Ndfip-mediated degradation of Jak1 tunes cytokine signalling to limit expansion of CD4+ effector T cells

Claire E. O’Leary1, Christopher R. Riling1, Lynn A. Spruce2, Hua Ding2, Suresh Kumar3, Guoping Deng2, Yuhong Liu4, Steven H. Seeholzer2 & Paula M. Oliver1,2

Nedd4 family E3 ubiquitin ligases have been shown to restrict T-cell function and impact T-cell differentiation. We show here that Ndfip1 and Ndfip2, activators of Nedd4 family ligases, together limit accumulation and function of effector CD4+ T cells. Using a three-part proteomics approach in primary T cells, we identify stabilization of Jak1 in Ndfip1/2-deficient T cells stimulated through the TCR. Jak1 degradation is aborted in activated T cells that lack Ndfips. In wild-type cells, Jak1 degradation lessens CD4+ cell sensitivity to cytokines during TCR stimulation, while in Ndfip-deficient cells cytokine responsiveness persists, promoting increased expansion and survival of pathogenic effector T cells. Thus, Ndfip1/Ndfip2 regulate the cross talk between the T-cell receptor and cytokine signalling pathways to limit inappropriate T-cell responses.
Integration of signals from T-cell receptor (TCR), co-receptors and cytokine receptors directs proliferation, survival and differentiation of T cells. Cross talk among these pathways is essential to prevent aberrant T-cell responses. One example of such cross talk is TCR-induced downregulation of cytokine receptor signalling to limit cytokine responses\(^ {1-4}\). Ubiquitylation of protein substrates by E3 ubiquitin ligases can regulate both the binding and ubiquitylation assays suggest that Ndfip1 and Ndfip2 are both sufficient to activate the catalytic function of Nedd4-family E3 ligases. However, whether Ndfip2 might also play a role in T cells, we generated Ndfip2 knockout mice by insertion of GFP into exon 2 of the Ndfip2 gene, putting subsequent exons out of frame (Supplementary Fig. 1a–c). We observed Mendelian frequencies of Ndfip2\(^ {\text{+/+}}\) mice (Supplementary Fig. 1d), in contrast to the sub-Mendelian frequency of Ndfip1\(^ {\text{+/+}}\) mice (Supplementary Fig. 1e), at weaning.

Using Ndfip2\(^ {+/+}\) mice, which express one copy of GFP under the Ndfip2 promoter, we analysed GFP as a reporter of Ndfip2 expression. In splenocytes, we observed the highest GFP expression in T cells (Supplementary Fig. 2a). In stimulated Ndfip2\(^ {+/+}\) T cells, GFP\(^ {\text{high}}\) cells increased in abundance over the course of stimulation and corresponded with CD44 expression, high CD25 expression and cell division (Supplementary Fig. 2b,c).

**Ndfip2**\(^ {+/+}\) mice do not show signs of inflammation. As our GFP reporter indicated high Ndfip2 expression in T cells, we focused our analysis of Ndfip2\(^ {+/+}\) mice on the T-cell compartment. Ndfip2\(^ {+/+}\) mice had normal thymic populations and normal splenic CD4\(^ +\) and CD8\(^ +\) T cell percentages in lymph nodes and spleens (Fig. 1a; Supplementary Fig. 3a). Ndfip2\(^ {+/+}\) mice had no increase in percent of CD44\(^ +\) T cells or cytokine production upon ex vivo analysis (Fig. 1b,c; Supplementary Fig. 3a). Helper T-cell differentiation in vitro, measured by expression of lineage defining transcription factors and cytokine production, was similar between Ndfip2\(^ {+/+}\) and control cells (Supplementary Fig. 3b,c).

If Ndfip1 and Ndfip2 have overlapping molecular functions, expression of Ndfip1 might mask effects of Ndfip2 in immune cells. Ndfip1 mRNA expression is increased on T-cell activation, consistent with its role limiting aberrant activation and cytokine production in stimulated naive CD4\(^ +\) T cells.\(^ {3,20}\) Comparing expression of Ndfip1 and Ndfip2 mRNA during CD4\(^ +\) T cell stimulation revealed that Ndfip1 was more robustly induced on initial stimulation than Ndfip2 (Fig. 1d). However, Ndfip1 and Ndfip2 both increased in expression following re-stimulation. Together with our GFP reporter data, these data support that Ndfip2 expression is increased in newly activated CD4\(^ +\) T cells, but more strongly induced during stimulation of previously activated T cells.

**Ndfip2** deficiency exacerbates inflammation in Ndfip1\(^ {\text{KO}}\) mice. To test whether Ndfip1 expression in Ndfip2\(^ {+/+}\) mice masked effects of Ndfip2 deficiency, we generated mice doubly deficient in Ndfip1 and Ndfip2. The number of double knockout (DKO) pups was unexpectedly low at weaning, and foetal analysis indicated sub-Mendelian frequencies (Supplementary Table 1). We next generated mice doubly deficient for Ndfip1 and Ndfip2 in T cells by crossing Ndfip2\(^ {+/+}\) with CD4\(^ +\) Cre\(^ +\) mice. Ndfip1\(^ {fl/fl}\)CD4\(^ +\) Cre\(^ +\) mice (cKO) exhibit T-cell-mediated, T\(_{\text{H}2}\)-biased inflammation similar to that observed in Ndfip1\(^ {+/+}\) mice.\(^ {20}\) We hypothesized that loss of Ndfip2 in Ndfip1 cKO mice would worsen the Ndfip1 cKO phenotype if Ndfip2 acts to prevent aberrant T-cell responses.

We observed that Ndfip2\(^ {+/+}\)Ndfip1\(^ {fl/fl}\)CD4\(^ +\) Cre\(^ +\) (referred to as cDKO) mice showed increased inflammation at barrier surfaces, increased spleen size and decreased body weight relative to age-matched cKO, Ndfip2\(^ {+/+}\) and control mice (Fig. 2a–c). Splenic CD4\(^ +\) T cells from cDKO mice were more likely to express CD44 (Fig. 2d) and produce IL-4 (Fig. 2e,i). We observed a significantly increased number of CD4\(^ +\) T cells in spleens from cDKO mice (Fig. 2f) due largely to an increased number of CD44\(^ +\) CD8\(^ +\) T cells (Fig. 2g); the number of CD62L\(^ +\) CD4\(^ +\) T cells in cDKO spleens was not statistically different from cKO and control mice (Fig. 2h).

