IMMUNOLOGY

Interleukin-2 shapes the cytotoxic T cell proteome and immune environment–sensing programs

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Interleukin-2 (IL-2) and Janus kinases (JAKs) regulate transcriptional programs and protein synthesis to promote the differentiation of effector CD8⁺ cytotoxic T lymphocytes (CTLs). Using high-resolution mass spectrometry, we generated an in-depth characterization of how IL-2 and JAKs configure the CTL proteome to control CTL function. We found that IL-2 signaling through JAK1 and JAK3 (JAK1/3) increased the abundance of a key subset of proteins to induce the accumulation of critical cytokines and effector molecules in T cells. Moreover, IL-2 maintained the concentration of proteins that support core metabolic processes essential for cellular fitness. One fundamental insight was the dominant role for IL-2 in stimulating effector T cells to detect microenvironmental cues. IL-2–JAK1/3 signaling pathways thus increased the abundance of nutrient transporters, nutrient sensors, and critical oxygen-sensing molecules. These data provide key insights into how IL-2 promotes T cell function and highlight signaling mechanisms and transcription factors that integrate oxygen sensing to transcriptional control of CD8⁺ T cell differentiation.

INTRODUCTION

Interleukin-2 (IL-2) is a member of the γ_c cytokine family, which activates receptors containing the common γ_c subunit. IL-2 has numerous roles in orchestrating immune responses, including stimulating the proliferation and differentiation of CD4⁺ and CD8⁺ effector T cells (1-5). This vital role in controlling T cell fate has made manipulation of IL-2 signaling an attractive aim for immunotherapies. Hence, IL-2 was one of the first cytokines used in immunotherapy to increase T cell responses. IL-2 is also used to expand tumor-specific T cells and chimeric antigen receptor-redirected T cells ex vivo before adoptive transfer into patients (6, 7). IL-2 signals through the tyrosine kinases JAK1 and JAK3; hence, inhibitors of both JAK1 and 3 (JAK1/3), such as tofacitinib, have been developed to modulate IL-2 immunoregulatory pathways to treat autoimmune and inflammatory conditions. Moreover, the pleiotropic role of IL-2 in promoting both proinflammatory effector T cell responses and the anti-inflammatory homeostasis of regulatory T cells has stimulated the development of strategies, using modified IL-2 proteins with altered receptor binding (8) and antibodies that target this cytokine (4, 9), to direct IL-2 activity toward specific T cell subsets to manipulate IL-2 signaling responses for therapies.

In terms of CD8⁺ cytotoxic T lymphocytes (CTLs), IL-2 stimulates T cell growth and T cell clonal expansion (6, 10, 11). Thus, IL-2 stimulates transcriptional programs that are required for cell cycle progression and proliferation. IL-2 also stimulates the production of interferon- γ (IFN- γ) and the effector molecules perforin and granzyme and directs the repertoire of adhesion molecules and chemokine receptors present on the plasma membrane of the CTL to promote trafficking to peripheral tissues. The outcome of these regulatory events is that IL-2 directs the differentiation of effector CTLs at the expense of the development of memory CD8⁺ T cells (12–15).

To induce this differentiation, IL-2 activates signal transducer and activator of transcription 5 (STAT5) (3, 16-18) and MYC (19) tran-

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scriptional programs. In addition, IL-2-stimulated JAK1/3 activates serine and threonine kinase signaling networks. For example, IL-2 activates mammalian target of rapamycin complex 1 (mTORC1)mediated signaling pathways, which promote the production of inflammatory cytokines, cytolytic effector molecules, and glucose transporters, and enhance glucose and fatty acid metabolism in CTLs (20-23). Moreover, the IL-2-JAK-regulated phosphoproteome of CTLs is dominated by proteins that control mRNA stability and components of the protein translational machinery (24). Hence, a key role for IL-2 is to sustain protein synthesis in CTLs. Consequently, IL-2 is a "growth factor" for antigen-activated T cells (12, 24, 25). By controlling protein synthesis (24, 25), IL-2 can modify the proteome of CTLs independently from its regulation of gene transcription. One example of this is the ability of IL-2 to stimulate the accumulation of the transcription factor MYC: IL-2 promotes the synthesis of MYC protein without increasing the abundance of MYC mRNA (19). Furthermore, IL-2-mediated regulation of mTORC1, which can promote both mRNA translation and cellular protein degradation pathways (23), is another means by which IL-2 can alter the cellular proteome independently from changes in the cell's transcriptional programs.

Although IL-2 activates JAKs to control T cell transcriptional programs, differences in the rates of protein production—translation and synthesis—and protein degradation—controlled by protein stability and rates of protein degradation—generate discordances between the cellular transcriptome and proteome. Hence, determining which proteins are sustained in CTLs to control T cell function requires mapping of IL-2–regulated proteomes. Here, we used high-resolution quantitative mass spectrometry (MS) to analyze how IL-2 maintains the proteome of differentiated CTLs so that we could generate global and in-depth insights into how this key cytokine controls CD8⁺ T cell identity and controls cell cycle progression, metabolism, and the abundance of effector molecules.

RESULTS

IL-2 regulation of the CTL proteome

To explore the role of IL-2 on effector CD8⁺ CTL function, we differentiated lymphocytes from transgenic mice that express a knock-in T cell receptor (TCR) specific for the gp-33 peptide from lymphocytic

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choriomeningitis virus (LCMV; P14 mice) (26) into effector CD8⁺ CTLs by culturing the cells in IL-2. IL-2–maintained CTLs are large, granular cells (Fig. 1A) that depend on IL-2 for sustained proliferation

and viability. After 24 hours of IL-2 deprivation, CTLs had similar viability to the IL-2–maintained cultures (Fig. 1A) but were decreased in size, as judged by flow cytometry analysis of forward scatter (Fig. 1A).

Fig. 1. IL-2 regulation of the CTL proteome. (A) Size and viability of CTLs maintained or deprived of IL-2 as judged by flow cytometric analysis of forward scatter (FSC) and side scatter (SSC). The percentages of viable CTLs are shown alongside. (B) Protein content of II -2-maintained and II -2deprived CTLs. (C) The Venn diagram shows the commonality of proteins identified in the proteomic analysis, and the volcano plot shows the ratio of the protein copy numbers in CTLs deprived of IL-2 for 24 hours compared to those in IL-2-maintained CTLs. Proteins that exhibited statistically significant changes in abundance (P < 0.05 by one-sample Student's t test) by greater than 1.5-fold are shown in red. A twofold decrease in protein abundance is indicated by the vertical dashed line. (D) Average protein mass frequency per cell. Bins containing the proteins that make up ~50% of the total CTL mass are shown in black. (E) Volcano plot as in (C) with surface receptors shown in red. (F) Molecule numbers, estimated from the proteomic data, for the abundance of IL-2Rα. (G) Flow cytometric analysis of the cell surface abundance of IL-2Rα. The graph alongside shows the geometric mean fluorescence intensity (GeoMFI) for three biological replicates. (H to J) Copy numbers estimated from the proteomic data. (K) The ratio of p27 to the total sum of cyclin D2 and cyclin D3 molecules was calculated and plotted. (L) Schematic representation of selected cell cycle proteins and DNA synthesis and repair enzymes regulated by IL-2. Data are, or



are representative of, three biological replicates. In bar charts, data points from biological replicates are color-matched; the bar shows the mean, and the errors bars show the SD. *P* values were calculated by two-tailed, one-sample Student's *t* test performed on the ratios of no IL-2 (IL-2–deprived)/IL-2 (IL-2–maintained).

After 48 hours of IL-2 deprivation, cell viability substantially decreased. The decrease in the size of IL-2–deprived CTLs observed by flow cytometry at 24 hours (Fig. 1A) correlated with a reduction in CTL mass (Fig. 1B).

To understand how IL-2 shapes the CTL phenotype, we used quantitative label-free high-resolution MS to compare the proteome of CTLs maintained in IL-2 and CTLs deprived of IL-2 for 24 hours (fig. S1A). Identified peptides were matched to a single protein or multiple proteins, termed protein groups, using MaxQuant software (*27*). We identified more than 6200 protein groups per biological replicate (fig. S1, B and C, and table S1), and there was >90% overlap in the proteins identified among the three biological replicates (fig. S1, B and C). We converted our label-free data into protein copy numbers per cell using "histone-ruler" methodology (*28*). The correlation between estimated copy numbers of proteins in different biological replicates of each condition was good; the lowest r^2 value was 0.89 (fig. S1, D and E).

