Equilibrative Nucleoside Transporter 3 Regulates T Cell Homeostasis by Coordinating Lysosomal Function with Nucleoside Availability

Highlights

- Lysosomal nucleoside transporter ENT3 is required for T cell survival upon activation.
- Absence of ENT3 leads to accumulation of surplus mitochondria and elevation of ROS.
- ENT3 regulates lysosomal integrity and nucleoside availability to support T cells.
- T cell lysosomes are an important source of salvaged metabolites.

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In Brief

A sufficient supply of biomaterials is essential during T cell differentiation and proliferation. Wei et al. reveal that the nucleoside transporter ENT3 is necessary for maintaining T cell homeostasis and survival by regulating lysosome function and nucleoside availability.
Equilibrative Nucleoside Transporter 3 Regulates T Cell Homeostasis by Coordinating Lysosomal Function with Nucleoside Availability

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S U M M A R Y

T cells are a versatile immune cell population responding to challenges by differentiation and proliferation followed by contraction and memory formation. Dynamic metabolic reprogramming is essential for T cells to meet the biosynthetic needs and the reutilization of biomolecules, processes that require active participation of metabolite transporters. Here, we show that equilibrative nucleoside transporter 3 (ENT3) is highly expressed in peripheral T cells and has a key role in maintaining T cell homeostasis by supporting the proliferation and survival of T cells. ENT3 deficiency leads to an enlarged and disturbed lysosomal compartment, resulting in accumulation of surplus mitochondria, elevation of intracellular reactive oxygen species, and DNA damage in T cells. Our results identify ENT3 as a vital metabolite transporter that supports T cell homeostasis and activation by regulating lysosomal integrity and the availability of nucleosides. Moreover, we uncovered that T cell lysosomes are an important source of salvaged metabolites for survival and proliferation.

I N T R O D U C T I O N

Metabolism and immunity are considered distinct physiological activities: metabolism is responsible for the processing and disposal of nutrients, whereas immunity guards the body from pathogen invasion. Nevertheless, both act to regain body homeostasis. Recent advances suggest a strong link between immunity and metabolism (O’Neill et al., 2016) because their balance affects each other. Although inflammation contributes to metabolic dysregulation (Hotamisligil and Erbay, 2008), starvation impairs immune responses (Demas et al., 2003), and over-nutrition, such as type 2 diabetes, induces chronic inflammation (Donath and Shoelson, 2011; Shu et al., 2012). It is becoming apparent that steady energy flux and metabolic homeostasis are crucial for the immune system to function properly.

Immune cells are a unique cell population in the body. The clonal expansion nature of the immune response makes them one of the few cell populations that respond to external stimuli by drastic proliferation followed by contraction, providing a physiological cellular model to understand how cells initiate metabolic change and coordinate it with cell growth and proliferation. It has been estimated that each antigen-reactive T cell can give rise to 20 daughter cells within a 48-hr period (Gudmundsdottir et al., 1999). Although naive T cells are metabolically inert, activating signals, through T cell receptor and co-stimulatory receptors, switch them from quiescence to the proliferative and active effector state (Jones and Thompson, 2007). This shift is a costly decision; the cell has to increase ATP production and acquire or synthesize the substrates (e.g., glucose, amino acids, nucleotides, fatty acids) that are needed for differentiation and proliferation. Activated T cells transit their energy production from oxidative phosphorylation (OXPHOS) to glycolysis (Buck et al., 2015), and the ones that cannot fulfill this bioenergetic demand die by apoptosis (Michalek and Rathmell, 2010). The connection between cellular metabolism, such as sensing and acquiring nutrients, and activation and differentiation of T cells has become clear with recent advances (Wei et al., 2017). The molecular machinery responsible for the drastic intracellular metabolic reprogramming in T cells, such as the role of metabolite transporters in this dynamic but regulated process, has started to gain attention. For example, glucose is transported into activated T cells via high-affinity surface glucose transporters (Wofford et al., 2008) and metabolized by glycolysis, which both provides energy and directs the use of glucose for macromolecular biosynthesis. Similarly, the expression of amino acid transporters, which are responsible for obtaining large neutral amino acids that are required for T cell differentiation, is directly regulated by antigen receptor-mediated activating signals (Sinclair et al., 2013). These results emphasize the contribution of metabolite transporters in regulating the accessibility of crucial metabolites during T cell responses. However, how the availability of nucleosides, the major building blocks for DNA/RNA synthesis, and their respective transporters is regulated in activated T cells is largely unexplored.

The importance of nucleosides and their derivatives in T cells has been demonstrated by various studies. For example, purine...
nucleoside phosphorylase (PNP) deficiency causes a selective intracellular accumulation of deoxyguanosine triphosphate (dGTP) and leads to T cell lymphopenia and abnormalities in T cell function (Papinazath et al., 2011). Deletion of nucleoside salvage pathway enzymes causes dNTP pool depletion and results in genomic instability because of replication stress and eventually leads to a lymphoproliferative phenotype in animals (Austin et al., 2012). Moreover, T cells deficient in cytidine triphosphate (CTP) synthase 1 fail to proliferate upon T cell receptor (TCR) activation, and patients with mutations in this enzyme develop immunodeficiency (Martin et al., 2014). Together, these results strongly suggest that the availability of nucleosides and their derivatives has a vital role in the activation and survival of T cells.

