represses translation via eIF4A1 binding\(^2\). Interestingly, 4E-BPs were only detected in activated but not naïve T cells (Fig. 5c). Increased expression of translational repressors from naïve to activated cells seems inconsistent with the increased translation capacity in activated T cells, but critically 4E-BPs are only expressed at a maximum of 2 × 10^4 copies
per cell, whereas eIF4E (their target) has $>10^6$ copies in immune-activated CD8$^+$ cells and over $5 \times 10^5$ copies in activated CD4$^+$ cells. eIF4E is thus always in large excess of 4E-BPs (Fig. 5c). Hence, any modeling of 4E-BP translation repression needs to consider repressor and effector stoichiometry: any effects will be restricted to a subpopulation of eIF4Es and not relevant in naïve T cells where these repressors cannot be detected. Next, we considered PDCD4:eIF4A1 ratios since, to repress translation by eIF4F complexes, one PDCD4 molecule binds two eIF4A1 molecules. Naïve T cells have $3-5 \times 10^5$ PDCD4 copies per cell, which is sufficient to saturate the majority of eIF4A1 (Fig. 5d). In activated T cells, PDCD4 is downregulated (Fig. 5d), whereas eIF4A1 copies increase to over $4 \times 10^6$ copies per effector cell (Fig. 5b), leaving very low PDCD4:eIF4A1 ratios and free eIF4A1 to promote cap-dependent translation (Fig. 5d).

Immune activation shapes environment-sensing pathways in T cells. T lymphocyte function is regulated by environmental factors, such as glucose, amino acids, oxygen and iron availability. Moreover, activated T cells must actively take up nutrients to fuel metabolic processes and macromolecule biosynthesis, and to

Fig. 5 | Regulation of mRNA translation in T cells. a, Number of ribosomes in naïve, TCR-activated and effector CD4$^+$ and CD8$^+$ T cell populations. Numbers of ribosomes were estimated by calculating the mean number of ribosomal subunits within each cell using the Kyoto Encyclopedia of Genes and Genomes (annotation 03010). b, Expression profiles of key components of the eIF4F mRNA translation initiation complex during differentiation. eIF4F consists of eIF4G1, eIF4A1, eIF4E and PABPC1 (poly(A) binding protein cytoplasmic 1). CAP is the 5' cap on the mRNA. Data are presented as protein copies per cell in naïve, TCR-activated and effector CD8$^+$ and CD4$^+$ T cell populations. c, Stoichiometry of 4E-BP1 and 4E-BP2 (4E-BP1 + 2) to eIF4E in T cell populations. Copy numbers for 4E-BP1 and 4E-BP2 were combined. 4E-BP1 + 2 numbers are also plotted adjacent to copy numbers for eIF4E to assess whether inhibitor levels are adequate to block translation initiation. Ratios of 4E-BP1 + 2 to eIF4E are also presented to the right. ND, not detected. d, Left: abundance of PDCD4 during T cell differentiation. Right: ratios of PDCD4 to eIF4A1 in naïve, TCR-triggered and effector CD4$^+$ and CD8$^+$ T cells are shown adjusted, to account for one molecule of PDCD4 binding two molecules of eIF4A1 (middle), to inhibit CAP-dependent translation. In a–d, n = 6 biologically independent samples for CD8$^+$ naïve cells and n = 3 biologically independent samples for each of the other T cell populations. Histogram bars represent means ± s.d.
control nutrient-sensing kinase activity. The present data show that immune activation increases the expression of a limited repertoire of 12 amino acid transporters, two glucose transporters and three lactate transporters to fuel and regulate activated T cells (Fig. 4a). Nutrient transporters are essential components of T cell environment-sensing machinery as they act as ‘gatekeepers’ to control the activity of nutrient-sensing kinases. However, one other key insight herein is that immune activation quite comprehensively regulates the expression of other environment-sensing molecules (Fig. 6).

The expression of oxygen-sensing proline hydroxylase proteins and hypoxia-inducible factor-1α (HIF-1α), along with iron transport and iron-sensing proteins, is thus predominantly restricted to activated and effector T cells (Fig. 6a,b). Antigen activation also modulates the expression of DNA-sensing pathways. The cyclic guanosine monophosphate–adenosine monophosphate synthase–stimulator of IFN genes (STING) DNA-sensing pathway is present in naïve T cells but highly enriched in effector T<sub>H1</sub> cells and CTLs (Fig. 6c). One other noteworthy observation is that immune activation...
increases the expression of the nutrient-regulated protein kinases AMPKα1 and mTORC1 and the amino acid–sensing kinases GCN2 and PERK1 of the integrated stress response pathway\textsuperscript{95} (Fig. 6d). There is also immune control of components of the nutrient-sensing regulatory pathways that control mTORC1 activity, including the lysosomal arginine sensor SLC38A9, the cytosolic arginine and leucine sensors CASTOR1 and Sestrin2, the GTPase RHEB, and GATOR complexes\textsuperscript{11,31–35} (Supplementary Fig. 3). Collectively, these data show that immune activation comprehensively shapes T cell responsiveness to environmental stimuli.

mTORC1 control of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell proteomes. The nutrient-sensing kinase mTORC1 is a critical regulator of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell differentiation, and one current objective was to explore the consequences of mTORC1 inhibition in different T cell populations\textsuperscript{4,15,36–38}. The data reveal that mTORC1 controls cell growth in both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells: in TCR-activated CD8\textsuperscript{+} T cells, mTORC1 inhibition with rapamycin caused a 26% loss of mass compared with a 16% loss of cell mass in the TCR-activated CD4\textsuperscript{+} T cells (Fig. 7a). Using a >1.5-fold cut-off, mTORC1 controlled the expression of more than 2,300 proteins in the TCR-activated CD8\textsuperscript{+} T cells compared with 600 in the CD4\textsuperscript{+} T cells (Fig. 7b). However, it is evident that immune activation still induces a substantial increase in cell mass in the absence of mTORC1 activity (Fig. 7a).

