Intracellular metabolic adaptation of intraepithelial CD4⁺CD8αα⁺ T lymphocytes

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Highlights

Microbes induce hypoxic conditions in the intraepithelial compartment

Induced IELs show a lower OCR, glucose uptake, and mitochondrial membrane potential

DPIEL show reduced expression of Hif1α/Hif2α during development

Downregulation of Rptor and Hif1α/Hif2α expression in CD4⁺ T cells induces DPIEL

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Intracellular metabolic adaptation of intraepithelial CD4⁺CD8αα⁺ T lymphocytes

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SUMMARY

Intestinal intraepithelial lymphocytes (IELs), the first line of defense against microbial and dietary antigens, are classified as natural or induced based on their origin and receptor expression. Induced CD4⁺CD8αα⁺ T cells (double positive, DPIELs) originated from CD4⁺CD8α⁺ T cells (single positive, SPIELs) increase with aging. However, the metabolic requirements and the metabolic-related genes in IEL development remain unclear. We determined that the intraepithelial compartment is hypoxic in the presence of microbes and DPIELs increased more than natural IELs in this location. Moreover, DPIELs consumed less oxygen and glucose and exhibited unique alterations in mitochondria. Using inhibitors and genetically modified mice, we revealed that DPIELs adapt to their surrounding oxygen-deprived environment in peripheral tissues by modulating specific genes, including hypoxia-inducible factor, mammalian target of rapamycin complexes (mTORC), phosphorylated ribosomal protein S6 (pS6), and other glycolytic factors. Our findings provide valuable insight into the metabolic properties of IELs.

INTRODUCTION

The intestinal mucosal barrier, which has a poor blood supply, is formed by a dense mucus layer, organized epithelial cells, and intraepithelial lymphocytes (IELs) (Cheroutre et al., 2011). IELs are located in the intraepithelial compartment and are the first line of defense against bacterial or food antigens. IELs are classified as natural IELs and induced IELs. Natural IELs, including TCRγδ+ cells and CD8α⁺ T cells, develop in the thymus, and induced IELs, such as CD8αβ⁺ TCRβ⁺ cells and CD4⁺CD8αα⁺ TCRβ⁺ T cells (double positive cells, DPIELs), develop in the periphery (Olivares-Villegomez and Van Kaer, 2018; Van Kaer and Olivares-Villegomez, 2018). DPIELs originate from CD4⁺CD8α⁺ T cells (single positive IELs, SPIELs) and accumulate with aging (Konkel et al., 2011). DPIELs express both CD4 and CD8α via the downregulation of T helper POZ/Kruppel like factor (Thpok also known as Zbtb7b) and upregulation of Runt-related transcription factor (Runx3) during their development (Bilate et al., 2016; Cervantes-Barragan et al., 2017; Sujino et al., 2016). IELs express activation markers such as CD44 and CD69 (Cheroutre et al., 2011), also they express enzymes and cytokines, such as granzyme B and interleukin (IL)-17A, depending on the cell type. They proliferate less with other T cells, and originate from long-lived tissue-resident effector memory cells (Groux et al., 1997; Konjar et al., 2018; Mucida et al., 2013; Reis et al., 2013; Sydora et al., 1993; Vandereyken et al., 2020; Yu et al., 2008).

Metabolic reprogramming is essential for T cell activation and function. The activation of naïve CD4⁺ T cells in lymphoid tissues leads to metabolic reprogramming from oxidative phosphorylation (OXPHOS) to anaerobic metabolism. For instance, Foxp3-positive regulatory T cells (Tregs) preferentially use OXPHOS, and effector T cell subsets (T helper type (Th) 1, Th2, Th17, and CD8⁺ T cells) preferentially undergo glycolysis with lactate production (Jung et al., 2019; MacIver et al., 2013; Pearce and Pearce, 2013; Shi et al., 2011). The metabolic requirements of T cell subsets were previously studied using in vitro cultured cells or cells isolated from spleen and lymphoid tissues. In addition, cells alter their energy usage in response to environmental factors. Tregs mainly use OXPHOS in oxygen-rich conditions, whereas in glucose-deprived and oxygen-deprived environments, such as cancer or peripheral tissues, Tregs rely on lactate as an alternate energy source (Angelin et al., 2017; Newton et al., 2016). Hypoxia inducible factor 1 subunit alpha (Hif1α) is...
DPIELs showed a reduced OCR compared with naive CD4+ T cells, and DPIELs, develop in low oxygen conditions. We measured the OCR in splenic naive CD4+ T cells increase by 3- to 5-fold in SPF mice compared with GF mice (Figure 1D). Furthermore, induced IELs, such as CD4+ TCRgd T cells, increased by more than a thousand times in SPF mice compared with GF mice. These data indicate that induced IELs exhibit reduced oxygen consumption, glycolysis, and glucose uptake.

Among IELs, glucose uptake was increased in TCRgd T cells compared with CD8β+ T cells, and DPIELs, develop in low oxygenic conditions. DPIELs were increased by more than a thousand times in SPF mice compared with GF mice. These data indicate that peripheral tissue-resident cells develop by adapting to the environmental niche, such as hypoxic conditions, with alterations in metabolic-related gene expression.

RESULTS

Intestinal intraepithelial compartment is hypoxic in SPF mice

We first analyzed the intestinal oxygen supply in the presence of microbes. Although the oxygen concentration of the lamina propria compartment was equivalent in specific-pathogen-free (SPF) mice and germ-free (GF) mice, the oxygen concentration in the intraepithelial compartment was decreased in SPF mice compared with GF mice (Figures 1A and 1B). These data indicate that the intraepithelial compartment is deprived of oxygen in the presence of microbes. Intraepithelial cells develop and reside in a suitable location with low oxygenic conditions in SPF mice. Therefore, we questioned which cell types develop in the low oxygenic intraepithelial compartment in SPF mice. The numbers of cells in the intraepithelial compartment in GF mice and SPF mice were analyzed (Figure 1C). We divided the cell number in SPF mice and GF mice in each population. The numbers of natural IELs, including TCRγδ+ T cells and CD8αα+ TCRβ+ T cells, were increased by 3-fold to 5-fold in SPF mice compared with GF mice (Figure 1D). Furthermore, induced IELs, such as CD4+ TCRβ+ T cells and CD8αβ TCRβ+ T cells, were increased by 15-fold to 20-fold in SPF mice compared with GF mice (Figure 1D). CD4+ TCRβ+ IELs are divided into two populations: terminally differentiated IELs that express CD4+ CD8αα+ TCRβ+ (DPIELs) and CD4+ CD8αα+ TCRβ+ cells (SPIELs). Significantly, DPIELs were increased by more than a thousand times in SPF mice compared with GF mice. These data indicate induced IELs, especially DPIELs, develop in low oxygenic conditions.

