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Tonic LAT-HDAC7 signals sustain *Nur77* and *Irf4* expression to tune naïve CD4 T cells

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Summary

CD4⁺ T cells differentiate into T helper cell subsets in feed-forward manners with synergistic signals from the T cell receptor (TCR), cytokines, and lineage-specific transcription factors. Naïve CD4⁺ T cells avoid spontaneous engagement of feed-forward mechanisms but retain a prepared state. T cells lacking the adapter molecule LAT demonstrate impaired TCR-induced signals yet cause a spontaneous lymphoproliferative T helper 2 (T_H2) cell syndrome in mice. Thus, LAT constitutes an unexplained maintenance cue. Here we demonstrate that tonic signals through LAT constitutively export the repressor HDAC7 from the nucleus of CD4⁺ T cells. Without such tonic signals, HDAC7 target genes *Nur77* and *Irf4* are repressed. We reveal that *Nur77* suppresses CD4⁺ T cell proliferation and uncover a suppressive role for *Irf4* in T_H2 polarization; halving *Irf4* gene-dosage leads to increases in GATA3⁺ and IL4⁺ cells. Our studies reveal that naïve CD4⁺ T cells are dynamically tuned by tonic LAT-HDAC7 signals.

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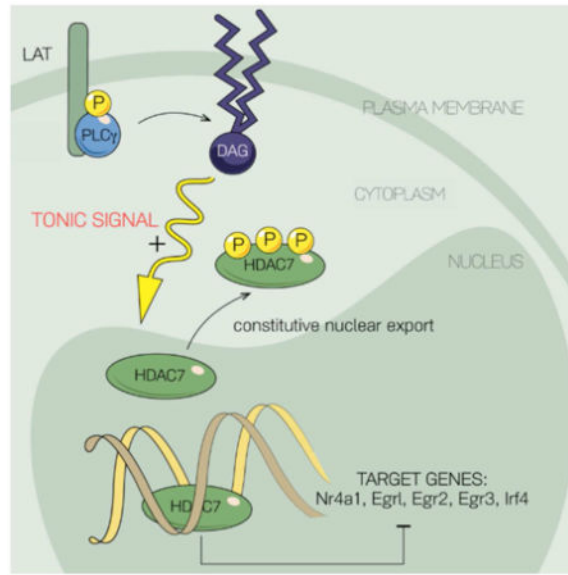
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Author Contributions: MZ provided material from LAT mouse models. AB performed the array in figure 1C. HK performed the analyses in figure 2. HWL executed figures 3, 4B, and 5A. JH performed experiments in Figure 5H. DRM, TL, and EM performed all other experiments and interpreted data. JZ, DJE, EV, and WZ provided reagents and technology and edited the manuscript. JPR conceived the study, oversaw all work, the collaboration, and wrote the manuscript with DRM and TL.

Accession Numbers: Array data can be found in GEO (mouse array accession number GPL7207; human array accession number GPL18142).

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Graphical abstract



Introduction

CD4⁺ T cells can polarize into T helper cell (T_H) subsets, such as T_H1 and T_H2 (T helper 2) subsets that produce either interferon- γ (IFN- γ) or IL-4, -5, and -13 cytokines (Mosmann et al., 2005). T cell receptor (TCR) signals, cytokine receptor signals, and transcription factors cooperate to establish these lineages via a feed-forward loop (Zhu et al., 2010); TCR signals in the presence of IFN- γ result in the activation of STAT4 and induction of the transcription factor T-bet (*Tbx21*) that induces more IFN- γ expression (reviewed in (Ansel et al., 2006; Murphy and Reiner, 2002)). Analogously, in a IL-4/STAT6/GATA3/IL-4 induction-, reinforcement-, and maintenance- model for T_H2, an initial TCR signal leads to upregulation of *IL-4* (termed “early IL-4”) so that subsequent TCR signals with a low-level IL-4/STAT6 signal lead to very robust induction of IL-4, -5, and -13 (Ansel et al., 2006; Paul, 2010). An initial model emerged with T-bet, GATA-3, Foxp3, Ror γ t, and Bcl6 transcription factors directing T cells to T_H1, T_H2, T_{REG} (regulatory T cells), T_H17, and T_{FH} (follicular helper T cells) lineages (Figure 1A), though it is clear that additional modes of regulation must exist (Locksley, 2009; O’Shea and Paul; Zhou et al., 2009; Zhu and Paul, 2010).

It is not completely understood how CD4⁺ T cells remain in a “resting”, naïve state that is permissive to the above-mentioned feed-forward mechanisms of T_H cell differentiation. Epigenetic control mechanisms of cytokine loci can impact the effect of lineage transcription factors (Hirahara et al., 2011; Kanno et al., 2012), and additional transcription factors with broader expression patterns operate in transcriptional networks with the lineage-specific transcription factors (Li et al., 2014). One of these, Interferon regulatory factor 4 (Irf4), is expressed in different T_H subsets (Biswas et al., 2010; Huber and Lohoff, 2014). Irf4 plays a critical role in T_H2 differentiation; Irf4 cooperates with NFATc2 to promote IL-4 production, is critical for GATA3 upregulation, and GATA3 overexpression partially rescues IL-4 production by Irf4-deficient T_H2 cells (Biswas et al., 2010; Huber and Lohoff, 2014).

Tonic or constitutive signals in B lymphocytes rely on surface IgM and are critical for survival (Lam et al., 1997). In T cells, tonic signaling also occurs (Monroe, 2006), but its physiological role is largely unknown (Hogquist et al., 2003). Survival of naïve CD4⁺ T cells is only modestly impacted following inducible deletion of *TCRα* (T cell receptor α chain) (Polic et al., 2001). Instead, tonic signals in T cells have been reported as immuno-modulatory, either enhancing (Stefanova et al., 2002) or blunting (Bhandoola et al., 2002; Smith et al., 2001) subsequent TCR responses to foreign antigen. Biochemically, tonic signals such as TCRζ phosphorylation can be detected in T cells rapidly isolated from peripheral lymphoid organs but not when isolated from peripheral blood, and these tonic signals quickly dissipate when cells are culture *in vitro* in non-stimulatory conditions (Stefanova et al., 2002; van Oers et al., 1994; van Oers et al., 1993). We previously established that the adapter molecule LAT (Linker for Activation of T cells) is critical for sending tonic Ras-ERK kinase signals, which can repress (Roose et al., 2003) or maintain (Markegard et al., 2011) expression of genes.

Crippling LAT's phospho-tyrosine docking site for PLCγ in the mouse germline via mutation of tyrosine 136 into phenylalanine (termed LAT^{Y136F} here) results in a spontaneous T_H2 hyperproliferative syndrome (Aguado et al., 2002; Sommers et al., 2002). Sophisticated mouse models with inducible LAT deletion (termed LAT^{NEG} here) or inducible switching from wildtype LAT to LAT^{Y136F} demonstrated that the spontaneous T_H2 hyperproliferative syndrome also develops when these LAT perturbations occur exclusively in peripheral T cells (Mingueneau et al., 2009; Shen et al., 2009) (Figure 1B). T cells with perturbed LAT function demonstrate impaired TCR-induced PLCγ activation and decreased PLCγ-dependent calcium- and ERK-signaling (Mingueneau et al., 2009; Shen et al., 2009), yet paradoxically take on a CD44⁺CD62L^{LOW} activated/memory T cell phenotype and produce high levels of intracellular IL-4 (Chuck et al., 2010; Mingueneau et al., 2009; Shen et al., 2009). Whereas it is known that MHC class II and CD28 expression are required for the development of the T_H2 immune pathology (Mingueneau et al., 2009), no further mechanistic insights relating loss of LAT in naïve peripheral T cells to immune abnormalities have been established.