Because IL-2–deprived CTLs decreased in both size and mass (Fig. 1, A and B) compared to IL-2–maintained CTLs, a key question was whether IL-2 removal resulted in an equal and uniform reduction in all proteins in CTLs. In total, ~6500 proteins were identified in both IL-2–maintained and IL-2–deprived CTLs (Fig. 1C), with protein abundance spanning several orders of magnitude. About 3000 proteins were not substantially affected by IL-2 deprivation. The unaffected proteome included cytoskeletal and structural proteins— β -actin (ACTB), one of the most highly abundant proteins in CTLs, vimentin, and laminin b—and signaling molecules—the phosphotyrosine phosphatase (GTPase) RAP1B (table S1). Furthermore, the abundances of a few proteins were statistically significantly increased in response to IL-2 deprivation (Fig. 1C). Thus, the loss of IL-2 signals did not result in a universal and uniform loss in protein.

About 100 proteins, including histones, cytoskeletal proteins, ribosomal proteins, glycolytic enzymes, and the cytolytic effector molecules granzyme A and granzyme B, contributed to ~50% of the mass of IL-2-maintained CTLs (Fig. 1D). IL-2 could control CTL biomass by regulating only the amount of these abundant proteins. However, the overall reduction in the mass of CTLs deprived of IL-2 was between 1.5- and 2-fold (Fig. 1B). Although this decrease included a reduction in some of the abundant proteins, the effect of IL-2 deprivation on the CTL proteome was more complex. We found ~900 proteins that decreased proportionally to the overall loss in mass, indicative of some general cell scaling down of protein content. However, most IL-2-regulated proteins were changed in abundance by more than twofold (Fig. 1C and table S2). A subset of CTL proteins was also highly sensitive to IL-2 deprivation: ~140 proteins became undetectable (table S2), and other proteins changed >20-fold in abundance. These results indicate that the effect of IL-2 on CTL cell mass is not just a simple reduction and scaling down in overall cellular protein content, but a complex reshaping of the proteome.

Dominant effects of IL-2 on the CTL proteome

To understand the dominant consequences of IL-2 deprivation on the CTL proteome, we focused on the proteins that were most decreased in abundance by IL-2 deprivation. About 140 proteins were only reproducibly identified in IL-2–maintained CTLs: These proteins included the effector cytokines lymphotoxin α (LT- α) and IFN- γ (table S2). The cytokine receptor protein TNFRSF9 (also known as CD137 or 4-1BB) was one of the proteins that decreased the most in abundance in CTLs deprived of IL-2. We detected ~300,000 mole-

cules per cell in IL-2–maintained CTLs and ~2000 molecules per cell in IL-2–deprived CTLs (table S2). Other examples of cell surface receptors markedly decreased in abundance by the lack of IL-2 include IL-4R, IL-12 β 1, and the inhibitory receptor Tim-3 (also known as HAVCR2) (Fig. 1E). The abundance of IL-2R α (also known as CD25) subunit is controlled by IL-2 (29), and we identified a two- to fivefold reduction in the amount of IL-2R α after IL-2 deprivation of CTLs (Fig. 1F). This decrease was consistent with the observed changes to IL-2R α , as determined by flow cytometry analysis of the CTLs before proteomic analysis (Fig. 1G). We also noted that IL-2R β and IL-2R γ , the other components of the IL-2R, were decreased in abundance in IL-2–deprived CTLs (Fig. 1E). Together, the proteomic data highlight the key role for IL-2 in sustaining the abundance of proteins that enable CTLs to respond to external stimuli, including cytokines and inhibitory proteins.

IL-2 regulation of the cell cycle machinery

A quintessential biological function of IL-2 is to control the clonal expansion of antigen receptor-activated T cells. The commitment of T cells to DNA synthesis depends on the activity of cyclin D-cyclindependent kinase 2 (CDK2) complexes or cyclin D-CDK4 complexes. We quantified the effect of IL-2 deprivation on the numbers and stoichiometry of the key cell cycle regulators to understand how IL-2 regulates T cell proliferation. We found that IL-2 increased the abundance of cyclin D2 (Fig. 1H) and cyclin D3 (Fig. 1I). Conversely, IL-2 reduced the abundance of the CDK inhibitor CDKN1B (also known as p27 or p27kip) (Fig. 1J). IL-2-deprived CTLs showed increased abundance of CDKN1B molecules relative to the numbers of cyclin D2 and D3 molecules (Fig. 1K). This would result in decreased abundance of active cyclin D-CDK complexes and would be consistent with inhibition of progression through the G₁ to S phase of the cell cycle. We also found that IL-2 deprivation caused a decrease in the abundance of essential components of the DNA replication machinery (Fig. 1L), which would limit the ability of CTLs to perform DNA synthesis. IL-2 also sustained the abundance of DNA damage repair proteins (Fig. 1L). Because cyclin D-CDK4 and cyclin D-CDK6 complexes stimulate the activity of the transcription factor E2F, which then stimulates the expression of genes encoding DNA replication proteins (30), the reduction in core DNA synthesis machinery may result from loss of cyclin D-CDK4/6 activity. Thus, our proteomic data indicate that there are multiple mechanisms by which loss of IL-2 signals limits DNA synthesis and repair in effector T cells, enabling IL-2 to control cell cycle progression and the proliferation of CTLs.

IL-2 regulation of the CTL cytolytic machinery

The key cytolytic effector molecules of CTLs are perforin, granzymes, and Fas ligand (FasL or CD95L), all of which are stored in granules. Granules are specialized secretory lysosomes, which, like other lysosomes, are positive for the lysosomal proteins LAMP-1 (also known as CD107a) and LAMP-2 (*31*). IL-2 did not substantially affect LAMP-1 and LAMP-2 abundance in CTLs (Fig. 2, A and B). However, cells deprived of IL-2 exhibited a reduction in the abundance of the cytolytic granule cargo molecules perforin, granzyme A, and granzyme B (Fig. 2, C to E). Cells maintained in IL-2 had an increased abundance of proteins required for cytolytic granule fusion with the plasma membrane, such as the GTPase RAB27A, STXBP2 (MUNC18-2), and UNC13D (MUNC13-4) (Fig. 2, F to H). These data indicate that IL-2 may regulate the protein content and release of lytic granules.

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Fig. 2. IL-2 regulation of CTL cytolytic machinery. (**A** to **H**) Copy numbers estimated from the proteomic data for LAMP-1 (A), LAMP-2 (B), perforin (C), granzyme A (D), granzyme B (E), RAB27A (F), STXBP2 (G), and UNC13D (H). In graphs, the three biological replicates are color-matched; the bar shows the mean, and the error bars show the SD. *P* values were calculated by two-tailed, one-sample Student's *t* test performed on the ratios of no IL-2/IL-2. N.S., not significant.

IL-2 control of transcriptional regulators and core protein synthesis machinery

Another key function of IL-2 is to regulate and maintain T cell transcriptional programs, in part through the activation of STAT5. Our data indicate that IL-2 may influence gene transcription in both general and specific ways in CTLs by increasing the abundance of multiple transcription factors and proteins involved in transcription and RNA processing, including some components of the general transcriptional machinery, such as TAF1, TAF8, and TCEB1 (Fig. 3A). Moreover, histone and DNA methylases, DNA and RNA helicases, and some RNA-processing proteins (Fig. 3A), with the exception of proteins involved in methylation of the mRNA cap (Fig. 3A), were all statistically significantly decreased in abundance in CTLs deprived of IL-2. These decreases suggested that the loss of IL-2 signaling would generally reduce gene expression. However, some of the transcription factors reduced in IL-2-deprived cells are gene-specific transcription factors (Fig. 3A and table S2). These included MAFK, NFIL3, the AP-1 family proteins (JUN, JUNB, JUND, and FOSL2), eomesodermin (EOMES), IRF8, and TCF25 (Fig. 3, A and B). However, the abundance of two transcription factors, FOXO1 and TBET (Fig. 3C), which are both important for the maintenance of T cell identity (32, 33), was not changed by the loss of IL-2 signals. These data highlight that the ability of IL-2 to control gene transcription and the production and stability of mRNAs in T cells goes beyond stimulation of STAT5 phosphorylation and activity.

