



Published in final edited form as:

Cell Rep. 2018 July 17; 24(3): 577–584. doi:10.1016/j.celrep.2018.06.060.

The Ubiquitin Ligase Adaptor NDFIP1 Selectively Enforces a CD8⁺ T Cell Tolerance Checkpoint to High-Dose Antigen

Mayura V. Wagle^{1,2}, Julia M. Marchingo^{3,4,11}, Jason Howitt^{5,6}, Seong-Seng Tan⁵, Christopher C. Goodnow^{1,2,7,10,*}, and Ian A. Parish^{1,8,9,10,12,*}

¹John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia

²Garvan Institute of Medical Research, Sydney, NSW, Australia

³Division of Immunology, The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia

⁴Department of Medical Biology, The University of Melbourne, Parkville, VIC, Australia

⁵The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, VIC, Australia

⁶Department of Health and Medical Sciences, Swinburne University, Melbourne, VIC, Australia

⁷St Vincent's Clinical School, University of New South Wales, Sydney, NSW, Australia

⁸Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville, VIC, Australia

⁹Cancer Immunology Program, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia

¹⁰Senior author

¹¹Present address: Division of Cell Signalling and Immunology, School of Life Sciences, University of Dundee, Dundee, UK

¹²Lead Contact

SUMMARY

Escape from peripheral tolerance checkpoints that control cytotoxic CD8⁺ T cells is important for cancer immunotherapy and autoimmunity, but pathways enforcing these checkpoints are mostly uncharted. We reveal that the HECT-type ubiquitin ligase activator, NDFIP1, enforces a cell-intrinsic CD8⁺ T cell checkpoint that desensitizes TCR signaling during *in vivo* exposure to high antigen levels. *Ndfip1*-deficient OT-I CD8⁺ T cells responding to high exogenous tolerogenic

*Correspondence: c.goodnow@garvan.org.au (C.C.G.), ian.parish@petermac.org (I.A.P.). M.V.W., I.A.P., and J.M.M. conducted experiments; M.V.W., C.C.G., and I.A.P. designed experiments; J.H. and S.-S.T. provided important experimental resources; and M.V.W., C.C.G., and I.A.P. wrote the paper.

DECLARATIONS OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

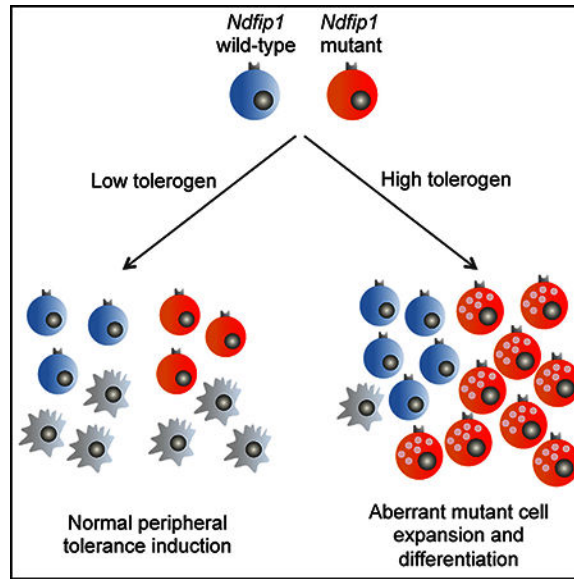
Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.06.060>.

antigen doses that normally induce anergy aberrantly expanded and differentiated into effector cells that could precipitate autoimmune diabetes in RIP-OVA^{hi} mice. In contrast, NDFIP1 was dispensable for peripheral deletion to low-dose exogenous or pancreatic islet-derived antigen and had little impact upon effector responses to *Listeria* or acute LCMV infection. These data provide evidence that NDFIP1 mediates a CD8⁺ T cell tolerance checkpoint, with a different mechanism to CD4⁺ T cells, and indicates that CD8⁺ T cell deletion and anergy are molecularly separable checkpoints.

In Brief

Ndfip1 restrains CD4⁺ T cell differentiation, but its role in CD8⁺ T cells is unclear. Wagle et al. show that *Ndfip1* selectively enforces peripheral CD8⁺ T cell tolerance to abundant antigen while minimally affecting both CD8⁺ T cell tolerance to scarce antigen and effector expansion and differentiation during acute infection.

Graphical Abstract



INTRODUCTION

Activated CD8⁺ T lymphocytes are key effector cells of the adaptive immune system that produce inflammatory cytokines and lytic granule proteins to kill infected or neoplastic cells. However, potentially pathogenic self-reactive CD8⁺ T cells escape thymic selection, and peripheral tolerance checkpoints have thus evolved to control these cells and to enable tolerance to food, commensal microbiota, and fetal antigens. These peripheral checkpoints must respond to a range of antigen levels because of variation in antigen amount released by different tissues. Malignant cancer cells can exploit these checkpoints to prevent immune recognition of mutated neo-antigens, and checkpoint inhibitors have emerged as a third pillar of cancer treatment alongside chemotherapy and radiotherapy.

Peripheral CD8⁺ T cells undergo deletion or anergy when resting naive T cells encounter antigen in the absence of infection or inflammation. In this context, the responding T cells do not become cytotoxic effectors and adopt a transcriptional profile that is distinct from other differentiation states (Hernandez et al., 2001; Parish et al., 2009). CD8⁺ T cell deletion occurs when cells undergo BIM-dependent apoptosis but largely retain T cell receptor (TCR) signaling capacity (Davey et al., 2002; Parish et al., 2009; Redmond et al., 2005; Wagle and Parish, 2016), whereas CD8⁺ T cell anergy is characterized by persistence of cells with diminished TCR signaling, with tolerogenic antigen levels thought to determine outcome (Redmond et al., 2005). The molecular pathways that enforce CD8⁺ T cell anergy *in vivo* are poorly defined, and it is unknown whether anergy checkpoint disruption interferes with CD8⁺ T cell deletion or if the two processes are molecularly distinct.

NDFIP1, a Golgi and intracellular vesicle localized transmembrane protein, plays a selective checkpoint role within CD4⁺ T cells (Altin et al., 2014; Oliver et al., 2006). NDFIP1 binds to and activates HECT-type E3 ubiquitin ligases (Mund and Pelham, 2009; Riling et al., 2015), thereby triggering ubiquitin-mediated degradation of key T cell differentiation regulators, including JUNB, ROR γ t, and JAK1 (Layman et al., 2017b; O'Leary et al., 2016; Oliver et al., 2006). In T cells, NDFIP1 primarily recruits and activates the HECT-type E3 ligase ITCH (Oliver et al., 2006). *Ndfip1*-deficient CD4⁺ T cells resist both *in vitro* anti-CD3 induced anergy and *in vivo* tolerance to low or high antigen levels because of excessive interleukin (IL)-2 production, a failure to exit the cell cycle, and aberrant differentiation into T helper (Th) 2 or Th17 cells (Altin et al., 2014; Layman et al., 2017b; Oliver et al., 2006; Ramos-Hernández et al., 2013). Mice lacking NDFIP1 develop a fatal T cell-mediated inflammatory disease associated with T cell activation, regulatory T cell dysfunction, and Th2-mediated organ pathology (Altin et al., 2014; Beal et al., 2011; Layman et al., 2017a; Oliver et al., 2006). NDFIP1 likely plays similar roles in humans, because *NDFIP1* polymorphisms and *ITCH* deficiency are associated with inflammatory and autoimmune diseases (Ferreira et al., 2011; Franke et al., 2010; Hu et al., 2011; International Multiple Sclerosis Genetics et al., 2011; Lohr et al., 2010; Ramon et al., 2011).