**Results**

Generation of Ndfip2 knockout/GFP knock-in mice. Given that deficiency in either Itch or Ndfip1 leads to hyperactive T cells and T\(_{\text{H}2}\)-mediated pathology,\(^ {5,13,15}\) and knowing that Ndfip1 and Ndfip2 have similar functions in vitro,\(^ {2,16-19}\) we investigated whether Ndfip2 might also play a role in T cells. We generated Ndfip2 knockout mice by insertion of GFP into exon 2 of the Ndfip1 and Ndfip2 promoters, analysed Ndfip2 expression in T cells, and validated that Jak1 is aberrantly degraded in T cells lacking Ndfip1/Ndfip2. Jak1 is ubiquitylated on multiple lysine residues and degraded following TCR stimulation of WT cells; this degradation is aborted in Ndfip-deficient cells. Consistent with published data, this proteomic approach confirmed that the E3 ligases Itch and Nedd4-2 (also called Ndfip1 and Ndfip2) are both sufficient to activate the catalytic function of Nedd4-family E3 ligases.\(^ {2,16-19}\) Here we establish a role for Ndfip2 in regulating immune responses. Although Ndfip2\(^ {+/+}\) mice show no overt immunopathology, animals with T cells lacking both Ndfip1 and Ndfip2 have a dramatically increased pool of activated CD4\(^ +\) T cells compared with mice with T cells lacking only Ndfip1. To determine the mechanism whereby loss of Ndfip1/Ndfip2 drives expansion of effector cells, we developed an unbiased and reproducible proteomic workflow for use in primary T cells. Consistent with published data, this proteomic approach confirmed that the E3 ligases Itch and Nedd4-2 (also called Nedd4L) are active in an Ndfip-dependent manner in stimulated effector T cells.\(^ {2,16-19}\) We identified several candidate substrates for Ndfip-dependent degradation, and validated that Jak1 is aberrantly degraded in T cells lacking Ndfip1/Ndfip2. Jak1 is ubiquitylated on multiple lysine residues and degraded following TCR stimulation of WT cells; this degradation is aborted in Ndfip-deficient cells. Consistent with increased stability of Jak1, T cells lacking Ndfip1/Ndfip2 fail to undergo TCR-mediated downregulation of cytokine responsiveness, a Jak1-dependent process, as measured by STAT5 phosphorylation following exposure to IL-2. Increased Jak1 signalling led to increased survival and proliferation of these cells in vitro, while in vivo this drives an expanded population of pathogenic effector T cells. Our data reveal that TCR-induced cytokine non-responsive ness requires Ndfip-dependent degradation of Jak1. This is a previously unknown function for Ndfips in restricting cytokine signalling to limit expansion, and, consequently, pathogenicity, of CD4\(^ +\) effector T cells.
unstimulated CD4 T cells could lead to expansion of pathogenic Th2 effector T cells, even among CD44+ T cells lacking only Ndfip1 to be activated in vivo, and, once activated, are more proliferative and more likely to produce cytokine.

The increased percent of activated Ndfip-deficient CD4+ T cells suggested a competitive advantage of Ndfip deficiency among activated cells. We analysed this by determining the relative frequencies of CD45.2/CD45.1 cells in different T-cell populations, normalizing to the chimerism observed in IgM+ B cells from bone marrow. We found a significant increase in the relative frequency of CD44+ CD4+ DKO T cells isolated from spleen or lung (Fig. 3g). We observed a similar pattern for Ndfip1−/− and, surprisingly, Ndfip2−/− CD4+ T cells, although this did not achieve statistical significance. Thus, the
population of previously activated CD4+ T-effector cells is significantly expanded when both Ndfip1 and Ndfip2 are lacking.

**Ndfip1/Ndfip2-deficient CD4+ T cells cause increased colitis.**

Our data indicate that both Ndfip1 and Ndfip2 control effector cell numbers and pathogenicity. To test this we transferred naïve WT, Ndfip2−/−, Ndfip1 cKO and cDKO CD4+ T cells into RAG1−/− recipients to induce colitis. cDKO CD4+ T cells caused severe pathology: recipients had high spleen/body weight ratios, contracted colons and increased colon crypt depth (Fig. 4a–d). Recipients of Ndfip2−/− CD4+ T cells showed equivalent pathology to Ndfip1 cKO recipients—both cohorts developed more severe inflammation than WT cell recipients as measured by inflammation index, although colon histopathology, as quantified by crypt depth, was similar. Ndfip2−/− T cells, like WT cells, were IL-17A producers, while cDKO cells, like Ndfip1-deficient T cells, produced IL-4 (Fig. 4e). Thus, while Ndfip1 plays a dominant role in limiting Th12 differentiation, after initial activation both Ndfip1 and Ndfip2 limit the pathogenic potential of activated CD4+ T cells by limiting their expansion and function.

**Ndfip1/Ndfip2-deficient T cells outcompete WT cells in vitro.**

We next sought to model these phenotypic findings in vitro. When naïve CD4+ T cells were examined after 5 days of in vitro stimulation, cDKO cells showed increased GATA3 expression and proliferation relative to control cells (Supplementary Fig. 4a–c). We also observed an increase in cDKO cell viability relative to experiment-matched control cells (Supplementary Fig. 4d).

We next tested whether Ndfip-deficient CD4+ T cells could outcompete WT cells within the same cytokine environment. WT cells co-cultured with cDKO CD4+ T cells had increased GATA3 expression and proliferation, but this was significantly
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**Figure 3 | Ndfip deficiency causes intrinsic CD4+ effector T-cell expansion.** (a-g) Mixed foetal liver chimeras using CD45.2 WT, Ndfip1−/−, Ndfip2−/− or Ndfip1/Ndfip2 DKO foetal liver were analysed 6 weeks following reconstitution. (a) Representative CD44 and CD62L staining of splenic CD4+ T cells from Ndfip1−/− (top) and DKO mixed chimaera (bottom), previously gated on live, singlet CD3+CD4+CD45.1 or CD45.2+ cells. (b,c) Flow cytometry analysis of CD45.1+ and CD45.2+ splenic T cells from chimeras showing (b) percentages of CD44+ cells and (c) percentages of CD44+ that are Ki67+. (d-f) Ex vivo stimulated splenocytes were stained for IL-4. (d) Representative flow plots of IL-4+CD4+ T cells, (e) combined data from d, (f) percentages of CD44+CD4 T cells that are IL-4+. (g) Percentages of CD44.2+ cells among various T-cell subsets, normalized for reconstitution, as determined by the ratio for CD45.2:CD45.1 IgM + B220+ B cells in the bone marrow. Compartments analysed are as follows: A = IgM+B cells in bone marrow, B = double positive thymocytes, C = single positive CD4+ thymocytes, D = CD44+CD4+ T cells in spleen, E = CD44+CD4+T cells in lung. Data shown in b and e were pooled from two experiments, 7-8 chimeras per group; (c,e,f) have 4-5 chimeras per group. Quantifications are average ± s.e.m. P values calculated by two-way ANOVA (b,c,e,f) or repeated measures one-way ANOVA (g), with Holm-Sidak test for multiple comparisons: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

decreased relative to CDKO cells within the same culture (Supplementary Fig. 4e-i). cDKO continued to show enhanced viability—on day 5 cDKO CD4+ T cells significantly out-numbered WT cells in the same culture (Supplementary Fig. 4h,i). Thus, increased cytokine production in the absence of Ndfips is insufficient to explain increased viability and proliferation.

