Asymmetric metabolism by sibling lymphocytes coupling differentiation and self-renewal

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ABSTRACT

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After naïve lymphocytes are activated by foreign antigens, they yield cellular progeny with diverse functions, including memory cells, effector cells, and precursors of germinal center B cells. However, it remains unclear whether a naïve lymphocyte is capable of generating daughter cells with multiple fates or multiple naive cells are activated and each give rise to daughter cells with different cell fates. This dissertation analyzes the role of asymmetric cell division in the generation of effector lymphocytes and maintenance of progenitor cells. Our data provide evidence that daughter cells exhibit differential mitochondrial stasis and inherit different amounts of glucose transporters, which is coupled to distinct metabolic and transcriptional program in the sibling cells.

To uncover the links between mitochondrial stasis, transcription network reprogramming and cell fate, we perturbed mitochondrial clearance with pharmacological and genetic approaches. I found that the treatments, which impaired mitochondrial function, increased the differentiation of B cells and T cells into effector subsets. Thus, we hypothesize that mitochondrial stasis could be a trigger for effector cell differentiation. To further explore the mechanism for aged mitochondria-induced shifts in transcriptional and metabolic programs, we used ROS scavengers and glycolysis inhibitors to demonstrate that mitochondria function and the

expressions of lineage-specific transcription factors crosstalk through ROS-mediated signaling and activating AMPK. ROS scavenger treatments helped to maintain the progenitor population and suppressed the differentiation of effector subsets, whereas effector cell differentiation was boosted in the AMPK-α1 knockout. These results suggest mitochondrial stress-induced ROS is required for repressing Pax5 and increasing IRF4.

In addition to showing mitochondrial stasis' connection to cell fate, this dissertation also demonstrates the linkage between phosphatidylinositol-3-kinases and glucose transporter 1 (Glut1) in establishing polarity in dividing cells and in transcriptional reprogramming. The PI3K/mTORC1 pathway is required for maintenance of surface and intracellular Glut1 levels. By using retroviral reporters and Myc-Tagged Glut1 transgenic mice, we found that activated B cells and $CD8^+$ T cells up-regulate surface and intracellular Glut1 while shifting their transcriptional programs toward effector cell differentiation. PI3K and Rab11a are required to establish the polarity of Glut1 near MOTC in interphase through metaphase, and to asymmetrically segregate Glut1 into the two daughter cells, suggesting that PI3K signaling not only induces the different transcriptional programs in the daughter cells but also establishes the self-amplifying mechanism of aerobic glycolysis in the daughter cell that will become an effector cell.

In sum, this dissertation suggests that asymmetric mitochondrial stasis and nutrient up-take could be part of the driving force of cell fate owing to self-reinforcement and reciprocal inhibition between anabolism and catabolism. These results shed light on the deterministic mechanism of effector cell differentiation and provide clues to the basis of maintenance of self-renewal by activated lymphocytes. These findings could be beneficial for producing memory cells and preventing effector cell exhaustion phenotype in a chronic infection or in cancer microenvironment.

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Chapter 1: Introduction

1.1 Models for generating effector and memory cell heterogeneity

Two opposing models have been proposed to explain how lymphocytes generate robust effector-memory responses. The “one cell–one fate” model predicts that early stimulation specifies a homogeneous fate among the cellular progeny of an antigen-specific cell, whereas the “one cell–multiple fates” model predicts that the cellular progeny of an antigen-specific cell can adopt different fates. We have observed asymmetric segregation of fate determinant transcription factors, such as T-bet and Bcl-6, in B cells and T cells. If asymmetric division can generate progenies with different fates, our results will strengthen the one cell-multiple fate model. Visualizing the process of differentiation in vivo can, therefore, help to resolve this controversial issue. Here, I would like to combine multi-fluorescent transgenic mouse model, confocal microscopic analysis, and intravital imaging to determine where, when, and how pre-GC B cells become committed to a specific fate.

1.2 B cell maturation and differentiation

1.2.1 B cell development in the bone marrow

The development of B cells can be divided into two repertoires based on genetic modification events and where the event take place (Sagaert, Sprangers, & De Wolf-Peeters, 2007). A primary B-cell repertoire is derived from the hematopoietic stem cells (HSCs) in the bone marrow. HSCs differentiate into hematopoietic multipotential progenitors, which are the precursor of lymphoid-primed multipotential progenitors (LMPPs) (Adolfsson et al., 2005). LMPPs have the potential to become monocytes and common lymphoid progenitors (CLPs). The branch point of B cell and T cell developments starts from CLPs, which can give rise to both B cells and T cells but not

myeloid-lineage cells (Kondo, Weissman, & Akashi, 1997). The development of B-cell precursors is guided by cytokines secreted by different bone-marrow cells, the surface receptor repertoire dynamics, and the coordination of several transcription factors, such as Pax5, forkhead box O1(Foxo1), PU.1, E2A, and early B-cell factor 1 (Ebf1) (Y. C. Lin et al., 2010).

However, not until the up-regulation of Pax5 expression do pro-B cells become lymphoid or myeloid lineage committed. Indeed, pro-B cells derived from Pax5 knockout mice were capable of differentiating into macrophages, dendritic cells, or natural killer cells with the proper cytokine inductions, suggesting pro-B cells are not committed to B cell lineages in the absence of Pax5 (Nutt, Heavey, Rolink, & Busslinger, 1999). In the next section, we will discuss their function and how their interaction with other transcription factors directs and maintains B cell commitment.

Immunoglobulin (Ig) gene rearrangement is the major genetic event during differentiation stages in bone marrow. Progenitor B-cells rearrange Ig heavy chain genes to become pre-B cells, which then rearrange their Ig light chain genes to become immature B-cells (Hardy, Carmack, Shinton, Kemp, & Hayakawa, 1991). Immature B cells are capable of expressing functional B-cell receptors in IgM isotype, whereas mature B cells carry two receptor isotypes, namely IgM and IgD (Geisberger, Lamers, & Achatz, 2006; Pernis, Chiappino, & Rowe, 1966; Rowe, Hug, Forni, & Pernis, 1973).

To ensure proper rearrangement of both heavy and light chains of B cell receptors, and to prevent further rearrangement during cell proliferation, B cell development involves sequential cell proliferation and cell differentiation events. The alternating expression of different receptors is regulated by activating PI3K-mediated cell cycle program and negative feedback circuits that antagonize PI3K signals.

Flt3 is highly expressed on LMPPs but lost by the late pro-B cell stage (Wasserman, Li, & Hardy, 1995). This is because Pax5 starts to be expressed in the pro-B cells and to suppress Flt3 (Holmes, Carotta, Corcoran, & Nutt, 2006). CLPs can be sub-divided into two populations based on the expression of lymphocyte antigen 6 complex, locus D (Ly6D). In $Ly6D^-$ CLPs, E2A and HEB activate the expression of Foxo1, which then cooperate with E2A and IL7 receptor signals to induce the expression of Ebf1 in $Ly6D^+$ CLPs (Welinder et al., 2011). The $Ly6D^-$ CLPs are called all-lymphoid progenitors, which are biased toward differentiation into B and T cells but still can produce small numbers of NK cells or dendritic cells. By contrast, the $Ly6D^+$ CLPs only generate B cells, and thus they are known as B cell biased lymphoid progenitors (Inlay et al., 2009). In CLPs co-expressing Flt3 and IL7R, the binding of Flt3 ligand to Flt3 receptor signals through the ERK and AKT pathways, whereas IL7R signals through STAT5 (Ahsberg et al., 2010; Kikuchi, Lai, Hsu, & Kondo, 2005).

In pro-B cells, establishment of a positive feedback circuit composed of FoxO1 and Ebf1 promotes B-cell development into pre-B cells. The expression of FoxO1 strengthens the expression of Ebf1, and vice versa (Mansson et al., 2012). ChIPseq data reveals FoxO1 and Ebf1 binds to different enhancer region of Pax5 and work cooperatively to enhance Pax5, Igll1, and Rag1 expression. These genes are important for rearrangement of immunoglobulin heavy chain and assembling of pre-BCR, and thus are essential for pro-B cells to become pre-B cells.

Pre-B cells express IL7R and pre-BCR, which is composed of a successfully rearranged immunoglobulin heavy chain and a heterodimeric surrogate light chain. The IL7R signals through the STAT5 and PI3K/AKT pathways to stimulate cell proliferation and to inhibit Igκ gene recombination, whereas the pre-BCR signals through Syk kinases and B cell linker protein (BLNK) to facilitate immunoglobulin light chain gene rearrangement and through

Ras/extracellular signal-regulated kinase (ERK) to inhibit cyclin D3-controled cell proliferation. Therefore, there are a series of feedforward and feedback regulatory loops downstream the two receptors to ensure one of the two receptors become at a given time. The STAT5 activates cell cycle and pro-survival genes by inducing the expression of cyclin D3 and by pro-survival factor myeloid cell leukaemia sequence 1 and B cell lymphoma 2, respectively (Cooper et al., 2006; Malin et al., 2010).

To become immature B cells, pre-B cells need to successfully rearrange their immunoglobulin light chain gene, which again requires the expression of Rag1/Rag2 genes. This event requires attenuation of IL7R signals through reduction of PI3K/AKT activity and enhancement of FoxO1 expression, enabling FoxO1 to enter the nucleus (Ochiai et al., 2012). In the pre-B cell stage, FoxO1 has been found to bind to the regulatory region of a wide range of genes involved in immunoglobulin recombination such as *Rag1* and *Rag2*, cell-cycle inhibitors (*Cdkn1a* and *Cdkn1b*), and adaptor proteins downstream of pre-BCR signaling (*Blnk* and *Syk)* (Ochiai et al., 2012). By activating Blnk (also known as SLP-65) and Syk, stimulated pre-BCRs up-regulate the expression of IRF4 and p38 (Ishiai et al., 1999; Thompson et al., 2007).

The augmented IRF4 expression then stimulates the expression of chemokine receptor CXCR4, which directs the pre-B cells toward the chemokine CXCL12 and away from IL7-producing stroma cells, which may serve as a mechanism for attenuating the IL7 receptor signaling pathway (Johnson et al., 2008). IRF4 expression also increases Igk and Igλ germline transcripts as well as Rag1 and Rag2 gene transcripts. Therefore, the expression of IRF4 induced by pre-BCR signaling promotes the immunoglobulin light-chain recombination in pre-B cells (Johnson et al., 2008). Unlike AKT-mediated phosphorylation on FoxO1, p38 activates FoxO1's transcriptional activity by phosphorylation (Asada et al., 2007). In sum, FoxO1 and Pax5

cooperatively promote a positive feedback loop, including Blnk, IRF4 and p38, to antagonize the repression effect of the PI3K/AKT pathways on immunoglobulin light-chain recombination and inactivation of Foxo1 and thus facilitate the transition of pre-B cell to immature B cells.

1.2.2 Extra follicular plasma cell and germinal center B cell differentiation

During development, immature B cells are trapped in the bone marrow under the guidance of their CXCR4 and chemokine stromal-derived factor-1 (also known as CXCL12), which is expressed by immature osteoblasts and endothelial cells (Ponomaryov et al., 2000). Thus, immature B cells egress from bone marrow is regulated by CXCR4 down-regulation (Beck, Gomes, Cyster, & Pereira, 2014). After immature B cells leave the bone marrow and enter the bloodstream, they are delivered to peripheral lymphoid organs in which a secondary B-cell repertoire develops. Naïve B cells are defined as the B cells that have left the bone marrow but not yet experienced external antigens (Banchereau, Briere, Liu, & Rousset, 1994). After exposure to foreign antigens, mature, naïve B cells acquire diverse cellular fates. Activated B cells start to express C-C chemokine receptor type 7 and migrate to the border of T-cell zone (Forster et al., 1999). Some of them proliferate and differentiate into short-lived plasma cells secreting low affinity antibodies in the extra-follicular region (MacLennan et al., 2003; Smith, Hewitson, Nossal, & Tarlinton, 1996). Others migrate back into the primary B cell follicles and become GC B cells, which give rise to memory B cells and long-lived plasma cells secreting high affinity antibodies (Nossal, 1992).

My study focused on understanding the molecular mechanism balancing the memory and effector cell repertories during mature B cell differentiation. In the next section, I will discuss the key transcription factors required for maintaining B cell commitment and directing mature B cell

differentiation.

1.2.3 Transcription factors that determine memory B cell and plasma cell differentiation

The Pax5 gene encodes a member of the paired box family of transcription factors. Pax5 is also called B-cell specific activator protein, because it is a mammalian homolog of the tissue-specific activator protein in sea urchins (Barberis, Widenhorn, Vitelli, & Busslinger, 1990). Pax5 is a key transcription factor required in early differentiation of B cells including progression beyond the pro-B-cell stage and repression of B-lineage-inappropriate genes (Nutt et al., 1999). Thus, B cell identity is maintained by expression of Pax5 throughout immature and mature B cell stages, until their terminal differentiation into antibody-secreting plasma cells (Delogu et al., 2006; Nera et al., 2006). In Pax5 knockout mice, B-cell development is blocked at the pre-B cell stage (Rolink, Schaniel, Bruno, & Melchers, 2002).

The IRF4 gene encodes a transcription factor of interferon regulatory factor (IRF) superfamily. In accordance with its name, IRF4 is an important regulator in defending against viral infection (D. Xu, Zhao, Del Valle, Miklossy, & Zhang, 2008). However, IRF4 also modulates the development of several adoptive immune cells. IRF4 expression in both B cells and T cells is required for the formation of germinal center and follicular helper T cells (Willis et al., 2014). Furthermore, germinal center B cells and memory cells derived from IRF4 conditional knockout mice failed to differentiate into plasma cells, implying IRF4 is a key transcription factor of B cell commitment into plasma cells (Klein et al., 2006). IRF4-deficient B cells also lack class-switch recombination and expression of activation-induced deaminase (Klein et al., 2006).

The relationship between Pax5 and IRF4 is complex and differs in each differentiation stage. In the precursors of germinal center B cells or naïve B cells that have been stimulated in vitro,

IRF4 expression is low and Pax5 expression is high (Shukla & Lu, 2014). Thus, IRF4 interacts with Ets and AP-1 family transcription factors to bind the EICE/AICE motifs on the promoters of Bcl6, Obf1 and AID genes (Brass, Zhu, & Singh, 1999; Glasmacher et al., 2012; Ochiai et al., 2013). The co-expression of Bcl6 and Pax5 then stimulates the expression of AID and UNG, which are required for somatic hyper-mutation and class switch recombination, initiating the germinal center reaction (Alinikula, Nera, Junttila, & Lassila, 2011). At these early stages, Pax5 and IRF4 have synergistic function on activated B cells. The levels of IRF4 gradually augment as the B cells undergo several rounds of division.

However, when B cells undergo terminal differentiation into plasma cells, the high level IRF4 is required for down-regulation of Pax5, leading to the expression of plasma cell specific transcription factors, such as XBP1 and Blimp-1 (Jourdan et al., 2009; Shaffer et al., 2004). In the light zone of the germinal center, antigen presented by follicular dendritic cells and follicular helper T cells increases the expression level of IRF4 in centrocytes. At the higher cellular concentration, IRF4 can bind to the low affinity ISRE motifs. This shift in binding pattern allows IRF4 to stimulate the expression of Blimp-1, which represses Bcl-6 and Pax5 expression and initiates plasma cell differentiation (Klein et al., 2006; K. I. Lin, Angelin-Duclos, Kuo, & Calame, 2002).

1.2.4 The regulators of Pax5 and IRF4 during B cell activation

In the previous section, we talked about the dynamics of Pax5 and IRF4 expression during B cell maturation and differentiation. Here, we are going to further discuss the regulators and signal pathways that modulate the expression of Pax5 and IRF4. The most important signaling pathway associated with immune cell activation is the PI3K/AKT/mTOR pathway.

Phosphoinositide 3-kinase (PI3K) can be activated by several kinds of receptors, including integrins, receptor tyrosine kinases, B and T cell receptors, cytokine receptors and G-protein-coupled receptors (Okkenhaug & Vanhaesebroeck, 2003). In B cells, cross-linking B-cell antigen receptors leads to CD19 phosphorylation, which provides a docking site for class I_A PI3K (Koyasu, 2003; Otero, Omori, & Rickert, 2001). The class I_A PI3K phosphorylates the 3'-hydroxol group in phosphatidylinositol and phosphoinositides to produce phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2), and phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3 (Okkenhaug, 2013) at the plasma membrane. PI(3)Ps serve as docking sites for the Pleckstrin Homology domain (PH domain)-containing protein kinases: 3-phosphoinositide dependent protein kinase-1 (PDK1) and the serine/threonine kinase AKT (also called protein kinase B) (Bayascas et al., 2008; Franke, Kaplan, Cantley, & Toker, 1997). The co-localization of PDK1 and AKT enables PDK1 to phosphorylate and to activate AKT (Alessi et al., 1997).

AKT activates mTORC1 through multiple parallel mechanisms. AKT phosphorylates tuberous sclerosis protein 2 (TSC2) and proline-rich Akt substrate of 40 kDa (PRAS40), which are negative regulators of mTORC1 function. The phosphorylated TSC2 switches its partner from TSC1 to cytosolic anchoring protein 14-3-3, disrupting the TSC1/TSC2 dimer, which inhibits mTORC1 function (Cai et al., 2006; Inoki, Li, Zhu, Wu, & Guan, 2002). Similarly, phosphorylated PRAS40 breaks apart with the regulatory-associated protein of mTOR (Raptor) protein in mTORC1, enabling Raptor to recruit mTORC1's substrates: S6K1 and 4E-BP1 (Oshiro et al., 2007; Vander Haar, Lee, Bandhakavi, Griffin, & Kim, 2007).

The PI3K/AKT pathway represses Pax5 expression through sequestering its activator Foxo1 from the nucleus and supporting the expression of its inhibitor IRF4 (W. H. Lin et al., 2015).

AKT phosphorylates the transcription factor forkhead box O1 (FoxO1), leading Foxo1 to be bound by 14-3-3 protein (Biggs, Meisenhelder, Hunter, Cavenee, & Arden, 1999). The 14-3-3 proteins are a family of proteins that prefer to bind to specific phosphorylated serine and threonine motifs (Obsil & Obsilova, 2011). One of the 14-3-3 interacting motifs in FoxO1 overlaps with the C-terminal basic region of the DNA-binding domain. Because this region also includes the nuclear localization signal of FoxO1, the binding of 14-3-3 protein inactivates FoxO1's DNA binding activity and promotes its shuttling from the nucleus to cytoplasm (X. Zhao et al., 2004).

In addition to hijacking the activator of Pax5, activation of the PI3K/AKT/ mTOR pathway also augments the expression of IRF4, which represses Pax5 expression at higher concentrations (Sciammas et al., 2006). An early study showed that overexpression of IRF4 in Oci-Ly7, a cell line derived from human germinal center B cells, repressed a group of genes highly expressed in germinal center B cells, including Pax5, Bcl6, and Aicda (Sciammas et al., 2006). Similarly, our group has used IRF4 knockout mice to demonstrate that IRF4 is essential for the downregulation of Pax5. In LPS-stimulated mature B cell cultures, IRF4 knockout B cells retain their Pax5 high phenotype, even after they have undergone sufficient numbers of cell divisions that Pax5 repression is observed in wild-type cells under the same conditions (W. H. Lin et al., 2015). In the same study, we also demonstrated that the chemical inhibitors PI3K, AKT, or mTOR could suppress the Pax5 downregulation and IRF4 up-regulation in wild-type B cell cultures. Taken together, PI3K signaling pathway-induced Pax5 suppression is partially mediated by strengthening the expression of IRF4.

1.3 T cell maturation and differentiation

1.3.1 Early T cell development in the thymus

Despite the progenitors of B cells and T cells sharing some key transcription factors, such as PU.1, Runx1/CBFβ, and Ikaros, their lineage commitment pathways differ significantly in several respects. First, the developmental events for B cells happen predominantly in the bone marrow, whereas those for T cells happen in the thymus. Therefore, bone marrow-derived progenitors need to exit the bone marrow, move within the bloodstream, and home to the thymus throughout the life span. However, studies about the identification of multi-potent progenitors that migrate through the blood stream to populate the thymus have remained controversial. Perhaps more than one progenitor population contributes to replenish the progenitors in thymus through distinct homing mechanisms (Zlotoff & Bhandoola, 2011). The most promising candidates are LMPP and CLP.

Second, the progenitors of T cells have relatively broad potential to further differentiate into other lineages. B cell commitment occurs after Pax5 upregulation in pro-B cells, whereas the T cell commitment is not complete until after sequential lineage exclusion steps block the potential to become macrophages, granulocytes, DCs, and NK cells (Cobaleda, Schebesta, Delogu, & Busslinger, 2007). The early thymic progenitors derived from LMPP and CLP are characterized by $Kit^{hi}LI\text{-}7R^{low}$. They differentiate into $Kit^{hi}LI\text{-}7R^{low}$ double-negative (DN)2a cells. The T cell commitment happens during the transition from the DN2a to DN2b stage. In the DN2a stage, the expression of transcription factor Bcl11b is induced by the Notch pathway and multiple transcription factors, including CSL, Tcf-1, GATA3 and Runx1 (Kueh et al., 2016). The expression of Bcl11b restricts the potential of T cells to differentiate into NK cells, myeloid and dendritic cells (Li, Leid, & Rothenberg, 2010). Once they enter the DN2b stage, these pro-T cells

start to rearrange the TCRβ, TCRδ and TCRγ loci.

Third, Notch1 signaling is essential for T cell lineage commitment from bone marrow progenitors stages until they have passed the β-selection checkpoint. By contrast, the function of Notch signaling during early B-cell development remains unclear, but Pax5-mediated repression of Notch1 is part of the mechanism of B cell lineage commitment and repression of T cell lineage commitment (Souabni, Cobaleda, Schebesta, & Busslinger, 2002).

Only after their transition from DN3a to DN3b stage can pro-T cells stop relying on Notch signaling. It is during this transition that the successfully rearranged TCRβ chain pairs with pre-T cell receptor alpha to form pre-TCR. The cells that generate functional pre-TCR are stimulated by pre-TCR signals for survival and proliferation, whereas those cells that fail to produce functional pre-TCR are eliminated by apoptosis. Thus, this checkpoint of T cell development is called β selection.

1.3.2 Effector and memory T cell-fate decisions

Until now, the mechanism and time point for generation of memory T cell after infection has remained unclear. Indeed, how one defines a memory T cell lies at the heart of this dispute. One of the most widely used definitions is based on the time that the antigen specific cells persist. That is, antigen specific memory cells can be detected months after infection or exposure to antigen. However, this definition cannot be applied to study the early development of effector and memory cells after infection. Therefore, some studies have defined memory cells by their functional characteristics, including their ability to undergo rapid proliferation in response to antigen and to undergo homeostatic proliferation when they are stimulated by cytokines (such as IL15 and IL-7) (Kaech, Hemby, Kersh, & Ahmed, 2002). Other studies have proposed that the

memory T cells can be subdivided into two groups with different homing potentials and effector functions by measuring their surface expression of CCR7 and CD62L (Sallusto, Lenig, Forster, Lipp, & Lanzavecchia, 1999). The $CD62L^-/CCR7^-$ effector memory T (T_{EM}) cells are attracted to inflamed tissues and can perform immediate effector function, whereas the $CD62L^+$/ $CCR7^+$ central memory T (T_{CM}) cells home to lymph nodes but lack immediate effector function. In addition, T_{CM} can differentiate into $CCR7^-$ effector cells when re-challenged by antigens.

As previously discussed in Section 1.1, one of the basic developmental questions about the generation of effector and memory cells is whether a single cell is sufficient to generate daughters with distinct function and longevity. The "one cell, one fate" hypothesis states that one activated T cell can only give rise to daughter cells with the same cell fate; thus, distinct fates should emerge only from different naïve T cells. By contrast, the "one cell, multiple fate" hypothesis states that a single T cell can generate progenies with diverse phenotypes and functions that one observes in a T cell response. This question is now partially resolved by single-cell adoptive transfer technology. Using an injection system monitored by microscopy, Stemberger and colleagues demonstrated that an adoptively transferred single naive OT-I T cell (a FACS-enriched $CD45.1^+$ $CD44^{low}$ TCR-transgenic cell) expanded into diverse populations as characterized by surface expression levels of CD62L and CCR7 and by differential expression of cytokines, including IFN-γ, IL-2, and TNF-α, in response to *Listeria* infection (Stemberger et al., 2007). The pattern of surface marker and cytokine production in a single cell-derived population is indistinguishable from that of endogenous epitope-reactive T cells, suggesting a single T cell can generate the full repertory of functional T cell families after infection; thus, this result is a strong support for the one cell, multiple fate hypothesis.

Although the single cell transfer assay is a useful tool for mapping the fate of the founding

ancestor at single-cell resolution, it can only capture a snapshot of the daughters at a specific time point. Thus, this technique cannot record the branching process of all daughters derived from a single T cell in vivo. Therefore, the next question is when and how fate determination happens during the immune response. Traditionally the development of memory T cells has been described by the linear differentiation model, which proposes that the branch point for differentiation into effector or memory T cells does not happen until the population enters the contraction phase (Ahmed & Gray, 1996; Wherry et al., 2003). This model proposed a linear differentiation pathway: naïve T cells become effector T cells, which give rise to T_{EM}, and then T_{EM} become T_{CM}. However, several recent discoveries have challenged this model and alternate models have been proposed to explain the new observations.

The linear differentiation model is based on observations made in the settings of acute lymphocytic choriomeningitis virus (LCMV) and Listeria monocytogenes (LM) infections, which are characterized by clearance of pathogens and generation of $CD8^+$ memory cells, which can perform recall proliferation and differentiation into effector cells after reencountering antigen. However, this model did not explain the mechanism for generating heterogeneous effector and memory T cells and failed to explain why $CD8^+$ T cells become defective in chronic infections where antigen persists (D'Souza & Hedrick, 2006; Renno, Hahne, Tschopp, & MacDonald, 1996).

An alternative model is the decreasing potential model, which holds that activated $CD8^+$ T cells progressively lose their ability to differentiate into memory cells and become more terminally differentiated during rounds of division (Ahmed & Gray, 1996). The number of divisions is positively correlated with the strength and duration of antigen stimulation. Therefore, only optimal stimulation strength leads to enough T cell expansion for the generation of

functional memory cells and effector cells (Sarkar et al., 2007). Weak stimulation leads to limited T-cell expansion and impaired memory development, whereas persistent stimulation leads to generation of exhausted effector T cells(Renno et al., 1996; Williams & Bevan, 2004). The generation of T_{CM} and T_{EM} is also associated with the duration of antigenic stimulation. The T_{CM}s are generated by short antigenic stimulation, whereas T_{EM}s and effector T cells are generated by prolonged antigenic stimulation (van Faassen et al., 2005).

1.3.3 Transcription factors of memory or effector T lymphocyte differentiation

Despite the developmental pathway and mechanism for generating memory and effector T cells not being fully understood, many key transcription factors that participate in the early development of T cells in thymus have been found to play a role in maintaining memory cells or in differentiation into effector cells. Some factors are required for promoting memory cell development, survival, and self-renewal, whereas others support the effector function of T cells. The former includes TCF1 and FoxO1, and the latter includes Bcl11b, T-bet, and Blimp-1.

For example, the T cell lineage commitment is regulated by Tcf-1 and Bcl11b. TCF1 knockout T cells fail to generate and maintain the memory T cells (Zhou et al., 2010), whereas the deletion of Bcl11b impairs the function of effector $CD8^+$ T cells and affects the differentiation of $CD4^+$ T cell subsets (Avram & Califano, 2014).

In addition, the protein level of Foxo1 in memory-precursor T cells is higher in comparison with that in effector T cells. Mice with conditionally-silenced Foxo1 in $CD8^+$ T cells fail to elicit an efficient secondary immune response against *Listeria monocytogenes* (M. V. Kim, Ouyang, Liao, Zhang, & Li, 2013). Moreover, Foxo1 has been demonstrated to bind to the regulatory region of the Tcf-1 gene by chromatin immunoprecipitation sequencing assay (M. V. Kim et al.,

2013). These data collectively suggest that Foxo1 is required for the differentiation and maintenance of long-lived memory $CD8^+$ T cells.

1.4 Immunometabolism

1.4.1 The connection between metabolism and the immune system

An effective immune response requires different types of immune cells to reprogram their metabolic configurations, enabling them to adopt distinct requirements of nutrient catabolism, anabolism, cell proliferation, and longevity. Immunometabolism is an area of study aimed to decipher the connection between metabolism and the immune system (Loftus & Finlay, 2016; O'Neill, Kishton, & Rathmell, 2016a). Understanding how distinct metabolic configurations in various cell types facilitate pathogen defense and support immune function holds tremendous hope for improving the efficiency of vaccination and immune therapy as well as the prevention and treatment of auto-immune disease.

Metabolic reprogramming is essential for fulfilling various cellular activities, including energy supply, anabolism, translation, transcription, posttranslational modifications, secretion, migration, proliferation, survival, redox balance. It is thus associated with several immune functions such as differentiation, the production and secretion of antibody and cytokine, maintenance of memory populations, and rapid proliferation after activation by antigens. For example, the metabolism of glucose is essential for supporting several immune functions of cytotoxic $CD8^+$T cells. Glycolysis is one of the basic metabolic pathways for cells to generate ATP and to prepare materials for anabolism. Moreover, the synthesis of cytokine interferon-γ (IFN-γ) relies on glucose availability, because glucose deprivation inhibits IFN-gamma transcription and de novo protein synthesis (Cham & Gajewski, 2005). The secretion of IFN-γ is also dependent on glycolysis (Cretenet et al., 2016).