Despite elevated activated effector CD8⁺ T cells in *Ndfip1*^{-/-} mice and high *Ndfip1* expression in CD8⁺ T cells (Altin et al., 2014), CD8⁺ T cell activation in *Ndfip1*^{-/-} mice is largely an indirect consequence of excessive IL-4 production by *Ndfip1* mutant CD4⁺ T cells (Altin et al., 2014; Kurzweil et al., 2014). However, excessive bystander inflammation in *Ndfip1*^{-/-} mice may mask a CD8⁺ T cell-intrinsic role for NDFIP1. Here we circumvent these complications by transferring *Ndfip1* mutant and wild-type OT-I TCR transgenic CD8⁺T cells and tracing their response. We reveal that NDFIP1 is a critical checkpoint against CD8⁺ T cell expansion and effector formation during chronic exposure to high tolerogenic antigen levels.

RESULTS

Ndfip1 Is Dispensable for CD8⁺ T Cell Deletional Tolerance to a Pancreatic Self-Antigen

Bystander CD8⁺ T cell activation confounds analysis of any CD8⁺ T cell-intrinsic role of NDFIP1 in *Ndfip1*^{kru/km} mice homozygous for an NDFIP1-truncating null mutation (Altin et al., 2014). Activated or effector CD44^{hi} CD8⁺ T cell accumulation was reduced in

Ndfip1^{kru/kru} mice bearing a rearranged TCR transgene encoding OT-I, an ovalbumin (OVA)-specific major histocompatibility complex class I (MHC class I)-restricted TCR, and was abolished in *Rag1^{-/-}* OT-I *Ndfip1^{kru/kru}* mice in which no other TCRs can be expressed (Figure S1A). Thus, *Rag1^{-/-}* *Ndfip1^{kru/kru}* OT-I mice provided a homogeneous source of naive *Ndfip1*-deficient CD8⁺ T cells.

We first tested a peripheral CD8⁺ T cell deletion checkpoint triggered by low self-antigen from pancreatic islet β cells, because NDFIP1 loss disrupts a similar CD4⁺ T cell checkpoint (Altin et al., 2014). A 50:50 mix of *Ndfip1^{kru/kru}* (CD45.1/CD45.2) and *Ndfip1^{+/+}* (CD45.1/CD45.1) *Rag1^{-/-}* OT-I CD8⁺ T cells was labeled with the cell division dye Cell Trace Violet (CTV). The cells were injected into CD45.2/CD45.2 *Ndfip1^{+/+}* *Rag1^{+/+}* recipients bearing the rat insulin protein (RIP)-OVA^{hi} transgene or non-transgenic (B6) controls (Figure S1B). In RIP-OVA^{hi} mice, soluble OVA expressed in insulin-secreting pancreatic islet β cells is constitutively cross-presented by dendritic cells in the draining pancreatic lymph nodes. Transferred OT-I cells divide in response to cross-presented OVA but do not form cytotoxic effector cells and undergo BIM-dependent apoptotic death (Davey et al., 2002; Parish et al., 2009; Wagle and Parish, 2016). However, co-transferred *Ndfip1^{kru/kru}* and *Ndfip1^{+/+}* OT-I cells proliferated comparably, as assessed by cell ratios, numbers, and CTV dilution (Figures S1C-S1E), and failed to differentiate into effector cells (data not shown) in RIP-OVA^{hi} recipients. RIP-OVA^{hi} mice given either *Ndfip1^{kru/kru}* or *Ndfip1^{+/+}* OT-I cells also did not develop diabetes, with both wild-type and mutant cells deleted normally by 6 weeks post-transfer (Figures S1F-S1H). Thus, in contrast to CD4⁺ T cells, *Ndfip1* is dispensable for a peripheral CD8⁺ T cell deletion checkpoint to a tissue-restricted self-antigen.

Ndfip1 Deficiency Disrupts CD8⁺ T Cell Tolerance to a Pancreatic Self-Antigen in the Context of Higher-Antigen Doses

Islet-specific *Ndfip1* mutant CD4⁺ T cells do not cause diabetes unless simultaneously exposed to a large, systemic bolus of tolerogenic antigen (Altin et al., 2014). To test whether a similar NDFIP1-dependent checkpoint exists in CD8⁺T cells, RIP-OVA^{hi} mice injected with *Ndfip1* mutant or wild-type OT-I cells as in Figure S1F (day 0) were given either a high dose (10 mg) of OVA peptide (SIINFEKL) or saline (PBS) on days -1, 1, and 2 (Figure 1A). As in Figure S1G, neither wild-type nor mutant cells caused diabetes without peptide treatment (Figure 1B). Peptide-treated wild-type OT-I cells also did not cause diabetes (Figure 1B). However, 84% of RIP-OVA^{hi} mice given mutant OT-I cells and systemic OVA peptide developed diabetes associated with pancreatic islet infiltration and destruction (Figures 1B and 1C), with peptide treatment augmenting mutant cell numbers (Figures 1D and 1E). Thus, NDFIP1 cell autonomously restrains effector CD8⁺ T cell accumulation and target tissue cell destruction, but only in response to high systemic tolerogen levels.

Tolerogen Concentration Governs Ndfip1 Restraint of CD8⁺ T Cell Expansion and Differentiation

Low tolerogen doses induce peripheral CD8⁺ T cell deletion, whereas higher doses cause CD8⁺ T cell persistence in an anergic state (Redmond et al., 2005). To test whether NDFIP1 is important for the CD8⁺ T cell anergy—but not low-dose deletion-checkpoint, we co-transferred a 50:50 mix of CD45- marked *Ndfip1^{kru/kru}* and *Ndfip1^{+/+}* OT-I cells into wild-

type B6 mice as before (day -1) (Figure 2A). Recipient mice were then given daily intravenous (i.v.) injections of either PBS or a low (1 mg) or high (10 mg) dose of OVA peptide on days 0, 1, and 2. The mutant and wild-type OT-I cell numbers increased comparably in the spleen by day 3 after the first peptide injection (Figures 2B–2D). However, a striking increase in *Ndfip1*^{kru/kru} relative to *Ndfip1*^{+/+} OT-I cells occurred by day 6 after high-dose peptide, with mutant cells making up 86% of cells (Figures 2B–2D). By contrast, mutant cell ratios and numbers exhibited little difference at day 6 after low-dose peptide (Figures 2C and 2D). Thus, NDFIP1 acts cell autonomously to inhibit CD8⁺ T cell expansion to high systemic tolerogen levels.

