# Toll-like receptor 2/7 activation enhances the metabolism and functions of CD8+ T and B cells

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# 1. Introduction

## 1.1 T/B Lymphocytes and host immunity

### 1.1.1 CD8+ T cells and host immunity

CD8+ T cell response is extremely important to protect our body from various pathogens. It is crucial for the clearance of viruses, protozoan pathogens, intracellular bacteria, and cancerous cells. Depletion of CD8+ T cells results in impaired host defense, prolonged viral infection, sustained tumor growth and delayed disease progression.[1,2] Despite lacking inflammatory cytokine secretion, CD8+ T cell deficiency also impairs the control of viral infections and leads to fatal inflammation.[3,4] On the other hand, immunologically naïve individuals adoptively transferred with CD8+ T cells can survive lethal infection. Also, immunocompromised recipients adoptively transferred with CD8+ T cells are able to control viral infection.[5] Thus, CD8+ T cell responses provide protective immunity, but the absence of CD8+ T cell response leads to impaired immunity of infected individuals.

CD8+ T cells mediate non-cytolysis and cytolysis mechanisms to promote viral clearance at the site of infection or accelerate the elimination of tumor cells.[6-10] Direct cytolysis of target cells is mediated by direct binding of Fas and Fas-L or perforin release on CD8+ T cells.[11] CD8+ T cells mediate non-cytolysis by secreting cytokines such as interferon-γ (IFN-γ), tumor necrosis factor (TNF) and Interleukin-2 (IL-2) against pathogens. They also release chemokines, therefore attracting inflammatory cells to the sites of infection.[5]

### 1.1.2 Regulation of CD8+ T cell response

T cells that exert effector function usually rely on three signals: the involvement of the T cell receptor, the presence of costimulatory molecules, and the supply of pro-inflammatory cytokines.[12,13] The T cell receptor binding can be triggered by either non-specific or antigen-specific stimulation. The activation of T cells is restricted by T cell receptor binding affinities and kinetics.[14,15]

The costimulatory molecule, such as CD28, is required for the complete activation of T cells via decreasing the threshold of T cell receptor binding affinity.[16,17] Conversely, insufficient T cell receptor signaling results in T cell anergy, leading to immune tolerance or immune evasion.[18-20] In the past few years, the CD28-C80/86 axis has been considered to be a unique co-stimulatory pathway.[21] The CD27-CD70 axis has been defined as another constitutively

expressed costimulatory receptor on T cells later.[22] Furthermore, it has been demonstrated that a set of molecules induced by TCR signaling provides a second wave of costimulation. Classical examples are as follows: CD30, 4-1BB, and Ox40 of the TNF receptor family and inducible co-stimulator (ICOS) of the immunoglobulin family.[23] Upon activation, T cells express a number of receptors with a co-stimulatory capacity, which is important for sustained activation, proliferation, and differentiation.[24]

In the dynamic activation procedure, CD69 is the initial up-regulated surface marker when pathogens encounter T cell receptor stimulation in CD8+ T cells. Thereafter, at the early stage of stimulation, the expression of CD25 is increased on the surface of T cells.[25,26] In addition, CD44 can also serve as an immunological checkpoint for the control of CD8+ T cell activation.[27] To exert the effects of CD8+ T cells, they produce high levels of cytokines, such as pro-inflammatory molecules IFN-γ, TNF-a, and IL-2 against infections and tumorigenesis. If two or more cytokines are released, they are called more potent effector T cells.[28-30]

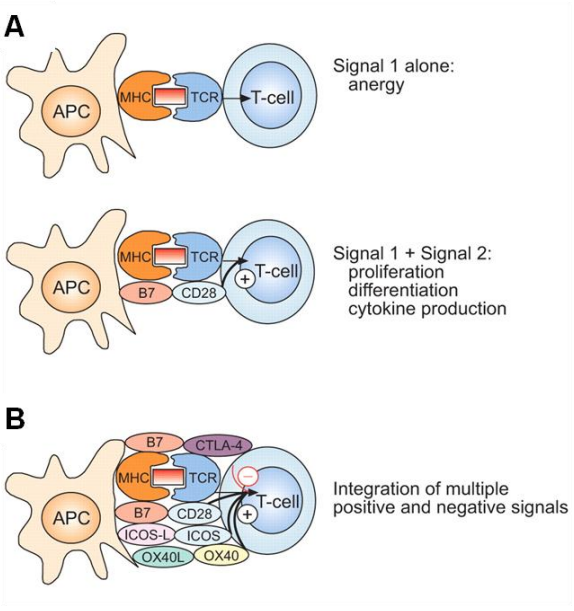



**Figure1.1 The principles of full T cell activation.** Fig. 1.1 shows the detailed knowledge of a full T cell activation. (A) T cells cannot be fully activated without additional co-stimulators. Activation of T cells by TCR alone induces the anergy of T cells. (B) Additional signals delivered by the co-stimulatory molecules were able to fully activate T cells.[31] (APC: antigen-presenting cell; MHC: major histocompatibility complex; TCR: T cell receptor; CTLA: cytotoxic T lymphocyte antigen; ICOS: inducible co-stimulator; L: ligand.)

### 1.1.3 B cells and host immunity

B cells have been characterized as the other arm of the adaptive immunity. They are also indispensable for the clearance of viruses, protozoan pathogens, intracellular bacteria, and endogenous tumor cells. B cells are specialized in the production of protective high-affinity antibodies.[32] In B cell-mediated humoral immunity, the neutralized antibodies can act directly to a certain antigen. In the case of Hepatitis B virus（HBV）infection, for example, they induce the secretion of HBV antibodies which are specific to HBV and have potent neutralization activities.[33]

Moreover, B cells also play an important role in the innate immune response. B cells can also be activated through innate immune response via an antigen-independent manner. Innate immune activation of B cells is crucial for producing antibodies, promoting B cells maturation and maintaining immunomodulatory functions to provide protection against autoimmunity.[34-40]

### 1.1.4 Regulation of B cell response

B cells can be activated in either T cell-dependent (TD) activation or T cell-independent (TI) manner, depending on whether T cells are involved in the antigen presentation directly.[41] In the T cell-dependent manner, B cells are activated by an antigen which is presented by MHC and co-stimulatory (CD40-CD40L) signals from Th2 cells.[42] After that, B cells mutate and differentiate into either high-affinity memory B cells or plasma cells.[43] The so-called plasma cells are matured B cells that generate their antigen-specific receptors in the form of antibodies.[44] The T cell-independent procedure involves multivalent polymerized antigens, such as LPS and bacterial flagellin. TI antigens can be divided into type 1 and 2 and activate B cells by different mechanisms. Most TI-1 antigens are polyclonal B cell activators, such as mitogens, which can activate B cells directly regardless of their antigenic specificity. At higher TI-1 antigen concentrations, one-third of all B cells undergo rounds of proliferation and antibody secretion while in lower concentrations of TI-1 antigens, the activation only allows for antigen epitope-specific B cells. TI-2 antigen activating B cells are determined by extensive crosslinking of membrane-bound immunoglobulin (Ig) receptor.[41,45,46]

Similar to the procedure of T cells, B cells also undergo somatic hypermutation to generate antigen receptors, while B cells require isotype switching, which allows a functional switch of

different classes of antibodies (i.e. IgM and IgG). Isotypes determine the effector function of antibodies, and antibodies originate from the IgM isotype.[47,48] It has been reported that several cytokines, such as IL-4, induce the activated B cells to undergo isotype switching to IgE; TGF-β induces the activated B cells to undergo isotype switching to IgG2b and IgA, and IFN-γ induces the activated B cells to undergo isotype switching to IgG2a and IgG3.[49-52]

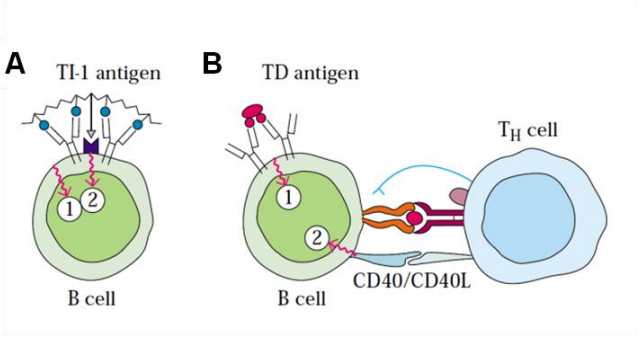



**Figure1.2 Two manners of B cell activation.** Fig. 1.2 shows B cell activation in detail. (A) The T cell-dependent manner requires the antigen presentation of Th cells, not simply exposure to Th-derived cytokines. (B) B cells can also be activated in the absence of T cells. The TI antigens can be divided into type1 and 2 based on different B cell epitopes which they are binding.[42]

## 1.2 Toll-like receptors (TLRs)

### 1.2.1 Structural biology, agonists and TLR pathway

It is well established that mammals have four classes of pattern-recognition receptors (PRRs) that are responsible for detecting pathogens: Toll-like receptors (TLRs), NOD-like receptors (NLR), retinoic acid-inducible gene I (RIG-I)-like receptors, and C-type lectin receptors (CLRs).[53,54] They recognize both exogenous pathogens and self-derived molecules from damaged cells. The exogenous pathogens are also known as the pathogen-associated molecular patterns (PAMPs), and the self-derived molecules are also known as damage-associated molecular patterns (DAMPs). The specific recognition in organisms starts both the innate and adaptive immune system to resist the infecting microbes.[55,56]

The TLR family consists of TLR1-TLR10 in human beings, and TLR1-9, TLR11-TLR13 in the murine system.[57,58] Based on their different localization, TLRs can be divided into two subfamilies: TLR1, TLR2, TLR5, TLR6, and TLR10 are localized on the plasma membrane. TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13 are localized intracellularly, for example in the endosomal compartment, lysosome-related organelle. Whereas TLR4 is reported to be localized on both the cell surface and endosome.[59]

Ligands of TLRs not only derive from a microorganism, such as bacterial, fungal and viral components, but also from chemical synthetic drugs, such as the TLR2 agonist Pam3CSK4 (P3C), the TLR7 agonists Imidazoquinoline (R837) and Resiquimod (R848). For their corresponding ligands, it has been demonstrated that TLR2, TLR1 or TLR6 can recognize PAMPs such as lipoproteins, peptidoglycans, lipoteichoic acids, glycolipids, and zymosan. TLR4 recognizes bacterial lipopolysaccharide, which is also known as LPS. TLR5 recognizes bacterial flagellin which is also known as Flagellin as well as Dicyllipopeptides. The intracellular compartment TLR3, TLR7, TLR8, and TLR9 can recognize the microbial nucleic acids. Specifically, TLR3 is responsible for viral double-stranded RNA (dsRNA), siRNA, and TLR7/8 is responsible for single-stranded RNA (ssRNA) derived from viruses. TLR9 recognizes viral or bacterial DNA (Figure 1.3). [55,59,60,61,62]

| TLRs | localization | Bacterial ligands | fungal ligands | viral ligands |
|---|---|---|---|---|
| TLR1 | cell surface | Triacyl lipopeptides | | |
| TLR2 | cell surface | Diacyl lipopeptides, Triacyl lipopeptides, Peptidoglycans, Lipoteichoic acid,Glycolipids | Zymosan, Phospholipomannan | envelope proteins of Measles virus, human cytomegalovirus, herpes simplex virus type I |
| TLR3 | endosome | | | Viral dsRNA, synthetic |
| TLR4 | cell surface | LPS | Mannan, Glucuronoxylomannan | F protein of respiratory syncytial virus (RSV), Envelope protein of mouse mammary |
| TLR5 | cell surface | Flagellin | | |
| TLR6 | cell surface | Diacyl lipopeptides, | Zymosan | |
| TLR7 | endosome | | | ssRNA, synthetic imidazoquinoline derivatives (anti-viral drugs) |
| TLR8 | endosome | phagocytized bacterial RNA | | ssRNA, synthetic imidazoquinoline derivatives ( Imidazoquinoline ,bropirimine, Resiquimod) |
| TLR9 | endosome | CpG DNA | | Oligodeoxynucleotide DNA |
| TLR10 | cell surface | triacylated lipopeptides | | |
| TLR11 | endosome | Not determined | | |
| TLR12 | endosome | Not determined | | |
| TLR13 | endosome | bacterial ribosomal | | virus RNA sequence "CGGAAAGACC" |

**Figure1.3 The localization and ligands of all TLRs.**

Under the stimulation of TLR agonists, the TLR signaling can be conducted from two distinct pathways, the MyD88-dependent pathway, and the MyD88-independent signaling pathway.[63] In the MyD88-dependent manner, ligands that bind to the corresponding receptors cause a conformational change of TLRs, which facilitates the recruitement of the adaptor protein myeloid differentiation primary response protein 88 (Myd88).[59] Adaptor proteins such as TIR-domain containing adapter protein (TIRAP) and Myd88 can further recruit interleukin-1 receptor-associated kinase 4 (IRAK4), interleukin-1 receptor-associated kinase 1(IRAK1), and interleukin-1 receptor-associated kinase 2(IRAK2). IRAK was phosphorylated to activate the tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6).[64] Subsequently, TRAF6 dissociates from the adaptor protein and forms a new complex with transforming

growth factor beta-activated kinase 1 (TAK1), TGF-β -activated kinase 1 (TAB1) and TGF-β-activated kinase 2(TAB2).[65] On the one hand, the complex activates the IκB kinase (IKK) complex consisting of IκB kinase α (IKKα), IκB kinase β (IKKβ), and NF-κB essential modifier (NEMO)/IκB kinase γ (IKKγ) to further induce phosphorylation of IκB proteins.[66] Phosphorylated IκB proteins are finally degraded by the proteasome. This process allows NF-κB to translocate to the nucleus.[67] On the other hand, TAK can activate mitogen-activated protein kinase (MAPK), allowing the activation of activator protein (AP-1).[68] In the last step, the inflammatory gene starts transcription, producing a series of inflammatory cytokines, such as TNF-α, IL-6, IL-1, IL-12 and etc.[69] In the Myd88-independent signaling pathway, TLR3 and TLR4 recruit the TIR-domain-containing adaptor proteins including TRIF (TIR-domain-containing adaptor protein) and TRAM (TRIF-related adaptor molecule).[70] TRIF and TRAM are crucial molecules involved in the activation of nuclear factor NF-κβ, MAPKs, and the transcription factor interferon regulatory factor (IRF3). Thereafter, the secretion of cytokines such as IFN-β is induced.[71]

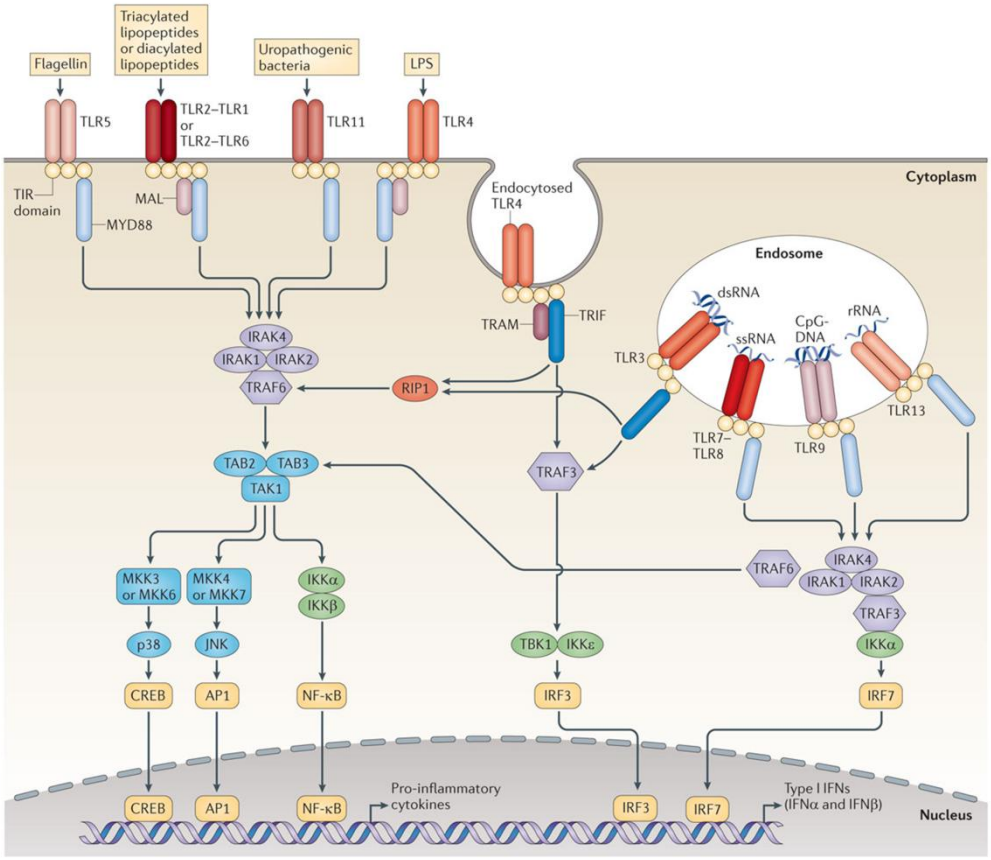



**Figure1.4 The distinct TLR pathway.** Fig. 1.4 shows the detailed knowledge of the Toll-like receptor (TLRs) signal pathway. The heterodimers of TLR1/2 or TLR2/6, TLR4, TLR5, TLR11 bind to their corresponding ligands on the cell surface. TLR3, TLR7/8, TLR9, and TLR13 are expressed on endosomes, binding their corresponding ligands, such as microbial and host-derived nucleic acids in endosomes. TLR4 localizes at both the cell surface and the intracellular organelle.[72] TLR ligands initiate the pathway by activating the Toll–IL-1-resistance (TIR) domain. Toll/interleukin-1 receptor (TIR) in turn recruits adaptor proteins, such as myeloid differentiation primary response protein 88 (MyD88), MyD88-adaptor-like protein (MAL), TIR domain-containing adaptor protein including IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM).[73] These adaptors then stimulate downstream molecules, such as IL-1R-associated kinases (IRAKs) and the adaptor molecules TNF receptor-associated factors (TRAFs). The cascade procedure finally leads to the activation of the Jun N-terminal kinase (JNK) and p38 and further activates transcription factors, such as the nuclear factor-κB (NF-κB), the interferon-regulatory factors (IRFs), the cyclic AMP-responsive element-binding protein (CREB) and the activator protein 1 (AP1).[74]