The phenotype of a cell is controlled by its transcriptional program; however, gene expression cannot be executed without protein synthesis. IL-2 has a key role in promoting the rate of protein synthesis in CD8⁺ T cells (24, 25). Our proteomic analysis revealed that IL-2 influenced protein synthesis on many levels. IL-2 increased the abundance of ribosomal proteins (Fig. 3D) such that CTLs maintained in IL-2 contained $\sim 2.8 \times 10^6$ ribosomes, whereas CTLs deprived of IL-2 only had $\sim 1.2 \times 10^6$ ribosomes (Fig. 3D). IL-2 also controlled the abundance of transfer RNA (tRNA) synthases, including the methionyltRNA synthase, and sustained the abundance of critical components of the EIF translational initiation complex (Fig. 3E). IL-2 also maintained the abundance of proteins that enable the transport of newly synthesized proteins into the endoplasmic reticulum (ER) and the abundance of proteins involved in protein folding and protein degradation in both the ER lumen and the cytosol (Fig. 3E). These data suggest that IL-2 coordinates the abundance of ribosomes, translation complexes, and protein quality control mechanisms.

IL-2 regulation of amino acid transporters and amino acid sensors

A supply of amino acids is required for cells to sustain high rates of protein synthesis. System L transporters are hetero-

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dimeric, sodium-independent amino acid transporters whose substrates include large neutral amino acids, such as leucine, tryptophan, and methionine (34). IL-2 sustains system L-mediated amino acid transport in CTLs (35). CD8⁺ T cells lacking the system L amino acid transporter SLC7A5 fail to differentiate into CTLs (35). Our proteomic data identified SLC7A5 as the most abundant amino acid transporter in CTLs (with ~170,000 copies per cell) and determined that its abundance depended on IL-2 (Fig. 4A). We quantified about six other amino acid transporters in CTLs, including an arginine transporter (SLC7A1) (Fig. 4B) and a transporter for glutamine and serine (SLC1A5) (Fig. 4C). The abundance of all seven amino acid transporters was maintained by IL-2 (Fig. 4D). IL-2 also increased the abundance of proteins required for amino acid biosynthesis (Fig. 4E) and proteins involved in the recovery of amino acids by autophagy (Fig. 4, F to I). These results indicate that IL-2 maintains the supply of amino acids to CTLs through multiple mechanisms.

In addition to amino acids being vital for the protein biosynthetic capacity of T cells, specific amino acids are also necessary for other essential metabolic processes. For example, serine fuels the biosynthesis of the purine nucleotides needed for T cell proliferation (*36*); glutamine fuels protein O-GlcNAcylation in T cells (*37*); and arginine promotes oxidative phosphorylation over glycolysis and increases the survival of T cells (*38*) and, together with leucine, stimulates the activity of mTORC1. Differentiation and maintenance of the cellular mass of CD8⁺ T cells depend on mTORC1 (*20–22*). Our data suggest that

Fig. 3. IL-2 regulation of gene expression. (A) Schematic rep-

resentation of selected transcription factors, DNA-modifying enzymes, and mRNA-binding proteins in CTLs identified by MS. (B and C) Copy numbers estimated from the proteomic data for eomesodermin (EOMES), IRF8, TC25, TBET (TBX21), and FOXO1. (D) The number of ribosomes per cell was estimated by calculating the median copy number of all the proteins annotated with the Kvoto Encvclopedia of Genes and Genomes (KEGG) pathway "ribosome" for each biological replicate and each condition and was plotted in a bar chart. (E) Schematic representation of selected proteins involved in ribosomal biogenesis, translation, and protein processing in the ER. In graphs, individual data points from the three biological replicates performed for the proteomic analysis are shown and colormatched, with the bar showing the mean and the errors bars showing the SD of the experiments. P values were calculated by two-tailed, one-sample Student's t tests performed on the ratios of no IL-2/IL-2.



Fig. 4. IL-2 regulation of amino acid uptake and biosynthesis and mTORC1.

(A to C) Copy numbers estimated from the proteomic data for the amino acid transporters SLC7A5 (A), SLC7A1 (B), and SLC1A5 (C). (D and E) Volcano plots of the IL-2dependent proteome: ratio of the protein abundance (copy number) in IL-2-deprived CTLs compared to that in IL-2-maintained CTLs was plotted against P value (as determined by one-sample Student's t test). Proteins with ratios that were statistically significantly regulated (P < 0.05) by >1.5-fold are shown in dark gray, with amino acid transporters highlighted in red in (D) and proteins involved in amino acid biosynthesis in (E). (F to I) Bar graphs show estimated copy numbers calculated using the proteomic data for VPS34(F), VPS15 (G), ATG4B (H), and ATG5 (I). (J) Schematic representation of proteins involved in the activation of mTORC1 signaling. Proteins decreased in abundance by the loss of IL-2 are highlighted in purple. (K and L) Estimated copy numbers per cell for RHEB (K) and SESTRIN2 (L). In graphs (A) to (C), (F) to (I), and (K) and (L), individual data points from the three biological replicates performed for the proteomic analysis are shown and color-matched, with the bar showing the mean and the errors bars showing the SD of the experiments. P values were calculated by two-tailed, one-sample Student's t test performed on the ratios of no IL-2/IL-2.



one mechanism by which IL-2 can sustain high mTORC1 activity in effector CTLs is by maintaining the supply of leucine and arginine. Another mechanism suggested by the data is that IL-2 increases the abundance of proteins that regulate mTORC1 activity (Fig. 4, J to L) (39). Although some of the proteins that IL-2 sustained are parts of negative regulatory complexes, others are positive regulators of mTORC1 (Fig. 4J). For example, IL-2 maintained the abundance of the GTPase

RHEB (Fig. 4K) and the cytosolic leucine sensor SESTRIN2 (Fig. 4L).

IL-2 control of glucose metabolism and oxygen sensing

CTLs have high rates of glucose transport, exhibit a high rate of glycolysis, and have a high rate of oxidative phosphorylation. These metabolic programs are required for effector function (23). In our data set, we found that proteins mapped to oxidative phosphorylation comprised ~1% of the IL-2-maintained CTL proteome by mass (Fig. 5A); proteins of the tricarboxylic acid (TCA) cycle were ~0.8% of the CTL proteome (Fig. 5B); and glycolytic enzymes were ~9% of the CTL proteome (Fig. 5C). IL-2 deprivation reduced the amount of proteins involved in glucose metabolism, but they retained a similar relative abundance within the cell (Fig. 5, A to C). The rate-limiting step for glucose metabolism in CTLs is glucose transport, and our data showed that IL-2 maintains the abundance of the glucose transporters GLUT1 (also known as SLC2A1) and GLUT3 (also known as SLC2A3) in CTLs (Fig. 5, D and E). This IL-2-dependent increase in glucose transporters had functional consequences: IL-2-deprived CTLs had reduced glucose uptake compared to that of CTLs maintained in IL-2 (Fig. 5F).

The expression of genes encoding glucose transporters in CTLs is controlled by the hypoxia-inducible factor 1 transcriptional complex comprising of HIF1a and HIF1B (21). IL-2 deprivation caused a decrease in the abundances of HIF1B (Fig. 5G) and HIF1 α , which we measured by Western blotting because HIF1α was not detected by MS (Fig. 5H) (21). We previously described HIF1 target genes in CTLs (21). Here, we showed that, in addition to a reduction in the abundance of GLUT1 and GLUT3, other proteins that are encoded by genes stimulated by HIF1, such as perforin (Fig. 2C), TIM-3 (also known as HAVCR2) (Fig. 5I), and NFIL3 (Fig. 5J), were decreased upon IL-2 deprivation. In addition, we previously established that HIF1 complexes repress the

 2.0×10^{4}

 1.0×10^{4}

0

expression of the gene encoding the cell adhesion molecule CD62L (L-selectin, SELL) (21). The increase in the abundance of CD62L (Fig. 5K) in IL-2-deprived CTLs was thus consistent with a reduction in the HIF1 transcriptional program.