In CD4⁺ T cells, NDFIP1 promotes cell-cycle exit as measured by Ki67 loss in T cells responding to low- or high-tolerizing antigen doses (Altin et al., 2014). By contrast, the fraction of Ki67⁺ OT-I cells induced by high-dose peptide was comparable in mutant and wild-type cells (Figure 2E). We next measured the replication rate of responding OT-I cells on days 4 and 5 through incorporation of the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU), and a higher percentage of *Ndfip1*^{kru/kru} OT-I cells had incorporated EdU by day 6 (Figure 2F). By contrast, the pro-apoptotic protein BIM, which mediates CD8⁺Tcell death intolerance models (Davey et al., 2002), was comparably expressed in mutant and wild-type OT-I cells (Figure S2A). Paradoxically, the pro-survival BCL2 protein was downregulated in *Ndfip1* mutant T cells (Figure S2B), but this likely reflects greater effector differentiation (described later). However, when mutant and wild-type OT-I cell death was analyzed with the cell viability dye 7AAD and Annexin V (AxV), there was a small decrease in early apoptotic (AxV+7AAD-) mutant OT-I cells, with a corresponding increase in AxV-7AAD- live cells (and no change in late apoptotic or necrotic 7AAD+ cells) (Figure 2G). Thus, NDFIP1 restrains both CD8⁺ T cell replication rate and survival in response to high tolerogen doses.

We next tested whether *Ndfip1* mutant OT-I cells aberrantly became effectors, because CD8⁺ T cell peripheral tolerance checkpoints normally inhibit cytotoxic effector cell differentiation by limiting cytolytic protease Granzyme B (GzmB) expression and cytokine production (Hernandez et al., 2001; Parish et al., 2009). In response to high-dose peptide, a higher percentage and number of GzmB⁺ CD62L⁻ *Ndfip1* mutant OT-I cells were seen on day 6 (Figure 2H). To assess whether this translated into increased target killing by mutant cells, we conducted an *in vivo* cytotoxicity assay on day 6. After 4 hr, we observed significantly more killing by splenic mutant versus wild-type OT-I cells (Figure 2I). Because the assay was saturated in the mutant condition (i.e., all peptide-pulsed targets were killed), this likely underestimates mutant OT-I cell killing. In contrast, mutant cells did not exhibit elevated cytokine production at any time point (Figure S2C). OT-I cells variably maintained the capacity to de-granulate (as assessed by surface CD107a expression), but mutant and wild-type degranulation was similar (Figure S2D). Elevated GzmB⁺ CD62L⁻ *Ndfip1* mutant OT-I cells were also seen at low peptide doses, although this was less pronounced than at high peptide doses (Figure S2E) and no GzmB⁺ CD62L⁻ mutant OT-I cells were seen in RIP-OVA^{hi} mice not treated with peptide (data not shown). Thus, in addition to increased expansion, NDFIP1-deficient CD8⁺ T cells aberrantly form cytolytic effectors.

Because naive precursor frequency can affect CD8⁺ T cell differentiation, we next examined whether *Ndfip1* mutant OT-I cells aberrantly expanded and differentiated at low precursor frequencies. Although more variability was observed at lower precursor frequencies, we still observed augmented expansion and effector differentiation of mutant OT-I cells (Figures S2F and S2G), suggesting that the mutant phenotype is largely independent of precursor frequency.

Ndfip1 Limits TCR Signaling in Anergic CD8⁺ T Cells

We next asked whether exaggerated effector cell formation in *Ndfip1*-deficient OT-I cells was via the same mechanism as in CD4⁺ T cells. In *Ndfip1*^{-/-} CD4⁺ T cells, exaggerated Th2 differentiation is accompanied by greater levels of the Th2 transcription factor JUNB, which is a direct target of ITCH (Altin et al., 2014; Oliver et al., 2006). *Ndfip1*^{-/-} CD4⁺ T cells also produce more IL-2 and express more of the high-affinity IL-2 receptor (CD25) (Ramos-Hernández et al., 2013). By contrast, there was no increase in IL-2, CD25, or JUNB in NDFIP1-deficient OT-I cells responding to high peptide doses (Figures S2C, S3A, and S3B). Thus, the NDFIP1-mediated CD8⁺ T cell checkpoint is distinct from the checkpoint mechanism in CD4⁺ T cells.

We next examined whether NDFIP1 restrains TCR signaling in CD8⁺ T cells, because the ITCH ubiquitin ligase inhibits TCR signaling during *in vitro* ionomycin-induced CD4⁺ T cell anergy (Heissmeyer et al., 2004), and TCR-induced ERK phosphorylation is inhibited in CD8⁺ T cells exposed to high tolerogen doses (Redmond et al., 2005). After *in vitro* peptide restimulation, 76% of control OT-I cells from PBS-treated mice phosphorylated ERK (denoted ppERK; PBS group) (Figure 3A). By contrast, on day 3, only 15% of OT-I cells treated with high-dose peptide were DDERK⁺ after restimulation, regardless of *Ndfip1* genotype. However, on day 4, *in vitro* restimulation induced ppERK in 30% of OT-I cells, with significantly more ppERK⁺ *Ndfip1*-deficient versus wild-type cells (Figure 3A). By day 6, ERK signaling in mutant and wild-type cells had recovered comparably. Recovery of function upon antigen withdrawal within peptide anergy models has been observed by others (Pape et al., 1998; Redmond et al., 2005).

TCR-induced mTOR signaling is impaired during CD4⁺ T cell anergy (Zheng et al., 2007), and this was seen in OT-I cells exposed to 10 mg of OVA peptide. 79% of restimulated OT-I cells from PBS-treated mice phosphorylated ribosomal protein S6 (pS6), an mTOR target, compared to only 37% of wild-type OT-I cells on day 3 (Figure 3B). *Ndfip1*-deficient OT-I cells again had a partial rescue in signaling, with significantly greater pS6 responses at this time point and on days 4 and 6 (Figure 3B).

Because ERK and mTOR signaling promote anabolic growth, we compared forward scatter as a measure of cell size in *Ndfip1*-deficient and wild-type OT-I cells before restimulation. Forward and side scatter were significantly increased in *Ndfip1* mutant versus wild-type OT-I cells on day 4 (Figures 3C and 3D). Thus, an inability to fully dampen TCR signaling in *Ndfip1* mutant OT-I cells may explain their exaggerated expansion and effector differentiation.

To test whether augmented mTOR signaling causes the *Ndfip1* mutant OT-I cell phenotype, mice containing peptide stimulated wild-type and mutant OT-I cells were treated with rapamycin from days 3 to 5 of the experiment. Although rapamycin treatment reduced overall OT-I expansion and effector differentiation (Figures S3C and S3D), augmented expansion and differentiation of mutant relative to wild-type OT-I cells was still observed (Figures S3E and S3F). Thus, elevated mTOR signaling alone cannot explain the mutant phenotype.