These new genetic mouse models ruled out altered T cell developmental or selection processes as the predominant causes for the T_H2 hyperproliferative syndrome, which implies that there is an unappreciated role for LAT in providing some cue to curb CD4⁺ T cells in their naïve state (Brownlie and Zamoyska, 2009). Here we describe how tonic signals through LAT facilitate constitutive export of the transcriptional repressor histone deacetylase 7 (HDAC7) from the nucleus, and thereby promote expression of immune-modulatory genes like *Nur77* and *Irf4*. We reveal repressive roles for *Nur77* and *Irf4* and demonstrate that these act to tune the naïve state of CD4 T cells.

Results

Progressively altered gene expression in naïve CD4 T cells with LAT perturbations

CD4⁺ T cells take on an activated/memory CD44^{HIGH}CD62L^{LOW} phenotype when LAT is deleted or mutated via tamoxifen-induced Cre recombination (Chuck et al., 2010). In search of a direct molecular mechanism that underlies the hyperproliferative T_H2 syndrome when T

cells lack the adapter LAT or express LAT^{Y136F} (Figure 1B), we profiled gene expression in sorted naïve CD4⁺ T cells that are CD44^{LOW}. Triplicate samples of purified naïve CD4⁺ T cells from mice treated for either 1- or 4- weeks with tamoxifen resulting in LAT-negative (LAT^{NEG}), or point-mutated LAT (LAT^{Y136F}) status revealed altered expression for 188 genes when compared to wild type naïve CD4⁺ T cells. Naïve, CD4⁺ T cells that express LAT^{Y136F} for 4 weeks demonstrated the most striking changes in gene expression (Figure 1B and Supplemental Figure S1). These changes could reflect selection of particular T cell clones that occurs over the 4-week period in the LAT^{Y136F} model with a diverse TCR repertoire. LAT protein turnover takes 4 days (Ou-Yang et al., 2012), therefore one week of tamoxifen treatment effectively results in 3 days of LAT^{NEG} or LAT^{Y136F}. In search of the immediate and direct effects of LAT perturbation in naïve CD4 T cells, we focused on this short period of LAT perturbation.

HDAC target genes are repressed 3 days after LAT deletion or Y136F mutation

Examination of expression levels of transcription factors that regulate helper T cell differentiation (Figure 2A) revealed no conspicuous changes when CD44^{LOW}CD4⁺ T cells are LAT^{NEG} or LAT^{Y136F} for three days. By contrast, expression of a cluster of genes including *Egr1*, *Egr2*, and *Egr3*, *Irf4*, as well as *Nr4a1* (encoding Nur77), *Nr4a2*, and *Nr4a3* was greatly attenuated in both LAT^{NEG} and LAT^{Y136F} T cells (Figure 2B and Supplemental Figure S2). These genes are immediate early response genes downstream of mitogenic signals in many cell types. Several of these, *Nr4a1* in particular, have also been described as target genes of HDAC7 in both thymocytes (Dequiedt et al., 2003) and in DO11.10 T cell hybridoma cells (Kasler and Verdin, 2007).

HDACs deacetylate histone tails, which correlates with gene repression. HDACs are subdivided into four classes (I, IIa, IIb, and IV) (Haberland et al., 2009), and class I and IIa can be inhibited by Trichostatin A (Verdin et al., 2003). We previously reported that gene repression in a Jurkat T cell lymphoma line without LAT could be reversed by Trichostatin A (Markegard et al., 2011) and postulated that there may be a functional connection between LAT, HDACs and the unexplained T_H2 hyperproliferative syndrome. HDAC7 belongs to class IIa, and class IIa members (HDACs 4, 5, 7, and 9) display unique and tissue-specific expression patterns (Haberland et al., 2009; Verdin et al., 2003). Through Taqman analyses we established that HDAC7 is the predominant class IIa HDAC expressed in CD4⁺ T cells (Figure 2C) and therefore centered our attention on HDAC7.

We confirmed the reduced expression of *Nr4a1* (Nur77) in sorted naïve, CD4⁺ T cells by quantitative PCR (Figure 2D). We utilized our previously published gene expression sets from thymocytes that either lack HDAC7 or express HDAC7^P, a constitutively nuclear, super-repressor version of HDAC7 with mutated serine phosphorylation sites (Kasler et al., 2012; Kasler et al., 2011) to generate a list of potential thymic HDAC7 targets. We applied an arbitrary threshold of 2.0-fold differential expression between loss of HDAC7 and the HDAC7^P super-repressor, which resulted in 369 differentially expressed genes (Supplemental tables 1 and 2). The Venn diagram in Figure 2E demonstrates that the cluster of immediate early response genes expressed at lower levels in LAT^{NEG} or LAT^{Y136F} naïve CD4⁺ T cells overlap with HDAC7 targets in this analysis. In sum, the expression levels of a

set immediate early response genes are maintained by LAT and repressed by HDAC7; we will focus on the functional roles of *Nur77* and *Irf4* in CD4⁺ T cells later.

HDAC7 effects, expression, and phosphorylation

Early support for a role for HDAC's in T_H cell function came from studies where T cells were treated with HDAC inhibitors, which enhanced the expression of both IFN- γ and T_H2-type cytokines (Bird et al., 1998; Valapour et al., 2002). To test if HDAC7 may, in principle, impact T_H cell function, we isolated T cells from mice with HDAC7 deletion or expression of HDAC7^P, the super-repressor version of HDAC7 (Kasler and Verdin, 2007; Kasler et al., 2011). Direct *ex vivo* stimulation followed by intracellular FACS staining for cytokines revealed that HDAC7 deficiency led to increased percentages of CD4⁺ T cells producing IFN- γ (Figure 3A and Supplemental Figure S3A). Fewer HDAC7^P-expressing T cells expressed IFN- γ compared to WT (Figure 3B and Figure S3B). HDAC7^P did not cause spontaneous increases in IL-4-producing cells under short-term stimulatory conditions (Figure 3B). Transduced purified wildtype T cells with a retroviral construct for HDAC7^P-GFP also revealed suppression of IFN γ production (Supplemental Figure S3C).

We next explored the possibility that HDAC7 levels could impact Th2 differentiation. Microarray expression data revealed no statistically significant differences in *HDAC7* mRNA levels between WT, LAT^{NEG}, and LAT^{Y136F} CD44^{LOW}CD4⁺ T cells (Figure 3C). Relative HDAC7 protein levels fell when thymocytes developed into naïve T cells and rose when these were stimulated (T_H0) or stimulated and polarized into different effector populations (Figure 3D). Comparisons of HDAC7 levels between *in vitro* generated T_H2 versus T_H1, T_H2, T_H17, and T_{REG} or T_H0, populations did not show striking differences (Figure 3D). These results argue that altered expression levels of HDAC7 are not the source of the LAT^{NEG} and LAT^{Y136F} T cell abnormalities.