In CTLs, the abundance of HIF1 α is increased by the activity of the mTORC1 pathway (21). In the presence of high oxygen, the oxygen-dependent proline hydroxylase (PHD) proteins hydroxylate



and IL-2-deprived CTLs. Estimated mass per protein was determined using estimated copy numbers calculated from the proteomic data using the histone ruler method. (D and E) Bar charts show estimated protein copy numbers per cell for GLUT1 (SLC2A1) (D) and GLUT3 (SLC2A3) (E). (F) Glucose uptake in IL-2-maintained and IL-2-deprived CTLs was measured using radiolabeled 2-deoxyglucose. Data were normalized to glucose uptake in IL-2-maintained CTLs. (G) Bar charts show estimated protein copy numbers

HOH? per cell for HIF1β. (H) HIF1α abundance in CTLs in the presence and absence of IL-2 was measured by Western blotting. Quantification of HIF1 α detected by Western blot is shown alongside with data normalized to SMC1 intensity and HIF1 a protein abundance in IL-2-maintained CTLs. (I to L) Bar charts show estimated protein copy numbers per cell for TIM-3 (I), NFIL3 (J), L-selectin (CD62L) (K), and PHD2 (L). In bar charts, individual data points from the three biological replicates are shown and color-matched, with the bar showing the mean and the errors bars showing the SD of the experiments. P values were calculated by two-tailed, one-sample Student's t test performed on the ratios of no IL-2/IL-2. prolines within HIF1 α , targeting HIF1 α for ubiquitylation by the ubiquitin ligase VHL. This limits the abundance of HIF1 α in CTLs when environmental oxygen is high but enables a rapid increase in HIF1 α abundance in response to a reduced amount of oxygen in the environment. The oxygen-sensing pathways mediated by PHD proteins and VHL are important for restraining CD8⁺ T cell immune responses (40). We identified PHD2 as the dominant PHD in CTLs and did not detect PHD1 or PHD3. The abundance of PHD2 was maintained by IL-2 (Fig. 5L), highlighting IL-2 as a critical regulator of oxygen-sensing pathways in CTLs.

IL-2 control of the CTL proteome through JAKs

IL-2-induced activation of JAK1 and JAK3 is essential for IL-2 signal transduction. Consequently, we predicted that pharmacological inhibition of JAKs would mimic the effect of IL-2 deprivation and similarly reshape the CTL proteome. We used MS to compare the proteomes of CTLs maintained in IL-2 alone or in the presence of the JAK1/3 inhibitor tofacitinib, which is now used clinically to limit JAK1/3 activity in autoimmune diseases (41). We treated CTLs with 100 nM tofacitinib, which is within the range of tofacitinib concentrations found in the plasma in patients (42-45). Consistent with our previous work (24), 100 nM tofacitinib decreased the tyrosine phosphorylation of STAT5 in CTLs (Fig. 6A), prevented T cell cycle progression (Fig. 6B), and decreased the size (Fig. 6C) and protein content of CTLs (Fig. 6D), indicating suppression of protein synthesis. CTLs exposed to 100 nM tofacitinib were viable after 24 hours; higher concentrations of the drug caused losses in cell viability (fig. S2A). Using MS, we identified ~7000 protein groups in the IL-2-maintained control and tofacitinibtreated CTLs (fig. S2, B and C, and table S3). In each of these data sets, there was good correlation and >87% overlap in the identified proteins between the replicates for each condition (fig. S2, B to E).

Inhibition of JAK1/3 affected the composition of the CTL proteome (Fig. 6E). Tofacitinib decreased the abundance of the IL-2R α subunit (Fig. 6, F and G) in a response that was very similar to that observed in cells deprived of IL-2 (fig. S2F and table S4). There were some quantitative differences in the magnitude of protein regulation between tofacitinib treatment and IL-2 deprivation, with IL-2 deprivation tending to produce a stronger reduction in abundance (fig. S2, F to J, and table S4). Treatment of IL-2-maintained CTLs with 100 nM tofacitinib decreased the protein content of CTLs by ~25% (Fig. 6D), whereas IL-2 deprivation caused a ~40% reduction in protein mass (Fig. 1B). However, despite these quantitative differences, inhibition of JAK1/3 with tofacitinib induced changes to the CTL proteome that were qualitatively comparable to those we observed in response to IL-2 deprivation. For example, JAK1/3 inhibition resulted in a loss of the cell surface proteins IL-4Ra, IL-12RB1, and TNFRSF9 (also known as 4-1BB and CD137) (Fig. 6E and fig. S2F). The abundance of key cell cycle regulatory proteins and DNA synthesis machinery was also reduced by JAK1/3 inhibition (Fig. 7A and fig. S2G), with the exception of p27, which increased in abundance in response to both tofacitinib (Fig. 7A) and IL-2 deprivation (Fig. 1L). Tofacitinib also replicated the effect of IL-2 deprivation on the protein synthesis machinery and decreased the abundance of ribosomal proteins and proteins required for translation (Fig. 7A and fig. S2, H and I). Inhibition of JAK1/3 mimicked the effect of IL-2 deprivation on the abundance of amino acid transporters (Fig. 7B and fig. S2J), and similar to IL-2-deprived CTLs, CTLs treated with tofacitinib decreased the abundance of the mTORC1 pathway leucine sensor SESTRIN2 (Fig. 7C and fig. S2J). Tofacitinib also decreased the abundances of the HIF1 targets GLUT1 and GLUT3 in

CTLs (Fig. 7, D and E, and fig. S2J). This correlated with tofacitinibtreated CTLs having reduced glucose uptake (Fig. 7F) and glycolysis, as determined by measurement of lactate output (Fig. 7G), as compared to IL-2–maintained CTLs. Tofacitinib-treated CTLs also decreased the abundance of other HIF targets, such as perforin (Fig. 7H and fig. S2J) and the transcription factor NFIL3 (Fig. 7I and fig. S2J). The reduction in proteins encoded by HIF1-stimulated genes indicated that JAK inhibition also reduced HIF1 α abundance. We confirmed the dependency of HIF1 α abundance on JAK activity by Western blotting analysis with selective HIF1 α antibodies (Fig. 7J). Together, these data highlight how the ability of IL-2 to shape the T cell proteome depends on JAK catalytic activity; the data also reveal the extent to which a clinically relevant inhibitor of JAK signaling modulates T cell responses to IL-2.

Identification of NFIL3 as a key effector of an IL-2-mediated oxygen-sensing pathway

A challenge with large, unbiased data sets is identifying data that give new biological insights. We applied two strategies to gain new insights. We examined the proteins that had the largest change in protein abundance in IL-2-deprived or tofacitinib-treated CTLs. We also took a targeted approach based on our previous studies that mapped signaling pathways mediated by mTORC1 and HIF1 complexes in CTLs (21, 23). By applying both filters, the transcription factor NFIL3 emerged as a target for IL-2-JAK1/3-mTORC1-HIF1 signaling. Our proteomic data indicate that NFIL3 was statistically significantly decreased in copy number (Figs. 5J and 7I) and relative abundance (Fig. 8A) in CTLs in response to IL-2 deprivation or tofacitinib. We validated these results using the orthogonal approach of Western blotting (Fig. 8B). IL-2 deprivation and tofacitinib treatment also decreased the relative amount of Nfil3 mRNA (Fig. 8C), suggesting that transcriptional regulation of NFIL3 contributes to its sustained abundance in IL-2-maintained CTLs.

NFIL3 has critical functions in natural killer (NK) cells (46), other innate lymphoid cells (ILCs) (47), and CD4⁺ T cells (48), but *Nfil3* mRNA has not been previously reported as being detected in CD8⁺ T cells. Therefore, we evaluated the abundance of NFIL3 at the protein and transcript level in naïve and antigen-activated P14 CD8⁺ T cells. Both NFIL3 protein and *Nfil3* mRNA were either undetectable or at very low abundance in naïve CD8⁺ T cells (Fig. 8, D and E) and were low, albeit reproducibly detectable, in antigen receptor–activated T cells (Fig. 8, D and E). However, when antigen-activated CD8⁺ T cells were clonally expanded in IL-2 to produce effector CTLs, *Nfil3* mRNA and protein increased and were sustained at high abundance (Fig. 8, D and E). This is consistent with the model that the induction of NFIL3 is not controlled by TCR activity but depends on IL-2– JAK1/3 signaling.

