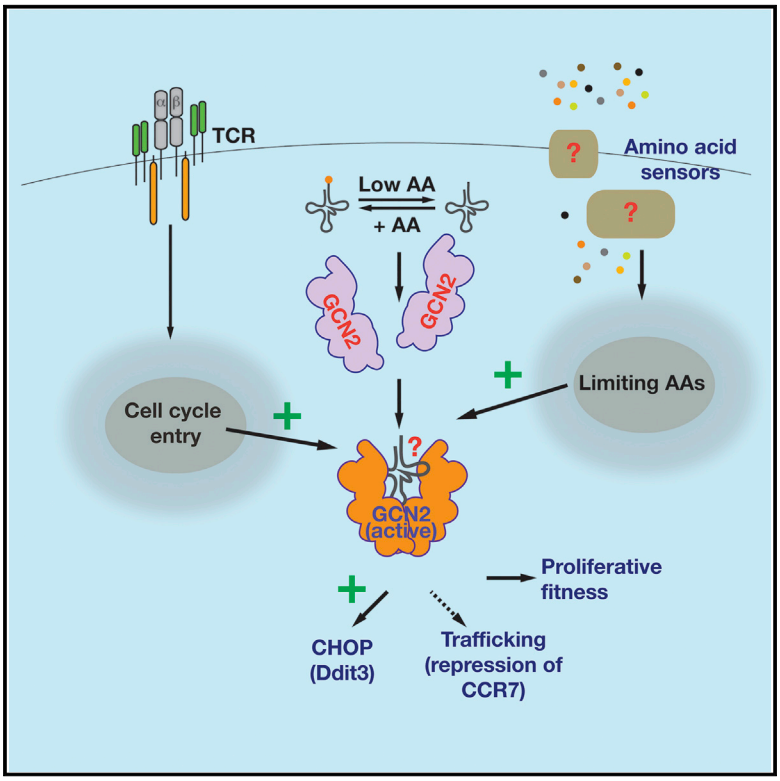
Article

Cell Reports

Stress Kinase GCN2 Controls the Proliferative Fitness and Trafficking of Cytotoxic T Cells Independent of Environmental Amino Acid Sensing

Graphical Abstract



Authors

Lee-Ann Van de Velde, Xi-Zhi J. Guo, Lidija Barbaric, Amber M. Smith, Thomas H. Oguin III, Paul G. Thomas, Peter J. Murray

Correspondence

peter.murray@stjude.org

In Brief

Van de Velde et al. show that the stress kinase GCN2, previously considered an amino acid "sensor" that blocks T cell proliferation in low amino acids, has a more complex role, including regulation of cytotoxic T cell trafficking in the body.

Highlights

- T cells do not require GCN2 to block proliferation in low amino acids
- Cytotoxic T cells require GCN2 for efficient proliferation
- GCN2 controls trafficking of cytotoxic T cells in vivo
- Multiple signals including proliferative stimuli activate stress transcription

Van de Velde et al., 2016, Cell Reports *17*, 2247–2258
November 22, 2016
http://dx.doi.org/10.1016/j.celrep.2016.10.079

Cell Reports
Article

Stress Kinase GCN2 Controls the Proliferative Fitness and Trafficking of Cytotoxic T Cells Independent of Environmental Amino Acid Sensing

Lee-Ann Van de Velde,[1,2] Xi-Zhi J. Guo,[2] Lidija Barbaric,[1,2] Amber M. Smith,[1,2] Thomas H. Oguin III,[2] Paul G. Thomas,[2] and Peter J. Murray[1,2,3,*]
[1]Department of Infectious Diseases
[2]Department of Immunology
St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA
[3]Lead Contact
*Correspondence: peter.murray@stjude.org
http://dx.doi.org/10.1016/j.celrep.2016.10.079

SUMMARY

GCN2 is one of four "stress kinases" that block translation by phosphorylating eIF2α. GCN2 is thought to bind uncharged tRNAs to "sense" amino acid availability. In mammals, myeloid cells expressing indoleamine dioxygenases locally deplete tryptophan, which is detected by GCN2 in T cells to cause proliferative arrest. GCN2-deficient T cells were reported to ectopically enter the cell cycle when tryptophan was limiting. Using GCN2-deficient strains crossed to T cell receptor (TCR) transgenic backgrounds, we found GCN2 is essential for induction of stress target genes such as CHOP. However, GCN2-deficient CD8$^+$ T cells fail to proliferate in limiting tryptophan, arginine, leucine, lysine, or asparagine, the opposite of what previous studies concluded. In vitro and in vivo proliferation experiments show that GCN2-deficient CD8$^+$ T cells have T cell-intrinsic proliferative and trafficking defects not observed in CD4$^+$ T cells. Thus, GCN2 is required for normal cytotoxic T cell function.

INTRODUCTION

GCN2 (encoded by *Eif2ak4* in mouse) is one of four "stress"-sensing kinases that phosphorylate a single known substrate: serine 51 on the translation factor eIF2α (Donnelly et al., 2013). Seminal work by Hinnebusch and colleagues using yeast showed GCN2 is activated by uncharged tRNAs when cells are starved for essential amino acids (Dong et al., 2000; Lageix et al., 2014). Amino acid starvation causes a rise in uncharged tRNAs, triggering dimerization and activation of GCN2's kinase activity, which leads to phosphorylation of serine 51 on eIF2α to block global translation and protect cells under nutrient duress. The other three members of the stress kinase family are activated by heme stress (HRI), double-stranded RNA (PKR) and ER stress (PERK) (Donnelly et al., 2013). In addition to targeting eIF2α, all four stress kinases activate a parallel gene and protein expression pathway mediated by activation (via translation) of the transcription factor ATF4 (Harding et al., 2000). The net effect of stress kinase activation is thought to be cellular protection and resource conservation.

In the immune system, GCN2 appears to play several distinct roles. For example, GCN2 is required for effective dendritic cell activation and antigen presentation (Ravindran et al., 2014). In T cells, a key finding by Munn, Mellor, and their colleagues (Munn et al., 2005) found CD8$^+$ T cells lacking GCN2 failed to integrate signals from tryptophan starvation and ectopically entered the cell cycle when tryptophan was limiting. Thus, rather than arresting growth when an essential resource was absent, GCN2-deficient cells initiated growth (Munn et al., 2005).

Tryptophan is an important amino acid in immune regulation because two enzymes, IDO1 and IDO2 (indolamine 2,3-dioxygenases), degrade tryptophan into kynurenines and their downstream metabolites (Munn and Mellor, 2013). Another tryptophan-degrading enzyme, called TDO2, is expressed predominantly in the liver and is thought to contribute to kynurenine production (Ball et al., 2014; Bessede et al., 2014). Both kynurenine production and local tryptophan starvation are immunoregulatory, although the precise contribution of each pathway to other different immune responses is unresolved (Moffett and Namboodiri, 2003; Murray, 2016). In the Munn et al. (2005) study, CD8$^+$ T cells lacking GCN2 were exposed to conditions where tryptophan amounts were artificially (via the culture media) or naturally (via other cells expressing IDO proteins) manipulated. In another study, concordant results were reported for arginine-starved T cells (Rodriguez et al., 2007). Thus, it is widely accepted that T cells, like yeast, use GCN2 as an information processor for environmental amino acid amounts, causing cessation of proliferation when essential amino acids are limiting.

Here we challenge the findings concerning the link between amino acid starvation and GCN2 in T cells. We use antigen-specific genetic systems to show that GCN2-deficient CD4$^+$ and CD8$^+$ cells have overtly similar responses to control T cells when starved of the essential amino acids leucine, lysine, arginine, and asparagine. We found GCN2 was