### 1.2.2 Promoting CD8+ T cell responses by TLR ligands

#### 1.2.2.1 TLR ligands up-regulate CD8+ T cell responses indirectly

TLRs are expressed on a series of antigen-presenting cells such as DCs, macrophages, neutrophils and B cells.[75,76,77,78,79] They serve as key PRRs in innate immune cells. TLR activation leads to the maturation of APCs, therefore enhancing the function of T cells indirectly.[80] DCs are a good example to explain the mechanism: When mDCs encounter

pathogens, they up-take pathogenic antigens, digest antigens into peptides and load them onto MHC molecules.[81,82] Normally, MHC class I-bound peptides can be further recognized by CD8+ T cells based on the T cell receptor (TCR) recognition.[83] Thereafter, TLRs up-regulate the expression of MHC-I molecules and costimulatory molecules (CD40, CD80, CD86, and CD70) on DCs, ultimately increasing the effective function of CD8+ T cells.[84] Besides, CCR7 acts as chemokine receptor that can be up-regulated by TLRs and contribute to the migration of lymphocytes.[85] Furthermore, TLR activation on APCs induces the release of cytokines, such as Interferon-γ (IFNg) and Interleukin 12 (IL-12), which are necessary for the sustainability and survival of T cells.[86]

TLR activation on APC induced Th response also contributes to enhancing the CD8+ T cell response. On the one hand, TLR ligand stimulation on APCs enhances the antigens cross-presenting to MHC-I molecules, thereby activating a specific CD8+ T cell response.[87] The elevated CD8+ T cell response is required for anti-viral effects during acute or chronic infections such as Hepatitis B and C virus (HBV/HCV) infection.[1,88] On the other hand, TLR activation on APCs induces the secretion of inflammatory cytokines resulting in the crosstalk between CD8+ T cell response and other cells. For example, TLR activation enhances IL-12p70 production, mediating crosstalk between Th1 responses and CD8+ T cell responses.[89] Different TLR activations are able to induce either pro-inflammatory or anti-inflammatory cytokines. For example, in contrast to LPS, myeloid DCs activated by TLR2 heterodimers (TLR2/1 or TLR2/6) are capable to produce more IL-10, which favors Th2 responses rather than Th1 responses,[90] whereas TLR7 and TLR9 are also highly expressed in the phagolysosomes of ER in plasmacytoid DC (pDCs).[91] TLR7/9 activation on pDCs leads to the secretion of pro-inflammatory cytokines, resulting in an enhanced Th1 response, thereby further inducing cytotoxic T-cell (CTL) responses against viral infections.[92,93] Based on all these studies, TLR ligands are thought to indirectly upregulate T cell responses.

#### 1.2.2.2 TLR ligands mediate T cell responses directly

Numerous studies have found that naïve CD8+ T cells express mRNA for TLR1, 2, 6, 9 and only little TLR7, but not TLR4 in C57/BL6 (B6) mice.[94,95] It has been verified that TLR expression on T cells appears to be regulated by TCR-dependent activation. ACD3 activated

CD8+ T cells express more TLR2 than naïve CD8+ T cells.[96] This is consistent with the fact that TLRs are highly expressed on certain subtypes of purified T cells, such as effector CD8+ T cells and memory CD8+ T cells. This finding suggests that TLR can serve as co-stimulatory molecules of CD8+ T cells and that TLRs can enhance the activation, proliferation and cytokine production of effector T cell in the presence of TCR signals.[97,98]

It has been well established that the TLR2 ligand can act as the co-stimulator of TCR signaling in CD8+ T cells.[99] TLR2 engagement on CD8+ T cells significantly reduces their threshold of co-stimulatory signals primed by APCs, lowers the antigen (Ag) concentration, which is necessary for optimal activation, and augments the functionality of memory cells even if exposed to a low TCR signal.[95] In favor of this concept, our previous study found that Pam3CSK4 (P3C) can enhance the therapeutic efficacy of the DNA vaccine in WHV Tg mice.[100] Besides, P3C is also efficient for the therapy of tumors due to the enhanced tumor-specific CD8+ T cells response.[101]

In the case of TLR7, early studies have demonstrated that the TLR7 ligand significantly increases the activation of purified CD8+ T cells in HIV-1-infected individuals compared to healthy controls.[102] The TLR7 ligand was also confirmed to enhance specific CD8+ T cell responses in an accessory cell-dependent manner.[103] Recently, the TLR7 ligand GS-9620 was shown to be potentially inhibiting the secretion of serum viral DNA and antigens in the chimpanzee and woodchuck models of CHB.[104,105] The enhanced anti-viral response is believed to be associated with the aggregated intra-hepatic CD8+ T cells.[104] Clinical studies further demonstrated that the Toll-like receptor 7 agonist GS-9620 induces a prolonged inhibition of HBV via intra-hepatic aggregates of T cells.[106] Another study revealed that the Toll-like receptor 7 agonist R848 in combination with radiation therapy (RT) leads to the expansion of tumor antigen-specific CD8+ T cells and the clearance of tumor in the T lymphoma mouse model.[107] These findings all supported the idea that the up-regulation of TLR7 in CD8+ T cells directly contribute to both anti-viral and anti-tumor immunity.

Based on these findings, the physiological significance of TLR7 engagement on T cell responses might support new approaches for developing effective immunotherapies in the field of the tumor and viral infection.

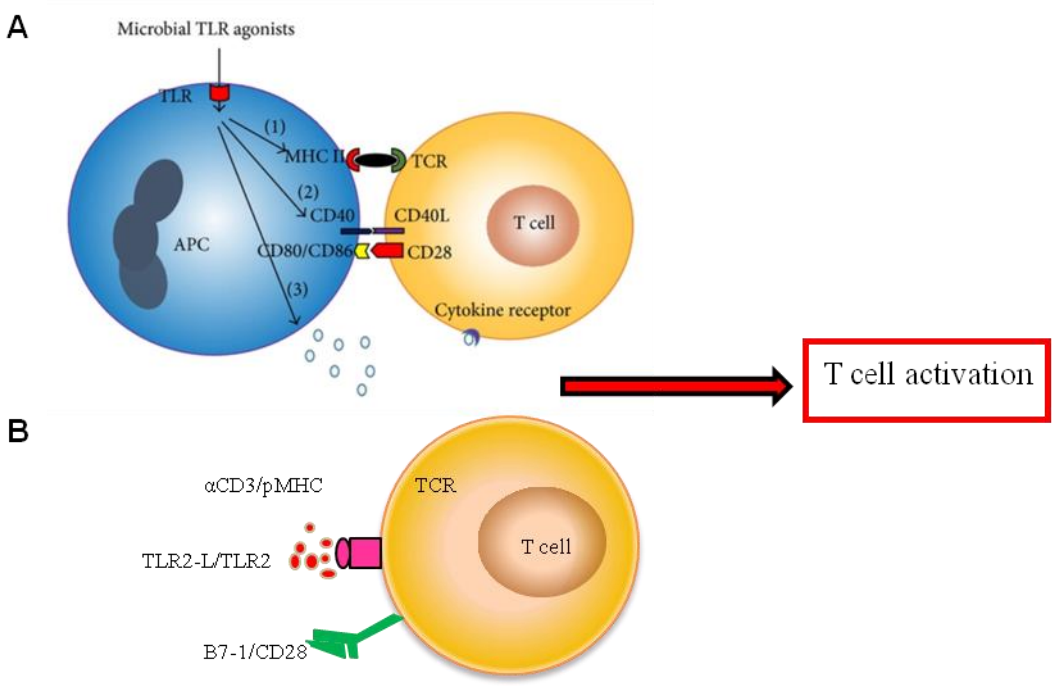



**Figure1.5 TLR activation on T cells.** Indirect activation of T cells by TLRs. The APCs are stimulated by TLR agonists to express more co-stimulatory molecules and cytokines, therefore enhancing the Ag presentation and stimulation signaling to T cells. (B) Direct activation of T cells by TLRs. As TLRs are expressed on both memory and activated T cells, they are said to serve as a co-stimulatory receptor of CD8+ T cells.[99]

### 1.2.3 Promoting B cell responses by TLR ligands

TLRs are lowly expressed on naïve human B cells, while TLR1, TLR6, TLR7, TLR9, and TLR10 are highly expressed on activated or memory B cells.[108-111] Expression of TLRs varies a lot from different subsets of murine B cells, therefore they respond differently to their corresponding ligands.[112,113] It has been reported that activation of TLR2, TLR7, and TLR9 increases the proliferation of follicular and MZ B cells, whereas activation of TLR4 can only induce the proliferation of MZ B cells. TLR ligands also induce antibody secretion in B cells. TLR2, TLR4, TLR7, and TLR8 activation induces a strong secretion of immunoglobulin M (IgM) in B1-B cells.[113] TLR2, TLR4 agonists, but not TLR7 or TLR9 ligands, induce significant IgM and IgG secretion of follicular B cells.[114] Furthermore, TLR stimulation in B cells also induces a wide range of cytokines, such as IL-1α , IL-1β , IL-6, IL-8, and IL-10.[110,115,116]

As to the activation of B cells, it has been proved that B cells require three different signals for initial activation: (1) antigen-triggered BCRs signals, (2) T-cell co-stimulation signal through CD40 activation, (3) TLR/Cytokine-induced innate immune signal. In the procedure

of B cell activation, TLRs can interact and synergize with the stimulation of BCRs by antigen or stimulation of CD40 by CD40L.[117,118] Interestingly, different TLRs mediate different immune responses when co-stimulated with BCRs or CD40 of B cells. It has been reported that BCR or CD40 stimulation together with TLR3, TLR4 or TLR9 promotes activation and proliferation, while co-stimulation together with TLR1/2, TLR2/6, TLR4, and TLR7 promotes the maturation of plasma cells.[118] In contrast to this, many other researchers have reported that human naïve B cells can be activated by only the TLR signaling. For example, TLR9 agonist-ODN stimulation can induce more effective functionality alterations on B cells.[119-123]

### 1.3 Cell catabolism and metabolic regulation

#### 1.3.1 Biochemistry

Sustained energy supply is necessary for the basic activities of organisms such as muscle contractions, transport of molecules and cellular movements. The free energy origins from hydrolyzing the energy carriers such as adenosine triphosphate (ATP).[124-126]

One molecule of ATP is made up of one molecule of adenosine and three phosphate groups. Hydrolysis of ATP to ADP is an energetically unfavorable enzymatic process.[127] Glucose is a significant free energy source of ATP hydrolysis.[128] The initial process of glucose metabolism is glycolysis. It is well known that glycolysis is a ten-step enzymatic procedure. The organism can get two molecules of three-carbon compound pyruvate and ATPs from each enzymatic reaction. The first step of glycolysis is to phosphorylate glucose by hexokinase, which needs ATP to produce Glucose-6-phosphate. Glucose-6-phosphate is then isomerized by glucose phosphate-isomerase and the consumption of ATP, thereby forming fructose-6-phosphate. The next step is to phosphorylate fructose-6-phosphate to fructose-1,6-bisphosphate by phosphofructokinase. The fructose-1,6-bisphosphate is cleavaged into two isomers dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate by aldolase subsequently. These two metabolites isomers are capable to convert to each other, and the glyceraldehyde-3-phosphate can also be finally metabolized to 1,3–bisphosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase. The energy conversion in this step is from NAD+ to NADH. Then, 1,3–bisphosphoglycerate is catalyzed by phosphoglycerate kinase and metabolized to 3-phosphoglycerate, which leads to the generation of ATP. The following

steps are catalyzed by phosphoglycerate-mutase and enolase. 3-phosphoglycerate is metabolized to 2-phosphoglycerate firstly, followed by forming phosphoenolpyruvate. Subsequently, phosphoenolpyruvate is converted to 2 molecules of pyruvate, which can be catalyzed by pyruvate kinase after that. ATPs are also generated during this step. Finally, the consumption of one molecule of glucose and one NAD+ in the glycolytic pathway produces 2 molecules of pyruvate, 2 ATP, and one NADH. Pyruvate can either be further utilized for ATP generation by entering the tricarboxylic acid (TCA) cycle under the aerobic environment or be metabolized for lactate production under the anaerobic environment.[129]

### 1.3.2 Abnormal cellular metabolism during disease

The energy that origins from basic metabolism is required for maintaining cellular homeostasis. However, more energy and nutrients are required for the rapidly growing cells such as the tumor cells for their abnormal proliferation. The famous observation in cancer metabolism was known as the "Warburg effect", named after the researcher Otto Heinrich Warburg, and was first mentioned in the 1920s.[130] The "Warburg effect" refers to the observation that cancer cells favor to utilize glucose via glycolysis and produce more lactate rather than through the TCA-cycle even in the presence of oxygen. One possible explanation for the abnormal metabolism are multiple alterations in the tumor microenvironment. The glycolysis was upregulated within the tumor microenvironment in certain tumor cells and increased glucose transporters and glycolytic enzymes were observed in it.[131-133] Signaling pathways such as the PI3k-Akt signaling pathway are upregulated to accelerate glycolysis.[134,135] Transcription factors such as Myc and HIF are also elevated to regulate glycolysis in the tumor cells.[136-138]

However, the "Warburg effect" is not only found in tumor cells but can also be observed in most highly proliferating or activating cells such as cells T lymphocytes.[139] During viral infection, T cells recognize the specific antigen and proliferate within hours to form an effector population. The effector T cells are quickly recruited to the site of infection, which is similar to the microenvironment of tumor cells.[140] Therefore, metabolism is abnormal in the microenvironment of infection and tumor progress. The "Warburg effect" applies to both tumor cells and immune cells.[141,142]

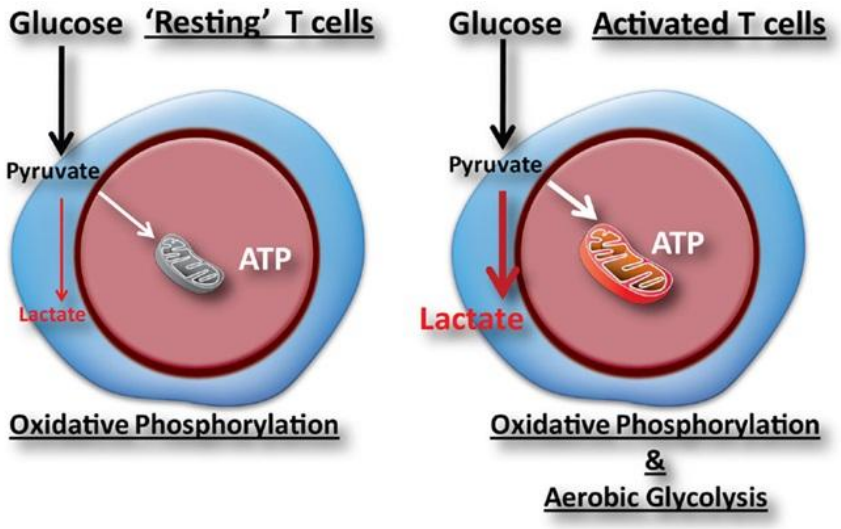



**Figure1.6 The glucose metabolic procedure modulates T cell activation.** The resting T cells rely mainly on oxidative phosphorylation for their energy supply. Once they are activated by the TCR signaling, T cells increase the expression of the glucose transporter, thereby enhancing cellular glucose uptake, which finally promotes oxidative phosphorylation and glycolysis to sustain cell function and proliferation.[143]

Researchers found that the specific CD8+ T cells in the PBMC from chronically HBV-infected individuals, for example, were functionally exhausted and their metabolism altered.[144,145] Depolarization of mitochondrial membrane potential and mitochondrial membrane peroxidation were increased, genes that are involved in cellular procedures, such as electron transport, oxidative phosphorylation, fatty acid and amino acid metabolism were decreased in these CD8+ T cells from the PBMC of chronically HBV-infected individuals. Mitochondrial-targeted antioxidants are reported to rescue the dysfunction of mitochondria in the CD8+ T cells of chronically HBV-infected individuals. Moreover, these antioxidants treated CD8+ T cells show a higher cytokine production, such as IFN-γ and TNF-α.[145] This indicates that targeting the metabolism can be a strategy to rescue the function of CD8+ T cells.