Ndfip1 Restrains CD8⁺ T Cell Expansion and Effector Differentiation during Continuous Antigen Exposure

Ndfip1-deficient CD8⁺ T cells underwent exaggerated expansion and effector differentiation after the last peptide injection. We thus asked whether NDFIP1 loss would disrupt this CD8⁺ T cell checkpoint in the face of sustained antigen by continuing the high-dose peptide injections for the duration of the experiment (Figure 4A). *Ndfip1* mutant OT-I cells exposed to sustained antigen still expanded to higher numbers than co-transferred wild-type cells (Figure 4B) and formed more GzmB⁺ CD62L⁻ effector cells (Figure 4C). As with transient peptide treatment, the NDFIP1-deficient cells retained higher TCR-induced ppERK and pS6 (Figures 4D and 4E) and displayed an increase in forward and side scatter (Figures 4F and 4G).

Ndfip1 Does Not Limit CD8⁺ T Cell Expansion and Differentiation during Infection

To test whether NDFIP1 also repressed effector T cell expansion and differentiation during an acute infection, *Ndfip1* mutant or wild-type OT-I cells were transferred into B6 mice that were then infected with *Listeria monocytogenes*, using a strain that transgenically expresses OVA (Figure S4A). Wild-type and mutant OT-I cell expansion was similar at day 10 post-infection (Figure S4B). mTOR signaling promotes effector CD8⁺ T cell terminal differentiation (Araki et al., 2009), but we did not see an increase in terminal effectors (KLRG1^{hi}CD127^{lo}) or loss of memory precursors (KLRG1^{lo}CD127^{hi}) in mutant cells, suggesting no functionally relevant increase in mTOR signaling (Figure S4C). Thus, *Ndfip1* loss does not influence effector CD8⁺ T cell differentiation and expansion during *Listeria* infection.

To better delineate effector and memory differentiation of *Ndfip1*^{-/-} CD8⁺T cells, we examined the response to acute lymphocytic choriomeningitis virus Armstrong strain (LCMV-Arm) infection. To avoid bystander autoimmunity in *Ndfip1*^{krv/krv} mice, we used mice bearing a floxed *Ndfip1* allele (*Ndfip1*^{fl/fl}) and a GranzymeB-cre (GzmB-cre) transgene. GzmB-cre mice delete floxed alleles in activated effector CD8⁺ T cells during LCMV infection but only minimally flox genes in activated CD4⁺ T cells (Rutishauser et al., 2009). Consistent with minimal gene deletion in CD4⁺ T cells, no dermatitis or fatal autoimmune disease was seen in GzmB-cre⁺ *Ndfip1*^{fl/fl} mice up to 3–4 months of age, and unlike *Ndfip1*^Δ mice, there was no accumulation of activated T cells in the blood of 8 week old mice (data not shown). GzmB-cre⁺ *Ndfip1*^{fl/fl} (conditional knockout, or cKO) mice or GzmB-cre⁺ *Ndfip1*^{+/+} littermate (wild-type, or WT) controls were infected with LCMV-Arm, and the CD8⁺ T cell response was tracked in the blood to an early memory time point (day 42), after which splenic memory T cell number, phenotype, and function was assessed. There were no changes in expansion, contraction, and persistence of tetramer⁺ CD8⁺ T cells specific for the

immunodominant LCMV GP₃₃₋₄₁ or NP₃₉₆₋₄₀₄ peptides in cKO versus WT mice (Figures S5A and S5B). Although the proportion of KLRG1^{hi} and KLRG1^{lo}CD127^{hi} cells was also unchanged at the peak of expansion (day 8), a significant decrease in KLRG1^{lo}CD127^{hi} cells was seen within GP₃₃₋₄₁ and (to a lesser extent) NP₃₉₆₋₄₀₄-specific cells (Figure S5B) (data not shown), although accumulation of CD62L^{hi} central memory cells was normal (Figure S5C). Similar trends were observed within splenic memory cells (Figures S5D-S5F). However, memory T cell function was unchanged. The same number of interferon γ (IFN γ)-producing cells was recovered in WT and cKO mice (Figure S5G), with no alteration in IFN γ produced per cell or proportions of IFN γ + tumor necrosis factor α + (TNF- α +) cells and IFN γ +TNF- α +IL-2+ cells (Figure S5H). Thus, *Ndfip1* loss minimally affects the CD8⁺ T cell response to an acute systemic infection.

DISCUSSION

In this study, we reveal a CD8⁺ T cell-intrinsic, highly context- dependent NDFIP1-mediated tolerance checkpoint. *Ndfip1* deficiency dysregulates CD8⁺ T cells by a different mechanism than in CD4⁺ T cells. *Ndfip1*^{-/-} CD4⁺ T cell hyper-proliferation is due to elevated IL-2 production and an inability to exit the cell cycle (Altin et al., 2014; Ramos-Hernández et al., 2013), and it is accompanied by excessive IL-4 production and Th2 differentiation due to JUNB accumulation (Oliver et al., 2006). By contrast, IL-2 production and JUNB were not increased in *Ndfip1*-deficient OT-I cells, and augmented expansion was via more rapid proliferation and diminished death rather than cell-cycle exit deficiencies. Elevated GzmB expression in *Ndfip1*-deficient CD8⁺ T cells demonstrates that NDFIP1 can inhibit type 1 immune responses, in contrast to CD4⁺ T cells, in which NDFIP1 loss has little effect on Th1 differentiation (Layman et al., 2017b; Oliver et al., 2006).

The primary biochemical effect of NDFIP1 deficiency in highzone CD8⁺T cell tolerance was partial recovery of TCR signaling. Other mechanisms, such as the ubiquitin ligase CBLB (Heiss-meyer et al., 2004; Jeon et al., 2004) or diacylglycerol kinase alpha (Olenchock et al., 2006), must also contribute to TCR desensitization, because signaling in NDFIP1-deficient CD8⁺ T cells remained lower than in non-tolerized control T cells. NDFIP1 may directly target mTOR signaling pathway components, and increased mTOR signaling was proposed to augment *Ndfip1*^{-/-} regulatory T cell (Treg) proliferation and differentiation (Layman et al., 2017a); however, our data suggest that elevated mTOR signaling alone cannot explain the mutant CD8⁺ T cell phenotype. Our data also do not rule out a role for pathways beyond partially rescued TCR signaling in the *Ndfip1* mutant cell phenotype, and further work is required to pinpoint the mechanism.