This limited effect of mTORC1 inhibition on T cell growth was also seen in CTLs and T\textsubscript{H}1 cells, where the impact of 24 h mTORC1 inhibition was an approximate 20% loss of cell mass, but cells did not return to naive cell sizes (Fig. 7a). In T\textsubscript{H}1 cells, 800 proteins decreased expression, whereas in CTLs, the effects were restricted to around 260 proteins (Fig. 7b). One key point is that there were quantitative differences in the impact of mTORC1 inhibition on CD4\textsuperscript{+} and CD8\textsuperscript{+} proteomes, but no obvious qualitative differences. mTORC1 thus controls the production of the effector molecules IFN-γ, perforin and granzymes in both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (Supplementary Fig. 4), but does not radically prevent the dynamic remodeling of transcription factors that drives T cell differentiation (Supplementary Fig. 4). T cells activated in the presence of mTORC1 inhibition thus show effectively normal patterns of expression of T-bet, IRF4, BATE, MYC and BLIMP-1 (Supplementary Fig. 4). mTORC1 control of HIF-1α has been described, and the present data show that this is a consistent effect in CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells\textsuperscript{52} (Supplementary Fig. 4).

The main contribution to the loss of cell mass caused by mTORC1 inhibition in all populations was the reduced expression of metabolic proteins—notably, ribosomes, glycolytic enzymes, mitochondrial proteins, glucose and lactate transporters, and fatty acid and sterol metabolism proteins (Fig. 7c,d and Supplementary Fig. 4). It has been reported that loss of mTORC1 activity decreases the expression of mitochondrial ribosomal proteins and oxidative phosphorylation enzymes in CD3- and CD28-activated CD4\textsuperscript{+} T cells\textsuperscript{51}. The current data showed statistically significant decreased expression of mitochondrial proteins in both CD4\textsuperscript{+} and CD8\textsuperscript{+} populations, but these differences were very low in magnitude, such that mitochondrial proteins were highly abundant in mTORC1-inhibited T cells and unlikely to be rate limiting (Fig. 7c and Supplementary Fig. 5). Moreover, in no population did mTORC1 inhibition revert the expression levels of mitochondria or metabolic proteins back to naive cell levels, indicating that the expression of these key components in T cells is also controlled by mTORC1-independent pathways. For example, rapamycin treatment caused expression of the glucose transporter SLC2A1 to drop from around 50,000 to 17,000 copies per cell in TCR-activated CD8\textsuperscript{+} cells, and from 137,000 to 77,000 copies per cell in CTLs (Supplementary Fig. 4). Glucose transport is rate limiting for glucose metabolism in T cells, so reducing glucose transporter abundance would allow mTORC1 to control glucose metabolic pathways in T cells\textsuperscript{50}. Nevertheless, a key insight is that mTORC1 is not an on or off switch for glucose metabolism, nor is it an absolute on or off switch for cell growth.

Hence, a salient point is that immune-activated T cells still increase cell mass when mTORC1 activity is suppressed, and loss of mTORC1 activity in effector cells does not revert their mass to that of a naive T cell. In this context, the selectivity of mTORC1 control of T cell proteomes is emphasized by the large number of proteins that were unchanged in copy number in all rapamycin-treated populations, as well as the examples of proteins that increased in abundance and concentration in mTORC1-inhibited cells (Fig. 7e and Supplementary Data 4). Proteins increasing in concentration included the translational repressor PDCD4, which increased in both TCR-activated and effector cells treated with rapamycin (Fig. 7e). However, eIF4A1 did not decrease pro-rata, and remained in excess of PDCD4 in these populations, allowing continued protein synthesis (Fig. 7f and Supplementary Fig. 5). Another protein consistently showing increased copy number in mTORC1-inhibited cells was the adhesion molecule CD62L (Fig. 7e). The increased expression of CD62L in mTORC1-inhibited CTLs restores the ability of these cells to traffic into secondary lymphoid tissue\textsuperscript{53}. mTORC1 repression of CD62L expression in CD4\textsuperscript{+} T cells argues that mTORC1 could control CD4\textsuperscript{+} T cell positioning within lymphoid tissues.

Cell context effect of mTORC1 inhibition. One objective of the present study was to identify cell context effects of mTORC1 signaling. In this respect, Gene Ontology term enrichment analysis indicated dominant mTORC1 control of lipid metabolism in effector T\textsubscript{H}1 cells and CTLs (Table 1). mTORC1 control of lipid metabolism was also seen in antigen-activated CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, but Gene Ontology term enrichment analysis indicated that the dominant effect of mTORC1 inhibition in these populations was on cell cycle and DNA replication pathways (Table 1). These data are consistent with early studies showing that rapamycin delayed the first cell cycle entry of phytohemagglutinin-activated human T cells but did not block proliferation once cells had committed to the cell cycle\textsuperscript{54,55}. To orthogonally challenge these predictions, we monitored DNA synthesis in TCR-activated and effector CD8\textsuperscript{+} populations, and found that rapamycin inhibited DNA synthesis of antigen-activated naive CD8\textsuperscript{+} T cells but not CTLs (Fig. 8a). This is consistent with a decrease in the expression of proteins implicated in DNA replication in rapamycin-treated TCR-activated cells but not in CTLs (Supplementary Fig. 6). In this respect, a critical cell cycle check point is mediated by D-type cyclins and their associated kinases cyclin-dependent kinases 4 and 6 (CDK4 and CDK6). Naive T cells have low levels of cyclin D3 and CDK4/6 but high levels of the cyclin-dependent kinase inhibitor protein 1B (CDKN1B or p27) (Fig. 8b and Supplementary Fig. 6). Antigen activation increased cyclin D2 and D3 and CDK4/6 expression, resulting in excess D-type cyclins, relative to the inhibitor CDKN1B. Interestingly, rapamycin treatment caused decreased cyclin D expression in both TCR-activated CD8\textsuperscript{+} T cells and effectors (Fig. 8c), but why then were there no cell cycle progression defects in effectors? Here, insights come from considering cyclin D copy numbers in different T cell populations relative to numbers of CDK4/6 and CDKN1B. For example, naive CD8\textsuperscript{+} T cells express high levels of CDKN1B, undetectable cyclin D3, and low cyclin D2 and CDK4/6 (Fig. 8b). CD8\textsuperscript{+} cells activated for 24 h upregulate cyclin D expression, increase CDK4/6 levels and downregulate CDKN1B: simple modeling predicts that they have free cyclin D and CDK4/6 complexes. In this experiment, TCR-activated CD8\textsuperscript{+} cells had approximately 3,000 and 8,000 copies per cell of cyclin D2 and D3, respectively, and only 2,000 copies of CDKN1B. As 60% of these cells were replicating DNA (Fig. 8a), this level of ‘free’ cyclin D and CDK complex (estimated as approximately 9,000) must be sufficient to drive S-phase entry. mTORC1 inhibition caused cyclin D2 and D3 levels to drop to 2,000 copies per cell
Fig. 7 | Impact of mTORC1 inhibition on CD4+ and CD8+ T cell proteomes. 