CD4+CD8αα+ TCRβ+ IELs exhibit a reduced OCR and ECAR

We then determined the OCR of IELs in the intraepithelial compartment because CD4+ TCRβ+ T cells, especially DPIELs, develop in low oxygen conditions. We measured the OCR in splenic naive CD4+ T cells, TCRγδ+ T cells, CD8αβ T cells, SPIELs, and DPIELs from small intestine epithelium. SPIELs and DPIELs showed a reduced OCR compared with naive CD4+ T cells and TCRγδ+ T cells. DPIELs displayed a slightly lower OCR compared with SPIELs, but the result was not significant (Figure 2A). CD8αβ+ T cells tended to show a higher OCR than SPIELs and DPIELs. We next measured glycolysis by determining the ECAR and glucose intake using 2-NBDG in each cell type. SPIELs and DPIELs showed a lower ECAR than naive CD4+ T cells and increased OCR compared with naive CD4+ T cells (Figure 2B). The ECAR in CD8αβ+ T cells was higher than in SPIELs and DPIELs, although not significantly. Glucose uptake in naive CD4+ T cells was higher than in IEL cells. Among IELs, glucose uptake was increased in TCRγδ+ T cells compared with CD8αβ+ T cells, SPIELs, and DPIELs (Figure 2C). These data indicate that induced IELs exhibit reduced oxygen consumption, glycolysis, and glucose uptake.

Given that CD8+ IELs showed an increased number of mitochondria with altered lipid metabolism compared with naive CD4+ T cells (Konjar et al., 2018), we next counted the number and size of mitochondria in naive CD4+ T cells and induced IELs. SPIELs, and DPIELs. The total number of mitochondria was increased in SPIELs and DPIELs compared with naive CD4+ T cells (Figures 2D and 2E). Interestingly, DPIELs...
displayed a higher number of mitochondria than SPIELs. Of note, the area of each mitochondrion in DPIELs was smaller than in naive CD4+ T cells and SPIELs (Figures 2D and 2F). Mitochondrial fission controls the number and the size of mitochondria in memory T cells (Buck et al., 2016). To support our findings, we analyzed the mitochondrial fission-related genes dynamin-related protein 1 (Drp1) and mitochondrial fission 1 gene (Fis1). The expression levels of Drp1 and Fis1 were increased in SPIELs and DPIELs compared with naive CD4+ T cells (Figure 2G). The expression levels of Drp1 and Fis1 were comparable in DPIELs and SPIELs. These data indicate that SPIELs and DPIELs have a lower OCR with an increased number of small mitochondria, and mitochondrial fission genes are upregulated in SPIELs and DPIELs.

CD4⁺CD8αβ⁺TCRβ⁺ IELs are a mitochondrial membrane potentiallo population

We then measured mitochondrial size and membrane potential in SPIELs and DPIELs using MitoTracker. Among CD4⁺ T cells in spleen and mesenteric lymph node (mLN) cells, CD4⁺CD62L⁺ cells were classified as mitochondria sizehi and membrane potentialhi (gated as Q1) populations, and CD4⁺CD44⁺ effector and memory cells were identified as mitochondria sizelo and membrane potentiallo (Q2) populations (Figure S1A). We analyzed the mitochondrial fission-related genes dynamin-related protein 1 (Drp1) and mitochondrial fission 1 gene (Fis1). The expression levels of Drp1 and Fis1 were increased in SPIELs and DPIELs compared with naive CD4⁺ T cells (Figure 2G). The expression levels of Drp1 and Fis1 were comparable in DPIELs and SPIELs. These data indicate that SPIELs and DPIELs have a lower OCR with an increased number of small mitochondria, and mitochondrial fission genes are upregulated in SPIELs and DPIELs.
measured the mitochondrial size and membrane potential of CD4+ T cells in the intraepithelial (CD4IELs) and lamina propria (CD4LPLs) compartment. The percentage of Q1 and Q3 in CD4IELs of SPF mice was reduced compared with GF mice, whereas the percentage of Q1 and Q3 in CD4LPLs was comparable between SPF mice and GF mice (Figures 3C–3F). In CD4LPLs, the percentage of Q2 was slightly increased in GF mice than in SPF mice. These data indicate that CD4IELs but not CD4LPLs have a lower mitochondrial potential in SPF mice compared with GF mice. We then divided CD4IELs into two populations (SPIELs and DPIELs) because DPIELs were increased in SPF mice (Figures 1C and 1D). The percentage of Q1 and Q2 in DPIELs was decreased compared with that in SPIELs (Figures 3G and 3H). We also confirmed that DPIELs exhibited a reduced mitochondrial membrane potential compared with SPIELs by tetramethylrhodamine ethyl ester (TMRE) staining (Figures 3I and 3J). Taken together, induced IELs, especially DPIELs, are a population of cells with small mitochondria and low mitochondrial membrane potential.
2DG inhibits the induction of CD4+CD8αα+TCRβ+ IELs

Because DPIELs consume less glucose than naïve CD4+ T cells (Figure 2C), we next investigated whether glucose uptake affects the development of DPIELs. We administered 6-week-old mice with or without 2DG via daily intraperitoneal injection for 5 weeks (Figure 4A) (Hamanaka and Chandel, 2012). 2DG administration did not reduce the percentage of TCRβgd+ T cells in CD45+ cells but reduced the percentage of TCRβ+ T cells in CD45+ cells which contained the DPIELs cells (Figure S2A). Among TCRβ+ T cells, the percentage of CD8αα+CD8β+ cells was slightly increased in 2DG mice and the percentage of CD8β+ T cells was similar between with or without 2DG administration. Of note, the percentage of total CD4+ T cells in TCRβ+ T cells was reduced in 2DG administration. The proportion of DPIELs among total CD4+ cells before 2DG treatment was approximately 20%. The percentage of DPIELs was 40% without 2DG treatment and decreased to 20% in 2DG treated mice (Figures 4B and 4C). Moreover, the percentage of DPIELs in total TCRβ+ cells and CD45+ cells was decreased with 2DG treatment (Figure S2B).