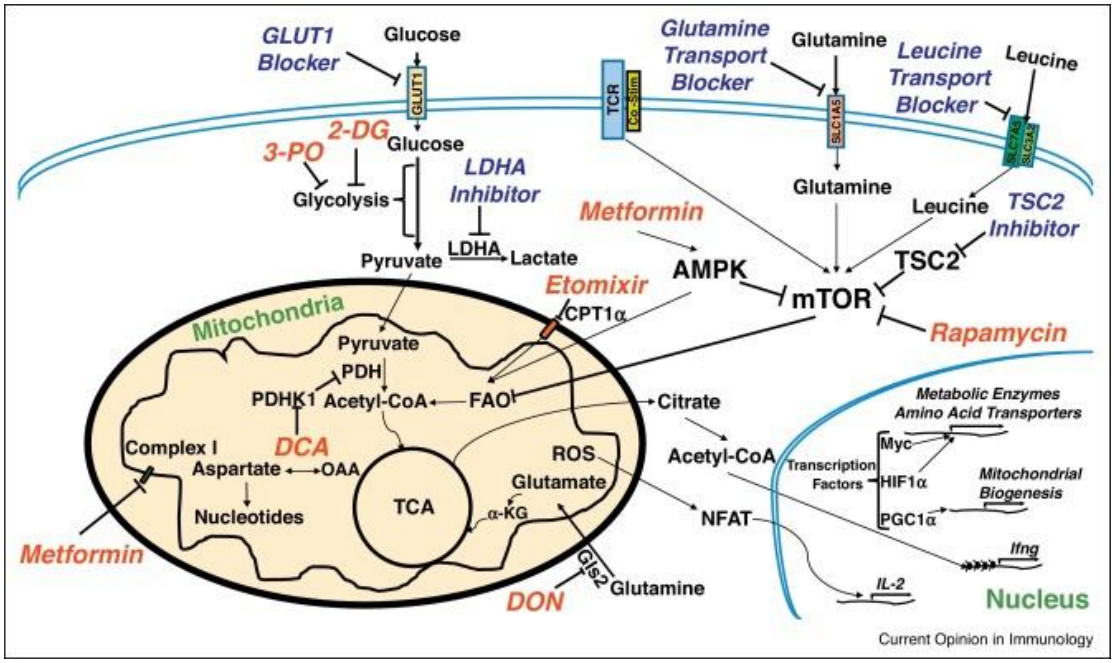



**Figure1.7 Regulating T cell fate and function via metabolic reprogramming.** Targeting T cell metabolism is believed to be a strategy to regulate T cell activation, differentiation, and function. This figure refers to several metabolism targets that facilitate the modulation of T cell functions as discussed. Several metabolic pathway inhibitors were marked in red. [146]

### 1.3.3 Signal pathway regulation of metabolism in T cells

Recently, evidence has emerged that TLRs are tightly associated with metabolism. TLR activation leads to the so-called "Warburg effect" in immune cells, involving a switch to glycolysis, similar to that occurring in tumors.[147] Studies on the mechanism of TLR-induced changes have shown that the metabolic pathway of TOR signaling and its related molecules protein kinase B (Akt) and protein kinase C (PKC) are involved in the activation of CD8+ T cells by using TLR ligand. The PI3K-mTOR signaling pathway is believed to control the TLR-induced cytokine production on DCs. [148,149,150,151] Furthermore, the PI3K-mTOR signaling is verified to be tightly associated with the glycolytic pathway on innate immune cells such as DCs, natural killer cells (NKs) and macrophages.[152,153,154] This indicates that the mammalian target of Rapamycin (mTOR) signaling plays an essential role in the regulation of metabolism.

In our unpublished study, the transcriptomic analysis revealed that thousands of mRNAs were up-regulated on TLR1/2 agonist P3C treated CD8+ T cells, of which more than 25% were metabolically related genes, and immune response related genes were lower than 7%. Compared with αCD3-activated CD8+ T cells, P3C+αCD3-activated cells were significantly

up-regulated in glycolysis, glutamine metabolism, and lipid synthesis. Cells showed a more active metabolic state when treated with TLR ligands. Therefore, we concluded that the TLR2 pathway can not only enhance the effector function of CD8+ T cells but also activate a certain metabolic pathway to provide enough energy for the activation and differentiation of immune cells.

### 1.3.4 Transcription factors regulate metabolism in T cells

Given that TLRs link inflammation to metabolism,[155] TLRs enhance glycolysis by promoting the association of the glycolytic enzyme HK-II with mitochondria. The kinases TBK1, IKKε, and Akt are involved in TLR-enhanced metabolic alteration of DC cells.[156] Furthermore, TLR agonist treatment induces a profound metabolic transition to aerobic glycolysis via the PI3K/Akt pathway, which can be antagonized by the adenosine monophosphate (AMP)-activated protein kinase (AMPK).[157] Up-regulation of AMPK in DCs led to decreased LPS-induced IL-12p40 expression and glucose consumption, whereas suppression of AMPK by shRNA resulted in increased IL-12p40 and CD86.[158]

TCR signaling induces the up-regulation of the transcription factor MYC. It initiates cell division, as well as metabolic reprogramming. MYC is proved to be essential in setting up the metabolic program for T cell proliferation and differentiation.[159] Importantly, another transcription factor, Interferon regulatory factor 4 (IRF4), which can be induced by TCR signal, is also crucial for maintaining the metabolic activity of activated T cells.[160] IRF4 regulates the expression of multiple immune-related genes as well as some transcription factors such as Forkhead box protein O1 (Foxo1) and Hypoxia-inducible factor 1-alpha (Hif1α) that are required for glycolysis. Furthermore, IRF4 together with transcription factor B cell-activating transcription factor (BATF) can promote the expression of Hif1α, therefore enhancing glycolysis and glutamine decomposition.[160,161] On the other hand, IRF4 cooperates with BATF to promote the expression of transcription factors T-bet and Blimp-1, which facilitate the effector function and differentiation of CD8+ T cells.[162,160,161] Thus, IRF4/BATF is required for maintaining the cellular metabolism of CD8+ T cells as well as regulating the effector function of CD8+ T cells.

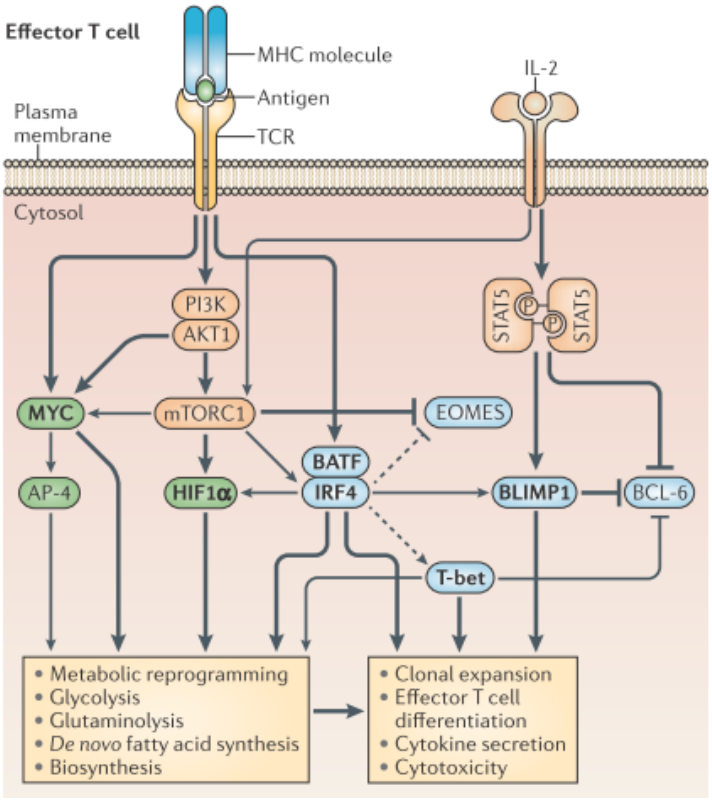



**Figure1.8 Synchronizing transcriptional regulatory T cell metabolism and function.**The schematic picture shows the interaction between immune pathways and metabolic pathways. It has been established that the PI3K/Akt-mTOR pathway is crucial to both the metabolic and immune procedure. The T cell receptor (TCR)-dependent transcription factors interferon-regulatory factor 4 (IRF4) and B cell-activating transcription factor (BATF) work as a transcriptional hub that coordinate both cell metabolism and the functionality of T cells. Besides, some other transcription factors, such as MYC and hypoxia-inducible factor-1α (HIF1α), are also the downstream molecules of the TOR signaling pathway. They are verified to regulate cellular glycolysis and finally leading to the transcription of T-bet tightly.[163]

### 1.3.5 Metabolic reprogramming in B cells

Similarly to the metabolic reprogramming in T cells, the enhanced effective immunity of B cells is also highly associated with metabolic reprogramming. CD40L/IL-4-stimulated B cells showed increased processes of oxidative phosphorylation (OXPHOS), tricarboxylic acid (TCA) cycle, and nucleotide biosynthesis rather than glycolysis by coupling RNA sequencing (RNA-seq) data with glucose isotopomer tracing. Instead, glucose is utilized for the synthesis of ribonucleotides. Thus, the function of B cells cannot be affected by glucose inhibition during OXPHOS or glutamine inhibition.[164]

The β isoform of protein kinase C (PKCβ), as the downstream molecular of BCR signaling, mediates proliferative signaling of B cells. Tsui *et al*. have proved that the formation of GCs and plasma cells was impaired in PKCβ-deficient B cells. Also, PKCβ-deficient B cells have

an impaired capacity at presenting antigens to helper T cells. More importantly, the activation of mTORC1 is reduced in PKCβ-deficient B cells, resulting in impaired metabolism and mitochondrial remodeling, which led to decreased differentiation of plasma cells.[165]

R-Ras2 was another downstream molecule of both the BCR and the costimulatory protein CD40. Mendoza *et al*. showed that GTPase R-Ras2-deficient B cells are unable to proliferate. The lack of R-Ras2 in B cells led to complex metabolic reprogramming processes, including the inhibition of the activation of the PI3K-Akt-mTORC1 pathway, reduction of the replication of mitochondrial DNA and the expression of glycolytic-related genes.[166]

## 2. Aims of the study

TLRs are major components of the innate immune system. They play an important role in the recognition of a wide range of endogenous and exogenous pathogens by the so-called pattern recognition process. The TLR-initiated innate immune response is able to mediate the adaptive immune response. It indicates that TLRs contribute to the adaptive immune system indirectly.

Evidently, TLRs are expressed not only in the innate immune cells but also the adaptive immune cells such as T/B cells. Other studies have reported that the TLR2 ligand P3C directly enhances murine CD4+ and CD8+ T cell proliferation, survival, and cytokine (IL-2, IFN-α, TNF-β) production. Besides, TLR2-activated CD8+ T cells require a lower antigen concentration and costimulatory signals delivered by APCs and induce the effector function regardless of a low TCR signal. Our previous *in vivo* studies have found that the TLR2 ligand Pam3CSK4 could enhance the therapeutic efficacy of a DNA vaccine against chronic hepadnaviral infection. As other studies identified, TLR7 ligands (GS-9620) also augment CD8+ T cell aggregation intrahepatically during chronic hepatitis B virus infection. It is likely that TLR2/7 is able to promote CD8+ T cell response *in vivo*. However, the direct effects of TLR2/7 ligands on CD8+ T cells have not been fully investigated.

B cells serve as the other arm of the adaptive immunity. It is well established that TLR signaling synergizes with BCR signaling increased B-cell activation and proliferation *in vitro*, while TLR engagement on B cells by the corresponding ligands is more likely to promote B-cell activation *in vivo*. However, the functional alteration of TLR2/7-activated B cells has also not been fully investigated.

Given that the enhanced function of CD8+ T cells and B cells may be associated with metabolic reprogramming, we addressed the question whether TLR2/7 ligands increase the function of CD8+ T and B cells and analyzed the underlying mechanism. Next, we further explored the definite mechanisms of how metabolic reprogramming affects the function of TLR2/7-activated CD8+ T/B cells.

To elucidate these questions, we have investigated the following steps:

1. Verification whether the effector function of CD8+ T cells can be enhanced by TLR7 costimulation *in vitro*.
2. Investigation whether the TLR7-enhanced effector function of CD8+ T cells depends on the MyD88-dependent manner by using certain gene knockout mice.
3. Clarification of the metabolic alterations in TLR7-activated CD8+ T cells.
4. Detecting the regulatory signaling pathways and transcription factors involved in the TLR7-induced metabolic alterations.
5. Comparison of whether TLR7 and TLR2 induce different functional and metabolic changes in CD8+ T cells.
6. Confirmation whether the TLR2/7 agonists can enhance B cell function.
7. Comparison of different metabolic changes in different TLRs-activated B cells.
8. Answering the question of whether Toll-like receptor-2/7 activation enhances the metabolism and functions of CD8+ T and B cells.

# 3. Materials and Methods

## 3.1 Materials

### 3.1.1 Reagents for cell culture

| | |
|---|---|
| RPMI 1640 Medium, W/O Glucose, W/O L-Glutamine | PAN biotech, Wimborne Minster, UK |
| RPMI 1640 medium | Gibco, Edinburgh, UK |
| Fetal Bovine Serum, dialyzed | Thermo Fisher, Waltham, USA |
| Fetal Bovine Serum (FBS) | Gibco, UK |
| Sodium pyruvate | Gibco, UK |
| L-glutamine | Gibco, UK |
| Penicillin-Streptomycin | Sigma-Aldrich, St. Louis, USA |
| Glucose | Thermo Fisher, USA |
| 2-Mercaptoethanol | Thermo Fisher, USA |