**a**. Protein content of T cells in response to mTORC1 inhibition. Naïve CD4+ and CD8+ T cells were TCR triggered for 24 h with or without rapamycin, while effector CD4+ and CD8+ cells were incubated for 24 h with rapamycin.

**b**. Volcano plots show the protein ratios for rapamycin-treated cells versus controls (plus rapamycin/control copy numbers). Proteins highlighted in red had a P value < 0.05 and a fold change > 1.5, while proteins highlighted in gray did not change significantly.

**c**. Impact of rapamycin on the glycolytic pathway, ribosomes and mitochondria in CD4+ and CD8+ cells. 

**d**. Summary of cellular processes impacted by mTORC1 inhibition. Arrows pointing downwards indicate decreased abundance while arrows pointing upwards indicate increased abundance. Proteins/processes changing in TCR-stimulated cells only are labelled ‘TCR’, while those changing in TCR and effector populations are labelled ‘TCR + eff’. 

**e**. Impact of inhibiting mTORC1 on protein concentrations. Volcano plots were generated as described for **b**. 

**f**. Ratios of PDCD4 to eIF4A1. One molecule of PDCD4 binds two molecules of eIF4A1, and the ratios have been adjusted to account for this binding stoichiometry. For **a–f**, n = 6 biologically independent samples for CD8+ naïve cells and n = 3 biologically independent samples for each of the other T cell populations. Histogram bars represent means ± s.d. For **b** and **e**, P values were calculated using a two-tailed t-test with unequal variance.

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### Table: Adjusted Ratio of PDCD4 to EIF4A1

| Protein | Naive | TCR | TCR + rapamycin | Effector | Effector + rapamycin |
|---------|-------|-----|-----------------|----------|---------------------|
| CD8+    | 1 to 1.3 | 1 to 55 | 1 to 21 | 1 to 16 | 1 to 5 |
| CD8+    | 1 to 1.5 | 1 to 68 | 1 to 19 | 1 to 16 | 1 to 7 |
environmental cues is not intrinsic but shaped by immune activation through sensors and nutrient transporters, revealing that T cell sensing of essential amino acid and glucose transporters in response to immune activation highlights a fundamental mechanism that ensures the targeted supply of nutrients to T cells participating in an immune response. Only T cells triggered by cognate antigen will be able to fuel the cell cycle. This study characterized murine naïve CD4+ T cells as a system with relatively small and mTORC1-independent pathways also controlling cell cycle progression. A comparison of previous studies of how raport-deficient CD4+ T cells responded to CD3 and CD28 antibodies versus how CD8+ effector CTLs responded to rapamycin indicated that there might be differences between how mTORC1 controls comparable CD4+ and CD8+ T cell populations. mTORC1 inhibition had an impact on glucose transporters, glycolysis, mitochondria, fatty acid metabolism, ribosomes, translational machinery and cell adhesion molecules in both antigen-activated and effector CD4+ and CD8+ T cells.

**Discussion**

This study characterized murine naïve CD4+ and CD8+ T cell proteomes and mapped the impact of immune activation and mTORC1 inhibition on the expression of more than 9,000 proteins. The data show how environment signaling pathways are integrated with antigen and cytokine selective pathways to ensure the immune specificity of T cell activation. New, biologically relevant insights include that antigen exposure increases the expression of key oxygen and nutrient sensors and nutrient transporters, revealing that T cell sensing of environmental cues is not intrinsic but shaped by immune activation and restricted to activated T cells. The considerable upregulation of for each protein, while CDKN1B remained at 2,000 copies, leaving few free-cyclin complexes for cell cycle progression (Fig. 8c). In contrast, CTLs have high cyclin D2 and D3 levels: 60,000 and 140,000 copies per cell compared with 7,000 CDKN1B copies. Rapamycin treatment decreased cyclin D levels twofold, but cyclin D:CDKN1B stoichiometry remained >10:1 (that is, there were more than 85,000 ‘inhibitor’-free cyclin D and CDK complexes, which would be sufficient to drive cell cycle progression) (Fig. 8c). These data illustrate that, to fully understand the consequences of a change in expression of each protein, it is necessary to understand protein quantity and the relative levels of expression of its regulatory partners.

One experimental aim was to compare the impact of mTORC1 inhibition on CD4+ and CD8+ T cells, since there are often general comments made about what mTORC1 does to control lymphocyte function, based on experiments performed in a single cell population, and assuming that observations can be extrapolated to all cells. In this context, a general comment frequently made about mTORC1 is that it regulates cell growth. The present data show this to be true, but with the caveat that mTORC1 growth effects are relatively small and mTORC1-independent pathways also control T cell growth. Indeed, the selectivity of mTORC1 shaping of CD4+ and CD8+ T cell proteomes was striking. The present data give insights as to why mTORC1 effects on T cell protein mass are limited. For example, mTORC1 is proposed to control cell growth by phosphorylating and inactivating the translational repressors 4E-BP1 and 4E-BP2 (refs. 11,40). However, we show that 4E-BP copy numbers are very low relative to their target eIF4E, making it unlikely that releasing any 4E-BP repression would have a major impact on protein translation, and would rather be highly selective to a small subset of proteins. Similarly, we have shown that mTORC1 inhibition increases expression of the translational repressor PDCD4, but that the PDCD4 target eIF4A1 remained in large excess to PDCD4 in rapamycin-treated T cells. These considerations highlight the value of quantitative data that model protein complex stoichiometry for an understanding of how immunomodulatory stimuli effect T cell phenotypes.