These data indicate that 2DG inhibited the development of DPIELs. 2DG might affect Treg populations because some ex-Tregs differentiate into DPIELs. However, the percentages of Tregs were comparable between mice with and without 2DG (Figure 4D). These data indicate that 2DG inhibits the development of DPIELs without changing the Treg population. To exclude the possibility of other immune cells being involved, we then questioned the oxygen concentration and 2DG directly affecting the development of DPIELs. We cultured splenic naïve CD4+ T cells with or without 2DG in the presence of TGF-β, retinoic acid (RA), and IFN-γ (DPIEL condition) in vitro under normoxic (O2 20%) or hypoxic conditions (O2 8%) (Figures 4E–4G). As expected, the proportion of CD4+CD8αα+ T cells in total CD4+ T cells was increased with the hypoxic condition compared with normoxia. These data indicate the hypoxia induces CD4+CD8αα+ T cells in DPIEL condition in vitro. 2DG decreased the proportion of CD4+CD8αα+ T cells in CD4+ T cells...
and S3C). Among LPLs, the percentages of Tregs and RORγt+Foxp3- (Vhl) are known to be upregulated under hypoxic conditions. We questioned whether the expression of HIF was upregulated in CD4IELs. We FACs sorted naive CD4+ T cells (Hif1a+/fl/fl mice) and analyzed the cell populations at the age of 12 weeks. The percentage of DPIELs was increased by almost 2-fold in Hif1a+/fl/fl mice (Figure S3A). The percentage of DPIELs was increased by almost 2-fold in Hif1a+/fl/fl mice compared with Hif1a+/fl/+ mice. Von Hippel–Lindau (Vhl) is a negative regulator of HIF. The RNA expression level of Vhl in naïve CD4+ T cells isolated from wild-type mice are cultured for 4 days with plate-bound α-CD3 and α-CD28 in the presence of TGF-β+RA + IFN-γ. ns: not significant, **p < 0.01, ***p < 0.001 (unpaired Student’s t test with Welch’s correction).

**Hif1a/Hif2α are reduced during the development of CD4*CD8αα*TCRβ+ IELs**

The intestinal epithelium is an oxygen-poor environment with no blood vessels separating anaerobic intestinal bacteria from the intestinal epithelium. Hif1a and Hif2α are known to be upregulated under hypoxic conditions. We questioned whether the expression of HIF was upregulated in CD4IELs. We FACs sorted naive CD4+ T cells, SPIELs, and DPIELs and analyzed the expression of HIF-related genes (Figure 5A). As previously reported, DPIELs expressed higher levels of Runx3 than SPIELs and naïve CD4+ T cells. Interestingly, the RNA expression levels of Hif1α and Hif2α in DPIELs were lower than in SPIELs. We tested whether the prevention of glucose uptake inhibits the development of DPIELs and they consume less glucose than naïve CD4+ T cells.

**Hif/Vhl gene expression regulates the development of CD4*CD8αα*TCRβ+ IELs**

We next questioned whether the downregulation of Hif1α/Hif2α expression was essential for the development of DPIELs. We crossed Hif1αα/Cre+Hif2αα/Cre+ mice with Cdp4+/− mice to generate mice lacking Hif1α and Hif2α in CD4+ T cells (Hif1α−/−Hif2α−/−). The total number of IELs in Hif1α−/−Hif2α−/− mice was not increased compared with Hif1α+/−Hif2α+/− mice (Figure S3A). The percentage of DPIELs was increased by almost 2-fold in Hif1α−/−Hif2α−/− mice compared with Hif1α+/−Hif2α+/− mice, whereas the percentage of Tregs was decreased in Hif1α−/−Hif2α−/− mice (Figures S3B and S3C). As HIF1α also regulates retinoic acid-related orphan receptor gamma t (RO Ryt) expression (Shi et al., 2011), we confirmed that the proportion of RO Ryt+Foxp3- IELs was decreased in Hif1α−/−Hif2α−/− mice compared with Hif1α+/−Hif2α+/− mice (Figures S3B and S3C). Among LPLs, the percentages of Tregs and RO Ryt+Foxp3- T cells were comparable between Hif1α−/−Hif2α−/− mice and Hif1α+/−Hif2α+/− mice (Figures S3D and S3E). These data indicate that the downregulation of Hif1α/Hif2α expression in CD4 T cells induces DPIELs. Then, we crossed Vhl−/− mice with Cdp4+/− mice to generate Cdp4+/−Vhl−/− (Vhl−/−) mice and analyzed the cell populations at the age of 12 weeks. The percentage and total number of CD4+ T cells in the thymus were comparable in Vhl−/− mice and Vhl−/− mice.
SPIELs. 

The expression of Rptor gene was also comparable in DPIELs and SPIELs from small intestine epithelium of wild-type mice (n = 4). The data were shown as fold change normalized to naive CD4+ T cells. Interestingly, TKO mice showed an increased proportion of DPIELs compared with SPIELs. In addition, mTORC regulates the development of DPIELs and SPIELs. In other words, the expression level of mTORC in DPIELs was comparable to that in SPIELs. However, DPIELs displayed reduced phosphorylation level of S6 compared with splenic naive CD4+ T cells (Figures S4A–S4C), as previously reported (Zhu et al., 2019). The total number of CD4+ T cells in IELs was also comparable in DPIELs and SPIELs from small intestine epithelium of wild-type mice (n = 4). The data were shown as fold change normalized to naive CD4+ T cells. However, DPIELs displayed reduced phosphorylation level of S6 compared with splenic naive CD4+ T cells (Figures S4A–S4C), as previously reported (Zhu et al., 2019). The total number of CD4+ T cells in IELs was also comparable in DPIELs and SPIELs from small intestine epithelium of wild-type mice (n = 4). Unique information included here.