Our proteomic data indicate a role for oxygen-sensing pathways in the control of *Nfil3* expression. Transcriptional microarray analysis found that *Nfil3* mRNA was decreased in abundance in HIF1βnull CTLs (21). Thus, we evaluated *Nfil3* expression in HIF1α-null (*Hif1* α^{-1-}) CTLs. Western blotting analysis indicated that the abundance of NFIL3 in effector CTLs depended on HIF1 α and was responsive to changes in oxygen (Fig. 8F). T cells switched from 21% oxygen to 1% oxygen exhibited an increase in the abundance of HIF1 α and the expression of HIF1 target genes, including those encoding the glucose transporter GLUT1 (SLC2A1) and perforin (21). We found that NFIL3 abundance increased when wild-type, but not *Hif1* $\alpha^{-/-}$, CTLs were switched from 21% oxygen to 1% O₂ (Fig. 8F). This correlated with an





Fig. 6. The effect of tofacitinib on CTLs. (A) Western blotting analysis of STAT5A and STAT5B phosphorylation in CTLs in response to 100 nM tofacitinib (Tof). Quantification of phosphorylated STAT5A and STAT5B is shown below. **(B)** Flow cytometric analysis of cell cycle stages using 5-ethynyl-2'-deoxyuridine (EdU) incorporation into DNA. The percentages of CTLs identified as in G₁ and S are shown alongside. **(C** and **D**) Flow cytometry profiles (C) and protein content (D) of IL-2-maintained CTLs treated with or without tofacitinib for 24 hours. **(E)** The Venn diagram shows the commonality of proteins identified in the proteomic analysis, and the volcano plot shows the ratio of protein copy numbers in IL-2-maintained CTLs treated with 100 nM tofacitinib for 24 hours compared to those in IL-2-maintained CTLs plotted against *P* value (as determined by one-sample Student's *t* test). Proteins with ratios that were statistically significantly regulated (*P* < 0.05) by greater than 1.5-fold are shown in red with selected surface proteins

labeled. (**F** and **G**) IL-2R α abundance in CTLs treated with or without tofacitinib for 24 hours as measured by proteomic analysis (F) and flow cytometry (G). The graph alongside shows the normalized GeoMFI for three biological replicates. Data are, or are representative of, three biological replicates. In bar charts, data points from biological replicates are color-matched; the bar shows the mean, and the errors bars show the SD. *P* values were calculated by two-tailed, one-sample Student's *t* test performed on the ratios of IL-2 + tofacitinib/IL-2.

increase in *Nfil3* mRNA in CTLs cultured in 1% O₂ compared to that in CTLs cultured in 21% O₂ (Fig. 8G). The effect on *Nfil3* transcripts was comparable to the effect on the abundance of transcripts for GLUT1 in response to a reduction in oxygen (Fig. 8H). Together, these data show that the abundance of NFIL3 in CTLs is regulated by oxygen-sensing pathways controlled by the HIF1 transcription factor complex.

To explore the functional relevance of the regulation of NFIL3 in CTLs, we isolated CD8⁺ naïve T cells from the spleens of NFIL3-deficient (*Nfil3^{-/-}*) mice, activated the TCR, and clonally expanded the cells in IL-2. Activated *Nfil3^{-/-}* CD8⁺ T cells proliferated normally in response to IL-2 (fig. S3A). Moreover, IL-2-maintained *Nfil3^{-/-}* CD8⁺ effector T cells increased their amounts of activation markers, such as IL-2R α , CD69, and CD44 (fig. S3, B to D). They also showed normal activity of AKT and mTORC1, key regulators of CTL differentiation (fig. S3, E and F). We previously reported that HIF1 α -null effector CD8⁺ T cells fail to decrease the abundance of CD62L, a key adhesion

molecule that controls lymphocyte migration into secondary lymphoid tissue, and are negative for perforin (21). $Nfil3^{-/-}$ CD8⁺ T cells phenocopied HIF1 α -null CD8⁺ T cells, retaining high amounts of CD62L (*Sell*) mRNA and protein (Fig. 8, I and J) and failing to induce perforin (*Prf1*) mRNA and protein (Fig. 8, K and L). Together, these data identify NFIL3 as a transcription factor whose abundance in CTLs is regulated by HIF1-mediated oxygen-sensing pathways. The data also suggest that NFIL3 controls perforin abundance in T cells and has a role in the repression of CD62L in antigen- and cytokine-activated CD8⁺ T cells.

DISCUSSION

Our study provides an in-depth insight into the role of IL-2 in configuring and maintaining the proteome of differentiated CTLs. One key perspective from the proteomic analysis was the extent to which IL-2–JAK1/3 signaling shapes the ability of effector CTLs to respond



biological replicates. In bar charts, data points from biological replicates are color-matched; the bar shows the mean, and the errors bars show the SD. P values were calculated by two-tailed, one-sample Student's t test performed on the ratios of IL-2 + tofacitinib/IL-2. Fig. 8. Regulation of NFIL3 by IL-2-JAK1/3 signaling. (A) Average frequency of protein copy numbers per cell calculated from the proteomic data sets: The bin that contains NFIL3 is indicated in black. (B) Western blotting analysis of NFIL3 abundance in CTLs after IL-2 deprivation or treatment with 100 nM tofacitinib. Ouantification is shown alongside as data normalized to SMC1 intensity and NFIL3 protein abundance in IL-2-maintained CTLs. (C) Abundance of Nfil3 mRNA in CTLs. (D and E) Abundance of NFIL3 protein (D) and mRNA (E) in naïve CD8⁺ T cells, TCR-activated CD8⁺ T cells, and CTLs activated for 48 hours and then maintained in IL-2 for 1, 3, or 5 days. (F) Analysis of NFIL3 protein abundance in HIF1 α -expressing (*Hif1a*^{+/+}) and HIF1 α -deficient (*Hif1a^{-/-}*) CTLs maintained in normoxia (21% O₂) or switched into hypoxic (1% O₂) conditions for 8 hours. Quantification of NFIL3 protein is shown alongside. (G and H) Relative abundances of Nfil3 (G) and GLUT1 (Slc2a1) (H) mRNAs in CTLs maintained in normoxia or switched into $1\% O_2$ for 4 hours. (I and J) Abundances of CD62L (Sell) mRNA (I) and cell surface protein (J) in IL-2-maintained Nfil3^{+/+} and Nfil3^{-/-} CTLs. (J) Percentages of CD8⁺ CD62L^{hi} CTLs is shown alongside. (K and L) Abundances of perforin (Prf1) mRNA (K) and protein (L) in $Nfil3^{+/+}$ and Nfil3^{-/-} CTLs. (L) Quantification of NFIL3 protein abundance, normalized to SMC1 and perforin protein abundance in IL-2-maintained CTLs, as detected by Western blotting is shown alongside. In (C), (G), and (H), the quantitative polymerase chain reaction (gPCR) data were normalized to Tbp and the mRNA abundance in IL-2maintained CTLs under normoxia. In (E), gPCR data were normalized to Tbp and Cd8 and all are shown relative to day 5 IL-2maintained CTLs under normoxia. In (I) and (K), mRNA was normalized to Hprt, and data are shown relative to the abundance in Nfil3^{+/+} CTLs. Data in (A) to (E),



(G), and (H) are representative of three biological replicates. Data in (F) are representative of six $Hif1a^{+/+}$ and five $Hif1a^{-/-}$ biological replicates from four independent experiments. Data in (I) to (L) are representative of four $Nfil3^{+/+}$ and three $Nfil3^{-/-}$ biological replicates. Bar charts show data from individual biological replicates, colormatched where appropriate. The bar shows the mean, and the error bars show the SD. *P* values in (B) to (H) were calculated from two-tailed, one-sample Student's *t* tests, whereas those in (I) to (L) were calculated from two-tailed, unpaired unequal variance Student's *t* tests.

to their immune environment. IL-2 controlled the abundance of multiple cell surface molecules, including receptors for the cytokines IL-4 and IL-12, the inhibitory receptor Tim-3, and the costimulatory receptor 4-1BB. The signaling pathways controlled by these receptors can substantially modulate T cell responses. A 4-1BB agonist antibody, utomilumab, is in clinical trials to promote effector CD8⁺ antitumor T cell therapy. The recognition that the abundance of 4-1BB is increased by IL-2 is a useful insight into how therapeutic IL-2 treatments may be able to enhance the function of effector T cells.