Both HDAC-5 and -7 are regulated via phosphorylation of N-terminally located serine residues; phosphorylation leads to nuclear export and cytoplasmic retention of HDAC-5 and -7 and counteracts their repressive effects on gene expression (Dequiedt et al., 2003; Parra et al., 2005; Vega et al., 2004; Verdin et al., 2003; Zhang et al., 2002). B cell receptor (BCR) induced phosphorylation of HDAC-5 and -7 had previously been reported in avian- and murine- B cell lines and primary B cells (Matthews et al., 2006). We observed that TCR stimulation could induce phosphorylation of HDAC-5 or -7 transfected into a Jurkat T cell leukemia cell line (Figure 3E and 3F). However, we noted a substantial amount of tonic HDAC phosphorylation in the unstimulated Jurkat samples, which had also been observed in a B cell line system (Matthews et al., 2006). Examination of primary lymph node cells revealed that TCR-induced HDAC7 phosphorylation was very modest and contrasted with the robust induction of ERK phosphorylation. Instead, tonic HDAC-7 phosphorylation was very pronounced in these primary cells (Figure 3G). Given our previous studies on LAT's role in tonic signals in non-stimulated cells and gene regulation (Markegard et al., 2011; Roose et al., 2003), we next investigated if the adapter LAT regulates HDAC7 phosphorylation, localization, and function in a tonic fashion.

LAT-dependent, tonic nucleocytoplasmic shuttling of HDAC7

HDAC7 has nuclear (N) localization in CD4⁺CD8⁺ double positive thymocytes but becomes predominantly cytoplasmic (C) in more developed CD4⁺ or CD8⁺ single positive thymocytes (Kasler et al., 2011). Unstimulated, naïve CD4⁺ T cells maintain the predominant cytoplasmic distribution of HDAC7 seen in their predecessor CD4-single positive thymocytes (Figure 4A), which agrees with the evident tonic phosphorylation of HDAC7 in primary lymph node cells (Figure 3G). These data show that tonic signals in CD4⁺ T cells constitutively keep HDAC7 phosphorylated and exported from the nucleus. Uniform cytoplasmic localization of HDAC7 in T_H0, T_H1, T_H2, T_H17, and T_{REG} cells demonstrated that nucleocytoplasmic trafficking occurs efficiently in all T_H subsets (Figure 4A).

Primary cells rapidly lose tonic signals *in vitro* (Stefanova et al., 2002; van Oers et al., 1993) making biochemical studies on tonic signaling and pathway mapping challenging; we therefore used a model cell line to investigate biochemical details of the tonic signals. To examine LAT, we used a LAT-deficient Jurkat T cell line, J.CaM2. We determined that tonic phosphorylation of HDAC-5 and -7 is decreased when cells lack LAT and is partially restored when J.CaM2 are stably reconstituted with a wildtype LAT cDNA construct (Figures 4B and 4C). Our results with the LAT^{Y136F} mouse model point to a role for PLC γ 1 in the tonic signaling pathway, since PLC γ 1 normally docks at phosphorylated Y136. We previously utilized overexpression of DGK ζ to reduce tonic PLC γ 1-diacylglycerol (DAG) signals as DGK ζ converts DAG to phosphatidic acid (Markegard et al., 2011). Overexpression of FLAG-tagged DGK ζ expression reduced tonic phosphorylation of HDAC-5 in unstimulated Jurkat T cells (Figure 4D), indicating that a LAT-PLC γ 1-DAG pathway is an important component of the tonic signaling pathway. Concomitant with decreased tonic HDAC phosphorylation in LAT-deficient J.CaM2 cells, its nucleocytoplasmic ratio increased 4.8 fold when cells do not express the adapter LAT (Figure 4E).

Tonic regulation of HDAC7 impacts Nur77 expression and proliferation of CD4⁺ T cells

To substantiate our findings in figure 4, we now exploited the fact that tonic signals rapidly dissipate when primary cells are rested *ex vivo* in non-stimulatory medium (Stefanova et al., 2002; van Oers et al., 1993). Resting lymph node cells for 30 or 60 minutes in PBS at 37 °C resulted in a 70-80% decrease of tonic HDAC7 phosphorylation as well as in previously reported decreases of tyrosine-phosphorylated proteins detected by 4G10 immunoblotting (Figure 5A). Analysis of HDAC7 with CD4-costaining on cytopspins of cells fixed immediately after isolation revealed a predominantly cytoplasmic HDAC7 distribution in CD4⁺ T cells (Figure 5B) with the highest pixel intensity for HDAC7 at the cell perimeter (Figure 5C). Resting cells for 30 minutes in PBS before fixation resulted in a translocation to predominantly nuclear HDAC7 localization (Figures 5D and 5E), as expected based on our phosphorylation data (Figure 5A).

CD5 expression is a sensitive reporter of TCR affinity (Azzam et al., 1998) and can be used as a marker of tonic TCR signaling (Mandl et al., 2013). Naïve CD4⁺ T cells display a range of CD5 expression in which CD5^{HIGH} cells received most tonic signal input (Mandl et al., 2013). We used CD44 and CD5 markers to sort CD44^{LOW} naïve CD4⁺ T cells into the most

bright and most dim for CD5 while keeping cells ice cold and established that CD5^{HIGH} naïve CD4⁺ T cells have substantially more tonic HDAC7 phosphorylation than CD5^{LOW} naïve CD4⁺ T cells (Figure 5F). Furthermore, CD5^{HIGH} naïve CD4⁺ T cells expressed significantly higher mRNA levels of the HDAC target *Nur77* compared to their CD5^{LOW} counterparts (Figure 5G). Single deficiency for *Nur77* leaves T cell development largely intact (Lee et al., 1995). More recently it has been reported that loss of *Nur77* results in increased proliferation of stimulated CD8⁺ T cells (Nowyhed et al., 2015), implying that *Nur77* has suppressive functions. In support of this notion, we found that stimulation of sorted naïve CD4⁺ T cells resulted in more proliferation measured by CTV-dilution when these cells lack *Nur77* (Figure 5H), indicating that *Nur77* is not only simply under tonic signal control but provides a negative feedback loop to suppress T cell proliferation. In sum, by utilizing three systems – induced LAT perturbation, PBS rest, and CD5-based sorted populations – we demonstrate that naïve CD4⁺ T cells constitutively export HDAC7 from the nucleus in a dynamic manner that relies on tonic LAT-PLC γ 1-DAG signals. Loss of this tonic control signal results in reduced expression of immediate early response genes; one of these, *Nur77*, functions to suppress T cell proliferation (Figure 5I).

Tonic regulation of *Irf4* expression limits T_H2 polarization of CD4⁺ T cells

Our gene expression analyses also indicated that tonic LAT-HDAC7 signals maintain *Irf4* expression in naïve T cells (Figure 2). *Irf4* is a member of the Irf (interferon regulatory factor) family of transcription factors (Lohoff and Mak, 2005; Tamura et al., 2008) and plays a critical role in the function and homeostasis of mature but not developing T cells (Mittrucker et al., 1997). *Irf4* function in T cells is complex. First, specific ablation of *Irf4* in Foxp3-positive regulatory T cells yielded the surprising phenotype of a spontaneous T_H2 immune disorder (Zheng et al., 2009); thus, *Irf4* fulfills a T_{REG}-intrinsic role to prevent effector T cells from polarizing towards T_H2 (Figure 6A). Multiple studies identified *Irf4* as a critical factor promoting differentiation of T_H2 cells and production of T_H2 cytokines under T_H2 polarizing conditions *in vitro* and demonstrated that *Irf4* is required for T_H2 responses *in vivo* (reviewed in (Biswas et al., 2010; Huber and Lohoff, 2014)). However, it has also been observed that *Irf4* inhibits IL-4 production in naïve CD4⁺ T cells from BALB/c mice (Honma et al., 2008). Thus, *Irf4* may have both T_H2-stimulatory and T_H2-suppressing functions in CD4⁺ T cells depending on their state (Figure 6B).