Studies on CD8⁺ T cell checkpoints largely focus on exhaustion, in which established effector cells lose inflammatory function when chronically stimulated. By contrast, *in vivo* tolerance checkpoints that regulate initial CD8⁺ T cell activation are poorly defined in molecular terms. Our findings provide evidence that molecularly distinct tolerance pathways operate in the context of high and low tolerogen doses. Because high and low antigen doses favor anergy and deletion, respectively (Redmond et al., 2005), and only high-zone tolerance depends on NDFIP1, these data imply that deletion and anergy are molecularly separable states. This difference may be due to the 6 day delay in *Ndfip1* mutant OT-I cell phenotype;

responding cells may die in deletion models before a phenotype can manifest. Alternatively, because *Ndfip1* is TCR induced, it may only enforce tolerance during strong TCR engagement. However, there may be a delay in the kinetics of peripheral deletion that was missed in our study. Moreover, because a hallmark of anergy is cell persistence, the long-term persistence of *Ndfip1*-deficient anergic cells needs to be examined.

The NDFIP1-mediated tolerance checkpoint may maintain CD8⁺ T cell tolerance to abundant environmental antigens (e.g., commensal and food antigens), fetal antigens once the fetus has increased in mass, or abundant peripheral self-antigens not efficiently expressed in the thymus. Thus, dysregulated CD8⁺ T cell tolerance could contribute to the autoimmune and inflammatory diseases seen in patients with *NDFIP1* polymorphisms. NDFIP1 may also limit CD8⁺ T cell priming against high-burden tumors and could have utility in cancer immunotherapy. Finally, although *Ndfip1* loss has little effect on the effector T cell response to acute infection, our data do not rule out a role for *Ndfip1* in effector CD8⁺ T cell exhaustion during chronic stimulation within tumors or persistent viral infection. Future work is needed to evaluate these possibilities.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6, OT-I, *B6.129S7-Rag1^{tm1Mom}/J (Rag1^{-/-})*, and B6.SJL-PfpreaPep3b/ BoyJ (CD45.1) mice were purchased from the Australian Phenomics Facility, Australian National University (ANU), Australia. RIP-OVA^{hl} (Kurts et al., 1998), *Ndfip1^{kru/kru}* (Altin et al., 2014), GzmB-cre (Jacob and Baltimore, 1999), and *Ndfip1^{lfl}* (Howitt et al., 2012) mice have been previously described. All animal work was in accordance with protocols approved by the ANU Animal Experimentation Ethics Committee and current guidelines from the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Flow Cytometric Analysis

Cell suspensions were stained in PBS containing 2.5% fetal calf serum and 0.1% azide. Intracellular staining was conducted using the Foxp3 Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's instructions. Samples were collected on a BD LSRII, Fortessa, or X20 flow cytometer (BD Biosciences), with data analyzed using FlowJo Software (Tree Star).

Statistical Analysis

Data analysis was conducted using Prism Software (GraphPad). Data were analyzed using paired or unpaired t tests or two-way ANOVA with a Bonferroni post-test (Figures S5A-S5C).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Debbie Howard and Sarp Kaya for technical assistance. This work was funded by NIH grant U19-AI100627, by an Australian Government Research Training Program Domestic Scholarship (to M.V.W.), by a Sydney Parker Smith Postdoctoral Research Fellowship from the Cancer Council of Victoria (to J.M.M.), and by the National Health and Medical Research Council (NHMRC) through Program Grants 1016953,1113904, and 1054925, Australia Fellowship 585490 (to C.C.G.), Senior Principal Research Fellowship 1081858 (to C.C.G.), CJ Martin Early Career Fellowship 585518 (to I.A.P.), and Independent Research Institutes Infrastructure Support Scheme Grant 361646. Florey Institute of Neuroscience and Mental Health and WEHI acknowledge the strong support from the Victorian Government and in particular funding from the Operational Infrastructure Support Grant.

REFERENCES

- Altin JA, Daley SR, Howitt J, Rickards HJ, Batkin AK, Horikawa K, Prasad SJ, Nelms KA, Kumar S, Wu LC, et al. (2014). Ndfip1 mediates peripheral tolerance to self and exogenous antigen by inducing cell cycle exit in responding CD4+ T cells. *Proc. Natl. Acad. Sci. USA* 111, 2067–2074. [PubMed: 24520172]
- Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP, and Ahmed R (2009). mTOR regulates memory CD8 T-cell differentiation. *Nature* 460, 108–112. [PubMed: 19543266]
- Beal AM, Ramos-Hernandez N, Riling CR, Nowelsky EA, and Oliver PM (2011). TGF- β induces the expression of the adaptor Ndfip1 to silence IL-4 production during iTreg cell differentiation. *Nat. Immunol* 13, 77–85. [PubMed: 22080920]
- Davey GM, Kurts C, Miller JF, Bouillet P, Strasser A, Brooks AG, Carbone FR, and Heath WR (2002). Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. *J. Exp. Med* 196, 947–955. [PubMed: 12370256]
- Ferreira MA, Matheson MC, Duffy DL, Marks GB, Hui J, Le Souéf P, Danoy P, Baltic S, Nyholt DR, Jenkins M, et al.; Australian Asthma Genetics Consortium (2011). Identification of IL6R and chromosome 11q13.5 as risk loci for asthma. *Lancet* 378, 1006–1014. [PubMed: 21907864]
- Franke A, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, Lees CW, Balschun T, Lee J, Roberts R, et al. (2010). Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat. Genet* 42, 1118–1125. [PubMed: 21102463]
- Heissmeyer V, Macián F, Im SH, Varma R, Feske S, Venuprasad K, Gu H, Liu YC, Dustin ML, and Rao A (2004). Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat. Immunol* 5, 255–265. [PubMed: 14973438]
- Hernandez J, Aung S, Redmond WL, and Sherman LA (2001). Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. *J. Exp. Med* 194, 707–717. [PubMed: 11560988]
- Howitt J, Lackovic J, Low LH, Naguib A, Macintyre A, Goh CP, Call-away JK, Hammond V, Thomas T, Dixon M, et al. (2012). Ndfip1 regulates nuclear Pten import in vivo to promote neuronal survival following cerebral ischemia. *J. Cell Biol* 196, 29–36. [PubMed: 22213801]
- Hu X, Kim H, Stahl E, Plenge R, Daly M, and Raychaudhuri S (2011). Integrating autoimmune risk loci with gene-expression data identifies specific pathogenic immune cell subsets. *Am. J. Hum. Genet* 89, 496–506. [PubMed: 21963258]
- International Multiple Sclerosis Genetics Consortium; Wellcome Trust Case Control Consortium 2; Sawcer S, Hellenthal G, Pirinen M, Spencer CC, Patsopoulos NA, Moutsianas L, Dilthey A, Su, Z, Freeman C, Hunt SE, et al. (2011). Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 476, 214–219. [PubMed: 21833088]
- Jacob J, and Baltimore D (1999). Modelling T-cell memory by genetic marking of memory T cells *in vivo*. *Nature* 399, 593–597. [PubMed: 10376601]
- Jeon MS, Atfield A, Venuprasad K, Krawczyk C, Sarao R, Elly C, Yang C, Arya S, Bachmaier K, Su L, et al. (2004). Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. *Immunity* 21, 167–177. [PubMed: 15308098]