A further insight from our data was that IL-2 maintained the abundance of proteins that stimulate the differentiation and proinflammatory actions of CTLs in parallel with those that may keep these activities in check, thus potentially maintaining cellular fitness. IL-2 induces a proinflammatory gene expression program by regulating the activity of transcription factors such as STAT5, HIF1α, and NFIL3, by promoting glucose metabolism, and by enhancing protein synthesis. IL-2 stimulates the production of proteins on many levels by regulating the abundance of amino acid transporters, tRNA synthases, ribosomal subunits, core translational machinery, and proteins that coordinate the assembly of translationally competent ribosomal complexes. Maintaining glucose metabolism is likely to be a critical determinant of sustaining the highly energy-consuming processes of ribosomal biogenesis and protein translation in CTLs. Thus, by enhancing metabolism and protein synthesis, IL-2 modulates an indirect regulation of cell phenotype independently of transcriptional networks. In this respect, the data reveal that IL-2 equally sustained the abundance of proteins that destroy misfolded proteins. Maintaining these protein degradation pathways may be required to prevent the accumulation of damaged proteins and preserve the cellular fitness of these CTLs as they perform the high rates of protein biosynthesis required for rapid cell proliferation and effector function. In a similar way, IL-2 finely tunes the abundance of proteins to prevent aberrant immune responses. For example, IL-2 increased the abundance of 4-1BB, which boosts CTL function, but also increased the abundance of Tim-3, which can inhibit CTL function. The ability of IL-2 to control the abundance of stimulatory and inhibitory receptors would, therefore, control the balanced activity of effector T cells.

One fundamental insight from the current data set is the dominant role for IL-2 in controlling how effector T cells respond to their nutrient environment. We found that IL-2 regulated a diverse repertoire of nutrient transporters, including those required for the uptake of glucose, iron, methionine, leucine, serine, arginine, and glutamine. These amino acids are necessary for protein synthesis and for other critical metabolic pathways. IL-2 also maintained the abundance of the amino acid-sensing pathways mediated by the leucine sensor SESTRIN2, which controls mTORC1 activity. The importance of nutrient supply for effector T cell function is now well recognized, and the effects of IL-2 on maintaining nutrient transporter abundance, therefore, enable IL-2 to have a major role in co-regulating metabolism and protein production in effector CD8⁺ T cells. In this respect, the fact that more than 3000 proteins did not undergo changes in abundance and that some proteins increased in abundance as a result of IL-2 deprivation seems contradictory to the marked effect of IL-2 on amino acid transport and overall protein synthesis. However, proteomic data provide a snapshot of cellular protein concentrations that result from the balanced rates of protein synthesis and protein degradation. The proteins that are IL-2-insensitive may well be very stable proteins. In addition, although IL-2 controls amino acid uptake from the environment, it is well established that amino acid-

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deprived cells switch on autophagy to recycle amino acids. Moreover, the overall metabolic stress caused by IL-2 deprivation could result in preferential translation of mRNAs that enable the cell to maintain some core protein constituents.

Another finding from our study was the integration of IL-2 signaling and oxygen-sensing signaling pathways. The importance of oxygen sensing in regulating effector T cell function is increasingly recognized. The HIF1 signaling pathway is a crucial regulator of T cell differentiation and controls the production of glucose transporters, glycolytic enzymes, chemokine receptors, adhesion molecules, and the cytolytic effector molecule perforin (21, 49). An increase in the abundance of HIF1 α is linked to enhanced effector CD8⁺ T cell function (49), whereas reduced PHD protein abundance improves the efficacy of effector T cells in the lung to clear tumor metastases (40). The expression of PHDs in T cells in the lung also suppresses effector T cells to prevent immune-mediated tissue damage (40). Therefore, it is important to understand that IL-2 drives the accumulation of both HIF1 α and PHD2.

In this respect, another finding from this study was the convergence of the IL-2 and oxygen-sensing signaling pathways. An example of this was the ability of both IL-2 and HIF1 to control the expression of NFIL3, a bZIP family transcriptional repressor, in CTLs. Previous studies showed that NFIL3 is important for the development of NK cells (46) and other ILCs (47). Our finding that NFIL3 is regulated by HIF1 downstream of IL-2 and hypoxia reveals how the expression of this important transcription factor is controlled. Note that we found that the abundance of NFIL3 in CTLs was markedly decreased in response to the JAK1/3 inhibitor tofacitinib, which is in clinical use. Thus, our data give insights into the molecular details of how these drugs control immune cell function. For example, it would be important to understand whether the JAK-mediated control of oxygen-sensing and HIF1 transcription factors determines NFIL3 expression in NK cells or ILCs. It will also be important to understand the molecular basis for NFIL3 control of CD8⁺ T cell transcriptional programs in more detail. NFIL3 inhibits FOXO-mediated transcriptional programs in human embryonic kidney (HEK) 293 cells (50), and FOXOs are critical regulators of CD8⁺ T cell differentiation and, similar to NFIL3, can control the expression of perforin and CD62L (51, 52). Thus, it will be important to explore whether NFIL3 acts to form a functional link between oxygen sensing and the control of FOXO transcription factors in T cells.

In summary, our data highlight the multiple ways in which IL-2– JAK signaling can shape the proteomic landscape of effector T cells to control protein metabolism and other key cellular metabolic programs. These data offer a comprehensive understanding of the effect of IL-2 signaling on effector CD8 T cells that explains the powerful immunomodulatory actions of this cytokine. IL-2 controls the fate of regulatory and effector CD4⁺ T cells; a future challenge will be to understand how IL-2 configures the proteomes of these cells to see if there are distinct effects in different populations.

MATERIALS AND METHODS

Study plan

This study aimed to define the proteome of in vitro–generated CTLs in the presence or absence of IL-2 and the JAK inhibitor tofacitinib. Three biological replicates were used for proteomic studies. Each biological replicate was generated from the spleen of one mouse. Mice were age- and sex-matched and were used between 12 and 15 weeks of age. Where possible, cage mates were used within each experiment. Other experiments were performed as indicated in the figure legends.

Mice

P14 mice have been described previously (26). Female P14 TCR transgenic mice were used for proteomic studies, and both male and female mice were used for other experiments. Mice with floxed *Hif1a* alleles were crossed with mice expressing Cre recombinase under the control of the *Vav* promoter to generate mice with deletion of HIF1 α in Vavexpressing cells. *Nfil3^{-/-}* mice have been described previously (46). All mice were bred and maintained in compliance with UK Home Office Animals (Scientific Procedures) Act 1986 guidelines.

In vitro cultures of CTLs

CTLs were generated as described previously (24). Briefly, naïve CD8⁺ T cells taken from the spleens of mice were activated by stimulation of the TCR for 48 hours in the presence of IL-2 (20 ng/ml; Proleukin, Novartis) and IL-12 (2 ng/ml; R&D Systems). P14 mice were activated with gp-33 peptide (100 ng/ml) from LCMV, and TCR nontransgenic T cells were activated with anti-CD3 (0.5 µg/ml; BioLegend) and anti-CD28 (0.5 µg/ml; eBioscience). After 48 hours, the activated CD8⁺ cells were removed from TCR stimulation and were then cultured in RPMI 1640 (Life Technologies) with 10% fetal bovine serum (Life Technologies), supplemented with penicillin G (50 U/ml; Gibco), streptomycin (50 µg/ml; Gibco), 50 µM β-mercaptoethanol (Sigma-Aldrich), and IL-2 (20 ng/ml; Proleukin, Novartis). CTLs were differentiated in IL-2 for 4 days before any 24-hour treatments were performed. Samples were considered biological replicates if the CTLs were generated from separate spleens. When required, CD8⁺ T cells were purified and isolated with an EasySep Mouse CD8⁺ T Cell Isolation kit (STEMCELL Technologies), as directed by the manufacturer's instructions.