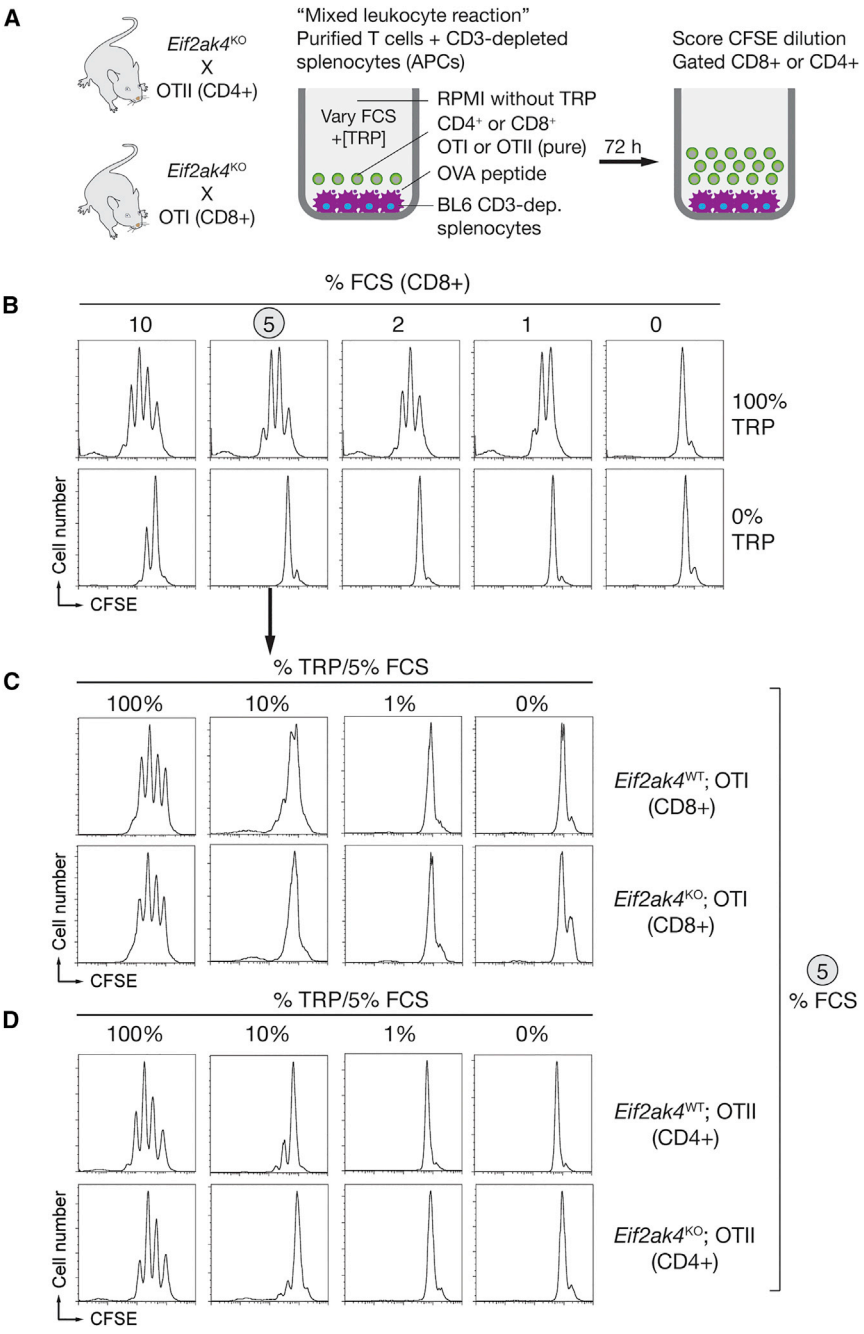


Figure 1. GCN2^{KO} T Cells Fail to Proliferate in Limiting Tryptophan

(A) Schematic of the generation of TCR transgenic mice and the co-culture conditions.

(B) Titration assay to determine the lowest amount of tryptophan required to induce T cell proliferation. Note that 10% FCS contains sufficient tryptophan to allow one cycle of division. Therefore, 5% FCS was chosen for the subsequent studies.

(C) CFSE proliferation analysis of CD8$^+$ division in limiting tryptophan.

(D) CFSE proliferation analysis of CD4$^+$ division in limiting tryptophan.

(C and D) Representative experiments from three independent experiments each using individual mice are shown.

RESULTS

Antigen-Specific CD4$^+$ or CD8$^+$ T Cells Lacking GCN2

We generated ovalbumin (OVA)-specific CD4$^+$ (OT-II transgene) or CD8$^+$ (OT-I transgene) T cell receptor (TCR)-specific transgenic mice on GCN2-deficient backgrounds (GCN2^{KO}) using mice available from The Jackson Laboratory (B6.129S6-*Eif2ak4*$^{tm1.2Dron}$/J) (Maurin et al., 2005; Munn et al., 2005). This *Eif2ak4* allele lacks exon 12 encoding an essential part of the kinase domain. Splicing of exons 11–13 would result in a frameshift and premature stop codon (Figure S1A) that is equivalent to a null (Harding et al., 2000; Maurin et al., 2005). Using qRT-PCR, we confirmed a complete absence of GCN2 mRNA in activated CD8$^+$ or CD4$^+$ T cells (Figure S1B).

GCN2-Deficient T Cells Have Intact Tryptophan-Sensing Mechanisms

One of the key assertions of the link between GCN2 and tryptophan sensing is that IDO1 expression in antigen-presenting cells (APCs) locally depletes tryptophan. This causes activation of the GCN2 pathway, leading to growth arrest and activation of the stress response (Muller et al., 2008; Munn and Mellor, 2013; Munn et al., 2005). We used an amino acid starvation system whereby tryptophan is titrated out of the culture media and T cell proliferation was measured (Munn et al., 2005) (Figure 1A). Because serum contains tryptophan bound to serum proteins, we used tryptophan-free RPMI with serum supplements to titrate out the amount of tryptophan available to anti-CD3/CD28-activated T cells (Munn et al., 2005). However, we were unable to observe T cell proliferation using serum substitutes in combination with the tryptophan-free media. We reasoned serum was dispensable for tryptophan sensing that blocks cell-cycle entry when amino acids are limiting. Instead, GCN2 was required for the optimal proliferation of CD8$^+$ T cells after antigen stimulation in vitro. Loss of GCN2 had minimal effects on CD4$^+$ T cell proliferation and selective effects on CD8$^+$ proliferation, especially in competitive assays. We further show the GCN2 stress pathway is necessary for CD8$^+$ T cells to correctly traffic to lymphoid organs, and that GCN2 pathway activation requires independent signals: an environmental signal from low amino acids and a second, internal signal from entry into the cell cycle.

required for T cells to proliferate, and therefore performed cross-titration experiments between serum and tryptophan amounts to arrive at a minimal serum concentration where control $CD8^+$ cell growth arrests in low tryptophan (5% serum; Figure 1B). In this assay both control and GCN2-deficient T cells failed to proliferate in low concentrations of tryptophan (Figures 1C and 1D), whereas viability was unaffected (Figure S2). Therefore, GCN2 was dispensable for $CD4^+$ or $CD8^+$ T cells sensing limiting tryptophan to arrest cells prior to entry into the cell cycle. These data differ from those of Munn et al. (2005), where GCN2-deficient T cells proliferated in low tryptophan.

GCN2 Is Dispensable for Sensing Environmental Amino Acids Required for Cell Cycle Entry

We next tested whether GCN2 had general roles in T cell-mediated environmental amino acid sensing because they require exogenous supply of most amino acids (Murray, 2016). Using $CD4^+$ or $CD8^+$ $GCN2^{KO}$ cells, we tested whether T cells sensed limiting arginine, lysine, or leucine in a GCN2-dependent way. The expectation, based on other work (Munn et al., 2005; Rodriguez et al., 2007), was that T cells would enter the cell cycle following TCR and co-stimulation (via CD28) engagement. This is because GCN2 would fail to "sense" low amino acid concentrations in the media, and thus fail to block global translation. We used an assay where peritoneal macrophages were loaded with peptide and used as APCs with titrations of the amino acid concentration in the media (Figure S3A). $GCN2^{KO}$ $CD4^+$ or $CD8^+$ T cells sensed arginine, lysine, or leucine were limiting and failed to proliferate (Figures S3B and S3C). Similar to the approach in Figure 1, we adapted the same culture system with lowered FCS to measure tryptophan sensing in the macrophage co-cultures (Figure S3D) and observed limiting tryptophan sensing was intact in both $CD4^+$ and $CD8^+$ cells (Figures S3E and S3F).

Lymphocytes are auxotrophs for asparagine, and this property has led to the successful development of injectable recombinant bacterial asparaginases (ASNase) for leukemia treatment (Rubnitz et al., 2009). We therefore tested whether GCN2 was required for T cell sensing of asparagine. Using an assay where ASNase was titrated into anti-CD3/CD28-stimulated $CD8^+$ T cells, we observed no differences in the ability of ASNase to block T cell growth (Figure S3G). Thus, GCN2 was dispensable for a T cell-mediated sensing pathway that blocks cell-cycle entry when asparagine, leucine, lysine, tryptophan, and arginine are limiting.

GCN2 Controls $CD8^+$ Expansion and Trafficking

Given that our experiments had yet to reveal obvious relationships between GCN2 and environmental amino acid sensing, we turned to in vivo systems of proliferative T cell expansion. To test the role of GCN2 in in vivo expansion of $CD4^+$ T cells, we used a $CD4^+$-dependent colitis model. In this model, transferred $CD4^+$ cells depleted of effector T cells (T_{eff}) expand in vivo and eventually cause colitis. We sorted T_{eff} ($CD4^+$, $CD45RB^{hi}$, $CD25^-$) from $GCN2^{KO}$ or $GCN2^{WT}$ mice (without TCR transgenes) and transferred 5×10^5 cells into *Rag1*$^{-/-}$ recipients, which lack lymphocytes (Workman et al., 2011) (Figure 2A). At the endpoint of the experiment (8 weeks), the number and phenotype of $CD4^+$ T cells repopulating the lymphopenic hosts were measured. Recipient mice reconstituted with $GCN2^{WT}$ or $GCN2^{KO}$ $CD4^+$ T cells manifested fulminant diarrhea, colitis, and weight loss. Most importantly, the number of $CD3^+$ T cells in the colon and cecum was equivalent between genotypes (Figure 2B). Therefore, GCN2 was not required for $CD4^+$ expansion in vivo and was not required for the acquisition of pathogenic properties that cause colitis in this model.

To measure $CD8^+$ expansion, we transferred naive, purified, CSFE-labeled $CD8^+$ $GCN2^{KO}$; OT-I (CD45.2) and $GCN2^{WT}$; OT-I (CD45.1) into *Rag1*$^{-/-}$ recipients at a 1:1 ratio (Figure 2C). After 3 or 6 days, cohorts of mice were sacrificed and the number of T cells in different organs enumerated by flow cytometry. GCN2-deficient T cells were enriched in the spleen, but not the cervical or mesenteric lymph nodes (MLNs) (Figures 2C and 2D). In the spleen, higher numbers of $GCN2^{KO}$ T cells were recovered, which was coincident with increased proliferation as measured by CFSE dye dilution (Figure 2E). Therefore, a fraction of $GCN2^{KO}$ $CD8^+$ T cells selectively trafficked to the spleen relative to control cells injected at the same time. To confirm and extend these observations, we performed two types of experiments. First, we repeated the *Rag1*$^{-/-}$ reconstitution studies where mice were footpad immunized with OVA emulsified in Incomplete Freund's Adjuvant (IFA) 24 hr later and left to expand for 6 days and then harvested, providing a readout of Ag-specific proliferation. The popliteal LN, spleen, and cervical LNs were harvested and the total number of cells counted, along with the total number of divided cells. Although the ratio of $GCN2^{KO}$ to $GCN2^{WT}$ cells remained 1:1 in the draining LNs, we observed enrichment of $GCN2^{KO}$ T cells in the spleen rather than the popliteal LN (Figure 2F).