TCR stimulation results in strong upregulation of *Irf4* expression (Biswas et al., 2010; Huber and Lohoff, 2014). In CD8⁺ T cells, *Irf4* expression levels increase in a graded manner directly proportional to the strength of incoming TCR receptor signal (Nayar et al., 2012; Nayar et al., 2014; Yao et al., 2013). Taqman analysis revealed that *Irf4* levels are reduced by roughly 60% in both LAT^{NEG} and LAT^{Y136F} CD44^{LOW}CD4⁺ T cells (Figure 6C). Using the CD5-sorting strategy, we confirmed that baseline *Irf4* expression levels are impacted by tonic signaling; CD5^{LOW} naïve CD4⁺ T cells with the lowest level of tonic signaling reproducibly expressed roughly half of the *Irf4* mRNA and *Irf4* protein compared to CD5^{HIGH} naïve counterparts (Figure 6D and 6E).

To specifically address how decreases in *Irf4* expression levels impact CD4⁺ T cells, we generated an *Irf4* allelic series of two wildtype copies, one wildtype copy, or zero wildtype

copies of *Irf4* by crossing floxed *Irf4* mice to CD4-Cre mice (Figure 6F). *Irf4* Taqman revealed a 40% reduction in *Irf4* expression in heterozygous *Irf4^{+/-}* CD4 T cells (Figure 6G). CD3/CD28 stimulation of sorted *Irf4^{+/+}* naïve CD4⁺ T cells (CD44^{LOW} with exclusion of CD25⁺ cells) induced upregulation of *Irf4* expression, in agreement with published work (Biswas et al., 2010; Huber and Lohoff, 2014), but *Irf4* levels lagged behind in *Irf4^{+/-}* cells (HET), measured after 10 hours (Figure 6H). Functionally, an increased percentage of *Irf4^{+/-}* T cells expressed GATA3 compared to wildtype cells stimulated for 10 hours in T_H2-polarizing culture conditions (Figure 6I). Thus, *Irf4* plays a suppressive role during the T_H2 initiation phase, when cells are still more naïve. This suppressive effect of *Irf4* was still detectable in 5-day polarization assays: while full *Irf4* deletion resulted in the complete absence of IL-4 induction, we observed an increase in percentage of IL-4 producing cells when the gene dose of *Irf4* is halved (*Irf4^{+/-}*), compared to cells with WT levels of *Irf4* (Figure 6J). Using CD4-Cre, these results unequivocally demonstrate that some (expression level of) *Irf4* is critical for any polarization towards T_H2 cells but also revealed that normal expression levels of *Irf4* that are sustained by tonic signals function to curb T_H2 polarization.

Discussion

Mouse models with T cell-specific LAT perturbation develop a T_H2 hyperproliferative syndrome through unknown mechanisms (Chuck et al., 2010; Mingueneau et al., 2009; Shen et al., 2009). Utilizing three distinct experimental systems, we demonstrate the existence of dynamic maintenance cues in the form of tonic signals in naïve CD4⁺ T cells that rely on the presence of an intact LAT adapter molecule. We reveal that these tonic signals are critical for constitutive phosphorylation and nuclear export of the repressor HDAC7 in naïve CD44^{LOW}CD4⁺ T cells; without tonic HDAC7 regulation, expression of target genes become repressed. We particularly focused on the LAT-HDAC7 target genes *Nur77* and *Irf4* and revealed that these are immunosuppressive; this data offers mechanistic insights into how tonic signals tune the naïve state of CD4⁺ T cells.

We show that *Nur77* fulfills a negative feedback function to suppress proliferation of CD4⁺ T cells. In addition, one of *Nur77*'s reported targets is *FasL* (Fas ligand) (Rajpal et al., 2003), which regulates T cell homeostasis via induction of apoptosis of activated T cells. *FasL* is also a target of Egr transcription factors (Rengarajan et al., 2000) and we found *Egr-1*, *Egr-2*, and *Egr-3* expressed at reduced levels in T cells with perturbed LAT function (Figure 2B). LAT^{Y136F} T cells fail to upregulate *FasL* in response to TCR engagement (Ou-Yang et al., 2012; Sommers et al., 2002). The transcription factor *Irf4* has received a lot of interest as a T cell fate-determining factor (Biswas et al., 2010; Huber and Lohoff, 2014). We were inspired by the reported linear correlation between strength of TCR signal and *Irf4* expression levels (Nayar et al., 2012; Nayar et al., 2014; Yao et al., 2013), and our studies revealed that *Irf4* expression in naïve CD4⁺ T cells is under tonic control. Sustained and optimal *Irf4* expression functions to curb T_H2 differentiation, as a larger portion of *Irf4^{+/-}* T cells, which have a 40% reduction in *Irf4* mRNA, express GATA3 and IL4. Recent work uncovered that *Irf4* interacts with Fos/Jun heterodimers of the activator protein-1 (AP1) complex to regulate genes that contain AICE elements (AP-1/IRF composite elements) (Glasmacher et al., 2012; Li et al., 2012; Murphy et al., 2013). It is possible that reduced *Irf4*

levels result in altered composition of transcription factor complexes and/or altered regulation of AICE elements, which is an area for future investigation. Together, our studies with emphasis on *Nur77* and *Irf4* offer an explanation how perturbation of a tonic signal via LAT can cause a T_H2 hyperproliferative syndrome.

Our studies here focused on tonic LAT-HDAC signals in naïve CD4⁺ T cells but it is possible that such signals are important for regulatory T cells as well. We previously demonstrated that perturbations in LAT lead to impaired T_{REG} function (Chuck et al., 2010; Shen et al., 2010) and recent studies support the idea of functional constitutive signals in T_{REG}; deletion of the TCR in T_{REG} resulted in impaired homeostasis of T_{REG} (Levine et al., 2014; Vahl et al., 2014). Notably, TCR deletion in T_{REG} did not impact expression of FoxP3 or other T_{REG} signature genes, but instead, expression of *Egr1*, *Egr2*, *Nr4a1* (*Nur77*), *Irf4*, and *Ctla4* was reduced in the TCR-ablated T_{REG} (Levine et al., 2014; Vahl et al., 2014). Since we find that these same genes are under tonic LAT-HDAC7 control in naïve CD4⁺ T cells, this suggests that tonic LAT-HDAC7 signals may also regulate this gene set in T_{REG}. Furthermore, *Nur77* and the two other Nr4a family members are critical for the generation of T_{REG} (Sekiya et al., 2013) and suppression of T_H2 by T_{REG} (Sekiya et al., 2015) and *Irf4* was shown to have a T_{REG}-intrinsic role to suppress T_H2 differentiation as well (Zheng et al., 2009).

Together, our studies presented here combined with the above-cited work put forth a model in which loss of tonic LAT-HDAC7 signals alters the tuning of naïve CD4⁺ T cells (as well as the homeostasis of T_{REG}) and that tonic regulation of *Nur77* and *Irf4* levels are important mediators and feedback regulators for maintaining the naïve, undifferentiated state. We propose that dynamic maintenance cues in T cells should be considered in the context of cancer immunotherapy and immune-diseases, where these tonic signals may either be influenced by checkpoint blockade therapy or be altered through single nucleotide polymorphisms (SNPs). In support of the latter idea, it is of interest to note that three *Nur77* SNPs have been associated with severity of bronchial hyperresponsiveness in asthma patients (Kurakula et al., 2015) and that a SNP in the 3' UTR region of *Irf4* has been associated with recurrent bronchitis in children (Pinto et al., 2013).