These data suggest that loss of both Ndfip1 and Ndfip2 leads to increased survival and proliferation of activated CD4+ T cells, resulting, in vivo, in an expanded population of previously activated effector cells. Furthermore, Ndfip-deficient CD4+ T cells outcompete WT T cells within the same cytokine milieu, suggesting that Ndfip-deficient T cells are more competent to respond to cytokines promoting division and survival.

**Identification of differential ubiquitylation by proteomics.** Having determined that Ndfip1 and Ndfip2 both negatively regulate activated effector CD4+ T cells, we wanted to understand the mechanism underlying the increased proliferation and survival of Ndfip-deficient CD4+ T cells. Both Ndfip1 and Ndfip2 have been shown in *vitro* to bind and activate several members of the Nedd4 family of E3 ubiquitin ligases. While putative substrates of Nedd4 family E3 ligases have been described in targeted studies, in primary lymphocytes unbiased screens to identify substrates of these and other ubiquitin ligases are lacking. To address this need, we developed a three-part proteomic workflow to identify Ndfip-dependent ubiquitylation in activated CD4+ T cells (Fig. 5a).

We first used stable isotope labelling of amino acids in cell culture (SILAC) in combination with tandem ubiquitin binding entities (TUBE)s to enrich polyubiquitylated proteins from mixed WT and Ndfip-deficient cell lysates. We reproducibly obtained SILAC ratios (WT/DKO) for ∼2,500 proteins (Fig. 5b). In parallel, we performed whole proteome analysis of DKO and WT CD4+ T cells. We then calculated the unenriched ‘input’
ratio (DKO/WT) for each protein and applied this correction to the TUBE-enriched SILAC ratio for the same protein across each biological replicate (Fig. 5c; Supplementary Fig. 5a,b and Supplementary Data 1).

Peptides derived from trypsin cleavage of ubiquitylated proteins contain a di-glycine remnant (K-e-GG) on modified lysine residues\(^\text{22}\). The vast majority of such diglycine peptides are derived from ubiquitin linkages\(^\text{23}\). To limit our analysis to proteins directly modified by ubiquitin, we utilized an antibody against the K-e-GG motif\(^\text{24,25}\) to enrich modified peptides from activated WT CD4\(^+\) T cells (Supplementary Data 2).

Analysing proteins with at least three TUBE–SILAC ratios and at least one modified lysine (~1,000 proteins, Fig. 5d) indicated good reproducibility across replicates (Fig. 5e; Supplementary Data 3). Thus, despite technical limitations imposed by analysing primary lymphocytes, this approach yields reproducible data with good depth of proteome coverage.

### Ndfip-dependent function of Nedd4 family ligases in T cells.

Ndfip1 and Ndfip2 bind and activate the enzymatic function of several Nedd4 family E3 ubiquitin ligases in vitro; once active, these ligases promote their own ubiquitylation\(^\text{12,16–18,26}\). Thus, in effector T cells K-e-GG peptides should be observed for active Nedd4 family ligases; if these same ligases depend on Ndfips for function, increased ubiquitylation in WT cells would result in high WT/DKO TUBE–SILAC ratios. We therefore sought to validate our proteomics screen by identifying Nedd4 family E3 ligases with Ndfip-dependent function in effector T cells.

Among Nedd4 family members, we consistently observed Itch, Nedd4-2 and WWP2 after TUBE immunoprecipitation; analysis of the whole proteome revealed increased abundance of Itch and Nedd4-2 in DKO T cells, while WWP2 was not consistently identified (Supplementary Data 1). Of the Nedd4 family ligases, only Itch and Nedd4-2 were observed following diglycine remnant immunoprecipitation (Supplementary Data 2). Correcting the TUBE–SILAC ratios (WT/DKO) for both Itch and Nedd4-2 to account for the increased abundance of these ligases in Ndfip-deficient T cells indicated significantly more ubiquitylation in WT cells compared with Ndfip-deficient cells (Fig. 5c; Supplementary Fig. 5c).

Itch is known to interact with, and be activated by, Ndfip1 in T cells, while Nedd4-2 has been shown to work with either Ndfip1 or Ndfip2 (refs 8,13). In Ndfip doubly deficient T cells, decreased activity of Itch/Nedd4-2 could indicate E3 ligase dependence on both Ndfip1 and Ndfip2, or either Ndfip1 or Ndfip2 alone. We first assessed binding of Ndfips to Nedd4 family E3 ligases using the cytoplasmic domains of either Ndfip1 or Ndfip2 to isolate the endogenous E3 ligases from T-cell lysates. Both Ndfip1 and Ndfip2 were capable of isolating Itch and Nedd4-2 from WT CD4\(^+\) T cells in this pulldown assay (Fig. 6a). Analysis of Ndfip1/2-deficient cells yielded similar results (Fig. 6a).

Next, we tested the ability of Ndfip1 and Ndfip2 to activate Itch enzymatic function, which is restrained through...
Figure 5 | Identification of differential ubiquitylation by proteomics. (a) Schematic of the three proteomics methods used. (b) Area proportional Venn diagram illustrating the reproducibility of proteins identified as having SILAC ratios after TUBE enrichment in three out of four biological replicates. (c) SILAC–TUBE ratios (WT/DKO) for each protein were corrected using ‘input’ ratio (DKO/WT) as calculated by label-free quantification of whole proteome DKO and WT data sets. The average corrected ratio is plotted against the average number of unique peptides observed per protein across four whole proteome LC-MS/MS experiments. Plot limited to 40 average unique peptides for clarity. Itch and Nedd4-2 (indicated) have log2 transformed corrected ratio > 1 at the protein level. Itch = 1.57 ± 0.17 and Nedd4-2 = 3.18 ± 0.226. (d) Overlap of proteins with corrected SILAC–TUBE ratios (observed in at least three experiments) and proteins identified with at least one K-ε-GG peptide (in at least one of the three K-ε-GG immunoprecipitation experiments). (e) Heat map illustrating reproducibility of corrected SILAC–TUBE ratios (WT/DKO, log2 transformed, observed in at least three of four experiments) for proteins identified with K-ε-GG peptides. Reverse = SILAC labeling swapped.