Nucleosides are hydrophilic molecules that need specialized transporter proteins to facilitate their translocation across membranes. There are two families of nucleoside transporters: concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs) encoded by the solute carrier (SLC) family genes Slc28a2 and Slc29a2, respectively. CNTs transport nucleosides unidirectionally, whereas ENTs facilitate bidirectional movement. ENTs are abundant on intestinal epithelia as nucleoside pumps. ENTs have received much attention because of their ubiquitous expression and ability to facilitate the uptake of anti-cancer nucleoside analogs (Young, 2016; Young et al., 2013). There are four ENT family members: ENT1, ENT2, and ENT4 are situated on the plasma membrane, whereas ENT3 has been reported to have endosomal/lysosomal (Baldwin et al., 2005) and mitochondrial membrane localization (Govindarajan et al., 2009). Although ENTs are suggested to be involved in the activation of immune cells, little is known about the underlying mechanisms (Junger, 2011). ENT1 is considered a major metabolite transporter responsible for nucleoside analog drug delivery, and its mutation affects the prognosis of cancer treatment (Spratlin et al., 2004; Zimmerman et al., 2009), alcohol dependency (Kim et al., 2011), and ectopic mineralization of bones (Daniels et al., 2015). On the other hand, ENT3 mutations have been linked to a group of heterogeneous hereditary diseases in humans, including H syndrome (MoIho-Pessach et al., 2008), Faisalabad histiocytosis (Morgan et al., 2010), pigmentary hypotrichosis and non-autoimmune insulin-dependent diabetes mellitus syndromes (Cliffe et al., 2009), and Rosai-Dorfman disease (Morgan et al., 2010). Human ENT3 mutants have a wide spectrum of clinical symptoms, but patients commonly suffer from histiocytosis, an accumulation of monocyte cells in affected tissues. Previously we have identified ENT3 as a crucial lysosomal nucleoside transporter that participates in maintaining macrophage (Mø) function and homeostasis and shown that ENT3 deficiency results in a myeloproliferative phenotype (Hsu et al., 2012). However, it is noteworthy that, even though patients carrying ENT3 mutations have an increased number of Møs, they still experience recurrence of infection (Campeau et al., 2012), suggesting that ENT3 dysfunction may also affect adaptive immunity in the patients. Currently, the biological relevance of ENTs in adaptive immune cells is largely unknown.

Here, we show that the nucleoside transporter ENT3 contributes to the homeostasis of T cells at multiple levels. We found that ENT3 has a dynamic expression profile in T cells and is up-regulated in peripheral T cells, particularly the effector populations. The absence of ENT3 does not influence T cell development but affects the homeostasis of the peripheral T cell pool. ENT3 knockout (ENT3<sup>−/−</sup>) T cells have an effector-like surface phenotype and fail to proliferate and survive upon stimulation. We found that, as an intracellular nucleoside transporter, ENT3 participates in maintaining the quantity and integrity of lysosomes as well as regulating the availability of nucleosides. These results suggest that ENT3 is a key metabolite transporter that supports the homeostasis and activation of T cells.

RESULTS

ENT3 is Highly Expressed in Peripheral T Cells and Has a Distinct Expression Profile among T Cell Subpopulations

To investigate whether nucleoside transporters contribute to the development and/or function of T cells, we performed expression profiling of ENTs in thymocytes and peripheral T cells. ENT1 was abundant, whereas ENT3 had relatively low expression in the thymus (Figure S1A). However, the trend was reversed in peripheral T cells (Figure S1B). Among the ENT family members, no detectable level of ENT4 was found in both thymus and peripheral T cells (data not shown). We further examined the expression of ENT3 in T cell subpopulations. Thymocytes at different developmental stages were sorted by their surface phenotypes, whereas mature peripheral T cells were sub-fractionated into naive and effector populations. ENT3 was highly expressed in double-negative (DN) and double-positive (DP) populations and decreased significantly at single-positive (SP) stages in the thymus. Among SP thymocytes, CD4<sup>+</sup> SP cells had significantly higher expression than CD8<sup>+</sup> SP cells (Figure S1C). In the periphery, effector cells showed significantly higher ENT3 expression than naive cells in both CD4 and CD8 T cells (Figure S1D). It is noteworthy that, in both the thymus and periphery, ENT3 was highly expressed in the stages that require active proliferation, such as DN-to-DP and naive-to-effector transitions. These data show that ENT3 has a dynamic expression profile in different T cell subpopulations.

ENT3 Deficiency Does Not Affect T Cell Development but Affects the Homeostasis of T Cells in the Periphery

The dynamic expression profile of ENT3 in T cells prompted us to examine the development and homeostasis of T cells in ENT3-deficient animals. ENT3<sup>−/−</sup> mice had altered T cell phenotype in the periphery; there was a nearly 70% decrease in the proportions of TCRβ<sup>+</sup> cells in both the spleen (Figure 1B) and lymph nodes (data not shown) of ENT3<sup>−/−</sup> mice, with slightly decreased TCRβ<sup>+</sup> cell numbers compared with wild-type (WT) littermates. The ENT3<sup>−/−</sup> TCRβ<sup>+</sup> population had a skewed CD4 to CD8 T cell ratio (Figure 1C) with a significant decrease in CD4<sup>+</sup> and increase in CD8<sup>+</sup> populations. When examined in detail, the majority of ENT3<sup>−/−</sup> CD4 T cells showed an effector phenotype (CD62L<sup>−</sup>CD44<sup>+</sup>), whereas few remained naive (Figure 1D). A similar loss of the naive and gain of the effector phenotype was found in ENT3<sup>−/−</sup> CD8 T cells (Figure 1E). In addition, Foxp<sup>+</sup> regulatory T cells were also increased (Figure S2A). Together,
these data suggest that ENT3 may be dispensable for thymocyte development but may play an important role in maintaining the homeostasis of peripheral T cells.