The byproducts of metabolism are also involved in signaling pathways. Reactive oxygen species (ROS) are generated during cellular energy production, especially by aerobic

metabolism and mitochondrial respiration (Giorgio, Trinei, Migliaccio, & Pelicci, 2007). ROS play crucial roles in several biology functions of T cells (Belikov, Schraven, & Simeoni, 2015). Activated phagocytes are the extrinsic ROS source for T cells. Activated T cells trigger respiratory burst in phagocytes, which produce H_2O_2 that penetrates into T cells or oxidizes proteins on the plasma membrane of T cells (Cemerski, Cantagrel, Van Meerwijk, & Romagnoli, 2002; Flescher et al., 1994). In addition, there are several sources producing ROS in T cells; H_2O_2 is produced by DUOX-1 and NOX-2, and superoxide anion ($O_2^{\bullet-}$) is generated by the electron transport chain of the mitochondria. Carefully controlled equilibrium between ROS and antioxidants is required for a normal immune response. First, NOX-2-derived ROS suppress the differentiation of Th17 but support the differentiation of Th2 T cells. In T cell-specific NOX-2 (p47 subunit) knockout mice, the expressions of T-bet, STAT-1, and STAT-4 are diminished, but expression of phosphorylated STAT-3 is enhanced, suggesting NOX-2 deficiency favors differentiation into Th17 but suppresses that of Th2 (Shatynski, Chen, Kwon, & Williams, 2012; Tse et al., 2010). Second, ROS generation by complex I after TCR signaling induces expression and secretion of cytokines IL-2 and IL-4 and activation of transcription factors NF-κB and AP-1 (Kaminski et al., 2010). Third, ROS amplify the TCR signal in activation-induced cell death, which is an important mechanism to terminate the immune response after foreign antigens are cleared (Belikov, Schraven, & Simeoni, 2014).

1.4.2 Distinct metabolic programs in effector and memory lymphocytes

The detailed molecular mechanism and developmental pathway of effector and memory T cells has remained under debate. Several models have been proposed, each supported by different experiments. The three most commonly compared models are the dedifferentiation

model, the decreasing potential model, and the divergent lineage model (Joshi & Kaech, 2008).

The rapid proliferation and enhanced extracellular acidification rates of activated T cells suggest these cells undergo dramatic remodeling of their metabolic and transcriptional programs to fulfill the demand for energy and macromolecular building blocks during the generation of effector cells. In the previous sections, we have discussed several key transcription factors that have been shown to be essential for establishing lineage commitment and for maintaining the identity of effector and memory T cells. Here, we are focused on metabolic reprogramming and its link to the function and survival of effector and memory T cells.

Naïve T cells mainly rely on mitochondrial-dependent oxidative phosphorylation and fatty acid β oxidation to support their basal energy consumption. By contrast, activated, fast-dividing T cells rely on elevated aerobic glycolysis and glutamine oxidation together with oxidative phosphorylation. Most of the effector T cells are dead at the end of immune response; however, some of them survive and become memory cells by adopting a metabolic program similar to that of naïve T cells, and return to a quiescent state like adult stem cells.

The impact of perturbation of the metabolic programs on the differentiation of effector and memory T cells has been studied with mTORC1 and AMPK. Blocking mTORC1 with rapamycin promotes the differentiation of the memory-liked T cell population in in vitro and in vivo studies (Araki et al., 2009). On the other hand, stimulating AMPK, which is a negative regulator of mTORC1, with metformin also promotes the formation of memory T cells, whereas deletion of AMPK blocks the transition to a memory T cell fate (E. L. Pearce et al., 2009).

Directly targeting some rate-limiting enzymes also results in dramatic changes in the development of memory T cells. The over-expression of carnitine palmitoyl-transferase (CPT1), a key enzyme of β oxidation, augments the fraction of memory T cells (van der Windt et al., 2012), whereas over-expression of the glycolytic enzyme PGAM1 limits the capacity of activated T cells to generate immune cells (Sukumar et al., 2013).

Unfortunately, the link between metabolic and transcriptional reprogramming remains unclear. There is evidence suggesting that the metabolic status is associated with intracellular AMP to ATP ratio, which can be sensed by AMPK. A high AMP condition activates AMPK, which suppresses and antagonizes the effect of mTOR, accelerating translation and proliferation (W. H. Lin et al., 2015). Also, previous reports and our data have both demonstrated that inhibition of glycolysis by 2-deoxyglucose (2-DG) during T cell activation prevents the expression of the effector-like transcriptional program and the production of antigen specific memory cells (Sukumar et al., 2013).

1.4.3 The PI3K/AKT/mTOR pathway, AMPK and metabolism

In the earlier sections, we have introduced AKT's ability to activate mTOCR1 by multiple pathways. In this section we are going to focus on the different functions of mTPCR1 and mTORC2 and their agonist AMPK on fine-tuning the metabolic program.

Both mTOR, a serine-threonine kinase, and AMPK, a serine-threonine kinase protein complex, are evolutional conserved. mTORC1 and mTORC2 share three proteins: the catalytic subunit mTOR, the DEP domain-containing mTOR-interacting protein (Deptor), and mammalian lethal with SEC13 protein 8 (mLST8) (Peterson et al., 2009). However, mTORC1 includes Raptor and proline-rich AKT substrate 40 kDa (PRAS40) (Hara et al., 2002; Sancak

et al., 2007). By contrast, mTOCR2 encompasses rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1), and the protein observed with Rictor-1 (Protor-1) (L. R. Pearce et al., 2007; Sarbassov et al., 2004; Yang, Inoki, Ikenoue, & Guan, 2006).

An early study showed that rapamycin treatment also enhances memory T-cell responses, suggesting that memory T-cell differentiation is regulated by mTOCR1 (Araki et al., 2009). Indeed, later studies have demonstrated that mTORC1 influences $CD8^+$ T cell effector responses, whereas mTORC2 activity regulates $CD8^+$ T cell memory. Deletion of TSC2, a repressor of mTORC1, prevents $CD8^+$ T cells from differentiating into memory cells and helps them retain effector characteristics. In contrast, mTORC2 inhibition helps to increase the number of $CD8^+$ memory cells (Pollizzi et al., 2015).

Moreover, when memory $CD8^+$ T cells are activated by antigens, a rapid reprogramming of their glycolytic activity occurs, providing a subsequent ability to re-acquire effector functions. Such immediate early glycolysis in memory $CD8^+$ T cells is inhibited by OSI-027, an mTORC1-mTORC2 inhibitor, but not by rapamycin, suggesting that mTORC2, rather than mTOCR1, is associated with the glycolytic switch (Gubser et al., 2013).

1.4.4 AMPK and metabolism

AMPK is an important sensor and regulator of cellular energy production and response to various stress conditions, including a low level of ATP, high amounts of reactive oxygen or nitrogen species, and a high calcium concentration (Cardaci, Filomeni, & Ciriolo, 2012). AMPK is the major regulator of transcription factor PGC1-alpha, which is the key regulator that controls the production of genes related to mitochondria renewal (Canto & Auwerx, 2009). AMPK also

antagonizes the activation effect of AKT on Foxo1 and mTOR by activating the TSC1/TSC2 complex and phosphorylating Foxo1, respectively (Fay, Steele, & Crowell, 2009).

1.5 Mitochondria and immunity

1.5.1 The regulators of mitochondrial motion, fusion, and fission

Mitochondria not only play a role in energy production but also in diverse cellular activities, including anabolism, calcium and iron homeostasis, ROS production, apoptosis, and the immune response. To date, our knowledge about regulatory proteins involved in mitochondrial shaping, motion, and morphology is largely from studies of *Saccharomyces cerevisiae* (Altmann & Westermann, 2005; Dimmer et al., 2002). Although several homologous genes have been found in higher eukaryotes, their functions do not exactly recapitulate their homologs in yeast. For example, mitochondrial transportation in mammalian cells relies mainly on microtubules, whereas in yeast, mitochondrial transportation is more dependent on the actin cytoskeleton (Boldogh, Fehrenbacher, Yang, & Pon, 2005; Chada & Hollenbeck, 2004). Detailed mechanisms for how mitochondria segregate into daughter cells remain largely unclear in mammalian cells. In this introduction section, I mainly focused on mammalian systems.

Mitochondrial transportation and quality control are regulated by three activities: mitochondrial movement, fission, and fusion. The transportation of mitochondria along microtubule networks relies on two families of motor proteins: the kinesins and dyneins (Tanaka et al., 1998; Varadi et al., 2004). In addition, Milton and Miro (RhoT1/2) are two important adaptor proteins that form the mitochondrial motor/adaptor complex, which links the motor proteins to the mitochondria. Milton is the adaptor protein that connects Miro to the motor proteins, whereas the Miro is a transmembrane protein that anchors to the mitochondrial outer membrane through its C-terminal transmembrane domain (Fransson, Ruusala, & Aspenstrom, 2006; Glater, Megeath, Stowers, & Schwarz, 2006). Miro has two GTPase domains, which are

distantly related to the Ras superfamily of small guanosine triphosphatases (GTPases), separated by a pair of EF hand domains, which indicates that the activity of Miro can be regulated by the level of Ca^{2+} (Fransson et al., 2006). One of the ways to regulate the motion of mitochondria is that when Ca^{2+} binds to these EF hands domains in Miro protein, Miro reversibly disengages from microtubules. Using cultured embryonic hippocampal neurons derived from mice and rats, PINK1 and Parkin have been reported to induce proteasomal degradation of Miro by phosphorylated Miro and by ubiquitinylate Miro, respectively (X. Wang et al., 2011).

Mitochondrial morphology, fusion, and fission are regulated by Dynamin-related large GTPases: dynamin-related protein 1 (Drp-1), the mitofusins (Mfn1 and Mfn2), and Opa1 (Olichon et al., 2002; Santel et al., 2003; Smirnova, Griparic, Shurland, & van der Bliek, 2001). Drp-1 proteins are in the cytosol and are recruited to mitochondria by mitochondrial outer membrane proteins, such as mitochondrial fission 1 (Fsi1), mitochondrial fission factor (MFF), Mitochondrial dynamics protein of 49 kDa (MID49), and 51 kDa (MID51) (Loson, Song, Chen, & Chan, 2013). When assembling on and wrapping around the mitochondrial outer membrane, oligomeric Drp-1 proteins cause the constriction of mitochondria and thus facilitate mitochondrial fission (Yu, Fox, Burwell, & Yoon, 2005). By contrast, mitochondrial fusion is regulated by mitochondrial outer membrane proteins Mfn1 and Mfn2 and by mitochondrial inner membrane protein Opa1, which mediate the effusion of mitochondrial inner membrane (Olichon et al., 2002; Santel et al., 2003).

Mitochondrial morphology is coordinated with the cell cycle. In G1 phase, mitochondria form an interconnected network, which is fragmented when cells enter mitosis (Taguchi, Ishihara, Jofuku, Oka, & Mihara, 2007). This transition occurs because cyclin–CDK activity not only drives cell cycle progression but also regulates the transcription, stability, and activity of

mitochondrial fission proteins. On the transcriptional level, when CDK activates the transition from G1 to S phase, *pRb* is phosphorylated by CDKs. Phosphorylated pRB breaks apart from the *pRB/E2F* complexes, which enables E2F to activate the transcription of several mitochondrial function genes (Ambrus et al., 2013; Blanchet et al., 2011; Sankaran, Orkin, & Walkley, 2008). Moreover, when the cyclin-B-CDK complex is activated as a cell enters metaphase, it activates Drp-1 through phosphorylation (Taguchi et al., 2007). Also, during metaphase, mitotic kinase Aurora A phosphorylates RALA, which forms a complex with RALBP1 and cyclin B–CDK1 on mitochondria that mediates the phosphorylation of Drp-1 (Kashatus et al., 2011). The fission activity is inactivated at the end of telophase, when the anaphase-promoting complex (APC) targets the Drp-1 protein for degradation (Horn et al., 2011).

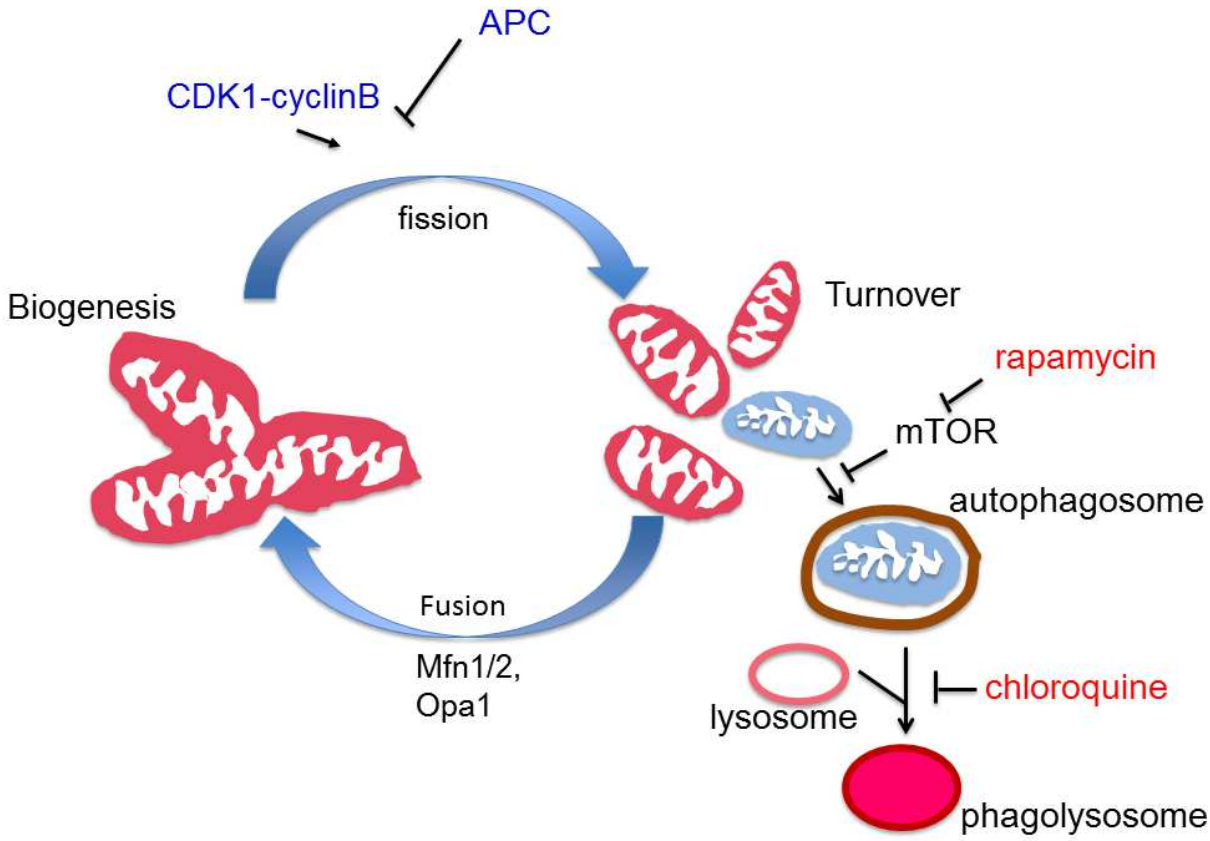


Figure 1.1 The regulators of mitochondrial motion, fusion and fission

The mitochondrial life cycle involves biogenesis, fission, turnover, and fusion. The fission and fusion of mitochondria is regulated by Cyclin-dependent kinases (CDKs) and anaphase-promoting complex (APC). In S phase, CDK1-cyclinB complex phosphorylates Drp-1 and triggers mitochondrial fission. Therefore, the mitochondrial network is fragmented at the G2 and M phase. When APC triggers the completion of metaphase, APC targets Drp-1 for degradation. The pharmacological inhibitors are colored red.

1.5.2 The linkage between mitochondrial function and the immune system

Mitochondria are multifunctional organelles. They serve not only as energy factories and biosynthetic factories but also as the major mediators of apoptosis and a hub in the innate immune system (Ernster & Schatz, 1981; X. Wang, 2001; West, Shadel, & Ghosh, 2011). Recent studies, however, have also implicated that mitochondria are signaling organelles influencing differentiation, proliferation, and aging. In both innate and adaptive immune cells, mitochondria are necessary for guiding lymphocyte differentiation and for fulfilling cell-type specific metabolic demands of different immune cells. Indeed, the development and differentiation of NK cells, T cells, and B cells are all reportedly linked with mitochondrial functions (Jang et al., 2015; van der Windt et al., 2012; S. Wang et al., 2016).

It has been demonstrated that memory T cell and memory NK cell formation can be enhanced by treating an immunized animal with rapamycin, which is a pharmacologic molecule that induces autophagy by inhibition of mTOR (Araki et al., 2009; T. E. O'Sullivan, Johnson, Kang, & Sun, 2015). Moreover, an early study on immune response to *S. aureus* infection found that rapamycin treatment blocked the differentiation of human B lymphocytes into antibody-secreting cells (Aagaard-Tillery & Jelinek, 1994).

1.5.3 The mitochondrial quality control system

Despite mitochondria playing an important role in providing energy and nutrient homeostasis, as well as in signaling pathways, accumulation of damaged mitochondria causes cell death and tissue dysfunction and increases oxidative stress by producing excessive reactive oxygen species (ROS) (Cleeter, Cooper, Darley-Usmar, Moncada, & Schapira, 1994). Therefore, a sophisticated quality control mechanism has been evolved to repair and to replace damaged mitochondria

(Ashrafi & Schwarz, 2013).

Because mitochondrial DNA only encodes less than 40 genes, the majority of the mitochondrial proteins are encoded in the nucleus (Taanman, 1999). These proteins are translated in the cytosol and are transported through mitochondrial membranes to their destination: to integrate into the outer/inner membranes, the intermembrane space, or the matrix. The first line of defense to prevent the accumulation of misfolded or damaged protein in mitochondria is through the mitochondrial unfolded protein response. In the matrix, damaged or misfiled proteins are degraded by soluble protease complexes (Lon and Clp family proteases) (de Sagarra et al., 1999; Q. Zhao et al., 2002), metallopeptidase Oma1 (Kaser, Kambacheld, Kisters-Woike, & Langer, 2003), and m-AAA proteases (Arlt, Tauer, Feldmann, Neupert, & Langer, 1996). In the inter-membrane space, undesired proteins are removed by the i-AAA protease, which is also a member of the AAA proteases. These proteases degrade their substrates to oligopeptides, which are further degraded to amino acids by mitochondrial oligopeptidases (Kambacheld, Augustin, Tatsuta, Muller, & Langer, 2005) or expelled from mitochondria (Augustin et al., 2005).

Damaged mitochondrial proteins can also be removed by a mitochondria–lysosome transport pathway. Vesicles budding from the mitochondria have been found to carry selected proteins to target the peroxisomes or lysosomes (Neuspiel et al., 2008; Soubannier et al., 2012). These mitochondria-derived vesicles (MDVs) are coated with mitochondrial outer membrane protein Tom20, rather than autophagosome marker LC3, suggesting that they are not generated from an autophagy or mitophagy pathway. This pathway remains unaffected by silencing the mitochondrial fission Drp-1 or deleting the autophagy protein ATG5. Therefore, the mechanism functions independently of the quality control of the mitochondrial fission.

As mentioned above, one of the ways to repair a damaged mitochondrion is through fusion with a healthy mitochondrion, whereby mixing components from the two mitochondria replenishes the damaged part of the malfunctioning mitochondria. However, for those instances where the damaged part cannot be fixed by fusion, or for mitochondria that lose their ability to undergo fusion, the fission mechanism enables the chopping away of the dysfunctional part for lysosome-mediated degradation: autophagy. Mitochondrial fusion and fission rates determine the lengths of mitochondria. Both fusion and fission involve members of the Dynamin GTPase family. In mammals, Mitofusin-1 and Mitofusin-2 redundantly support the fusion of mitochondrial outer membranes (H. Chen et al., 2003), whereas Opa1 governs the fusion of mitochondrial inner membranes (Delettre et al., 2000). By contrast, mitochondrial fission in mammalian cells is mediated by a single Dynamin family protein−Drp-1 (Smirnova et al., 2001). In response to various types of stimulation, Drp-1 proteins are recruited to the mitochondrial outer membrane by interacting with its mitochondrial adaptors (such as Fis1, Mff and MIEF1) (Hoppins, Lackner, & Nunnari, 2007). During fission, these Drp-1 proteins are then self-assembled into spirals encircling the mitochondria (Smirnova et al., 2001). Recent live cell imaging studies have proposed that the Drp-1 ring divides the mitochondria by a twisting mechanism rather than by constriction (Rosenbloom et al., 2014).

The entire mitochondria can be removed by macro-autophagy and mitophagy. Macro-autophagy (generally referred to as autophagy) is a multi-step catabolic process in which cytoplasmic components and organelles are encapsulated by double-membrane vesicles called autophagosomes. Mature autophagosomes ultimately fuse with lysosomes for degradation of their contents by acidic hydrolases. Macro-autophagy non-selectively degrades mitochondria together with other cellular components. By contrast, damaged mitochondria can also be

removed by a more selective turnover mechanism called mitophagy. Mitophagy is triggered by signals from damaged mitochondria through Parkin-dependent or Parkin-independent pathways. In the Parkin-dependent pathway, the E3-ubiquitin ligase Parkin and mitochondrial serine/threonine-protein kinase Pink1 cooperatively work in a feed-forward amplification loop to regulate mitophagy (D. Narendra, Tanaka, Suen, & Youle, 2008; D. P. Narendra et al., 2010). In coupled mitochondria, PINK1 is constitutively imported into the inner mitochondrial membrane and degraded by Rhomboid protease PARL (Jin et al., 2010). In depolarized mitochondria, PTEN-induced putative kinase 1 (PINK1) accumulate on the outer mitochondrial membrane and activate Parkin, an E3-ubiquitin-protein ligase, by phosphorylating Parkin and ubiquitin (Kazlauskaite et al., 2014; Kondapalli et al., 2012). Phosphorylated Parkin ubiquitinates itself and other substrates of PINK1 (Shimura et al., 2000), leading to the amplification of signals for mitophagy (D. Narendra et al., 2008).

Although mitophagy has been shown to remove mitochondria that have lost membrane potential when Parkin is overexpressed in HeLa cells, in mice, deletion of PARK2, which encodes Parkin protein, did not alter mitochondrial membrane potential, evoke significant neurological dysfunction or result in a prominent cardiac phenotype (Damiano et al., 2014; D. Narendra et al., 2008; Perez & Palmiter, 2005). These data suggest the presence of Parkin-independent mitophagy pathways. In Parkin-independent mitophagy, damaged mitochondria recruit autophagy receptor proteins (such as NIX, BNIP3, FUNDC1, and Cardiolipin) to re-localize to the outer membrane, and then these receptors interact with LC3 and summon autophagosomes to remove the mitochondria (Bellot et al., 2009; Chu et al., 2013; Liu et al., 2012; Schweers et al., 2007; Zhang et al., 2008).

1.5.4 The regulation of mitochondrial homeostasis by AMPK and mTORC1

In meeting shifting energy needs, mitochondrial biogenesis and clearance are regulated by two protein complexes—5' AMP-activated protein kinase (AMPK) and mammalian target of rapamycin complex 1 (mTORC1)—which sense and integrate different stress signals to regulate mitochondria activity. Although both protein complexes have a role in stimulating mitochondrial biogenesis, they work under distinct cellular conditions. AMPK is activated under a variety of stress conditions, including nutrient-poor conditions, whereas mTORC1 is activated in the presence of growth factors and abundant nutrients (Shaw, 2009).

mTORC1 regulates mitochondrial biogenesis at both the transcriptional and translational levels. One the one hand, mTORC1 controls mitochondrial biogenesis through facilitating the formation of the transcriptional complex of yin-yang 1 (YY1) and peroxisome-proliferator-activated receptor coactivator-1α (PGC-1α) (Cunningham et al., 2007). The Raptor in mTORC1 serves as a docking site for YY1 (Cunningham et al., 2007). mTORC1 phosphorylates YY1 at T30 and S365, which enhances the interaction between YY1 and PGC-1α (Blattler, Cunningham, et al., 2012; Blattler, Verdeguer, et al., 2012) and thus stimulates the expression of their targeted mitochondrial genes.

One the other hand, mTORC1 mediates mitochondrial biogenesis through phosphorylating the eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BPs) and ribosomal protein S6 kinases (S6Ks). Phosphorylated 4E-BPs release eIF4E (Morita et al., 2013), enabling the assembling of eIF4F complex, which is composed of eIF4E, eIF4A, and eIF4G, at the 5’ cap of mRNAs (Sonenberg, Rupprecht, Hecht, & Shatkin, 1979; Tahara, Morgan, & Shatkin, 1981). Phosphorylated S6Ks then phosphorylate S6, eIF4B, and PDCD4 (Dorrello et al., 2006; Fenton & Gout, 2011; Shahbazian et al., 2006). The phosphorylated PDCD4s are marked for

degradation (Dorrello et al., 2006), releasing eIF4A for translation initiation (Suzuki et al., 2008). Taken together, mTORC1 facilitates the assembly of the translation initiation complex and several essential genes required for cell growth, cell proliferation and anabolic processes (Laplante & Sabatini, 2012).

At the first glimpse, AMPK and mTORC1 share some similar functions, as they both promote glucose uptake and mitochondrial biogenesis (through phosphorylating PGC-1α). However, AMPK is activated by hypoxia, oxidative stress, and nutrient starvation, which is the inverse pattern of mTORC1 (Cardaci et al., 2012). Therefore, unlike mTORC1, AMPK suppresses protein synthesis and stimulates autophagy to overcome the energy crisis. Indeed, AMPK can repress mTORC1 activity by directly phosphorylating regulatory-associated protein of mTOR (Raptor) and Tuberous Sclerosis Complex 2 (TSC2), an upstream inhibitor of the mTORC1 complex (Gwinn et al., 2008; Inoki et al., 2006).

AMPK activation favors autophagosome formation in multiple ways. First, AMPK represses mTORC1 directly by inhibiting Ratptor and indirectly by activating TSC2. Second, mTORC1 inhibits Unc-51-like-kinase1 (ULK1) (Hosokawa et al., 2009), which is an initiating kinase of autophagosomes, whereas AMPK activates ULK1 by phosphorylating its Ser^{317} and Ser^{777} (J. Kim, Kundu, Viollet, & Guan, 2011). Third, AMPK induces mitochondrial fission by phosphorylating the Drp-1 receptor MFF on Ser^{155} and Ser^{172}. These phosphorylations are required for MFF to recruit Drp-1 to the mitochondrial outer membrane and mediate mitochondrial fission (Toyama et al., 2016). Four, AMPK activates forkhead-box transcription factors, especially Foxo1 and Foxo3, by phosphorylation (Greer et al., 2007; Yun et al., 2014). Whereas AKT-mediated phosphorylation of the forkhead-box family of transcription factors prevents them from entering the nucleus (Brunet et al., 1999), AMPK-mediated phosphorylation

enhances the transcriptional activity of Foxo1 and Foxo3, which regulate numerous genes associated with autophagy and mitophagy. These genes include LC3b, Ulk2, BINP3, BINP3L, Atg5, Atg12, and Atg14 (Webb & Brunet, 2014). In sum, AMPK induces mitochondrial biogenesis and autophagy, and links mitochondrial fission with mitophagy to maintain cellular energy homeostasis and mitochondrial homeostasis.

1.5.5 Mitochondrial dynamics in memory and effector cells

In recent years, accumulating studies have pointed out that the remodeling of mitochondrial morphology and function is responsible for guiding lymphocytes to differentiate into effector cells or memory cells. For $CD8^+$ T cells, effector T cells ($CD44^{hi}$, $CD62L^{low}$) have punctate mitochondria, whereas memory T cells ($CD44^{hi}$, $CD62L^{hi}$) have a network of long and fused mitochondria. Optic atrophy 1 (Opa1) is a protein required for fusion of the mitochondrial inner membrane (Olichon et al., 2002). Opa1 knockout T cells have a survival defect and cannot form memory T cells after adoptive transfer into recipient animals (Buck et al., 2016). Moreover, activated T cells have elevated mitochondria ROS production, which is a message to activate nuclear factor of activated T cells (NFAT)-mediated IL-2 production (Sena et al., 2013).

For B cells, activated B cells increase mitochondrial mass and membrane potential, but when they gradually differentiate toward plasma cells, mitochondrial mass and membrane potential decrease. Moreover, activated B cells with higher ROS (staining by CM-H2DCFDA) tend to undergo class-switch recombination, whereas those that have lower ROS tend to differentiate into plasma cells (Jang et al., 2015).

Finally, the persistence of long-lived natural killer cells requires BNIP3- and BNIP3L-mediated mitophagy. The number and frequency of long-lived natural killer cells can be increased by treating them with rapamycin to inhibit mTOR, or by treating them with metformin

to activate AMPK (T. E. O'Sullivan et al., 2015). Taken together, in lymphocytes, including B cells, T cells, and NK cells, activation and effector function are associated with increased mitochondrial mass, membrane potential, and ROS production, whereas memory function is associated with reduced mitochondrial mass, membrane potential, and ROS production.

In this study, I uncover a more detailed molecular mechanism linking mitochondrial status to the transcriptional program in both B cells and T cells. I demonstrate that the mitochondrial status is sensed by AMPK and signaled through mitochondrial-derived ROS to alter the expression of Pax5 and Tcf7 in B cells and T cells, respectively.

1.5.6 Mitochondrial stasis is associated with stem cell identity, self-renewal, and fate decisions

Similar to the observations made in memory and effector lymphocytes, stem cells and committed progenitors have been found to display different mitochondrial morphology. During neurogenesis in embryos, neural stem cells with self-renewal ability exhibit relatively elongated mitochondrial networks, whereas differentiated neural progenitor cells have relatively fragmented mitochondrial networks. Silencing mitochondrial inner membrane fusion protein OPA1 in $Sox2^{+}$ uncommitted neural stem cells reduced the ability to form neurospheres in vitro and increased the frequency of commitment to nerve cells. Knockdown of mitochondrial outer membrane fusion proteins Mfn1 and Mfn2 in uncommitted cells results in similar defects in neurosphere formation. Knockdown of these mitochondrial genes results in increased mitochondrial ROS and cytoplasmic ROS as measured by MitoSox and dihydroethidium fluorescence, respectively (Khacho et al., 2016).

Mitochondrial stasis not only plays a role in guiding the differentiation of neural stem cells

during embryonic development but is also required for preventing senescence of the adult quiescent stem cells. In muscle tissues, defective autophagy causes stem cell senescence through the ROS-induced $p16^{INK4a}$ axis, and thus declined autophagy function has been linked to the impaired muscle stem cells' (satellite cells') regenerative capacity in aged mice and humans (Garcia-Prat et al., 2016). Inhibiting autophagy in young cells by bafilomycin leads to mitochondrial accumulation and an increase in ROS. By contrast, inhibiting ROS with Trolox or stimulating autophagy with rapamycin prevents the senescence phenotype and rescues the proliferative and regenerative abilities of geriatric satellite cells.