As DPIELs exhibit reduced Hif1α/Hif2α expression during development, and the downregulation of Hif1α/Hif2α expression is important for the development of DPIELs, we hypothesized that the downregulation of Hif1α/Hif2α expression in CD4IELs is not only regulated by hypoxia but also by mTORC signaling, which controls Hif1α/Hif2α expression. mTORC exists as mTORC1 and mTORC2 complexes. Interestingly, although the expression of Hif1α/Hif2α was reduced in DPIELs compared with SPIELs, the expression of the mTORC1 gene Rptor in DPIELs was comparable to that in SPIELs (Figure 6A). Both DPIELs and SPIELs expressed higher levels of Riptor than splenic naive CD4+ T cells. We then analyzed the RNA expression of Riptor-related genes, including isoform 2 of the glycolytic enzyme hexokinase (Hk2) (enhances glucose metabolism) and arginase-2 (Arg2) (regulates L-arginine in the urea cycle). The expression of Hk2 in DPIELs was slightly but not significantly increased compared with SPIELs. The expression of Arg2 was comparable in DPIELs and SPIELs. The expression of Rictor was also comparable in DPIELs and SPIELs. In addition, mTORC regulates sterol regulatory binding protein (SREBP), which mediates fatty acid metabolism. The expression of Srebp1 and Srebp2 in DPIELs was higher than in naive CD4+ T cells and SPIELs (Figure 6A). These data indicate that the expression level of mTORC in DPIELs was comparable to that in SPIELs. However, DPIELs displayed...
reduced Hif1α/Hif2α and increased Srebp1 and Srebp2 expression. Given that T cell receptor signaling induces the Akt/mTOR/pS6 pathway, we next analyzed pS6 in CD4IELs. DPIELs expressed lower phosphorylation levels of S6 than SPIELs (Figure 6B). Of note, CD4+CD8αα+ LPLs and CD4+CD8αα/C0LPLs expressed similar levels of pS6. Our findings are consistent and show reduced mTORC1/pS6 signaling in DPIELs.

Downregulation of Rptor but not Rictor induces CD4ααCD8αβ TCRβ+IELs

We next questioned whether mTORC1 or 2 was important for the induction of DPIELs. We first administered rapamycin, which modulates mTORC1 signaling, to 6-week-old mice for 5 weeks (Figure 6C) (Neff et al., 2013). Interestingly, the percentage of DPIELs was increased in mice treated with rapamycin compared with control mice, whereas the percentages of Tregs in IEL were comparable in both groups (Figures 6D and 6E). The percentage and total number of Rorγt/Foxp3+ cells in LPL were decreased in rapamycin-treated mice, whereas the
percentage and total number of Rorγt Foxp3+ cells were increased in rapamycin-treated mice (Figures S5A and S5B). The total number of Rorγt Foxp3+ cells in rapamycin-treated mice was reduced, but the percentage was comparable. These data indicate that the inhibition of mTORC1 function is important for the development of DPIELs. To confirm mTORC1 involvement in the induction of DPIELs, we generated Cd4CreERT2, Rptorfl/fl (referred to as RptorACD4) mice with CD4-specific Rptor downregulation after tamoxifen administration because the downregulation of Rptor in the thymus impairs peripheral CD4+ T cells (Hoshii et al., 2014). We administered tamoxifen to mice at the age of 6 weeks and analyzed them 5 weeks later. No spontaneous intestinal inflammation was observed in Rptorfl/fl or RptorACD4 mice (data not shown). DPIELs accounted for approximately 60% of CD4IELs, and the proportion of DPIELs was significantly increased in iRaptorACD4 mice (Figures 6F and 6G). We then generated Cd4cre:Rictorfl/fl mice (referred to as RictorACD4). The proportion of DPIELs was comparable between Rictorfl/fl and RictorACD4 mice (Figures 6H and 6I). Moreover, we cultured naive CD4+ T cells from Rictorfl/fl and RictorACD4 mice under the DP condition with or without Torin 1. As observed in vivo, the percentage of DPIELs in CD4+ T cells in the absence of Torin 1 was comparable between Rictorfl/fl and RictorACD4 cells in vitro. Torin 1 increased the percentage of DPIELs development in both Rictorfl/fl and RictorACD4 cells (Figures 6J and 6K).

Taken together, the inhibition of mTORC1 functionally but not mTORC2 is important for the development of DPIELs, whereas the expression levels of Rptor and Rictor are not downregulated during the development of DPIELs.

**DISCUSSION**

CD4-induced IELs, especially CD4+CD8αα+ T cells, in the intestinal epithelium are not increased in GF mice, indicating that the involvement of bacteria in the intestinal epithelium plays a major role in the induction of CD4-induced IELs (Sujino et al., 2016). CD4+CD8αα+ T cells are induced by aryl hydrocarbon receptor ligands from food or intestinal bacteria and the surrounding environment, such as IL-15, food antigens, and MHC class II molecules in the intestinal epithelium (Bilate et al., 2016; Mucida et al., 2013). Environmental cues shape induced IELs; however, how the cells adapt in oxygen rich or poor circumstances remains poorly understood. Similarly, how induced IELs adapt to their environmental conditions by altering gene expression related to intracellular metabolism is unknown (Almeida et al., 2016; Buck et al., 2015; Dumitru et al., 2018; Konjar et al., 2018; Phan et al., 2016; Raud et al., 2018; Shi et al., 2011). Induced CD4IELs exist as long-lived effector memory cells and express enzymes, such as granzyme B. Effector T cells are induced by elevated glycolysis, and they upregulate Hif1α/Hif2α expression. In contrast, memory T cells survive long-term in peripheral tissue by using OXPHOS. Here, we determined that induced CD4IELs, especially DPIELs, adapt to their unique metabolic conditions and exhibit a low OCR with reduced glucose uptake and anaerobic glycolysis.

We first show that oxygen saturation is decreased in organs with poor blood flow, including the intestinal epithelium, not only because of cell oxygen consumption but also the presence of intestinal bacteria. Previously the number of TCRγδ+ cells was comparable in the GF and SPF conditions (Hoytema van Konijnenburg et al., 2017). As we counted the intestinal live cells in GF and SPF condition, we observed the increased number of both TCRγδ+ cells and TCRβ+ cells in SPF condition. Moreover, the total number of CD4+ T cells, especially DPIELs T cells, increased dramatically in the SPF condition.