autoinhibition.12,27,28 Using a time-resolved fluorescence resonance energy transfer (TR-FRET) assay21, we observed Itch ubiquitylation activity only in the presence of Ndfip1 or Ndfip2 (Fig. 6b). While unequal purity of the GST fusion Ndfips made quantitative comparisons of activation difficult (Supplementary Fig. 5d), we can conclude that both Ndfip1 and Ndfip2 are sufficient to activate the E3 ligase function of Itch. We recently determined that Ndfip1 activates Itch catalytic activity by promoting ubiquitin charging of Itch29. However, the role of Ndfip2 in this process is unknown. We found that either Ndfip1 or Ndfip2 is sufficient for E2-mediated ubiquitin charging of Itch (Fig. 6c), indicating that Ndfip1 and Ndfip2 activate Itch via the same molecular mechanism. Thus, both Ndfips could promote the ubiquitylation activity of Itch observed in our proteomic analysis.

We then assessed whether Ned4-2 is activated by Ndfips. Under our cell-free in vitro conditions using the human homologue of Ned4-2, Ned4L, we observed that Ned4L was not autoinhibited; nevertheless, an increased rate of Ned4L activity was evident when Ndfip1/Ndfip2 were included in the reaction (Fig. 6d,e). Thus, while Ndfips are not required for function of Ned4L in these assays, they potentiate its catalytic activity. Both Itch and Ned4-2/Ned4L have been published to work with Ndfips in T cells, and are known to autoubiquylate when activated, indicating that our assay can identify Ndfip-dependent ligase activity and changes in Ndfip-dependent ubiquitylation in an unbiased fashion8,12,13,15,19,29.

Jak1 degradation is dependent on Ndfip1/Ndfip2. Having validated that our screen can identify Ndfip-dependent ubiquitylation, we turned to substrate identification. Candidate substrates of Ndfip-dependent ubiquitylation (Supplementary Data 4) had positive corrected TUBE–SILAC ratios (WT/DKO) in all replicates, low error across replicates, and multiple K-ε-GG peptides. We identified Jak1 and Jak2 as differentially ubiquylated in Ndfip-deficient cells, and observed K-ε-GG peptides from all Jak family members (Supplementary Fig. 6a,b). Analysis of Jak2, which is known to be monoubiquylated, and for which suppressor of cytokine signalling (SOCS)-mediated degradation has been demonstrated30, revealed that Jak2 is not robustly degraded during TCR stimulation (Supplementary Fig. 6c,d). Although Jak1 ubiquitylation has never been shown in T cells, it is known to have a short half-life3. Consistent with ubiquitylation of Jak1, we observed that several distinct lysines in Jak1 had the K-ε-GG motif. Jak1 is a key component in signalling via common γ chain containing cytokine receptors, and can drive survival, proliferation and differentiation of
consistent with published data. Thus increased levels ofJak1 and STAT5 did not show cycloheximide sensitivity (Fig. 7e,f),

Jak1 degradation was aborted (Supplementary Fig. 7a,b). As withstimulation was intact in Ndfip-deficient cells; however, furtherJak1 degradation during the first hours of TCR

deficient cells.
p-STAT5 are indicative of increased Jak1 activity in Ndfip-
cDKO CD4

STAT5 were not significantly increased in cDKO CD4
affected Jak1 stability and STAT5 activity (Fig. 7a–d). Levels of total

Jak1 is degraded in TCR-stimulated WT cells

of a minimum of three independent experiments.

Therefore, we hypothesized that Jak1 degradation in CD4+ T cells depends on Ndfip1 and Ndfip2.

Jak1 was robustly degraded in TCR-stimulated WT cells treated with cycloheximide, a translation inhibitor (Fig. 7a). Ndfip-deficient CD4+ T cells showed significantly less degradation (Fig. 7a,b). We also observed increased phosphorylation of STAT5a/b after stimulation of Ndfip-deficient cells compared with WT cells (Fig. 7c,d). We did not observe any change in Jak1 stability or p-STAT5a/b signal in CD4+ T cells from Ndfip1 cKO mice, suggesting that loss of Ndfip1 alone is not sufficient to affect Jak1 stability and STAT5 activity (Fig. 7a–d). Levels of total STAT5 were not significantly increased in cDKO CD4+ T cells, and STAT5 did not show cycloheximide sensitivity (Fig. 7e,f), consistent with published data. Thus increased levels of p-STAT5 are indicative of increased Jak1 activity in Ndfip-deficient cells.

A balance of protein synthesis and degradation controls Jak1 levels—during T cell activation, Jak1 translation is halted and existing Jak1 is degraded. For both WT and Ndfip-deficient cells, relative levels of Jak1 remaining after stimulation were not altered by cycloheximide (Fig. 7a,b,g). Ndfip-deficient cells showed increased Jak1 after stimulation in either condition, indicating that Jak1 protein synthesis is terminated normally in stimulated cDKO cells. Furthermore, this indicates that Jak1 stability in cDKO T cells is not impacted by new protein synthesis (e.g., production of cytokines). Overall, Jak1 degradation was significantly faster in WT CD4+ T cells compared with cDKO cells (Fig. 7h). Jak1 degradation during the first hours of TCR stimulation was intact in Ndfip-deficient cells; however, further Jak1 degradation was aborted (Supplementary Fig. 7a,b). As with Ndfip1, loss of Ndfip2 alone was not sufficient to affect Jak1 stability during TCR stimulation (Supplementary Fig. 7c).

If Jak1 degradation is promoted by Ndfip-dependent E3 ubiquitin ligases, either directly or indirectly, then unstimulated cells, with ongoing Jak1 synthesis, should show increased Jak1 over time in the absence of Ndfips. In unstimulated cells, we found that Jak1 in WT cells was stable, but in the absence of Ndfip1/Ndfip2 Jak1 levels steadily increased (Fig. 7i). After 24 h in the absence of presence of TCR—afters translational recovery occurs in TCR-stimulated cells—Jak1 was significantly increased in cDKO CD4+ T cells as compared with WT cells (Fig. 7j).

Ndfip-deficient T cells show persistent cytokine signalling. High Jak activity can lead to increased cell viability and proliferation through STAT5 activation and subsequent transcription of STAT5 target genes. Consistent with this, we observed increased expression, but not aberrant degradation, of the STAT5 target cyclin D2 (refs 31,43,44) in stimulated Ndfip1/Ndfip2-deficient CD4+ T cells (Supplementary Fig. 7j). Similarly, surface expression of the IL-2 receptor a (IL-2Ra, aka CD25), another STAT5 target, was increased on stimulated cDKO cells compared with WT CD4+ T cells, even when these cells were co-cultured (Supplementary Fig. 7e,f).