What about cell context effects of mTORC1? A comparison of previous studies of how raport-deficient CD4+ T cells responded to CD3 and CD28 antibodies versus how CD8+ effector CTLs responded to rapamycin indicated that there might be differences between how mTORC1 controls CD4+ and CD8+ T cell proteomes. The present study enabled direct comparisons between populations, and revealed no major qualitative differences in how mTORC1 controls comparable CD4+ and CD8+ T cell populations. mTORC1 inhibition had an impact on glucose transporters, glycolysis, mitochondria, fatty acid metabolism, ribosomes, translational machinery and cell adhesion molecules in both antigen-activated and effector CD4+ and CD8+ T cells. There were differences in how mTORC1 inhibition impacted antigen-activated lymphocytes as they exit quiescence versus mTORC1 control of effector T cells. In particular, mTORC1 inhibition had a dominant effect on cell cycle progression in antigen-activated naive CD4+ and CD8+ cells but not in Tθ,1 or CTL effector populations. The basis for this difference was that key cell cycle regulators are so highly abundant in effectors compared with T cells progressing into their first cell cycle that decreases in expression caused by a

**Table 1 | Gene Ontology term enrichment analysis for proteins that significantly dropped in abundance in rapamycin-treated cells**

| Enriched Gene Ontology term | Fold enrichment | P value |
|-----------------------------|-----------------|---------|
| CD8+ TCR                    |                 |         |
| 0007049: cell cycle          | 6               | 1.7 × 10−35 |
| 0051301: cell division       | 7               | 5.5 × 10−35 |
| 0007067: mitotic nuclear division | 8   | 6.4 × 10−31 |
| 0007059: chromosome segregation | 10  | 6 × 10−34 |
| 0007018: microtubule-based movement | 12 | 1.2 × 10−9 |
| CD4+ TCR                    |                 |         |
| 0006260: DNA replication     | 10              | 1.2 × 10−12 |
| 0007049: cell cycle          | 3               | 6.5 × 10−8  |
| 0006270: DNA replication initiation | 14  | 2.9 × 10−4  |
| 0006974: response to DNA damage | 2    | 8.5 × 10−4  |
| 0051726: regulation of cell cycle | 5   | 1.2 × 10−3  |
| CTL                         |                 |         |
| 0006636: unsaturated fatty acid biosynthetic process | 35 | 1.3 × 10−4 |
| 0006633: fatty acid biosynthetic process | 10 | 2.3 × 10−4 |
| 0006629: lipid metabolic process | 3    | 1.0 × 10−3 |
| 0030154: cell differentiation | 3   | 1.3 × 10−3 |
| 0016239: regulation of macroautophagy | 14 | 2.3 × 10−3 |
| Tgfβ1                      |                 |         |
| 0001626: sterol biosynthetic process | 12  | 9.3 × 10−5 |
| 0002802: steroid metabolic process | 6    | 6.2 × 10−4 |
| 0006695: cholesterol biosynthetic process | 11  | 7.9 × 10−3 |
| 0006694: steroid biosynthetic process | 7   | 8.2 × 10−4 |
| 0035455: response to IFN-α | 18              | 1.1 × 10−3 |

The top-five enriched Gene Ontology terms for biological processes are presented. Significance was defined as a P value of <0.05 (two-tailed t-test with unequal variance) and a fold change of >2 s.d. from the mean fold change.
loss of mTORC1 function are not rate limiting. These data indicate that for mTORC1 inhibitors to prevent T cell cycle progression it will be necessary to deliver the inhibitor before the T cell has accumulated high levels of D-type cyclins. The data reveal cell-context-dependent functions of mTORC1, and show that these can be understood when there is knowledge and understanding of the stoichiometry of critical protein complexes and their positive and negative regulators, plus some understanding of functional thresholds for different pathways. The current data provide comprehensive quantitative information about protein copy number and concentration in T cell populations that are used extensively as models to probe T cell biology. We provide an easily interrogated resource for exploring the protein landscape of key immune cells and for predicting T cell responses to the environment and other immune modulators.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41590-019-0495-x.

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Methods

Mice. For proteomics experiments P14 (ref. 41) and OT-II+, transgenic mice along with C57BL/6 (wild-type) B6 mice were used. For the single-cell amino acid transport assay, CD4Cre Skl7a5+/− (CD45.2+) and C57BL/6 Lys1.5 (CD45.1-) were used. Male and female mice aged between 50 and 120 d were used. Further details can be found in the Life Sciences Reporting Summary. All mice were maintained in the Biological Resource Unit at the University of Dundee using procedures that were approved by the University Ethical Review Committee and under the authorization of the UK Home Office Animals (Scientific Procedures) Act 1986.

Flow cytometry. Forward/ side scatter analysis of naïve CD4+ and CD8+ T cells. Lymph node–derived naïve CD4+ and CD8+ cells were stained with CD8-PE and CD8-Pacific Blue. Forward and side scatter profiles were acquired on a LSRFortessa II with DIVA software, and data were analyzed using FlowJo software version 9 (Tree Star). Four biologically independent samples were analyzed.