Next, we transferred naive, purified, CSFE-labeled $CD8^+$ $GCN2^{KO}$; OT-I (CD45.2) or $GCN2^{WT}$; OT-I (CD45.1) into Thy1.1 recipients at a 1:1 ratio and infected them with influenza H3N2 strain X31-OT-I, which expresses the peptide recognized by OT-I T cells (Jenkins et al., 2006) (Figures 3A, S3A, and S3B). Similar to what we observed in the *Rag1*-deficient reconstitution model, $GCN2^{KO}$; OT-I were enriched relative to control cells in both number and percentage, especially in the spleen, but not in the bronchoalveolar lavage (BAL), where antigen-specific proliferation occurs (Figures 3C and 3D). Taken together, $GCN2^{KO}$ $CD8^+$ cells appeared to have a preferential propensity to migrate to the spleen relative to other secondary lymphoid organs. Next, we measured the kinetics of $CD8^+$ trafficking in the X31-OT-I infection system. Thy1.1 mice were reconstituted as detailed earlier, and the percentage and number of T cells in the spleen and BAL were measured at 3, 6, or 12 days post-infection. At day 3, insufficient numbers of cells were recovered from either genotype to make accurate estimates of T cell number. However, by day 6, $GCN2^{WT}$; OT-I cells were detected on spleen and BAL, and thereafter declined in number and percentage in the former, but not latter (Figures 3E–3H). By contrast, $GCN2^{KO}$; OT-I accumulated in the spleen at day 12. The percentage and number of $GCN2^{KO}$; OT-I was lower in the BAL relative to the co-injected control cells (Figures 3E–3H). These data, together with the experiments in the *Rag1*-deficient reconstitution model, suggested that a side-by-side test of the proliferative expansion of $CD8^+$ $GCN2^{KO}$; OT-I versus $GCN2^{WT}$; OT-I cells at the site of

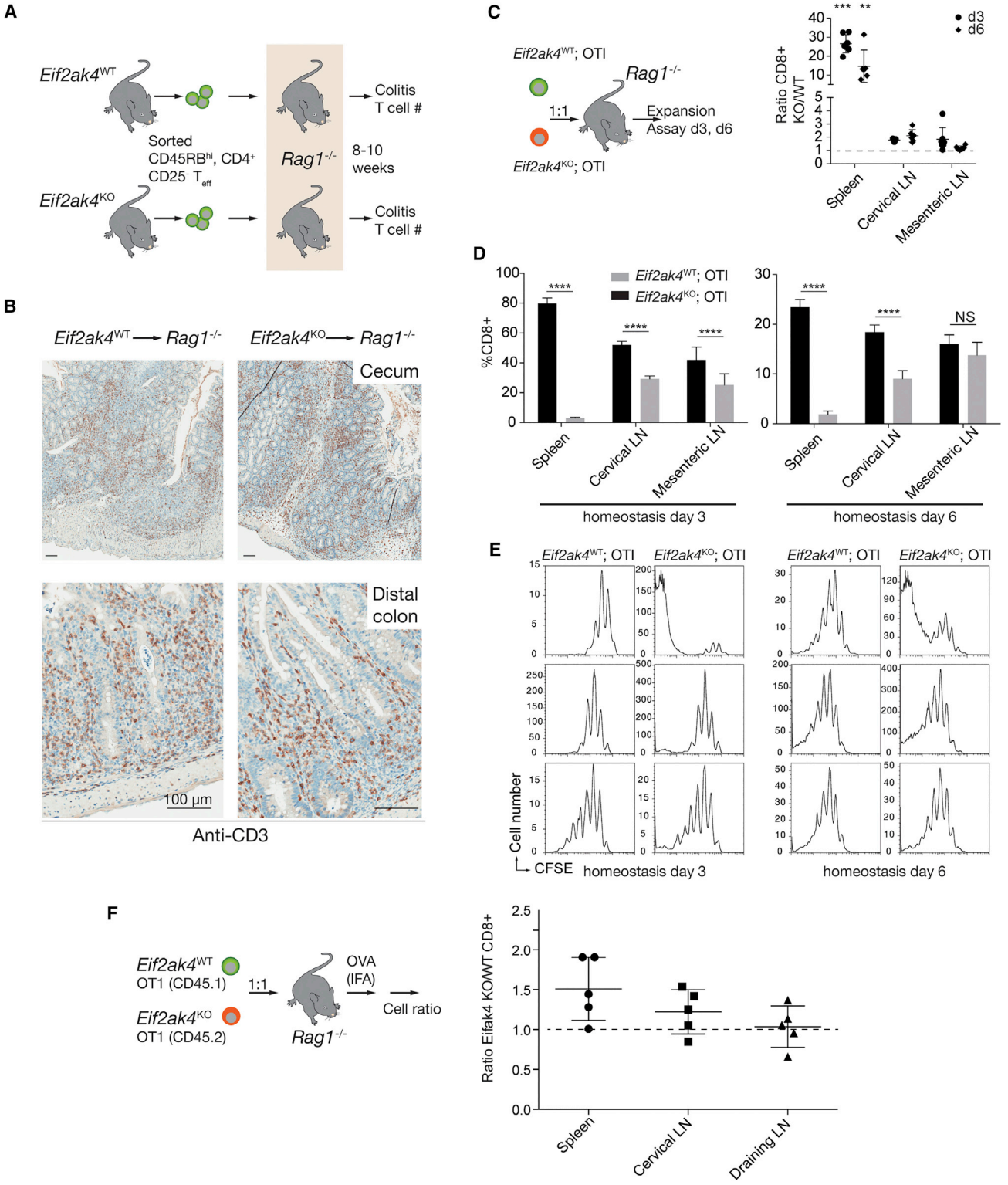


Figure 2. GCN2 Controls In Vivo Proliferation and Splenic Trafficking of CD8+ T Cells

(A) Schematic of the experimental design to test CD4+ T cell expansion in vivo.

(B) Anti-CD3 staining of T cells in the colon and cecum of reconstituted mice. Data are representative of two independent experiments using two to seven mice per group.

(legend continued on next page)

antigen enrichment was not possible, because most GCN2^{KO} T cells emigrated to the spleen.

CCR7 mediates the major, but not exclusive, pathway for CD8^{+} T cell enrichment in secondary lymphoid organs (Förster et al., 1999; Sharma et al., 2015; Unsoeld et al., 2004). Therefore, we hypothesized that GCN2 may have a cell-intrinsic influence on CCR7 expression. Consistent with this idea, activated GCN2^{KO} CD8^{+} had increased CCR7 mRNA (Figure 3I). We next repeated the *Rag1*-deficient reconstitution model where splenic enrichment of GCN2^{KO} CD8^{+} occurs (Figure 3J) and found increased CCR7 expression on GCN2^{KO} CD8^{+} relative to the co-injected controls (Figures 3K and 3L), which was confirmed in the influenza infection model (Figure S4C). Taken together, our data suggest GCN2 has limited effects on CD4^{+} T cell expansion in vivo, but plays a key role in suppressing CCR7 expression in CD8^{+} cells, which leads to increased relative accumulation in the spleen.

T Cell-Intrinsic Requirement for GCN2 in Antigen-Induced Proliferation

In the experiments shown in Figures 1, S2, and S3, we consistently observed minor defects in GCN2^{KO} CD8^{+} proliferation, generally manifested under conditions of lowered amino acid availability. Recent data have pointed to key roles for GCN2 in regulating antigen presentation in myeloid cells (Ravindran et al., 2014). Thus, a defect in APC function could account for the low proliferative activity of GCN2^{KO}; OT-I cells under conditions where GCN2^{KO} APCs were present. An alternative possibility is that GCN2 is required for T cell proliferation in a T cell-intrinsic way. To gain more experimental control over the relative contributions of GCN2 to antigen presentation versus proliferation in our systems, we established a "mixed leukocyte reaction" (MLR) culture system where "wild-type" LN cells (containing T cells and APCs) were titrated with LN cells from GCN2^{KO}; OT-I or GCN2^{WT}; OT-I controls (Figure 4A), allowing us to simultaneously control for APC effects and T cell proliferation within the same well. When we compared the proliferation of GCN2^{KO}; OT-I with GCN2^{WT}; OT-I cells mixed with different ratios of CD45.1 cells, a striking pattern emerged where GCN2^{KO}; OT-I failed to proliferate efficiently, regardless of the number of input CD45.1 (GCN2^{WT}) cells (Figure 4B). By comparison, GCN2^{WT}; OT-I cells titrated with "doped" CD45.1 LN cells proliferated efficiently under all conditions (Figures 4B and 4C). We next performed a variant of this experiment where purified CD8^{+} T cells from GCN2^{KO}; OT-I to GCN2^{WT}; OT-I mice were mixed in different ratios of CD45.1 LN cells. Wild-type OT-I T cells proliferated to a greater degree than GCN2^{KO}; OT-I cells (Figure 4D). The results of these experiments suggest GCN2 is required for CD8^{+} T cells to proliferate in a T cell-intrinsic way. The data also suggest GCN2 is not absolutely required for T cell proliferation; rather, GCN2 contributes to efficient proliferation especially in a competitive environment where other cells are dividing, or in conditions where amino acids are limiting.

Another possibility to account for the data shown in Figure 4 was that GCN2^{KO}; OT-I cells could have had an increased propensity to die following activation, and the differential rate of death between GCN2^{WT}; OT-I and GCN2^{KO}; OT-I cells could account for the decreased proliferation observed in the GCN2^{KO}; OT-I cultures. Therefore, we stained activated CD8^{+} cells with a viability dye across time (Haverkamp et al., 2014) and noted minimal differences in death between GCN2^{KO}; OT-I and controls (Figure 4E).