- Kurts C, Miller JF, Subramaniam RM, Carbone FR, and Heath WR (1998). Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. *J. Exp. Med* 188, 409–414. [PubMed: 9670054]
- Kurzweil V, LaRoche A, and Oliver PM (2014). Increased peripheral IL-4 leads to an expanded virtual memory CD8+ population. *J. Immunol* 192, 5643–5651. [PubMed: 24795452]
- Layman AAK, Deng G, O’Leary CE, Tadros S, Thomas RM, Dybas JM, Moser EK, Wells AD, Doliba NM, and Oliver PM (2017a). Ndfip1 restricts mTORC1 signalling and glycolysis in regulatory T cells to prevent autoinflammatory disease. *Nat. Commun* 8, 15677. [PubMed: 28580955]
- Layman AAK, Sprout SL, Phillips D, and Oliver PM (2017b). Ndfip1 restricts Th17 cell potency by limiting lineage stability and proinflammatory cytokine production. *Sci. Rep* 7, 39649. [PubMed: 28051111]
- Lohr NJ, Molleston JP, Strauss KA, Torres-Martinez W, Sherman EA, Squires RH, Rider NL, Chikwava KR, Cummings OW, Morton DH, and Puffenberger EG (2010). Human ITCH E3 ubiquitin ligase deficiency causes syndromic multisystem autoimmune disease. *Am. J. Hum. Genet* 86, 447–53. [PubMed: 20170897]
- Mund T, and Pelham HR (2009). Control of the activity of WW-HECT domain E3 ubiquitin ligases by NDFIP proteins. *EMBO Rep.* 10, 501–507. [PubMed: 19343052]
- O’Leary CE, Riling CR, Spruce LA, Ding H, Kumar S, Deng G, Liu Y, Seeholzer SH, and Oliver PM (2016). Ndfip-mediated degradation of Jak1 tunes cytokine signalling to limit expansion of CD4+ effector T cells. *Nat. Commun* 7, 11226. [PubMed: 27088444]
- Olenchock BA, Guo R, Carpenter JH, Jordan M, Topham MK, Koretzky GA, and Zhong XP (2006). Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat. Immunol* 7, 1174–1181. [PubMed: 17028587]
- Oliver PM, Cao X, Worthen GS, Shi P, Briones N, MacLeod M, White J, Kirby P, Kappler J, Marrack P, and Yang B (2006). Ndfip1 protein promotes the function of itch ubiquitin ligase to prevent T cell activation and T helper 2 cell-mediated inflammation. *Immunity* 25, 929–940. [PubMed: 17137798]
- Pape KA, Merica R, Mondino A, Khoruts A, and Jenkins MK (1998). Direct evidence that functionally impaired CD4+ T cells persist *in vivo* following induction of peripheral tolerance. *J. Immunol* 160, 4719–4729. [PubMed: 9590217]
- Parish IA, Rao S, Smyth GK, Juelich T, Denyer GS, Davey GM, Strasser A, and Heath WR (2009). The molecular signature of CD8+ T cells undergoing deletional tolerance. *Blood* 113, 4575–4585. [PubMed: 19204323]
- Ramon HE, Riling CR, Bradfield J, Yang B, Hakonarson H, and Oliver PM (2011). The ubiquitin ligase adaptor Ndfip1 regulates T cell-mediated gastrointestinal inflammation and inflammatory bowel disease susceptibility. *Mucosal Immunol.* 4, 314–324. [PubMed: 20962770]
- Ramos-Hernández N, Ramon HE, Beal AM, Laroche A, Dekleva EA, and Oliver PM (2013). Ndfip1 enforces a requirement for CD28 costimulation by limiting IL-2 production. *J. Immunol* 191, 1536–1546. [PubMed: 23851689]
- Redmond WL, Marincek BC, and Sherman LA (2005). Distinct requirements for deletion versus anergy during CD8 T cell peripheral tolerance *in vivo*. *J. Immunol* 174, 2046–2053. [PubMed: 15699134]
- Riling C, Kamadurai H, Kumar S, O’Leary CE, Wu KP, Manion EE, Ying M, Schulman BA, and Oliver PM (2015). Itch WW domains inhibit its E3 ubiquitin ligase activity by blocking E2-E3 ligase trans-thiolation. *J. Biol. Chem* 290, 23875–23887. [PubMed: 26245901]
- Rutishauser RL, Martins GA, Kalachikov S, Chandele A, Parish IA, Meffre E, Jacob J, Calame K, and Kaech SM (2009). Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31, 296–308. [PubMed: 19664941]
- Wagle MV, and Parish IA (2016). FOXO3 is differentially required for CD8+ T-cell death during tolerance versus immunity. *Immunol. Cell Biol* 94, 895–899. [PubMed: 27323690]
- Zheng Y, Collins SL, Lutz MA, Allen AN, Kole TP, Zarek PE, and Powell JD (2007). A role for mammalian target of rapamycin in regulating T cell activation versus anergy. *J. Immunol* 178, 2163–2170. [PubMed: 17277121]

Highlights

- *Ndfip1*-deficient CD8⁺ T cells breach tolerance to abundant tolerogenic antigen
- *Ndfip1* loss has little impact on tolerance to low tolerogen doses
- *Ndfip1* loss during high-zone tolerance increases proliferation and TCR signaling
- *Ndfip1* deletion minimally affects the response to acute systemic infection

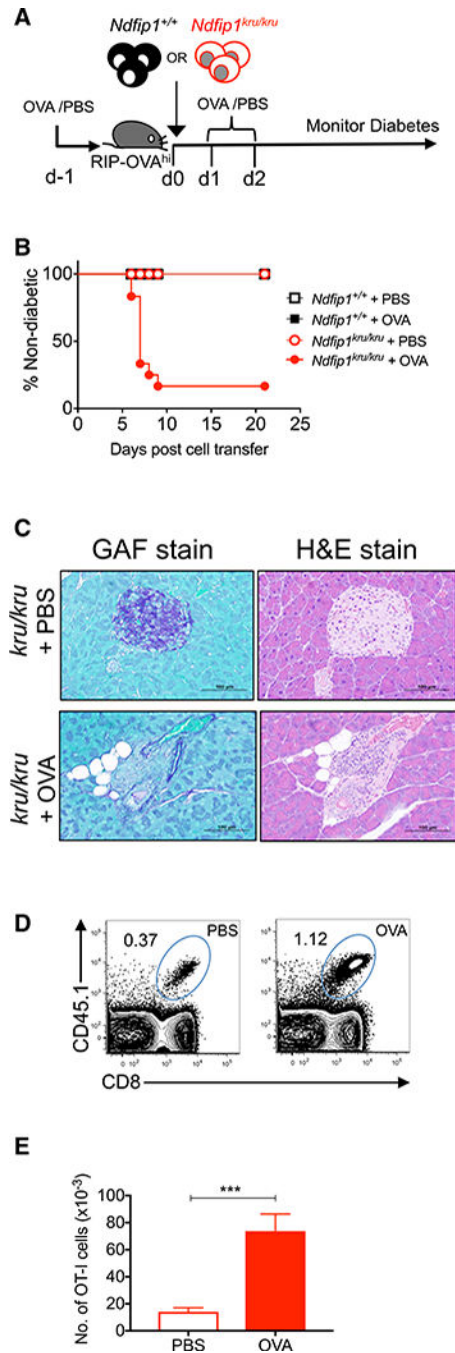


Figure 1. NDFIP1 Limits Autoimmune Pathology after High Antigen Dose Stimulation

(A) 2×10^6 *Ndfip1*^{+/+} or *Ndfip1*^{*kru/kru*} OT-I cells were injected into RIP- OVA^{hi} mice that did or did not receive 10 mg OVA peptide on days -1,1, and 2.