DNA synthesis assay. To measure DNA synthesis, cells were fed 10 μM Click-iT EdU (Thermo Fisher Scientific) for 30 min. Cells were then harvested, stained with CD8-FITC (for TCR-activated cells), fixed with 1% paraformaldehyde and permeabilized with 0.1% Triton X-100 before undergoing a copper–catalyzed click chemistry reaction with Alexa 647-azide (Thermo Fisher Scientific). Cells were analyzed by flow cytometry to determine the degree of incorporation of EdU. All flow cytometry data were acquired on either an LSRFortessa II with DIVA software or a FACSVerse flow cytometer with FACSsuite software (BD Biosciences). Data were analyzed using FlowJo software version 9 (Tree Star). Three biologically independent samples were analyzed for each condition. The gating strategy for flow cytometry analysis is provided in Supplementary Fig. 7.

Monitoring System L amino acid transport. A single-cell assay to monitor System L amino acid transport, with kynurenine as a fluorescent system L transport substrate, was performed as described previously42. In brief, wild-type (C57BL/6) Lys1.5; CD45.1+ and CD4Cre Skl7a5+/− (CD45.2+) cells were fixed and washed in warmed HBSS (approximately 2.5 × 106 cells in 50 μl HBSS) and then incubated in 200 μl warmed HBSS (approximately 2.5 × 106 cells in 200 μl HBSS) with 20 μM kynurenine (α-ketoglutarate analogue) and 1% paraformaldehyde. Subsequently, cells were washed three times in prewarmed HBSS before being snap frozen in liquid nitrogen. After 24 h, cells were harvested by centrifugation and prepared for cell sorting to isolate a pure population of CD8+ cells. Fluorophores were blocked using 1 μg Flc block (BD Pharmingen) per million cells. Cells were stained with lymph node isolates from OT-II transgenic mice to first gate effective TCR activation. A preparation of antigen-presenting cells was first generated. Splenocytes from wild-type B6 mice, and T lymphocytes were removed using anti-TCR-biotin antibody and an immunomagnetic isolation kit (Miltenyi Biotec). To create an antigen-restimulated T cell, naïve CD8+ T cells were incubated with lipopolysaccharide (2 μg ml−1) and OVA peptide (323–339; 1 μM; Sigma– Aldrich) overnight before being combined with cells from OT-II lymph nodes. Antigen-presenting cells were combined with OT-II lymph node cells at a ratio of 1:2 in RPMI media. Cells were incubated with OVA peptide (1 μg ml−1) plus 2 ng ml−1 IL-12 (PeproTech) and added to naïve CD8+ T cells with or without 20 nM rapamycin. Over 24 h, cells were subjected to cell sorting to generate a pure population of activated CD4+ cells. Fluorophores were blocked using 1 μg Flc block (BD Pharmingen) per million cells. Cells were stained with CD4-PerCP Cy 5.5, Vio2-PE and CD8 PE before cell sorting. CD4+ and Vio2+ cells were collected. Purified cells were washed twice in HBSS before being snap frozen in liquid nitrogen.

To generate CTLs, spleens were extracted from P14 mice and washed in red blood cell lysis buffer before being suspended in RPMI media supplemented with GP33 peptide and IL-2 and IL-12 (as described for TCR activation above). Cells were activated for 48 h, washed out of activation buffer and then cultured for 3 d in media supplemented with 20 ng ml−1 IL-2 and 2 ng ml−1 IL-12. Cells were treated with or without 20 nM rapamycin for 24 h before being harvested. Dead cells were removed using a dead cell removal kit (Miltenyi Biotec), washed twice with HBSS and snap frozen. TCR+ T cells were cultured from wild-type C57BL/6 mice. Spleen and lymph nodes were extracted from mice, washed and filtered through a 70-μm cell strainer, then CD8− cells were depleted using immunomagnetic selection (Miltenyi Biotec). Cells were activated on 2% CD3 (3 μg ml−1), CD3 antibody (2 μg ml−1), IL-2 (20 ng ml−1) and IL-12 (10 ng ml−1). Cells were cultured for a total of 6 d, with the last 24 h with or without rapamycin at 20 nM. Dead cells were removed as described above, and cells were washed twice with HBSS before being snap frozen in liquid nitrogen.

Proteomics sample preparation and tandem mass tag (TMT) labeling. Cell pellets were lysed in 400 μl lysis buffer (4% sodium dodecyl sulfate, 50 mM tetrathiomolybdenum bromide (pH 8.5) and 10 mM tris(2-carboxyethyl)phosphine hydrochloride). Lysates were boiled and sonicated with a BioRuptor (30 cycles: 30 s on and 30 s off) before alkylation with 20 mM iodoacetamide for 1 h at 22 °C in the dark. The lysates were subjected to the SP3 protocol for protein clean-up before elution into digest buffer (0.1% sodium dodecyl sulfate, 50 mM tetrathiomolybdenum bromide (pH 8.5) and 1 mM CaCl2) and digested with LysC and Trypsin, each at a 1:50 enzyme:protein ratio. TMT labeling and peptide clean-up were performed according to the SP3 protocol. T cell populations were TMT labeled in the following six batches: naïve CD4+ cells (mass tag 126, 127C and 128C), TCR-activated CD4+ cells with or without rapamycin (mass tag 126, 127N and 128C for control and 129N, 130C and 131N for rapamycin); TCR+CD8+ T cells with or without rapamycin (mass tag 126, 127N, 127C for control and 128N, 128C and 129N for rapamycin); naïve CD8+ cells (mass tag 128, 128C, 129N, 129C, 130C and 131C); TCR-activated CD8+ cells with or without rapamycin (mass tag 126, 127C, 128C for control and 128N, 128C and 129N for rapamycin); and naïve T cells with or without rapamycin (mass tag 126, 127C, 128C for control and 129N, 130C and 131C for rapamycin). After labeling, samples were eluted into 2% DMSO in water, combined and dried in vacuo.