We repeated an antigen-specific proliferation experiment where plasmacytoid DCs were isolated from the tumor draining lymph node (LN) of mice bearing a flank B16 melanoma. In this system, plasmacytoid dendritic cells (pDCs) express IDO1, causing local tryptophan depletion and blocking T cell proliferation in a tryptophan- and IDO1-dependent way (Munn et al., 2005). Using the B16 tumor model (we note Munn et al., 2005, used a B16 tumor expressing GM-CSF, but did not report results from cognate controls), the same pDC sorting scheme, culture conditions, and T cell responder system (GCN2^{KO}; OT-I or GCN2^{WT}; OT-I controls) (Figures S5A and S5B), we found that GCN2^{KO}; OT-I failed to proliferate when exposed to tumor draining lymph node pDCs, whereas control T cells had robust proliferation (Figure S5C). These data are the opposite from what Munn et al. (2005) reported. We also performed a similar study with a different tumor system, the EG7 thymoma, again with the same results: GCN2-deficient CD8^{+} T cells had a proliferative defect rather than ectopically entering into the cell cycle as previously reported (data not shown).

GCN2-Mediated Stress Pathway Activation in T Cells Requires Two Signals

To gain insight into the GCN2-dependent pathways activated in cytotoxic T cells, we quantified GCN2- and stress-dependent gene expression in activated GCN2^{KO}; OT-I or GCN2^{WT}; OT-I cells in limiting tryptophan. CHOP expression (encoded by *Ddit3*), a cardinal stress kinase-activated transcription factor, was GCN2 dependent as expected and induced ~100-fold at the mRNA level by limiting tryptophan (Figure 5A). CHOP expression is therefore a sensitive readout of both GCN2 activity and environmental amino acid amounts. Atf3, another transcription factor activated by the stress signaling pathway, was also GCN2 dependent, but maximally induced later in the proliferative process (72 h) relative to CHOP (Figure 5B).

To test whether CHOP expression was regulated by amino acid starvation alone, we next performed experiments where purified OT-I cells were co-cultured with APCs in the presence or absence of antigen across time (24–72 h) and under tryptophan

(C–E) Proliferative and trafficking defects in GCN2-deficient CD8^{+} T cells transferred into lymphopenic hosts. A total of 5 × 10^{6} CFSE-labeled CD8^{+} CD45.1 control and GCN2^{KO} CD45.2 T cells were transferred into *Rag1*$^{-/-}$ mice, and the ratio (C), percentage (D), and proliferation (E) of CD45.1:CD45.2 CD8^{+} cells were assessed in the spleen, cervical, and mesenteric LNs at days 3 and 6 after transfer. Reconstitution experiments were performed twice using six to seven mice per group. Data from one complete experiment is shown.

(F) Ratio of CD45.1:CD45.2 CD8^{+} OTI^{+} cells in the spleen, cervical, and draining LNs from *Rag1*$^{-/-}$ reconstituted mice 6 days after footpad immunization with OVA/IFA. One of two experiments using five to six mice per group is shown.

*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

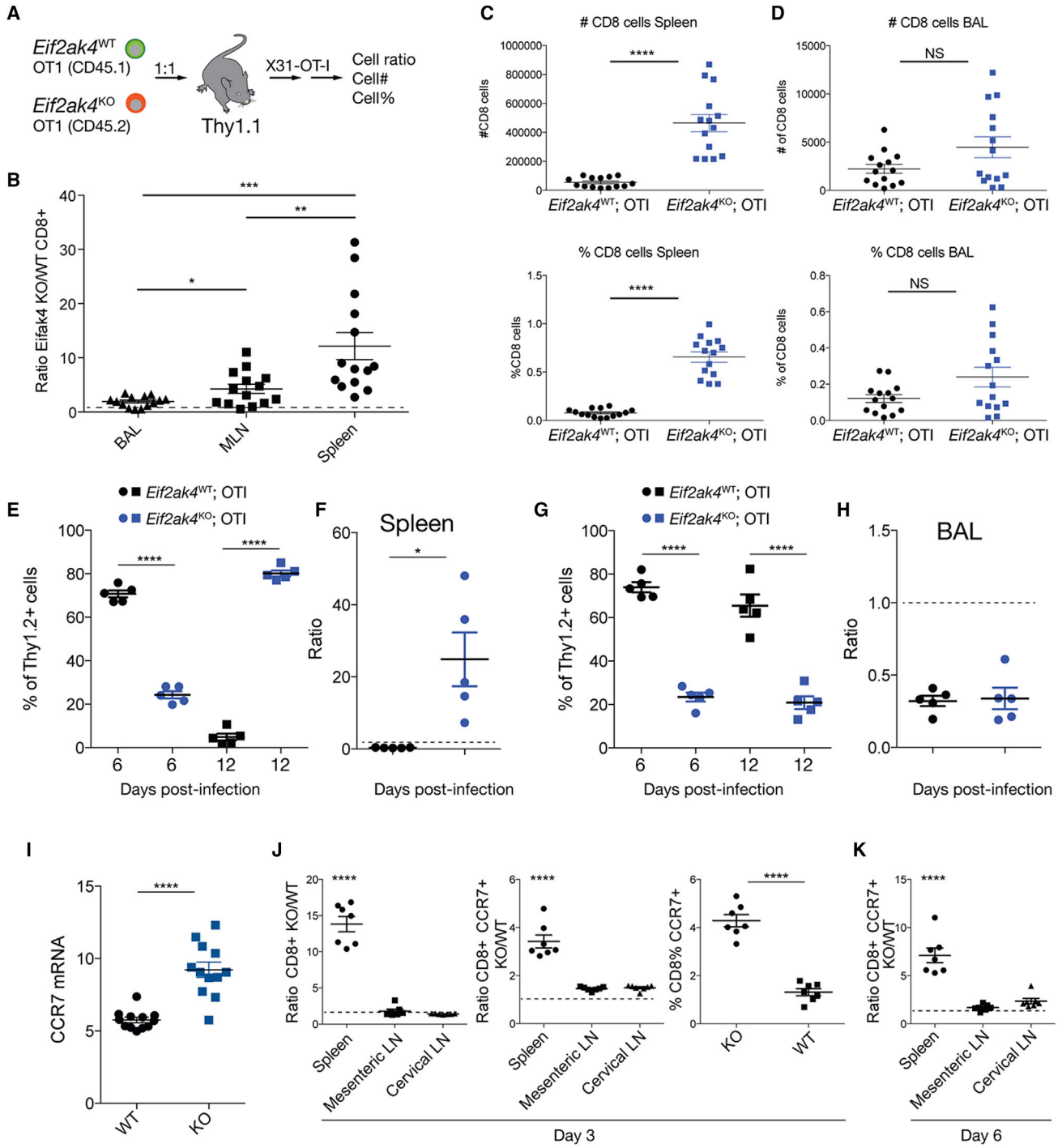


Figure 3. GCN2 Controls Trafficking and CCR7 Expression of CD8⁺ T Cells

(A) Congenically marked (CD45.1 or CD45.2) GCN2-deficient and WT OVA-specific CD8 T cells were mixed at equal numbers (10^4 each), transferred in Thy1.1 animals, and infected with influenza X31-OT-I the next day.

(B) Six days after X31-OT-I influenza infection, OVA-specific CD8⁺ expansion was quantified in the indicated organs and plotted as the ratio of the two cell types in BAL, MLN, or spleen. The dotted line indicates a ratio of 1, where equal numbers of cells from both genotypes would be recovered.

(C) Percentages of CD8⁺ OVA-specific T cells in the BAL.

(D) Percentages of CD8⁺ OVA-specific T cells in MLN.

(E and F) Percentages and ratios of CD8⁺ T cells were quantified at day (d) 6 and d12 in the spleen.

(G and H) Percentages and ratios of CD8⁺ T cells were quantified at d6 and d12 in the spleen.

(legend continued on next page)

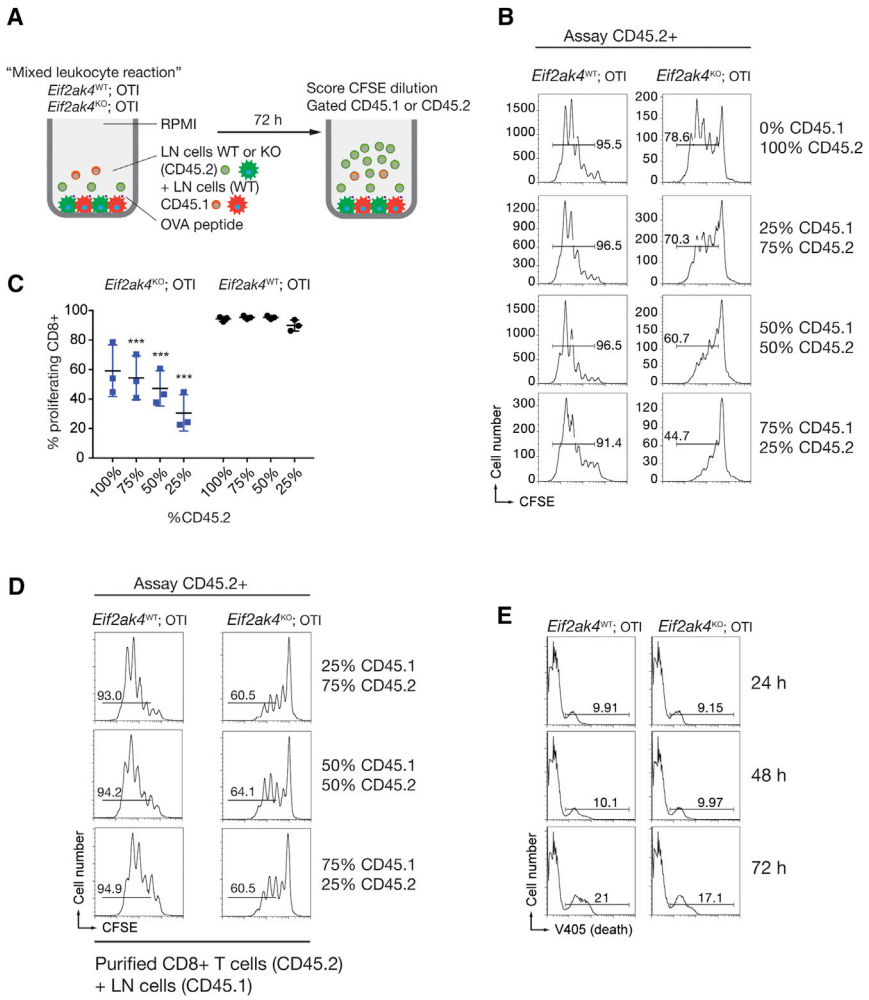


Figure 4. GCN2 Is Required for CD8$^+$ T Cell-Intrinsic Proliferation

(A) Schematic of the co-culture conditions used to mix CD45.1 and CD45.2 cells.