### 3.1.2 Antibodies, characteristics of fluorochromes

| Antibody | Clone | Manufacturer |
|---|---|---|
| 7AAD | | BD Pharmingen, Heidelberg, Germany |
| Annexin V-FITC | | BD ebioscience, Heidelberg, Germany |
| Fixable Viability Dye eFluor™ 780 | | Thermo Fisher, Dreieich, Germany |
| Alexa Fluor® 647 Mouse anti-S6 (pS235/pS236) | N7-548 | BD ebiosciences, Germany |
| Alexa Fluor® 488 anti-IRF4 Antibody | IRF4.3E4 | Biolegend, Fell, Germany |
| CD16/CD32 rat anti-mouse antibody | 2.4G2 | BD Pharmingen, |

| | | |
|---|---|---|
| | | Germany |
| CD8a Monoclonal Antibody, eFluor 450 | (53-6.7) | Thermo Fisher, USA |
| CD8a Monoclonal Antibody, Apccy7 | (53-6.7) | Thermo Fisher, USA |
| CD25 APC rat anti-mouse antibody | PC61.5 | eBioscience, Germany |
| CD44 Monoclonal Antibody, FITC | IM7 | eBioscience, Germany |
| CD3 monoclonal hamster anti-mouse antibody | 145-2C11 | eBioscience, Germany |
| CD28 Monoclonal Antibody, Functional Grade | 37.51 | Invitrogen, Darmstadt, Germany |
| EOMES Monoclonal antibody, ef450 | Dan11mag | eBioscience, Germany |
| Glut1 Antibody | ---- | NOVUS, Bonn, Germany |
| IFNγ FITC rat anti-mouse antibody | XMG1.2 | eBioscience, Germany |
| IFNγ APC rat anti-mouse antibody | XMG1.2 | BD Pharmingen, Germany |
| mTOR antibody | pAb | Cell Signaling, Danvers, USA |
| PE anti-human CD69 Antibody | FN50 | Biolegend, Germany |
| PE/Cy7 anti-mouse CD62L Antibody | MEL-14 | Biolegend, Germany |
| PE anti-mouse CD98 Antibody | (4F2) | Biolegend, Germany |

| | | |
|---|---|---|
| PE mouse anti-Akt ( pS473 ) | 55/PKBα/AKt | BD biosciences, Germany |
| PE Mouse anti-BATF | s39-1060 | BD Bioscience, Germany |
| PE/Cy7 anti-T-bet Antibody | 4B10 | Biolegend, Germany |
| Purified Mouse IgG1 isotype | MOPC-21/P3 | eBioscience, Germany |
| phospho-Mtor | pAb | Cell Signaling, USA |
| Purified anti-human CD3 Antibody | OKT3 | Biolegend, Germany |
| AF488 Anti-Mouse CD19 | 6D5 | BD Bioscience, Germany |
| PE anti-mouse CD43 | S11 | Biolegend, Germany |
| Brilliant Violet 510™ anti-mouse/human CD45R/B220 Antibody | RA3-6B2 | Biolegend, Germany |
| PE anti-human CD282 (TLR2) Antibody | TL2.1 | Biolegend, Germany |
| FITC TLR7 Polyclonal Antibody | | Thermo Fisher, USA |
| PE/Cy7 anti-mouse I-A/I-E Antibody | M5/114.15.2 | Biolegend, Germany |
| APC/Cy7 anti-mouse CD86 Antibody | GL-1 | Biolegend, Germany |
| FITC anti-mouse IgM Antibody | II/41 | Biolegend, Germany |
| PE anti-mouse IgD Antibody | RMG1-1 | Biolegend, Germany |

| | | |
|---|---|---|
| FITC Mouse Anti-Human CD25 | M-A251 | BD Pharmingen, Germany |
| PE Mouse Anti-Human CD44 | G44-26 | BD Bioscience, Germany |
| APC anti-human CD8 Antibody | SK1 | Biolegend, Germany |

| Fluorochrome | Abbreviation | Absorption (nm) | Emission (nm) |
|---|---|---|---|
| 7-aminoactinomycin D | 7AAD | 488 | 647 |
| Allophycocyanin | APC | 633 | 660 |
| Carboxyfluorescein succinimidyl ester | CFSE | 488 | 520 |
| Fluorescein isothiocyanate | FITC | 488 | 518 |
| Peridinin-chlorophyll-protein complex | PerCP | 488 | 675 |
| Phycoerythrin | PE | 488 | 575 |
| Alexa Fluor 700 | AF700 | 696 | 719 |
| eFluor 450 | eF450 | 405 | 450 |
| FVD | eF780 | 633 | 780 |
| Brilliant Violet | BV510 | 405 | 510 |
| Alexa Fluor 488 | AF488 | 488 | 519 |
| Phycoerythrin/Cyanine7 | Pecy7 | 488 | 774 |
| Allophycocyanin/Cyanine7 | Apccy7 | 633 | 750 |

### 3.1.3 Chemical reagents

| | |
|---|---|
| TLR7 ligand Resquimod | InvivoGen, Germany |
| TLR2 ligand P3C | InvivoGen, Germany |
| EDTA solution pH 8.0<br>SDS (sodium dodecyl sulfate) | AppliChem, Darmstadt, Germany |

| | |
|---|---|
| Tween 20 | Sigma Aldrich, USA |
| FACS Rinse, FACS Clean, FACS Flow, | BD biosciences, Germany |
| Trypan Blue | Santa Cruz Biotechnology, Texas, USA |
| Liberase™ TM Research Grade | GE Healthcare, Solingen, Germany |
| BSA (bovine serum albumin) | Serva, Heiderberg, Germany |
| Brefeldin A | Sigma, USA |
| Penicillin/Streptomycin | PAA Laboratories, Pasching, Austria |
| Erythrocyte Lysis Buffer | Qiagen, MD, USA |
| Fetal Calf Serum (FCS) | Biochrom AG, Berlin, Germany |
| Liver Perfusion Medium | Gibco, USA |
| Gey's Balanced Salt Solution | Sigma-Aldrich, UK |
| 2-Deoxy-D-glucose (2-DG) | Sigma-Aldrich, UK |
| Rapamycin | Sigma-Aldrich, UK |
| Akti-1/2 | Sigma-Aldrich, UK |
| 6-Diazo-5-oxo-L-norleucine (DON) | Thermo Fisher, USA |
| Oligomycin | Thermo Fisher, USA |
| 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino) -2-Deoxyglucose (2-NBDG) | Thermo Fisher, USA |
| JC-1 (Mitochondrial Membrane Potential Probe) | Thermo Fisher, USA |

### 3.1.4 Buffers

| | |
|---|---|
| PBS (pH 7.4) | 4 g NaCl<br>0.1 g KCl<br>0.77 g Na2HPO4<br>0.12 g KH2HPO4<br>500 mL H2O |

| | |
|---|---|
| T-PBS washing buffer (ELISA) | 250 μL Tween 20<br>500 mL PBS |
| STOP solution (ELISA) | 0.5 M $H_2SO_4$ |
| FACS flow buffer | 0.5 g BSA<br>0.1 g NaN3<br>500 mL PBS |
| MACS buffer | 10 mL FCS<br>2 mL 0.5M EDTA<br>488 mL PBS (adjust pH to 7.2) |
| Murine/human T lymphocytes culture medium | RPMI 1640 medium<br>10% FCS<br>1% Penicillin/Streptomycin |
| Murine B lymphocytes culture medium | RPMI 1640 medium<br>10% FCS<br>1% Penicillin/Streptomycin<br>0.5% 2-Mercaptoethanol |
| 10% / 12% Separation gel (8 mL) | 2.72 mL / 3.2 mL 30% (w/v) acrylamide<br>2.08 mL / 2.08 mL 1.5M Tris (pH 8.8)<br>80 μL 10% (w/v) SDS<br>80 μL 10% (w/v) APS<br>3.2 μL TEMED<br>3.04 mL / 2.56 mL ddH2O |
| 5% Concentration gel (4 mL) | 2.52 mL deionized water<br>576 μL 30 % (w/v) acrylamide<br>432 μL 1.0 M Tris（pH6.8）<br>36 μL 10 % (w/v) SDS<br>20 μL 10% (w/v) APS<br>4 μL TEMED |
| 10×Transfer buffer (1 L) | 142.6 g Glycine<br>30 g Tris-base |

| | |
|---|---|
| Lysis buffer for protein (RIPA lysis buffer, 5 mL) | 0.75 mL NaCl<br>0.05 mL NP-40<br>0.025 mL Sodium deoxycholate<br>5 μL SDS<br>2.5 mL Tris(50 mM, pH7.4)<br>1.67 mL ddH2O |
| 10×Running buffer(500 mL) | 15.6 g Tris-base<br>72 g Glycine<br>5 g SDS |
| TBST (500 mL) | 12.114 g Tris-base<br>43.83 g NaCl<br>PBS 500 mL （adjust PH to 7.2-7.4） |

### 3.1.5 Commercial kits

| | |
|---|---|
| Qiagen q-PCR kits | Qiagen, Germany |
| Cytofix / Cytoperm Kit | BD Pharmingen, Germany |
| Mouse IL-2 ELISA Ready-SET-Go | eBioscience, USA |
| Mouse IFNγ ELISA Ready-SET-Go | eBioscience, USA |
| Mouse TNF-α ELISA Ready-SET-Go | eBioscience, USA |
| Anti-PE beads | Miltenyi Biotec, Bonn, Germany |
| Transcription Factor Staining Buffer Set | eBioscience, USA |
| $CD8a^+$ T Cell Isolation Kit II | Miltenyi Biotec, Germany |
| Lactate Colorimetric/Fluorometric Assay Kit | Biovision, Califolia, USA |

| | |
|---|---|
| Fixation/Permeabilization Solution Kit | BD Bioscience, USA |
| CellTrace™ CFSE Cell Proliferation Kit | Thermo Fisher, USA |
| CD8+ T cell isolation kit, human | Miltenyi Biotec, Germany |
| IgM Mouse Uncoated ELISA Kit with Plates | Thermo Fisher, USA |
| IgG Mouse Uncoated ELISA Kit with Plates | Thermo Fisher, USA |
| CD43 (Ly-48) MicroBeads, mouse | Miltenyi Biotec, Germany |
| Seahorse XF kit | Agilent, Santa Clara, CA |

### 3.1.6 Instruments used in this study

| | |
|---|---|
| Cell culture plates (48 well) | Thermo Fisher, USA |
| Cell strainers (70, 100 µm) | Falcon BD, Kleberg, Germany |
| Combitips (0.5 mL; 2.5 mL; 5 mL) | Eppendorf, Wesseling, Germany |
| Centrifuge Megafuge 1.0R | Heraeus, Cologne, Germany |
| Centrifuge Avanti J-26Xpi | Beckman Coulter, Munchen, Germany |
| Centrifuge: Ultracentrifuge Optima L-70K | Beckman Coulter, Germany |
| CO2 incubator | Thermo, Germany |
| Dishes | Greiner bio-one, Frickenhausen, Germany |
| Disposable syringes (1 mL) | TERUMO, Leuven, Belgium |
| Disposable syringes (2 mL; 5 mL; 10 mL) | BBraun, Glandorf, Germany |
| FACS tubes | Becton Dickinson, Germany |
| ELISA Expert Plus Micro-plate Reader | Biochrom, Cambrige, UK |
| Flat-bottom 96-well micro-plates | Thermo Fisher, USA |
| Forceps (pointed and curved) | Oehmen Maschinenmontagen, Duren, |

| | |
|---|---|
| | Germany |
| FACS Calibur flow cytometer (CantoII, LSRII) | Becton Dickinson, Germany |
| Fridge / Freezer (-20℃) | Bielmeier Hausgerate, Germany |
| Freezer (-80℃) | Thermo, Germany |
| Microscope (inverted) | ZEISS, Bochum, Germany |
| MACS Column: LS Columns | Miltenyi Biotec, Germany |
| MACS Separator (70 nm, 100 nm) | Miltenyi Biotec, Germany |
| Mini Trans-Blot Cell | BIO-RAD, USA |
| Needles (0,4 x 19 mm ; 0,9 x 40 mm) | Becton Dickinson, Germany |
| NUNC Elisa plates (96-well) | NUNC, Roskilde, Denmark |
| Pipette tips (10 µL ; 200 µL ; 1000 µL) | STARLAB, Hamburg, Germany |
| Plastic sterile pipettes (5 mL; 10 mL; 25 mL) | Greiner bio-one, Germany |
| Reaction tubes (1.5 mL; 2 mL) | Eppendorf, Germany |
| Screw-cap tubes (15 mL; 50 mL) | Falcon BD, Germany |
| U-bottom 96-well micro-plates | Thermo Fisher, USA |
| Scissors | Oehmen, Essen, Germany |
| Single, multichannel pipettes | Eppendorf, Germany |
| Shaker (Duomax 1030) | Heidolph, Nurnberg, Germany |
| Cell counting chamber (Thoma) | Marienfeld, Cologne, Germany |
| U-bottom 96-well micro-plates | Thermo Fisher, USA |
| Seahorse XFe96 Analyzer | Agilent, Santa Clara, CA |

### 3.1.7 Mice

C57BL/6 wild type (WT) mice were purchased from Harlan Winkelmann Laboratories (Borchen, Germany). TRIF$^{-/-}$, MyD88$^{-/-}$, TRIF/MyD88$^{-/-}$ mice and DbGagL T-cell receptor tg (FV T-cell receptor tg) mice were bred at specific pathogen-free conditions in the Institute for Virology of the University Hospital Essen. The FV-T-cell receptor tg mice are of C57BL/6 or B6.SJL (CD45.1 congenic) background, and 90% of the CD8+ T cells respond to the Friend virus (FV) epitope. IRF4 and BATF-deficient mice were transferred from the University Health Network, Canada. All mice were at 6-8w age. All mice experiments were conducted under the guidance of the animal facility at the University Hospital Essen.

## 3.2 Methods

### 3.2.1 Splenocyte isolation

Splenocytes were isolated and stimulated with αCD3 ± R848 for 24h, after which the function and metabolism were detected according to the manufacturer's instructions.

1. Isolate the spleen from mice, hold on a sterile container (70nm) with PBS.
2. Put and smash the tissue on the surface of the cell strainer, flushing it with PBS into a 50 mL tube.
3. Centrifuge at 1800 rpm, 10-15 minutes.
4. Lyse red cells with 5 mL EL buffer for each spleen, hold on 5 min at room temperature.
5. Stop lysis immediately with 45 mL PBS, wash again with 50 mL PBS.
6. Centrifuge again at 1800 rpm, 10-15 minutes.
7. Suspend the cells with a cell culture medium and count all the spleen cells.

### 3.2.2 Purification of splenic CD8+ T cells

1. Prepare splenocytes as above.
2. Add MACS buffer to resuspend the cell pellet with a ratio of 40 μL MACS buffer/$10^7$ cells.
3. Add 10 μL of Biotin-Antibody Cocktail/$10^7$ cells into the resuspension.
4. Mix them well and incubate for 10 minutes at 4℃.
5. Resuspend them with 30 μL MACS buffer/$10^7$ cells.
6. Add 20 μL of Anti-Biotin Micro-beads/$10^7$ cells for further conjunction with the Biotin-Antibody Cocktail.

7. Incubate them again for 15 minutes, 4℃.
8. Resuspend cells with 5 mL MACS. Prepare the magnetic cell separation.
9. Prepare the LS Column in the magnetic field of a MACS separator by rinsing it with 3 mL MACS buffer.
10. Add cell suspension onto the column. Wash it with 3 × 3 mL MACS buffer. Collect the buffer which flows through the separator, representing the enriched CD8a+ T cells.

**3.2.3 Purification of splenic B cells**

1. Prepare splenocytes as told before.
2. Add MACS buffer to resuspend the cells with a ratio of 40 μL MACS buffer/$10^7$ cells.
3. Add 10 μL of CD43 Micro-beads/$10^7$ cells for further conjunction.
4. Mix them well and incubate for 15 minutes in the refrigerator.
5. Add 30 μL MACS buffer/$10^7$ cells.
6. Centrifuge at 1800 rpm, 10-15 minutes. Discard the supernatant.
7. Resuspend cells in 5 mL MACS buffer. Proceed to subsequent magnetic cell separation.
8. Prepare the LS Column in the magnetic field of a MACS separator by rinsing it with 3 mL MACS buffer.
9. Add cell suspension onto the column and wash column with 3 mL MACS buffer. Collect the buffer which flows through the separator, representing the enriched B cells.

**3.2.4 Stimulation and inhibition experiment *in vitro***

1. Purified CD8+ T cells were isolated by negative selection by using the mouse/human CD8+ T Cell Isolation Kit II (MiltenyiBiotec); procedure as above. Purified B cells were isolated by CD43 Microbeads as mentioned before.
2. The CD8+ T cells were further activated with the αCD3 antibody (mouse-clone 145-2C11; human-clone OKT3; 5 μg/mL) coated plate alone or with anti-CD28 antibody (2 μg/mL) for 24-48 hours. In some experiments, TLR2 ligand P3C (2 μg/mL) or TLR7 ligands R837/R848 (10 μg/mL) were added into the culture medium. Finally, the cells were collected for the measurement of FACS, and the supernatants were collected for the detection of cytokines by ELISA and the production of lactate.
3. In some experiments, different doses of metabolic pathway inhibitors (2-DG, DON, Oligomycin, Akti, Rapamycin) were added into the culture medium. In addition, in the

glucose blockade experiment, the cells can also be cultured directly in glucose deprived medium which is free of glucose.

### 3.2.5 Surface staining, intracellular staining, and intra-nuclear staining

1. All molecules expressed on the cell surface were incubated with fluorescent-labeled antibodies in 50μL FACS buffer at 4℃ for 30 minutes. The dead cells and cellular debris were excluded by staining with either 7-aminoactinomycin (7AAD) or Fixable Viability Dyes (FVD), which are fluorescent chemical compounds with a strong affinity for DNA. 7AAD/ FVD-positive cells suggest dead cells. After that, cells were washed with 150 μL FACS buffer.
2. For the staining of intracellular molecules, cells were fixed and permeabilized for 20-30 minutes at 4℃ with 50μL of Cytofix/Cytoperm solution. Then cells were washed with 150 μL 1xPerm/Wash Buffer. Discard the supernatant. Stain the cells for 30 minutes at 4℃.
3. For the staining of intra-nuclear molecules, the cells were fixated and permeabilized with 50 μL 1x Perm/Wash Buffer after the surface staining overnight.
4. After washing, the cells should be blocked with 50 μL/well FcR (1:200 in perm buffer) at 4℃ for 30 min. After that, 150 μL/well FACS buffer was added to wash them again. Intra-nuclear molecules were stained with antibodies (antibody: FACs buffer=1:200), at 4℃, 30 min.
5. After washing, cells were suspended in 250 μL FACS flow buffer and measured by flow cytometry. The data were analyzed using FlowJo V10.