Peptide fractionation. The TMT samples were fractionated using off-line high-pH reverse-phase chromatography: samples were loaded onto a 4.6 × 250 mm XbridgeTM BEH130 C18 column with 3.5 μm particles (Waters). Using a Dionex BioRS system, the samples were separated using a 25-min multistep gradient of solvents A (10 mM formate at pH 9 in 2% acetonitrile) and B (10 mM ammonium formate at pH 8 in 80% acetonitrile), at a flow rate of 1 ml min−1. The mobile phases were: 2% acetonitrile, incorporating 0.1% formic acid (solvent A) and 80% acetonitrile incorporating 0.1% formic acid (solvent B). The spray was initiated by applying 2.5 kV to the
EASY-Spray emitter, and the data were acquired under the control of Xcalibur software in a data-dependent mode using the top speed and 4 s duration per cycle. The survey scan was acquired in the Orbitrap covering the m/z range from 400–1,400 Thomson units (Th), with a mass resolution of 120,000 and an automatic gain control (AGC) target of 2.0 × 10⁶ ions. The most intense ions were selected for fragmentation using collision-induced dissociation in the ion trap with 30% collision-induced dissociation energy and an isolation window of 1.6 Th. The AGC target was set to 1.0 × 10⁶, with a maximum injection time of 70 ms and a dynamic exclusion of 80 s. During the MS3 analysis for more accurate TMT quantifications, ten fragment ions were co-isolated using synchronous precursor selection, a window of 2 Th and further fragmented using a higher-energy collisional dissociation energy of 55%. The fragments were then analyzed in the Orbitrap with a resolution of 60,000. The AGC target was set to 1.0 × 10⁶ and the maximum injection time was set to 300 ms.

Processing and analysis of proteomics data. The data were processed, searched and quantified with the MaxQuant software package (version 1.5.8.3). Proteins and peptides were identified using the UniProt mouse database (SwissProt and Trembl) and the contaminants database integrated in MaxQuant, using the Andromeda search engine47,48 with the following search parameters: carbamidomethylation of cysteine, as well as TMT modification on peptide amino termini and lysine side chains, were fixed modifications; methionine oxidation and acetylation of amino termini of proteins were variable modifications. The false discovery rate was set to 1% for positive identification at the protein and peptide-to-spectrum match level. The dataset was filtered to remove proteins categorized as ‘contaminants’, ‘reverse’ and ‘only identified by site’. Copy numbers were calculated as described18 after allocating the summed MS1 intensities to the different experimental conditions according to their fractional MS3 reporter intensities. The accuracy of quantitation was established using the following guidelines: proteins categorized as high accuracy had more than eight unique and razor peptides and a ratio for unique/unique + razor of ≥0.75; proteins categorized as medium accuracy had at least three unique and razor peptides, and a ratio for unique/unique + razor of ≥0.5; and any proteins below these thresholds were classified as low accuracy.

Statistics and calculations. P values were calculated via a two-tailed, unequal-variance t-test on log-normalized data. Elements with P values < 0.05 were considered significant. Fold-change thresholds were established using two methods. Method 1 established a fold-change cut-off > 1.5 or < 0.67. Method 2 used the standard deviation of the log,[fold change]. A cut-off was then set as two standard deviations from the mean log,[fold change], allowing us to focus on those proteins showing the greatest change in expression. The mass of individual proteins was estimated using the following formula: CN × MW/Nₐ = protein mass (g cell⁻¹), where CN is the protein copy number, MW is the protein molecular weight (in Da) and Nₐ is Avogadro’s Constant. Heat maps were generated using the Morpheus tool from the Broad Institute (https://software.broadinstitute.org/morpheus). Proteins included in heat maps had a copy number of at least 1,000 copies per cell in at least one population, and were found in at least one CD4⁺ and CD8⁺ T cell population. Heat maps were arranged with T-bet positioned at the top and with all proteins ranked according to similarity in expression using nearest-neighbor analysis and Pearson correlation. CD4⁺ and CD8⁺ T cell heat maps were aligned in the same order to enable side-by-side comparison. Proteomics data were uploaded to the EPD (www.pepttracker.com/epd), allowing public interrogation of the full dataset.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

- Proteomics data were acquired on a Orbitrap Fusion Tribrid mass spectrometer running Xcalibur software (version 4.1) (Thermo Scientific).
- Flow cytometry data were acquired on a LSR Fortessa II with DIVA software (version 8.0.1) or a FACSVerse flow cytometer with FACSuite software (version 1.0.5.3841) (BD Biosciences).

Data analysis

- Mass spec data was analysed using the MaxQuant software package version 1.5.8.3.
- Statistical tests were performed using Microsoft Excel version 16.16.6.
- Heat maps were generated using the Morpheus tool from the Broad Institute (https://software.broadinstitute.org/morpheus).
- All flow cytometry analysis performed using Flowio software V9 (TreeStar).

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All proteomics data is available for interrogation using the Encyclopedia of Proteome Dynamics (EPD – https://peptracker.com/epd). Analysed proteomics data used to generate figures is available in Supplementary Files 1-5. Raw mass spec data files and MaxQuant analysis files are available on the ProteomeXchange data repository (http://proteomecentral.proteomexchange.org/cgi/GetDataset) and can be accessed with identifier PXD012058. Flow cytometry data that support the findings of this study are available from the corresponding author upon request.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | For all proteomics experiments at least 3 biological replicates were generated. Pilot experiments performed within the lab have revealed that 3 replicates is sufficient to perform statistical analyses and identify significant changes in protein expression using mass spectrometry. |
| Data exclusions | Data was not excluded from the analysis. |
| Replication | For proteomics experiments 6 biologically independent samples were generated for CD8+ naive cells and 3 biologically independent samples were generated for all other T cell populations. Proteomic experiments have been orthogonally validated within the lab by looking at the expression profile of known markers of T cell activation and differentiation. We have also compared our data set with publicly available data sets (where available) and other data sets within the lab. For all flow cytometry experiments at least 3 replicates were generated (from biologically independent samples) producing similar results. |
| Randomization | Randomization was not possible for this study. Where cells were treated with rapamycin (mTORC1 inhibition), control and inhibitor treated cells came from the same starting pool of cells. |
| Blinding | Blinding was not performed for this study. T cell populations were analyzed at defined stages in activation and differentiation and blinding the samples would have been challenging. The proteomics approach is unbiased, and statistics were performed (as detailed in the text) to identify significant changes in protein abundance. |