(B and C) Purified CSFE-labeled LN cells from GCN2^{KO} or GCN2^{WT} mice crossed to the OT-I TCR background were mixed with CFSE-labeled LN cells from CD45.1 mice in the ratios shown with OT-I peptide. After 72 hr, cells were stained for CD45.1 or CD45.2, proliferation was scored in either population, and data from the CD45.2 fraction are shown. Data are representative of three independent experiments (C).

(D) As in (B) but using purified CD8$^+$ T cells from GCN2^{KO} or GCN2^{WT} mice crossed to the OT-I TCR background mixed with bulk LN cells as APCs. This experiment was performed twice with similar results. Shown is a representative experiment where each data point represents an individual mouse.

(E) V405 staining for cell death quantification. Experiments are representative examples of two independently performed experiments.

GCN2 Pathway Activation in T Cells Is Not Specific for Tryptophan or TCR Activation

Previous work has closely linked GCN2 and tryptophan starvation induced by IDO proteins. Because GCN2 has been proposed to bind uncharged tRNAs (Gallinetti et al., 2013), one possible route to GCN2 activation is selective recognition of the uncharged tryptophan tRNA. To test specificity of GCN2 activation, we incubated GCN2^{WT} or GCN2^{KO} cells on an OT-I background to antigen-specific stimulation in the presence of limiting tryptophan, arginine, leucine, or lysine (Figure 6A). In each case, CHOP expression was induced in a GCN2-dependent way, and only when exposed to each limiting amino acid. Therefore, GCN2 activation by limiting amino acids is not specific for the tryptophan pathway in T cells, consistent with data from other cellular systems demonstrating GCN2 is a pan-amino acid sensor (Gallinetti et al., 2013).

As shown in Figure 5, GCN2-dependent CHOP expression requires activation through the TCR. These data raised the possibility that GCN2 activation was specific for TCR-mediated signals, or could be a consequence of proliferation, and unrelated to specific events downstream of the TCR (or co-stimulation pathways). To differentiate between these possibilities, we

limitation (100%, 10%, 1%, or 0% of the tryptophan concentration of RPMI). As expected from the results shown in Figure 5A, CHOP expression was GCN2 dependent and dependent on limiting tryptophan. However, CHOP expression was highly dependent on TCR activation coincident with tryptophan limitation (Figures 5C and 5D). We performed similar experiments in OVA-specific CD4$^+$ T cells and found a similar pattern to CD8$^+$ T cells: detection of low tryptophan required GCN2 to activate CHOP, but only when cognate TCR stimulation was present (Figure 5E). These data suggest activation of GCN2, and subsequent activation of stress kinase transcription, is coupled to cell activation in addition to amino acid amounts, but remains independent of the signals that block entry into the cell cycle when amino acids are limiting.

(I) Relative CCR7 mRNA in GCN2^{KO} and GCN2^{WT} CD8$^+$ after 24 hr of TCR activation. Values were normalized to GAPDH mRNA.

(J and K) A total of 5 × 10^6 CFSE-labeled CD8$^+$ CD45.1 control and GCN2^{KO} CD45.2 T cells were transferred into *Rag1*$^{-/-}$ mice and the ratios, percentage (third panel), and surface CCR7 expression on CD8$^+$ T cells were assessed on transferred cells in the spleen, cervical, and mesenteric LNs at d3 (J) and d6 post-transfer (K).

(A–H) Data are representative of three independent experiments where four to six recipient Thy1.1 mice were used per time point. (I) Data are pooled data from four experiments where each data point represents an individual mouse. (J and K) Data are combined from two experiments replicating the reconstitution conditions in Figures 2C–2E. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

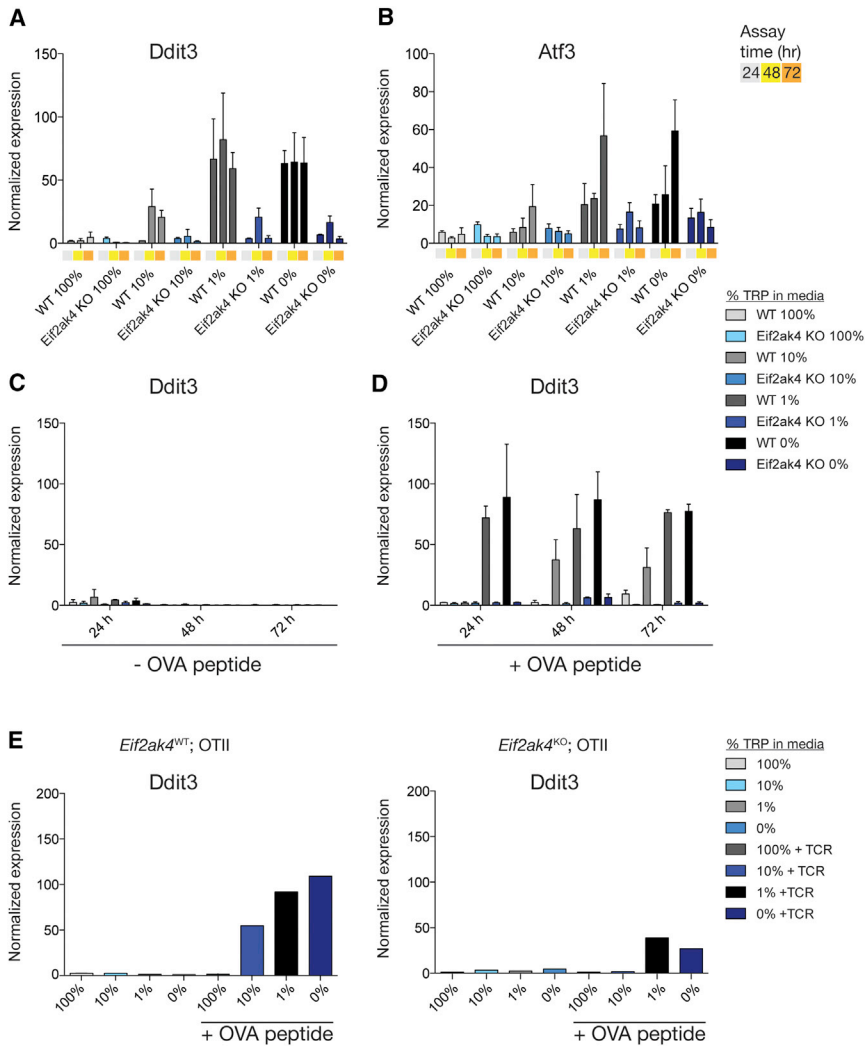


Figure 5. GCN2 Controls Stress-Response Gene Expression to Amino Acid Starvation and TCR Stimulation

(A) CHOP (Ddit3) and (B) Atf3 mRNA in CD8$^+$ T cells across time of culture (24, 48, and 72 hr shown under the abscissa) in limiting tryptophan. Color code indicates the sampling times (24, 48, or 72 hr). Data are combined from three to four independent experiments (mean ± SEM).

(C and D) Expression of CHOP (Ddit3) in CD8$^+$ T cells without (C) or with (D) T cell activation provided by APCs. Data are combined from two to three individual experiments (mean ± SEM).

(E) As in (C) and (D), but with CD4$^+$ T cells. Shown are the averaged data from three independently performed experiments.

Collectively, the data in Figures 5 and 6 demonstrate that stress kinase activation through the GCN2 pathway requires two signals in T cells; one is mediated by limiting amino acids and is not specific for any one amino acid, and a second signal is linked to proliferation.

DISCUSSION

We established GCN2 has specific effects in regulating T cell proliferation and trafficking. Three significant findings from our study were that proliferating CD8$^+$ T cells have an obligate, but not absolute, requirement for GCN2 that was not observed in CD4$^+$ T cells. A second finding concerned the independent nature of amino acid-sensing mechanisms in T cells. A general GCN2-independent pathway detects when environmental amino acids are limiting and blocks cell-cycle entry after activation. However, this pathway is unconnected, or operates in parallel to the GCN2 pathway, which activates the canonical stress kinase response including CHOP and Atf3 expression in low amino acids. A third and unanticipated finding was that the transcriptional program activated by GCN2 in low amino acids was dependent on entry into the cell cycle, suggesting the GCN2 stress kinase response is required for coordinating events linked to proliferative cycles in T cells.

We noted from in vitro experiments that GCN2-deficient CD8$^+$ T cells did not always proliferate in an identical way to controls on the same TCR background. Recent studies showed GCN2 has an important role in TLR-mediated dendritic cell activation and antigen presentation, suggesting part of the defect observed in GCN2^{KO} CD8$^+$ cells could have been a property of a requirement for GCN2 in the APCs in the culture (Ravindran et al., 2014). To distinguish between T cell-intrinsic versus APC-intrinsic effects of GCN2, we used experiments where CD45.1 splenocytes were mixed in defined ratios with GCN2^{KO}; OT-I or GCN2^{WT}; OT-I controls. The results showed GCN2 is intrinsically required

"bypassed" the TCR by stimulating with PMA and ionomycin, which supplies a strong TCR-independent proliferative signal to T cells. We observed CHOP expression was activated only in limiting tryptophan as before and required a general proliferative signal, because either the TCR or PMA/ionomycin was able to induce CHOP expression in 1% tryptophan (Figure 6B).