**ENT3 Supports the Survival and Proliferation of Peripheral T Cells**

Given the significant loss of naive T cells in ENT3−/− mice, we hypothesized that ENT3 supports the survival and/or proliferation of mature peripheral T cells. We used an Annexin V binding assay to examine whether the absence of ENT3 leads to increased apoptosis of T cells. An increased percentage of Annexin V+ cells was found in ENT3−/− CD4 naive and effector cells (Figure 2A) as well as CD8 naive cells (Figure 2B) compared with the WT. We further measured the expression of the anti-apoptotic protein Bcl-2, and, to our surprise, we detected a higher amount of Bcl-2 in ENT3−/− T cells (Figures 2C and 2D). Expression of Bcl-2 in T cells is regulated by survival cytokines such as interleukin-2 (IL-2), IL-7, and IL-15 (Rathmell et al., 2001). Although ENT3−/− mice had normal transcription of these cytokines (Figure 2E), elevated amounts of IL-2 and IL-15 were detected in the serum (Figure 2F), suggesting a T cell lymphopenic environment. Thus, despite high levels of pro-survival cytokines, ENT3−/− T cells failed to expand. To test whether ENT3 has a vital role in supporting T cell proliferation, naive CD4 T cells were harvested, labeled, and activated by anti-CD3/CD28 stimulation. We noted a clear defect of ENT3−/− T cells responding to CD3/CD28 stimulation by 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) dilution. While the majority of WT T cells underwent 2 to 3 divisions upon activation, significantly fewer dividing ENT3−/− T cells were observed (Figure 2G). Moreover, a viability analysis of the stimulated T cells at various time points showed that ENT3−/− T cells not only failed to proliferate but also underwent cell death upon activation (Figure 2H). Our data show that ENT3-deficient T cells fail to maintain their homeostasis; they neither survive nor expand in response to activation. These results suggest that ENT3 is pivotal in supporting T cell survival and proliferation.

**ENT3−/− T Cells Have Intrinsic Defects and a Homeostasis-Driven Activation Phenotype**

The results reported above raised the possibility that the shift of T cells naive to the effector surface phenotype in ENT3−/− mice...
is the consequence of homeostatic proliferation-driven activation. To test this possibility and delineate the intrinsic versus extrinsic effects, total T cells were purified from WT and ENT3−/− donors, labeled, mixed at a 1:1 ratio, and adoptively transferred to WT, ENT3−/− or RAG2−/− hosts. We found that ENT3−/− T cells were at disadvantage in all hosts tested (Figures 3A and 3C). In the WT host, the majority of the WT T cells retained their naive surface phenotype as expected, whereas ENT3−/− T cells retained their effector-like phenotype (Figures 3B and 3D). A significantly higher proportion of transferred WT T cells showed an effector-like phenotype in the ENT3−/− and RAG2−/− host (Figure 3B and D), suggesting that the ENT3−/− and RAG2−/− host environment contributed to development of the effector-like phenotype. Although both transferred WT and ENT3−/− T cells remained quiescent in the WT host, WT T cells proliferated in the ENT3−/− host (Figure 3E), although these cells failed to survive and reconstitute the T cell compartment (Figure 3C). Collectively, these data suggest that the T cell phenotype of ENT3−/− mice can be attributed to both intrinsic and extrinsic factors and that ENT3−/− T cells have intrinsic defects in survival and proliferation. We also identified that homeostasis-driven activation is one of the major contributors to the activated phenotype of ENT3−/− T cells.

### ENT3 Is Required for Maintaining T Cell Size and Complexity

Our data showed that ENT3 is necessary for the survival and proliferation of T cells, but the question of how ENT3 participates in T cell homeostasis remained. To address it, we assessed the morphology of T cells in detail. Electron microscopy images showed that both naive and effector ENT3−/− T cells had altered morphology compared with WT cells (Figures 4B and S3B). Moreover, the nucleus-to-cytosol ratio (N/C ratio) was substantially lower in naive (Figure 4C) but not effector ENT3−/− T cells (Figure S3C). To determine whether the decreased N/C ratio is a result of increased cell size or nucleus shrinkage, we estimated

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**Figure 2. Survival and Proliferation ofPeripheral T Cells Require ENT3**

(A and B) The survival of (A) CD4+ or (B) CD8+ T cells was evaluated by Annexin V binding assay. *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired t test); n = 10, pooled results from 4 independent experiments.

(C and D) The expression of Bcl-2 in CD4+ (C) and CD8+ (D) T cell was examined by intracellular staining. *p < 0.05 (unpaired t test).

(E and F) Transcription of IL-2, IL-7, and IL-15 in lymph nodes was evaluated by qPCR (E), and the serum levels of these cytokines are shown in (F). *p < 0.05 (unpaired t test). N.D., not detected.

(G) Naive CD4 T cells were purified from WT and ENT3−/− mice, labeled with CFSE, and stimulated with anti-CD3/CD28. The proliferation response was determined by CFSE dilution 48 hr after stimulation.

(H) The stimulated cells were subjected to propidium iodide (PI) staining at various time points to determine viability. *p < 0.05 (unpaired t test). Data are means ± SEM of 3 independent experiments.
the size of T cells using flow cytometry. \textit{ENT3}\(^{-/-}\) T cells were substantially larger compared with WT cells, with increased internal complexity (Figure S3A). These observations showed that, in the absence of ENT3, the T cells experienced cellular disturbances, including cell enlargement and the accumulation of excess vacuoles and organelles. These results suggest that ENT3 plays an important intrinsic role in maintaining the cellular homeostasis of T cells.