Reporting for specific materials, systems and methods

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| □ □ Human research participants | |
| □ □ Clinical data | |

Antibodies

Antibodies used

FC receptors were blocked using FC Block (BD Pharmingen Cat # 553141) at 1 ug /million cells. The following antibodies were used for flow cytometry:

- TCRB PerCP Cy 5.5 (clone H57-597, eBioscience, Cat # 45-5961-82, dilution 1:200)
- CD8 PE (clone S3-6.7, BD Pharmingen, Cat # 553032, dilution 1:200)
- CD44 APC (clone IM7, BD Pharmingen, Cat # 559250, dilution 1:200)
- CD62L FITC (clone MEL-14, BioLegend, Cat # 104406, dilution 1:200)
- CD4 PeCy7 (clone RM4-5, BD Pharmingen, Cat # 552775, dilution 1:200)
- Va2 PE (clone B20.1, BD Pharmingen, Cat # 553289, dilution 1:200)
- CD8 FITC (clone S3-6.7, BioLegend, Cat # 100705, dilution 1:200)
- CD4 PerCP Cy 5.5 (clone GK1.5, BioLegend, Cat # 100434, dilution 1:200)
- CD4 PE (clone RM4-5, BioLegend, Cat # 100512, dilution 1:200)
- CD8 PeCy7 (clone S3-6.7, BioLegend, Cat # 100722, dilution 1:200)
- CD45.1 PerCP Cy 5.5 (clone A20, BioLegend, Cat # 110728, dilution 1:200)
- CD45.2 FITC (clone 104, BioLegend, Cat # 109806, dilution 1:200)

The following antibody was used for T cell depletion:

- TCR β Biotin (clone H57-597, BD Pharmingen, Cat # 553169, dilution 1:100)
The following antibodies were used for T cell activation:
- **CD3e Monoclonal Antibody** (clone 145-2C11, BioLegend, Cat # 100331, final concentration 2 micrograms/ml)
- **CD28 Monoclonal Antibody** (clone 37.51, ThermoFisher, Cat # 16-0281-82, final concentration 3 micrograms/ml)

### Validation

Validation of the specificity of these antibodies can be found at the manufacturers websites. In summary:

| Antibody Name | Description |
|---------------|-------------|
| TCR\(\beta\) PerCP Cy 5.5 (clone H57-597) | The H57-597 monoclonal antibody reacts with the beta chain of mouse TCR. Applications Tested: This H57-597 antibody has been tested by flow cytometric analysis of mouse splenocytes. This antibody has been cited in 37 publications on the manufacturers website. |
| CD8 PE (clone 53-6.7) | The 53-6.7 antibody monoclonal antibody specifically binds to the 38 kDa \(\alpha\) and 34 kDa \(\alpha^\prime\) chains of the CD8 differentiation antigen (Ly-2 or Lyt-2) of all mouse strains tested. This antibody has been routinely tested for flow cytometry and has been cited in 25 publications on the manufacturers website. |
| CCD44 APC (clone IM7) | The IM7 antibody specifically recognizes an epitope on both alloantigens and all isoforms of the CD44 glycoprotein (Pgp-1, Ly-24). This antibody has been routinely tested for flow cytometry and has been cited in 22 publications on the manufacturers website. |
| CCD62L FITC (clone MEL-14) | Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. This antibody has been cited in 11 publications on the manufacturers website. |
| CD4 PeCy7 (clone RM4-5) | The RM4-5 monoclonal antibody specifically binds to the CD4 (L3T4) differentiation antigen expressed on most thymocytes, subpopulations of mature T lymphocytes. This antibody has been routinely tested for flow cytometry and has 15 citations on the manufacturers website. |
| Va2 PE (clone B20.1) | The B20.1 monoclonal antibody specifically binds to most members of the Va2 T-cell Receptor (TCR) subfamily in mice having the \(a\), \(b\), and \(c\) haplotypes of the Tcrb gene complex. B20.1 antibody may crossreact with V\(\delta\) TCR, which shares >90% sequence homology with Va2 TCR. This antibody has been routinely tested for flow cytometry and has 3 citations on the manufacturers website. |
| CD8 FITC (clone S3-6.7) | Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. This antibody has been routinely tested for flow cytometry and has 47 citations on the manufacturers website. |
| CD4 PerCP Cy 5.5 (clone GK1.5) | Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. This antibody has been routinely tested for flow cytometry and has 28 citations on the manufacturers website. |
| CD4 PE (clone RM4-5) | Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. This antibody has been routinely tested for flow cytometry and has 16 citations on the manufacturers website. |
| CD8 PeCy7 (clone S3-6.7) | Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. This antibody has been routinely tested for flow cytometry and has 37 citations on the manufacturers website. |
| CD45.1 PerCP Cy 5.5 (clone A20) | Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. This antibody has been routinely tested for flow cytometry and has 12 citations on the manufacturers website. |
| CD45.2 FITC (clone 104) | Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. This antibody has been routinely tested for flow cytometry and has 33 citations on the manufacturers website. |
| TCR \(\beta\) Biotin (clone H57-597) | The H57-597 antibody reacts with a common epitope of the \(\beta\) chain of the T-cell Receptor (TCR) complex on \(\alpha\beta\) TCR-expressing thymocytes, peripheral T lymphocytes, NK1.1+ thymocytes, and NK-T cells of all mouse strains tested. This antibody has been tested for flow cytometry and immune histochemistry. |
| Anti-CD3 (clone 145-2C11) | Clone 145-2C11 is used in T cell activation assays, inducing proliferation and cytokine production. Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. This antibody has 4 citations on the manufacturers website. |
| Anti-CD28 (clone 37.51) | The 37.51 antibody has been reported for use in flow cytometric analysis. 37.51 has also been reported in costimulation of T cells in vitro and in vivo. This antibody has 106 citations on the manufacturers website. |