Finally, we tested the effect of GCN2 on eIF2α Ser51 phosphorylation. Recent studies in fibroblasts lacking all four stress kinases (GCN2, PERK, HRI, and PKR) have confirmed that no other kinases phosphorylate eIF2α (Taniuchi et al., 2016). However, the phosphorylation of eIF2α is countered by the Ppp1r15a/Gadd34 phosphatase, which terminates stress kinase signaling (Tsaytler et al., 2011). We therefore isolated CD8$^+$ T cells from GCN2^{WT} or GCN2^{KO} cells on an OT-I background and measured p-Ser51 on eIF2α across time of stimulation using validated and highly specific mAbs (Taniuchi et al., 2016). We found p-Ser51 eIF2α in all samples at time zero, which may reflect stress from processing and purifying the T cells. Over time, however, we found p-Ser51 eIF2α was largely dependent on GCN2, but independent of low amino acid amounts.

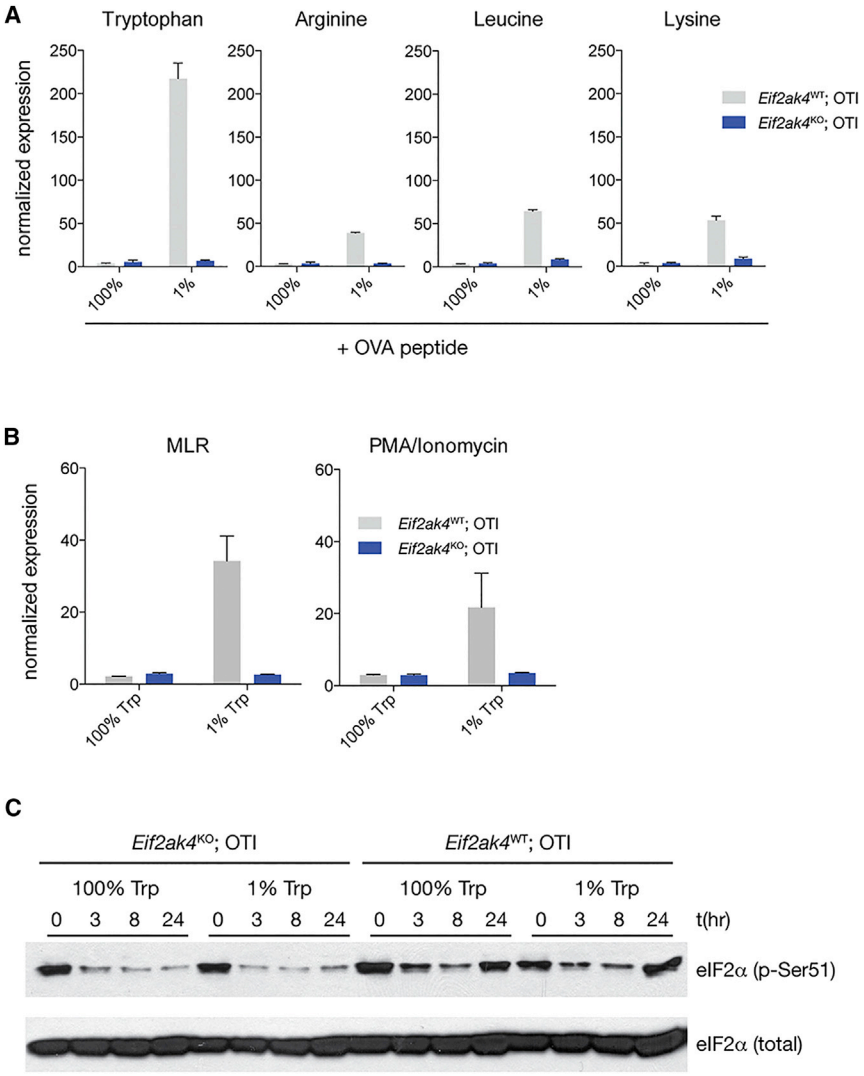


Figure 6. CHOP Expression Requires Proliferation and GCN2

(A) Purified CD8$^+$ cells from GCN2^{KO} or GCN2^{WT} mice crossed to the OT-I TCR background were incubated in control (normal RPMI with 10% serum, or 5% serum when limiting tryptophan was used) or in RPMI containing 1% tryptophan, arginine, leucine, or lysine were 1% in the presence of cognate OVA peptide. CHOP expression was measured after 24 hr. Data are the combined results of three independent experiments.

(B) As in (A) using limiting tryptophan, but activating T cells with PMA/ionomycin for 24 hr. Data are pooled from two to three independent experiments.

(C) Activation of p-Ser51 on eIF2α in CD8$^+$ T cells activated in RPMI containing 100% or 1% tryptophan. Data are representative of two independent experiments.

for CD8$^+$ proliferation under conditions of direct competition in a closed system. Our data are consistent with another study where retroviral-mediated transfer of dominant-negative GCN2 killed T cells undergoing antigen stimulation (Scheu et al., 2006). In that study, activation of the stress kinase response was closely tied to TCR stimulation. When considered together, our data and that of Scheu et al. (2006) argue for a model where GCN2, and possibly other stress kinases, are activated by multiple pathways including the stress of proliferation.

In vivo, the effects of the absence of GCN2 in CD8$^+$ cells were more complex. Using different transfer models into immune-deficient and immune-intact backgrounds, we found GCN2 is required to regulate the trafficking of CD8$^+$ T cells. In the absence of GCN2, CD8$^+$ T cells predominantly home to the spleen, which we discovered was linked to increased CCR7 expression in GCN2-deficient cells. Homing of GCN2^{KO} cells to the spleen was a universal finding in all transfer models we tested and in part obscured our initial investigation into Ag-specific proliferation in lungs or draining lymph nodes. In the lung, GCN2^{KO} CD8$^+$ T cells had lowered proliferative capacity compared with co-injected controls. However, these data must be interpreted in light of the fact that most GCN2^{KO} cells migrate to the spleen and are not in numerically equal competition with wild-type cells in the same organ. Further work will be necessary to tease apart the molecular pathways that emanate from GCN2 to cause the specific defects we observed. Clearly, GCN2 is required for more than amino acid sensing.

We found GCN2 has a greater cell-intrinsic role in CD8$^+$ T cells compared with CD4$^+$ cells. Consistent with our data, another group showed CD4$^+$ GCN2 had no apparent in vivo role in amino acid sensing (Cobbold et al., 2009). However, Scheu et al. (2006) also used CD4$^+$ T cells to demonstrate the vital role of GCN2 in allowing T cell proliferation. One explanation for these data concerns the way the experiments were performed. In the genetic systems we used, CD4$^+$ T cells may compensate for the loss of GCN2 as they develop and populate lymphoid tissue. By contrast, Scheu et al. (2006) used wild-type CD4$^+$ T cells and introduced a dominant-negative GCN2; under these conditions CD4$^+$ T cells may not be able to adjust their stress responses quickly enough and die. Therefore, we propose GCN2 is a component of an overlapping and partially redundant network of kinases that work in a lymphocyte-specific way.

Contradictory data have been published on the GCN2:IDO connection. The Munn and Mellor model proposed that the absence of GCN2 should cause T cells to ectopically enter into the cell cycle in low tryptophan, which through IDO protein expression in neighboring cells leads to a central immunoregulatory checkpoint (Munn and Mellor, 2013; Munn et al., 2005). Yet the GCN2^{KO} mice, or GCN2 mice with a complete deficiency (Costa-Mattioli et al., 2005), have not been reported to manifest

autoimmune diseases. Further, in our hands, mice lacking IDO1 and IDO2 are immunologically normal after ~1.5 years in conventional housing (Van de Velde et al., 2016). Furthermore, IDO1 was shown to have an irreplaceable role in protecting the "host" from the extreme inflammation associated with T cell-mediated graft-versus-host disease. However, the effect of IDO1 in this setting was independent of GCN2 expression in T cells (Jasperson et al., 2009). Other data have argued GCN2 is required for arginine sensing (Highfill et al., 2010; Rodriguez et al., 2007); like our experiments with tryptophan, we were unable to confirm a link between GCN2 and arginine sensing that permits cell-cycle entry. Instead, we suggest GCN2 functions to regulate the stress of T cell proliferative expansion specifically in cytotoxic T cells rather than functioning as a "sensor" of environmental amino acids necessary for coupling to cell-cycle entry. In this model GCN2 may determine amounts of uncharged tRNA inside the cell, although there is no direct evidence to suggest this is true: GCN2 may have different mechanisms to control cell division. From our experiments, we can exclude the possibility that GCN2 is exclusively required for sensing uncharged tryptophan tRNAs, because CHOP expression was GCN2 dependent under arginine, leucine, or lysine starvation.

Recent studies using fibroblasts lacking all four stress kinases were used to confirm that this kinase family is solely responsible for phosphorylation of Ser51 on eIF2α, the key event in activation of stress kinase signaling. Using these cells reconstituted with each individual stress kinase, Taniuchi et al. (2016) established that multiple signals activate GCN2, including salt, arsenic, and hydrogen peroxide. These signals may eventually impinge on the ability of cells to make enough charged tRNAs and thus activate GCN2. Nevertheless, the activation of GCN2 appears more complex than previously assumed. Indeed, in our experiments, some eIF2α p-Ser51 was GCN2 dependent, but was independent of tryptophan amounts in the cultures. To precisely connect the molecular events linking low amino acids, GCN2 activation, eIF2α phosphorylation, and eventual activation of stress transcription, a systematic comparison of mutants in each step of the pathway in antigen-specific T cells, including T cells bearing eIF2α Ser51 > Ala51, will be necessary.