In addition to maintain the normal stem cell populations, mitochondrial renewal is associated with the stem-like cells in breast cancer. Using Snap-tag chemistry or photoactivatable green fluorescent protein to monitor the dynamics of mitochondrial apportioning in dividing stem-like cells showed that one of the daughter cells received more old mitochondria than the other. The daughter cells receiving less old mitochondria exhibit superior mammosphere-forming capacity compared with those cells accumulating more old mitochondria (Katajisto et al., 2015).

Collectively, these experiments point out the importance of maintaining mitochondrial quality and dynamics in preserving the self-renewal ability and potential to differentiate in stem cells and stem-like cells. Memory lymphocytes with self-renewal capacity are thought to be the fundamental population to establish long-term immunological memory. They remain quiescent until being re-challenged by antigen and differentiating into effector cells. In this study, I identify that lymphocytes may apply mitochondrial quality control mechanisms to ensure that a limited number of activated lymphocytes can continue expansion and differentiation, after stimulation by antigen.

1.6 Glycolysis regulates lymphocyte functions

1.6.1 Metabolism and lymphocyte functions

How does the reprogramming of metabolic pathways in diverse immune cell types shape the immune response? Different metabolic pathways are required to provide energy for growth, proliferation, survival, and executing immune functions. For example, inflammatory and rapidly proliferating immune cells are often dependant on aerobic glycolysis, whereas regulatory T cells, memory T cells, and many immune cells that exhibit longer lifespans tend to rely on fatty acid oxidation. Indeed, after activation, enhanced glycolysis can be observed in both adaptive and innate immune cells, including B cells, effector T cells, natural killer cells, macrophages and dendritic cells (O'Neill, Kishton, & Rathmell, 2016b).

Although aerobic glycolysis is not the most efficient way to generate ATP, it can produce NADH, which can serve as a cofactor for many enzymes. The biosynthetic intermediates in glycolysis also supply materials to support rapid cell growth. Similarly, the intermediates of the TCA cycle can provide UDP-GlcNAc for the glycosylation of cell surface receptors and secretory proteins. Moreover, many metabolic enzymes play a second role in addition to metabolism to regulate cellular functions, thus linking metabolism with immune function. For example, GAPDH suppresses the translation of IFN-gamma mRNA when it is not involved in glycolysis (C. H. Chang et al., 2013). Also, HK1 binds to mitochondrial VDAC1, which is required for activation of NLRP3 inflammasome in macrophages (J. S. Moon et al., 2015).

In addition, several critical transcription factors involved in immune function also regulate a variety of metabolic pathways, such as Foxo1 and HIF-1 alpha (Tannahill et al., 2013). Taken together, the metabolic program is not just downstream to the transcriptional networks. Metabolic program can feedback to reprogram the transcriptional networks in different immune

cell types and in response to different stimuli.

1.6.2 Autophagy, mTOR pathway, and memory cells

In 2009, Koichi Araki et. al. demonstrated that in mice infected with acute lymphocytic choriomeningitis virus, the number of virus-specific $CD8^+$ T cells could be enhanced by treatment with rapamycin, which is an immune suppressive drug that inhibits T cell activation and proliferation. Using retrovirus based RNA interference (RNAi), the authors found that knockdown of Raptor, which is part of the mTORC1 complex, also enhanced memory T cell formation, suggesting rapamycin regulates memory cell differentiation through inhibiting the function of the mTOCR1 complex (Araki et al., 2009).

How is mTORC1 function linked to maintaining the number of memory cells? mTOR has been found to negatively regulate autophagy, which is an adaptive mechanism for recycling cellular components to provide amino acids during nutrient depletion and starvation. mTOR phosphorylates ULK1 and Atg13, an essential protein for autophagosome formation, under nutrient-enriched conditions (Hosokawa et al., 2009). Starvation and rapamycin treatments lead to dephosphorylation of ULK1, and thus the kinase activity of ULK1 is activated. Activated ULK1 initiates autophagosome formation by phosphorylating Atg13 and FIP200 (Ganley et al., 2009; Jung et al., 2009). Conditionally knocking out either of the autophagy-related molecules Atg5 or Atg7 by Gzmb-Cre impairs the survival of virus-specific T cells during the contraction phase after LCMV Armstrong strain infection and prevents the formation of long-lived memory T cells (X. Xu et al., 2014). Metabolite analysis reveals that lipid and glycan-biosynthesis pathways ae altered in the Atg7 knockout T cells (X. Xu et al., 2014). A similar phenotype had been reported in transgenic mice in which tumour necrosis factor (TNF) receptor-associated

factor 6 (TRAF6) is conditionally knocked out in T cells. In these animals, effector T cell function remains intact, but the formation of memory T cells is abrogated. TRAF6-deficient $CD8^+$ T cells are found to have defective fatty acid metabolism (E. L. Pearce et al., 2009).

A later study found that memory T cells do not directly import long chain fatty acids, but uptake glucose to support de novo synthesis of fatty acids and use lysosomal acid lipase to provide substrates for fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS). Collectively, these experiments suggest that autophagy may be required for fueling mitochondrial FAO in memory T cells to supply the energy required for survival (D. O'Sullivan et al., 2014).

1.6.3 Glycolysis and effector function of T cells

The requirement of glycolysis to switch and to modulate the expression of glucose transporter has been reported in several studies. The mRNA of glucose transporter 1, 3, 6, and 8 are detectable in Th1, Th2, Th17, and Treg populations (Macintyre et al., 2014). In all of the cell types, Glut1 is the predominant glucose transporter. In naïve T cells, Glut1 and Glut3 are highly expressed. In T cells stimulated by anti-CD3 and anti-CD28 in vitro, the expression of Glut1 increases as stimulation time is prolonged, whereas the expression of Glut3 gradually decreases. The effects of conditionally knocking out Glut1 in $CD4^+$ and $CD8^+$ T cells have also been reported (Macintyre et al., 2014). Deletion of the loxP-flanked Glut1 alleles in early double-negative (DN) thymocytes by Lck-Cre results in a dramatic reduction of the total number of thymocytes to only 30% of that in a wild-type animal. This is because of the reduction of CD4 CD8 double-positive and CD4 single-positive cells, but not double negative cells. By contrast, deletion of the loxP-flanked Glut1 alleles in the DP-to-SP transition by CD4-Cre did not

significantly alter the number of thymocytes, suggesting that Glut1 is dispensable for the survival of the single positive thymocytes. Although the number of $CD4^+$ and $CD8^+$ T cells in the spleen and inguinal lymph nodes in CD4-Cre $Glut1^{fl/fl}$ mice remains similar to that of wild-type mice, the ability of Glut1-knockout $CD8^+$ and $CD4^+$ T cells to homeostaticly proliferate is reduced in in irradiated and lymphopenic recipients. For $CD8^+$ T cells, the effect of Glut1 deletion is less obvious. Although in vitro stimulated CD4 Cre-$Glut1^{fl/fl}$ $CD8^+$ T cells divide more slowly than wild-type $CD8^+$ T cells, the effector function of $CD8^+$ T cells to express and release granzyme B, IL-2, tumor necrosis factor alpha, and interferon gamma (IFNγ) remains unaffected by Glut1 deletion, implying that the function of Glut1 can be compensated by over-expression of other glucose transporters. Indeed, western blot and proteomic data reveal that Glut1 knockout $CD8^+$ T cells may up-regulate Glut3 and Glut6 expression (Hukelmann et al., 2016; Macintyre et al., 2014). For $CD4^+$ T cells, knocking out Glut1 not only affects the differentiation into Th1 and Th17 subsets but also impairs the proliferation of effector $CD4^+$ T cells in vitro and in vivo. Interestingly, the differentiation and in vivo functions of Tregs are less affected by Glut1 deletion, suggesting they may adopt alternative metabolic pathways (Macintyre et al., 2014).

1.6.4 The role of glycolysis in B cells and plasma cells

The phenotype of lost Glut1 in B cells is similar to that of T cells. The number of peripheral B cells is reduced in CD19-Cre $Glut1^{fl/fl}$ mice because of the reduction of mature B cells ($IgM^{hi}B220^+$). However, the frequency of immature B cells ($IgM^{low}B220^+$) in CD19-Cre $Glut1^{fl/fl}$ mice is increased, suggesting the presence of a developmental problem. The effector function of B cells is also affected by Glut1 deletion. The steady state serum IgM levels as well as the levels of serum IgM and IgG induced by immunization with NP-ovalbumin are dramatically reduced in

CD19-Cre Glut1$^{fl/fl}$ mice (Caro-Maldonado et al., 2014).

Another study focused on long-lived plasma cells (LLPCs) demonstrated an additional layer function of glucose metabolism for maintenance of the memory and function of antibody secretin plasma cells. The data showed that LLPCs constitutively import Glucose to generate pyruvate for mitochondrial energy production and to glycosylate antibodies (Lam et al., 2016).

1.6.5 HK2, AKT, and mTORC1

Hexokinase catalyzes the first reaction in glycolysis. It phosphorylates glucose and produces glucose-6-phosphate (G6P), which is the rate-limiting step in glycolysis. There are 4 hexokinase isozymes (hexokinase I to IV) encoded by the mammalian genome (Roberts & Miyamoto, 2015). The expressions of these isozymes are tissue specific. Among them, hexokinase II (HK2) is the dominate isozyme expressed in insulin-sensitive tissues and proliferating cancer cells (J. Chen, Zhang, Li, Tang, & Kong, 2014; Heikkinen et al., 2000).

In most cell types, such as in skeletal and cardiac muscle cells, the activity of HK2 is regulated by the PI3K/AKT/mTORC1 pathway from multiple ways. First, AKT and mTORC1 are positive transcriptional regulators of HK2 (Osawa, Sutherland, Robey, Printz, & Granner, 1996; Printz et al., 1993). Because HK2 is a constitutively activated enzyme, the expression level of HK2 determines its cellular activity. Second, AKT directly phosphorylates HK2 and targets HK2 to the voltage-dependent anion channels on the mitochondrial outer membrane (Nakashima, Mangan, Colombini, & Pedersen, 1986; Roberts, Tan-Sah, Smith, & Miyamoto, 2013). Therefore, HK2 can use the ATP produced by mitochondria for powering the energy donation steps in glycolysis (Arora & Pedersen, 1988). Third, AKT-mediated phosphorylation also lowers the sensitivity of HK2 to the feedback inhibition of its product G6P (Ardehali et al., 1996;

Roberts et al., 2013). G6P not only negatively regulates the activity of HK2 but also dissociates HK-II from mitochondria (Roberts et al., 2013; Rose & Warms, 1967). Finally, inhibition of mTORC1 activity with rapamycin has been shown to enhance the expression of micro-RNA 143, suggesting mTORC1 stabilizes HK2 mRNA by suppressing the micro-RNA that targets the HK2 mRNA for degradation (Fang et al., 2012).

On the other hand, HK2 also regulates mTORC1 activity through protein-protein interaction. In addition to its enzyme activity, HK2 has been shown to stimulate autophagy in a glucose-limited environment (Figure 1.2). Under glucose deprivation conditions, HK2 uses its TOS motif (TOR signaling motif) to directly bind to Raptor in mTORC1 and thus inhibits mTORC1-mediated repression of autophagy (Roberts, Tan-Sah, Ding, Smith, & Miyamoto, 2014; Schalm & Blenis, 2002).

1.6.6 The regulation of PI3K on glucose transporter Glut1

During the development, differentiation, and activation of lymphocytes, PI3K integrates activation signals from a wide range of receptors, such as BCR and CD19 on B cells, TCR and C28 on T cells, and different cytokine receptors, including IR2-R, IR7-R. One of the important consequences of PI3K activation is to reprogram the metabolic status of the cell, such as by increasing glucose transporters on the cell membrane, expressing glycolytic genes, and activating anti-apoptotic pathways. Several studies have proposed a role of PI3K in regulating glucose transporter 1 in B cells and T cells by using PI3K inhibitor LY294002. The first B cell paper describing PI3K and surface Glut1 was published in 2006 (Doughty et al., 2006). The authors found that anti-Ig–induced BCR engagement increased surface Glut1 expression and uptake of 2-[^{3}H]deoxyglucose, which can be compromised by LY294002 inhibitor or knockout of the PI3K

p85α subunit. A later study used the lymphoid cell line FL5.12 to study IL-3-stimulated up-regulation of surface Glut1 (Wieman, Wofford, & Rathmell, 2007). The authors showed that expression of a myristoylated and constitutively active oncogenic form of Akt is sufficient to maintain the level of surface Glut1 even in the absence of IL-3 stimulation. Moreover, expression of dominant-negative Rab11a, a Rab GTPase involved in endosomal recycling, leads to the accumulation of intracellular FLAG-Glut1 but reduces surface FLAG-Glut1. In sum, these results imply that the PI3/AKT pathway increases surface Glut1 levels through modulating the recycling of internalized Glut1-containing endosomes (Figure 1.2).

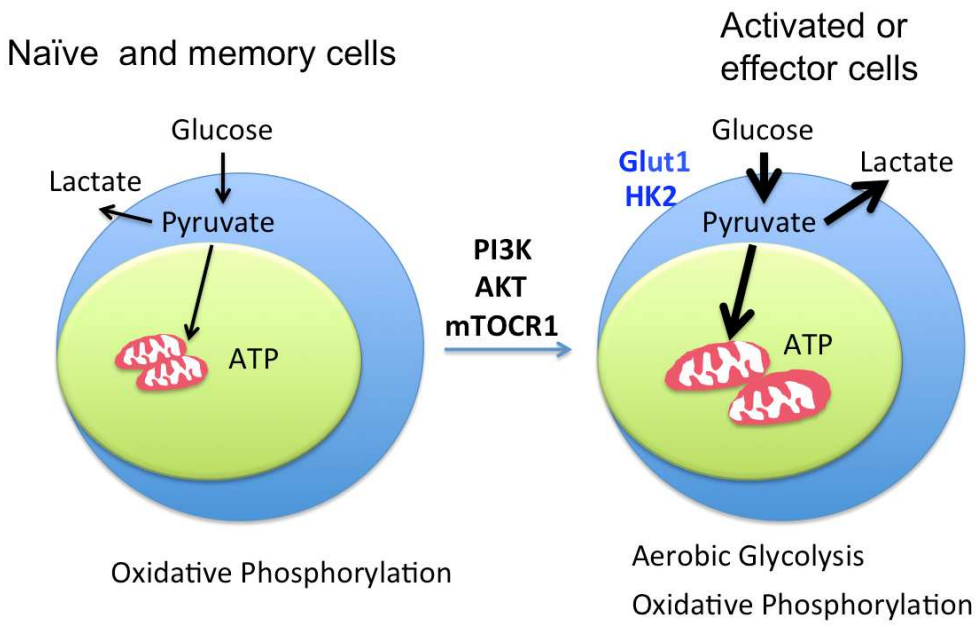


Figure 1.2 Metabolic re-programming during lymphocyte activation

Naïve and memory lymphocytes are more dependent on oxidative phosphorylation than activated or effector cells. When naïve and memory lymphocytes are activated by antigen, they up-regulate glucose transporter (Glut1), and hexokinase 2 (HK2), leading to higher aerobic glycolysis and higher oxidative phosphorylation rates.

1.6.7 Lis1 and dynein dependent vesicle transportation

Lis1 forms a complex with NUDE and NUDEL and binds the head domain of dynein heavy chain (most of the dyneins are microtubule minus-end motor protein) (Kardon & Vale, 2009; Yamada et al., 2008). There are four reported functions of Lis1. First, at the kinetochores and microtubule plus ends, Lis1 forms a complex with NDEL1 and dynein. These complexes involve the apical movement of nerve cells and are required for proper mitotic spindle organization and orientation in mitotic cells (H. M. Moon et al., 2014; Shu et al., 2004; Tai, Dujardin, Faulkner, & Vallee, 2002). Similarly, in developing mouse embryos, Lis1 modulates centriolar microtubule organization and directs microtubule-associated Tiam1 to the cell cortex, which leads to local activation of the Rac-PAK signaling pathway. The activation of Rac-PAK establishes a polarity cue to guide the position of the basal body (Sipe, Liu, Lee, Grimsley-Myers, & Lu, 2013). Third, in postnatal hair cells, Lis1 facilitates centrosome positioning by coordinate cortical dynein and pericentriolar matrix organization to generate tension on centriolar microtubules (Sipe et al., 2013). Finally, the interplay of Lis1, Nudel, and dynein affects the speed of minus end–directed cargo including lysosomes and is required for maintaining the integrity of Golgi cisternae (Liang et al., 2004).

1.6.8 Bifurcating nutrient signaling and cell fate

Our hypothesis is that a lymphocyte competent to undergo differentiation unequally licenses its daughter cells to utilize nutrients, resulting in a bifurcation of nutritive signaling, transcriptional network, and cell fate (Adams et al., 2016). In one of the daughter cells, PI3K signaling inactivates FoxO1 by AKT-mediated phosphorylation on FoxO1 (W. H. Lin et al.,

2015). PI3K and the mTOR pathway increase the transcription of glucose transporter 1 (Glut1) and the expression of IRF4 and c-Myc. PI3K/AKT and mTOR repress autophagy, which facilitates mitochondrial stasis. Accumulation of aged mitochondria and up-regulated aerobic glycolysis synergistically increase mitochondrial ROS production (Adams et al., 2016). The loss of FoxO1 protection and the augmentation of IRF4 function contribute to the repression of Pax5 or Tcf1 in one of the sibling B cells or T cells. On the other hand, one of the daughter cells has relatively lower PI3K activity and thus has higher AMPK activity. AMPK supports the translocation of FoxO1 into the nucleus and inhibits the activity of mTOCR1 (Macintyre et al., 2014). Inhibiting mTOR stimulates autophagy, but limits glucose uptake, and represses IRF4 and c-Myc. This results in lower glycolysis, whereas OXPHOS and FAO both increase. Therefore, this daughter cell can more efficiently renew mitochondria and produce less mitochondrial ROS. Altogether, higher FoxO1 and AMPK activity and lower ROS level allow the daughter cell to maintain Pax5 in B cells and Tcf1 in T cells (Figure 1.3).

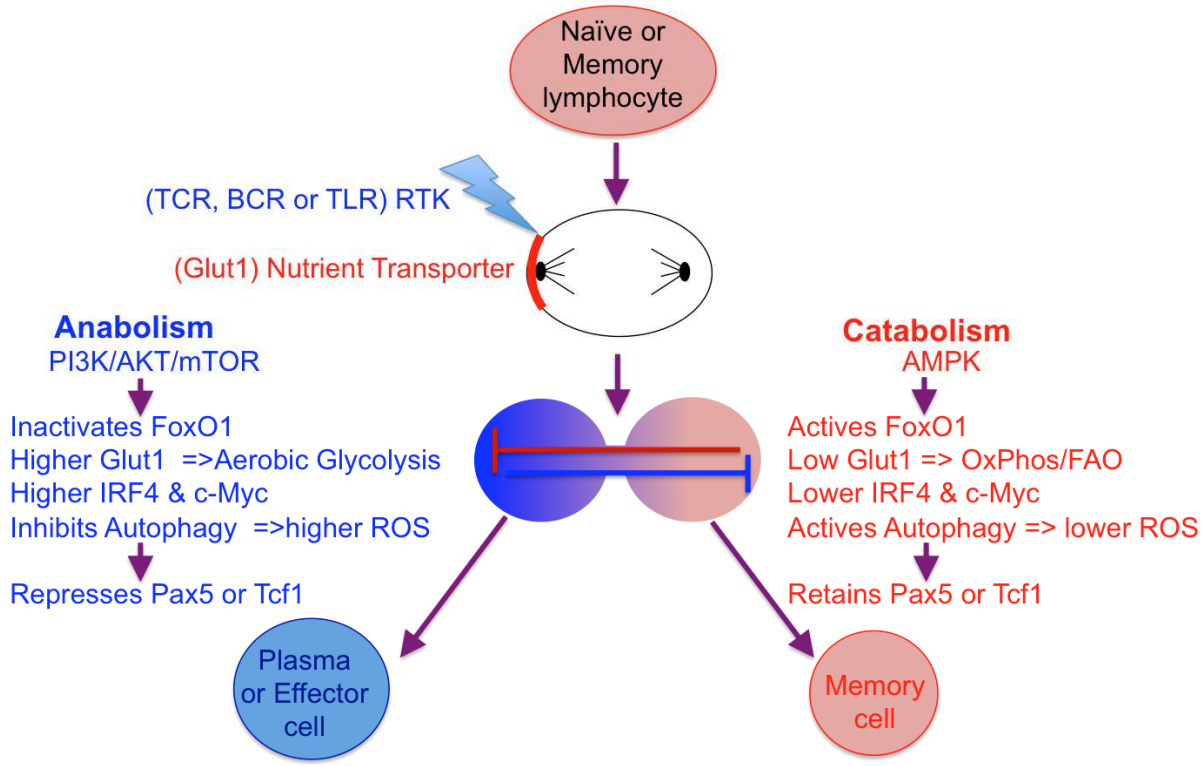


Figure 1.3 Bifurcating nutrient signaling and cell fate

We proposed a model for asymmetric metabolism by sibling lymphocytes coupling differentiation and self-renewal. In one of the daughter cells, PI3K/AKT activates mTOR, glycolysis, mitostasis, and elevated ROS, but inactivates FoxO1. Glycolytic flux and ROS are involved in a feedback loop that enforces the anabolic constellation and prepares the cells for rapid proliferation. Conversely, in the other sibling cell, inhibiting glycolytic flux activates AMPK, which shuts down glycolytic pathway genes and helps maintain mitoclearance and FoxO1 activity. These interrogating feed-forward and feed-back regulations and mutual inhibition of other states help to bifurcate nutrient signaling in the two sibling cells and diversify their cell fates.

Chapter 2: Materials and methods

2.1 Mice

All animal work was conducted in accordance with Institutional Animal Care and Use Guidelines of Columbia University. Mice were housed in specific-pathogen-free conditions. C57BL/6 mice, Pax5-IRES-hCD2 reporter mice (Fuxa & Busslinger, 2007), P14 TCR transgenic (P14) mice recognizing LCMV peptide gp33-41/Db, C57BL/6 OTII mice expressing an I-Ab-restricted TCR specific for ovalbumin amino acids 323-339, AMPKα1 knockout mice (Jorgensen et al., 2004) (Prkaa1tm1Vio), and IRF4 knockout mice (Klein et al., 2006) have been previously described. 8-12 week old mice were used for most experiments. Myc-tagged-Glut1 mice were from Dr. Jeffery Rathmell (Young et al., 2011), whereas $Glut1^{fl/fl}$ mice were from Dr. Gerard Karsenty (Wei et al., 2015).

2.2 Cell culture and lymphocyte activation

Spleens from mice were isolated and processed using established protocols. B cells and $CD8^+$ T cells were enriched from spleens by magnetic bead negative selection with the MACS B cell isolation kit (Miltenyi) and the MACS $CD8^+$ T cell isolation kit (Miltenyi), respectively. B cells (5×10^5 cells per well in a 48-well plate) were cultured in Iscove's (Corning) complete media, unless specified otherwise, with lipopolysaccharide (LPS) (20 μg/mL; InvivoGen) for up to three days. Enriched $CD8^+$ T cells (5×10^5 cells per well) were cultured in Iscove's complete media containing human recombinant IL-2 (100 units/mL) in 48 well plates coated with anti-CD3/anti-CD28 antibodies (1 μg/mL; Bio X Cell). For stimulation of $CD8^+$ T cells from P14 mice, single cell suspensions from whole spleens were prepared and cultured in Iscove's complete media,

unless specified otherwise, containing gp33-41 peptide (1μg/mL) plus IL-2 (100 units/mL). For glucose deprivation experiments, B cells or P14 $CD8^+$ T cells were cultured in glucose-free/2mM glutamine replete RPMI (Gibco) supplemented with Fetal Calf Serum (dialyzed overnight with SnakeSkin dialysis tubing 10K MWCO; Thermo Fisher) and where specified 10 mM D-(+)-glucose (Sigma) or 10 mM D-(+)-galactose (Sigma). The following compounds were used in cell culture experiments: mDivi-1 (Cayman), Chloroquine (Enzo), Metformin (AMPK activator; Calbiochem), Rapamycin (mTOR inhibitor; Selleckchem), N-Acetyl-L-cysteine (NAC; Sigma), 2-Deoxy-D-glucose (2-DG; Sigma), LY294002 (PI3K inhibitor; Cell Signaling), Triciribine (AKT inhibitor; Cayman), AS1842856 (FoxO1 inhibitor; Calbiochem), and Oligomycin (Sigma).

2.3 Retroviral transduction

Enriched B cells or $CD8^+$ T cells were stimulated with LPS or plate-bound anti-CD3/anti-CD28 antibodies plus IL-2, respectively, for 36 hours prior to retroviral transduction. Cells were then re-plated in supernatant collected prior to transduction. Retroviral constructs used were as follows: mCherry-alpha-tubulin fusion protein (Day et al., 2009); mouse dominant negative Drp-1 (K38A), a gift from David Chan (Addgene plasmid # 26049) that we subsequently cloned into the MSCV-IRES-Thy1.1 plasmid, a gift from Anjana Rao (Addgene plasmid # 17442); and hexokinase 2, made by subcloning the rat hexokinase 2 coding sequence into an MSCV-IRES-GFP vector. Empty MSCV-IRES- Thy1.1 or MSCV-IRES-GFP vectors were used as controls. The pMIG-IRES-truncated human NGFR and its derivative exofacial-FLAG epitope tagged rat Glut1 were gifts from Dr. J. Rathmell (Wieman et al., 2007); GFP-wild-type Rab11a and GFP-dominant negative Rab11a (S25N) were made by subcloning human Rab11a (Addgene plasmid #s 12674 and 12678, respectively) (Choudhury et al., 2002) into the

MSCV-IRES-Thy1.1 vector; and Far Red-tubulin by subcloning pmiRFP703-Tubulin (Addgene plasmid # 79991) into the MSCV-IRES-Thy1.1 vector. Empty MSCV IRES- Thy1.1 or MSCV-IRES-truncated NGFR (Addgene plasmid # 27489) vectors were used as controls.

2.4 Adoptive Transfers and Infectious Challenges

Resting $CD8^+$ T cells from P14 mice ($Thy1.1^+$) were purified with the $CD8^+$ T Cell Isolation Kit (Miltenyi Biotec). 3×10^6 $CD8^+$ T cells and adoptively transferred intravenously (i.v.) into wild-type congenic C57BL/6 mice ($Thy1.2^+$). To generate acutely resolved systemic infections, mice were infected with 5×10^3 PFUs of *Listeria monocytogenes* expressing gp33-41 (LMgp33) by intravenous injection. The animals were sacrificed by day 4 post-infection for sorting of $CD8^+$ $Thy1.2^-$ cells for immunofluorescence.

For surface Glut1 level experiments, P14 Myc-Tagged Glut1 transgenic mice were infected with 2×10^5 plaque-forming units (PFUs) of LCMV, Armstrong strain, by intraperitoneal (i.p.) injection. The animals were sacrificed by day 7 post-infection for FACS analysis.

2.5 Flow cytometry

For detection of intranuclear/intracellular antigens, cells were initially washed in PBS and stained by antibody against selected surface antigens and by LIVE/DEAD Fixable Green or Red Dead Cell Stain Kit (Thermo Fisher) and then treated with the Transcription Factor Buffer Staining Set (eBioscience) according to the manufacturer's instructions. Antibodies specific for the following antigens were used: Pax5 (clone 1H9 conjugated to Alexa Fluor 488 or PerCP-Cy5.5 from Biolegend or APC from eBioscience; 1:200), IRF4 (clone 3E4 conjugated to PE or eFluor660 from eBioscience; 1:100), TCF1 (clone C63D9 conjugated to Alexa Fluor 647 from

Cell Signaling; 1:100), GFP (clone 9F9.F9 conjugated to Alexa Fluor 488 from Sigma; 1:100), and Thy-1.1 (clone OX-7 conjugated to PerCP-Cy5.5 from Biolegend; 1:200). BD LSRII, Fortessa, and FACSAria II flow cytometers with FACSDiva software were used to analyze and/or purify cells. Flow cytometry data was analyzed using FlowJo software (versions 8 and 10).

2.6 Other dye staining protocols

Mitochondrial membrane potential was measured by incubating cells with MitoTracker® Red CMXRos (Cell Signaling) (100nM for flow cytometry; 200nM for confocal microscopy) for 15 to 20 minutes in the culture medium at 37 degrees Celsius. Autophagic organelles were labeled with CYTO-ID Autophagy Detection Kit (Enzo; 1:1000 dilution of stock) according to the manufacturer's instructions. Reactive Oxygen Species (ROS) were detected by incubating cells with MitoSOX Red Mitochondrial Superoxide Indicator (Thermo Fisher; 5 μM) for 15 minutes at 37 °C in media or CM-H2DCFDA (Thermo Fisher; 5 μM) for 30 minutes at 37 °C in media. Glucose uptake was measured by incubating cells in 2-NBDG (Cayman; 100 μM) for 45 minutes at 37 °C in glucose-free media.