Experimental Procedures

Mice

WT C57BL/6 mice were bred in house at UCSF. LAT^{f/-} and LAT^{Y136F} mice crossed to ER-Cre (Chuck et al., 2010; Markegard et al., 2011) and HDAC7 KO mice and HDAC7- P mice (Kasler et al., 2012; Kasler et al., 2011) have been described previously. *Nur77*^{-/-} mice were obtained from The Jackson Laboratory. *Irf4*^{fl/fl} (Jackson Labs) and CD4-Cre (Taconic Farms) mice were purchased and crossed. All mice used in experiments were between 8 and 14 weeks of age, and both males and females were analyzed. Mice were housed and treated in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) guidelines of the University of California, San Francisco (AN098375-03B for JPR and AN1110172-01 for EV) and Duke University (A160-14-06 for WZ).

Microarrays

Microarray analysis of CD4⁺ T cells was carried out using Agilent gene arrays using protocols optimized in our group and the UCSF Functional Genomics Core Facility. For details see the Supplemental Information.

Quantitative Real-Time PCR

RNA was extracted from harvested cells using Trizol, the RNeasy kit (QIAGEN) and treated with DNase I. Random primers (Invitrogen) and M-MLV Reverse Transcriptase (Invitrogen) were used to generate cDNA. For mRNA gene expression assays, TaqMan primers/probe were purchased from Life Technologies: *HDAC7*: Mm00469527_m1, *Nr4a1 (Nur77)*: Mm01300401_m1, *Irf4*: Mm00516431_m1, and *Actb*: Mm01205647_g1. TaqMan Real-Time PCR was performed using SensiMix II Probe Kit (Bioline). TaqMan reactions were run on a Mastercycler EP Replex system (Eppendorf) in triplicate. Values are represented as the difference in Ct values normalized to *Actb* for each sample.

Cell Lines, Transfections, Cell Isolation, Stimulations, and Fractionations

Jurkat T cells and derived J.CaM2 cells were grown, transfected, and stimulated as described before (Das et al., 2009; Markegard et al., 2011). In short, cells were transfected with 10 µg of HDAC7-GFP, HDAC5-GFP, or GFP, rested overnight and stimulated with C305 antibody to trigger TCR signaling. For biochemical analysis, lymph node CD4⁺ T cells were purified through MACS depletion (Miltenyi) using negative isolation. MACS isolation yielded cells with >95% purity. CD4⁺ T cells were stimulated *in vitro* with α-CD3 (2C11, UCSF mAb core) and α-CD4 (clone GK1.5, UCSF mAb Core) as before (Das et al., 2009) and lysed with SDS lysis buffer. Nuclear and cytoplasmic fractions were prepared from indicated T cell subsets using NE-PER Nuclear and Cytoplasmic Extraction Kit (ThermoFisher Scientific, Cat#: 78838).

Immunoblotting

Western blot analyses were performed and quantitated using a LAS3000 Imaging System (Fuji) and MultiGauge software as described previously (Das et al., 2009; Markegard et al., 2011). Antibodies to phosphorylated and total ERK, IRF4, and actin were from Cell Signaling; antibodies to HDAC7 and Grb2 were from Santa Cruz Biotechnology; antibody to GFP was from Clontech; antibody to lamin b was from Abcam, and antibody to histone 3 was from EMD Millipore. Rabbit antiserum to phosphorylated HDAC7 (S259) was a gift from Dr. Timothy McKinsey.

Microscopy

For microscopic analysis of HDAC7 distribution in cytospins, single cell suspensions were prepared from lymph nodes, washed with PBS, and were either immediately fixed in 2% PFA or were rested in PBS for 30 minutes prior to fixation. Cells were then washed and spun onto slides using a Cytospin (700rpm, 4 minutes) and stained with primary antibodies to HDAC7 (Santa Cruz Biotechnology) and CD4 (clone GK1.5, UCSF Hybridoma Core) followed by secondary antibodies; goat-anti-rabbit antibody conjugated to Alexa Fluor 568 (Invitrogen, A-11036) for HDAC7 and goat anti-rat antibody conjugated to Alexa Fluor 488

(A-11006) for CD4. Cells were counterstained with DAPI. Images were acquired on a Zeiss spinning disk confocal microscope. Images and pixel intensity were analyzed using ImageJ software.

Flow Cytometry Assays on Primary T Cells

CD4 T cells used in polarization assays were purified from lymph nodes using MACS negative isolation and, where indicated, were subsequently sorted to >98% purity on a MoFlo XDP (Beckman Coulter) or a FACS Aria (BD Biosciences) in the UCSF flow cytometry core. Cells were stimulated in α -CD3 (2C11) and α -CD28 (37.51) coated plates in the presence of Th2 polarizing media (50ng/ml rIL-4 (Peprotech) and 10ug/ml α -IFN- γ (XMG1.2, UCSF mAb core)). After 5 days, cells were restimulated for 4 hours with PMA (5ng/ml) and ionomycin (0.67nM) in the presence of monensin (BD GolgiStop). Following restimulation, cells were labeled with Live/Dead fixable viability dye (LifeTechnologies) and antibodies to CD4 (GK1.5), IFN- γ (XMG1.2), and IL-4 (11B11). For Gata3 assays, cells were stimulated on coated plates for up to 48 hours in Th2 media before fixation and permeabilization in Foxp3/Transcription Factor buffer set (eBioscience) and staining with Gata3 (TWAJ, eBioscience).

For proliferation assays, CD4⁺ T cells from *Nur77*^{-/-} and WT B6 lymph nodes were enriched using MACS negative isolation, labeled with Cell Trace Violet (Thermo Fischer Scientific) and subsequently sorted to >98% purity. Sorted cells were cultured on CD3/CD28 coated plates for up to 96 hours prior to FACS analysis.

All antibodies used were from eBioscience, BD, or Tonbo Biosciences. All flow cytometry data was acquired on LSRII or Fortessa instruments (BD Biosciences) and analyzed using FlowJo (Tree Star). Statistical significance was determined using unpaired t tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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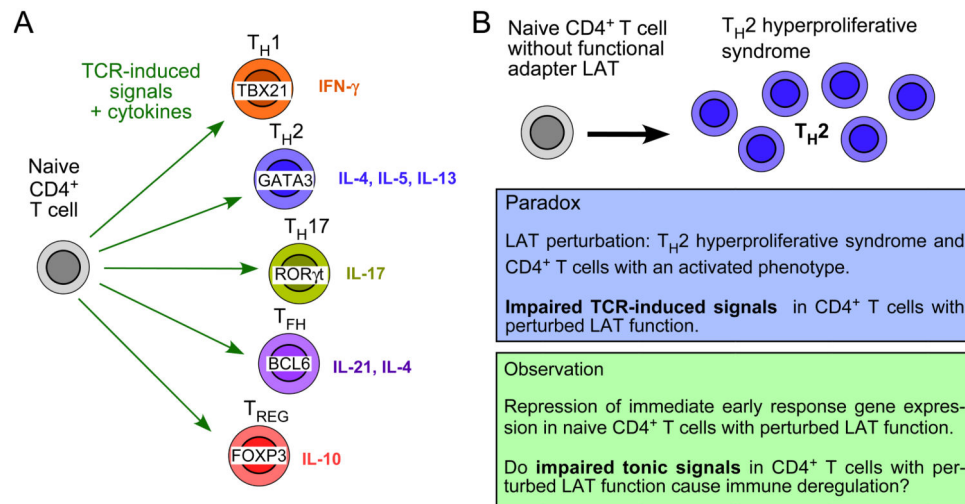


Figure 1. A paradoxical TH2 hyperproliferative syndrome

(A) Schematic of Th1-, Th2-, Th17-, Tfh-, and Treg- effector subset differentiation.

(B) Schematic of the Th2-biased hyperproliferative syndrome that occurs following perturbations of LAT function and explanation of the apparent paradox.