We note that the initial proposal that GCN2 controls the ability of mice to sense (and avoid) meals depleted of essential amino acids has recently been revisited (Leib and Knight, 2015; Maurin et al., 2005). Newer findings suggest mice cannot immediately discriminate between food lacking or replete with essential amino acids. After a few days of exposure of amino acid-deficient food, mice adapt their behavior to seek out normal food. However, this behavior modification is independent of GCN2 (Leib and Knight, 2015), in contrast with the initial reports that GCN2 controls feeding behavior (Maurin et al., 2005).

GCN2 has been connected to mTOR activation in some studies. For example, GCN2 was essential for leucine-mediated activation of mTORC1 in embryonic fibroblasts (Averous et al., 2016). However, in our experiments in primary T cells, mTORC1 activity is coupled to activation of the T cell receptor and independent of amino acid amounts in the media (L.V.d.V. and P.J.M., unpublished data), suggesting amino acid sensing and response pathways, including GCN2 and mTORC1, may have cell-type specificity in their activation and connection to the necessary downstream metabolic pathways (Lee et al., 2016; Murray, 2016). Therefore, biochemical approaches will be necessary to establish the cell-intrinsic effects of GCN2, how GCN2 cooperates with the other stress kinase members and the mTOR pathway, and how the precise connection between amino acid amounts and GCN2 activity are mediated.

EXPERIMENTAL PROCEDURES

Mice

GCN2^{KO} (B6.129S6-*Eif2ak4*$^{tm1.2Dron}$/J), OT-I [C57BL/6-Tg(*TcraTcrb*)1100Mjb/J], OT-II [B6.Cg-Tg(*TcraTcrb*)425Cbn/J], CD45.1 (B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ), and *Rag1*$^{-/-}$ (B6.129S7-Rag1^{tm1Mom}/J) mice were from The Jackson Laboratory. GCN2^{KO} mice were crossed with OT-I, OT-II, or DO11.10 mice and genotyped using primers described on The Jackson Laboratory website (https://www.jax.org/jax-mice-and-services). Results from the GCN2^{KO} genotyping PCRs were as described on The Jackson Laboratory website. The junction point of the GCN2^{KO} mutation was confirmed by cloning the recombination point generated by Ron and colleagues using PCR primers and sequencing of cloned fragments from cDNAs made from fresh bone marrow (Maurin et al., 2005). All animals were maintained and used according to the policies and procedures of the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee under approved protocols to P.J.M. or P.G.T.

Antibodies and Reagents

The following anti-mouse antibodies were used for flow cytometric analysis: anti-CD4 (clone RM4-5; BioLegend), anti-CD8a (clone 53-6.7; BioLegend), anti-CD45.1 (clone A20; BioLegend), anti-CD45.2 (clone 104; BioLegend), anti-CD25 (clone PC61; BioLegend), anti-CD45RB (clone 16A; BD Biosciences), anti-B220 (clone Ra3-6B2; BD Biosciences), anti-CD11c (clone L3; BD Biosciences), anti-Thy1.1 (clone OX-7; BioLegend), anti-Thy1.2 (clone 53-2.1; BioLegend), and anti-CCR7 (clone 4B12; BioLegend). Antibodies for total eIF2α and p-Ser51 eIF2α were from Cell Signaling (clones D7D3 and D9G8 rabbit XP mAbs). OVA 257–264 and OVA 323–339 peptides were purchased from American Peptide Company. Live/Dead Fixable Violet was purchased from Invitrogen.

Specialized Media Formulations and Modifications

For non-titration-related experiments, RPMI 1640 (Corning) was supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and penicillin-streptomycin (Gibco). Tryptophan-free RPMI 1640 was custom-made by Corning, by modifying item #10040 to exclude L-tryptophan. Tryptophan-free RPMI 1640 was supplemented with either 1 mg/mL BSA (HyClone), 10 μg/mL bovine insulin (Sigma-Aldrich), 5 μg/mL iron-saturated transferrin (Sigma-Aldrich) and penicillin-streptomycin, or 5% dialyzed FBS (Gibco) and penicillin-streptomycin. For L-arginine, L-leucine, and L-lysine titration experiments, SILAC RPMI 1640 (Sigma) was supplemented with 10% dialyzed FBS and penicillin-streptomycin. Cell culture grade amino acids were purchased from Sigma.

T Cell Isolation and Purification

Lymph nodes (LNs) were isolated from all regions of the body except the mesenteric LN. Single-cell suspensions were obtained by grinding the LN through a 70 μm nylon mesh. Purified CD4^{+} or CD8^{+} populations were obtained by negative selection. In brief, LN cells were incubated with biotinylated anti-CD45R (B220, clone RA3-6B2), anti-I-A/I-E (clone M5/114.15-2), anti-CD11b (clone M1/70), anti-CD49b (clone DX5), and either anti-CD4 (clone GK1.5) or anti-CD8α (clone 53-607) (BioLegend). Cells were washed and incubated with streptavidin microbeads (Miltenyi), washed and passed over a magnetic column (Miltenyi), and the negative fraction collected. Cells were then labeled with 2 μM CFDA-SE (Invitrogen) and resuspended in the appropriate media for the given assay.

Proliferation Assays

For Arg, Leu, or Lys titrations, thioglycollate-elicited peritoneal macrophages from C57BL/6 mice were plated out at a density of 1 × 10^{5} cells/well (OT-I

system) or 8.5 × 10^5 cells/well (OT-II system) in 24-well plates. Cells were allowed to adhere for 2 hr and the media were changed to remove non-adherent cells. The following day, the media were changed and complete SILAC RPMI containing the appropriate amino acid amounts, 0.1 μM OVA peptide, and 1 × 10^6 T cells were added per well. Proliferation was assessed at 72 hr by flow cytometry.

Mixed Leukocyte Reactions

Antigen-presenting cells (APCs) were obtained by depleting $CD3^+$ cells from C57BL/6 splenocytes using a CD3 microbead kit (Miltenyi) according to the manufacturer's instructions. In 96-well flat-bottom plates, either 5 × 10^4 APCs (OT-I system) or 2 × 10^5 APCs (OT-II system) were mixed with 5 × 10^5 T cells in media containing a final concentration of 0.1 μM OVA peptide and the appropriate concentration of L-tryptophan. Proliferation was assessed at 72 hr by flow cytometry.

pDC Assay

The pDC assay was performed as described earlier, with the exception being B16 cells were used in place of B16-GM-CSF cells (Munn et al., 2005). A total of 1.5 × 10^6 B16-OVA melanoma cells or 3 × 10^6 EG7 thymoma cells were implanted subcutaneously in the flank region of C57BL/6 mice. After 14 days, tumor draining LNs were harvested, and single-cell suspensions were treated with Fc block for 10 min prior to surface staining with anti-CD45R and anti-CD11c, and the pDC population ($CD45R^+$ $CD11c^+$) was sorted. In 96-well round-bottom plates, 2.5 × 10^3 pDCs were mixed with 1 × 10^5 $OT\text{-}I^+$ T cells in complete RPMI 1640 containing 0.1 μM OVA peptide. L-tryptophan was added in excess as indicated in the figure legends. Proliferation was assessed at 72 hr by flow cytometry.

Asparaginase Formulations and Assays

Asparaginase (10,000 U/vial; Lundbeck) was diluted in 1 mL of PBS and stored at −80°C. Lymph node cells from OT-I or OT-II transgenic mice were labeled with 2 μM CFSE, washed, and distributed in a 96-well plate at 1 × 10^6 cells/well. Cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies from eBioscience at final concentrations of 1.0 and 3.0 μg/mL, respectively. Asparaginase was immediately added at 0, 0.1, or 1.0 U/well (200 μL final volume). Proliferation of $CD8^+$ or $CD4^+$ T cells was evaluated by flow cytometry after 72 hr.

RNA Isolation and Analysis

$CD4^+$ or $CD8^+$ T cells were purified from MLR using negative selection or by cell sorting. Cells were lysed in TRIzol (Invitrogen) for subsequent RNA isolation according to the manufacturer's protocol. cDNA was synthesized using SuperScript II reverse transcriptase, random and oligo dT primers (Invitrogen). Eif2ak4, Ddit3, Atf4, and Ccr7 primer sets were purchased from Qiagen. SYBR Green incorporation (Applied Biosystems) was measured using an ABI Prisms 7300 thermocycler (Applied Biosystems). All values were normalized to GAPDH mRNA.

T_{eff} Colitis Assays

T_{eff} ($CD4^+$, $CD45RB^{hi}$, $CD25^-$) from $GCN2^{KD}$ or $GCN2^{WT}$ mice were sorted on an iCyt high-speed sorter and transferred into $Rag1^{-/-}$ recipient mice (5 × 10^5, intraperitoneally). Mice were monitored for signs of colitis (diarrhea, general body condition) over an ~8- to 10-week period and weighed three times per week. Any animal losing 20% of the starting weight was removed from the study and considered an endpoint. Mice were further analyzed by anti-CD3 immunohistochemistry of the intestines.

Rag1-Deficient Mice Reconstitution and Immunization

$Rag1^{-/-}$ mice were retro-orbitally injected with 5 × 10^6 $CD8^+$ OTI cells from both $GCN2^{KO}$ $CD45.2^+$ and $GCN2^{WT}$ CD45.1 mice. For homeostatic expansion experiments, spleens, cervical LNs, and mesenteric LNs were harvested 6 days post-transfer, and $CD8^+$ populations were assessed. For immunization experiments, 25 μL of a 1:1 emulsion of incomplete Freund's adjuvant (Thermo Fisher Scientific) and 1 mg/ml ovalbumin protein (Sigma-Aldrich) in PBS were injected into the footpad 24 hr post-cell transfer. At 6 days post-immunization, the draining popliteal LN, cervical LNs, and mesenteric LNs were harvested, and $CD8^+$ populations were assessed.