2.7 Confocal microscopy

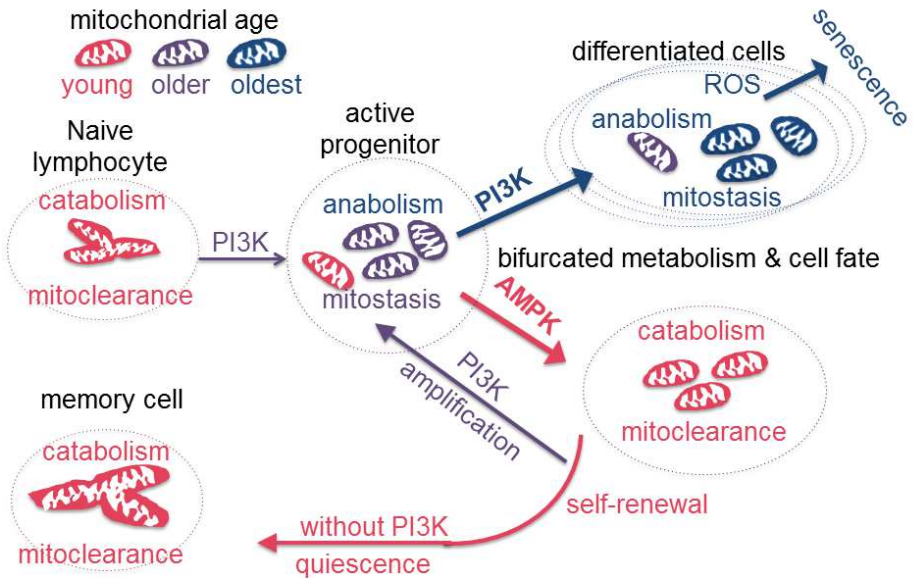
Confocal microcopy was performed as described (J. T. Chang et al., 2011; W. H. Lin et al., 2015). Using transmitted light morphology, tubulin bridge staining, and DNA staining, specific identification of telophase or cytokinetic sibling pairs with exclusion of any spurious neighboring-but-unrelated cell pairs is achieved (Lin et al., 2015). For fixed cell imaging, cells were adhered to poly-l-lysine (Sigma) coated coverslips (#1.5 Thermo Fisher) and treated with freshly prepared 3.7 % PFA (Sigma) diluted in cell culture media (pH 6.8) in order to preserve

mitochondrial morphology and MitoTracker Red FM fluorescence intensity. Following brief treatment with 0.1% Triton, cells were stained with antibody against α-Tubulin (clone YOL1/34 Abcam; 1:300) and then anti-rabbit antibody conjugated to Alexa Fluor 568 (Thermo Fisher) in a PBS solution containing 0.01 % saponin and 0.25 % fish skin gelatin (Sigma). For live cell imaging, cells were transferred to poly-l-lysine coated 8 chambered coverglass wells (#1 LabTek) in warm cell culture media and allowed to adhere briefly before imaging. Images were acquired on inverted confocal microscopes (Zeiss LSM710 or Nikon Ti Eclipse) and processed using ImageJ (v.1.46r) or Fiji (v.2.0) software to project z stacks, apply a minimal smooth filter for some displayed images, rotate, change pseudocolors, convert to RGB, and add scale bars or time scales. Total fluorescence in defined regions of single cells was quantitated using the integrated density function in ImageJ.

2.8 Statistical Analyses

Significance between experimental groups was determined using two-tailed t tests or two-tailed ratio t tests for paired data, two-tailed Mann-Whitney test for unpaired data, or repeated-measures one-way ANOVA. Two-tailed Fisher's exact test was used to determine significance for contingency statistics of microscopy data comparing asymmetric versus symmetric fluorescence intensity of molecules between sibling pairs. Statistical analyses and summary statistics to determine means and SDs were carried out using GraphPad Prism software v. 7 ($*p < 0.05$, $**p < 0.01$, and $***p < 0.005$).

Chapter 3: Asymmetric mitochondrial stasis in daughter cells guides the differentiation of effector cells and maintenance of progenitor cells



The results in this chapter have been published in a co-first author paper:

Anabolism-Associated Mitochondrial Stasis Driving Lymphocyte Differentiation over Self-Renewal.

Adams WC, Chen YH, Kratchmarov R, Yen B, Nish SA, Lin WW, Rothman NJ, Luchsinger LL, Klein U, Busslinger M, Rathmell JC, Snoeck HW, Reiner SL.

Cell Rep. 2016 Dec 20;17(12):3142-3152. doi: 10.1016/j.celrep.2016.11.065.

3.1 Pax5 repression represents an irreversible commitment to differentiation

After immunization, Pax5-positive B cells undergo clonal selection, and generate two exclusive cell populations: Pax5 high B cells and terminally differentiated, Pax5–silenced plasma cells. Using cells from Pax5-IRES-human CD2 reporter mice (Fuxa & Busslinger, 2007), we performed a re-plating assay to compare the ability of hCD2 $(Pax5)^{hi}$ and hCD2 $(Pax5)^{low}$ cells to self-renew and differentiate. Splenic B cells labeled with cell proliferation dye were activated with LPS. In cells having undergone several rounds of division, $hCD2^{hi}$ and $hCD2^{low}$ cells were sorted by flow cytometry and re-plated in LPS-containing media. The results showed that hCD2 $(Pax5)^{hi}$ cells are capable of generating both $Pax5^{hi}$ progeny (self-renewal) and of giving rise to $Pax5^{low}$ progeny, whereas Pax5$(hCD2)^{low}$ cells are unable to regain Pax5 expression and thus are only capable of giving rise to $Pax5^{low}$ progeny (Figure 3.1). These results suggest the Pax5 population, or a subset of this population, has stem-cell like activity: self-renewal and differentiation.

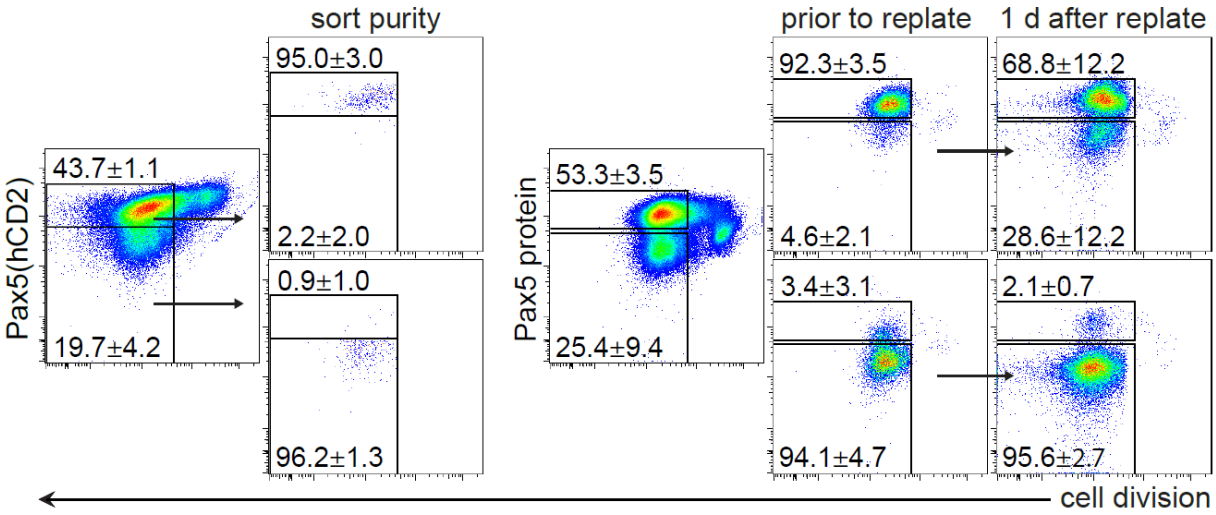


Figure 3.1 Pax5 repression represents an irreversible commitment to differentiation

(Left) LPS-activated, Pax5 (hCD2) reporter B cells were sorted by flow cytometry from later cell generations based on surface hCD2 expression. Post-sort purity of live cells is shown.

(Right) Pre-sort Pax5 protein staining of fixed cells next to Pax5 protein staining of sorted cells, followed by Pax5 protein staining of sorted populations one day after re-plating in fresh media containing LPS. Plots depict one representative experiment and numbers are summary statistics (mean ± SD, n=5) for the frequency of cells within the corresponding gate.

3.2 Pax5 repression is accompanied by reduced mitochondrial membrane potential

A successful immune response depends on the generation of effector and memory lymphocytes to provide disease-fighting cells as well as immunological memory. However, the detailed mechanism by which the balance of these two populations is maintained remains unclear. Our laboratory's earlier work supports a stem-cell model of clonal selection. That is, after antigen activation, an activated lymphocyte (such as a GC B cells or an activated memory cell) can generate differentiated effector and self-renewal progenitors at the same time through asymmetric cell division. Our earlier work has found that in dividing B cells and T cells, the two daughter cells asymmetrically express the lineage-maintaining transcription factors Pax5 and Tcf1, respectively (W. H. Lin et al., 2015). However, the detailed mechanism by which the polarized signal is provided remains unclear.

Effector and memory lymphocytes have been found to adopt distinct metabolic preferences. Also, mitochondrial dynamics and functional statuses have been linked to the differentiation of plasma cells. I hypothesis that asymmetric mitochondrial functional status in the two daughter cells provides guiding signals for establishing the asymmetric transcriptional program. To understand whether transcription programming is couple to mitochondrial status, I performed FACS analysis combining cell proliferation dye MitoTracker Red CMXRos (Mito-CMX), IRF4 or Pax5 specific antibodies, and mitochondrial membrane potential-dependent dye. To demonstrate that the staining of Mito-CMX is dependent on mitochondrial membrane potential, I performed Mito-CMX staining on activated B cells incubated with various concentrations of mitochondrial membrane potential uncoupler, carbonyl cyanide m-chlorophenyl hydrazone (CCCP). FACS analysis showed that Mito-CMX staining is inversely correlated with the concentration of CCCP in the media (Figure 3.2A), suggesting that the staining of Mito-CMX is

positively associated with mitochondrial membrane potential. When combining Mito-CMX staining with Pax5 and IRF4 staining, we found that the IRF4^{hi} population has relatively lower Mito-CMX staining, whereas the IRF4 intermediate population has higher Mito-CMX staining (Figure 3.2 B Left). Moreover, Mito-CMX high and IRF4 intermediate B cells are mainly Pax5 high, whereas Mito-CMX low and IRF4 high cells are Pax5 low (Figure 3.2 B Right). Taken together, these results suggest that Pax5 high, IRF4 intermediate subsets have relatively higher mitochondrial membrane potential, whereas more differentiated plasma blasts (Pax5 low, IRF4 high) exhibit reduced mitochondrial membrane potential. Gating on the number of cells division, we found that after B cells are activated, Pax5 is up-regulated together with increased mitochondrial membrane potential in the first two divisions. After the third division, subsets of cells start to down-regulate Pax5 and mitochondrial membrane potential. The reduction of mitochondrial membrane potential is more obvious in the Pax5 low population than in the Pax5 high population (Figure 3.2 C).

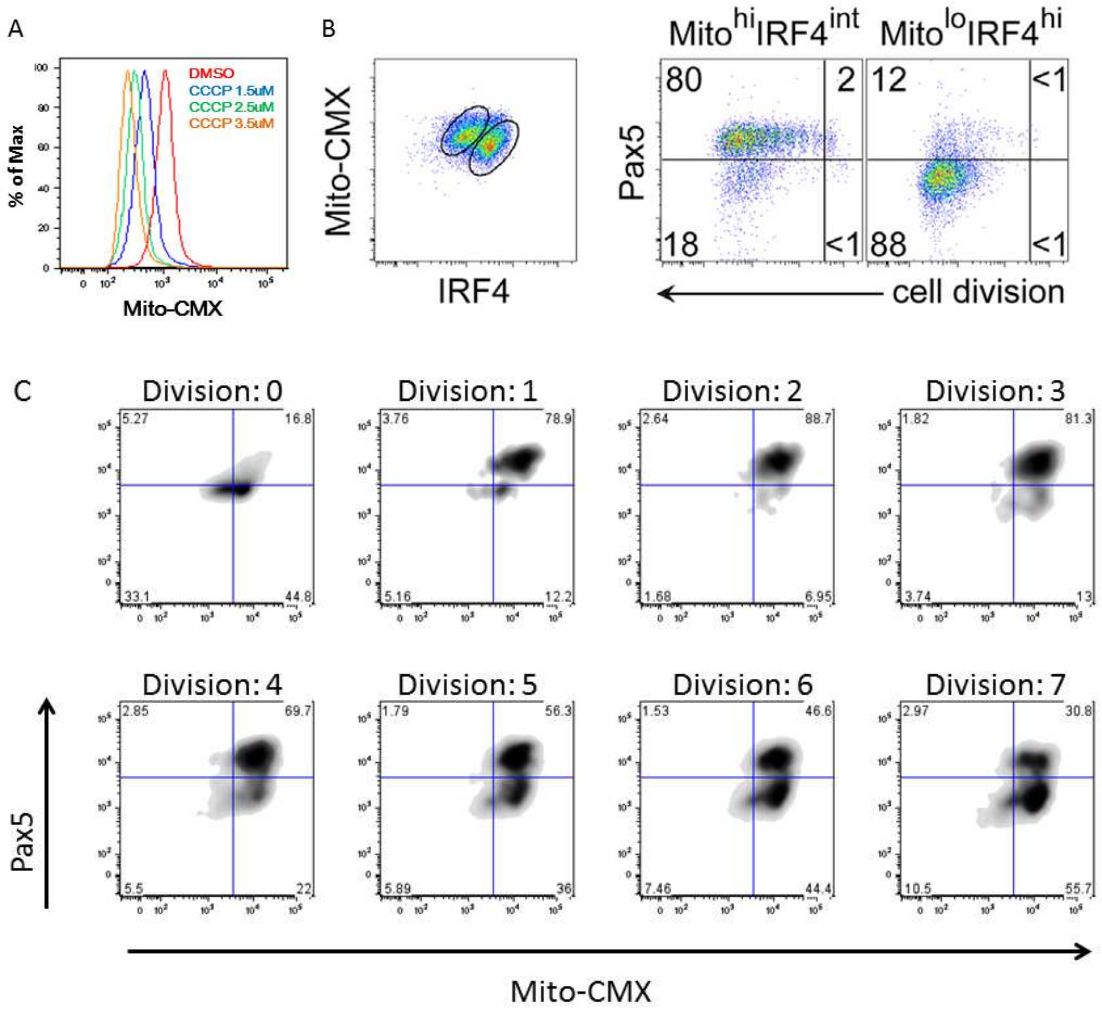


Figure 3.2 Pax5 repression is accompanied by reduction of mitochondrial membrane potential.

(A) A control experiment to demonstrate MitoTracker CMXRos dye (MitoCMX) staining is positively correlated with mitochondrial membrane potential. B cells were stimulated with LPS for 1.5 days. Before FACS analysis, CCCP was added into the media at indicated concentrations for 30 minutes, and then MitoTrackerCMXRos was added 20 minutes before FACS analysis. (B) CTV-labeled B cells were stimulated with LPS for 3.5 days and stained with MitoTracker CMXRos dye (MitoCMX) to measure mitochondrial membrane potential. Left plot: IRF4 versus MitoTracker CMXRos. Right plot: two populations of cells based on levels of IRF4 and

MitoTracker CMXRos dye (Mito) were analyzed for cell division versus Pax5.

(C) CTV-labeled naïve B cells stimulated with LPS for 3.5 days and stained with MitoTracker CMXRos dye (MitoCMX) to measure mitochondrial membrane potential. The plots are gated on each division, and show Pax5 versus MitoTracker CMXRos. Data are representative of two separate experiments.

3.3 Differential mitochondrial membrane potential in sibling cells is independent of cell-cell interaction

After observing the association of Pax5 expression and mitochondrial membrane potential, I aimed to visualize the dynamics of total mitochondria and mitochondrial membrane potential during each stage of cell division. In LPS-stimulated B cells, mitochondria are distributed around the two microtubule-organizing centers (MTOCs) in metaphase cells, whereas they are accumulated in the middle of division plane in anaphase. I found that sibling cells exhibit differential mitochondrial membrane potential in the cytokinetic stage, although the total amount of mitochondria is relatively equally distributed between the sibling cells (Figure 3.3 A). Staining for the mitochondrial outer membrane protein Tom20 reveals that the sibling cells that have lower mitochondrial membrane potential still have mitochondria, suggesting that the lower Mito-CMX staining in one of the sibling cell is not observed because of that sibling cell lacking mitochondria.

In our previous studies, we have found two groups of transcription factors that require different mechanisms to establish asymmetric patterns. The first group of transcription factors requires extrinsic signals for establishing polarity in cytokinetic cells. For examples, the asymmetric pattern of Bcl6 depends on cell-cell interaction through ICAM. These transcription factors are polarized in metaphase and exhibit asymmetric accumulation in sibling cells in vivo. However, they lose their asymmetric pattern and become equally distributed between the daughter cells when lymphocytes undergo homeostatic expansion in Rag1 knockout mice (Barnett et al., 2012). Because unequal mitochondrial membrane potential can be observed in LPS-stimulated B cells, I hypothesized that there is an intrinsic mechanism that is capable of establishing and maintain asymmetric mitochondrial status in sibling cells. If the hypothesis held,

I expected that differential mitochondrial membrane potential in sibling cells could be observed under a homeostatic expansion condition. The result of confocal analysis of transferred B1-8 high B cells to Rag1 knockout animals is in alignment with this prediction. That is, unequal mitochondrial membrane potential in sibling cells can still be observed during homeostatic expansion (Figure 3.3 B).

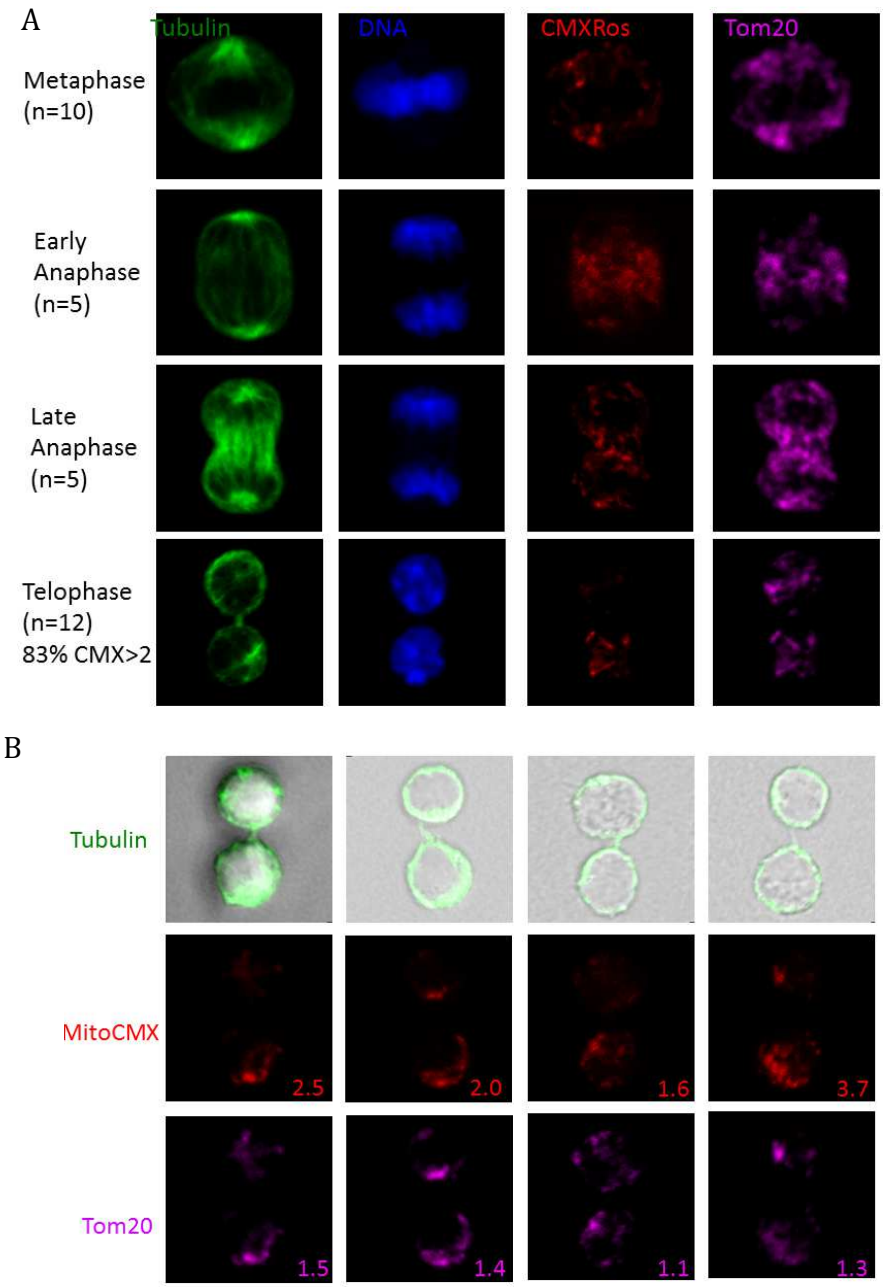


Figure 3.3 Sibling cells exhibit different mitochondrial membrane potential independent of cell-cell interaction

(A) Dynamics of mitochondria during B cell divisions**.** B cells were stimulated with LPS (20ug/ml) for 40 hours. The cells were stained with MitoTracker CMXRos dye (CMXRos) for 20 minutes before cell sorting, and then were fixed with 4% paraformaldehyde and permeabilized with ice cold methanol to enable staining of mitochondrial outer membrane

protein Tom20. The figures show representative events for metaphase (n=10), early anaphase (n=5), late metaphase (n=5) and telophase cells (n=12).

(B) MitoTracker CMXRos staining of homeostatically proliferating B cells (B1-8^{hi}, CD45.1^{+}) adoptively transferred into Rag1 knockout mice. By day 5 post-transfer, CD45.1^{+} cells are sorted for confocal analysis. Total number of imaged cytokinetic cells is 29.

3.4 Pax5 and healthy mitochondria are evident in the same daughter cell

To understand the dynamic relationships between Pax5 and mitochondrial membrane potential in dividing cells, in vitro stimulated B cells were co-stained with MitoTracker Red CMXRos dye and Pax5 and then subjected to confocal analysis. I found that the sibling cells with asymmetric Pax5 also exhibited greater differences in mitochondrial membrane potential, although sibling cells with different mitochondrial membrane could be observed in sibling cells with symmetric levels of Pax5 (Figure 3.4). The segregation pattern of Pax5 and mitochondria with higher membrane potential suggests that the daughter cells exhibit different mitochondrial activity before the establishment of asymmetric Pax5, and that the sibling cells with lower Pax5 levels also have a relatively lower mitochondrial proton gradient. Strong MitoTracker Red CMXRos dye staining is associated with three factors: greater mitochondrial membrane potential, higher OXPHOS activity, and higher mitochondrial mass. Because Tom20 staining reveals that the total mitochondrial mass is relatively equal in sibling cells with differential mitochondrial membrane potential (Figure 3.3), it is more likely that the sibling cells with lower Pax5 exhibit lower mitochondrial activity. There are two mechanisms resulting in low mitochondrial activity. First, aged, malfunctioning mitochondria have lower membrane potential, and thus they are removed by the Pink/Parkin pathway. Second, lower mitochondrial membrane potential may suggest the cells are less dependent on OXPHOS as an energy source and may be fueled by other metabolic pathways, such as glycolysis.

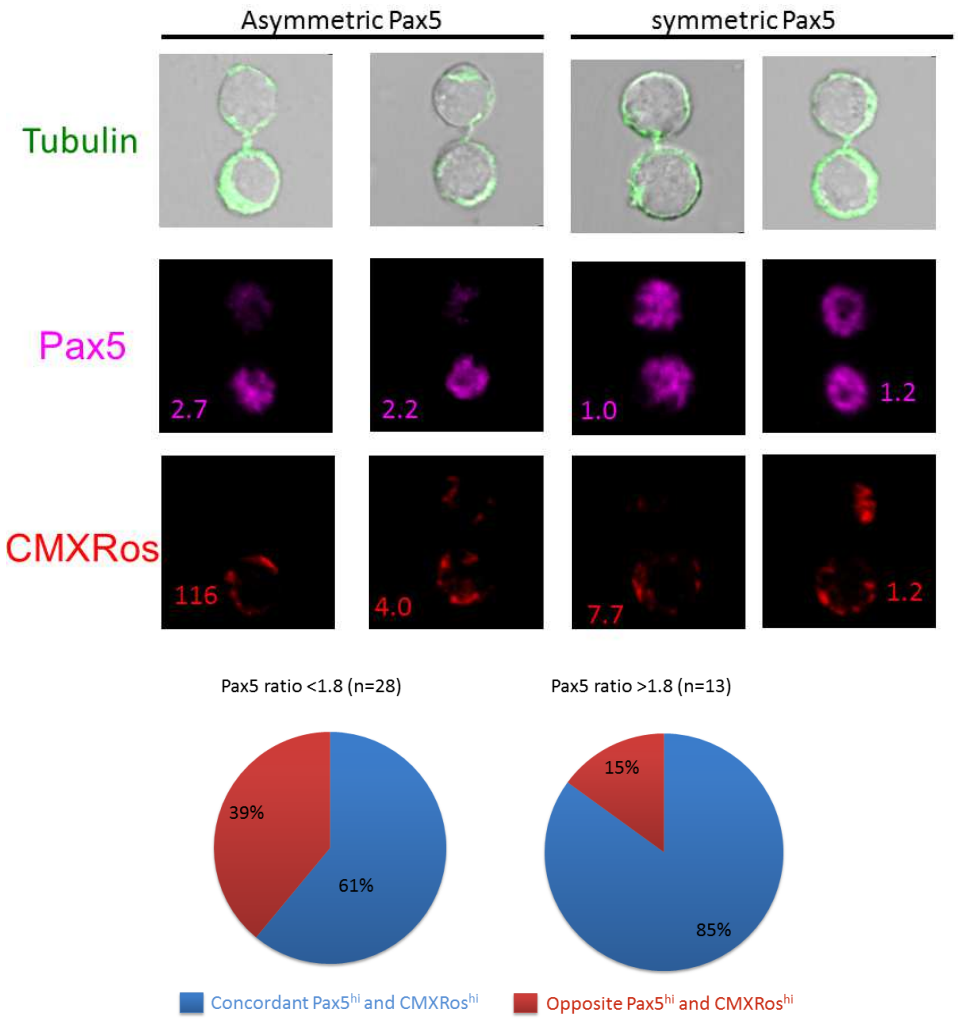


Figure 3.4 Pax5 and healthy mitochondria fall in the same daughter cell.

The cultured B cells were first labeled with MitoCMXRos for 20 minutes in the media before being harvested for methanol fixation. The samples were fixed with 3% PFA diluted in PBS, and then were permeabilized with methanol. After fixation, Pax5 protein was stained with rabbit anti-mouse Pax5 monoclonal antibody and goat-anti-rabbit IgG conjugated to Alexa Fluor® 647 dye. The tubulin was stained with rat monoclonal Tubulin antibody [YOL1/34] and goat-anti-rat IgG conjugated with Alexa Fluor® 488 dye. The number of imaged cytokinetic events is 41 (n=41 sibling pairs).

3.5 MitoTracker Pulse assay identifies unequal mitochondrial clearance in cytokinetic cells

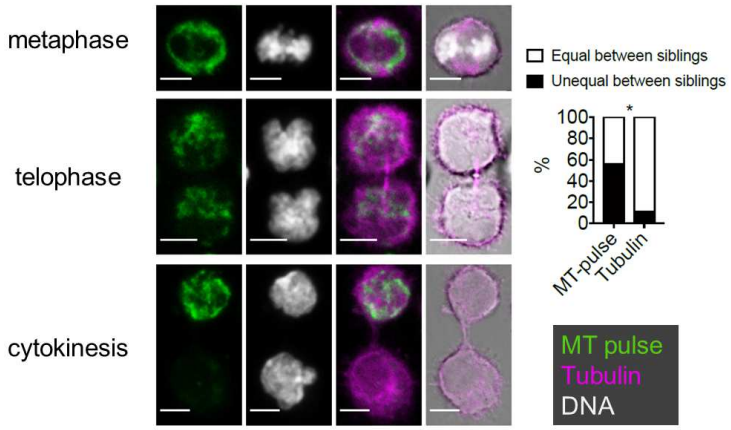
Maintaining healthy mitochondria has been linked to maintaining self-renewal ability and to preventing senescence of various type of stem cells, including muscle stem cells, neural stem cells, and cancer stem cells (Garcia-Prat et al., 2016; Katajisto et al., 2015; Khacho et al., 2016). Two different mechanisms have been reported for maintaining mitochondrial health in stem cells. One of the mechanisms is removing malfunctioning mitochondria through autophagy or mitophagy. This mechanism is found in muscle and neural stem cells (Garcia-Prat et al., 2016; Khacho et al., 2016). By contrast, studying the stem-like cells in a breast cancer cell line reveals daughter cells may inherit unequal amount of aged mitochondria, thus affecting their stem-like properties (Katajisto et al., 2015).

To determine whether these two mechanisms explain the asymmetric mitochondrial membrane potential phenotype in dividing B cells, we performed a MitoTracker Pulse assay to visualize the dynamics of clearing or accumulation of aged mitochondria. Imaging fixed cells by confocal microscopy reveals that in dividing lymphocytes, the subcellular distribution of aged (pulsed) mitochondria is fairly symmetric in metaphase until early telophase. However, when cells enter the cytokinetic stage, we find that in more than half of the sibling pairs, different amounts of aged (pulsed) mitochondria are inherited (Figure 3.5 A).

The same trend can also be observed in live cell imaging, implying that the observed differential accumulation of labeled mitochondria in one of the sibling cells is not due to an artifact of staining. Live cell imaging of anaphase cells or early cytokinetic cells reveals that the labeled mitochondria do not accumulate in a particular single daughter cell (Figure 3.5 B). Also, aged (pulsed) mitochondria do not exhibit a unidirectional movement pattern in time-lapse videos (Figure 3.5 B). However, unequal amounts of aged (pulsed) mitochondria can be

observed in late cytokinetic sibling cells (Figure 3.5 C). In sum, these data are more supportive of the hypothesis that asymmetric mitochondrial status is due to unequal ability to remove or renew mitochondria, rather than active partitioning of aged mitochondria into one of the daughter cells.