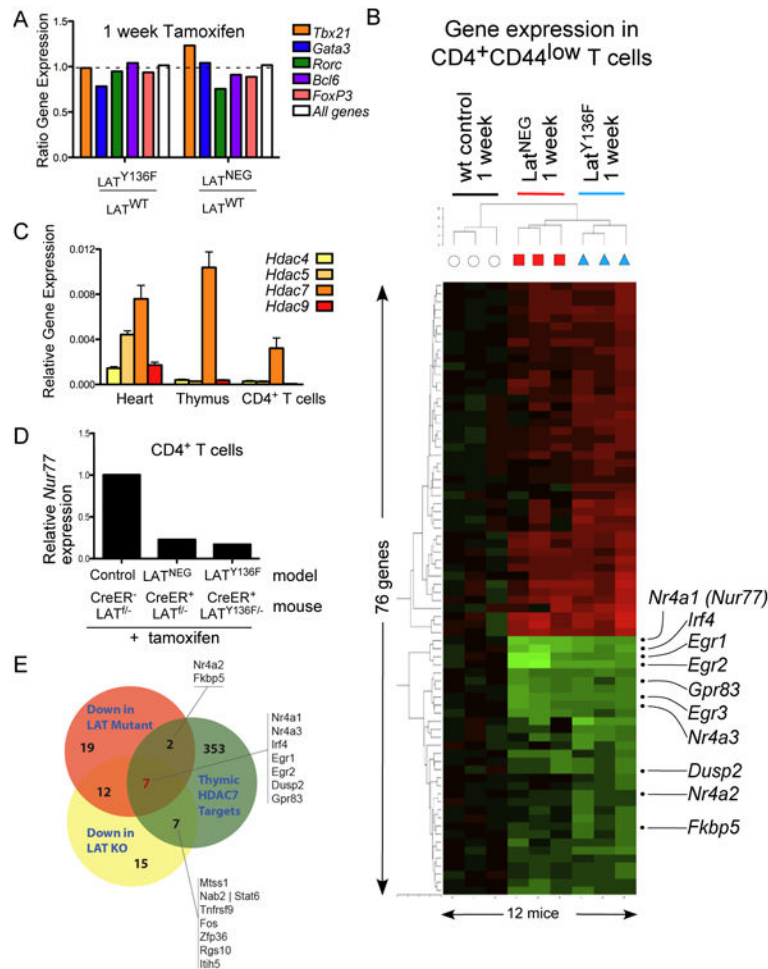


Figure 2. A panel of immediate early response genes is maintained by LAT and repressed by HDAC7

(A) Averaged gene expression levels of T_H cell transcription factors from microarray in Supplemental figure 1. Gene expression levels in LAT^{Y136F} and LAT^{NEG} were normalized to cells with intact, functional LAT (LAT^{WT}).

(B) Microarray analysis of gene expression changes in naïve CD4⁺ T cells with short term LAT perturbation. A specific cluster of HDAC7 target genes including *Egr1*, *Egr2*, *Egr3*, *Irf4*, *Nurr77* or *Nr4a1*, *Nr4a2*, and *Nr4a3* is highlighted. For more detail on the unsupervised clustering and all gene names, see Supplemental figures S1 and S2. Array data can be found in GEO (mouse array accession number GPL7207; human array accession number GPL18142).

(C) Taqman qPCR analysis of levels of class IIa HDAC transcripts in murine tissues, relative to *GAPDH* levels. Mean and error bars (SEM) are indicated of triplicate samples. A representative example of three independent experiments is shown.

(D) TaqMan qPCR analysis of *Nurr77* levels in purified CD4⁺ T cells isolated from tamoxifen-treated WT, LAT^{NEG} and LAT^{Y136F} mice. Representative example of two independent assays on these three samples.

(E) Venn diagrams identifying genes with shared regulation by LAT and HDAC7. For a complete overview of the analyses see Supplemental tables 1 and 2.

See also Supplemental tables 1 and 2

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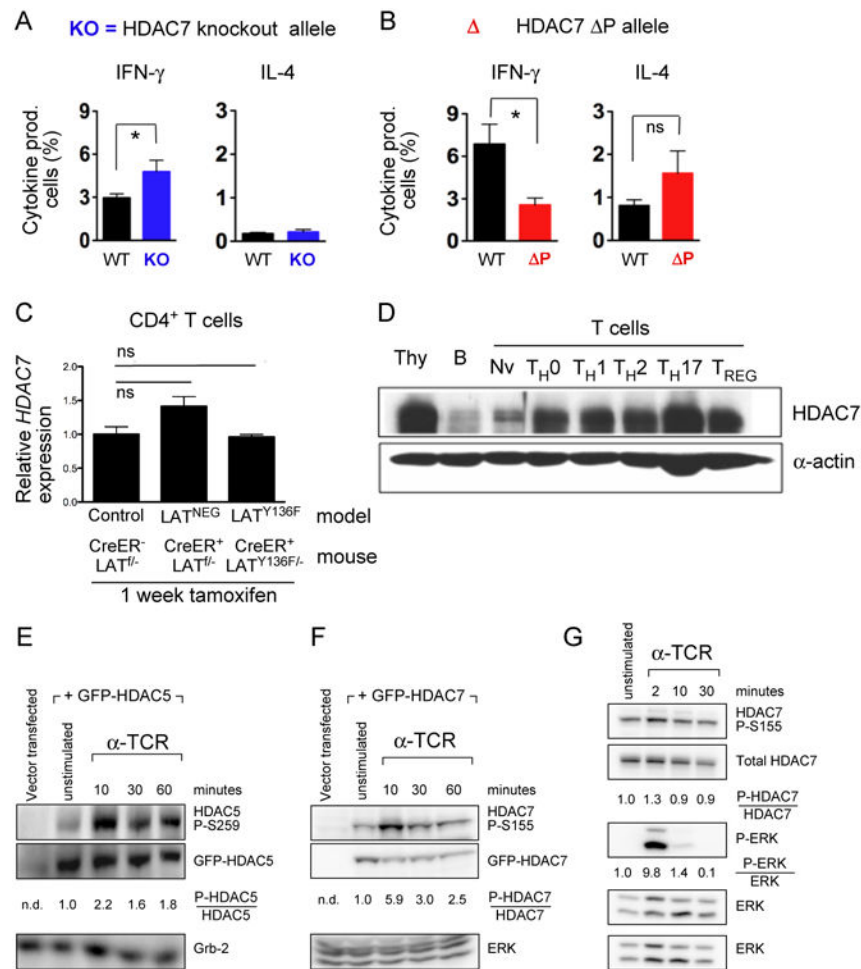


Figure 3. HDAC7 represses IFN- γ and is constitutively phosphorylated in CD4⁺ T cells (A and B) Bar graph representation of percentages of cytokine-producing cells isolated from wildtype mice compared to HDAC7-deficient (KO in A) and HDAC7 P-transgenic mice (P in B) that were acutely stimulated with PMA and ionomycin for 4 hours *ex vivo* in the presence of Brefeldin A. Data are representative of 3-6 (A) or 5 (B) independent experiments with 1-2 mice/group, and error bars represent the SEM. See also Supplemental figure 3

(C) Gene expression levels of HDAC7 in LAT^{WT}, LAT^{Y136F} and LAT^{NEG} mice. Gene expression levels (mean + SEM) were obtained from the 3 data points from the microarray with 1-week tamoxifen treatment. ns = not significant.

(D) Immunoblot for HDAC7 levels in thymocytes (Thy), B cells (B), Naive T cells (Nv), and *in vitro* stimulated or polarized helper T cell subsets (T_{H0}, T_{H1}, T_{H2}, T_{H17}, T_{REG}). α -actin functions as loading control.