### Animals and other organisms

**Policy information about studies involving animals**: [ARRIVE guidelines](https://arriveguidelines.org/) recommended for reporting animal research

**Laboratory animals**

| Group | Description |
|-------|-------------|
| Naïve CD8 cells – P14 transgenic mice, 50-65 days old, male | |
| Naïve CD8 cells TCR activated for 24 hours – P14 transgenic mice, 80-120 days old, female | |
| Effector CTL – P14 mice, 60 days old, male | |
| Naïve CD4 cells – wild-type C57BL/6 mice (Jackson Laboratories), 50-65 days old, male | |
| Naïve CD4 cells TCR activated for 24 hours – OTII transgenic mice, 80-120 days old, male | |
| Effector Th1 cells – wild-type C57BL/6 mice, 80-120 days old, male | |
| Proteomics-Experiments | |
| Naïve CD8 cells – P14 transgenic mice, 50-65 days old, male, | |
| Naïve CD8 cells TCR activated for 24 hours – P14 transgenic mice, 80-120 days old, female | |
Wild animals
This study did not involve wild animals.

Field-collected samples
This study did not involve samples collected from the field.

Ethics oversight
Mice were maintained in the Biological Resource Unit at the University of Dundee using procedures that were approved by the University Ethical Review Committee and under the authorisation of the UK Home Office Animals (Scientific Procedures) Act 1986.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots
Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
For flow cytometry based cell sorting lymph nodes (LN) were removed and disaggregated. FC receptors were blocked using 1 μg FC block (BD Phamigen) per million cells. Cells were stained and washed in PBS supplemented with 1% FCS. For cell sorting, naive CD8 cells were stained with the following fluorophore conjugated antibodies; TCRβ PerCP Cy 5.5, CD8 PE, CD44 APC, CD62L FITC and DAPI and sorted on an Influx cell sorter (Becton Dickinson). Naive CD8 cells were collected (CD8 positive, CD44 low and CD62L high). For naive CD4 cells, lymph node cells were isolated and processed as above but stained with the following antibodies; TCRβ PerCP Cy 5.5, CD4 PeCy7, CD8 PE, CD44 APC, CD62L FITC and DAPI. Naive CD4 cells were collected (CD4 positive, CD44 low and CD62L high). TCR activated CD8 cells were stained with CD8 PE and DAPI, while TCR activated CD4 cells were stained with CD4 PerCP Cy 5.5, Vα2 PE and DAPI.

For flow cytometry based DNA synthesis assay, cells were fed 10 uM Click-iT EdU (Thermo Fisher) for 30 mins. Cells were then harvested, stained with CD8 FITC (for TCR activated cells), fixed with 1 % paraformaldehyde and permeabilised with 0.5 % triton X 100 before undergoing a copper catalysed click chemistry reaction with Alexa 647-azide (Thermo Fisher). Cells were analysed by flow cytometry to determine the degree of incorporation of EdU.

Monitoring System L amino acid transport
Wild-type (C57BL/6J Ly5.1, CD45.1) and CD4Cre Slc7a5fl/fl (CD45.2) ex vivo lymph node cells were mixed. Surface antibody staining for CD45.1 (PerCP Cy 5.5), CD45.2 (FITC), CD4 (PE) and CD8 (PeCy7) was performed (15mins, at 37 °C). After surface antibody staining, cells were washed in pre-warmed HBSS, and resuspended in 200 μl warmed HBSS (approx. 2.5 × 106 cells in FACS tubes). Cells were kept in a water bath at 37 °C. 100 μl of HBSS or the System L transport inhibitor BCH (2-amino-2-norbornanecarboxylic acid, Sigma) was added to appropriate samples. Finally, 100 μl pre-warmed kynurenine (stock 800 μM freshly made in HBSS; final concentration 200 μM; final sample volume 400 μl) was added. To monitor kynurenine uptake in live cells, data was acquired on flow cytomer immediately following addition of kynurenine and fluorescence plotted against time. The 405 nm laser and 450/50 BP filter were used for kynurenine fluorescence detection. To calculate the ratio of System L uptake, kynurenine uptake was fixed after 5 mins by adding 125 μl 4% PFA for 30 min at room temperature, in the dark. Flow cytometry data was acquired on either a LSR Fortessa II with DIVA software or a FACSVerse flow cytometer with FACSuite software (BD Biosciences) and analysed using FlowJo software v9 (TreeStar) and following the gating strategy as shown in Supplementary Figures.

Instrument
Flow cytometry data were acquired on a LSR Fortessa II with DIVA software or a FACSVerse flow cytometer with FACSuite software (BD Biosciences).

Software
Flow cytometry data were acquired on a LSR Fortessa II with DIVA software (version 8.0.1) or a FACSVerse flow cytometer with FACSuite software (version 1.0.5.3841) (BD Biosciences). All flow cytometry analysis performed using FlowJo software V9 (TreeStar).

Cell population abundance
Between 2 and 5 million cells were sorted for proteomics experiments with a purity greater than 95% according to a post-sort purity check.

Gating strategy
The gating strategy for all flow cytometry experiments is provided as supplementary figures.
Gating for flow cytometry sorted cells:
All cells were gated for singlets and live cells (DAPI negative) in addition to markers below.
Naive CD8 cells were gated for TCRβ positive, CD8 positive, CD44 low and CD62L high.
Naive CD4 cells were gated for TCRβ positive, CD4 positive, CD44 low and CD62L high.
TCR activated CD8 cells were gated for CD8 positive.
TCR activated CD4 cells were gated for CD4 positive and Vα2 positive.

Gating for DNA synthesis assay:
TCR activated CD8 cells were gated for singlets and CD8 positive cells.
CTL were gated for singlets.

Gating for system L amino acid transport assay
Cells were gated for singlets and then CD45.1 (WT) and CD45.2 (SLC7A5 KO) positive cells. Cells were then gated for CD4 positive or CD8 positive populations.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.