In the small intestine, the presence of several commensal anaerobes that consume oxygen is consistent with the results. Long-lived memory CD8+ T cells exhibit an increased mitochondrial size and membrane potential, resulting in a higher level of oxygen consumption (Buck et al., 2016). CD4IELs consume less oxygen with increased mitochondrial fission and low mitochondrial membrane potential during development. Some IELs show an increased mitochondrial size and membrane potential after anti-CD3 antibody injection (Konjar et al., 2018). Anti-CD3 antibodies induce severe inflammation in the small intestine. As a result, cells use mitochondrial respiration because the oxygen supply might be increased in the epithelial compartment of the small intestine. Because DPIELs are diminished after anti-CD3 antibody injection, we cannot conclude that the size of mitochondria and membrane potential are increased in DPIELs after anti-CD3 antibody stimulation. We demonstrate hypoxic conditions induce more CD4+CD8αα+ T cells in DPIELs. However, the effect of oxygen saturation itself on the differentiation and maintenance of CD4IELs remains to be investigated. Our data suggest that induced CD4IELs are the population of cells that adapt to the hypoxic intraepithelial compartment.

DPIELs display enhanced gene expression of mTORC, which regulate HIF, mitochondrial biosynthesis, and lipid metabolism. However, DPIELs exhibit reduced HIF and pS6 expression during development, which are...
downstream factors of mTORC. Although DP_{IELs} reduce glucose uptake compared with naïve CD4^+ T cells, complete prevention of glucose intake with 2DG did not increase DP_{IELs}, indicating that glucose intake is indispensable for the development of DP_{IELs}.

DP_{IELs} do not show an increase in NUR77, indicating reduced T cell receptor signaling, but they express TCR_{B} (Bilate et al., 2020). In addition, previous studies have shown that MHC-II signaling in the epithelium is required for the induction of DP_{IELs} but dispensable for their expansion (Bilate et al., 2020; Mucida et al., 2013). Once naïve CD4^+ T cells activated via T cell receptor signaling import glucose, T cell activation continues in the late phase via Akt/pS6 and HIF upregulation with increased glucose uptake (Frauwirth et al., 2002; Jacobs et al., 2008; Menk et al., 2018). Interestingly, DP_{IELs} show reduced pS6 expression, HIF, and glucose uptake during their development from SP_{IELs}. Thus, DP_{IELs} might develop from cells that are activated temporally and quickly adapt to the tissue environments by reducing the expression of pS6 and HIF.

DP_{IELs} display reduced pS6 signaling and Hif1α expression but not decreased mTORC expression during their development from SP_{IELs}. These data indicate that mTORC gene expression in DP_{IELs} may play a role other than regulating glucose uptake or HIF. One possibility is that memory T cells residing in the peripheral tissue consume fatty acids (Fahrer et al., 2001; Konkel et al., 2011; Pan et al., 2017). Therefore, mTORC in DP_{IELs} might regulate lipid metabolism. In fact, the expression levels of the lipid biosynthesis genes Srebp1 and Srebp2 were increased in DP_{IELs} compared with naïve CD4^+ T cells and SP_{IELs}. The mechanisms by which mTORC regulates the balance between HIF, glucose uptake, and lipid metabolism in SP_{IELs} and DP_{IELs} remain unknown.

Compared with Hif2α, Hif1α is dominantly expressed in CD4^+ T cells. Hif1α expression might be a key factor that regulates DP_{IELs}. The specific factor that reduces mTORC1 or HIF expression and promotes DP_{IELs} development is still unknown. Further studies are needed because we cannot exclude the possibility that environmental autocrine or paracrine signals, such as cytokine production by the downregulation of mTORC1 or hypoxia in CD4^+ T cells, might induce DP_{IELs}.

Here, we demonstrate that CD4_{IELs} alter their metabolic status and express unique genes to adapt to hypoxic conditions, and the downregulation of Rptor or Hif1α/Hif2α expression induces DP_{IELs} (Figure S6). TCRγδ cells rapidly use glycolysis and migrate to protect the epithelium following bacterial invasion via MYD88 signaling. However, DP_{IELs}, which increase in number with aging, are dual function cells with the potential to suppress intestinal inflammation, produce IL-10, and prevent bacterial invasion (Bilate et al., 2020; Mucida et al., 2013). The presence of diverse cell populations dependent on various metabolic pathways and metabolic genes in the IEL may be significant in the intestinal epithelium, which is at the forefront of immune tolerance and immune responses against antigens.

Limitations of the study

We measure the OCR and ECAR in splenic naïve CD4^+ T cells and IELs. The OCR and ECAR in this study do not fully capture in each organ, because we analyze them after isolating each cell from peripheral tissue and the localization of each cell is different peripherally. We analyzed the frequencies and numbers of each immune cell in the intestine, but the right control of the metabolic study is not determined yet. DP_{IELs} are differentiated by transcriptional factors, such as Runx3, Zbtb7b, Tbet and by the environmental factors, such as IL-15 and IFN-γ produced by CD4^+ T cells or the other immune cells, but it is unknown how these transcriptional factor and environmental factors affect the metabolic status to develop DP_{IELs}. We measured mitochondrial size, membrane potential with fission related genes, but the mitochondrial content and the precise mechanism of mitochondrial fission related genes on DP_{IELs} are not elucidated yet.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
EXPERIMENTAL MODEL AND SUBJECT DETAILS
- Mice

METHOD DETAILS
- Preparation of intraepithelial lymphocytes and lamina propria mononuclear cells
- Preparation of spleen cell suspensions
- Flow cytometry
- Metabolic assays
- Quantitative real-time polymerase chain reaction
- Transmission electron microscopy
- In vivo tamoxifen treatment
- In vivo treatment of rapamycin and 2DG
- Hypoxyprobe-1 staining
- In vitro CD4⁺CD8αα⁺ T cell differentiation
- Analysis of glucose uptake

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104021.

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AUTHOR CONTRIBUTIONS
Y.H., E.N., K.M., and T.S. performed experiments. T.S. designed and supervised the experiments and wrote the manuscript. Y.Y., S.T., S.U., K.O., Y.M., N.N., K.T., N.H., and H.O. assisted with experiments. T.I., A.H., and Y.K. provided mice and assisted with generating mice. T.S. and T.K. conceived the paper.