### 3.2.6 FACS flow cytometry

Flow cytometry (FCM) is a technology that quickly detects and analyzes multiple physical characteristics of single particles such as cells. The single particles flow through a fluid stream and are given a beam of light. The particles' relative size, relative granularity, internal complexity, and relative fluorescence intensity can be measured and recorded. Normally, the flowed cells are labeled with monoclonal antibodies conjugated with a fluorescent dye; FCM is able to recognize the special populations according to their different markers.

### 3.2.7 Detection of cytokine production from in vitro cultured cells by ELISA

The protocol for the detection of cytokines is as follows:

1. Collect cell supernatant by differently treated cells.

2. Coat the plate (96-wells, ELISA plate) with 100 μL/well of capture antibody in Coating Buffer overnight at 4℃.
3. Wash the wells 3 times with >200 μL/well washing buffer before blocking.
4. Block the wells with 200 μL/well of 1 × Assay Diluent, incubate them at room temperature for 1 hour.
5. Dilute the samples with 1 ×Assay Diluent with a different ratio.
6. Wash the wells 5 times, add the diluted samples and dilute standards into the wells. Cover the plate and incubate them at room temperature for 2 hours with shaking.
7. Wash the wells for 5 times with >250 μL/well washing buffer. Add 100 μL/well of detection antibody diluted in 1 ×Assay Diluent buffer. Seal the plate and incubate at room temperature for 1 hour.
8. Aspirate and wash the wells 3-5 times. Add 100 μL/well of Avidin-HRP diluted in 1 × Assay Diluent. Incubate the plate at room temperature for 30 minutes.
9. Aspirate and wash the wells for more than 5 times, add 100 μL /well of Substrate Solution to each well and incubate at room temperature for 15 minutes
10. Add 50 μL of Stop Solution to each well and read the plate at the Elisa reader (OD 450 nm).

**3.2.8 RNA extraction**

Total cellular RNA was purified by Trizol. The method of RNA extraction is as follows:

1. Collect the cultured cells and resuspend them with 200 μL Trizol reagents.
2. Incubate samples for 5 min at room temperature to allow complete desolvation of the nucleoprotein.
3. Add 40 μL chloroform then shake tubes quickly by hand for 15s.
4. Incubate samples at room temperature for 3 min. Centrifuge at 12,000 g at 4 ℃ for 15 min.
5. Prepare a new tube, transfer the aqueous phase to it.
6. Add 250 μL isopropanol into the liquid.
7. Incubate at room temperature for 10 min. Centrifuge at 12,000 g at 4 ℃ for 10 min again.
8. Remove the supernatant and wash the RNA pellet with 40 μL fresh prepared 75% ethanol.
9. Shake the samples roughly, and centrifuge at 12,000 g at 4 ℃ for 5 min.

10. Remove the supernatant carefully and air dry the RNA pellet less than 10min.
11. Dilute RNA pellet with RNase free water.
12. Keep the storage concentration of RNA is 100 ng/μL and store at -80 ℃ refrigerator for further experiment.

**3.2.9 Real-time polymerase chain reaction (RT-PCR)**

Quantitative real-time PCR is a classical technology that analyzes the levels of relative mRNA expression in cells. β-actin is usually used for normalizing the mRNA levels.

The protocol of RT-PCR is as follows:

1. Prepare a reaction mixture system:

2 × SYBR Green RT-PCR

| | |
|---|---|
| Master Mix | 10 μL |
| 10 × primers | 2 μL |
| QuantiFast RT mix | 0.2 μL |
| Template RNA | 1 μL |
| RNase-free water | 6.8 μL |
| Total volume | 20 μL |

2. Start the procedure with the following steps:

A) 50 ℃, 10 min for RNA reverse transcription

B) 95 ℃, 5 min to activate the Taq DNA polymerase

C) 95 ℃, 10 s for denaturation

D) 60 ℃, 30 s, annealing and extension step 40 cycles for DNA (step 3 to 4)

**3.2.10 Western blotting**

The western blotting is a classical technology for analyzing the relative expression of a certain protein.

The protocol is as below:

1. Collect cells from the culture medium, wash the cells with cold PBS.
2. Lyse cells by adding 100 μL lysis buffer. Resuspend the cells immediately and transfer them to a new 1.5 mL tube on ice.

3. Boil samples at 95℃-100 ℃ for 5-10 min and put on ice.
4. Prepare gels previously according to the MW of the protein.
5. Load samples and protein markers into SDS-PAGE gels: 3 μL protein marker/well, 10 μL sample/well for purpose band, and 3 μL sample/well for β-actin.
6. Start gel electrophoresis at 80 V for 30 min, then change it to 120-140 V for 1.5 h in the running buffer.
7. Transfer gels to NC membrane, 200-250 mA for 1 h on ice.
8. Incubate NC membranes with 5% milk at room temperature for 1 h to block the unspecific brand.
9. Wash all membranes with 1 ×TBST.
10. Incubate all membranes with the primary antibody at 4 ℃ overnight with a gentle shake.
11. Wash membranes with 1 ×TBST 3 ×10 min.
12. Incubate membranes with HRP-conjugated secondary antibody with a gentle shake for 1-2 h.
13. Wash the membranes for 3 ×10 min.
14. Finish with ECL exposure.

**3.2.11 Glucose uptake experiment**

1. Sample preparation: The CD8+ T cells (5 x $10^5$ cells/well) are cultured in αCD3 pre-coated plate with or without TLR2/7 agonists for 24-48 h. The B cells (5 x $10^5$ cells/well) are stimulated with or without TLR2/7 agonists 24-48 h.
2. The cells are then harvested and washed 2 times with 1 ×PBS.
3. The cells are resuspended with 100 μL glucose-free medium and incubated at $37^0$C with 5% $CO_2$ for 30 min.
4. Prepare 200 nM 2-NBDG in 100 μL glucose-free medium for each well, mix them well and incubate at $37^0$C for another 20 min.
5. Afterward, the cells are washed 2 times with cold 1 ×PBS and stained with 100 μL FACS flow buffer containing cell surface staining antibodies.
6. The cells are then collected on ice and measured with Canto II as soon as possible. Mean fluorescence intensity in FL1 can be quantified and compared between cells treated with test compounds and untreated control cells.

### 3.2.12 Lactate measurement

1. Collect supernatants from the cultured cells. Dilute the supernatants with a proper concentration. Keep the final volume (50 μL/well) with Lactate Assay Buffer.
2. Make a curve for standards: Adjust different concentrations of standards. The concentrate of the L(+)-lactate standard are 0, 2, 4, 6, 8, 10 nM/well with the final volume 50 μL/well Lactate Assay Buffer.
3. Prepare reaction mix buffer.

   The 50 μL/well reaction mix buffer is prepared as follows.

| Components | Background Mix | Control Mix |
|---|---|---|
| Lactate Assay Buffer | 46 μL | 48 μL |
| Lactate Enzyme Mix | 2 μL | - |
| Probe | 2 μL | 2 μL |

Mix all components. Add 50 μL reaction mix buffer to each well containing the lactate standards & test samples and mix well again.

4. Incubate the reaction buffer at room temperature for less than 30 min in the dark. Detect the samples at the absorbance of OD 570 nm.
5. Calculate lactate concentration according to the standard curve and the OD 570 of each sample.

### 3.2.13 Seahorse XF Analyzers

Seahorse XF Analyzers are used to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of live cells, which represents the mitochondrial respiration and glycolysis of the cell metabolism.

The experiment is performed as follows:

1. Plate cells on the Cell-Talk-coated Seahorse Bioanalyzer XFe96 culture plates ($1\times 10^5$ Cells/well) in assay media. The assay media consists of basic cell culture media, 1% BSA, 25 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate for a special experiment.
2. Cells that are stimulated with αCD3±P3C/R848 are prepared as mentioned before. Basal rates are observed for 30 minutes.
3. Metabolic pathway inhibitors such as Oligomycin (1 mM), carbonyl cyanide

p-trifluoromethoxyphenylhydrazone (FCCP) (0.5 mM), 2-deoxy-d-glucose (10 mM) rotenone/antimycin A (0.5 mM) are injected to detect maximal respiratory and control values.

4. ECAR values vary among different stimulations. Obtain the figure panels including a representative trace and a normalized data (they are calculated as the maximal and basal ECAR values).

### 3.2.14 Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 6 (GraphPad Software Inc, San Diego, CA). Data between different groups were analyzed by a one-way ANOVA test. For the blockade experiments, data were analyzed by a two-way ANOVA test. The p-values $< 0.05$ were considered significant. Significant differences between different groups are marked as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All experiments are representative of three or two independent experiments.

## 4. Results

### 4.1 TLR7 stimulation directly enhances the effector function of CD8+ T cells

#### 4.1.1 R848 enhanced the functionality of Ag-specific CD8+ T cells

To initially assess the immunomodulatory properties of TLR7 on CD8+ T cells, splenocytes from naïve mice were stimulated with the TLR7 ligand R848 (resiquimod) in the presence of an activating αCD3 antibody. The results indicated that R848 could potently elevate the frequency of CD44+, CD69+, and IFN-γ+ CD8+ T cells. Costimulatory molecular R848 led to an increase of $CD44^{+}$CD8+ T cells from 41.9% to 58.1%, $CD69^{+}$CD8+ T cells from 11.6% to 55.3%, IFN-γ+CD8+ T cells from 0.42% to 6.23% (Figure 4.1).

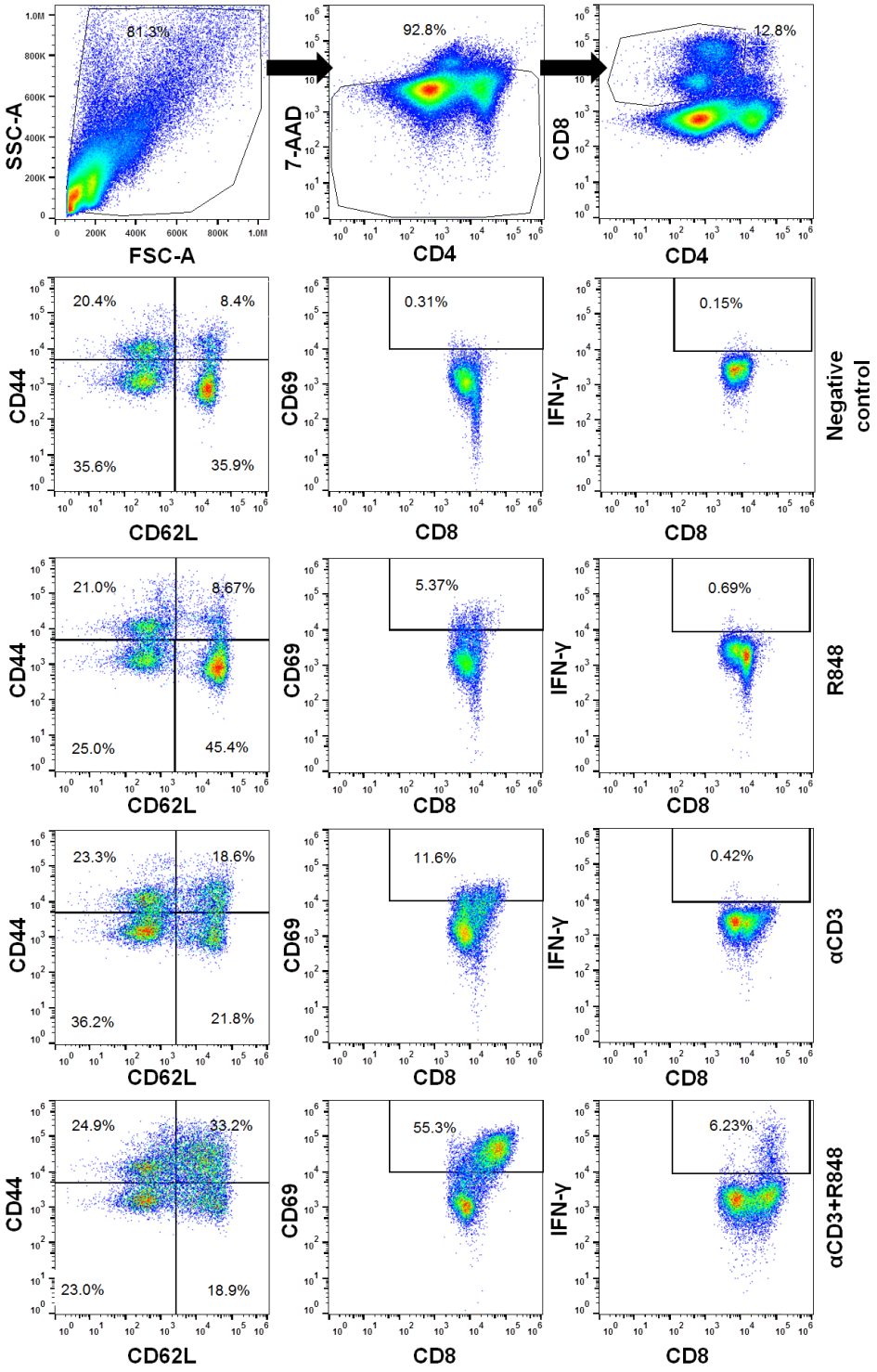



**Figure 4.1 TLR7 activation enhances the effector function of CD8+ T cells in mixed splenocyte cultures.** Splenocytes were isolated from WT mice and stimulated with αCD3 antibody (5 μg/mL) or/and R848 (10 μg/mL) for 24 h. The frequencies of CD44+CD8+ T and CD69+CD8+ T cells were detected by flow cytometry. IFN-γ production in CD8+ T cells was analyzed by intracellular cytokine staining. Data are representative of three independent experiments.

In addition, an increase in functionality including the enhanced CD25 expression on CD8+ T cells and the upregulation of IFN-γ secretion was also observed in FV-T-cell receptor-CD8+ T cells after co-culture with peptide-loaded DCs in the R848 group (Figure 4.2).

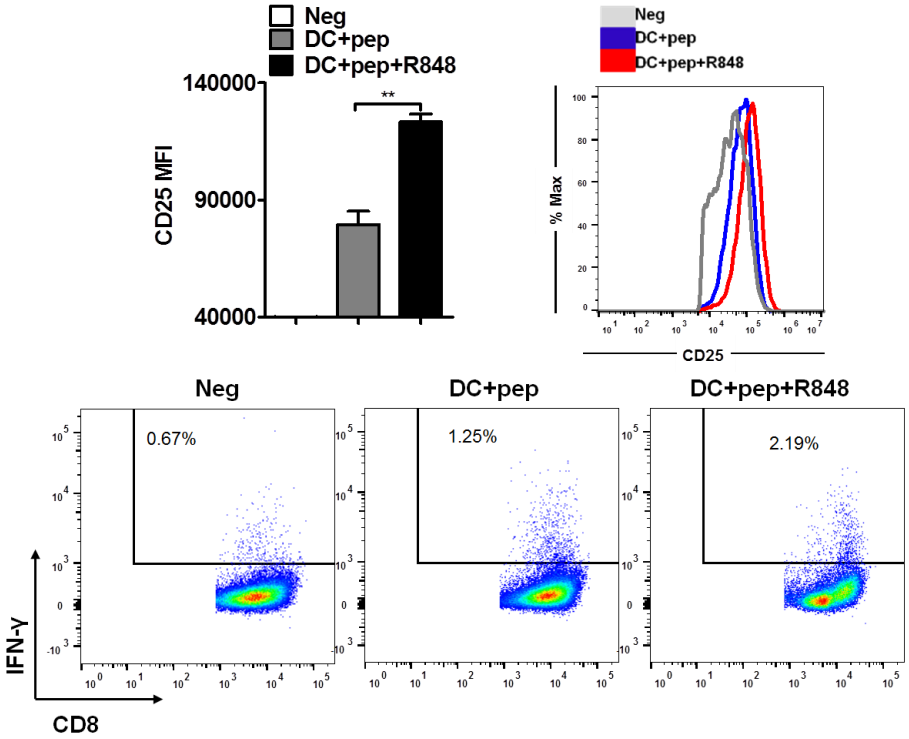



**Figure 4.2 TLR7 activation enhances the effector function of FV-specific CD8+ T cells.** CD8+ T cells were purified from FV-TCR Tg mice and co-cultured with Friend virus (FV)-peptide pre-coated dendritic cells (DCs) alone or in the presence of R848 (10 μg/mL). Mean fluorescence intensity (MFI) of CD25 and representative dot plots of IFN-γ are shown as determined by flow cytometry. Data are representative of two independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

### 4.1.2 Costimulation of R848 enhanced the functionality of splenic-CD8+ T cells

It has been reported that TLR7-activated APCs such as plasmacytoid dendritic cells mediate cross-talk with CD8+ T cells.[167-169] However, whether TLR7 ligands directly enhance the effector function of CD8+ T cells has not been examined to date. To test this, naïve splenic CD8+ T cells were highly purified from WT mice using magnet bead separation and then stimulated with an αCD3 antibody alone or in the presence of R848 for 24 h. Clearly, naïve CD8+ T cells could not be directly activated by TLR7 ligands unless they were stimulated synergistically with an αCD3 antibody (Figure 4.3). This result was consistent with the fact that TLR7 is expressed only on activated T cells.[103]

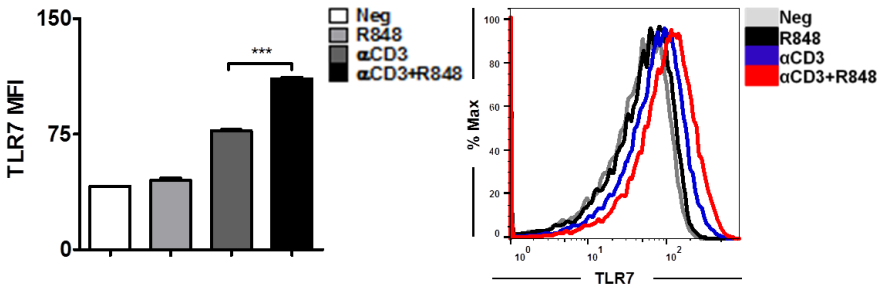



**Figure 4.3 TLR7 expression on activated CD8+ T cells.** Purified CD8+ T cells were stimulated in plates with bound αCD3 antibody (5 µg/mL) alone or with R848 (10 µg/mL) for 24 h. Mean fluorescence intensity (MFI) of TLR7 is shown as determined by flow cytometry. Data are representative of two independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

Upon TLR7 stimulation, the expression of CD25, CD44, and CD69 was significantly increased (Figure 4.4). Costimulatory molecular R848 led to an increase of $CD25^+$CD8+ T cells from 46.5% to 84%, $CD44^+$CD8+ T cells from 20.9% to 27.2%, $CD69^+$CD8+ T cells from 40.7% to 56.9%. This observation could also be confirmed by analyzing the mean fluorescence intensity of these molecules in CD8+ T cells.