To determine if Jak1 stabilization mediates the observed in vitro and in vivo hyperactivation and hyperviability of Ndfip-deficient CD4+ T cells, we tested whether Jak inhibition could normalize cDKO levels of proliferation/survival. cDKO CD4+ T cells stimulated in vitro in the presence of a Jak inhibitor showed reduced proliferation, down to WT levels, in a
dose-dependent manner; WT cell proliferation was responsive to Jak inhibition but did not show dose dependence, suggesting complete Jak inhibition occurred at a lower dose (Fig. 8a,b). CD25 decreased in both WT and cDKO CD4+ T cells in a dose-dependent manner (Supplementary Fig. 7g). Strikingly, cDKO CD4+ T cells showed a robust decline in viability, down to WT levels, when treated with the Jak inhibitor (Fig. 8a,c).

To determine if sensitivity to Jak inhibition was dependent on increased cytokine availability, we co-cultured WT and cDKO CD4+ T cells and treated with the Jak inhibitor. This revealed distinct regulation of viability and proliferation. WT cells cultured with cDKO cells now showed dose-dependent decreases in proliferation in the presence of Jak inhibition, although they still proliferated less than cDKO cells (Supplementary Fig. 8a,b). In contrast, the effect of Jak inhibition on viability was independent of increased cytokine production: the enhanced viability of cDKO cells was lost upon treatment with the Jak inhibitor, which had limited effect on WT cell viability (Supplementary Fig. 8c).

Therefore, following activation, the increased survival and proliferative capacity of cDKO CD4+ T cells is due to increased Jak-dependent cytokine signalling. Increased cell viability is independent of increased cytokine production, and can be normalized by Jak inhibition.

During acute TCR signalling, cytokine responsiveness decreases; Jak1 degradation is thought to be required for this desensitization⁴-⁶. In TCR stimulated CD4+ T cells, decreased induction of STAT5 phosphorylation downstream of the IL-2 receptor limits effector cell expansion by inducing growth arrest and apoptosis⁴. To test whether Jak1 stabilization in Ndfip-deficient T cells allows persistent cytokine signalling, we examined STAT5 phosphorylation after IL-2 treatment in cells that were or were not receiving TCR signals (Fig. 8d,e). In unstimulated cells, p-STAT5 was significantly increased in cDKO CD4+ T cells relative to WT cells on IL-2 treatment, consistent with the increased levels of Jak1 observed in cDKO cells at this time (Fig. 7j). Strikingly, WT CD4+ T cells stimulated through
their TCR failed to phosphorylate STAT5 in response to IL-2, while in cDKO cells there was no difference in the p-STAT5 response to IL-2 in the presence or absence of TCR signalling (Fig. 8d,e).

Indeed, cDKO cells exhibited strong STAT5 phosphorylation under TCR stimulation alone, without addition of exogenous IL-2 (Fig. 8e). This was significantly decreased when IL-2 was blocked in TCR stimulated cDKO CD4 + T cell cultures, indicating that cDKO cells persistently signal through IL-2 in an autocrine or juxtacrine fashion while actively signalling through the TCR (Supplementary Fig. 8d). Thus, Ndfip1 and Ndfip2 are required for TCR-induced cytokine desensitization, and, in the absence of Ndfip2, stabilization of Jak1 can drive persistent IL-2 signalling to promote cell survival and proliferation.

**Discussion**

Fine-tuned control of signalling downstream of the TCR, co-receptors and cytokine receptors is critical in activated T cells. Perturbed signalling can alter T cell function, differentiation, survival and proliferation with life-threatening consequences. One layer of control is degradation of key signal transducers via ubiquitylation to attenuate activating signals. Consistent with this, prior work has detailed the aberrant CD4 + T-cell responses that occur in the absence of the E3 ligase Itch and one of its known activators, Ndfip1 (refs 5,9,13–15,46).

Here we uncover a role for Ndfip2, another activator of Itch and related ligases, in limiting effector T-cell immunopathology by controlling effector cell expansion. Unlike Ndfip1, Ndfip2 does not impact naïve T-cell activation or differentiation; however, together Ndfip2 and Ndfip1 limit the expansion/persistence of effector CD4 + T cells. In previously activated CD4 + T cells, Ndfip1 and Ndfip2 are acting in a coordinated, but perhaps non-redundant, manner to prevent aberrant T cell responses. While expression of both Ndfip1 and Ndfip2 is increased during T-cell stimulation our data indicate distinct magnitude and kinetics, providing one possible explanation for why Ndfip2 knockout mice do not phenocopy mice lacking Ndfip1.

Nothing is known about how Ndfip2 intrinsically regulate T-effector cells. To investigate this, we developed a proteomics workflow in primary CD4 + T cells for unbiased identification of differential ubiquitylation in primary T cells. Using whole cell proteome analysis, polyubiquitin pulldown and diglycine remnant analysis, we examined differential ubiquitylation in primary T cells. Using this proteomics approach we identified Jak1 as one of nine Nedd4 family ligases, Nedd4-2 and Itch are likely robustness of this proteomic approach, our analysis identified Ndfip2 and Ndfip1 limit the expansion/persistence of effector CD4 + T cells. In previously activated CD4 + T cells, Ndfip1 and Ndfip2 are acting in a coordinated, but perhaps non-redundant, manner to prevent aberrant T cell responses. While expression of both Ndfip1 and Ndfip2 is increased during T-cell stimulation our data indicate distinct magnitude and kinetics, providing one possible explanation for why Ndfip2 knockout mice do not phenocopy mice lacking Ndfip1.

Using this proteomics approach we identified Jak1 as aberrantly ubiquitylated in the absence of Ndfip2. Jak1 has multiple ubiquitylated lysines, and was more stable in stimulated Ndfip-deficient CD4 + T cells. The hyperviability and hyperproliferation of Ndfip-deficient T cells was blocked by Jak inhibition. These inhibitor experiments revealed that, while hyperviability
was dependent on increased Jak signalling independent of cytokine production, the role of Jak in proliferation was proportionate to cytokine availability. Consistent with Jak1 stabilization, Ndfip-deficient cells failed to undergo TCR-mediated cytokine non-responsiveness—stimulated Ndfip-deficient cells remained sensitive to IL-2, activating signalling cascades downstream of Jak1 that promote survival and proliferation. These data support that Ndfip-dependent Jak1 degradation plays an important role in limiting cytokine responsiveness during TCR stimulation, although whether this is a direct effect of Ndfip-mediated activation of Jak-targeting E3 ligases remains to be seen.