A



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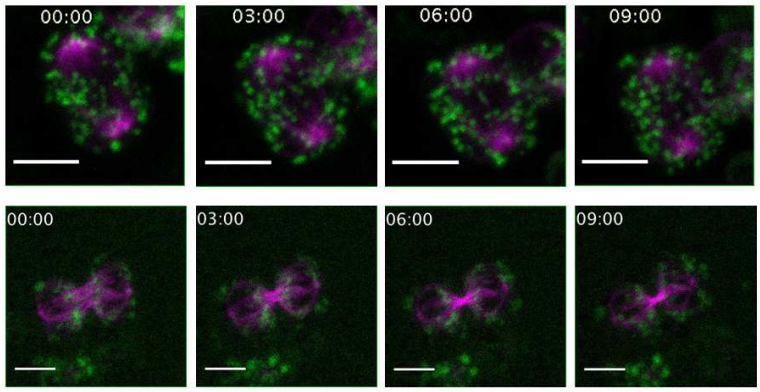

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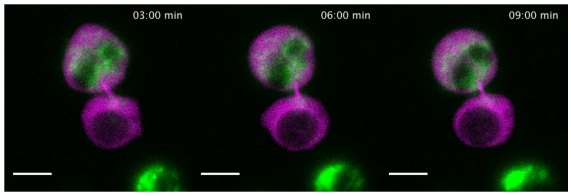


Figure 3.5 MitoTracker Pulse assay identified unequal mitochondrial clearance in cytokinetic cells. (A) Later in division, mitochondrial clearance is unequal. MitoTracker Red FM (MTR)-pulsed, LPS-stimulated B cells were fixed after 44 hours and stained with DNA dye (DAPI) and anti-tubulin antibodies. The plots show representative examples of metaphase, telophase, and cytokinetic cells. Experiment repeated three times (metaphase: n=11 cells, telophase: n=8 sibling pairs, and cytokinesis: n=18 sibling pairs). Graph depicts percentage of sibling cell pairs with equal or unequal ratios of 3D intensity of MTR and tubulin (*P<0.05,

Fisher's exact test). (B) Two representative time-lapse series of LPS-stimulated B cells labeled with SiR-tubulin dye (350nM, 15 hours) and pulsed with MTG (100nM). Frames represent 3-minute intervals. Scale bars = 6 mm. (C) A representative time-lapse series of LPS-stimulated B cells transduced with mCherry-alpha-tubulin RV and pulsed with MTG 16 hours prior to imaging. Frames, left-to-right, represent 3-minute intervals (n=3). Scale bars = 5 mm.

3.6 Inhibition of mitochondrial fission-induced effector cell differentiation

To study the effect of accumulation of aged mitochondria, we compared the effect of several mitochondrial inhibitors, which target different mitochondrial pathways, on the dynamics of mitochondrial morphology and on the transcriptional program of activated B cells. First, we treated the activated B cells with different inhibitors, and then labeled the total mitochondrial network with MitoTracker Green dye, which is a mitochondrial membrane potential-independent dye.

The results showed that rotenone, which is an inhibitor of electron transport form complex I to ubiquinone, did not significantly affect mitochondrial mass in treated B cells (Figure 3.6). By contrast, CCCP, which is a mitochondrial oxidative phosphorylation uncoupler, reduced the mitochondrial mass, which is in accordance with previous report suggesting that depolarized mitochondria are cleared by a mitophagy mechanism (D. Narendra et al., 2008). mDivi-1, which is a mitochondrial fission inhibitor, increased MitoTracker Green staining , suggesting inhibiting mitochondrial division extends the mitochondrial network and increases the total mitochondrial mass (Cassidy-Stone et al., 2008).

Activated B cells are characterized by high expression of Pax5 and B220, but low expression of CD138, Embigin, and IRF4. By contrast, plasma cells exhibit low levels of B220 and Pax5 but high levels of intracellular IRF4, and surface CD138 and Embigin (Fairfax, Kallies, Nutt, & Tarlinton, 2008; Kallies et al., 2007). Our previous work has established that PI3K inhibitor LY297004 suppresses the differentiation of plasma cells, whereas Foxo1 inhibitor induces differentiation toward plasma cells (W. H. Lin et al., 2015). By comparing the surface marker and intracellular transcription factor patterns, we found that mDivi-1 has a similar effect to Foxo1 inhibitor, whereas rotenone works like PI3K inhibitor. The CCCP-treated cells acquired

an intermediate phenotype, in which IRF4 and Embigin were upregulated but the down-regulation of Pax5 and B220 were inhibited (Table 1). These results suggest that inhibition of complex I in the electron transport chain has a similar effect to repressing PI3K, and that inhibition of mitochondrial fission mimics the effect of inactivating Foxo1 to stimulate differentiation into effector cells.

What is the result of inhibiting mitochondrial fission? We established a FACS staining protocol, called “mitoclearance”, combining MitoTracker Green pulse, cell proliferation dye and MitoSox staining. MitoTracker Green is a mitochondria-specific dye whose staining is less affected by mitochondrial membrane potential. MitoSOX is a mitochondria-specific superoxide detection reagent, whose oxidized products become fluorescent when binding to nucleic acid. The dilution of cell proliferation dye is mainly dependent on cell division, whereas the dilution of pulsed MitoTracker Green reflects both dilutions during cell division and clearance of labeled mitochondria. We found that cells exhibiting high MitoSOX staining retained more MitoTracker Green staining (mitoclearance low), whereas MitoSox low cells are a mixture of mitoclearance high and low cells. The MitoSox low and mitoclearance low cell may represent the cells that have repaired their aged mitochondria by fusion with new mitochondria (Figure 3.7).

Using the “mitoclearance” assay, we can see that mDivi-1-treated cell retained more aged mitochondria with MitoTracker Green pulse compared with the no drug control group (Figure 3.8 upper row). Also, MitoSox staining showed that mDivi-1 treatment produced more mitochondrial ROS. Taken together, the results suggest accumulation of aged mitochondria may enhance mitochondrial superoxide production (Figure 3.8 lower row).

To rule out the possibility that the observed effect of mDivi-1 on the differentiation of lymphocytes is the off-target effect of mDivi-1, we directly tested the result of disrupting Drp-1

function by over-expression of dominant negative Drp-1 (K38A) (DN Drp-1) using retroviral vector to transduce activated lymphocytes. FACS analysis showed that overexpression of DN Drp-1 has an even stronger effect on repressing Pax5 (and stimulating IRF4 expression) in B cells (Figure 3.9 A), and more obviously, Tcf1 repression in $CD8^+$ T cells (Figure 3.9 B). Moreover, the effect of overexpression of DN Drp-1 can be partially rescued by the addition of N-Acetyl L-Cysteine (NAC), a ROS scavenger, in the media, suggesting that the over-served repression effect involves the production of ROS (Figure 3.9 C)

In T cells, mitochondrial ROS has been reported to play a critical role in stimulating transcription factor nuclear factor of activated T cells (NFAT) and support interleukin-2 (IL-2) production (Sena et al., 2013). Here, our findings support the idea that mitochondrial ROS also plays a role in the repression of lineage-maintaining transcription factors in B cells and T cells, and thus guides the differentiation of effector cells and the limiting of the self-renewal ability of progenitor cells.

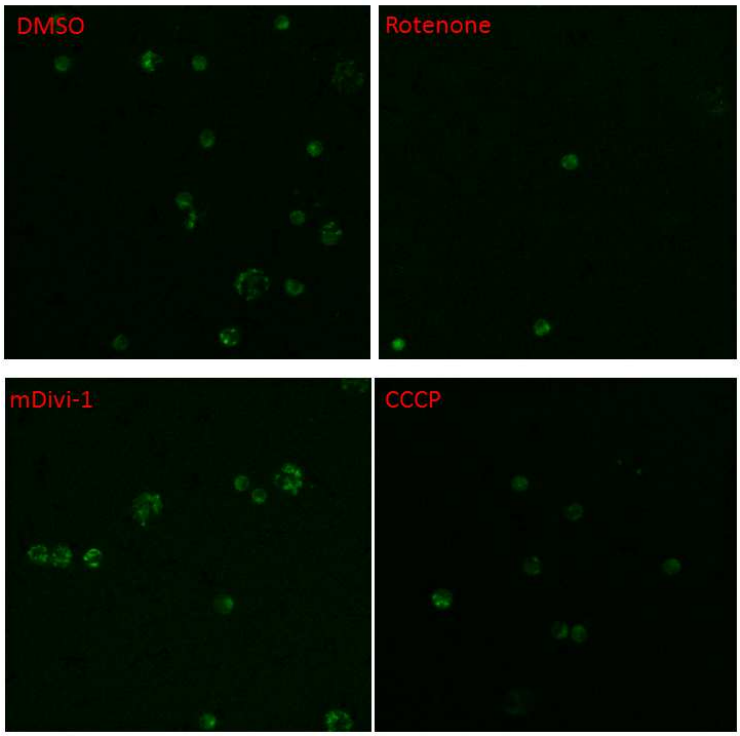


Figure 3.6 Mitochondrial fission inhibitor mDivi-1 treatment increased mitochondrial mass

B cells derived from wild-type B6 mice were stimulated with LPS (20ug/ml) for 3 days. The inhibitors were added (10μM mDivi-1; 2.5μM CCCP; 300nM Rotenone) into the culture media for 4 hours before live cell imaging by scanning microscopy. MitoTracker Green, which is a mitochondrial membrane potential-independent dye, was used for labeling mitochondria.

	B cell	Plasma cell	CCCP	mDivi-1	Rotenone	PI3K inhibitor	Foxo1 inhibitor
IRF4	Low	Hi	Up	Up	Down	Down	Up
Pax5	Hi	Low	Retain hi	Down	Retain hi	Retain hi	Down
B220	Hi	Low	Retain hi	Down	Retain hi	Retain hi	Down
CD138	Low	Hi	-	-	Down	Down	Up
Embigin	Low	Hi	Up	+/-	-	Down	Up
CMXRos	Hi	Low	Down	Up	Up		

Table 1 The effects of drugs on B cell transcriptional program and mitochondria membrane potential.

B cells were activated with LPS (20μg/ml), and drugs were added at the beginning of the culture (CCCP: 1.5μM, mDivi-1: 10μM, Rotenone: 150nM, PI3K inhibitor LY294002: 0.5μM, Foxo1 inhibitor AS1842856: 1μM). MitoTracker CMXRos was added to the media (200nM) 20 minutes before cells were harvested for FACS analysis.

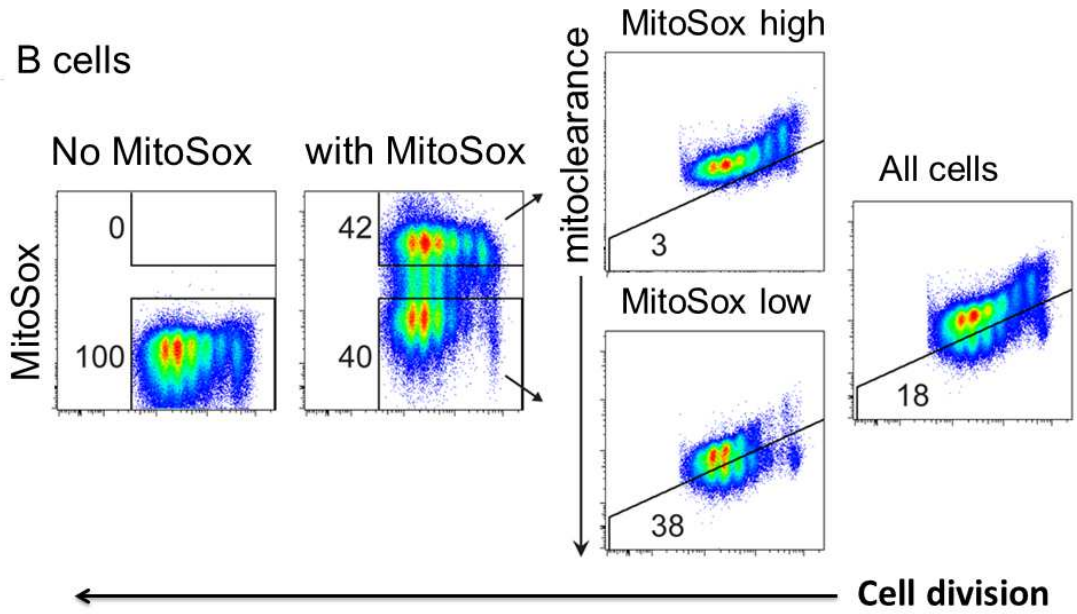


Figure 3.7 Mitochondrial stasis is associated with mitochondrial ROS production

MitoTracker GreenFM pulse-labeled B cells were activated with LPS for 3 days and then stained with MitoSOX. Cell division versus MitoSOX fluorescence in non-MitoSOX-labeled control cells (far left) versus MitoSOX-labeled cells (second-to-left). Cell division versus mitoclearance of MitoSOX^{hi} cells and MitoSOX^{lo} cells (third column), compared to all cells (far right) illustrates that MitoSOX^{hi} cells are enriched with stasis of aged (pre-pulsed) mitochondria, analogous to differentiated effector T cells enriched with mitochondrial stasis. Data are representative of two separate experiments.

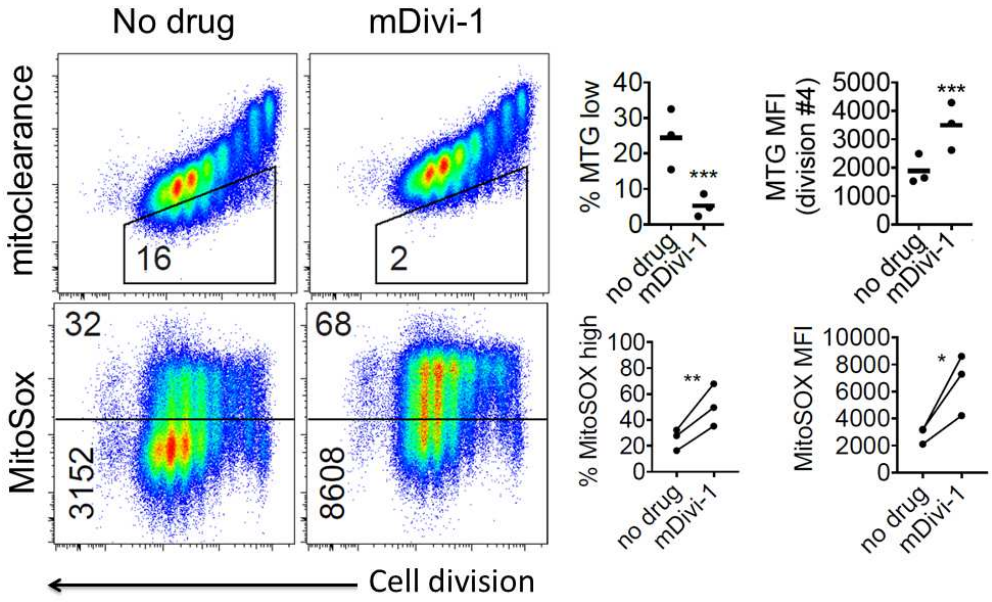


Figure 3.8 Inhibition of mitochondrial fission impedes mitochondrial turnover and increases mitochondrial ROS.

(Upper row) FACS of CTV-labeled and MitoTracker Green FM ("MTG") pulse-activated B cells (top row) and CTV-labeled and MitoSox labeled B cells (bottom row) stimulated with LPS for 66 hours. Cells were cultured in the absence or presence of mDivi-1 (10μM). A reverse arrow on the *y*-axis indicates dilution (and/or elimination) of pre-labeled (pulsed) mitochondria that occurs with each cell division (*x*-axis), termed mitoclearance. Upper row statistical graphs display the frequency of cells within the entire trapezoidal MTG low gate (left) and MFI of MTG signal in the fourth cell division (right) (n=3, **P<0.01 and ***P<0.005 for indicated treated groups compared to no drug control group, one-way repeated measures ANOVA).

(Bottom row) CTV-labeled B cells stimulated with LPS in the absence or presence of mDivi-1 (10 μM) and analyzed on day 3 for cell division versus mitochondrial ROS (MitoSOX). Horizontal numbers denote the frequency of cells within a bound area. Vertical numbers denote mean fluorescence intensity (MFI) of all cells, not simply those within bound area. Bottom row

statistical graphs display frequency of MitoSOX^{hi} cells (left) and MitoSOX MFI (right) among B cells stimulated in the absence or presence of mDivi-1 (n=3, *P<0.05 or **P<0.01, two-tailed paired t-test). FACS data are representative of three separate experiments.

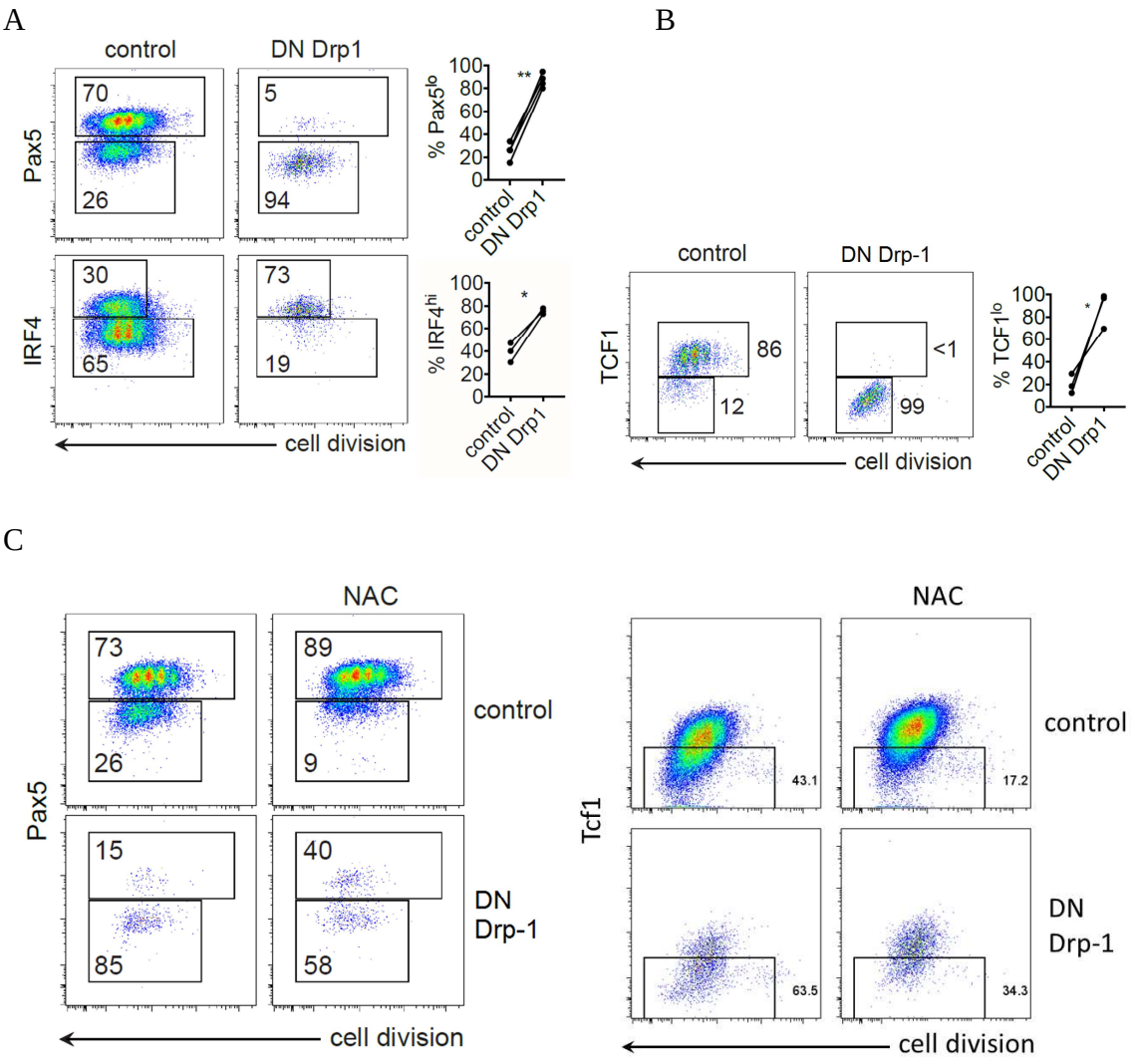


Figure 3.9 Overexpression of dominant negative Drp-1 promotes effector cell differentiation. (A) Overexpressing dominant negative Drp-1 (DN Drp-1) by transducing the B cells with retrovirus up-regulated IRF4 but repressed Pax5. B cells were activated with LPS for 40 hours, and then infected with retrovirus carrying DN Drp-1 or control vector, and then re-plating for another 24 hours before FACS analysis.

(B) Overexpressing dominant negative Drp-1 (DN Drp-1) by transducing the $CD8^+$ T cells with retrovirus repressed Tcf1. $CD8^+$ T cells enriched from spleen of wild-type B6 mice by MACS kit selection were activated by plate bound anti-CD3 and anti-CD28 and IL-2 (100 units/ml) for 40

hours, then infected with retrovirus carrying DN Drp-1 or control vector, and then re-plated in the presence of anti-CD3, anti-CD28, and IL-2 for another 24 hours before FACS analysis. (C) The addition of N-Acetyl L-Cysteine (NAC) (3mM) in the culture media suppressed the repression effect of DN Drp-1 on Pax5 and Tcf1 in B cells and T cells, respectively. Data are representative of two separate experiments for each cell type.

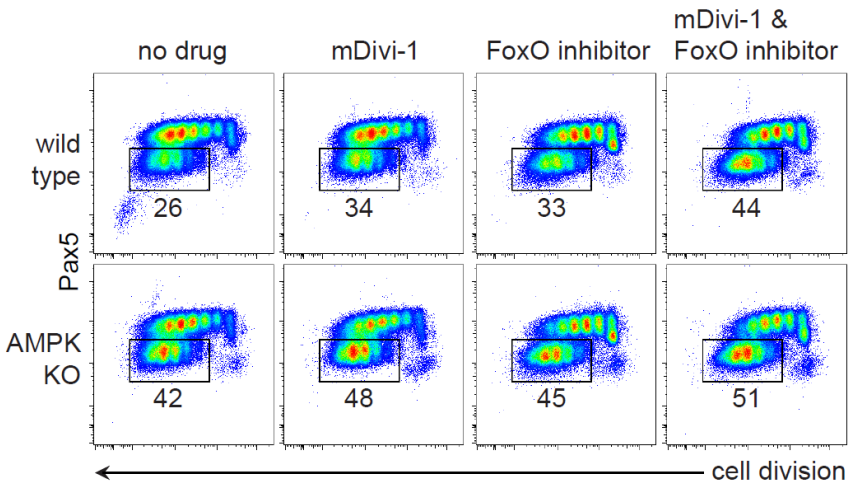
3.7 Inhibiting mitochondrial biogenesis and renewal promotes effector cell differentiation

Mitochondria quality control involves four major stages: fission, turnover, fusion, and biogenesis. So far, we have demonstrated that accumulation of aged mitochondria that produce more mitochondrial ROS accelerated the differentiation into effector cells by perturbing the fission and turnover mechanism. Next, I examined the effect of disrupting the key factors in the biogenesis pathway. If our model that accumulating aged mitochondria serves as the trigger for effector cell differentiation held, I expected that inhibiting biogenesis of mitochondria should accelerate the differentiation of effectors but reduce the population of self-renewing cells.

Two major transcription factor families that contribute to the biogenesis of mitochondrial genes are FoxO and PGC-1α (peroxisome-proliferator-activated receptor γ co-activator-1α). FoxO family transcription factors (especially FoxO1 and FoxO3) have been reported to participate in the biogenesis of mitochondria (Cheng et al., 2009; Peck, Ferber, & Schulze, 2013; Philip-Couderc et al., 2008). As previously discussed in the introduction section, the activity of Foxo1 can be inhibited by AKT-mediated phosphorylation. PGC-1α serves as co-transcriptional regulation factor modulating the activity of other key transcription factors, including nuclear respiratory factor 1 and nuclear respiratory factor 2, involved in mitochondrial gene biogenesis (Jornayvaz & Shulman, 2010). One of the key regulators of PGC-1α is AMPK (AMP-activated protein kinase). AMPK is a sensor of several stress signals, including low ATP level, ROS, nitric oxide, and reactive nitrogen species (RNS) (Cardaci et al., 2012). For mammalian cells, AMPK is a heterotrimeric complex comprised of α, β, and γ subunits. Each subunit has multiple isoforms (α1, α2, β1, β2, γ1, γ2, and γ3) and their expression is tissue- and cell-type-specific. For lymphocytes, α1 is the only α subunit expressed in B cells and T cells (Mayer, Denanglaire, Viollet, Leo, & Andris, 2008; Tamas et al., 2006).

We therefore tested whether knocking out the AMPK α1 subunit and treating cells with FoxO inhibitor would accelerate effector cell differentiation. Comparing the B cells derived from an AMPKα1 knockout animal to those from a wild-type animal, we found that more B cells exhibit a Pax5-low phenotype in the later divisions and that the Pax5 inhibition effect of the FoxO inhibitor and mDivi-1 is more significant in the AMKPα1 knockout (Figure 3.10). A similar synergistic effect of FoxO inhibitor and mDivi-1 on Tcf1 repression was also observed in AMPKα1 knockout $CD8^+$ T cells by using CD4-Cre to conditionally delete AMPKα1 in T cells only (Figure 3.11).

A



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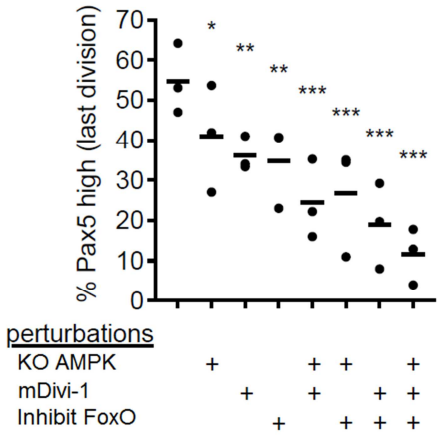


Figure 3.10 Inhibiting mitochondrial biogenesis and renewal synergistically promotes effector cell differentiation.

Cell division versus Pax5 expression of CTV-labeled B cells from wild-type and AMPKα1 knockout mice stimulated with LPS for 3 days in the absence or presence of mDivi-1 (10 μM), FoxO1 inhibitor (1μM), or both inhibitors. Graph contains frequency of $Pax5^{hi}$ cells in the last cell division among conditions indicated (n=3, *P<0.05 or **P<0.01 or ***P<0.005 comparing groups with one or more perturbations to group with no perturbation, repeated measures one-way ANOVA).

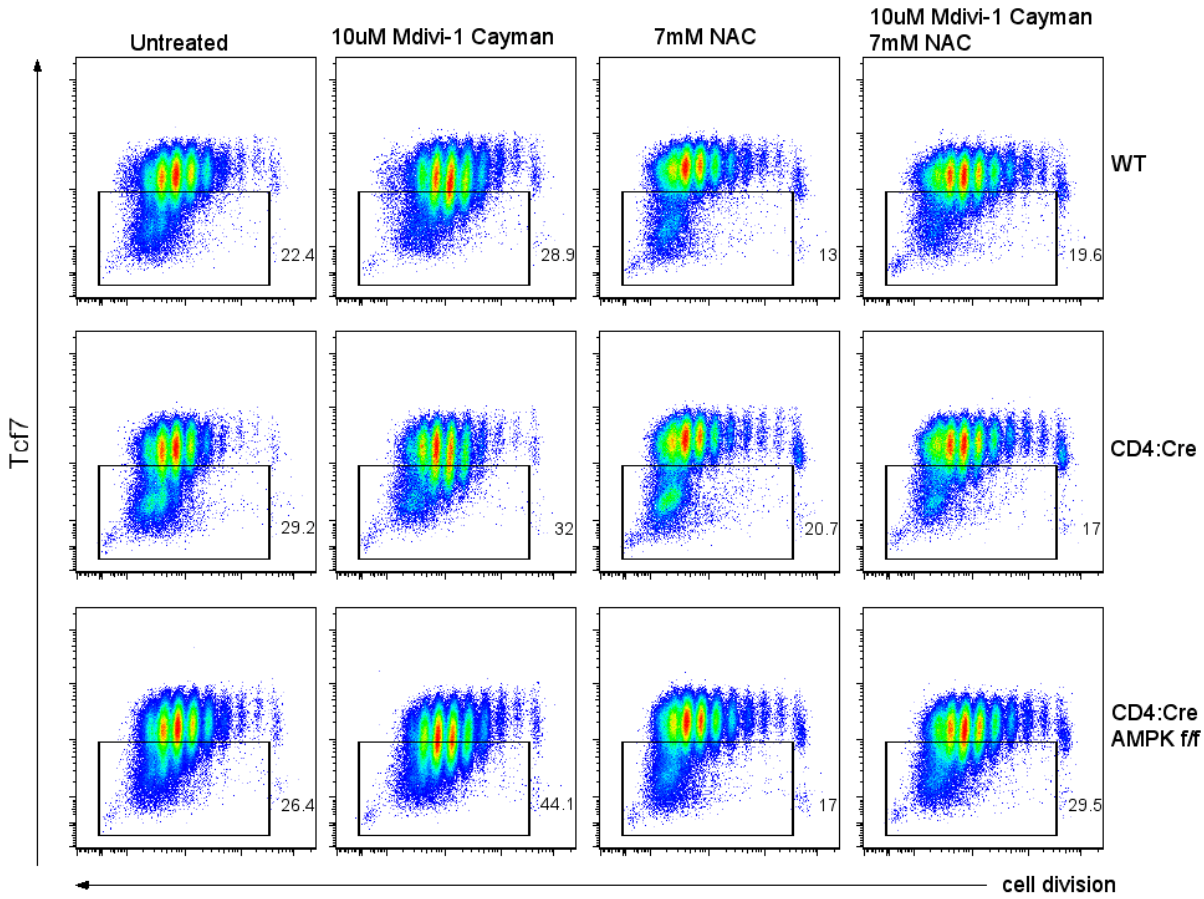
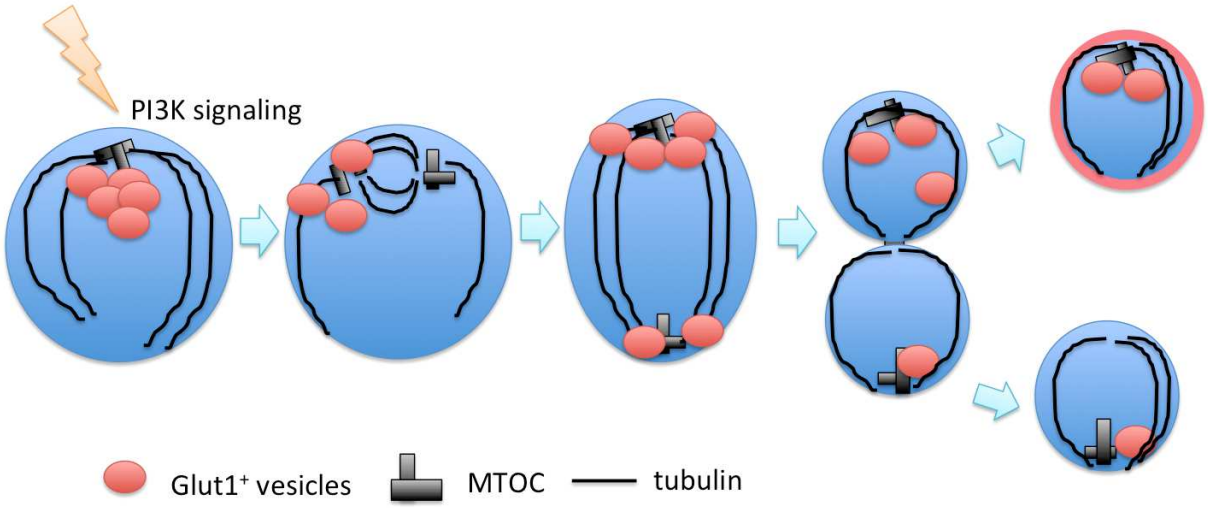


Figure 3.11 Inhibiting mitochondrial biogenesis and fission promotes effector cell differentiation. $CD8^+$ T cells were enriched from wild-type or CD4-Cre^+ animals with either loxP sites flanked Glut1 or wild-type Glut1 allele. Isolated $CD8^+$ T cells are labeled with cell proliferation dye (CTV) and then stimulated by plate-bound anti-CD3 and anti-CD28 in the presence of IL-2 in the culture media. Cells were harvested for FACS staining. The plots are representative of two separate experiments.