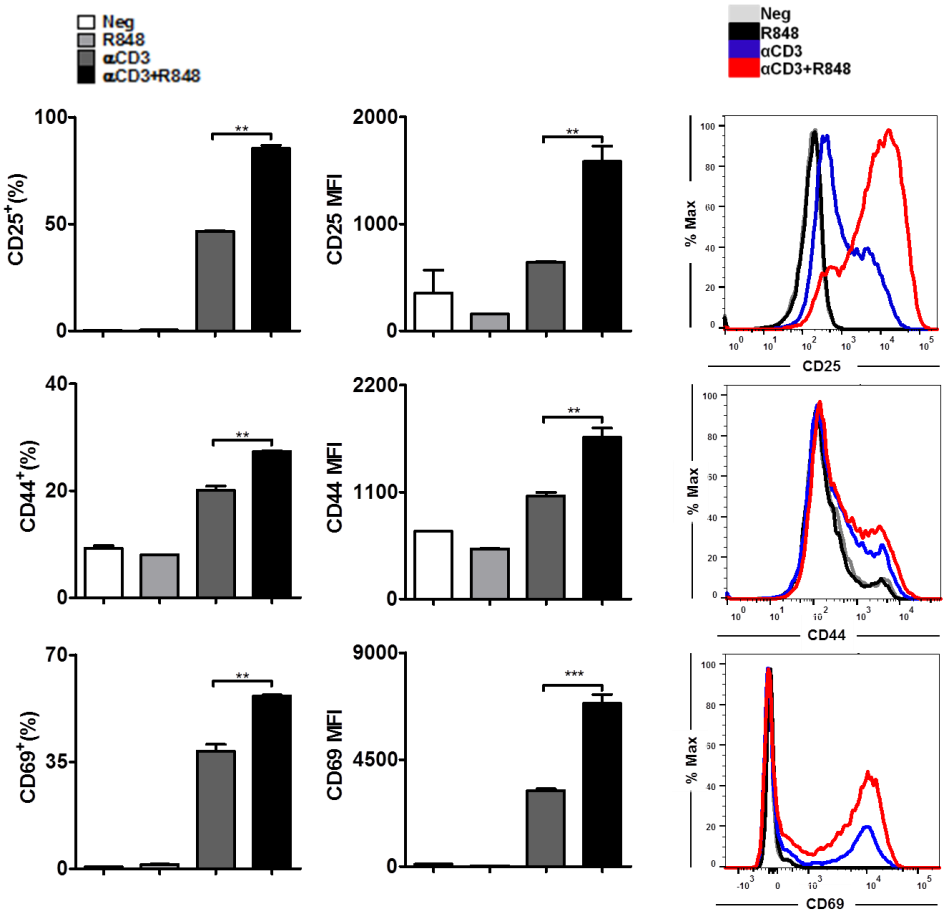



**Figure 4.4 TLR7 ligands enhance CD8+ T cell activation.** Purified CD8+ T cells were stimulated in plates with bound αCD3 antibody (5 μg/mL) alone or with R848 (10 μg/mL) for 24 h. CD25, CD44, and CD69 expression in CD8+ T cells was measured by flow cytometry. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

Moreover, the expression of the transcription factors T-bet and Eomes was also elevated by the costimulation of R848 (Figure 4.5). There was a significant increase in T-bet expression while only a mild increase in Eomes expression after costimulation with R848 for 24 h.

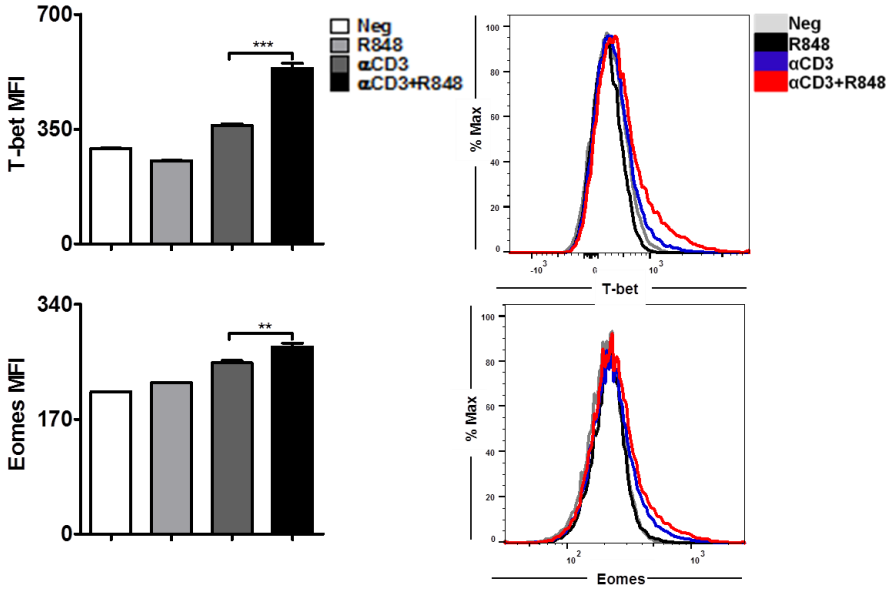



**Figure 4.5 TLR7 ligands enhance T-bet/Eomes expression.** Purified CD8+ T cells were stimulated in plates with bound αCD3 antibody (5 μg/mL) alone or with R848 (10 μg/mL) for 24 h. T-bet and Eomes expression in CD8+ T cells was measured by flow cytometry. Data are representative of three independent experiments. (* p<0.05; ** p<0.01; ns, not significant)

In addition, cytokine secretion including IFN-γ, TNF-α, and IL-2 was augmented in the R848 costimulated CD8+ T cells when compared to the αCD3 treatment group (Figure 4.6).

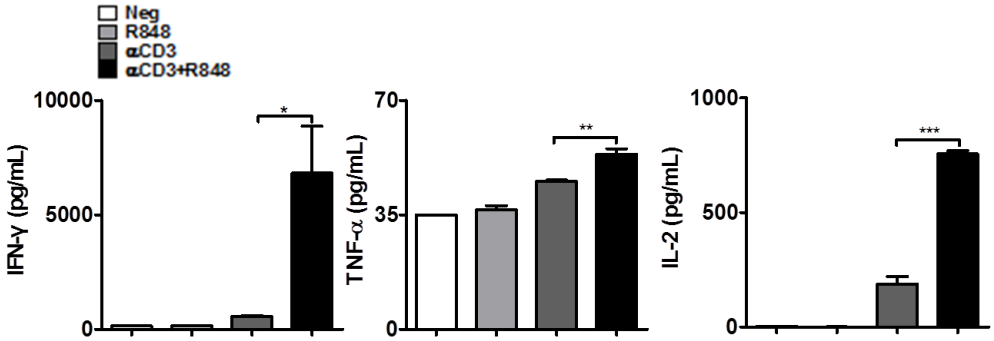



**Figure 4.6 TLR7 ligands enhance CD8+T cell cytokine production.** Purified CD8+ T cells were stimulated in plates with bound αCD3 antibody (5 μg/mL) alone or with R848 (10 μg/mL) for 24 h. Cytokine production including IFN-γ, TNF-α, and IL-2 was measured by specific ELISAs. Data are representative of three independent experiments. (* p<0.05; ** p<0.01; ns, not significant)

### 4.1.3 R848, a more potent agonist than R837

Imiquimod (R837), another TLR7 ligand, induced the maximum cell survival and cell activation at a dose of 1 μg/mL in the presence of αCD3 antibody compared to that with αCD3 antibody alone, whereas R848 at doses of 1–10 μg/mL appeared to improve cell

viability and cell activation compared to that in the presence of αCD3 antibody only (Figure 4.7).

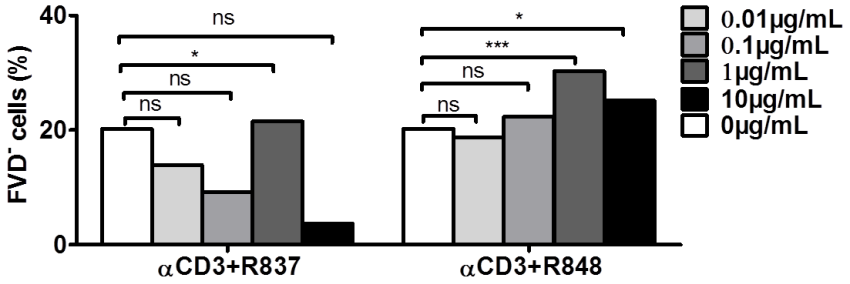



**Figure 4.7 Detection of dead cells in R848/ R837-stimulated CD8+ T cells.** Purified CD8+ T cells from WT mice were stimulated with αCD3 antibody (5 μg/mL) or/and R837/R848 (0, 0.01, 0.1, 1, and 10 μg/mL) for 48 h. The frequencies of dead cells were determined after staining and exclusion with FVD staining.

Comparing the two TLR7 ligands at the optimal concentration for cell survival and activation, R848 (10 μg/mL) induced better effector functions in CD8+ T cells than R837 (1 μg/mL). Specifically, a significantly enhanced CD25, CD44, and CD69 expression (Figure 4.8A) as well as IFN-γ and TNF-α release by CD8+ T cells (Figure 4.8B) was found with R848 co-stimulation compared to that with R837. These results suggest that TLR7 serves as a co-stimulatory receptor for T-cell receptor signaling. Moreover, R848 was verified as a more efficient TLR7 co-stimulatory molecule.

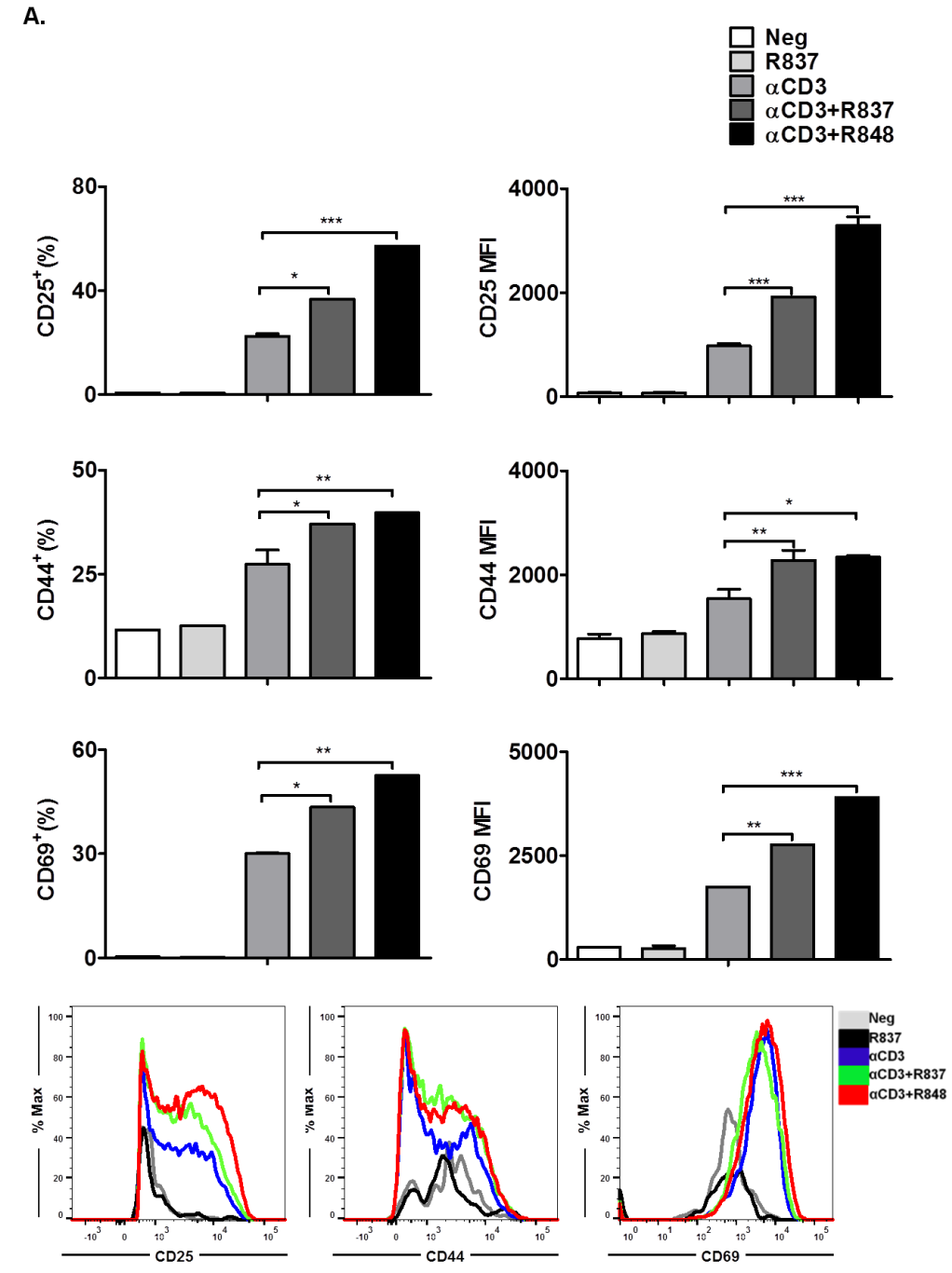

A.
Neg
R837
αCD3
αCD3+R837
αCD3+R848
CD25+ (%)
CD25 MFI
CD44+ (%)
CD44 MFI
CD69+ (%)
CD69 MFI
% Max
CD25
CD44
CD69
B.
IFN-γ (pg/mL)
TNFα (pg/mL)

**Figure 4.8 Comparison of the stimulatory effects of R848 and R837 on CD8+ T cells.** Purified CD8+ T cells from WT mice were stimulated with αCD3 antibody (5 μg/mL) or/and R837 (1 μg/mL) or R848 (10 μg/mL) for 48 h. The activation of CD8+ T cells was assessed by staining with αCD25, αCD44, and αCD69 antibodies. (B) IFN-γ and TNF-α secretion by CD8+ T cells was assessed by specific ELISAs. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

## 4.2 Enhancement of CD8+ T cell effector functions by R848 is MyD88-dependent

### 4.2.1 MyD88 deficiency abolished R848-elevated functionality of CD8+ T cells

Next, we investigated how TLR7 stimulation modulates the function of CD8+ T cells. It is known that intracellular TLR7 signaling occurs through the adaptor proteins MyD88 and TRIF.[170] After stimulation with αCD3, CD8+ T cell activation, differentiation, and cytokine production were increased in WT, $TRIF^{-/-}$, $MyD88^{-/-}$, or $TRIF/MyD88^{-/-}$ mice. CD25, CD44, and CD69 expression was further upregulated in WT and $TRIF^{-/-}$ CD8+ T cells after αCD3 stimulation in the presence of R848 (Fig. 4.9).