DECLARATION OF INTERESTS
Kentaro Miyamoto is an employee of Miyarisan Pharm. The other authors declare no competing interests.

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE                       | SOURCE                           | IDENTIFIER       |
|-------------------------------------------|----------------------------------|------------------|
| Antibodies                                |                                  |                  |
| CD4 (clone: RM4-5; BV421)                 | Biolegend                        | Cat# 100544; RRID: AB_11219790 |
| CD8a (clone: 53-6.7; PE-Cy7)              | BD Bioscience                    | Cat# 552877; RRID: AB_394506 |
| CD8b (clone: eBioH35-17.2; APC)           | eBioscience                      | Cat# 17-0083-81; RRID: AB_657760 |
| CD44 (clone: IM7; APC)                    | Biolegend                        | Cat# 103012; RRID: AB_312963 |
| CD45 (clone: 30-F11; BV510)               | Biolegend                        | Cat# 103138; RRID: AB_2563061 |
| CD62L (clone: MEL-14; FITC)               | Biolegend                        | Cat# 104406; RRID: AB_313093 |
| TCRb (clone: H57-597; APC-Cy7)            | Biolegend                        | Cat# 109220; RRID: AB_893624 |
| TCRg (clone: GL3; PerCP-Cy5.5)            | Biolegend                        | Cat# 118117; RRID: AB_10612572 |
| Foxp3 (clone: FJK-16s; PE)                | eBioscience                      | Cat# 12-5773-82; RRID: AB_465936 |
| Rorγt (clone: Q31-378; BV421)             | BD Bioscience                    | Cat# 562894; RRID: AB_2687545 |
| IL-17A (clone: eBio17B7; PE)              | eBioscience                      | Cat# 12-7177-81; RRID: AB_763582 |
| IFNγ (clone: XMG1.2; FITC)                | eBioscience                      | Cat# 11-7311-82; RRID: AB_465412 |
| CD16/32 (clone: 2.4G2)                    | BD Bioscience                    | Cat# 553142; RRID: AB_394657 |
| CD3e (clone: 145-2C11)                    | Biolegend                        | Cat# 100359; RRID: AB_2616673 |
| CD28 (clone: 37.51)                       | Biolegend                        | Cat# 102116; RRID: AB_11147170 |
| Phospho-S6 ribosomal protein (Ser235/236) | Cell Signaling                   | Cat# 4851; RRID: AB_10695457 |
| (clone: D57.2.2E, Alexa647)               |                                  |                  |

#### Chemicals, peptides, and recombinant proteins

|                         | Source                        | Cat#        |
|-------------------------|-------------------------------|-------------|
| Tamoxifen               | Sigma-Aldrich                 | TS648       |
| Corn oil                | Wako                          | P024-17016  |
| Phorbol 12-myristate 13-acetate (PMA) | Sigma-Aldrich                 | P8139       |
| Ionomycin               | Sigma-Aldrich                 | I9657       |
| Rapamycin               | Sigma-Aldrich                 | R0395       |
| Torin-1                 | Selleckchem                   | S2827       |
| 2-DG                    | Sigma-Aldrich                 | D8375       |
| Fixable Viability Dye eFluor 780 | eBioscience                   | 65-0865-14  |
| Dithiothreitol (DTT)    | Thermo Fisher Scientific      | P3232       |
| EDTA                    | Nacalai Tesque                | 06894-85    |
| Collagenase             | Wako                          | 032-22364   |
| DNase I                 | Sigma-Aldrich                 | DN25        |
| Percoll                 | GE Healthcare                 | 1789101     |
| Ammonium chloride       | Nacalai Tesque                | 02424-55    |
| 4% Paraformaldehyde Phosphate Buffer Solution | Wako                     | 163-20145   |
| 8% Glutaraldehyde       | Wako                          | 533-08681   |
| Agarose LM              | Nacalai Tesque                | 01161-54    |
| Bovine serum albumin (BSA) | Nacalai Tesque              | 01863-48    |
| Triton-X100             | Polysciences                  | 04605       |
| DAPI                    | Dojindo                       | D523        |
| Mouse Naive CD4+ T cell isolation kit | Miltenyi Biotech             | 130-104-453 |
| TGF-β                   | R&D systems                   | 7666-MB-005 |
| Retinoic acid (RA)      | Tokyo Chemical Industry       | 0064-1G     |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for the resources and reagents should be directed and will be fulfilled by the lead contact, Tomohisa Sujino (tsujino1224@keio.jp).

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| IFN-γ               | Peprotech | Cat# 315-05 |
| Fetal bovine serum (FBS) | Thermo Fisher Scientific | Cat# 10270-106 |
| HBSS                | Nacalai Tesque | Cat# 17460-15 |
| RPMI1640            | Nacalai Tesque | Cat# 30264-85 |
| DPBS                | Nacalai Tesque | Cat# 14249-24 |
| Penicillin/streptomycin | Nacalai Tesque | Cat# 09367-34 |
| Sodium pyruvate     | Thermo Fisher Scientific | Cat# 11360070 |
| MEM/NEAA            | Thermo Fisher Scientific | Cat# 11400050 |
| HEPES               | Thermo Fisher Scientific | Cat# 15630080 |
| β-mercaptoethanol   | Thermo Fisher Scientific | Cat# 21985023 |
| XF media            | Agilent Technologies | Cat# 102353-100 |
| TRIzol             | Invitrogen | Cat# 15596018 |

**Critical commercial assays**

| iScript cDNA Synthesis Kit | BioRad | Cat# 170-8891 |
| SYBR Green FAST qPCR Master Mix kit | Kapa Biosystems | Cat# KX4602 |
| HypoxyprobeTM-1 Plus Kit | Hypoxyprobe I | Cat# HP2-100KIT |
| XF Cell Mito Stress Test Kit | Agilent Technologies | Cat# 103015-100 |
| MitoTracker Green | Invitrogen | Cat# M7512 |
| MitoTracker CMXROS | Invitrogen | Cat# M7152 |
| TMRE-Mitochondrial Membrane Potential Assay Kit | Abcam | Cat# ab113852 |
| Foxp3/transcription factor staining buffer set | eBioscience | Cat# 00-5523-00 |
| 2-NBDG Glucose Uptake Assay Kit | Biovision | Cat# K682-50 |
| Anaeropack | Mitsubishi Gas Company | Cat# A-26 |
| GoldiStop Protein Transport Inhibitor | BD Bioscience | Cat# 554724; RRID: AB_2869012 |