**ENT3 Contributes to the Regulation of Lysosome Quantity and Integrity in T Cells**

The abnormal accumulation of intracellular vacuoles and organelles led us to suspect lysosomal dysfunction in \textit{ENT3}\(^{-/-}\) T cells. T cells are not phagocytes, but lysosomes are important for the degradation of endocytosed materials, release of luminal cytolytic granules, and autophagy in T cells (Luzio et al., 2007). To examine whether ENT3 deficiency leads to lysosomal disturbances in T cells, both the quantity and quality of lysosomes were measured. We observed a significantly higher LysoTracker signal in both CD4 and CD8 \textit{ENT3}\(^{-/-}\) T cells (Figures 5A and 5B), suggesting an increase of lysosome content in these cells, although no differences were found in \textit{ENT3}\(^{-/-}\) CD8 effectors. To investigate ENT3’s role in the maintenance of lysosomes, we examined lysosomal integrity by measuring lysosomal membrane permeabilization (LMP). Naive and effector T cells were stained for the lysosomal enzyme Cathepsin B to detect alterations in LMP. Although WT T cells showed lysosomal confinement of Cathepsin B and only had cytosolic distribution upon anti-CD3 activation (Figure S4A), a significant amount of cytosolic Cathepsin B was detected in \textit{ENT3}\(^{-/-}\) T cells (Figure 5C), suggesting a change of LMP in \textit{ENT3}\(^{-/-}\) T cells. We further applied a Galectin-3 translocation assay to detect lysosomal damage (Aits et al., 2015) and found that \textit{ENT3}\(^{-/-}\) T cells harbored a significantly increased number of damaged lysosomes (Figure 5D). The lysosomal alkalizing agent chloroquine enhanced Galectin-3 translocation in both WT and \textit{ENT3}\(^{-/-}\) T cells (Figure S4B), suggesting that the elevated lysosomal pH might be one of the factors contributing to the presence of damaged lysosomes in \textit{ENT3}\(^{-/-}\) T cells. In summary, our data suggest that...
ENT3 maintains the cellular homeostasis of T cells by regulating lysosome integrity.

**Accumulation of Surplus Mitochondria and Elevation of Reactive Oxygen Species in the Absence of ENT3**

Lysosomes are a crucial component of autophagy, a catabolic process that has been shown to be vital in T cell development, activation, and differentiation (Bronietzki et al., 2015). Autophagy not only plays pivotal role in mediating responses upon starvation but is also crucial for removing damaged proteins and organelles. We observed that ENT3−/− T cells experienced disturbances in autophagy, including accumulation of autophagic vacuoles, LC3-I, and p62 (Figure S5). One important role of autophagy in T cells is to remove surplus mitochondria. The reduction of mitochondrial content coincides with the transition from thymocytes to peripheral mature T cells (Pua et al., 2009) and is necessary for the survival of T cells in O2-rich blood circulation. The presence of disorganized mitochondria (Figure 4A) in ENT3−/− T cells implied defective autophagic clearance of mitochondria. We used MitoTracker to measure the amount of mitochondria in T cell populations (Figure 6A) and found a significant accumulation of mitochondria in all ENT3−/− T cells, except for CD8 effectors (Figure 6B). To evaluate whether ENT3 deficiency affects T cell energy-producing pathways, a metabolic flux analysis was performed. Naive and effector-like ENT3−/− T cells showed lower basal levels of oxygen consumption rate/extracellular acidification rate (OCR/ECAR) compared with the WT (Figure S6A) but higher ECAR values at maximal respiration (Figure S6B), results suggesting that ENT3−/− T cells had a distorted metabolic status in spite of higher mitochondrial content. Mitochondria are also responsible for reactive oxygen species (ROS) production via the electron transport chain (ETC), and activated phagocytes use ROS to fight pathogens. However, excess intracellular levels of ROS damage biomolecules (Sena and Chandel, 2012) and participate in activation-induced cell death (Belikov et al., 2015). We speculated that the abnormal accumulation of mitochondria and disrupted lysosomal membrane stability in ENT3−/− T cells might lead to increased intracellular ROS levels (Ghosh et al., 2011; Pua et al., 2009). Naive ENT3−/− T cells had a mild but significant increase in ROS levels compared with the WT (Figures 6C and 6D), likely as a result of accumulation of surplus mitochondria. However, a ROS scavenger failed to rescue the ENT3−/− T cell survival defects (Figure 6E), suggesting that an increased ROS level was not the sole contributor to enhanced ENT3−/− T cell death. Together, these data suggest that ENT3 has a key role in surplus mitochondrion removal, likely through lysosomal-mediated functions.
ENT3 Has a Major Role in Distributing Nucleosides that Are Needed for T Cell Survival

We have identified that ENT3 supports T cell homeostasis by mediating lysosome integrity. However, the function of ENT3 in the T cell lysosomal compartment remains to be investigated. Because ENT3 is a nucleoside transporter, we speculated that it might be responsible for supplying sufficient amount of nucleosides for DNA/RNA synthesis. We found that ENT3-/- T cells harbored DNA damage by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Figures 7A and 7B). To test whether nucleoside insufficiency contributes to the survival defect of ENT3-/- T cells, we supplemented exogenous nucleosides to T cells and measured their survival. As shown in Figure 7C, significant recovery of ENT3-/- T cell viability in the presence of exogenous nucleosides was observed. We then tested whether lysosomal degradation is involved in providing cellular nucleosides upon activation. We found that T cells treated with bafilomycin, a lysosome alkalization agent, greatly decreased their viability, whereas addition of nucleosides could partially rescue this effect (Figure 7D). These results suggest that ENT3 is vital in regulating nucleoside availability in T cells and revealed that lysosomal function is required for supplying cellular nucleosides.