PI3K maintains the recycling and surface expression of Glut1

4.1 Aerobic Glycolysis Linking an Anabolic Constellation to Differentiation

So far, our results suggest that the differing abilities of the two daughter cells to clear aged mitochondria is linked to the fate determination of effector cells and maintenance of self-renewal ability. We also demonstrated that accumulation of aged mitochondria results in augmentation of mitochondrial ROS production. Mitochondrial aging is associated with ROS induced-damage to proteins and mitochondrial DNA, which results in mitochondrial dysfunction (Cui, Kong, & Zhang, 2012). Why do activated lymphocytes generate high amounts of ROS? This is associated with the pathway that they use to produce energy. Resting and naïve lymphocytes and memory cells produce energy mainly through oxidation phosphorylation, whereas activated lymphocytes and effector cells produce energy by aerobic glycolysis and oxidation phosphorylation (C. H. Chang et al., 2013; Palmer, Ostrowski, Balderson, Christian, & Crowe, 2015; T. Wang, Marquardt, & Foker, 1976). After activation, both B cells and T cells display an augmented extracellular acidification rate (ECAR), which reflects aerobic glycolysis, and an augmented oxygen consumption rate (OCR), which represents mitochondrial respiration function (Caro-Maldonado et al., 2014). The self-reinforcing relationship between mitochondrial stasis and ROS prompted us to test the effects of metabolism on lymphocyte differentiation.

To clarify the relationships between glucose utilization and lymphocyte differentiation, we tested the result of perturbing the glycolytic pathway on the transcriptional program of B and T lymphocytes. Inhibiting glycolytic flux by treating activated lymphocytes with 2-Deoxy-D-glucose (2-DG) suppressed the downregulation of Pax5 and Tcf1 in proliferating B cells and T cells, respectively (Figure 4.1). That is, inhibition of glycolysis shifted the balance of lymphocyte fate toward self-renewal but suppressed effector cell differentiation. Indeed, 2-DG treatment has been reported to enhancing memory T cell formation in vivo by sustain FoxOx1

and Tcf1 activity(Sukumar et al., 2013).

In addition, 2-DG treatment also reduced the proliferation rate (Figure 4.1 A). Therefore, it is possible that the suppression of Pax5 and Tcf1 down-regulation is partially contributing to the reduced number of cell divisions. Thus, I tried to perform an opposite experiment to enhance glycolysis. If glycolysis is linked to effector cell differentiation, we predicted that enhancing glycolysis would induce more severe down-regulation of Pax5 and Tcf1 in B cells and T cells, respectively. Enhancing glycolysis is achieved by retroviral overexpression of hexokinase 2 (HK2), which is the rate-limiting enzyme in glycolysis (Roberts & Miyamoto, 2015), leading to an over-differentiation phenotype with more cells undergoing Pax5 and TCF1 repression (Figure 4.2). In B cells, IRF4 was also upregulated when HK2 was overexpressed, suggesting that enhancing glycolysis directs the differentiation toward plasmablasts and plasma cells.

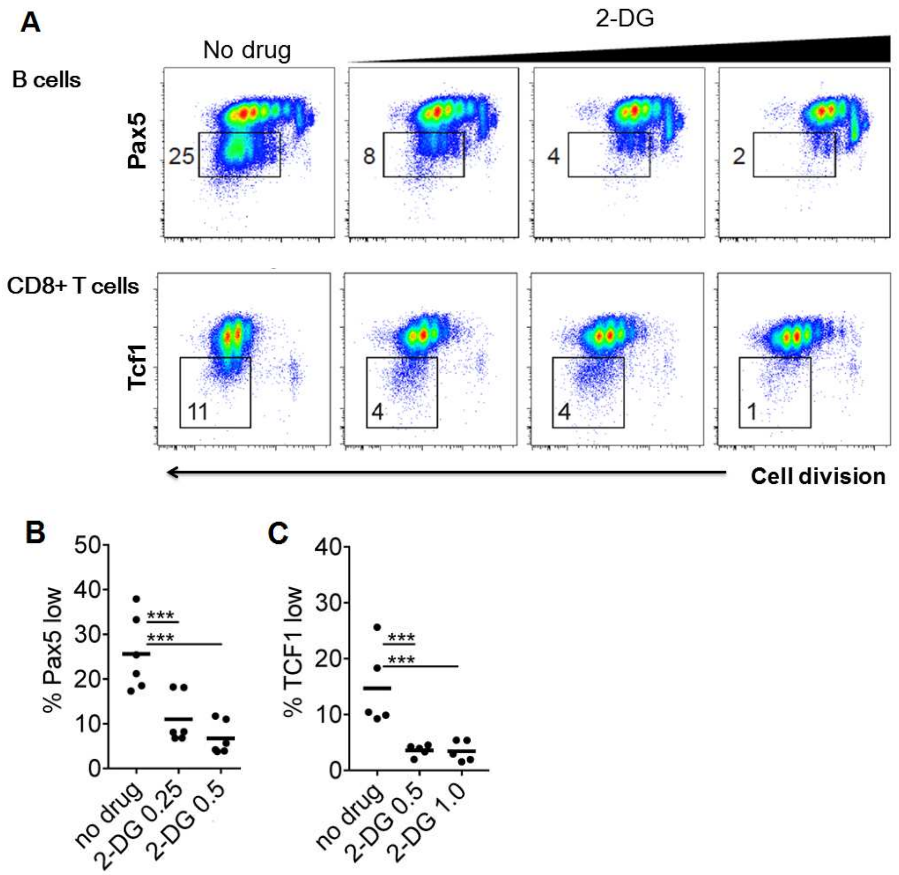
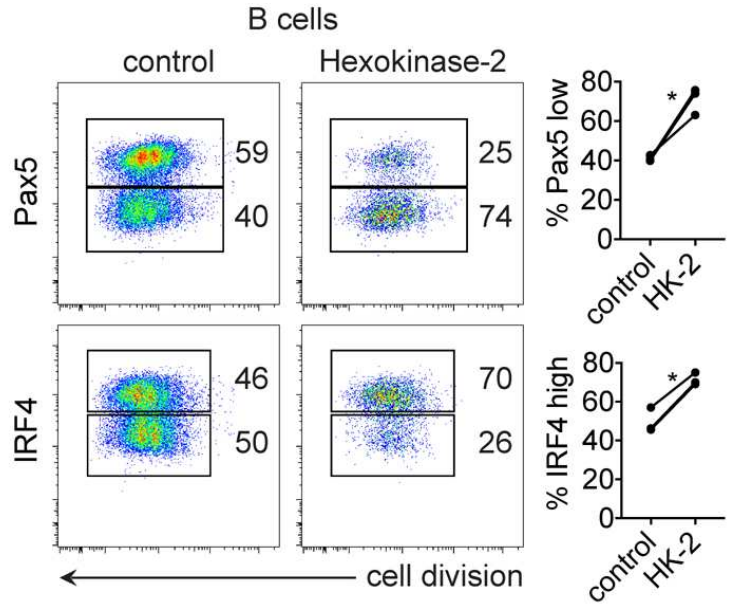


Figure 4.1 Suppression of aerobic glycolysis inhibits effector cell differentiation

(A) Upper panel: Cell division versus Pax5 expression of CTV-labeled B cells stimulated with LPS for 3 days in absence or presence of mDivi-1 (10 μM) and increasing doses of 2-Deoxy-D-glucose (2-DG) (0, 0.25, 0.50, and 0.75 mM left-to-right). In these B cell experiments, drug variables were added 18 hours after stimulation began. Lower panel: Cell division versus TCF1 expression of CTV-labeled P14 $CD8^+$ T cells stimulated for 4 days with gp33-41 peptide plus IL-2 in the absence or presence of mDivi-1 and increasing doses of 2-DG (0, 0.5, 1.0, and 2.0 mM), present from the beginning of stimulation. (B) The statistical graph depicts frequency of $Pax5^{lo}$ B cells in indicated conditions (n=6, ***P<0.005, repeated measures one-way ANOVA). (C) The statistical graph depicts frequency of $TCF1^{lo}$ $CD8^+$ T cells in indicated conditions (n=5, **P<0.01 or ***P<0.005, repeated measures one-way ANOVA).

A



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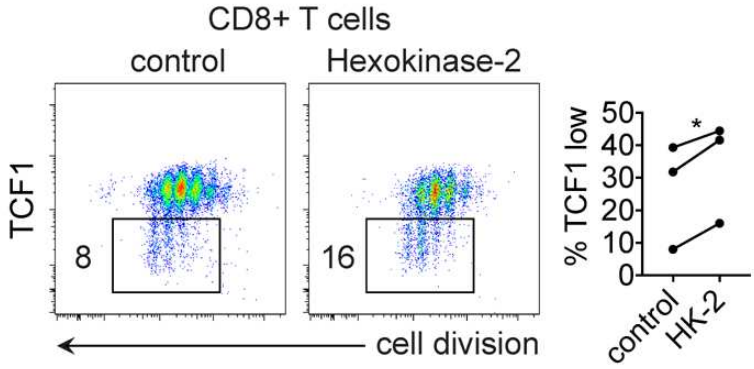


Figure 4.2 Enhancing aerobic glycolysis promotes effector cell differentiation

(A) CTV-labeled B cells stimulated for 36 hours with LPS prior to transduction with control or hexokinase-2 (HK-2) encoding retrovirus (RV) and analyzed 36 hours after transduction for cell division versus Pax5 and IRF4. Only transduced events are shown. Statistical graphs display frequency of $Pax5^{lo}$ and $IRF4^{hi}$ cells among cells transduced with control or HK-2 RV (n=3, *P<0.05, two-tailed paired t-test).

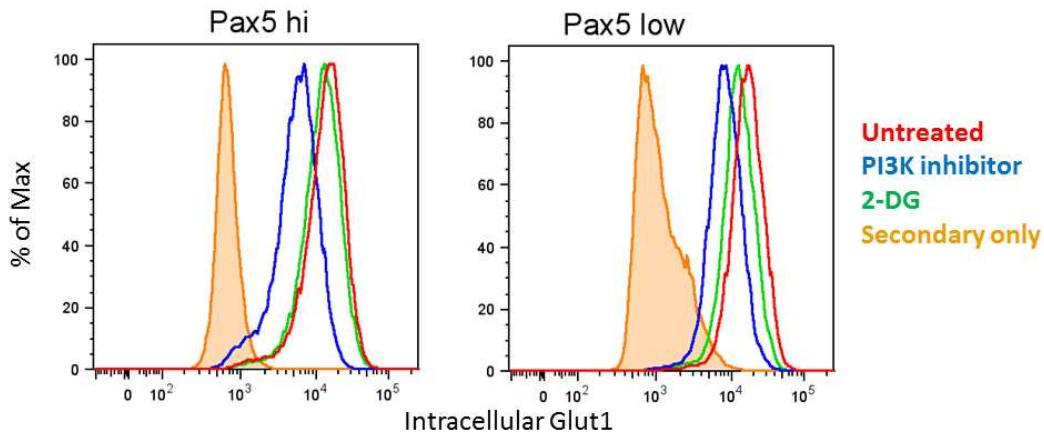
(B) CTV-labeled $CD8^+$ T cells stimulated with anti-CD3 and anti-CD28 antibodies with IL-2 for 36 hours prior to transduction with control RV or hexokinase 2 encoding RV and analyzed 36 hours after transduction for cell division versus TCF1. Only transduced events are shown. Statistical graph depicts frequency of $TCF1^{lo}$ cells among cells transduced with control or HK-2 RV (n=3, *P<0.05, two-tailed paired t-test).

4.2 Glycolysis regulates surface and intracellular glucose transporter level

Activated lymphocytes augment their glycolysis ability by increasing expression of HK2 and glucose transporters (Sukumar et al., 2013). Among the 13 glucose transporter family members, Glut1 is the predominant glucose transporter expressed in activated $CD8^+$ and $CD4^+$ T cells (Macintyre et al., 2014) as wells as activated B cells (Caro-Maldonado et al., 2014). Conditionally knocking out Glut1 in B cells impairs B cell homeostasis and antibody production (Caro-Maldonado et al., 2014), whereas knockdown Glut1 in $CD4^+$ T cells impairs proliferation, differentiation, and decreases survival. The result of knocking down Glut1 in $CD8^+$ T cells is less obvious than that observed in $CD4^+$ T cells, because $CD8^+$ T cells are able to compensate for the lack of Glut1 by upregulating the expression of Glut3, which is normally downregulated after activation (Macintyre et al., 2014).

Our recent publications provide evidence that treating activated lymphocytes with 2-DG reduces the levels of intracellular ROS (measured by DCFDA) and phosphorylated S6, which is an indication of mTORC1 activity. Therefore, we asked whether inhibiting glycolysis of 2-DG would affect glucose up-take. I performed intracellular staining with an anti-Glut1 antibody recognizing the intracellular domain in LPS stimulated B cells. The result demonstrated that the intracellular Glut1 level is strongly reduced by 2-DG in Pax5 low B cells (Figure 4.3 A). Moreover, 2-DG treatment also reduces the surface level of Flag-tagged Glut1 in transduced B cells (Figure 4.3 B), implying glucose deprivation may activate Glut1 transportation to the cell membrane. These results suggest an active glycolytic pathway is required for maintaining the intracellular and surface protein levels of Glut1, and that glycolysis can feedback to regulate the rate of glucose uptake, especially in effector cells.

A



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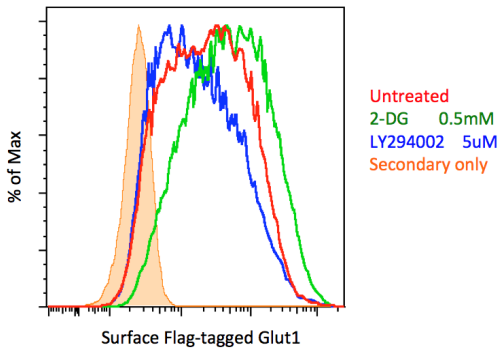


Figure 4.3 Inhibiting glycolysis reduces intracellular Glut1 but upregulates surface Glut1

(A) Glycolytic feedback regulates intracellular Glut1 levels

B cells are stimulated with LPS for 48 hours, before adding the drugs PI3K inhibitor LY294002 (5uM) and 2-DG (0.5mM), and then analyzed by day 3.5 after stimulation (36 hours after introduction of the drug treatments). The experiment has been repeated two times. **(B)** B cells were activated with LPS for 40 hours before spin infection. Activated B cells were infected with retroviral vector expressing Flag-Tagged-Glut1/NGFR or control NGFR vector and then further cultured for 24 hours before FACS analysis. Drugs were added by 6 hours after spin infection. The FACS plot represents NGFR positive events.

4.3 Glut1 polarization in dividing lymphocytes results in differential inheritance of Glut1

As discussed in an earlier section, we found that high membrane potential mitochondria are enriched in the daughter cell that acquires more Pax5. Live cell imaging using Cyto-ID, MitoTracker, and LysoTracker pulsed staining has provided evidence that the daughter cells have different abilities to remove old mitochondria. However, this result did not rule out another possibility: that that differential mitochondrial membrane potential may indicate the daughter cells have adopted different metabolic program.

To obtain biological macromolecules (such as DNA, polysaccharides, and proteins) for building new cells, proliferating cells need to uptake carbon and nitrogen from the environment. However, the cell membrane serves as a barrier to prevent nutrient molecules from diffusing in and out the cell. Most of the nutrient molecules (amino acids, peptides, fatty acids, and carbohydrates) pass through the cell membrane by way of a transporter complex. Our recent publications demonstrate that dividing lymphocytes are able to produce daughter cells with opposing cellular metabolisms, which is enabled by asymmetric expression/accumulation of metabolic regulators such as FoxO1 and IRF4. Given that 2-DG affects intracellular Glut1 levels, we want to know whether the daughters may inherit or express different amounts of nutrient transporters thus establishing and amplifying the different metabolic programs in the daughter cells. In this study, I imaged the subcellular localization of amino acid transporter heavy chain CD98 and the glucose transporter 1 (Glut1), respectively.

CD98, an early marker of T-cell activation, is a dual function protein. The protein is required to support rapid proliferation and lymphocyte clonal expansion by conducting extracellular integrin signals to cells to induce proliferation and to suppress apoptosis (Cantor, Slepak, Ege, Chang, & Ginsberg, 2011; Kurihara et al., 2015). It also serves as a subunit of the amino acid

transporter complex (Gottesdiener et al., 1988; Lindsten, June, Thompson, & Leiden, 1988). The uptake of branched-chain (valine, leucine, isoleucine) and aromatic (tryptophan, tyrosine) amino acids by lymphocytes is mediated by CD98 heterodimer, which served as the L-type amino acid transporter 1 (LAT1) (Kanai et al., 1998). The CD98 heterodimer is composed of a heavy chain (4F2 antigen heavy chain) with a type II single-pass transmembrane domain and one of the alternative multi-pass light chains. The CD98 heavy chain serves as an anchor to restrain the light chain on the cell membrane by disulfide linkage with the light chain (Nakamura et al., 1999).

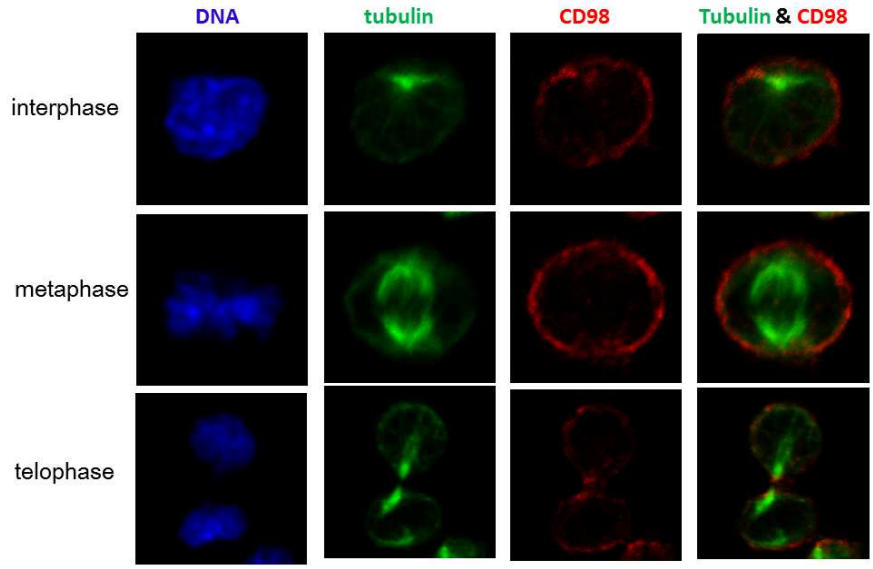
Knocking out the Slc3a2 gene, which encodes the heavy chain of CD98, blocks the proliferation of B cells and prevents their differentiation into plasma cells (Cantor et al., 2009). By contrast, knocking out Slc3a2 in T cells results in a reduced proliferation rate induced by plate bound antigens (anti-CD3 plus anti-CD28 Abs in the presence of IL-2) and modestly impaired homeostatic proliferation (Cantor et al., 2011). KN Pollizzi et. al. reported that when $CD8^+$ T cells are activated by antigen-presenting cells, the differential expression of CD98 in the cytokinetic stage contributes to asymmetric mTORC1 activity in daughter cells (Pollizzi et al., 2016).

To test whether asymmetric CD98 is associated with the observed asymmetric Pax5 in dividing B cells and Tcf1 in dividing T cells, I performed an immunofluorescence assay to trace the dynamics of CD98 in different cell cycle stages in B cells and T cells. The B cells were stimulated with LPS in vitro, whereas $CD8^+$ T cells derived from P14 transgenic mice were stimulated with gp33 peptide, because CD98 polarity has been reported to rely on antigen-presenting cell-mediated activation. In both LPS-stimulated B cells and gp33-activated P14 $CD8^+$ T cells, all of the CD98 staining is restricted to the cell membrane, and remains on the cell

surface through interphase, metaphase, and telophase (Figure 4.4). There is no evidence of CD98 polarization in metaphase or telophase cells.

On the other hand, we found that Glut1 staining was puncta or vesicle-like and localized to the microtubule-organizing center (MTOC) in interphase B cells and $CD8^+$ T cells. The most significant difference between the patterns of CD98 and Glut1 is that Glut1 is polarized to one of the MTOC in pro-metaphase cells, and such polarity can be also found in metaphase cells. We also find that in LPS-stimulated B cells and P14 $CD8^+$ T cells, telophase cells have acquired different amount of Glut1 (Figure 4.5). To demonstrate that the observation was not an artifact or staining problem, I used retroviruses expressing Glut1-GFP fusion protein and mCherry-tubulin to perform live cell imaging in LPS-stimulated B cells. We have obtained videos to validate that the GFP-containing intracellular vesicle was co-localized with the MTOC in the interphase cells (Figure 4.5, the cell on the left), and that a large GFP-positive vesicle containing Glut1 is polarized to one of the MTOC from metaphase through telophase (Figure 4.6 the cell on the right).

A



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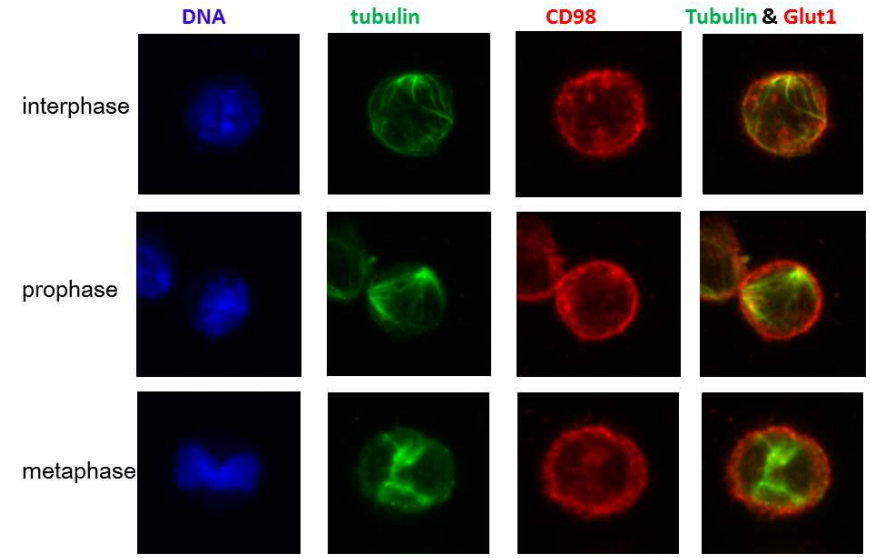
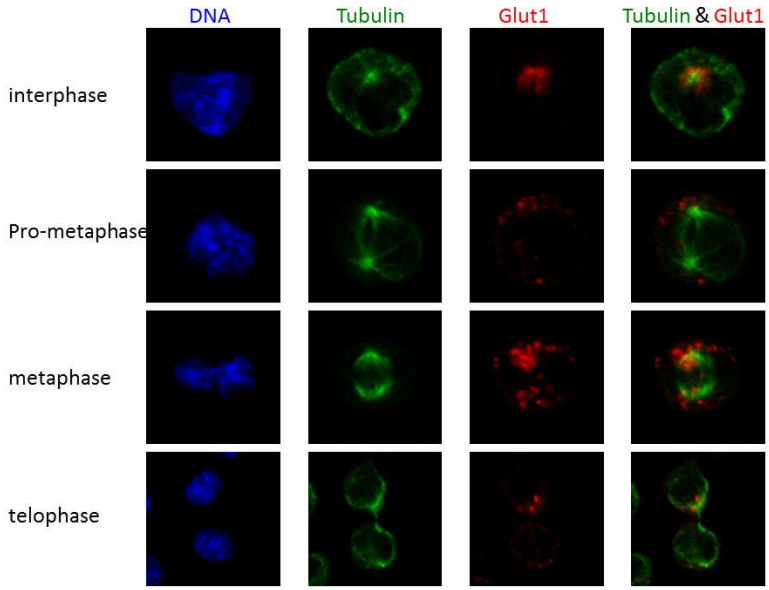


Figure 4.4 CD98 is not polarized in dividing B cells and $CD8^+$ T cells

(A) LPS-stimulated B cells are sorted for immunofluorescence staining by day 3.5 after stimulation. This plot shows representative events for interphase (n=2), metaphase (n=2) and telophase (n=6), respectively.

(B) Splenocytes derived from P14 transgenic mice were stimulated with gp33 peptide and IL-2 for 3.5 days. Enlarged splenocytes were sorted for immunofluorescence staining. This plot shows representative events for interphase (n=4), pro-metaphase (n=1) and metaphase (n=2), respectively.

A



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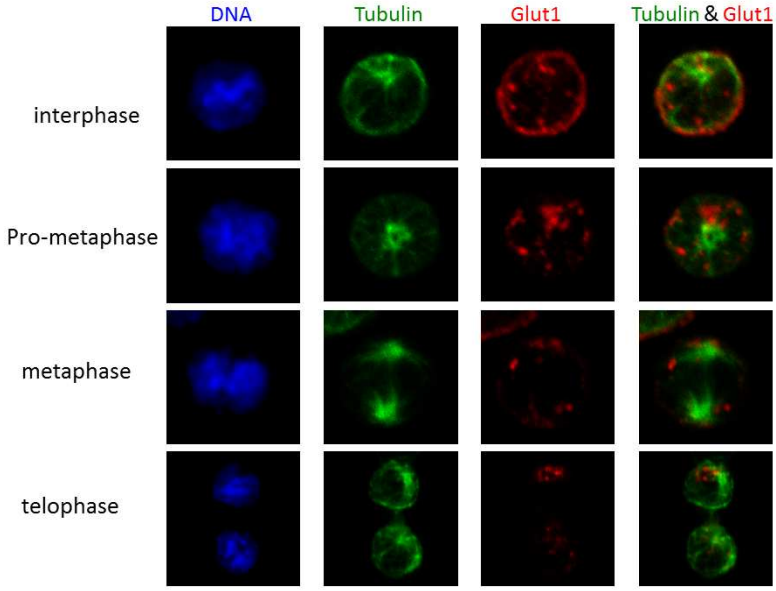


Figure 4.5 Glut1 is polarized in LPS-stimulated B cells and P14 CD8$^+$ T cells

(A) LPS (20ug/ml)-stimulated B cells were sorted for immunofluorescence staining by day 3 after stimulation. The B cells were fixed with 3% PFA in PBS, and then permeabilized with 0.3% Triton X100. After blocking, the samples were stained with rabbit monoclonal antibody to

Glut1 (EPR3915) and goat-anti-rabbit IgG Alexa Fluor® 568 dye. This plot shows representative events for interphase (n=2), metaphase (n=5), and telophase (n=9), respectively. (B) Thy 1.1^+ $P14^+$ $CD8^+$ T cells were transferred into a Thy 1.2^+ recipient animal, and then the recipient animal was infected with 5×10^3 PFUs of *Listeria monocytogenes* expressing gp33-41 (LMgp33) by i.v. injection. By 4 days after infection, enlarged, $CD8^+$, Thy 1.2^- cells were sorted for immunofluorescence analysis. This plot shows representative events for interphase (n=2), metaphase (n=5), and telophase (n=9), respectively.

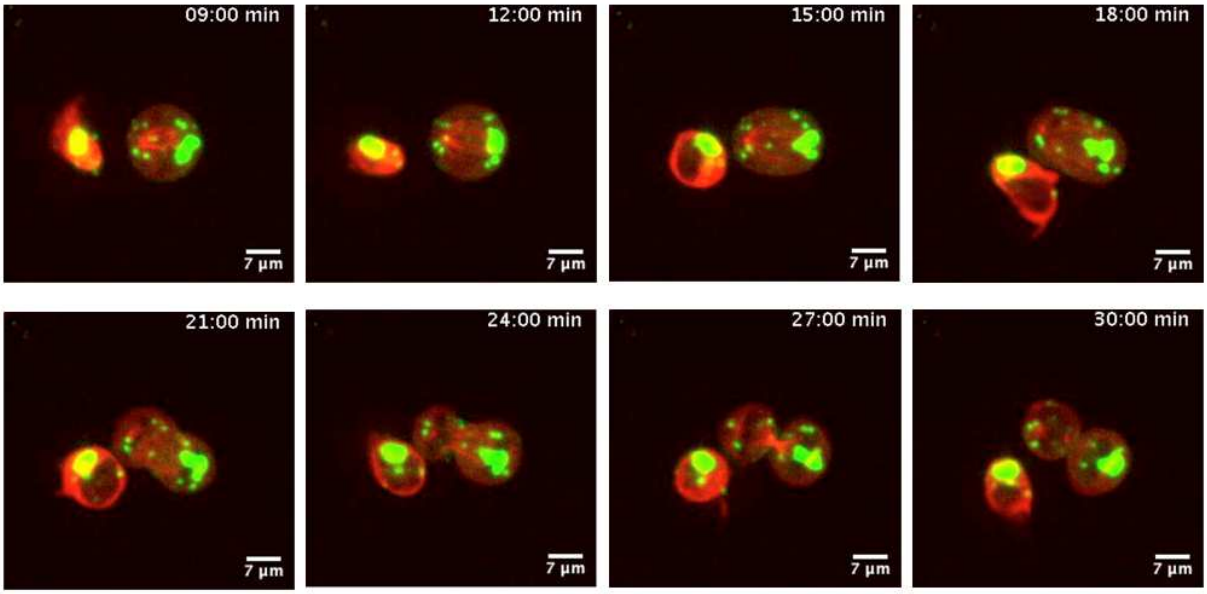


Figure 4.6 Live cell imaging reveals sibling cells inherit unequal amounts of Glut1 during cytokinesis

A representative time-lapse series of LPS-stimulated B cells (day 3) transduced with retrovirus expressing either mCherry-alpha-tubulin or Flag-tag-Glut1-GFP fusion protein 16 hours prior to imaging by spinning disk confocal microscopy. Frames, left-to-right, represent 3-minute intervals. Scale bars are 7 µm. The cell on the left is an interphase cell, whereas the cell on the right is a dividing B cell (from metaphase to cytokinesis).