**Experimental models: Organisms/strains**

| Mouse: CS7BL/6J | The Jackson Laboratory | JAX:000664 |
| Mouse: Cd4cre | The Jackson Laboratory | JAX:022071 |
| Mouse: Cd4creERT2 | The Jackson Laboratory | JAX:022356 |
| Mouse: Hif1afl/fl | Ryan et al., 2000 | N.A. |
| Mouse: Hif2afl/fl | Gruber et al., 2007 | N.A. |
| Mouse: Vhlfl/fl | Haase et al., 2001 | N.A. |
| Mouse: Riptorfl/fl | Hoshii et al., 2014 | N.A. |
| Mouse: Rictorfl/fl | Magee et al., 2012 | N.A. |

**Oligonucleotides**

| QPCR primers | Hokkaido System Science | Table S1 |

**Software and algorithms**

| GraphPad Prism 8 | GraphPad Software | https://www.graphpad.com/ |
| FlowJo Vx software | TreeStar | https://www.flowjo.com/ |
| ImageJ | ImageJ | https://imagej.net/Welcome |
| Imaris 8.4 | Bitplane | https://imaris.oxinst.com |
Materials availability
All the mouse lines used in this study are available upon request.
This study did not generate new unique reagents.

Data and code availability
All the detailed data in this paper are available upon request.
This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
Mice used in this study were of a C57BL/6 background. Cd4cre, Cd4creERT2, Raptorfl/fl, and Rictorfl/fl, Hif1afl/fl, Hif2afl/fl, and Vhlfl/fl mice were previously described (Gruber et al., 2007; Haase et al., 2001; Hoshii et al., 2014; Magee et al., 2012; Ryan et al., 2000) and were maintained under specific pathogen-free (SPF) conditions in the Animal Care Facility of Keio University School of Medicine. Germ-free (GF) mice (C57BL/6 background strain) were purchased from Sankyo Lab Service Corporation and were kept in the GF Facility of Keio University School of Medicine. Both female and male mice were used for this study and were aged between 6 and 12 weeks at the time of experiments. All experiments were approved by the Institutional Review Board for Animal Experiments of Keio University and were performed according to the institutional guidelines and home office regulations.

METHOD DETAILS

Preparation of intraepithelial lymphocytes and lamina propria mononuclear cells
Mice were euthanized by cervical dislocation, and the small intestines were collected. After removal of residual fat tissue and Peyer’s patches, the small intestine tissue was opened longitudinally and washed with Ca2+, Mg2+-free Hank’s balanced salt solution (HBSS) (Nacalai Tesque) to remove fecal content. After washing, the small intestine was further cut into small pieces and incubated with HBSS containing 1 mM dithiothreitol (Invitrogen) and 5 mM EDTA (Nacalai Tesque) for 30 min at 37°C to remove the epithelial layer. After removal of the epithelial layer, the mucosal pieces were washed with HBSS and digested by incubation with HBSS containing 1.5% fetal bovine serum (Thermo Fisher Scientific), 1 mg/mL collagenase (Wako), and 0.1 mg/mL DNase (Sigma-Aldrich) for 30 min at 37°C. The digested solution was centrifuged at 1,700 rpm for 5 min. The pellet was resuspended in 40% Percoll (GE healthcare) and overlaid on 75% Percoll. Percoll gradient separation was performed by centrifugation at 2,000 rpm for 20 min at 20°C. Cells at the interphase were collected as LPL. For intraepithelial lymphocytes, the supernatant containing the epithelial layers was collected and centrifuged at 1,700 rpm for 5 min. The pellet was resuspended in 40% Percoll and overlaid on 75% Percoll. Percoll gradient separation was performed by centrifugation at 2,000 rpm for 20 min at 20°C. Cells at the interphase were collected as IEL.

Preparation of spleen cell suspensions
Spleens were harvested from the mice after death and homogenized manually in HBSS. The lysates were filtered through a cell strainer. The passed cells were hemolyzed with 0.84% (vol/wt) ammonium chloride (Nacalai Tesque), washed with HBSS, and collected for analysis.

Flow cytometry
Nonspecific staining was inhibited by incubation with anti-CD16/32 (BD Bioscience) for 20 min. The surface antigens of isolated single-cell suspensions were stained with the following antibodies: CD4 (RM4-5), CD8α (53-6.7), CD8β (eBioH35-17.2), CD45 (30-F11), TCRβ (H57-597), TCRγδ (GL3). For intracellular Foxp3 (FJK-16s) and RORγt (Q31-378) staining, cells were permeabilized using fixation/permeabilization solution (eBioscience) before intracellular staining with antibodies. For IL-17A (eBio17B7) and IFNγ (XMG1.2) staining, cells were incubated in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Nacalai Tesque) with 50 ng/mL PMA (Sigma-Aldrich),
500 ng/mL ionomycin (Sigma-Aldrich), and GoldiStop (BD Bioscience). Cells were stained with antibodies against the indicated cell surface markers, and dead cells were stained with Fixable Viability Dye eFluor 780 (eBioscience). Cells were permeabilized using fixation/permeabilization solution before IL-17A and IFNγ staining. Spleen cell suspensions were stained with the following antibodies: CD45 (30-F11), TCRb (H57-597), CD4 (RM4-5), CD8α (53-6.7), CD44 (IM7), and CD62L (MEL-14). For flow cytometry analysis, the FACS Canto system (BD Biosciences) was used. Data were analyzed by FlowJo Vx software (Tree Star). Cell sorting was performed using the BD FACS Aria II sorter (BD Biosciences). For measurement of mitochondrial mass and membrane potential, purified cells were washed with prewarmed RPMI-1640 medium (Nacalai Tesque) supplemented with 10% FBS (staining buffer). Approximately, 1.5 x 10^6 cells were resuspended in 1 mL of staining buffer containing MitoTracker Green (20 nM; M7514; Invitrogen) and MitoTracker CMXROS (20 nM; M7152; Invitrogen) and incubated in a CO2 incubator at 37°C for 30 min. Stained cells were washed with 1 mL of prewarmed staining buffer and used for FACS analysis or further staining with antibodies. For pS6 staining, CD4IELs, CD4LPLs and spleen cells were stimulated for 30 min with soluble α-CD3 mAb (1 μg/mL), then fixed in phosphate-buffered saline (PBS)/2% paraformaldehyde for 20 min, permeabilized with 90% methanol for 30 min on ice, and then stained for CD4, CD45, TCRb, TCRγδ, CD8α, CD8β and pS6 in PBS. All the antibodies were used at 1:200 dilution. Alternatively, TMRE (Abcam) was used to monitor mitochondrial membrane potential. Cells were incubated with 5 μM TMRE for 30 min at 37°C and washed in PBS.