DISCUSSION

Our work reveals that ENT3, by preserving lysosomal function and regulating nucleoside availability, maintains the homeostasis of T cells. Deficiency of ENT3 leads to loss of lysosome integrity and results not only in accumulation of mitochondria and elevation of cellular ROS but also in nucleoside deficiency, eventually leading to T cell death. Together, our results place ENT3 as a crucial metabolite transporter that supports T cell immunity.

We found that ENT3 is vital for the homeostasis of peripheral T cells. Deficiency of ENT3 causes a biased effector over...
naive phenotype accompanied by excessive T cell death. This continuous T cell loss in ENT3−/− animals generates a vicious cycle. The T cell lymphopenic environment activates homeostasis-driven proliferation, leading to recruitment and activation of naive T cells into the effector pool, whereas activation of ENT3−/− T cells causes their death. These findings highlight that ENT3 is indispensable for T cell survival upon activation.

In contrast to mature T cells, ENT3−/− mice had normal thymocyte development despite the high expression of ENT3 in DP thymocytes. This could be due to compensation by other ENT members. Judging by the expression profile (Figure S1A), it is possible that ENT1 has a more prominent role in thymocytes and may compensate for loss of ENT3. However, ENT1, ENT2, and ENT4 are expressed mostly on the plasma membrane, whereas ENT3 has a distinct endosomal/lysosomal localization. It is unclear how ENT1, as a surface nucleoside transporter, amends ENT3 loss-of-function defects in the thymic microenvironment.

How does ENT3 support activation and proliferation of mature T cells? The available data point toward a major role of this nucleoside transporter in maintaining lysosomal function and integrity in T cells. We observed that ENT3 deletion results in changed cell morphology and disturbed lysosomal degradation in T cells. ENT3−/− T cells have an enlarged lysosomal compartment accompanied by aggregates and appear swollen with altered N/C ratios. Moreover, we found that ENT3−/− lysosomes are unable to maintain their integrity, as evidenced by Cathepsin B and Galectin-3 staining. Previously, we have demonstrated that ENT3−/− lysosomes have elevated intralysosomal pH values and are, thus, ineffective in degradation (Hsu et al., 2012). We speculate that the intralysosomal buildup of undigested materials ultimately leads to lysosome damage, as numerous studies using silica crystals have suggested (Hornung et al., 2008).

Although lysosomal degradation is not considered a core T cell function, our results suggest that lysosomes have a prominent role in the survival and activation of T cells. This notion is supported by studies with lysosomal acid lipase (LAL) knockout animals. The lack of LAL leads to intracellular lipid accumulation and, eventually, lysosomal dysfunction. LAL−/− mice showed a T cell paucity phenotype, suggesting that lysosomes might directly regulate T lymphocyte homeostasis (Qu et al., 2009).

One possibility of how lysosomes support T cell function is via autophagy. Autophagy is a major lysosomal degradation pathway of cellular materials and is induced upon CD4 T cell activation (Li et al., 2006). It has been considered a crucial regulator of memory CD8 T cell formation and survival (Puleston et al., 2014; Xu et al., 2014). Autophagy might provide metabolic intermediates that are required for T cell activation and proliferation (Bronietzki et al., 2015) or remove unnecessary organelles such as mitochondria. In this aspect, it is interesting that autophagy-deficient mice, such as CD4-Cre x Atg5−/− mice, also have normal T cell development but greatly increased apoptosis of mature T cells (Pua et al., 2007). These mutant mice fail to reduce the mitochondrial content of maturing T cells and suffer damage
from ROS when they enter the oxygen-rich bloodstream. Notably, ENT3−/− T cells also have increased levels of ROS. Thus, impaired mitophagy might underlie at least part of the ENT3−/− T cell phenotype.

Another distinct possibility for ENT3’s role in supporting activation and proliferation of mature T cells is through regulation of intracellular and extracellular nucleoside pools. As a transmembrane nucleoside transporter, its absence will cause accumulation of nucleosides on one side of the membrane and their lack on the other side. Nucleosides can affect the energy status of the cell through the availability of ATP and guanosine triphosphate (GTP), the synthesis of DNA and RNA, and numerous biosynthetic pathways through various uridine diphosphate (UDP) or guanosine diphosphate (GDP)-conjugated intermediaries. The importance of maintaining the intracellular nucleoside pools has been illustrated by the phenotype of mice with deletion of the cellular nucleoside pools. However, whether ENT3 supports cytokine-mediated T cell activation and differentiation should be further explored.

Can the defect in T cell survival in ENT3−/− mice be explained by extrinsic factors? Our T cell transfer experiments show the existence of an “empty” niche with an overabundance of naive T cell survival cytokines (Takada and Jameson, 2009) in ENT3−/− mice. These cytokines promote rather than impair T cell survival, thus they are unlikely to contribute to the demise of ENT3−/− T cells. However, whether ENT3 supports cytokine-mediated T cell activation and differentiation should be further explored.