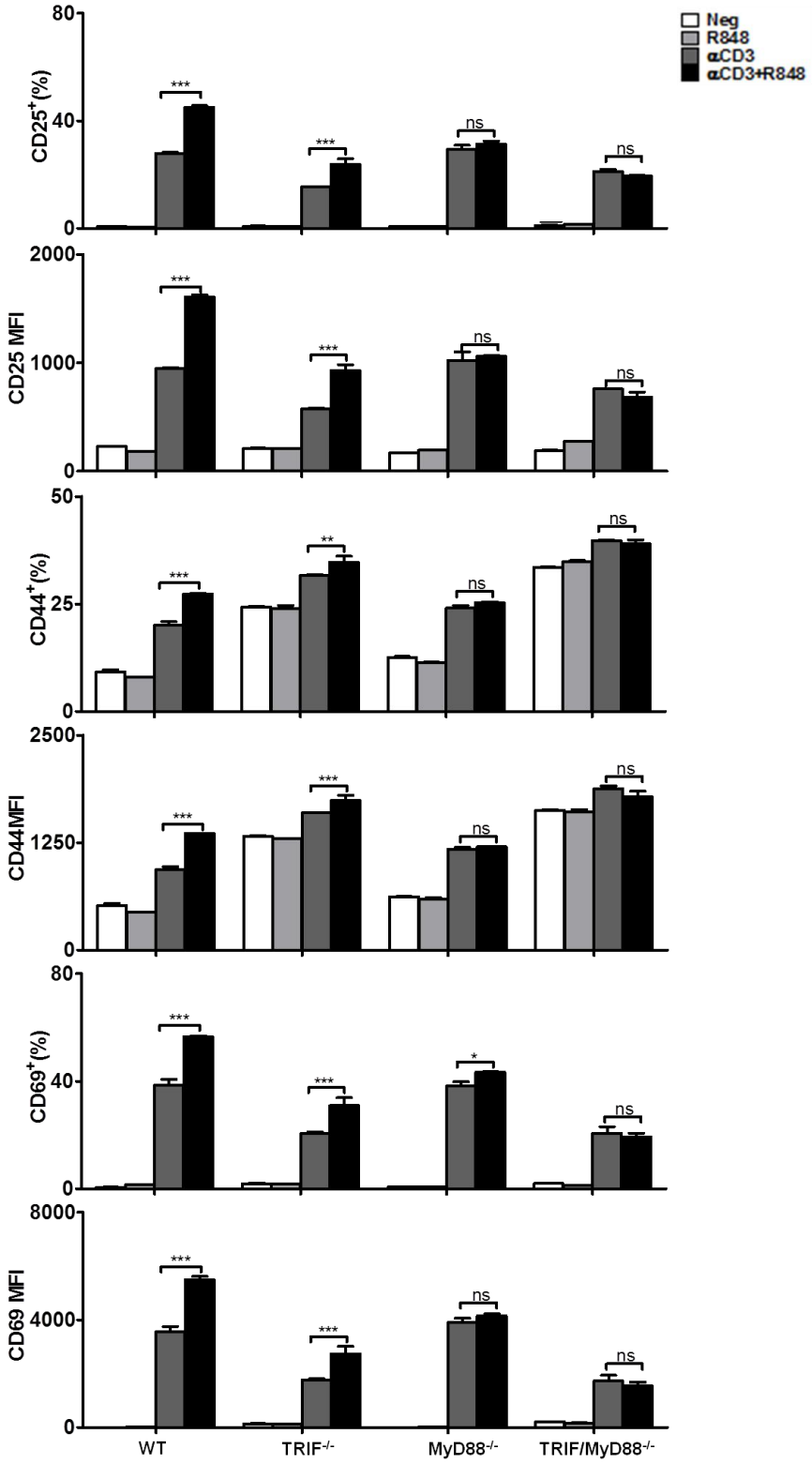



**Figure 4.9 Enhanced expression of activation markers in TLR7-activated CD8+ T cells is MyD88-dependent.** Purified CD8+ T cells from WT, TRIF$^{-/-}$, MyD88$^{-/-}$, and TRIF/MyD88$^{-/-}$ mice were stimulated in plates with bound αCD3 antibody (5 μg/mL) alone or with R848 (10 μg/mL) for 24 h. CD25, CD44, and CD69 expression in CD8+ T cells was analyzed by flow cytometry. Data are representative of two independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

The transcription factors T-bet and Eomes were also significantly increased in CD8+ T cells upon stimulation with αCD3+R848 only in WT and TRIF−/− CD8+ T cells (Fig. 4.10).

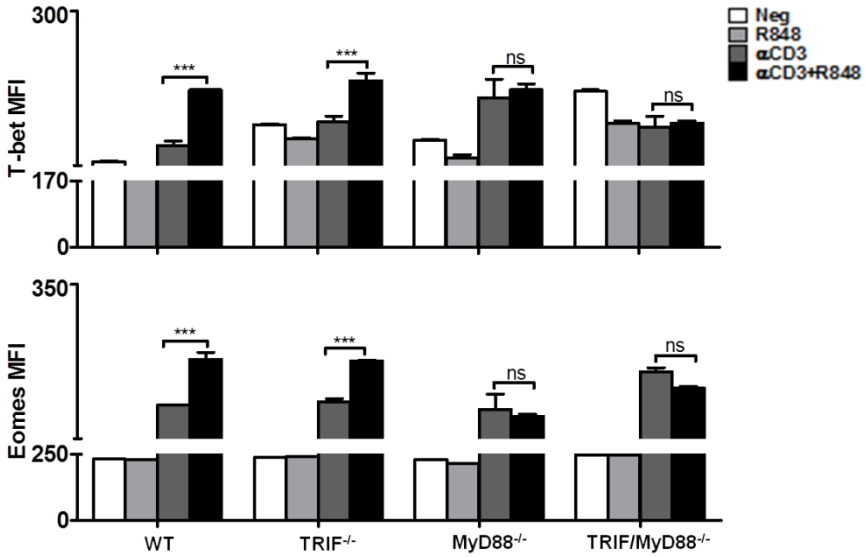



**Figure 4.10 Enhanced expression of transcription factors in TLR7-activated CD8+ T cells is MyD88-dependent.** Purified CD8+ T cells from WT, TRIF$^{-/-}$, MyD88$^{-/-}$, and TRIF/MyD88$^{-/-}$ mice were stimulated in plates with bound αCD3 antibody (5 μg/mL) alone or with R848 (10 μg/mL) for 24 h. T-bet and Eomes expression in CD8+ T cells were analyzed by flow cytometry. Data are representative of two independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

Moreover, IFN-γ and IL-2 secretion were elevated in WT and TRIF$^{-/-}$ CD8+ T cells under R848 co-stimulation (Fig. 4.11). However, R848 co-stimulation failed to further enhance the effector function of MyD88$^{-/-}$ and TRIF/MyD88$^{-/-}$ CD8+ T cells. MyD88 deficiency abolished R848-mediated enhanced CD8+ T cell effector function, confirming that this process is dependent on the MyD88 pathway.

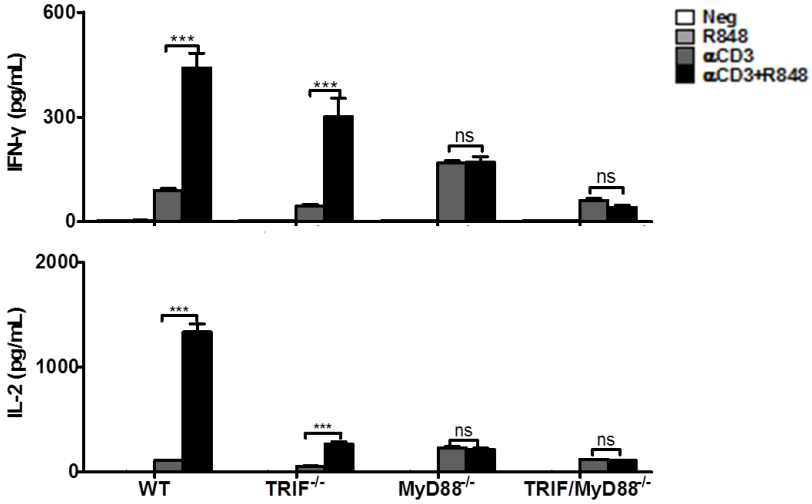



**Figure 4.11 Enhanced secretion of cytokine in TLR7-activated CD8+ T cells is MyD88-dependent.** Purified CD8+ T cells from WT, TRIF$^{-/-}$, MyD88$^{-/-}$, and TRIF/MyD88$^{-/-}$ mice were stimulated in plates with bound αCD3 antibody (5 μg/mL) alone or with R848 (10 μg/mL) for 24 h. IFN-γ and IL-2 production in CD8+ T cells were analyzed by flow cytometry. Data are representative of two independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

### 4.2.2 Direct activation of CD8+ T cells by R848 in the purification system

Although the purity of isolated CD8+ T cells exceeded 96% (Fig. 4.12), some residual non-CD8+ T cells were still present including APCs. Residual APCs in the fraction of purified CD8+ T cells may respond to R848, thus indirectly improving the functionality of CD8+ T cells.

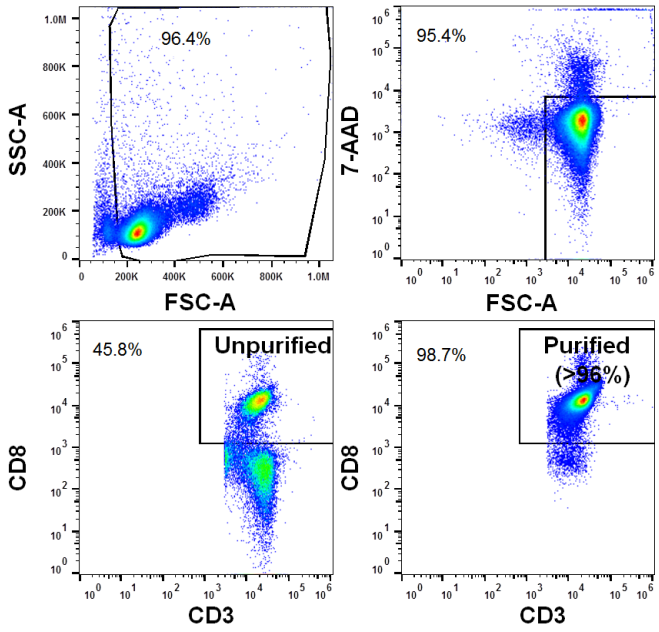



**Figure 4.12 Purification of CD8+ T cells by micro-beads and verification by flow cytometry.** CD8+ T cells were isolated from the spleens of WT mice using Miltenyi micro-beads. The purity of CD8+ T cells was verified by flow cytometry.

To exclude this possibility, MyD88−/− splenocytes were mixed with WT splenocytes at a ratio of 1:1. Equal numbers of naïve WT and MyD88−/− splenocytes were then subjected to CD8+ T cell purification by magnetic bead separation. MyD88−/− CD8+ T cells were labeled with the indicated fluorescence marker CFSE and thereby distinguished from WT CD8+ T cells by flow cytometric analysis (Fig. 4.13A). Purified WT and Myd88−/− CD8+ T cells with some residual APCs were further stimulated with αCD3 with or without R848. Results showed that R848 increased CD69 expression as well as IFN-γ production in WT CD8+ T cells but not in MyD88−/− CD8+ T cells. Therefore, R848 acted directly on CD8+ T cells in the in vitro culture system (Fig. 4.13B).

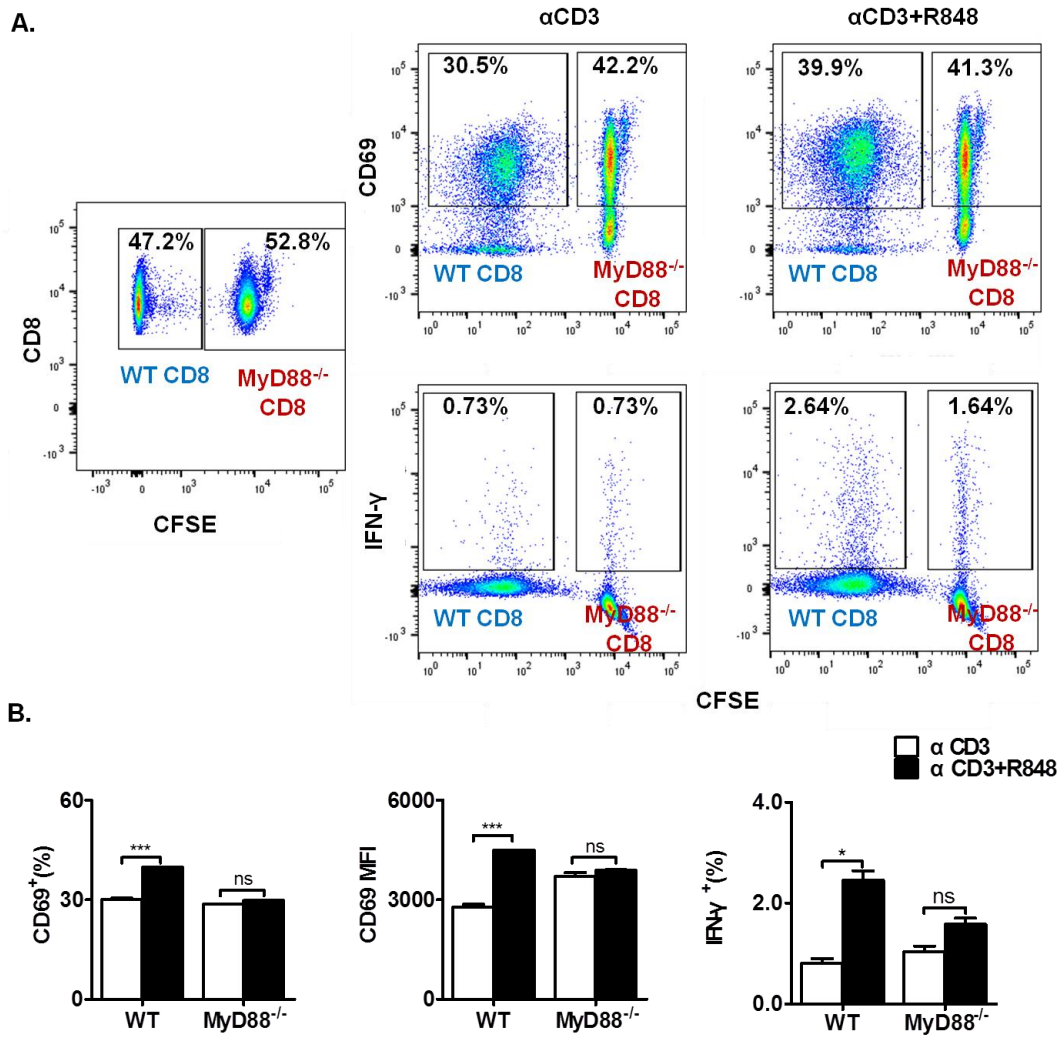


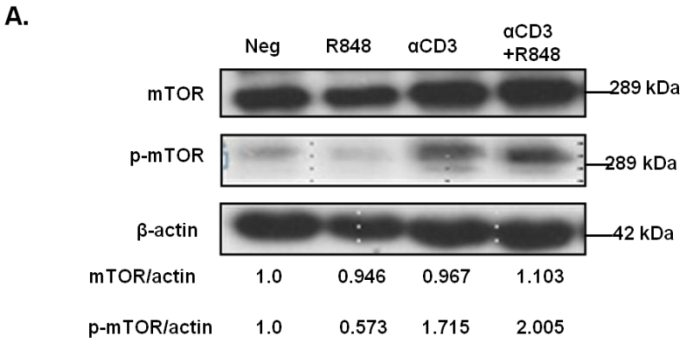

**Figure 4.13 Exclusion of the effect of residual non-CD8+ T cells on CD8+ T cell activation.** (A) Splenocytes from MyD88$^{-/-}$ mice were labeled with CFSE and mixed with the splenocytes from WT mice at a ratio of 1:1. They were then highly purified using a cocktail antibody in conjunction with bead separation. Purified CD8+ T cells were then stimulated with αCD3 antibody (5 μg/mL) either alone or in the presence of R848 (10 μg/mL) for 24 h. (B) CD69 expression and IFN-γ production in CD8+ T cells were analyzed by flow cytometry. The frequencies and/or MFI of CD69+ and IFN-γ+CD8+ T cells are presented respectively.