Inhibitors and cell treatments

Cells were treated with 100 nM tofacitinib (Selleckchem). Where appropriate, cells were treated with dimethyl sulfoxide as controls. To deprive cells of IL-2, cells were pelleted, washed in an excess of prewarmed medium lacking IL-2 for 5 min before being pelleted, and resuspended in prewarmed medium lacking IL-2. For the IL-2-maintained controls, the CTLs were subjected to the same washes, with IL-2 (20 ng/ml) being added back before further culturing.

Proteomic sample processing

After they were generated and treated, CTLs were lysed in urea buffer [8 M urea, 50 mM tris (pH 8.0), 1 mM TCEP] with protease (cOmplete Mini EDTA-free, Roche) and phosphatase inhibitors (PhosSTOP, Roche) at room temperature for 30 min. Samples were sonicated, and protein concentration was determined using a bicinchoninic acid assay (Pierce) according to the manufacturer's instructions. To reduce samples, 10 mM dithiothreitol was added for 30 min at room temperature. After reduction, 50 mM iodoacetamide was added to each sample and incubated for 45 min at room temperature in the dark. Samples were then diluted to 4 M urea with tris buffer [100 mM tris (pH 8.0), 1 mM CaCl₂]. LysC (Wako), reconstituted in tris buffer, was then added to each sample at a ratio of 50:1 protein/LysC, and the samples were incubated with LysC overnight at 30°C. Samples were then transferred to low-bind 15-ml Falcon tubes (Eppendorf) and further diluted with tris buffer to 0.8 M urea. Trypsin (Promega), re-

constituted with tris buffer, was then added to the samples at a ratio of 50:1 protein/trypsin, and the samples were incubated for 8 hours at 30°C. After digestion, the samples were desalted with C18 Sep-Pack cartridges (Waters) and dried down in a SpeedVac (Genevac). Dried peptide samples were resuspended in 10 mM sodium borate-20% (v/v) acetonitrile (pH 9.3) and fractionated into 16 fractions by strong anion exchange (SAX) chromatography. Peptide samples for proteomic analysis were fractionated with an UltiMate 3000 HPLC (high-performance liquid chromatography) system equipped with an AS24 SAX column as previously described (23). For the separation, the buffers used were 10 mM sodium borate (pH 9.3) (SAX buffer A) and 10 mM sodium borate (pH 9.3), 500 mM NaCl (SAX buffer B). Peptide samples were resuspended in 210 µl of 10 mM sodium borate-20% (v/v) acetonitrile (pH 9.3), injected onto the SAX column, and separated using an exponential elution gradient starting with buffer A. In total, 16 peptide fractions were collected and desalted with Sep-Pack C18 96-well desalting plates (Waters). Desalted peptides were dried down with a SpeedVac (Genevac). Fractions were desalted, dried, resuspended in 1% formic acid, and separated by nanoscale C18 reverse-phase chromatography (UltiMate 3000 RSLCnano System, Thermo Fisher Scientific) before being electrosprayed into the linear trap quadrupole-Orbitrap mass spectrometer (LTQ Orbitrap Velos Pro; Thermo Fisher Scientific). The chromatography buffers used were as follows: HPLC buffer A (0.1% formic acid), HPLC buffer B (80% acetonitrile and 0.08% formic acid), and HPLC buffer C (0.1% formic acid). Peptides were loaded onto an Acclaim PepMap100 nanoViper C18 trap column (100 µm inner diameter, 2 cm; Thermo Fisher Scientific) in HPLC buffer C with a constant flow of 5 µl/min. After trap enrichment, peptides were eluted onto an EASY-Spray PepMap RSLC nanoViper, C18, 2 µm, 100 Å column (75 µm, 50 cm; Thermo Fisher Scientific) using the following buffer gradient: 2% B (0 to 3 min), 2 to 40% B (3 to 128 min), 40 to 98% B (128 to 130 min), 98% B (130 to 150 min), 98 to 2% B (150 to 151 min), and equilibrated in 2% B (151 to 180 min) at a flow rate of 0.3 µl/min. The eluting peptide solution was automatically electrosprayed into the coupled linear trap quadrupole-Orbitrap mass spectrometer (LTQ Orbitrap Velos Pro; Thermo Fisher Scientific) using an EASY-Spray nanoelectrospray ion source at 50°C and a source voltage of 1.9 kV (Thermo Fisher Scientific). The mass spectrometer was operated in positive ion mode. Full-scan MS survey spectra (mass/charge ratio, 335 to 1800) in profile mode were acquired in the Orbitrap with a resolution of 60,000. Data were collected using data-dependent acquisition: the 15 most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalized collision energy, 35%; activation Q, 0.250; activation time, 10 ms) in the LTQ after the accumulation of 5000 ions. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. The lock mass option was enabled for survey scans to improve mass accuracy. MS was performed by the Proteomics Facility, University of Dundee, UK.

Proteomic data processing and analysis

The MS data files were processed with MaxQuant version 1.6.0.1, and the spectra were mapped to the reviewed UniProtKB mouse protein database and the contaminant database supplied by MaxQuant. The following search parameters were used: Trypsin and LysC were selected as the proteases; up to two missed cleavages were permitted; the minimum peptide length was set to six amino acids; protein N-terminal acetylation, methionine oxidation, glutamine to pyroglutamate, and glutamine and asparagine deamidation were selected as variable modifications; carbamidomethylation of cysteine residues was set as a fixed modification; MS tolerance of 20 parts per million (ppm) and MS/MS tolerance of 0.5 Da; and labelfree quantification was enabled. False discovery rates were set to 0.01 and based on hits against the reversed sequence database. This cutoff was applied to individual spectra and whole proteins in the MaxQuant output. The match between runs function was enabled. Proteins were quantified on the basis of unique (found only in a specific protein group) and razor peptides (peptides assigned to a specific protein group without being unique to that group) with the requantification feature enabled. After processing of the files by MaxQuant, Perseus software implementing the proteome ruler plugin was used to calculate estimated copy numbers of proteins per cell. This analysis assigns the copy numbers of histones in a diploid mouse cell to the summed peptide intensities of all histones in a sample to estimate copy numbers for all identified proteins in the data set (28). The accuracies of the quantification were annotated on the basis of the number of peptides and the percentage of unique peptides identified for the protein as follows: high: ≥ 8 peptides detected, a minimum of 75% unique peptides; medium: \geq 3 peptides detected, a minimum of 50% unique peptides; low: all other peptides. Data quality was assessed by evaluating peptide intensities for normal distribution, the overlap in protein identification, and the correlation of the estimated copy number between replicates. The output from MaxQuant was filtered to remove known contaminants, those proteins identified as part of a reversed protein library, and proteins identified only by a modified site. To evaluate changes to the proteome resulting from the loss of IL-2–JAK1/3 signaling, the estimated copy numbers were used to determine the ratio of the abundance of individual proteins within biological replicates. The statistical significance of these changes was determined by performing a one-sample t test, without further adjustment, on the ratios of the treated condition/IL-2-maintained condition. Proteins were defined as being statistically significantly regulated in abundance if they had a ratio of \geq 1.5 or \leq 0.67 and a *P* value of ≤ 0.05 .