Another aspect of ENT3−/− mice that can affect their T cell quantity is an increased number of macrophages in the tissues (histiocytosis). These extra phagocytes could engulf activated T cells (Albacker et al., 2013; Arandjelovic and Ravichandran, 2015). We also observed distorted B cell homeostasis (Figure S2B) and decreased natural killer (NK) cells (Figure S2C), suggesting that deficiency of ENT3 has a broad effect on immune cells. Nevertheless, we observed increased T cell death in vitro as well, suggesting that the defect is at least partly intrinsic. Finally, ENT3 could affect nucleoside equilibrium at both intracellular and extracellular levels. We showed that exogenous supplementation with nucleosides could rescue the viability of ENT3−/− T cells, suggesting that intracellular nucleosides are crucial for their survival. On the other hand, extracellular nucleosides are known to have immunomodulatory effects. For example, adenosine can suppress immune cells by activating the A2A receptor (A2aR) on them (Haskó et al., 2008). A2aR signaling maintains the quiescent state of naive T cells, whereas deletion of A2aR...
results in a decrease of naive T cells (Cekic et al., 2013). The predominant substrates of ENT3 are purine and pyrimidine nucleosides, with preferred selectivity for adenosine and inosine (Baldwin et al., 2003). It is, however, unclear whether there are alterations in extracellular nucleoside levels in ENT3−/− mice and whether they are sufficient to cause physiological effects, such as increased T cell apoptosis. Thus, based on the available data, we consider that the defect in T cell survival in ENT3−/− mice is mostly due to their defective lysosomal function and insufficient intracellular nucleoside pool, with extrinsic factors playing a less important role.

Even though ENT3−/− T cells are under cellular stress and prone to apoptosis, ENT3−/− animals do not experience complete T lymphocytopenia. An interesting question raised by this observation is how ENT3−/− T cells cope with the extra burden. Do they have an alternative program, adapting to the metabolic need and cellular stress? What are the sensing and feedback mechanisms to initiate this alternative program? The mechanistic target of rapamycin (mTOR) plays a central role in coordinating cell growth and metabolism with environmental cues (Saxton and Sabatini, 2017). Recent studies showed that mTORC1 increases MTHFD2, a key component of purine synthesis (Smid et al., 2015). Indeed, we observed that absence of ENT3 led to activation of the mTOR pathway (Figure S7), suggesting a potential role of the mTOR pathway in responding to nucleoside equilibrium. These results warrant future studies investigating the relationship between mTOR and nucleoside metabolism.

Activated T cells are highly proliferative cells similar to cancer cells. Many liver, pancreatic, gastric, renal, urothelial, and testicular cancers express high levels of ENT3 (Uhlen et al., 2015), suggesting that ENT3 may have functional importance in these cancer cells. Further understanding of the regulatory mechanism of ENT3 expression may shed light on how highly proliferative cells keep up with their metabolic needs and could present opportunities for therapeutic intervention.

**EXPERIMENTAL PROCEDURES**

**Mice**

8- to 10-week-old ENT3−/− mice (Tang et al., 2010) and their WT littermates were used in this study. ENT3−/− mice were obtained from the Mutant Mouse Regional Resource Center (MMRRC) repository and maintained in a specific-pathogen-free (SPF) facility. All experimental procedures of animal studies were approved and performed in accordance with the National Yang-Ming University (NYMU) Institutional Animal Care and Use Committee guidelines.

**Flow Cytometry (FACS) and Sorting**

Single-cell suspensions were prepared from spleens and thymi. To prevent non-specific binding, cells were incubated with anti-mouse CD16/32 antibody (24G2). The cells were then stained with fluorescence-conjugated antibodies (Supplemental Experimental Procedures) in PBS with 2% fetal bovine serum (FBS) and 5 mM EDTA (fluorescence-activated cell sorting [FACS] buffer), washed, and resuspended in FACS buffer with propidium iodide or Zombie viability dyes. Collection was done on an LSRFortessa (BD Biosciences) and analysis on Flowjo (Tree Star). To quantify the cellular compartments or intracellular ROS levels, cells were first incubated with 0.1 μM LysoTracker/Mitotracker or 5 μM CellROX at 37°C for 30 min, followed by surface staining as described above.

The Annexin V binding assay was performed using an Annexin V apoptosis detection kit following the manufacturer’s manual. Cell sorting was performed using Aria Ilu (BD Biosciences).

**qRT-PCR**

Total RNA was extracted using TRIzol reagent, and cDNA was synthesized with the SuperScript III First-Strand synthesis system. qRT-PCR was performed using the TaqMan Expression assay system or SYBR with the probes listed in the Supplemental Experimental Procedures on a StepOnePlus real-time PCR system (Applied Biosystems). The expression level of the gene of interest was normalized to RPL19.

**Enrichment of T Cells**

Single-cell suspensions of spleens were lysed of erythrocytes, incubated with the mouse Pan T Cell Isolation Kit II (Miltenyi Biotec), and negatively selected by magnetic-activated cell sorting (MACS) LS column. CD4+ T cells were purified using the mouse CD4+ T Cell Isolation Kit II (Miltenyi), CD62L+CD4+ naive T cells were purified from the CD4+ fraction by additional incubation with CD62L MicroBeads and positive selection on a MACS LS column. The purity of the cells was >90%.

**T Cell Proliferation**

To measure T cell division upon activation, purified naive CD4 T cells were labeled with 10 μM CFSE at 37°C for 5 min and incubated with DynaBeads Mouse T-Activator CD3/CD28 (Invitrogen) for various times as indicated in the figures. Cells were harvested and analyzed by FACS.

**T Cell Adoptive Transfer**

Total T cells were purified and labeled with 1 μM Cell Proliferation Dye eFluor 450 or eFluor 670 at 37°C for 15 min, washed, and mixed in equal ratios. 106 cells were delivered to each recipient mouse via tail vein injection. Mice were sacrificed 1 week after the transfer, and spleens were harvested for analysis.