OT-I Transfer Assays and Influenza Infections

A total of 10^4 $CD8^+$ $CD45.1^+$ OT-I wild-type or $CD45.2^+$ GCN2-deficient T cells purified as described earlier and labeled with CFSE were injected retro-orbitally into recipient Thy1.1 congenic animals. One day later, the mice were infected with 10^5 EID_{50} of X31 OT-I influenza virus intranasally. On days 3, 6, and 12 after infection, animals were euthanized and the draining mediastinal lymph node, bronchoalveolar lavage, and spleen were sampled and analyzed for the proliferation and phenotype of the transferred OT-I cells.

Statistics

All numerical data were processed in GraphPad Prism. Statistical comparisons were made using t testing, where 0.05 was considered significant and a normal distribution was assumed. The p values are reported as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.10.079.

AUTHOR CONTRIBUTIONS

L.V.d.V. did the majority of experiments. A.M.S. initiated the project. L.B. did the asparaginase experiments. L.V.d.V. and P.J.M. bred and analyzed mice. P.G.T., X.J.G., T.H.O., and L.V.d.V. did the influenza experiments. P.J.M. conceived the study, analyzed data, and wrote the manuscript with input from P.G.T. and L.V.d.V.

ACKNOWLEDGMENTS

We thank the St. Jude Children's Research Hospital Department of Immunology Flow Core for sorting and Linda Hendershot for discussion. This work was supported by NIH grant AI062921, Cancer Center Core Grant P30 CA21765, and the American Lebanese Syrian Associated Charities.

Received: January 13, 2016
Revised: October 6, 2016
Accepted: October 21, 2016
Published: November 22, 2016

REFERENCES

Averous, J., Lambert-Langlais, S., Mesclon, F., Carraro, V., Parry, L., Jousse, C., Bruhat, A., Maurin, A.C., Pierre, P., Proud, C.G., and Fafournoux, P. (2016). GCN2 contributes to mTORC1 inhibition by leucine deprivation through an ATF4 independent mechanism. Sci. Rep. *6*, 27698.

Ball, H.J., Jusof, F.F., Bakmiwewa, S.M., Hunt, N.H., and Yuasa, H.J. (2014). Tryptophan-catabolizing enzymes - party of three. Front. Immunol. *5*, 485.

Bessede, A., Gargaro, M., Pallotta, M.T., Matino, D., Servillo, G., Brunacci, C., Bicciato, S., Mazza, E.M., Macchiarulo, A., Vacca, C., et al. (2014). Aryl hydrocarbon receptor control of a disease tolerance defence pathway. Nature *511*, 184–190.

Cobbold, S.P., Adams, E., Farquhar, C.A., Nolan, K.F., Howie, D., Lui, K.O., Fairchild, P.J., Mellor, A.L., Ron, D., and Waldmann, H. (2009). Infectious tolerance via the consumption of essential amino acids and mTOR signaling. Proc. Natl. Acad. Sci. USA *106*, 12055–12060.

Costa-Mattioli, M., Gobert, D., Harding, H., Herdy, B., Azzi, M., Bruno, M., Bidinosti, M., Ben Mamou, C., Marcinkiewicz, E., Yoshida, M., et al. (2005). Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2. Nature *436*, 1166–1173.

Dong, J., Qiu, H., Garcia-Barrio, M., Anderson, J., and Hinnebusch, A.G. (2000). Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain. Mol. Cell *6*, 269–279.

Donnelly, N., Gorman, A.M., Gupta, S., and Samali, A. (2013). The eIF2α kinases: their structures and functions. Cell. Mol. Life Sci. *70*, 3493–3511.

Förster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Müller, I., Wolf, E., and Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell *99*, 23–33.

Gallinetti, J., Harputlugil, E., and Mitchell, J.R. (2013). Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR. Biochem. J. *449*, 1–10.

Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell *6*, 1099–1108.

Haverkamp, J.M., Smith, A.M., Weinlich, R., Dillon, C.P., Qualls, J.E., Neale, G., Koss, B., Kim, Y., Bronte, V., Herold, M.J., et al. (2014). Myeloid-derived suppressor activity is mediated by monocytic lineages maintained by continuous inhibition of extrinsic and intrinsic death pathways. Immunity *41*, 947–959.

Highfill, S.L., Rodriguez, P.C., Zhou, Q., Goetz, C.A., Koehn, B.H., Veenstra, R., Taylor, P.A., Panoskaltsis-Mortari, A., Serody, J.S., Munn, D.H., et al. (2010). Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. Blood *116*, 5738–5747.

Jasperson, L.K., Bucher, C., Panoskaltsis-Mortari, A., Mellor, A.L., Munn, D.H., and Blazar, B.R. (2009). Inducing the tryptophan catabolic pathway, indoleamine 2,3-dioxygenase (IDO), for suppression of graft-versus-host disease (GVHD) lethality. Blood *114*, 5062–5070.

Jenkins, M.R., Webby, R., Doherty, P.C., and Turner, S.J. (2006). Addition of a prominent epitope affects influenza A virus-specific CD8+ T cell immunodominance hierarchies when antigen is limiting. J. Immunol. *177*, 2917–2925.

Lageix, S., Rothenburg, S., Dever, T.E., and Hinnebusch, A.G. (2014). Enhanced interaction between pseudokinase and kinase domains in Gcn2 stimulates eIF2α phosphorylation in starved cells. PLoS Genet. *10*, e1004326.

Lee, J.H., Cho, U.S., and Karin, M. (2016). Sestrin regulation of TORC1: is Sestrin a leucine sensor? Sci. Signal. *9*, re5.

Leib, D.E., and Knight, Z.A. (2015). Re-examination of dietary amino acid sensing reveals a GCN2-independent mechanism. Cell Rep. *13*, 1081–1089.

Maurin, A.C., Jousse, C., Averous, J., Parry, L., Bruhat, A., Cherasse, Y., Zeng, H., Zhang, Y., Harding, H.P., Ron, D., and Fafournoux, P. (2005). The GCN2 kinase biases feeding behavior to maintain amino acid homeostasis in omnivores. Cell Metab. *1*, 273–277.

Moffett, J.R., and Namboodiri, M.A. (2003). Tryptophan and the immune response. Immunol. Cell Biol. *81*, 247–265.

Muller, A.J., Sharma, M.D., Chandler, P.R., Duhadaway, J.B., Everhart, M.E., Johnson, B.A., 3rd, Kahler, D.J., Pihkala, J., Soler, A.P., Munn, D.H., et al. (2008). Chronic inflammation that facilitates tumor progression creates local immune suppression by inducing indoleamine 2,3 dioxygenase. Proc. Natl. Acad. Sci. USA *105*, 17073–17078.

Munn, D.H., and Mellor, A.L. (2013). Indoleamine 2,3 dioxygenase and metabolic control of immune responses. Trends Immunol. *34*, 137–143.

Munn, D.H., Sharma, M.D., Baban, B., Harding, H.P., Zhang, Y., Ron, D., and Mellor, A.L. (2005). GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity *22*, 633–642.

Murray, P.J. (2016). Amino acid auxotrophy as a system of immunological control nodes. Nat. Immunol. *17*, 132–139.

Ravindran, R., Khan, N., Nakaya, H.I., Li, S., Loebbermann, J., Maddur, M.S., Park, Y., Jones, D.P., Chappert, P., Davoust, J., et al. (2014). Vaccine activation of the nutrient sensor GCN2 in dendritic cells enhances antigen presentation. Science *343*, 313–317.

Rodriguez, P.C., Quiceno, D.G., and Ochoa, A.C. (2007). L-arginine availability regulates T-lymphocyte cell-cycle progression. Blood *109*, 1568–1573.

Rubnitz, J.E., Onciu, M., Pounds, S., Shurtleff, S., Cao, X., Raimondi, S.C., Behm, F.G., Campana, D., Razzouk, B.I., Ribeiro, R.C., et al. (2009). Acute mixed lineage leukemia in children: the experience of St Jude Children's Research Hospital. Blood *113*, 5083–5089.

Scheu, S., Stetson, D.B., Reinhardt, R.L., Leber, J.H., Mohrs, M., and Locksley, R.M. (2006). Activation of the integrated stress response during T helper cell differentiation. Nat. Immunol. *7*, 644–651.

Sharma, N., Benechet, A.P., Lefrançois, L., and Khanna, K.M. (2015). CD8 T cells enter the splenic T cell zones independently of CCR7, but the subsequent expansion and trafficking patterns of effector T cells after infection are dysregulated in the absence of CCR7 migratory cues. J. Immunol. *195*, 5227–5236.

Taniuchi, S., Miyake, M., Tsugawa, K., Oyadomari, M., and Oyadomari, S. (2016). Integrated stress response of vertebrates is regulated by four eIF2α kinases. Sci. Rep. *6*, 32886.

Tsaytler, P., Harding, H.P., Ron, D., and Bertolotti, A. (2011). Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis. Science *332*, 91–94.

Unsoeld, H., Voehringer, D., Krautwald, S., and Pircher, H. (2004). Constitutive expression of CCR7 directs effector CD8 T cells into the splenic white pulp and impairs functional activity. J. Immunol. *173*, 3013–3019.

Van de Velde, L.A., Gingras, S., Pelletier, S., and Murray, P.J. (2016). Issues with the specificity of immunological reagents for murine IDO1. Cell Metab. *23*, 389–390.

Workman, C.J., Collison, L.W., Bettini, M., Pillai, M.R., Rehg, J.E., and Vignali, D.A. (2011). In vivo Treg suppression assays. Methods Mol. Biol. *707*, 119–156.