Notably, in the absence of Ndfips, Jak1 degradation did occur after TCR stimulation, but was limited temporally. Thus, initial Jak1 degradation is Ndfip-independent. If Ndfip-dependent ligases (for example, Ned4 family ligases) target Jak1 for degradation directly, then one possibility is that these ligases are activated by an Ndfip-independent mechanism downstream of early TCR signalling, allowing them to ubiquitylate Jak1. Activation of these ligases may occur via phosphorylation (Itch, Ned4, Ned4d-2) and calcium binding (Smurfl2, Ned4, Ned4d-2)67-51. However, after termination of early signalling events that could relase ligase autoinhibition in a calcium or phosphorylation-dependent manner, ligase activity may depend on Ndfips. Alternatively, Ned4 family E3 ligases may be dispensable for early Jak1 degradation. Nevertheless, Ndfip-independent degradation of Jak1 in CD4+ T cells is clearly not sufficient to terminate cytokine signals, as Ndfip-deficient cells fail to undergo TCR-mediated cytokine non-responsiveness.

Single deficiency in either Ndfip1 or Ndfip2 was not sufficient for Jak1 stabilization. These data suggest that Ndfip1 and Ndfip2 compensate for one another in promoting Jak1 degradation in restimulated effector CD4+ T cells. In these previously activated cells, expression of both Ndfips is induced equally well. One intriguing idea that increased sensitivity of previously activated T cells to TCR restimulation necessitates increased action of Ndfip-dependent E3 ligases, and therefore increased overall expression of Ndfips is required for efficient activation of a larger pool of these E3s. In addition, the sequence of Ndfip1 contains solvent lysine residues in close proximity to its PY motifs, which may be preferentially ubiquitylated when Ndfip1 interacts with and activates Ned4 family E3 ligases52. Ndfip2 does not have these lysines—while this may negatively impact its ability to activate Ned4 family E3 ligases52, it may also extend its half-life, thereby providing a longer lasting, although less efficient, Ndfip-dependent degradation of Jak1 stabilization.

When our work directly relates to IL-2 promoting survival and proliferation, increased IL-2 signalling has other effects on T cells. Increased phosphorylation of STAT5 downstream of IL-2 receptor signalling promotes T12 differentiation33,34 and limits T12 differentiation54. Previously published substrates of Ndfip-dependent ligases also play roles in promoting T12 differentiation and inhibiting T12 development3,8,9. While degradation of Ned4 family substrates could promote T12 and limit T12 differentiation, regulation of Jak1 levels, either directly or indirectly by Ndfip-dependent E3 ligases, and downstream cytokine signalling could augment these pathways in a cooperative or synergistic manner.

We propose a model in which Ndfip1 and Ndfip2 work together to limit cytokine signalling by promoting Jak1 degradation (Supplementary Fig. 9). In effector T cells not undergoing TCR stimulation, Ndfip1 and Ndfip2 maintain Jak1 levels in balance with protein synthesis. When T cells are activated via their TCR, Ndfip1 and Ndfip2 enforce a period of cytokine non-responsiveness, by promoting Jak1 degradation, to restrict the effector pool. Promoting TCR-mediated downregulation of cytokine signalling is a previously unknown role for Ndfip1 and Ndfip2 and their cognate E3 ligases. We propose that Ndfip-dependent degradation of Jak1 is critical in determining effector CD4+ T cell numbers that, when disrupted, leads to pathologic accumulation of effecter CD4+ T cells.

Methods

Mouse strains. Ndfip1−/− and Ndfip2−/−, C57BL/6 embryonic stem cells were generated by breeding Ndfip2−/−, Ndfip2+1/2, Ndfip1−/−, or Ndfip2−/−, and control mice used for flow cytometry analysis in Supplementary Fig. 1 are C57BL/6 embryonic stem cells, and are maintained on C57BL/6 background. Animals were used at 5–7 weeks of age with the exception of the 16-week-old Ndfip2−/− and control mice used for phenotypic analysis in Supplementary Fig. 1. Rag1−/− hosts for chimera experiments were 8 weeks old. Control animals were Ndfip1−/− Cre−, Ndfip1−/− Ndfip2−/− + /+, or CD45.1 as appropriate. Ndfip1/2 DKO mice were generated by breeding Ndfip2−/−, Ndfip1−/− + /− females to Ndfip2−/−, Ndfip1−/− + /− males. cDKO mice were generated by breeding Ndfip2−/− −/− Ndfip1−/− −/− Cre− mice to Ndfip1−/− −/− Cre− mice. We observed no sex differences in Ndfip2−/− or Ndfip1−/− cDKO mice and experiments were performed on both male and female mice. Control mice were maintained in a barrier facility at the Children’s Hospital of Philadelphia. All procedures were approved by the Institutional Care and Use Committee of the Children’s Hospital of Philadelphia. Genotyping primers for Rag1 KO/KI alleles were detected using the following primers on genomic DNA: WT_F 5′-CCCTTTGGCCACACCTTGTTACAGTG-3′; WT_R 5′-GTTAGAGCGATGGCGACAAGTTACT-3′; KO/KI_F 5′-CCTCAAGACGATACAGCAAG-3′; KO/KI_R 5′-CCCTTGTTATCCCTAGTGAAAG-3′. The FLP transgene was detected using the following PCR primers on genomic DNA: FLP_FWD 5′-GACACCGTGTTAGGGCAGGGCG-3′; FLP_FWD 5′-GACACCGTGTTAGGGCAGGGCG-3′; FLP_FWD 5′-GACACCGTGTTAGGGCAGGGCG-3′. All animals used in experiments described were FLP negative. Genotyping primers for Ndfip2 knockout alleles, Ndfip1/2 BOX alleles and CD4Cre were described previously.

Flow cytometry and immunoblot antibodies. All flow cytometry antibodies were used at 1:200 unless otherwise noted. The following flow cytometry antibodies were purchased from Biolegend: CD4 (GK1.5), CD8 (53-6.7), CD44 (IM7), IL-17A (TC11-18H10.1), CD3e (17A2), B220 (RA3-6B2), CD45.2 (104) and CD45.1 (A20). Antibodies against IFNγ (XMG1.2) and Ki67 (B62) were purchased from BD Biosciences. The remaining antibodies for flow cytometry were purchased from eBioscience: IgM (HI/4), CD25 (PC6.2), CD62L (MEC-14), GATA3 (TWAJ), IL-4 (11B11), IgM (HI/4), T-bet (4B10), Foxp3-biotin (FK-16a, 1:150), CD93 (AA4.1) (AA4.1). Biotinylated Foxp3 was detected using the following fluorophore-conjugated streptavidin (S32354 Invitrogen, 1:500). Stat5SAB (Scott) for flow cytometry was purchased from BD (p Y694, 47). The following primary antibodies were used for immunoblotting: rabbit antiJak1 (3332, Cell Signalling Technologies, 1:5000), monoclonal rabbit antiJak2 (D2E12, Cell Signalling Technologies, 1:5000), rabbit anti-Cyclin D2 (M-20, Santa Cruz Biotechnology, 1:1000) and monoclonal rabbit antiSTAT5a (E289, Abcam, 1:500).