(B) Diabetes incidence in the treatment groups (n = 17 mice per group from 2 experiments).

(C) Representative day 9 islet histology sections from peptide- or PBS-treated RIP-OVA^{hi} mice in (B) that received *Ndfip1*^{*kru/kru*} OT-I cells.

(D and E) Representative (D) and pooled data (E) (error bars are SEM) showing *Ndfip1*^{*kru/kru*} OT-I numbers at day 9 in the draining sacral and pancreatic lymph nodes of

peptide-treated RIP-OVA^{hi} versus PBS-treated control mice (pooled B6 and RIP-OVA^{hi} mice) (n = 7 mice per group from 2 experiments).

***p < 0.001.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

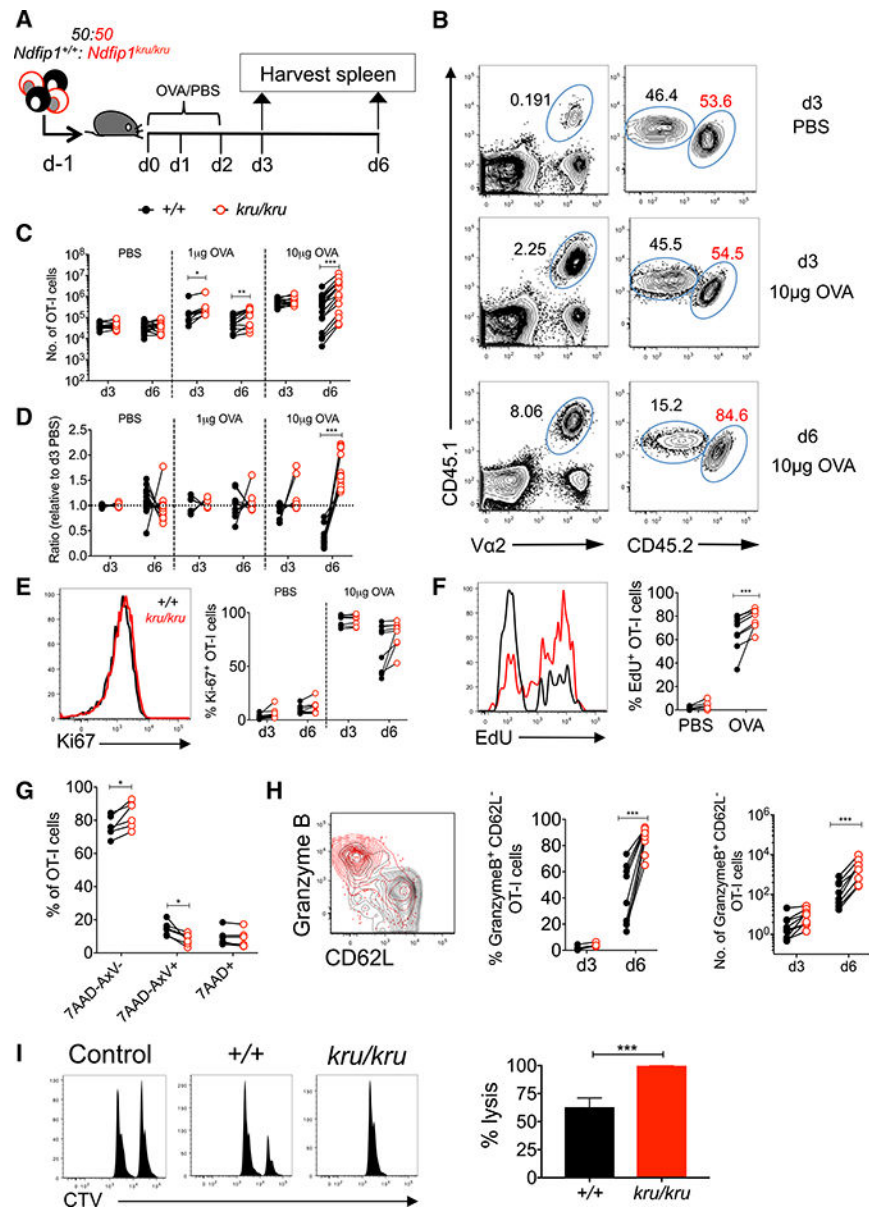


Figure 2. *Ndfip1* Mutant OT-I Cells Resist Tolerance to High Antigen Doses

(A) B6 mice were injected with 2×10^6 OT-I cells that were a 50:50 mix of *Ndfip1*^{+/+} and *Ndfip1*^{kru/kru} cells and then peptide treated and analyzed.

(B-D) Representative plots (B) and pooled data (C and D) showing the ratio (B and D) and number (C) of *Ndfip1*^{+/+} (CD45.1/CD45.1) and *Ndfip1*^{kru/kru} (CD45.1/CD45.2) OT-I cells after low- and high-dose OVA peptide treatment (n = 6–19 mice per group from 3 to 5 experiments).

(E) Percentage of Ki67⁺ *Ndfip1*^{+/+} and *Ndfip1*^{kru/kru} OT-I cells after high-dose peptide treatment on the indicated days (n = 7–10 mice per group from 3 to 4 experiments). Histogram shows data at day 6.