(E, F) Immunoblotting for phosphorylated HDAC-5 or -7 in Jurkat T cells transfected with vector alone, HDAC5-GFP (E) or HDAC7-GFP (F) and stimulated via the TCR for indicated time points. GFP indicates the level of transfected HDAC-GFP and Grb2- or ERK- blotting serves as loading control. The amount of phosphorylated HDAC was quantitated by normalizing for GFP levels, setting the unstimulated sample arbitrarily at 1.0.

(G) Immunoblotting of phosphorylated HDAC7 and phosphorylated ERK in primary CD4⁺ T cells isolated from mouse lymph nodes and stimulated via the TCR for indicated time points. Quantitation of phospho-HDAC7 or -ERK was determined by normalizing for total HDAC7 or ERK. Blots in panels D-G are representative examples of at least three independent experiments for each panel.

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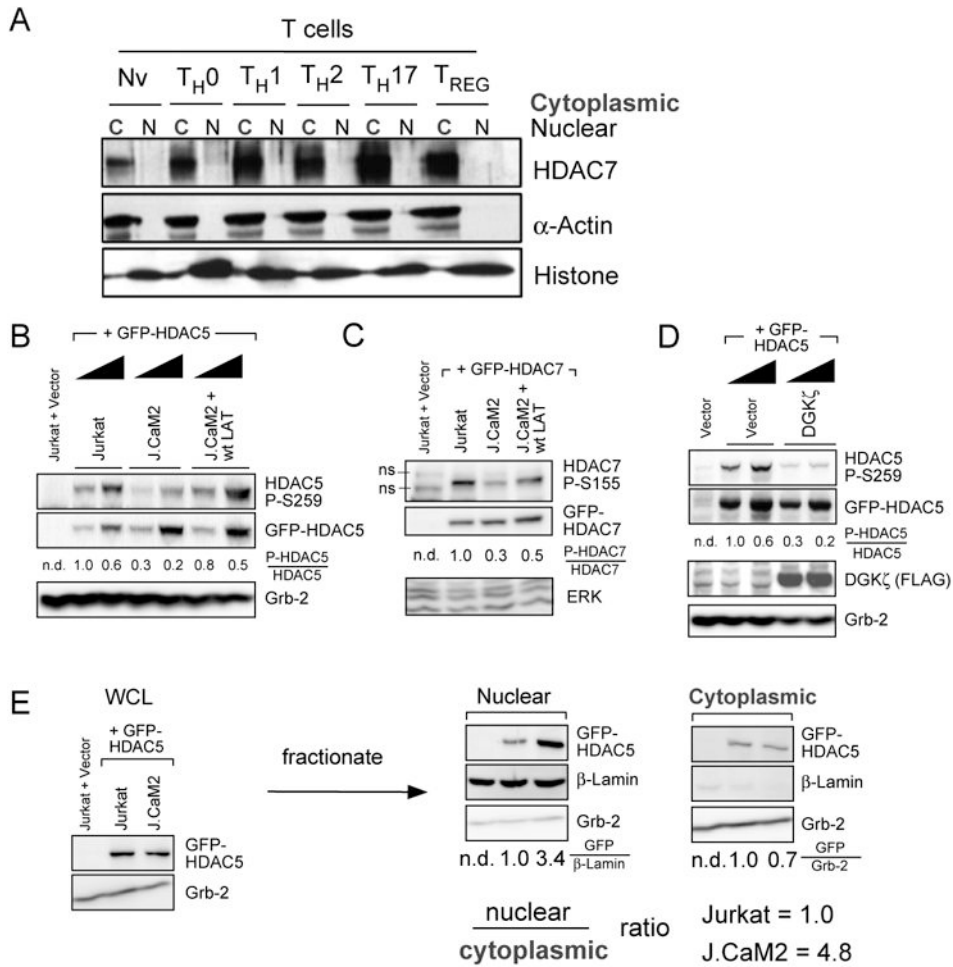


Figure 4. A tonic LAT-PLC γ 1-DAG signal regulates constitutive phosphorylation and nuclear export of HDAC-5 and -7

(A) Immunoblotting for HDAC7 in cytoplasmic (C) and nuclear (N) fractions obtained from naive T cells (Nv), and *in vitro* polarized helper T cell subsets (T_H0, T_H1, T_H2, T_H17, T_{REG}).

(B, C) Analysis of basal phospho-HDAC5 (in B) or HDAC7 (in C) levels in unstimulated Jurkat, J.CaM2 (LAT-deficient Jurkat T cells), and J.CaM2 + a WT LAT cDNA construct. Cells were transfected and analyzed as in Figure 3.

(D) Immunoblotting for basal phospho-HDAC5 in cells transfected with FLAG-tagged DGK ζ to counteract basal PLC γ 1-DAG signals.

(E) Analysis of basal HDAC5 localization in Jurkat and J.CaM2 cells transfected with HDAC5-GFP. Whole cell lysates (top) and nuclear (left) and cytoplasmic (right) fractions were assessed for HDAC5 by GFP blotting, β -lamin and Grb2 function as markers for the purity of the nuclear and cytoplasmic fractions, respectively. The fractions of GFP over β -lamin or Grb2 function were used to determine a nuclear/cytoplasmic ratio for HDAC5. This ratio was 1.0 for Jurkat and increased to 4.8 (3.4 over 0.7) for LAT-deficient J.CaM2 cells. Blots in panels 4A-E are representative examples of at least three independent experiments for each panel.

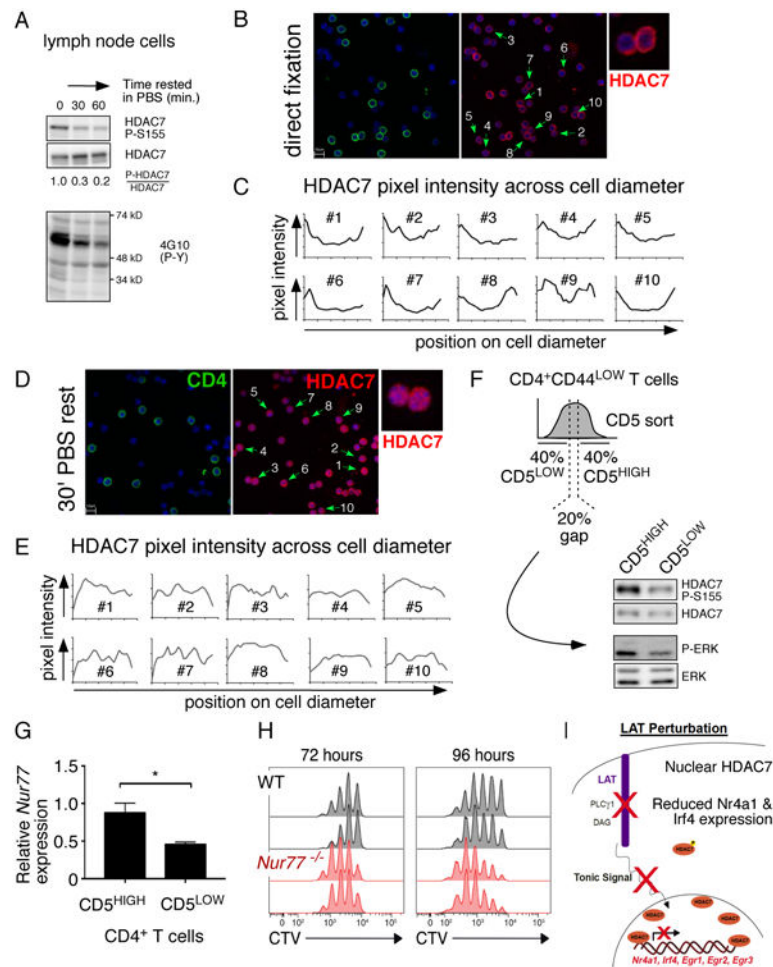


Figure 5. Tonic signals in CD4⁺ T cells dynamically regulate HDAC7 and its target Nur77 to limit proliferation

(A) Immunoblotting for phosphorylated HDAC7 (top) and total phosphotyrosine (4G10, bottom) in lymph node cells isolated from mice that were lysed directly or first rested in PBS for the indicated time points.