GST pulldown. Ndfip cytoplasmic domain constructs were prepared as previously described29. Plasmids were transformed into BL21(DE3) E. coli and purified by glutathione sepharose 4B (GE Life Sciences). WT and Ndfip1/Ndfip2 knockout CD4+ T cells were cultured as described below. Rested cells were resuspended for 4 h with PMA/ionomycin as described below, lysed in 1% NP40 (1% NP40, 150 mM NaCl, 50 mM Tris-HCl with phosphatase and protease inhibitors), precleared with GST, and then incubated with 10 μg recombinant GST or GST protein. Protein was collected with glutathione sepharose beads, and eluted by addition of 4× Laemmli sample buffer with boiling. Bound protein was analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting as described below.

Polyubiquitylation assay and ubiquitin charging assay. Polyubiquitylation was monitored using a homogeneous E3 ligase TR-FRET assay (Progena, Inc.) as described above. Briefly, ubiquitylation assay containing Itch, ubiquitin, glutathione sepharose 4B (GE Life Sciences). WT and Ndfip1/Ndfip2 knockout with varying doses of Ndfip1/2 and HTRF Detection Mix. TR-FRET was monitored in real time using a PerkinElmer Envision plate reader (Ex 340 nm; Em 480 nm); TR-FRET ratio was calculated as Em520/Em480. TR-FRET ratio was calculated as Em520/Em480. Ubiquitin charging assay and expression of recombinant Itch were described by Rügner et al.,25,26 recombinant E1, E2, and ubiquitin were incubated on ice in the presence of ATP and magnesium to generate pre-charged ubiquitin – E2 conjugates. This charging reaction was quenched by addition of EDTA, after which E3 ligase (Itch) was

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introduced to the reaction in the presence or absence of GST-Ndfip cytoplasmic domains. The reaction proceeded for 3 days before addition of reducing sample buffer. Ubiquitylated products were resolved by SDS-PAGE and western blotting as described below.

**Tissue processing and flow cytometry.** Spleens, thymi and lymph nodes were harvested and washed through 70 μm nylon filters using cold Hanks’ Balanced Salt Solution (HBSS). Spleens were red blood cell lysed using ammonium-chloride-potassium (ACK) lysis buffer, washed, filtered again and resuspended in cold HBSS. Bone marrow was isolated by flushing femur and tibia with cold HBSS, red blood cells were lysed using ACK lysis buffer and washed with PBS. Embryos were genotyped at the time of harvest. Cells were resuspended in freezing media (90% FCS, 10% DMSO) and kept at −80°C until use. Thawed cells were counted, mixed 1:1 with HBSS and injected i.v. into sublethally irradiated 6–8-week-old Rag1−/− recipients. Clean cage bedding was mixed with dirty bedding such that cages were only fully changed every 2 weeks. Mice were killed when recipients showed weight loss of 10% or more above baseline.

**Histology.** Skin, oesophagus and distal colon were dissected and fixed in 10% formalin for at least 24 h. Lung was perfused with formalin. All organs were embedded in paraffin, sectioned and stained with HE.

**Western blotting and cycloheximide treatment.** Bulk isolated CD4+ T cells were resuspended at 1 × 10^6 cells ml^−1 in complete DMEM and stimulated with ₵CD3/CD28 for 4 days. Cells were rested and expanded in recombinant human IL-2 at 50 U ml^−1 (3–4 days) prior to restimulation with plate-bound ₵CD3/CD28 for the indicated periods. Where noted, cycloheximide (Sigma) was added at 10 μg ml^−1 after 2 h of restimulation, and cells were incubated for an additional 2–4 h. Cells were collected with cold PBS, pelleted and lysed for 15 min on ice with digitonin lysis buffer13. Lysates were then centrifuged at 4,500 r.c.f. for 4 min, and separated from the pellet. Digitonin insoluble pellet was subsequently lysed in 1% SDS, 10 mM tris, 20 μg ml^−1 DNase I and Roche complete inhibitor tablet with boiling. A concentration of 4 × 10^6 Laemmli buffer was then added. Lysates were collected and stained with protein concentration and samples were boiled for 5 min. Lysates were loaded by cell number equivalents on pre-cast Criterion Tris-HCl gels (Biorad) and subjected to SDS–PAGE. Protein was transferred to polyvinylidene difluoride (Millipore) using a semi-dry transfer apparatus. Membrane was blocked in Roche blocking reagent (1% BSA/2% milk), incubated with antibody of interest (1:1000 dilution) and then probed with HRP conjugated secondary antibodies (1:5000 dilution) and visualized using an Odyssey imager (LICOR) using AF680 goat zaraabbit (Intronitrogen), IRDye 800 donkey ‘mouse’ (LICOR) and Rbdeye 800 donkey zygocat (LICOR) secondary antibodies. Bands were quantified using Image Studio Lite (LICOR). Western blots have been cropped for clarity in the figures. Uncropped blots with size markers can be found in Supplementary Figs 10–13.

**Foetal liver chimeras.** Foetal liver from WT (CD45.1−/+), Ndfip2−/− (CD45.2−/−), Ndfip2−/− (CD45.2−/−) and Ndfip2/2 DKO (CD45.2−/−) embryos was processed into a single-cell suspension by passing through a 35-μm filter. Embryos were genotyped at the time of harvest. Cells were resuspended in freezing media (90% FCS, 10% DMSO) and kept at −80°C until used. Thawed cells were washed, counted, mixed 1:1 with CD45.1−/+ or CD45.2−/− in PBS and injected i.v. into sublethally irradiated 6–8-week-old Rag1−/− recipients. Cells were washed weekly and mice were weighed every 2–3 days. Recipients were co-housed. Clean cage bedding was mixed with dirty bedding such that cages were only fully changed every 2 weeks. Mice were killed when recipients showed weight loss of 10% above baseline. After killing, mice were weighed, spleens were weighed and processed for flow cytometry as described above, colons were measured for contraction and distal colon sections were fixed for histology. Crypt depth was measured at three different points/slide on HE-stained colon sections, and averaged/animal.