Western blotting

Cells were lysed in buffer [50 mM tris (pH 7.4), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM TCEP] with cOmplete Mini EDTA-free protease inhibitor tablets (Roche) for 10 min at 4°C. The resulting lysates were sonicated and then mixed with NuPAGE LDS sample buffer supplemented with TCEP before being heated to 70°C for 10 min. Samples were resolved by SDS-polyacrylamide gel electrophoresis with the Mini-PROTEAN Tetra Cell system. The proteins were then transferred to nitrocellulose membranes (Hybond-C Extra, Amersham). Membranes were incubated with the following primary antibodies: STAT5A/B [Cell Signaling Technology (CST) #9363], STAT5A/B pTyr⁶⁹⁴/pTyr⁶⁹⁹ (CST #9359), NFIL3 (CST #14312), granzyme B (CST #4275), perforin 1 (CST #3693), HIF1α (clone 241809, catalog no. MAB1536; R&D Systems), SMC1 (catalog no. A300-055A; Bethyl Laboratories), S6 pSer²³⁵/pSer²³⁶ (CST #2211), S6 (CST #2217), S6K pThr³⁸⁹ (CST #9205), S6K (CST #9202), AKT pThr³⁰⁸ (CST #4056), AKT pSer⁴⁷³ (CST #4058), AKT (CST #9272), FOXO1/3A pThr²⁴/ pThr³² (CST #9464), and FOXO1 (CST #9454). Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, Thermo Fisher Scientific #31460; goat anti-mouse, Thermo Fisher Scientific #31430), and chemiluminescence was detected using x-ray films (Konica) or the Odyssey Fc Imaging System (LI-COR). ImageJ software (National Institutes of Health) was used to quantify chemiluminescence captured on film, and Image Studio Software (LI-COR) was used to quantify chemiluminescence detected using the Odyssey system. Total protein bands of interest were normalized to SMC1 loading controls, and then for each blot, each band was normalized to a control sample. Phosphorylated protein bands of interest were normalized to the total protein band and normalized to a control sample.

Cell staining and flow cytometry

Cells were stained with saturating concentrations of the following antibodies: IL-2R α [CD25; clone PC61 (BD Biosciences) or clone 7D4 (BD Pharmingen)], CD69 (clone HI.2F3; eBioscience), CD44 (clone IM7; BD Pharmingen), CD62L (clone MEL-14; BD Pharmingen), CD4 (clone RM4-5; eBioscience), and CD8 (clone 53-6.7; BioLegend). Data were acquired on a FACSVerse flow cytometer with FACSuite software (BD Biosciences) or a FACS LSR Fortessa flow cytometer with DIVA software (BD Biosciences). Viable cells were gated according to their forward and side scatter profiles. Data analysis was performed with FlowJo software (Treestar). To compare surface protein abundance from multiple experiments, the geometric mean fluorescence intensity (GeoMFI) was determined. If all data were recorded in the same experiment, then the raw GeoMFI data were shown. If data were normalized to controls for presentation.

RNA extraction and qPCR

RNA was isolated with a Qiagen RNeasy kit, and complementary DNA (cDNA) was generated using a Quantabio cDNA synthesis kit. Reverse transcription PCR was performed with the SYBR Green dve (Bio-Rad) on an iCycler iQ PCR machine. Tbp, Cd8a, and Hprt1 were used as the reference genes as indicated in the figure legends. Data were analyzed using the $\Delta\Delta C_{\rm T}$ method. Primers were as follows: Cd8a, 5'-GATATA-AATCTCCTGTCTGCCCATC-3' (forward) and 5'-ATTCATACC-ACTTGCTTCCTTGC-3' (reverse); Sell, 5'-CCTGTAGCCGTCATG-GTCAC-3' (forward) and 5'-GAATCAGTATGGATCATCCATC-3' (reverse); Slc2a1, 5'-CCAGCAGCAAGAAGGTGA-3' (forward) and 5'-ATGTTTGATTGTAGAACTCCTC-3' (reverse); Hprt1, 5'-TGAT-CAGTCAACGGGGGGACA-3' (forward) and 5'-TTCGAGAG-GTCCTTTTCACCA-3' (reverse); Nfil3, 5'-GCGATGGTAGCCGGA-AGTTGC-3' (forward) and 5'-CCTGTGCGGGGGCTTTCCTGAG-3' (reverse); Prf1, 5'-CGTCTTGGTGGGACTTCAG-3' (forward) and 5'-GCATTCTGACCGAGTGGCAG-3' (reverse); Tbp, 5'-GGGGA-GCTGTGATGTGAAGT-3' (forward) and 5'-CCAGGAAATAAT-TCTGGCTCAT-3' (reverse).

Glucose uptake

The measurement of radiolabeled glucose uptake has been described previously (21). Briefly, glucose-free RMPI 1640 medium labeled with radiolabeled 2-deoxy-D- $[1-^{3}H]$ glucose (0.5 µCi/ml; PerkinElmer) was layered over 500 µl of a 1:1 mixture of silicone oil (Sigma-Aldrich) and dibutyl phthalate (Sigma-Aldrich). Cells (1 × 10⁶ per measurement) were added and allowed to take up radiolabeled 2-deoxy-D- $[1-^{3}H]$ glucose for 4 min. The cells were then lysed in 100 mM NaOH, and the radioactivity was analyzed using the Beckman LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter). Assays were performed in duplicate or triplicate per biological replicate. For each biological replicate, data were normalized to the uptake of 2-deoxy-D- $[1-^{3}H]$ glucose in the IL-2-maintained CTL sample.

Lactate output

Lactate measurements were performed as described previously (21). CTLs were resuspended to a concentration of 2×10^6 cells/ml in RPMI with 10% (v/v) dialyzed serum and incubated for 4 hours, after which the culture medium was collected. Lactate concentration was determined using an enzymatic assay in which the oxidation of lactate by lactate dehydrogenase (LDH) is monitored by the change in absorption at 340 nm after the reduction of NAD⁺ (nicotinamide adenine dinucleotide) to NADH (reduced form of NAD⁺). To determine the lactate concentration, equal volumes of culture medium and master mix (320 mM glycine, 320 mM hydrazine, 2 U LDH, and 2.4 mM NAD⁺) were mixed and incubated for 10 min at room temperature. Absorbance of the reactions was then read at 340 nm with the CytoFluor II Fluorescence Multi-Well Plate Reader (Perceptive Biosystems). A lactate standard curve was generated, and the concentration of lactate in the test samples was inferred after data analysis with Prism software (GraphPad).

Statistical analyses

Statistical analyses of changes in protein abundance from the proteomic data were performed as outlined in the "Proteomic data processing and analysis" section. Further data derived from the proteomic studies (such as the estimated number of ribosomes per cell) and validation data were subjected to similar statistical analyses: The ratios of the treated condition/IL-2-maintained condition were determined and subjected to a two-tailed, one-sample *t* test, without further adjustment. For studies comparing T cells with gene knockouts to wild-type T cells, two-tailed unpaired, unequal variance *t* tests were used.

SUPPLEMENTARY MATERIALS

- www.sciencesignaling.org/cgi/content/full/11/526/eaap8112/DC1
- Fig. S1. Proteomic analysis of CTLs cultured in the presence or absence of IL-2.
- Fig. S2. Proteomic analysis of CTLs cultured with IL-2 in the presence or absence of tofacitinib.
- Fig. S3. Activation and generation of CTLs from *Nfil3^{-/-}* T cells. Table S1. The proteomes of IL-2–maintained and IL-2–deprived CTLs.

Table S2. IL-2–regulated proteome of CTLs.

Table S3. The proteomes of IL-2–maintained CTLs in the presence or absence of tofacitinib. Table S4. Comparison of the IL-2– and tofacitinib-regulated proteomes of CTLs.

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Interleukin-2 shapes the cytotoxic T cell proteome and immune environment-sensing programs

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Revealing how IL-2 sculpts T cells

The cytokine interleukin-2 (IL-2) is critical for the differentiation and proliferation of effector CD8⁺ cytotoxic T cells. IL-2 signaling is mediated through the tyrosine kinases JAK1 and JAK3. Because of the importance of this pathway in orchestrating immune responses, it is an attractive target for therapies, including tofacitinib, which inhibits JAK1/3 signaling and ameliorates autoimmune and inflammatory conditions. Rollings *et al.* performed proteomic analysis of cytotoxic CD8⁺ T cells that were cultured in the presence or absence of IL-2, as well as those treated with tofacitinib, and identified proteins in IL-2-treated cells that were critical to maintain their effector function. In addition, IL-2 shaped the ability of these cells to respond to their environment by inducing the production of proteins involved in nutrient sensing and uptake, as well as in oxygen sensing. A factor involved in oxygen sensing was decreased in response to tofacitinib, giving important insights into how such drugs control immune cell function.

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