4.4 Glut1 proteins are stored in intracellular vesicles

The asymmetric Glut1 level in daughter cells, prompted us to find out more details about the localization of Glut1 and the biological significance of Glut1 levels. Using retrovirus expressing Flag-tagged rat Glut1, we were able to stain surface Glut1 (by anti-Flag antibody) and intracellular Glut1 (by anti-Glut1 C-terminal antibody). The results showed that in interphase B cells, Glut1 was located in intracellular structures, whereas there was only some spot-like surface Flag-tagged Glut1 staining (Figure 4.7). Also, I found that the amount of surface Flag-tagged Glut1 in interphase cells was much higher than in metaphase cells and telophase cells (Figure 4.7). Although the staining of surface Flag-tagged Glut1 is lower than intracellular Glut1, the staining patterns of Glut1 and surface Flag-tagged Glut1 are very similar, suggesting there is a link between the localization of surface Glut1 and intracellular Glut1.

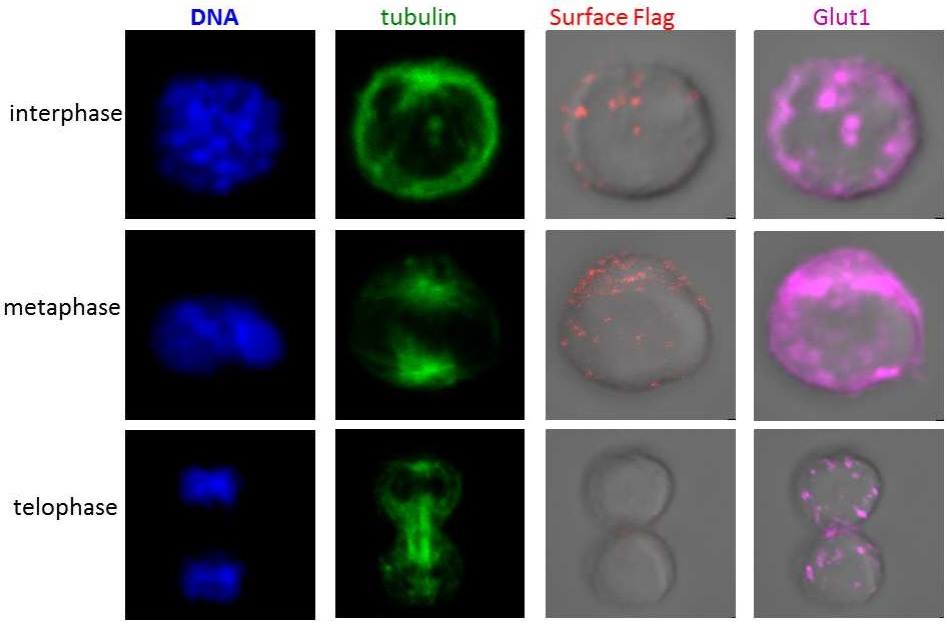


Figure 4.7 Immunofluorescence analysis of surface Glut1 and intracellular Glut1

B cells were stimulated with LPS (20ug/ml) for 40 hours before spin infection with retrovirus expressing Flag-tagged rat-Glut1 and truncated human NGFR. After being cultured for another 24 hours, the transduced cells were sorted by FACS for NGFR positivity and then subjected to immunofluorescence staining. Interphase (n=7), Metaphase (n=6), telophase (n=1).

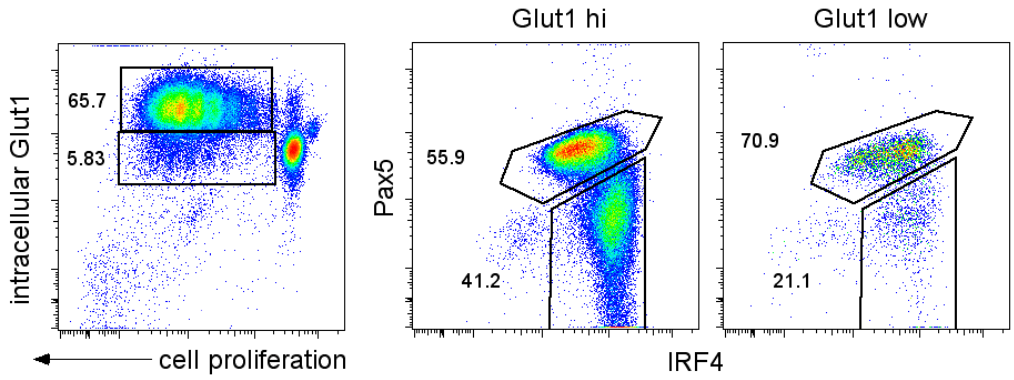
4.5 Upregulation of intracellular and surface Glut1 marks effector cell differentiation

Intracellular Glut1 staining demonstrated that the B cells which upregulated Glut1 were a heterogeneous population comprised of Pax5 high and Pax5 low cells, whereas the Glut1 low cells were enriched for Pax5 high cells, which suggests that increasing intracellular Glut1 may be associated with effector cell differentiation (Figure 4.8). We also performed confocal imaging on LPS-activated B cells and anti-CD3-, anti-CD28-, and IL- 2-stimulated $CD8^+$ T cells infected with Flag-tagged Glut1 retrovirus. Co-staining Flag-tagged Glut1 and Pax5 or Tcf1 in B cells and $CD8^+$ T cells, respectively, revealed that Pax5 high B cells exhibit low Glut, and that Tcf1 low $CD8^+$ T cells had a higher level of Glut1. Moreover, endosomal Glut1 could be found in Pax5 low B cells and Tcf1 low $CD8^+$ T cells (Figure 4.9).

Because confocal imaging has shown that surface Glut1 is localized near intracellular Glut1 vesicles, we tested whether surface Glut1 levels marked differentiation of effector cells. By using B cells and $CD8^+$ T cells derived from Myc-tagged Glut1 mice, we found that those cells which upregulate surface Glut1 have a greater probability of being identified as Pax5 low B cells or Tcf1 low $CD8^+$ T cells, suggesting that upregulated surface Glut1 may mark populations that exhibit a bias to differentiate into effector cells (Figure 4.10).

To further test whether in vivo activated T cells also upregulated Glut1 when differentiating into effector cells, we immunized mice expressing Myc-Tag-Glut1 with LCMV Armstrong strain, which is an acute infection model. At day 7 post-infection, animals were sacrificed for analysis of splenic $CD8^+$ T cells by flow cytometry. After been activated by antigen, $CD8^+$ T cells upregulated CD44 and a subset of the CD44 positive population further upregulated KLRG1 and downregulated Tcf1 to become effector T cells (W. W. Lin et al., 2016). Surface Myc-tag-Glut1 staining revealed that Tcf1 low, but not Tcf1 high, $CD8^+$ T cells upregulated surface Glut1

together with surface CD44 (Figure 4.11 A). In addition, in wild-type B6 mice infected with LCMV, we found that the intracellular Glut1 level was also positively correlated with the surface CD44 level, but not the KLRG1 level. Taken together, these data reveal that in vivo-stimulated CD8 T cells upregulated intracellular and surface Glut1 when they downregulated Tcf1, but before they upregulated surface KLRG1 (Figure 4.11 B).



Figurer 4.8 Intracellular Glut1 and Pax5 levels in B cells

B cells were activated with LPS (20ug/ml) for 3 days before FACS analysis.

Samples were stained with green live/dead dye before being fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set (eBioscience). After permeabilization, samples were stained with rabbit monoclonal antibody [EPR3915] to Glucose Transporter GLUT1 (Abcam), rat anti-Pax5 [1H9]-PerCP/Cy5.5 (BioLegend), and rat anti-IRF4 PE (eBioscience), and then with goat-anti-rabbit IgG conjugated to Alexa Fluor® 647 dye. Experiment has been repeated 2 times.

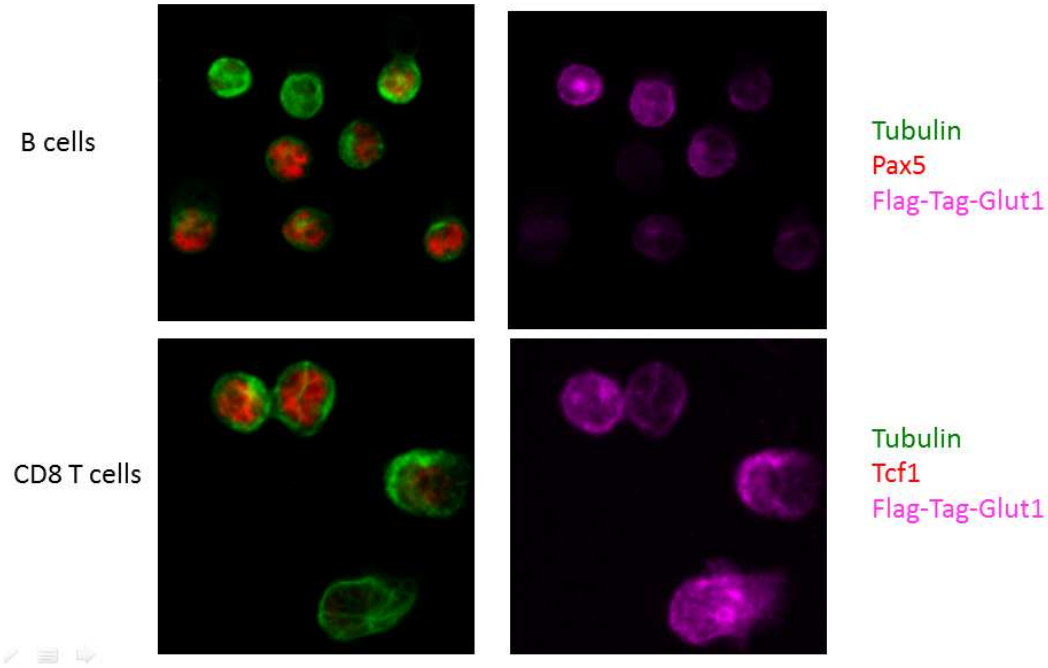
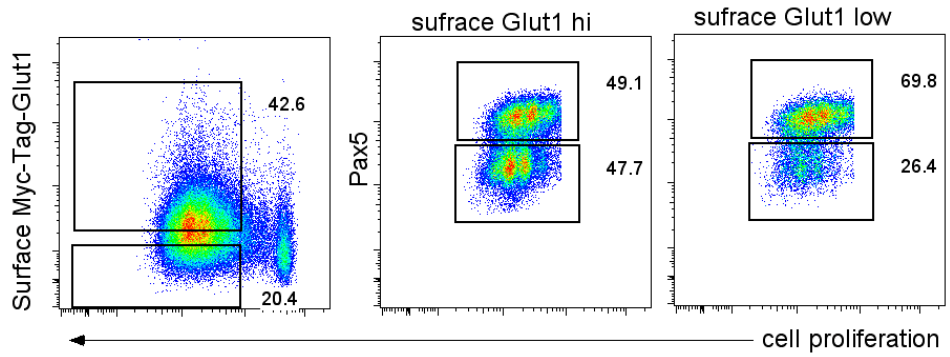


Figure 4.9 Effector cells exhibit higher Glut1 levels

B cells were activated with LPS (20ug/ml) for 40 hours before being infected with retrovirus expressing Flag-tagged Glut1 and truncated human NGFR. Similarly, $CD8^+$ T cells were stimulated by anti-CD3, anti-CD28 and IL-2 (100units/ml) for 40 hours and then infected with retrovirus expressing Flag-tagged Glut1 and truncated human NGFR. At 26 hours after retroviral infection, NGFR-positive B cells or $CD8^+$ T cells were sorted by flow cytometry for immunofluorescence staining. Flag-tagged Glut1 was stained with rat anti-DYKDDDDK-tag antibody [L5](BioLegend). Pax5 and Tcf1 were stained with rabbit anti-Pax5 and rabbit anti-Tcf1, respectively.

A



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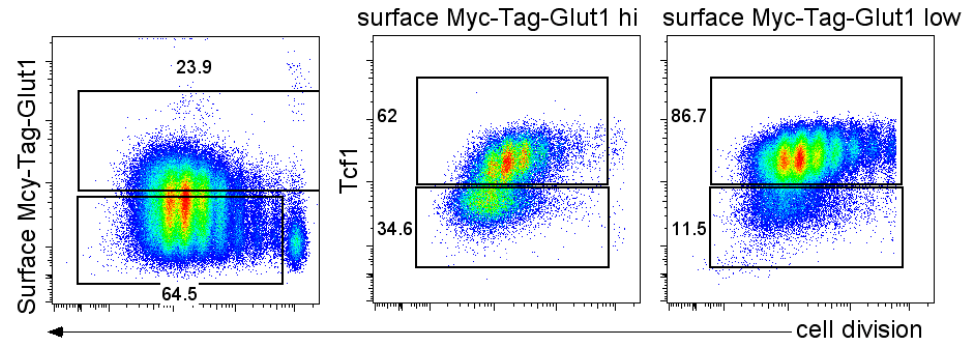
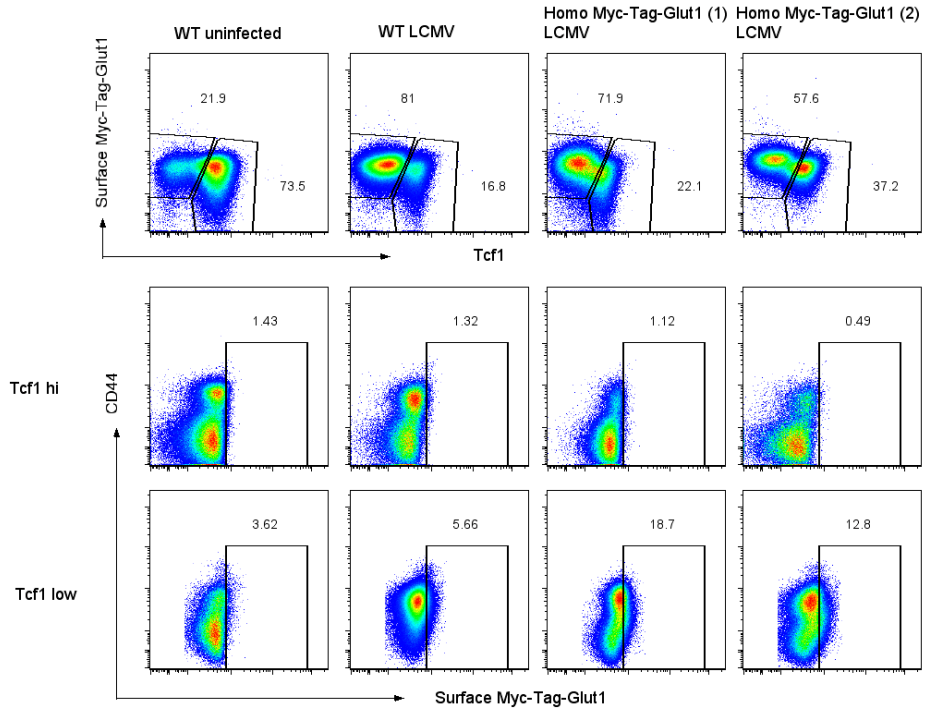


Figure 4.10 Upregulation of surface Glut1 marks effector cell differentiation

(A) B cells enriched from spleens of homozygous Myc-Tagged-Glut1 transgenic mice were stimulated with LPS (20ug/ml) for 3 days. The cells were stained with Red live/dead dye, and rabbit c-Myc antibody [71D10], and then goat anti-rabbit IgG conjugated to Alexa Fluor® 647 dye before fixation and permeabilization for intracellular anti-Pax5-PE staining.

(B) $CD8^+$ T cells enriched from spleens of homozygous Myc-Tagged-Glut1 transgenic mice were stimulated by plate-bound anti-CD3 and anti-CD28 antibody as well as IL-2 (100 units/ml) for 3 days. The samples were stained with Green live/dead dye, and mouse Myc Tag antibody [4A6], and then goat anti-Mouse IgG conjugated to Alexa Fluor® 647 dye before fixation and permeabilization for intracellular Tcf1 staining.

A



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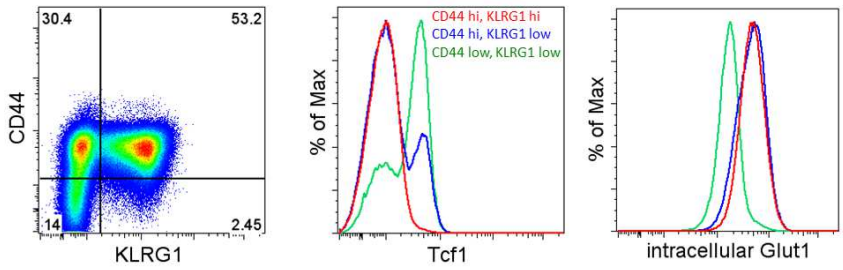

Figure 4.11 Surface Glut1 is upregulated in effector T cells in acute LMCV infection

Mice were infected with the Armstrong strain of LCMV ($2*10^5$ PFU) by intraperitoneal injection. The animals were sacrificed by day 7 post-infection. The experiments have been repeated twice. (A) In Myc-Tagged Glut1 transgenic mice (n=4), Tcf1 low $CD8^+$ T cells upregulate surface Glut1 during activation. The surface level of Glut1 increased when CD44 was upregulated. (B) Wild-type mice infected with LCMV for 7 days. The intracellular Glut1 increased as $CD8^+$ T cells increased CD44 expression. These experiments have been repeated twice.

4.6 The PI3K/mTORC1 pathway maintains intracellular and surface Glut1 levels

Three classes of PI3Ks generate different pools of the lipid second messenger, phosphoinositides 3 phosphates, on plasma membranes, endosomes, autophagosomes, and multivesicular bodies (MVBs) (Marat & Haucke, 2016). Class I PI3Ks produce predominately phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$). The assembly of the catalytic and regulatory subunits of Class I PI3Ks on membranes is directed by activated guanine nucleotide-binding protein coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs) (Jean & Kiger, 2014). $PI(3,4,5)P_3$ provides the docking sites for the pleckstrin homology (PH) domain of AKT, which then signals through mTOCRC1 and Foxo1 (Thomas, Deak, Alessi, & van Aalten, 2002; Tzivion, Dobson, & Ramakrishnan, 2011). Insulin stimulation has been reported to increase Glut1 protein content in 3T3-L1 adipocytes. This phenomenon occurs because the PI3K/PKB/mTOR cascade increases the transcription and translation of Glut1 mRNA (Taha et al., 1999).

To validate whether PI3K also regulates the total Glut1 protein level in B cells and $CD8^+$ T cells, we measured the intracellular Glut1 level by FACS after treatment with different inhibitors, including LY294002, a pan-PI3K inhibitor that targets PI3K class I, PI3K class III, and a subset of other PI3K-related kinases (such as mTOR and DNA-PK) (Gharbi et al., 2007); ZSTK474, a class I PI3K-specific inhibitor (Kong & Yamori, 2010); and rapamycin. We found that LY294002, ZSTK474, and rapamycin all exhibited a strong repression effect on intracellular Glut1 levels in both in vitro activated B cells and $CD8^+$ T cells, suggesting that the class I PI3K and mTORC1 pathway regulate the biogenesis and stability of Glut1 protein (Figure 4.12).

In addition to measuring the total intracellular Glut1 level, we also asked whether the PI3K pathway regulates the surface level of Glut1 protein. We measure the surface level of Glut1 by

using activated lymphocytes with retrovirus incorporating exofacial Flag-tagged rat Glut1 or lymphocytes derived from transgenic mice carrying Myc-Tagged–Glut1 alleles (Wardell et al., 2009; Wieman et al., 2007). The results of both assays indicated that suppressing pan-PI3K activities by LY294002, inhibiting class I PI3K, and inhibiting mTOCR1 all lead to the reduction of the surface Glut1 protein level (Figure 4.13, 4.14). Therefore, class I PI3K may act by stimulating the targeting of Glut1 to the plasma membrane, or may be required for intracellular Glut1 to be recycled back to the plasma membrane.

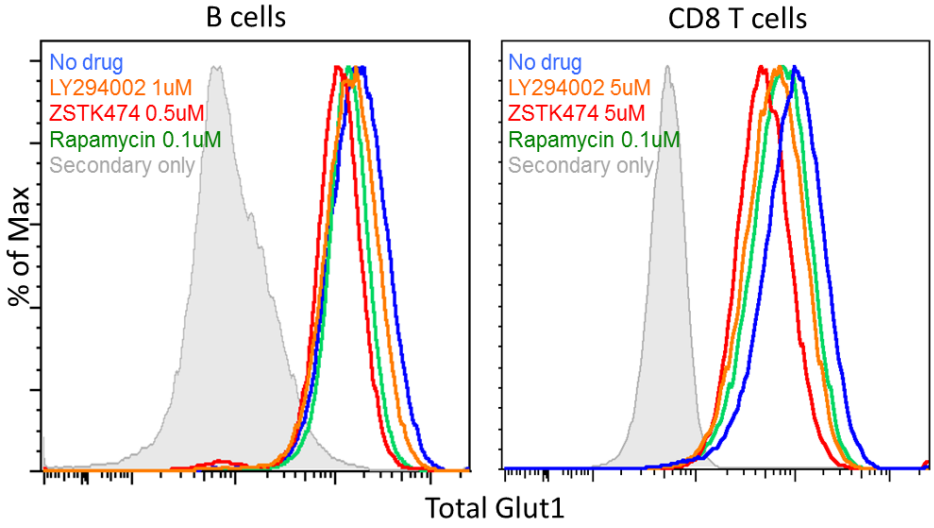

Figure 4.12 Class I PI3K and mTOCR1 regulate intracellular Glut1 levels

(Left) B cells were stimulated by LPS (20ug/ml) for 2.5 days before the addition of drugs and then harvested for FACS analysis 36 hours after the addition of the drugs. (Right) $CD8^+$ T cells derived from homozygous Myc-tagged Glut1 transgenic mice were stimulated with anti-CD3, anti-CD28, and IL-2 (100 units/ml). Drugs were added 2.5 days after activation, and then the cells were cultured for an additional 18 hours before being harvested for FACS analysis.

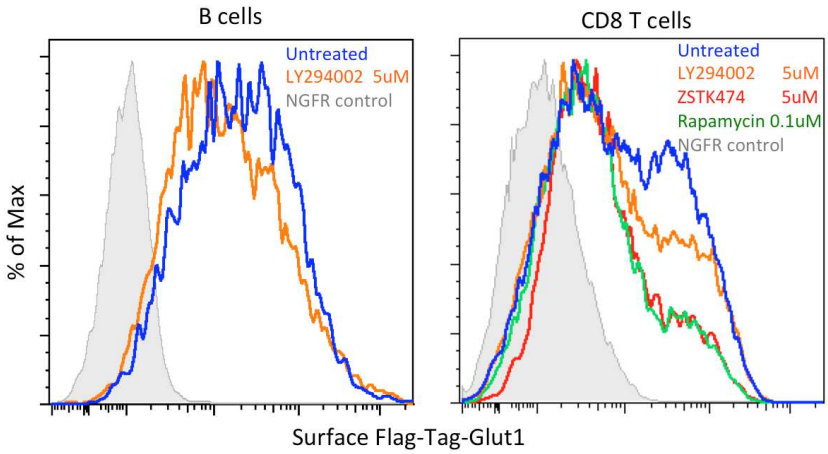

Figure 4.13 Class I PI3K and mTOCR1 regulate surface Glut1 levels

(Left) B cells were activated with LPS (20ug/ml) for 40 hours before infection with retrovirus carrying either Flag-tagged rat Glut1 IRES truncated human NGFR (tNGFR) or pMIG IRES tNGFR empty vector. Drugs were added 8 hours after spin infection. Infected cells were incubated with the drugs at indicated concentration for 18 hours before being harvested for FACS analysis.

(Right) $CD8^+$ T cells derived from wild-type B6 mice were stimulated with anti-CD3, anti-CD28, and IL-2 (100 units/ml) for 40 hours before spin infection. Activated $CD8^+$ T cells were transduced with retrovirus carrying either Flag-tagged rat Glut1 IRES tNGFR or pMIG IRES tNGFR empty vector. Drugs were added 8 hours after transduction and then the cells were cultured for 18 more hours before being harvested for FACS analysis.

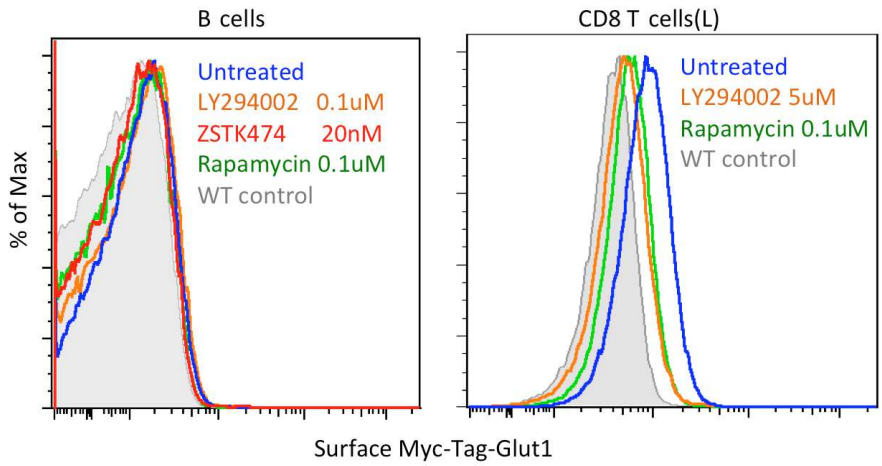

Figure 4.14 Class I PI3K and mTOCR1 regulate surface Myc-tagged Glut1 levels

(Left) B cells derived from homozygous Myc-tagged Glut1 transgenic mice were stimulated with LPS for 2 days before drug was added. After drug addition, the B cells were further cultured for 18 hours before FACS analysis with Rabbit Myc-Tag antibody and Goat-anti-Rabbit IgG conjugated to Alexa Fluor® 647 dye.

(Right) $CD8^+$ T cells derived from homozygous Myc-tagged Glut1 transgenic mice were stimulated with anti-CD3, anti-CD28, and IL-2 (100 units/ml). Drugs were added 2.5 days after activation, and cells were then cultured for additional 18 hours before being harvested for FACS analysis. The experiments have been repeated twice.

4.7 Inhibition of PI3K abolishes Glut1 polarity in dividing lymphocytes

To understand the effect of PI3K on Glut1 localization, we performed immunofluorescence staining to compare the localization of Glut1 in different stages of cell division. We found that PI3K inhibitor LY294002-treated B cells lost polarization of Glut1 vesicles to the MTOC in prophase (the two MTOCs were not positioned on the opposite sides of the dividing cells, and the chromatin has not yet condensed,) and metaphase (MTOC were positioned on the opposite sides of the dividing cells, and the chromatin has condensed and lined up in *the center of* the *cell*) (Figure 4.15). Moreover, PI3K inhibitor LY294002 abolished the asymmetric Glut1 phenotype in cytokinetic cells. In the presence of PI3K inhibitor, Glut1 vesicles are equally distributed into the two daughter cells, suggesting that the polarization of Glut1 in prophase and metaphase requires PI3K to establish polarization signals. Notably, in all of the images, PI3K-treated cells exhibit lower Glut1 staining, which is in consistent with the our FACS data, in which PI3K inhibitor also reduced the level of intracellular Glut1.

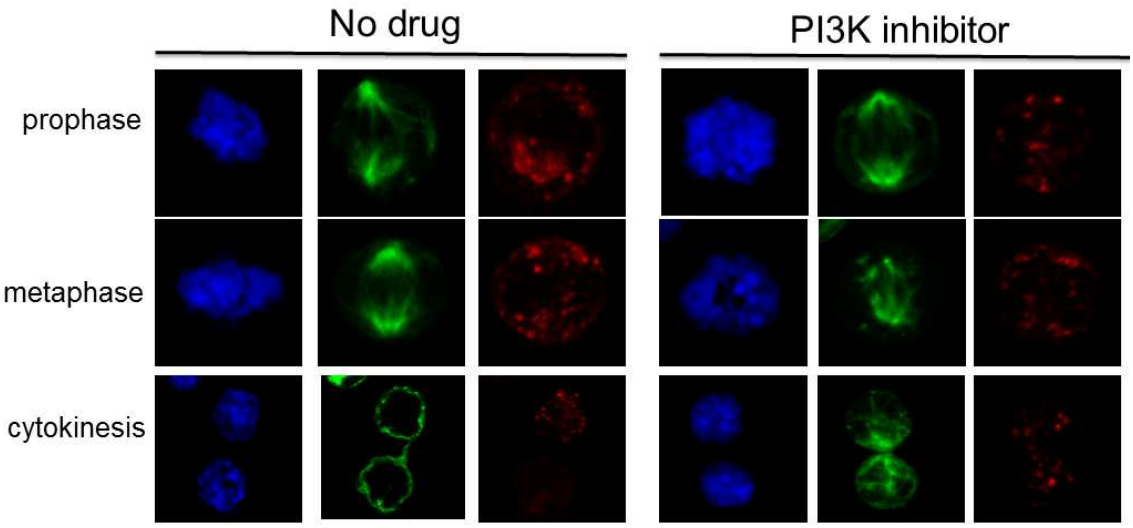

Figure 4.15 PI3K inhibitor abolishes asymmetric Glut1 localization

B cells derived from wild-type B6 mice were stimulated with LPS (20ug/ml) for 3 days before being sorted for immunofluorescence staining. PI3K inhibitor LY294002 (5uM) was added directly into the culture media 18 hours before sorting.