**Electron Microscopy**

Purified T cells were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer and post-fixed in 1% OsO4. Samples were dehydrated in acetone series, embedded in Spurr’s resin, and sectioned with a Reichert Ultrcut S or EM UC6 ultramicrotome (Leica). The ultra-thin sections (70-90 nm) were stained with uranyl acetate followed by lead citrate and then imaged using an FEI Tecnai Spirit transmission electron microscope at 80 kV. Data shown are representative transmission electron microscopy (TEM) images of cells at 11,000 x magnification. The obtained mages were analyzed using ImageJ (NIH).

**Immunofluorescent Staining**

Splenic T cells were left untreated or stimulated with 100 μM chloroquine or 10 μg/mL of plate-bound anti-CD3 in medium at 37°C for 4 or 24 hr, respectively. Detailed staining procedures and reagents used in the study can be found in the Supplemental Experimental Procedures.

**TUNEL Assay**

The APO-bromodeoxyuridine (BrdU) TUNEL assay was performed according to the manufacturer’s instructions. In brief, splenocytes were stained with fluorescence-conjugated antibodies as described above, fixed in 1% paraformaldehyde for 15 min on ice, and permeabilized with 70% ethanol at −20°C overnight. Cells were incubated with DNA labeling solution at 37°C for 60 min, stained with Alexa Fluor 488 anti-BrdU antibody at room temperature for 30 min, and analyzed by FACS.

**Statistical Analysis**

Prism 6 (GraphPad) was used for statistical analysis and making graphs. Data are presented as mean ± SEM. Comparisons for two groups were calculated using unpaired two-tailed Student’s t test.
SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.04.077.

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AUTHOR CONTRIBUTIONS
C.-W.W., D.-J.L., C.-Y.L., C.-F.C., J.-C.W., and T.-C.W. performed the experiments. Z.-F.C., C.-M.L., I.L.D., and C.-L.H. conceptualized the study. W.-N.J. performed the EM analysis. I.L.D. and C.-L.H. designed and supervised the experiments. C.-W.W. and C.-L.H. analyzed and interpreted the data and wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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REFERENCES


Supplemental Information

Equilibrative Nucleoside Transporter 3 Regulates T Cell Homeostasis by Coordinating Lysosomal Function with Nucleoside Availability

Chin-Wen Wei, Chia-Ying Lee, Ding-Jin Lee, Chang-Feng Chu, Ju-Chu Wang, Tien-Chiao Wang, Wann-Neng Jane, Zee-Fen Chang, Chuen-Miin Leu, Ivan L. Dzhagalov, and Chia-Lin Hsu
Figure S1. Distinct expression profiles of ENTs in various T cell sub-populations. Related to Figure 1. (A) Total thymocytes and (B) splenic T cells were harvested and subjected to qPCR analysis for the expression of ENTs. (C) Thymocytes were sorted by the surface expression of Thy1.2, CD4 and CD8 into four sub-populations: Thy1.2+CD4-CD8-: double-negative (DN); Thy1.2+CD4+CD8+: double-positive (DP); Thy1.2+CD4+CD8-: CD4 single-positive (CD4); Thy1.2+CD4-CD8+: CD8 single-positive (CD8). (D) Mature T cells were harvested from spleen and lymph nodes and were divided into TCRβ+CD4+ and TCRβ+CD8+ T naïve (CD62L+CD44-) and effector (CD62L-CD44+) cell populations. ENT3 expression in each cell populations was quantified by qRT-PCR. Relative mRNA levels were normalized to Rpl19. *p<0.05, **p<0.01, ***p<0.001 (unpaired t test). Data shown are means ± s.e.m. of 3 independent experiments.
Figure S2. Detailed examination of immune cell subpopulations in ENT3⁻/⁻ mice. Related to Figure 1. Splenocytes were harvested from WT littermates and ENT3⁻/⁻ mice and stained for the following surface markers or transcription factors to distinguish specific immune cell subpopulations. All the cells were pre-gated by FSC, SSC, and PI/Zombie viability dye negative populations. (A) T regulatory cells were defined as CD4⁺Foxp3⁺. (B) Follicular B cells (FOB): CD21⁻CD23⁺, marginal zone B cells (MZB): CD21⁺CD23⁻, Plasma cells (PC): B220⁺CD138⁺, Activated B cells: B220⁺CD69⁺. (C) NK1.1⁺ was used to identify Natural Killer cells (NK). *p<0.05, ***p<0.001 (unpaired t test). Data shown are means ± s.e.m. of 2 to 3 independent experiments, n=6-9.
Figure S3. ENT3 participates in maintaining the cellular homeostasis of T cells. Related to Figure 4. (A) Estimation of T cell size using flow cytometry. Mature T cells were harvested from spleen and divided into TCRβ⁺CD4⁺ and TCRβ⁺CD8⁺, naïve (CD62L⁺CD44⁻) and effector (CD62L⁻CD44⁺) cell populations. The size and cellular complexity were evaluated by FSC and SSC parameters. (B) and (C) To obtain sufficient number of effector cells for the analysis, WT effector cells were generated by treating CD4 or CD8 naïve cells with CD3/CD28 dynabeads for 72hr and subjected for TEM analysis. Images were acquired and analyzed by ImageJ, *p<0.05, ***p<0.001 (unpaired t test), n=10.
Figure S4. Lysosomal membrane permeability (LMP) in T cells was evaluated by Cathepsin B and Galectin-3 stainings. Related to Figure 5. To induce LMP, T cells were activated with 10μg/ml anti-CD3 for 24 hrs or treated with 100μM of Chloroquine for 4 hr to induce lysosomal damage. Naïve and effector WT and ENT3−/− T cells were stained for Cathepsin B (Red) and Hoechst (Blue) (A) or Galectin-3 (Red) (B), scale bar represented 5μm.
Figure S5. ENT3 participates in the regulation of autophagic flux. Related to Figure 5. Mature T cells were harvested from spleen and divided into TCRβ⁺CD4⁺ and TCRβ⁺CD8⁺ naïve (CD62L⁺CD44⁻) and effector (CD62L⁻CD44⁺) cell populations, and co-stained with Cyto-ID to measure the amount of autophagic vacuoles in the T cell subpopulations (A). The quantification of the Cyto-ID signal was shown in (B). The CD4 or CD8 T cells were purified and subjected to Western blot analysis to examine the expression of crucial autophagy pathway components LC3 (C) and p62 (D). β-actin and GAPDH were used as loading control.
Figure S6. Extracellular flux metabolic analysis of ENT3<sup>-/-</sup> T cells. Related to Figure 6. T cells were harvested and sorted into TCR<sup>β</sup>CD4<sup>+</sup> and TCR<sup>β</sup>CD8<sup>+</sup> naïve (CD62L<sup>+</sup>CD44<sup>-</sup>) cell populations. The naïve T cells were treated with CD3/CD28 Dynabeads for 72 hr to generate activated T cells. Both naïve and effector cells were then subjected to Seahorse analyzer for the extracellular flux analysis. The basal and maximal analysis was shown in (A) and (B) respectively. Since ENT3<sup>-/-</sup> failed to survive upon activation, the phenotypic effector-like (CD62L<sup>-</sup>CD44<sup>+</sup>) T cells were used for analysis. Shown representative results from two independent experiments.
Figure S7. Assess the T cell PI3K–AKT–mTOR pathway alterations in the absence of ENT3. Related to Figure 6. Mature T cells were harvested from spleen and divided into TCRβ⁺CD4⁺ and TCRβ⁺CD8⁺, naïve (CD62L⁺CD44⁻) and effector (CD62L⁻CD44⁺) cell populations, and co-stained with (A) p-AKT(Thr308), (C) p-mTOR, and (E) p-4EBP1. The quantification of positive signals was shown in (B, D, and F). *p<0.05, (unpaired t test), n=6.
Supplemental Experimental Methods and Materials:

Antibodies and reagents used in flow cytometric analysis:

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**Serum Cytokine measurements.** Whole blood was collected via cardiac puncture, and allowed coagulation at 37°C for 15 min. Samples were then centrifuged at 10,000g for 20 min at 4°C, the resulting supernatants were collected as serum samples. Serum levels of IL-2, IL-7 and IL-15 were measured by ELISA following manufacturers' manual. ELISA kits used in the study were listed as followed: IL-2 detection kit (BioLegend), IL-7 & IL-15 detection kits (R&D Systems).

**Autophagy pathway analysis by Western Blot.** Cells were lysed in 50 mM Tris-HCl , 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1X Protease Inhibitor Cocktail (Calbiochem) for 30 min on ice. Protein lysate were boiled in loading buffer (Biorad) for 10 min and electrophoresed on 10-15% SDS polyacrylamide gels and transferred onto PVDF membranes (Millipore). The membranes were incubated with anti-LC3 (Cell Signaling), and anti-p62 (GeneTex). Signals were visualized by incubating with horseradish peroxidase-conjugated (HRP) secondary antibody (GeneTex) followed by ECL reagent (Millipore). Loading controls were GAPDH and β-actin (GeneTex). Detection was performed with LAS-4000 (Fujifilm), and the relative level of protein was analyzed by ImageQuant TL.

**TEMPOL treatment to scavenge intracellular ROS.** Purified total T cells were pre-treated TEMPOL (membrane-permeable radical scavenger)(Sigma) at 0.1, 0.3, 1 mM for 1hr and stimulated with T-Activator CD3/CD28 dynabeads (ThermoFisher) for 72 hr, and the cell viability was evaluated by PI
staining.

**Intracellular staining for AKT/mTOR pathway analysis.** Splenocytes were stained with surface fluorescence-conjugated antibody as described before, and fixed in fixation buffer (Biolegend) for 20 min at room temperature, followed by permeabilization with intracellular staining perm wash buffer (biolegend). Cells were then stained with p-AKT (Thr308) (Cell Signaling) p-mTOR (S2448) (ebioscience), p-4EBP1 (Thr36, Thr45) (Thermo Fisher) or anti-Bcl-2 (Biolegend) antibodies for 20 min at room temperature, and subjected to FACS analysis.

**Generation of effector T cells.** WT naïve T cells were sorted according to surface phenotype by BD FACSMelody. 1*10^6/mL naïve T cells were stimulated with 20µl T-Activator CD3/CD28 dynabeads (ThermoFisher) with 20ng/ml IL-2 (biolegend) for 72 hours. Resultant cells were collected and used for further analysis (TEM, Seahorse analyzer).

**Metabolic flux analysis.** 3*10^5 cells were seeded to CellTak (Corning) pre-coated Seahorse plates. Basal oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured in non-buffered RPMI medium (containing 2% FBS without HEPES and sodium bicarbonate) by XF-96 Extracellular Flux Analyzer (Seahorse Bioscience), then cells were treated with 1µM oligomycin, 4µM FCCP, and 1µM Antimycin A sequentially to obtain various bioenergetics parameters.