(B, C) Immunofluorescence of HDAC7 localization in CD4⁺ T cells in a cytospin preparation co-stained for HDAC7 (red) and CD4 (green). Quantification of pixel signal intensity across the diameter of the 10 CD4⁺ T cells in B was obtained with Fiji image analysis software.

(D, E) Analysis of HDAC7 localization exactly as in B and C, except that lymph node cells were rested in PBS for 30 minutes prior to fixation and cytospin analysis. Note the switch to predominant nuclear HDAC7 localization following PBS rest.

(F) Schematic of CD5 sorting scheme of naïve CD44^{low} CD4⁺ T cells from lymph node and immunoblot analysis of phospho-HDAC7 and -ERK levels in sorted populations. An arbitrary 20% gap was chosen to segregate CD5^{HIGH} from CD5^{LOW} cells yet obtain sufficient cells for biochemistry. Total HDAC7 and ERK serve as loading controls.

(G) TaqMan qPCR analysis of *Nur77* mRNA levels in CD5 sorted cells. Taqman samples were run in triplicate. *Nur77* mRNA levels were arbitrarily set at 1.0 in one CD5^{LOW}

sample. Unpaired T test: $p = 0.018$ (indicated by *). All panels in A-G are representative examples of three independent experiments.

(H) Flow cytometric analysis of Cell Trace Violet (CTV) dilution in sorted ($\text{TCRb}^+ \text{CD4}^+ \text{CD25}^- \text{CD44}^{\text{LOW}}$) naïve CD4^+ T cells from two WT and two $\text{Nur77}^{-/-}$ mice. Cells were cultured for 72 and 96 hours on CD3/CD28 coated plates (0.5ug/ml each) at which point viable CD4^+ cells were analyzed for CTV dilution by flow cytometry. Data is representative of two independent experiments.

(I) Cartoon summarizing how tonic signals through LAT influence phosphorylation and nucleo-cytoplasmic shuttling of HDAC7 and expression of HDAC7 target genes.

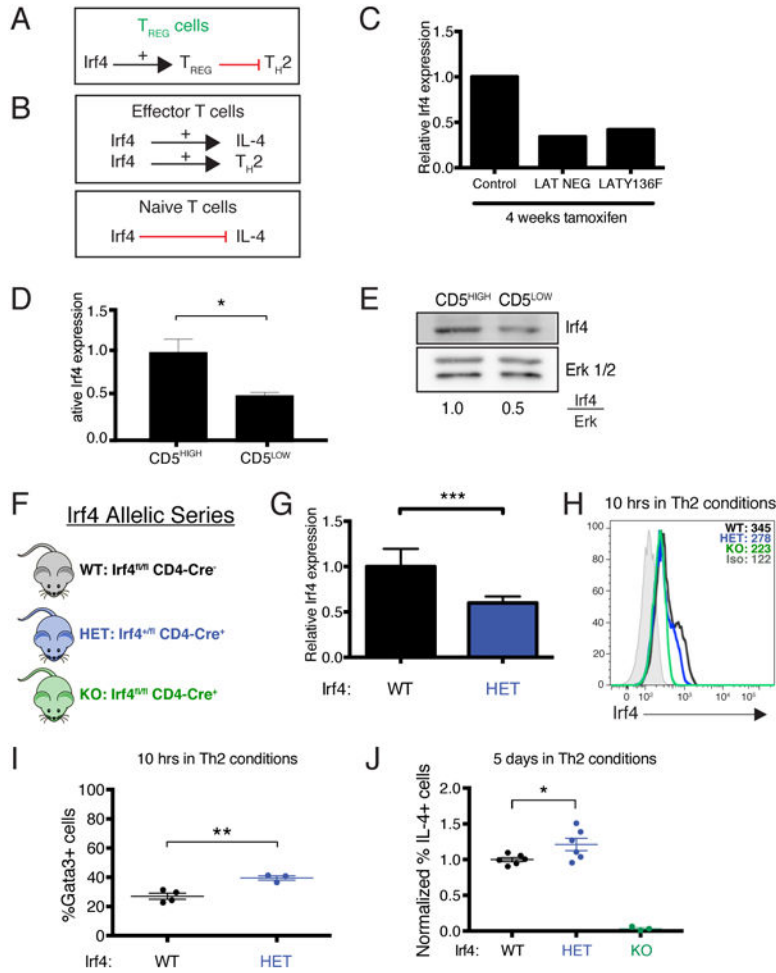


Figure 6. Irf4 expression is under tonic signal regulation and curbs TH2 polarization of CD4+ T cells

(A) Schematic of the role of Irf4 in regulatory T cells (T_{REG}) and suppressive effects on T_H2 differentiation.

(B) Schematic of the role of Irf4 in effector (top) and naïve (bottom) CD4⁺ T cells in controlling T_H2 differentiation and IL-4 production.

(C) TaqMan qPCR analysis of *Irf4* levels in T cells isolated from 4-week tamoxifen-treated WT, LAT^{KO} and LAT^{Y136F} mice. Representative example of three independent assays on these three samples.

(D) TaqMan qPCR analysis (n = 3) and (E) immunoblot analysis (n=2) of Irf4 levels in CD5 sorted cells. Taqman samples were run in triplicate. *Irf4* mRNA levels were arbitrarily set at 1.0 in one CD5^{LOW} sample. Unpaired T test: p = 0.034 (indicated by *).

(F) Cartoon of *Irf4* allelic series.

(G) TaqMan qPCR analysis of *Irf4* levels in T cells isolated from Irf4^{wt/wt} and Irf4^{+/-} CD4-Cremice. Representative example of two independent experiments performed in triplicate. UnpairedT test: p = 0.0005 (indicated by ***).

(H) Flow cytometry for Irf4 expression in sorted CD4⁺ CD25⁻ CD44^{LOW} T cells from Irf4 WT and HET mice cultured for 10 hours in T_H2-polarizing conditions on a-CD3/CD28

coated plates (0.5ug/ml each). Data is representative of two independent experiments with 2 mice/group.

(I) Flow cytometry for Gata3 in MACS-purified CD4⁺ CD25⁻ CD44^{LOW} T cells from Irf4 WT and HET mice cultured for 10 hours in T_H2-polarizing conditions. Data is representative of three independent experiments with 3-4 mice per group. Error bars represent SEM and significance was determined using an unpaired t-test, p=0.0056 (indicated by **).

(J) Flow cytometric analysis of IL-4 production in viable T_H2 polarized naïve CD4⁺ CD25⁻CD44^{LOW} T cells sorted from Irf4^{wt/wt} CD4-Cre mice (WT), Irf4^{+/-fl} CD4-Cre mice (HET), or Irf4^{fl/fl} CD4-Cre mice (KO) mice stimulated with 0.5ug/ml a-CD3 and a-CD28. Percentage of cytokine-positive cells were calculated and data was normalized and compiled from three independent experiments with 2 mice per group. Error bars represent SEM and significance was determined using an unpaired t-test, with Th2 WT vs HET p=0.0446 (indicated by *) and WT vs KO p<0.0001.