4.8 Rab11a regulates surface and intracellular Glut1 levels and is required for polarization and asymmetric segregation of Glut1 vesicles in dividing cells

The PI3K signaling pathway has been shown to enhance surface Glut1 levels in response to IL-3 stimulation through a Rab11a-mediated pathway in the murine prolymphocytic cell line FL5.12 (Wieman et al., 2007). To validate whether this pathway is also responsible for the trafficking of Glut1 in mature lymphocytes, we infected $CD8^+$ T cells derived from Myc-tagged Glut1 mice with retrovirus expressing wild-type or dominant negative Rab11a (S25N) fused to GFP to measure the surface Glut1 level with Myc-Tag antibody and the intracellular Glut1 protein level by anti-mouse Glut1 antibody. The results showed that expression of dominant negative Rab11a increased the intracellular Glut1 level at the expense of reducing the surface Glut1 level, suggesting that abolishing the GTPase activity of Rab11a (the GDP-locked form) led to the accumulation of intracellular Glut1 and reduced the recycling of Glut1 back to the plasma membrane (Figure 4.16) . This result is comparable to what has been reported in the IL-3 prolymphocytic cell line FL5.12.

In addition to Glut1 levels, we also measured the effect of expressing dominant negative Rab11a on the transcriptional program during lymphocyte proliferation and differentiation. Retrovirus expressing dominant negative Rab11a suppressed Pax5 but upregulated IRF4 in LPS-stimulated B cells (Figure 4.17 A). Similarly, overexpression of dominant-negative Rab11a suppressed Tcf1 in in vitro activated $CD8^+$ T cells (Figure 4.17 B). Thus, overexpressing dominant negative Rab11a promotes effector cell differentiation.

Finally, we performed confocal analysis to determine the effect of over-expression of dominant negative Rab11a on the localization of Glut1 vesicles in dividing lymphocytes. In B cells, overexpression of GFP-wild type Rab11a increased the amount of intracellular Glut1

vesicles. Although some the vesicles are not located near MTOC, most Glut1 vesicles were still polarized to the MTOC in interphase and metaphase. Also, daughter cells exhibiting asymmetric amounts of Glut1 could be found in 50% of the cytokinetic pairs. By contrast, overexpression of GFP-dominant negative Rab11a increased the amount of intracellular Glut1 vesicles, but these vesicles were not co-localized with the MTOC, and their distribution was more diffuse in the metaphase cells. In almost all of the observed cytokinetic cells, both daughter cells inherited equal amounts of Glut1, suggesting that asymmetric Glut1 distribution and polarization to the MOTC are both dependent on a Rab11a-mediated pathway (Figure 4.18). This loss of polarity and asymmetric Glut1 phenotype is similar to the observation in PI3K inhibitor Ly294002-treated cells, implying the PI3K/mTORC1 pathway may provide polarized signals for Glut1 vesicles through regulating the GTPase activity of Rab11a.

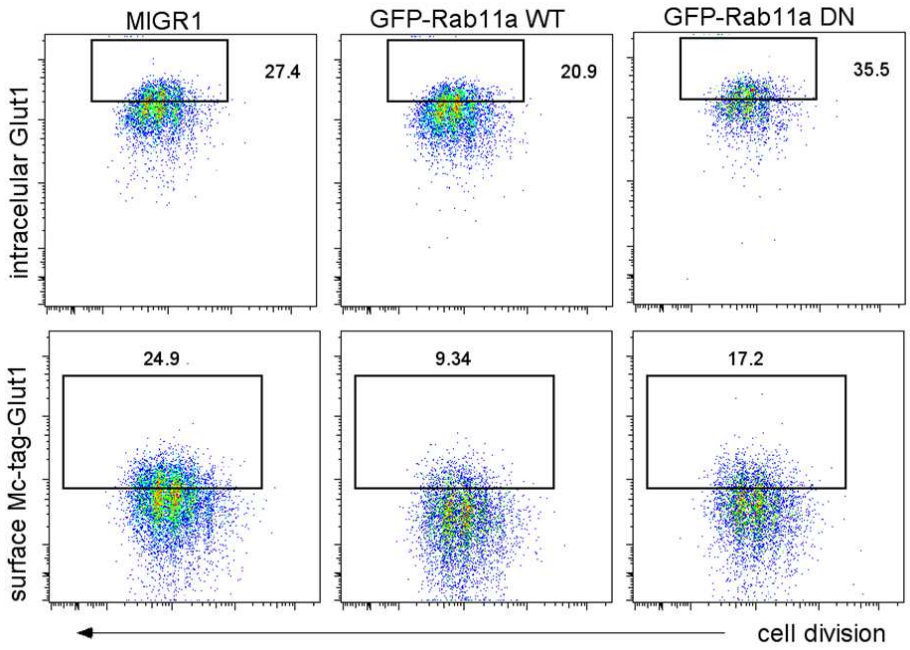

Figure 4.16 Rab11a regulates surface and intracellular Glut1 levels

$CD8^+$ T cells were enriched from the spleen of homozygous Myc-tagged Glut1 ansgenictr mice and stimulated for 40 hours by plate-bound anti-CD3, anti-CD28, and IL-2 (100 units/ml) in the media. Activated cells were infected with retrovirus carrying GFP-wild-type Rab11a, GFP-dominant negative Rab11a (S25N), or GFP-expressing control vector (MIGR1) and cultured with plate-bound anti-CD3, anti-CD28, and IL-2 in the media for 18 hours before FACS analysis. The surface Myc-tagged Glut1 was stained by Mouse Myc-Tag antibody (Clone 4A6) with Goat anti-mouse IgG conjugated to Alexa Fluor® 647 dye, whereas the intracellular Glut1 was detected by rabbit monoclonal [EPR3915] antibody to Glucose Transporter GLUT1 with Goat anti-rabbit IgG conjugated to Alexa Fluor® 647 dye.

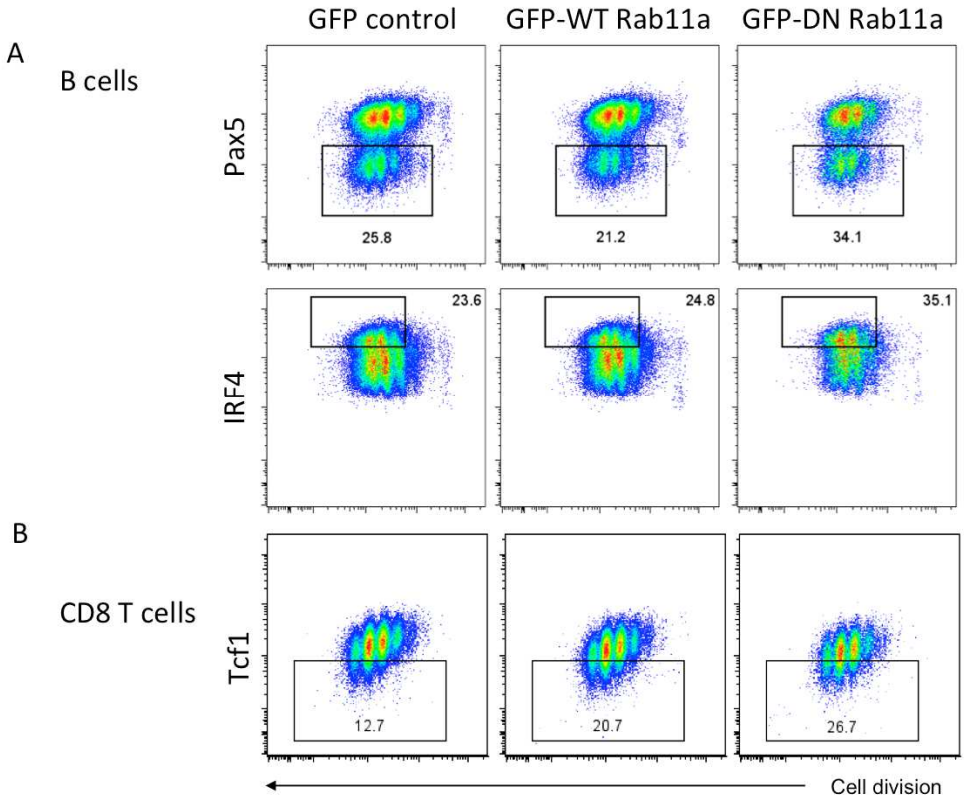

Figure 4.17 Dominant-negative Rab11a induced effector cell differentiation

B cells were stimulated by LPS (20ug/ml) and $CD8^+$ T cells were stimulated by plate bound anti-CD3 and anti-CD28 with IL-2 (100units/ml) in the media for 40 hours before retroviral transduction. Activated cells were transduced with MIGR1 (GFP control), GFP-Wild-type Rab11a, or GFP-dominant negative Rab11a. The expression of dominant-negative Rab11a inhibits Pax5 and Tcf1 in B cells and $CD8^+$ T cells, respectively. The experiments have been repeated twice.

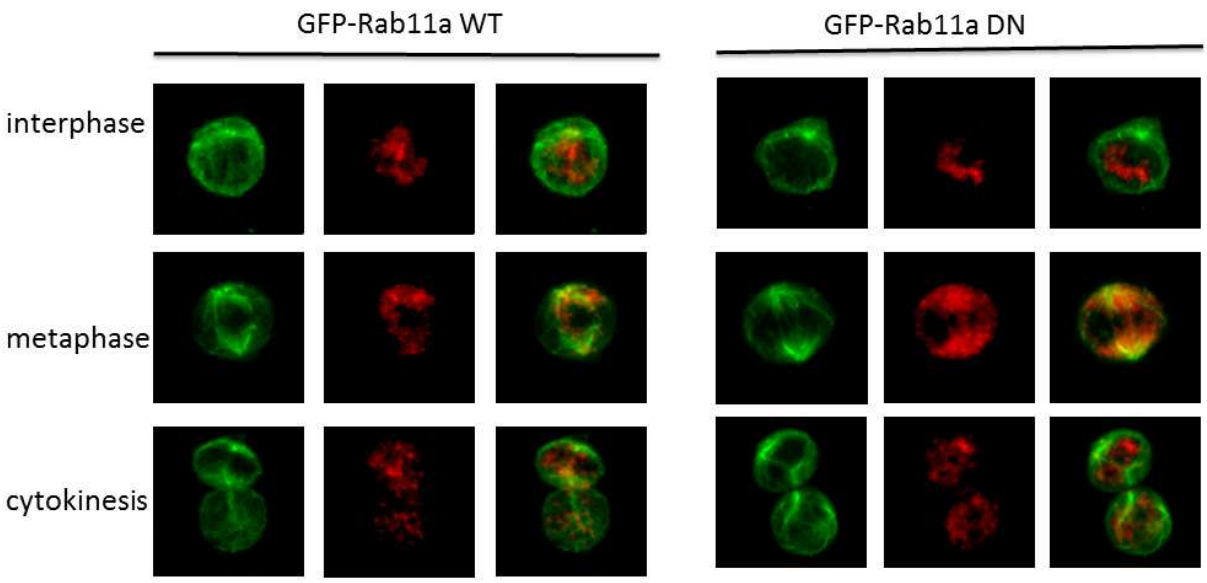

Figure 4.18 Overexpression of dominant-negative Rab11a abolishes the Glut1 asymmetric phenotype in dividing lymphocytes

B cells were activated with LPS for 40 hours before spin infection with retrovirus carrying GFP-Rab11a WT, GFP Rab11a-dominant negative, or MIGR1 (GFP control). The GFP-positive cells were sorted by day 3 after activation. This chart shows confocal image analysis of Glut1 protein staining in representative interphase (WT: n=9, DN: n=5), metaphase (WT: n=21, DN: n=16), and cytokinetic (WT: n=7, DN: n=3) B cells.

4.9 Lis1 regulates the polarization of Glut1-containing intracellular vesicles toward MTOC

By using CD4-Cre to conditionally delete Lis1 in $CD4^+$ and $CD8^+$ T cells, we found that P14+ $Lis1^{f/f}$ CD4-Cre $CD8^+$ T cells divided relatively slower compared to P14+ $Lis1^{f/f}$ $CD8^+$ T cells when stimulated by gp33 and IL-2. The $Lis1^{f/f}$ CD4-Cre $CD8^+$ T cells also showed relatively lower Tcf1 repression compared to $Lis1^{f/f}$ siblings (Figure 4.19).

Confocal analysis revealed that most of the cultured $P14^+$ $CD8^+$ T cells derived from the $Lis1^{f/f}$ CD4-Cre animal lack intracellular vesicles containing Glut1, but these T cells still have strong Glut1 staining near the cell membrane, suggesting knocking out Lis1 mainly affects recycling endosome but not surface Glut1 (Figure 4.20). In some of the interphase cells with intracellular Glut1 vesicles, these vesicles are not polarized toward the MTOC, which is similar to the phenotype of PI3K inhibitor-treated $CD8^+$ T cells. This result also confirms that the formation of large Glut1 vesicles near MTOC requires motor protein and vesicle transportation and therefore the large GFP-positive vesicles observed in live cell imaging of Glut1-GFP are not the result of GFP aggregation in the cells (Figure 4.7).

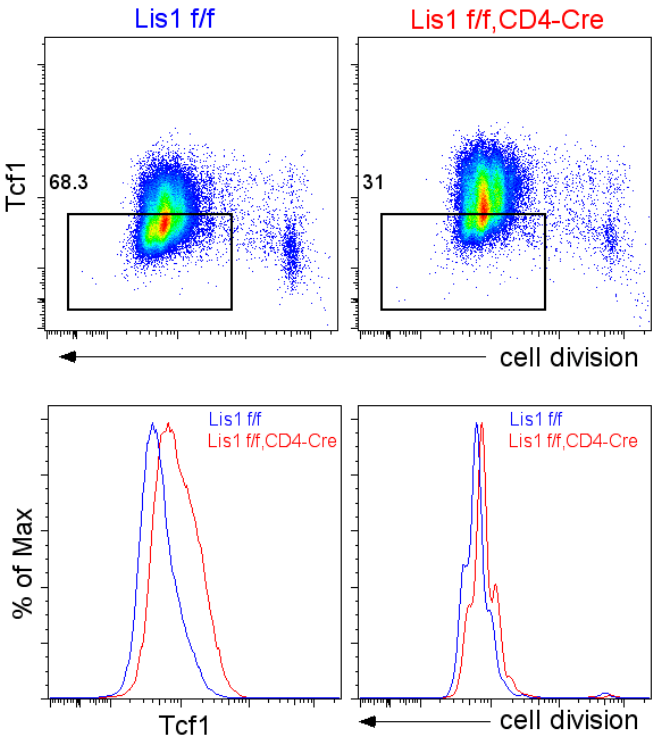

Figure 4.19 Knocking out Lis1 affects effector cell differentiation

The splenocytes derived from Lis1$^{f/f}$, CD4-Cre+, P14+ and Lis f/f, Cre negative, P14^{+} animals were labeled with CellTrace^{TM} Violet proliferation dye and then activated by gp33 (1ug/ml) and IL-2 (100 units/ml). Activated cells were harvested for analysis after being cultured for 80 hours. (Upper) FACS analysis of Tcf1 repression in Lis1 KO cells (Lis1$^{f/f}$, CD4-Cre+) and control cells (Cre-negative). Y-axis represents the Tcf1 staining, whereas the X-axis shows the cell proliferation dye staining (CellTrace^{TM} Violet).

(Lower Left): A histogram comparing the Tcf1 level in Lis1 KO cells and control cells.

(Lower Right): A histogram comparing the number of cell divisions in Lis1 KO cells and control cells.

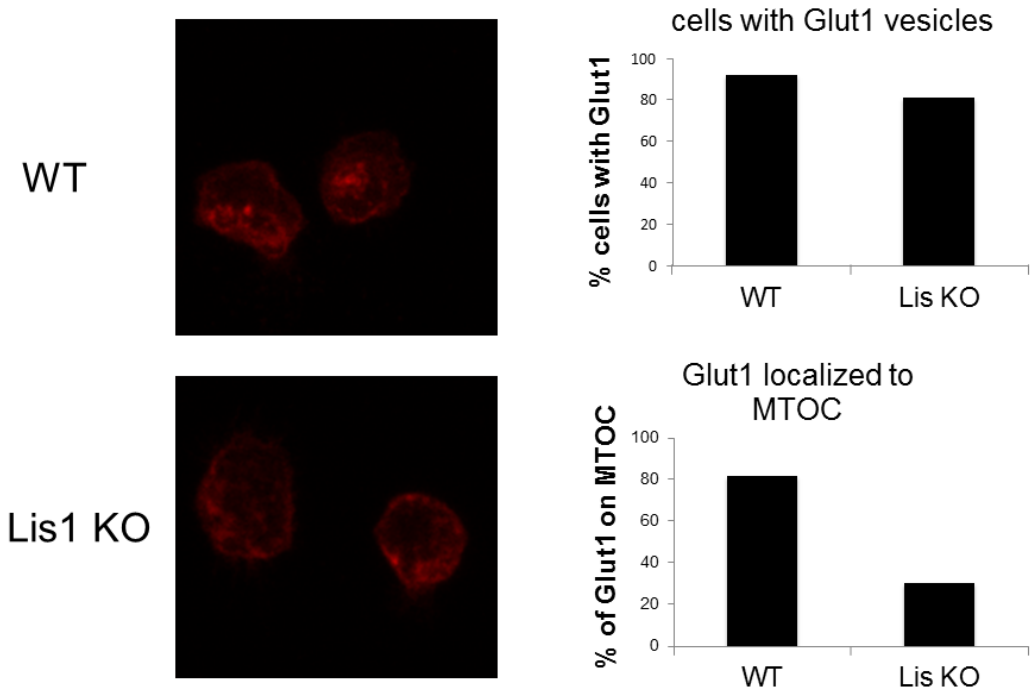

Figure 4.20 Lis1 is required for Glut1 vesicles to polarize to the MTOC

The splenocytes derived from Lis1$^{f/f}$, CD4-Cre+, P14+ (Lis1 KO) and Lis$^{f/f}$, Cre negative, P14+ (WT) animals were activated by gp33 (1ug/ml) and IL-2 (100 units/ml). Activated cells were harvested for analysis after 3 days of culture. (Left) Representative interphase cells in Lis1 KO and WT animals. (Right) The bar chart compares the frequency of cells positive for Glut1 staining (upper), and the percentage of cells with Glut1 vesicles polarized to the MTOC. The interphase cells in WT (n=38) and Lis1 KO (n=37) were imaged by confocal microscopy.

Chapter 5: Discussion

5.1 Is remodeling mitochondrial fusion and fission sufficient to alter effector and memory cell differentiation?

In a recent paper, Pearce and her colleagues proposed a model suggesting that enforcing mitocohondrial fission in effector cells reprogrammed their metabolism and imposed a memory cell phenotype(Buck et al., 2016). The authors stated that perturbing the fission and fusion of mitochondria is sufficient to reprogram the metabolism of effector cells and that mitochondrial morphology is a determining factor in T cell fate. Their data showed that the formation of a memory cell-like population was enhanced by adding mDivi-1 (10uM) to the culture media by day 3. However, our data indicate that treating cells with Mdivi-1 from the beginning of the culture accelerates the downregulation of Tcf1, implying that suppressing mitochondrial fission promotes effector cell differentation. At the first glance, these results are in conflict with each other. However, our experiment reveals that the efficiency of mDdivi-1 mediated repression of Pax5 or Tcf1 is dependent on the time point with which it is added to the culture. When Mdivi-1 or CCCP is added to the stimulated B cells or T cells within the first 36 hours after stimulation, the downregulation of Tcf1 in T cells and of Pax5 in B cells, respectively, is accelerated. The repression effect is less obvious when Mdivi-1 or CCCP is added 48 hours after stimulation. This data suggests that two important determinants for the effect of inhibiting mitochondrial fission are the number of divisions the cultured cells have undergone before drug treatment and the incubation time of the cultured cells with the drug.

We explained that Mdivi-1 did not cause acute damage to mitochondria with short-term treatment, but in the long-term, inhibition of the mitochondrial fission mechanism leads to accumulation of damaged mitochondrial compartments. Clearly, whereas fusion of relatively

healthy mitochondria may increase metabolic function, fusion of damaged mitochondria will not prevent the further accumulation of damage. Thus, we believe that the Pearce model which states that "fission favors effector and fusion favors memory" is oversimplified. The coordinated fission and autophagic turnover of mitochondria by AMPK are probably extremely important for memory.

5.2 Do AMPK and mTORC1 work cooperatively in rapidly proliferating lymphocytes?

It is well known that AMPK and mTORC1 are activated under opposite nutrient conditions. AMPK is activated under nutrient-deficient conditions, whereas mTORC1 is activated under nutrient-high conditions. Moreover, AMPK suppresses mTORC1 by phosphorylating Raptor and other mTORC1 associated proteins, such as TSC2 (Gwinn et al., 2008; Inoki et al., 2006). However, we found that even in normal culture conditions, B cells or T cells derived from an AMPK knockout animal exhibit a tendency for early downregulation of Pax5 or Tcf1, respectively, compared with B cells or T cells derived from wild-type animals. It is unlikely that this phenomenon is due to the cells running out of available nutrients after 4 days in culture, because the AMPK knockout cells start to downregulate Pax5 or Tcf1 after the first 2 divisions. These data suggest that even in the presence of sufficient nutrients and growth factors, rapidly proliferating cells may gradually subject to cellular stress responses. The stress may come from misfolding protein response of the endoplasmic reticulum and enhancing ROS generation during ATP production.

5.3 The linkage between PI3K and Glut1 trafficking

The detailed mechanism of how PI3K maintains intracellular and surface Glut1 protein levels remains unclear. Most of the earlier studies use LY294002 as a reversible PI3K inhibitor (Doughty et al., 2006; Wieman et al., 2007). Unfortunately, LY294002 has been found to inhibit several PI3Ks, including the PI3K p110 gamma isoform, and even other protein kinases (Gharbi et al., 2007). Therefore, we cannot rule out the possibility that the observed Glut1 alteration after LY294002 treatment is due to protein kinases other than PI3Ks. Also, LY294002 cannot selectively inhibit different PI3K isoforms; and thus more elegant experiments combining

inducible and conditional gene targeting should be performed to confirm which PI3K subunits are responsible for Glut1 trafficking, membrane docking, and degradation. Finally, LY294002 could also inhibit the effect of mTORC1 and therefore we cannot determine whether its effect was directly mediated by inhibition of PI3Ks or was through its off-target effect on mTORC1 (Brunn et al., 1996).

One of the potential links between PI3K and Glut1 trafficking may be WNK1. WNK1 is a protein kinase that regulates the Tre-2/USP6-BUB2-Cdc16 Domain Family Member 4 (TBC1D4)-Rab8A Complex, which is required for translocation of Glut1 to the surface of cell membranes (Mendes, Matos, Moniz, & Jordan, 2010). WNK1 can be phosphorylated on Thr60. In 3T3-L1 cells, insulin-like growth factor I-stimulated WNK1 phosphorylation could be blocked by the irreversible PI3K inhibitor wortmannin and RNA interference-directed depletion of Akt1 and Akt2, but not by rapamycin (Jiang et al., 2005). These results suggest the involvement of the PI3K/AKT pathway in WNK1 phosphorylation, but not mTORC1.

Interestingly, a recent study demonstrated the role of WNK1 in T cells to promote cell migration but to attenuate integrin-mediated adhesion. We are looking forward to testing whether targeting WNK1 would induce lost polarity of Glut1 in dividing B and T cells, just as in LY294002 treated cells. If so, the result will support a model that PI3K/AKT regulate WNK1-mediated Glut1 trafficking through phosphorylation of WNK1 (Kochl et al., 2016).

5.4 PI3K, AKT, mTORC1, and Glut1 surface expression

A further question about how PI3K regulates Glut1 surface expression is whether AKT and mTOCR1 are also involved in optimizing the trafficking of Glut1. The first paper which described that inhibiting PI3K activity blocks Glut1 surface expression in B cells was published in 2006 (Doughty et al., 2006). The authors demonstrated that the abundance of Glut1 protein in splenic B cells activated by F(ab')2 fragments of anti-mouse IgM is reduced by treatment with PI3K inhibitor LY294002 and deletion of p85α, a key subunit of PI3K, suggesting that the PI3K pathway is contributing to regulating the expression of Glut1. This paper also noted that the total Glut1 protein level in these anti-IgM activated wild-type B cells was not affected by rapamycin treatment.

The next year, J C Rathmell and his colleagues published a paper using the early hematopoietic myeloid/lymphoid cell line FL5.12 expressing exofacial-FLAG epitope tagged rat Glut1 to study the effect of altering PI3K and AKT activity on the surface expression of Glut1 (Wieman et al., 2007). The result of a pulse-chase assay showed that removing cytokine IL-3 from the media accelerated the turnover rate of labeled surface Glut1. Moreover, treatment with the PI3K inhibitor LY294002 blocked the IL-3 induced Glut1 surface expression, but did not affect the total protein level of FLAG-Glut1. Expressing myristoylated and constitutively active Akt (myrAkt) or phospho-mimetic (S473D/T308D; AktDD) forms of Akt1 is sufficient to maintain the surface FLAG-Glut1 level in the presence and absence of IL-3. Overexpressing myrAkt enhanced surface FLAG-Glut1 expression. Such phenotype could not be reverted by treatment with rapamycin, suggesting that it is the activity of AKT, but not mTORC1, that is responsible for modulating surface Glut1 trafficking. One of the caveats of this study is that they did not use wild-type lymphocytes and could not rule out the effect of rapamycin on the

efficiency of transient transfection of myrAkt.

5.5 The dynamics and localization of Glut1 vesicles

Our live cell imaging and immunofluorescence staining have demonstrated that Glut1-containing vesicles are polarized in dividing lymphocytes. The surface level of Glut1 is regulated by endocytosis and endocytic recycling. After endocytosis, cargo proteins can be transported through a series of endosome compartments. The major compartments are early endosomes, late endosomes, and the endocytic recycling compartment (ERC) and recycling endosomes (Grant & Donaldson, 2009). Early endosomes are coated with Rab5 and early endosome antigen 1 (EEA1), whereas recycling endosomes are marked by Rab11a. Late endosomes can be stained with LysoTracker, which is a dye that stains acidified vesicular structures in live cells.

Live cell imaging using B cells infected with retrovirus expressing Glut1-mCherry fusion proteins and GFP-Rab11a fusion proteins reveals that part of the Glut1 vesicles are co-localized with GFP-Rab11a, suggesting a subset of the Glut1 vesicles are in the recycling endosomes. Further experiments are needed to identify what kind of endosome is asymmetrically segregated in dividing lymphocytes, and how the PI3K/mTORC1 pathway regulates the polarity of such endosome.

5.6 New evidence of asymmetric cell division on regulating self-renewal versus effector cell differentiation

Traditionally, the primary role of asymmetric cell division in mature lymphocytes was understood to be in the first cell division, when lymphocytes are activated by antigen-presenting cells (Oliaro et al., 2010). Also, the driver of different fates of the sibling cells was thought to be the asymmetric abundance of transcription factors, such as T-bet and Tcf1 in T cells, or Bcl6, Pax5, and IRF4 in B cells (Barnett et al., 2012; J. T. Chang et al., 2011; W. W. Lin et al., 2016; Nish et al., 2016). However, in recent years, several studies and our work have suggested that signals regulating metabolism and nutrient sensing also exhibit differences between the sibling daughter cells and these asymmetric phenotypes are not limited to the first cell division. Such signals include PI3K and mTOCR1 signaling, resulting asymmetric Foxo1 activity, phosphorylated-S6 abundance, and c-Myc protein level (W. H. Lin et al., 2015; Pollizzi et al., 2016).

In this study, I further identify two mechanisms that contribute to establishing the differential fates of the sibling cells: asymmetry of mitochondrial stasis and glucose transporter. Both asymmetric phenotypes can be observed in the absence of antigen-presenting cells, suggesting these mechanisms can establish cell-intrinsic polarity cues. My experiments show that metabolism, mitochondria, and transcriptional programming interplay with each other to amplify the differences between the sibling cells. Thus, asymmetric cell division and cell differentiation are continuous and progressively changing processes.

Once the self-amplifying signals pass through a threshold, the cells enter a transition point, where further stimulation will generate siblings with distinct transcriptional programs. Some of the sibling cells will lose self-renewal ability, leading to irreversible effector cell differentiation,

whereas the other cells maintain self-renewal ability and differentiation potential. Therefore, a clone can be used, but not lost, to generate daughter cells with diverse functions.

We found that effector cells generate more ROS compared to self-renewing cells because they have a lower ability to renew aged mitochondria and a higher capacity for aerobic glycolysis. Therefore, my studies also provide methods to control the direction of effector cell differentiation. Treatment with ROS scavengers and glycolysis inhibitors during cell division can suppress the downregulation of lineage-maintaining transcription factors. This technique would be helpful in developing better methods for making memory-like lymphocytes for immune therapies, in identifying methods for preventing exhaustion of activated lymphocytes in a cancer micro-environment, and for improving the quality of lymphocytes derived from aged patients.

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Appendix

Primer list

number	Primer name	Primer sequence	usage	
001	Tbet-GFP-FGM-Fr	CCTTGAACCTCCTCGTTCGAC	Sequencing MIGR1 from upstream to CDS	
002	Tbet-GFP-FGM-Rev	TTGTGGCCGTTTACGTCGC	Sequencing CDS before the 5' end of EGFP, 80bp downstream to start	
003	BglII-ratGlut1-5e-Fr	ATGACAGATCTGCCACCATGGAGCCCAGCAGCAAGAAG	Cloning rat Glut1	
004	EcoR1-ratGlut1-3e-FGM	ATGACAGAATTCTCACTTGGGAGTCAGCCCCCAG	Cloning ratGlut1 to FGM	
005	EcoR1-ratGlut1-3e-CheFM	ATGACAGAATTCCACTTGGGAGTCAGCCCCCAG	Cloning rat Glut1 to CheFM	
006	BamHI-msDrp-1_5e-Fr	ATGACCAGGATCCGCCACCATGGAGGCGCTGATCCCGGTC	Cloning DN Drp-1	
007	msDrp-1-3e-XhoI-BamHI-Rv	ATGACCAGGATCCACCCTCGAGTTGTCACCAAAGATGAGTCTCTCGGATTTC	Cloning DN Drp-1	