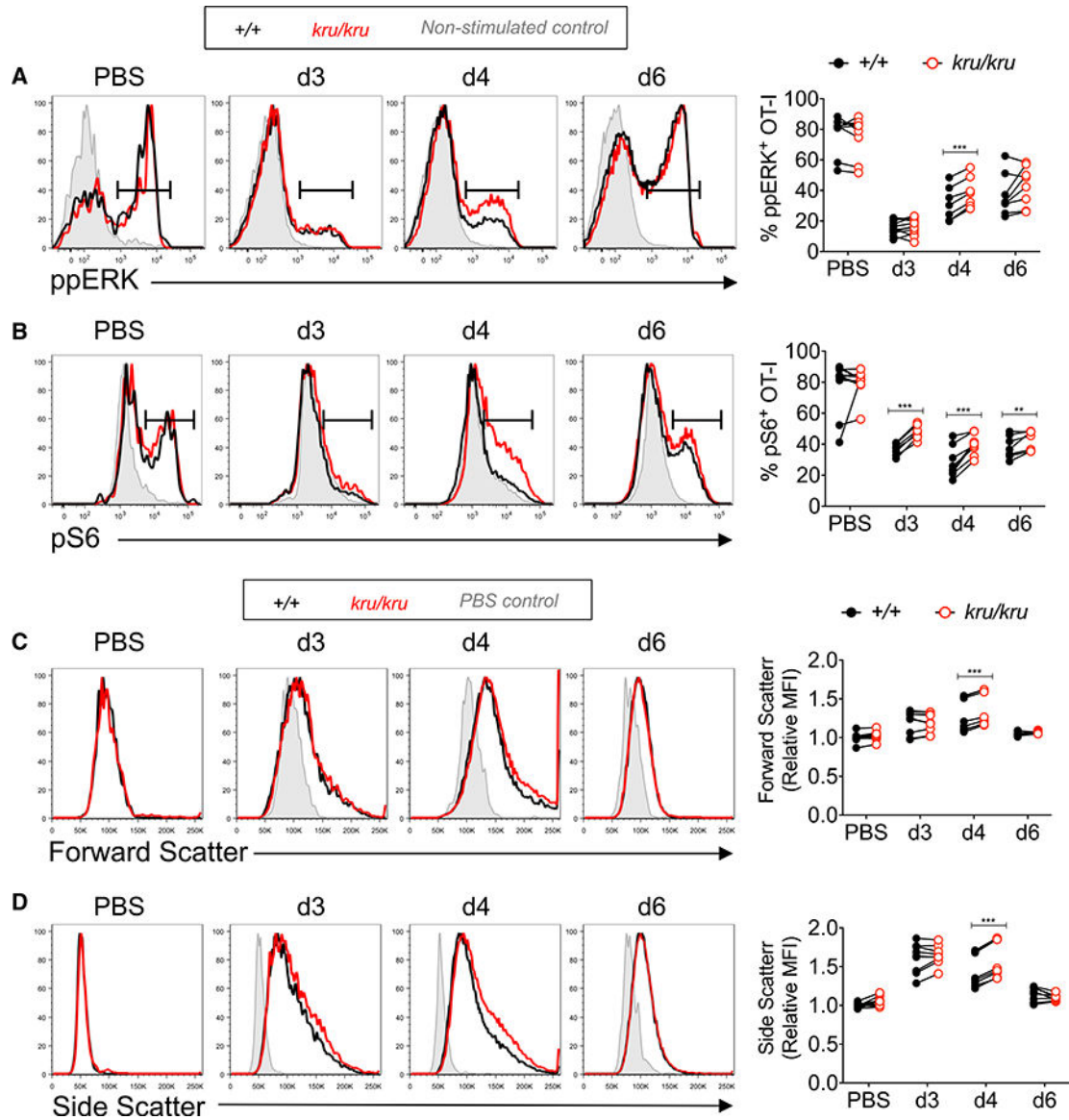
(F) EdU incorporation into *Ndfip1*^{+/+} and *Ndfip1*^{kru/kru} OT-I cells at day 6 after high-dose peptide treatment (n = 7–8 mice per group from 2 experiments). Histogram shows data at day 6.

(G) Percentages of live (7AAD–AxV–), early apoptotic (7AAD–AxV+), and late apoptotic/ necrotic (7AAD+) *Ndfip1*^{+/+} and *Ndfip1*^{kru/kru} OT-I cells at day 6 after high-dose peptide (n = 6 mice per group from 2 experiments).

(H) Percentage and number of GzmB⁺ CD62L⁺ *Ndfip1*^{+/+} and *Ndfip1*^{kru/kru} OT-I cells at days 3 and 6 after high-dose peptide (n = 8–9 mice per group from 4 experiments). Contour plot shows data at day 6.

(I) B6 mice given 2×10^6 *Ndfip1*^{+/+} or *Ndfip1*^{kru/kru} OT-I cells were treated with high-dose OVA peptide or PBS as in (A). At day 6, mice (including control untreated B6 mice) were injected with a 50:50 mix of OVA peptide-coated CTV^{hi} and untreated CTV^{lo} cytotoxic T lymphocyte (CTL) target cells, and splenic target cell lysis was measured after 4 hr. Representative histograms and the percentage of lysis are shown (n = 8 mice per group from 2 experiments, error bars are SEM).

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



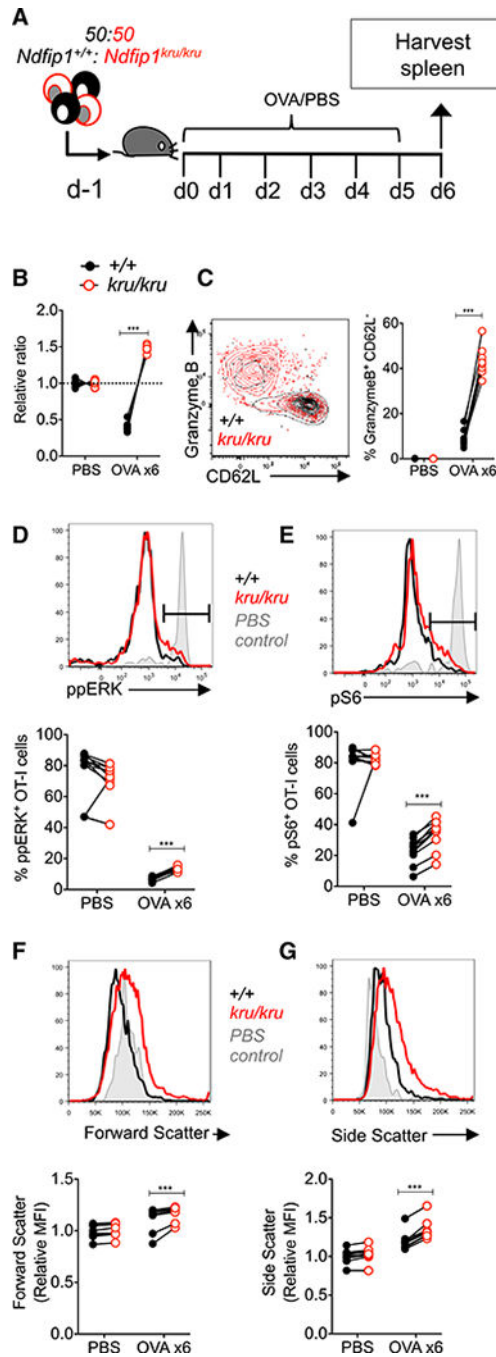


Figure 4. NDFIP1 Controls OT-I Energy to Sustained Antigen

(A) High-dose peptide energy experiment conducted as in Figure 2, except OVA peptide (or PBS) treatment was sustained.

(B-G) Ratios of WT to mutant cells (B), GzmB and CD62L expression (C), ppERK (D), and pS6 (E) after *in vitro* peptide restimulation, and forward (F) and side (G) scatter are shown (n = 10–11 mice per group from 2 experiments).

***p < 0.001.