**Metabolic assays**

OCR and ECAR were measured with an XF-24 analyzer (Seahorse Bioscience) according to the manufacturer’s protocols. FACS-sorted 5 x 10^5 T cells were cultured in XF media (non-buffered Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, and 2% fetal bovine serum, Agilent Technologies). The assay was performed using XF Cell Mito Stress Test Kit (Agilent Technologies). Three baseline recordings were taken, and this was followed by the sequential injection of the ATP synthase inhibitor oligomycin (2.5 μM), the mitochondrial uncoupler carbonyl cyanide-(trifluoromethoxy)phenyl-hydrazone (1 μM), and the respiratory chain inhibitors antimycin A (1 μM) and rotenone (1 μM).

**Quantitative real-time polymerase chain reaction**

RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. Complementary DNA was synthesized from the extracted RNA using the iScript cDNA Synthesis Kit (Bio-Rad). The obtained complementary DNA was amplified by quantitative real-time polymerase chain reaction (PCR) using primer sets and the SYBR Green FAST qPCR Master Mix kit (Kapa Biosystems). The house-keeping gene Rpl32 was used for normalization of samples. Primer sequences used were listed in Table S1.

**Transmission electron microscopy**

Freshly sorted naïve CD4+ T cells, SPIELs and DPIELs were immediately fixed with an equal amount of 4% glutaraldehyde and 4% parafomaldehyde in 0.1 M phosphate buffer pH 7.4 overnight. Fixed samples were dehydrated and transferred to a fresh 100% resin to polymerize at 60°C for 48 h. The polymerized samples were ultra-thin sectioned at 70 nm with an ultramicrotome (Ultracut UCT, Leica) and observed by a transmission electron microscope (JEM-1400Plus, JEOL Ltd.).

**In vivo tamoxifen treatment**

Mice were gavaged for two consecutive days at the beginning of the week with 5 mg of tamoxifen (Sigma-Aldrich) dissolved in corn oil (Wako) at 50 mg/ml. After 5 weeks from first administration, mice were used for experiments.

**In vivo treatment of rapamycin and 2DG**

Mice were injected intraperitoneally with vehicle, rapamycin (8 mg/kg, Sigma-Aldrich) or 2DG (500 mg/kg, Sigma-Aldrich) daily for 5 weeks. Rapamycin and 2DG were first reconstituted in DMSO and then diluted with PBS.

**Hypoxpyrobe-1 staining**

Mice were administrated pimonidazole (Hypoxyprobe, Burlington, MA) by intraperitoneal injection 30 min prior to sacrifice. Dissected pieces of mouse small intestine were washed in PBS and then fixed in 4% PFA.
for overnight, 4°C. Tissue was embedded in 4% low melting temperature agarose (Nacalai Tesque) and cut into 200 μm sections using a Vibratome (Leica). Vibratome sections were washed in PBS and incubated with Blocking buffer (1% BSA, 1% mouse serum, 0.1% Triton-X100 in PBS) for 2 hours, room temperature. Sections were incubated with FITC conjugated α-pimonidazole monoclonal antibody (Hypoxyprobe, 1:100) for 1 hour, room temperature. PBS washed sections were counterstained with DAPI and mounted to analyze by confocal microscopy (Olympus FV3000).

**In vitro CD4^+CD8αα^+ T cell differentiation**

Naïve CD4^+ T cells were isolated by negative selection with mouse Naïve CD4^+ T cell isolation kit (Miltenyi). 1 × 10^5 cells were cultured for 4 days in 96-well plates precoated with 1 μg/ml of α-CD3ε (145-2C11; Biolegend) and 1 μg/ml of soluble α-CD28 (37.51; Biolegend) in RPMI 1640 media supplemented with 10% FBS, 1% penicillin/streptomycin, 1% pyruvate (11360070; Thermo Fisher Scientific), 1% MEM/NEAA (11140050; Thermo Fisher Scientific), 2.5% HEPES (15630080; Thermo Fisher Scientific), and 55 μM β-mercaptoethanol (21985023; Thermo Fisher Scientific) under CD4^+CD8αα^+ T cell conditions: RA (10 nM, 0064-1G, Tokyo Chemical Industry), TGFβ (2 ng/ml, 7666-MB-005, R&D Systems), IFNγ (20 ng/ml, 315-05, Peprotech). For the treatment with inhibitors, Rapamycin (1 μM, Sigma-Aldrich), Torin-1 (1 μM, Selleckchem) and 2DG (1 mM, Sigma-Aldrich) were added into the cell culture. In some experiments, cells were placed inside a sealed air tight container which contains an anaeropack (Mitsubishi Gas Company, Tokyo, Japan). The anaeropack contains a gas-controlling reagent which absorbs oxygen and generates carbon dioxide resulting in a hypoxic atmosphere (8% O2).

**Analysis of glucose uptake**

Glucose uptake was determined with a fluorescently labeled deoxyglucose analog (2-NBDG) using a commercially available kit (Biovision, Milpitas, CA, USA). Purified cells were washed twice with RPMI 1640 media supplemented with 0.5% FBS. Cells were resuspended in 400 μl of glucose-free medium supplemented with 150 μg/ml 2-NBDG and incubated at 37°C for 30 min. The cells were washed with assay buffer, and glucose uptake was quantified by FACS analysis.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was performed using GraphPad Prism software. Data were analyzed by applying one-way analysis of variance or unpaired Student’s t-test whenever necessary. A P value < 0.05 was considered significant.