**TUBE affinity purification and whole proteome analysis.** Bulk isolated CD4+ T cells were isolated as described above. Dialysed foetal calf serum was used in SILAC RPMI for SILAC RPMI/sILAC RPMI (sILAC) using SILAC media with plate-bound ₵CD3/CD28 for 3 days. SILAC media components are as follows: sILAC RPMI (Hyclone), C13 or C12 L-arginine:HCl and L-lysine:2HCl (Cambridge Isotope Laboratory), 1% MEM NEAA (Intronitrogen), 1% sodium pyruvate (Intronitrogen), 1% Glutamx (Intronitrogen), 1% L-glutamine (Intronitrogen). 10% Dialysed FCS (Life Technologies), 2% HEPES (Fisher Scientific) and 0.12 μm betamercaptoethanol (Sigma). After stimulation, cells were rested in IL-2 for 3 days. Cells were restimulated at 4 × 10^6 ml^−1 SILAC media using PMA (30 ng ml^−1) and ₵CD3/CD28 (1 μg ml^−1) in 500 μl room temperature PBS and mixed at a 1:1 ratio with CFSE-labeled (4 × 10^6 ml^−1 in 1 ml PBS) and C13 or C12 L-lysine:2HCl (20 ng ml^−1) prior to stimulation. Cells were labelled with e670 cell proliferation dye (Invitrogen), Rbdeye 800 donkey ‘mouse’ (LICOR) and Rbdeye 800 donkey zygocat (LICOR) secondary antibodies. Bands were quantified using Image Studio Lite (LICOR). Western blots have been cropped for clarity in the figures. Uncropped blots with size markers can be found in Supplementary Figs 10–13.

**Quantitative PCR.** qPCR was performed as previously described14. In brief, 10 ng of complementary DNA was added to TaqMan Gene Expression Master Mix and TaqMan Gene Expression primer/probe mix specific for Ndfip1 or Ndfip2 (Applied Biosystems) for a final reaction volume of 20 μl. qPCR was performed using an Applied Biosystems 7500 Real-Time PCR system. Each sample was assayed in triplicate along with the endogenous control (actin). ACTB primer/probe (4352933E) was obtained from Applied Biosystems. Ndfip1 and Ndfip2 custom primers and probe sequences are as follows: Ndfip1_F (GATACTG-3′); Ndfip1_R 5′-CGATGGGCTTTGGAATACCG-3′; Ndfip2 TAACAGCACAC-3′; Ndfip2_R 5′-AGGACATGAGCTAACTACATACAC-3′; Ndfip2 TaqMan probe 5′-TTTTAGGAACATGATCCATTTGG-3′; Ndfip2_F 5′-AGACGATCACTGTTGGAAAGCT-3′; Ndfip2_R 5′-GGACAGGTTGAAAACATGCAATACAC-3′; Ndfip2 TaqMan probe 5′-CCTACACCTCTA-3′. ACTB primer/probe (4352933E) was used to normalize gene expression.

**T-cell isolation and culture.** Total CD4+ T cells were isolated from spleen and lymphnode by negative selection using rat tmouse CD86 (2.43; ATCC, 2.5 ml hybridoma supernatant per animal) and rat tmouse LFA-1 (31/6/11; BD Biosciences, 5 μl per animal). Cell suspensions were incubated with antibodies in complete DMEM for 1 h with end-over-end rotation at 4°C, washed and incubated with Biomag goat IgG (GAM) magnetic beads (Dynal) for 15 min with end-over-end rotation at room temperature; unbound cells were collected using a DynaMag magnet (Dynal). Purity after isolation and subsequent culture was > 95% CD4+ CD3+ T cells. To sort naive CD4+ T cells, single cell suspensions of spleen and lymphnode were stained with antibodies against CD44, CD86, CD44, CD25 and CD62L. Cells were filtered through 35 μm filter cap polystyrene FACS tube (BD Biosciences) and sorted under high speed on a MoFlo Astrios (Beckman Coulter) or a FACSAria (BD Biosciences). Naive cells were identified as follows: CD4+CD62L. Cells were incubated with ₵CD3/CD28 for 4 days. Cells were rested and expanded in recombinant human IL-2 (3–4 days) prior to restimulation with plate-bound ₵CD3/CD28.
K-GG peptide immunoprecipitation. Bulk CD4+ T cells were stimulated and expanded in IL-2, as above, prior to restimulation for 4 h with PMA/ionomycin (30 ng/μl each) including MG132 (10 μM) and chloroquine (50 μM) for 2 h, or restimulation for 4 h with mouse T cell activator beads (Invitrogen) at a 3:1 bead:cell ratio in the absence of inhibitors. Cell pellets were lysed in lysis buffer and protein concentration was measured via micro BC assay (Thermo), and peptides were prepared and immunoprecipitated as described. Approximately 250 × 106 total CD4+ T cells at the start of stimulation yielded ~3 mg of total peptide. Peptides were off-line basic reverse phase fractionated and recombined noncontiguously into three fractions of ~1 mg peptide per fraction for immunoprecipitation. Ubiquitin immunoreactive antibody, noncovalently conjugated to beads (Cell Signaling Technologies) was crosslinked as described. Crosslinking was validated by SDS-PAGE. 31 ug of crosslinked antibody was used for each 1 mg peptide fraction. The following modifications were made to the published protocol: step 6: alkylation was done with 20 mM IAM; step 10: peptides were acetylated at the N-terminus; step 37: beads were washed 2 × with IAP, followed by washing 2 × with IAP plus 0.05% Rapigest SF surfactant (Waters), followed washing 3 × with PBS; step 41: the eluted K-GG peptides were desalted via Oasis HLB columns. All mass spec samples were prepared in 0.1% TFA/water and analysed as described below. After LC-MS/MS analysis, >50% of peptides were modified.

LC-MS/MS. Tryptic digests were analysed by LC-MS/MS on a hybrid LTQ Orbitrap Elite mass spectrometer (Thermofisher Scientific) coupled with a nanoLC Ultra (Eksigent). Peptides were separated by reverse phase (RP)-HPLC on a reverse phase (RP) HPLC column (50 μm, 3 μm, 15 cm; Dr Maisch, Germany) in a Nanoflex chip system (Eksigent). Mobile phase A consisted of 0.1% formic acid (Thermofisher Scientific) and mobile phase B of 0.1% formic acid/80% acetonitrile. Peptides were eluted into the mass spectrometer at 300 nl min−1 with IAP plus 0.05% Rapigest SF surfactant (Waters), followed washing 3 × with PBS; step 41: the eluted K-GG peptides were desalted via Oasis HLB columns. All mass spec samples were prepared in 0.1% TFA/water and analysed as described below. After LC-MS/MS analysis, >50% of peptides were modified.
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