## 4.3 mTOR signaling is required for TLR7 ligands to regulate CD8+ T cell activation

### 4.3.1 mTOR signaling pathway is activated in R848-costimulated CD8+ T cells

Previous studies have reported that the mTOR pathway is linked to MyD88 signaling during the engagement of TLRs in CD4 or CD8+ T cells.[148] To investigate whether TLR7 activation regulates mTOR signaling, we determined mTOR expression and phosphorylation by western blotting and flow cytometry. The results showed that αCD3 stimulation led to a high level of phosphorylated mTOR in CD8+ T cells. R848 further slightly increased the levels of mTOR, phosphorylated mTOR and Akt in CD8+ T cells. Obviously, αCD3-stimulated CD8+ T cells

had a high expression of phosphorylated mTOR. Costimulatory molecular R848 led to an increased protein level of mTOR/β-actin from the ratio of 0.967 to 1.103, p-mTOR/β-actin from the ratio of 1.715 to 2.005 by western blot analysis. The dot of p-mTOR$^{+}$CD8+ T cells is 1.0% in the αCD3 group, while it is 2.85% in the costimulation group by FACS analysis. The dot of Akt$^{+}$CD8+ T cells is 0.25% in the αCD3 group, while it increased into 2.09% in the costimulation group by FACS analysis. It indicates that R848 further increased the expression of mTOR, phosphorylated mTOR, and Akt at the protein level (Figure. 4.14).



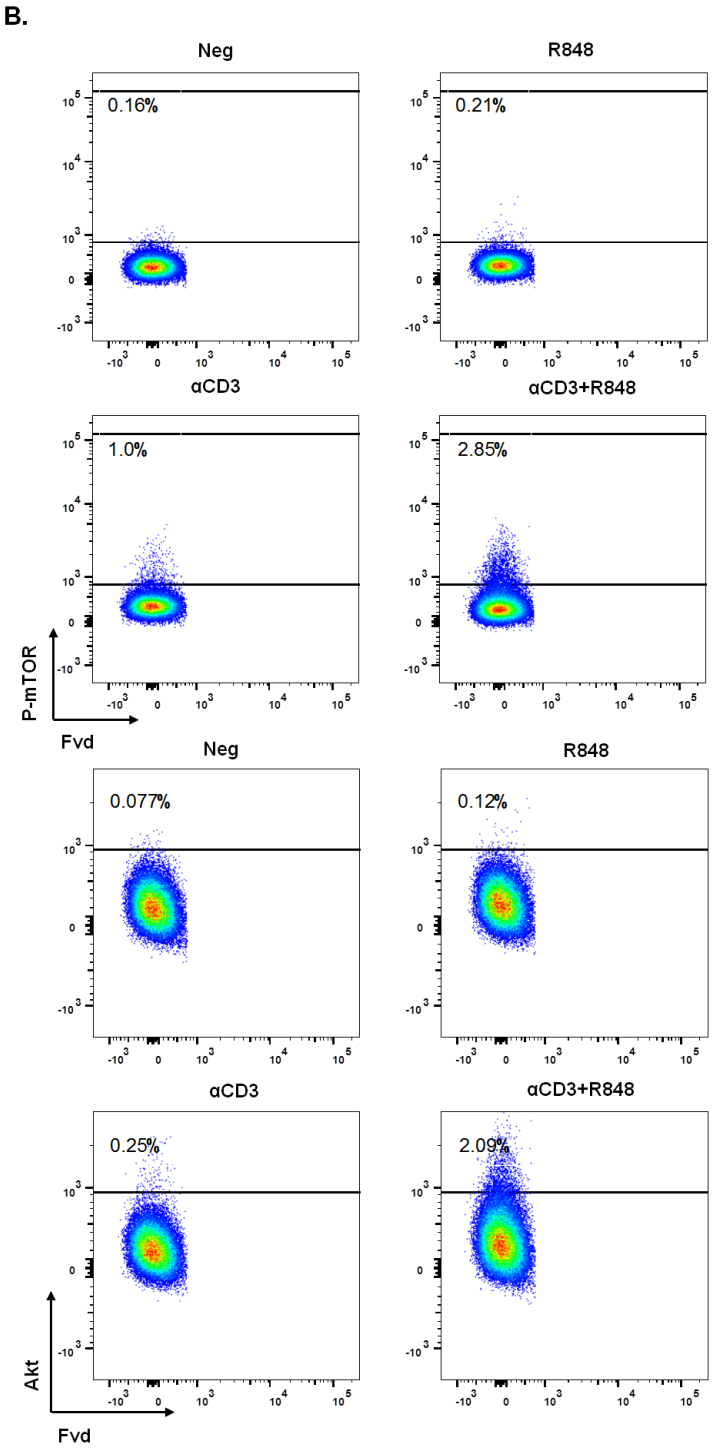



**Figure 4.14 R848 enhances the mTOR signaling in CD8+ T cells.** Purified CD8+ T cells were stimulated with αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL) for 48 h in the presence of Rapamycin (2 μM) or Akti (1 μM). (A) mTOR and phosphorylated mTOR in CD8+ T cells were detected by western blotting. (B) The level of phosphorylated mTOR and Akt were further determined by flow cytometry. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

### 4.3.2 Blocking mTOR pathway inhibited the enhanced functionality of R848-costimulated CD8+ T cells

To confirm that mTOR signaling is required to regulate TLR7-mediated CD8+ T cell activation, CD8+ T cells were stimulated by αCD3 and/or R848 and treated with two different mTOR pathway inhibitors, Rapamycin and Akti-1/2. Results indicated that despite stimulation by the polyclonal αCD3 antibody, inhibition of the mTOR pathway reduced the numbers of vital CD8+ T cells. However, we found that the numbers of vital CD8+ T cells were increased when R848 was co-applied in the presence of αCD3, depending on the inhibition of the mTOR pathway (Figure. 4.15).

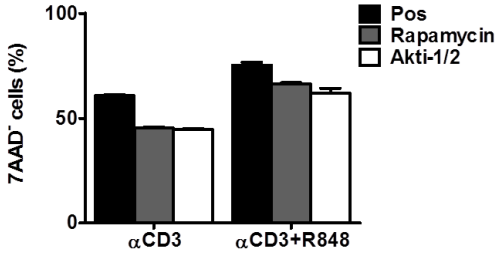



**Figure 4.15 mTOR signaling regulates the proliferation of CD8+ T cells.** Purified CD8+ T cells were stimulated with αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL) in the presence of either Rapamycin (2 μM) or Akti-1/2 (1 μM) for 48 h. Dead cells were labeled with 7AAD.

Consistent with previous studies, inhibition of mTOR slightly reduced the αCD3-induced T cell activation as indicated by a lower frequency of CD8+ T cells expressing activation markers, such as CD25 and CD44, and less IFN-γ production by CD8+ T cells. In the presence of Rapamycin, the use of R848 was still able to slightly upregulate the expression of CD25, CD44, and CD69. However, compared to the strong activation induced by R848 together with αCD3, the application of Rapamycin or Akti-1/2 significantly suppressed the upregulation of CD25, CD44, and CD69 in CD8+ T cells by R848 treatment (Figure. 4.16A). The decreased expression of these activation markers in the αCD3+R848 stimulated cells compared to the αCD3 only stimulated control could be clearly judged in the format of fold changes (Figure. 4.16B). Rapamycin and Akti-1/2 also abolished the further increase in IFN-γ and TNF-α production by CD8+ T cells in response to R848 (Figure. 4.16C).

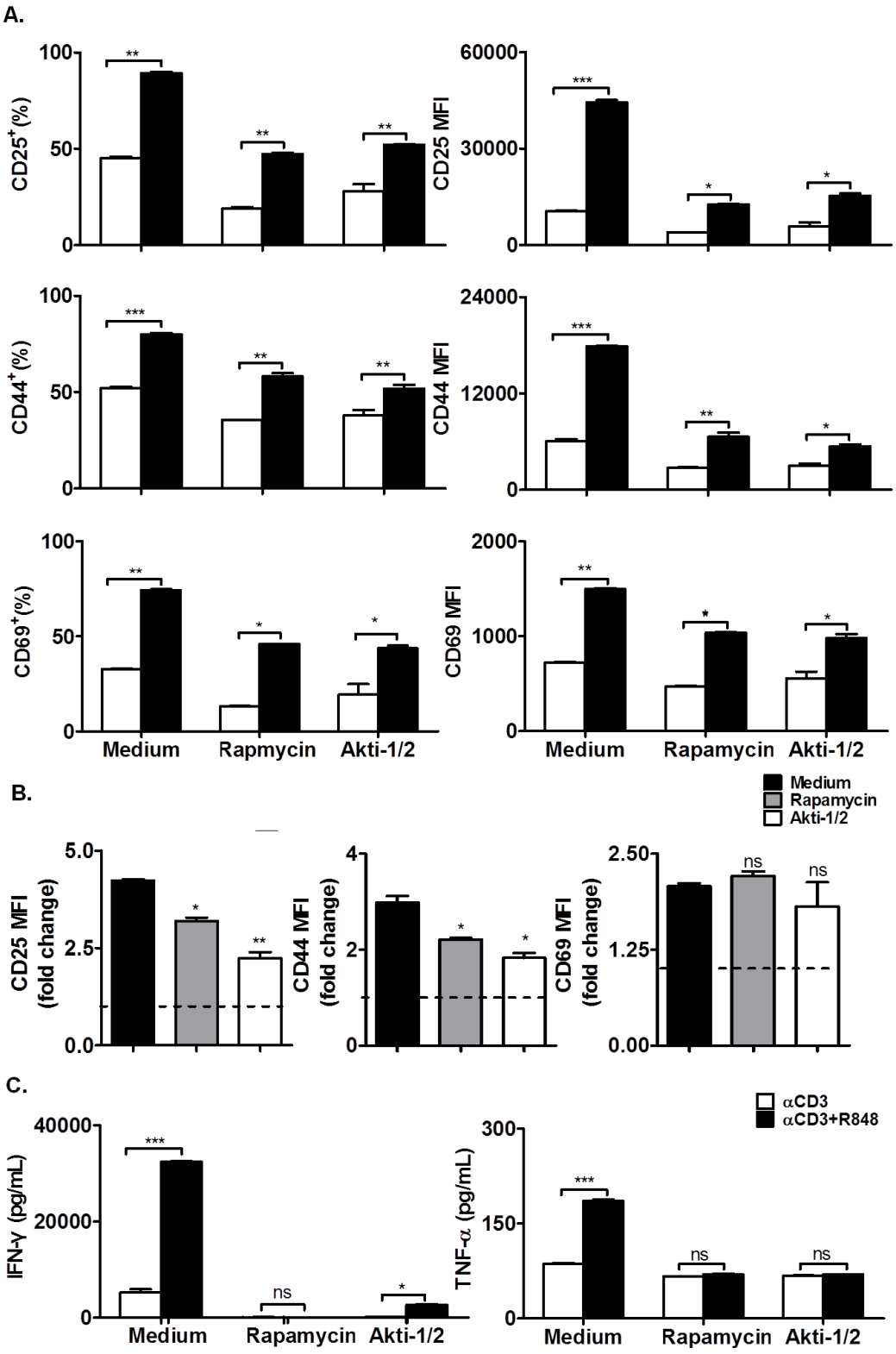

A.
CD25+(%)
CD25 MFI
CD44+(%)
CD44 MFI
CD69+(%)
CD69 MFI
Medium
Rapmycin
Rapamycin
Akti-1/2
B.
Medium
Rapamycin
Akti-1/2
CD25 MFI (fold change)
CD44 MFI (fold change)
CD69 MFI (fold change)
ns
C.
αCD3
αCD3+R848
IFN-γ (pg/mL)
TNF-α (pg/mL)

**Figure 4.16 mTOR signaling regulates the effector function of CD8+ T cells.** Purified CD8+ T cells were stimulated with αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL) for 48 h in the presence of Rapamycin (2 μM) or Akti (1 μM). (A) The activation of CD8+ T cells was assessed by staining with αCD25, αCD44, and αCD69 antibodies. (B) The expression of CD25, CD44, and CD69 in the αCD3+R848 stimulated cells was shown as fold changes compared to the αCD3 stimulated cells in the corresponding treatment of Rapamycin/Akti-1/2. (C) IFN-γ and TNF-α secretion were detected by specific ELISAs. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

The transcription factor IRF4 coordinates mTOR signaling to orchestrate immune activation and the function of T cells[171]. We detected the expression of this marker by intracellular staining and found that enhanced IRF4 expression in response to R848 was abolished by Rapamycin and Akti-1/2 treatment (Figure. 4.17). These results suggest that the blockade of Akt–mTOR pathway could significantly reduce or even abolish the R848-induced enhancement of T cell activation and function.

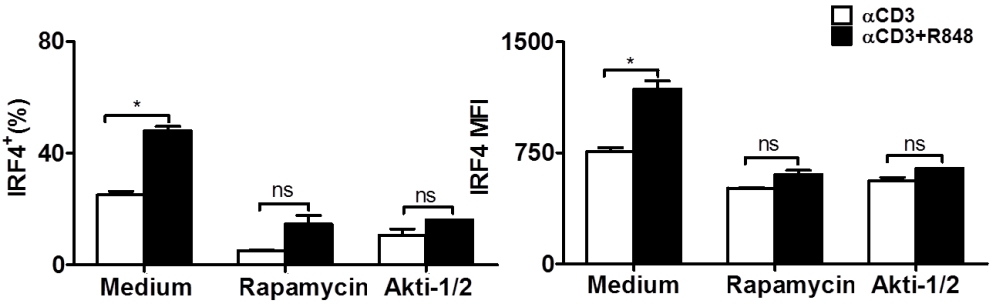



**Figure 4.17 mTOR signaling regulates IRF4 expression of CD8+ T cells.** Purified CD8+ T cells were stimulated with αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL) for 48 h in the presence of Rapamycin (2 μM) or Akti (1 μM). IRF4 expression was shown by representative dot plots and MFI in CD8+ T cells. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

Taken together, these data show that the Akt–mTOR pathway is required for the TLR7-mediated enhancement of immune functions in CD8+ T cells.

## 4.4 Akt-mTOR signaling pathway plays an important role in TLR7-mediated improvement of glycolysis in CD8+ T cells

### 4.4.1 Costimulation of R848 enhanced the glycolytic metabolism of CD8+ T cells

The modulation of metabolic pathways can significantly influence T cell activation and differentiation. TLR-driven early glycolysis reprogramming leads to the activation of DCs.[156] It is also known that mTOR-regulated T cell metabolism is required for the initial T cell activation, rapid proliferation, and

acquisition of effector functions. Further, mTOR signaling induces complex networks of reprogramming including enhanced aerobic glycolysis to facilitate rapid T cell clonal expansion.[163] To investigate TLR7-induced metabolic changes in CD8+ T cells, the expression of Glut-1 and the rate-limiting enzymes HK2 and LDH-α in the glycolytic pathway were determined by real-time RT-PCR (Fig. 4.18).

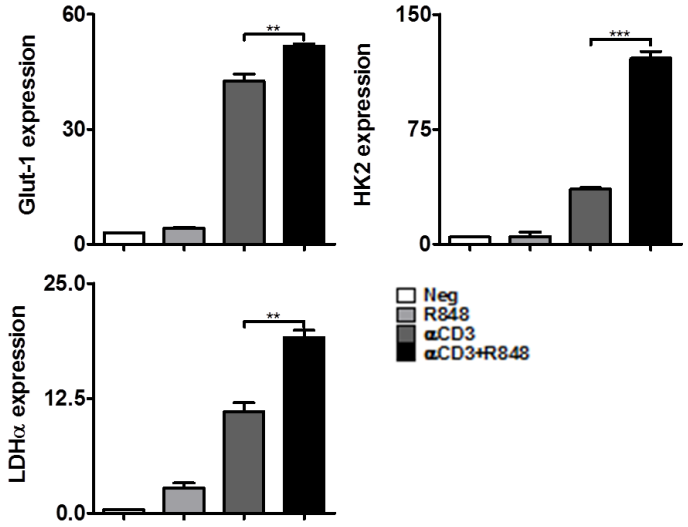



**Figure 4.18 TLR7 agonists improve glycolytic related gene expression via mTOR signaling in CD8+ T cells.** Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL) in the presence of Rapamycin (2 μM) or Akti-1/2 (1 μM). The expression of the glycolysis-related genes Glut-1, HK2, and LDHα was measured by real-time RT-PCR 24 h after stimulation. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

Increased expression of glycolysis-related genes was found in CD8+ T cells under R848 co-stimulation compared to that with αCD3 stimulation alone. Meanwhile, Glut-1 was also detected by flow cytometry. The uptake of glucose was measured by detecting the mean fluorescence intensity of the glucose analog 2-NBDG. A significantly higher Glut-1 expression level and increased MFI of 2-NBDG in CD8+ T cells were not achieved at 24 h but were detected at 48 h upon αCD3+R848 treatment compared to that with αCD3 alone (Fig. 4.19).

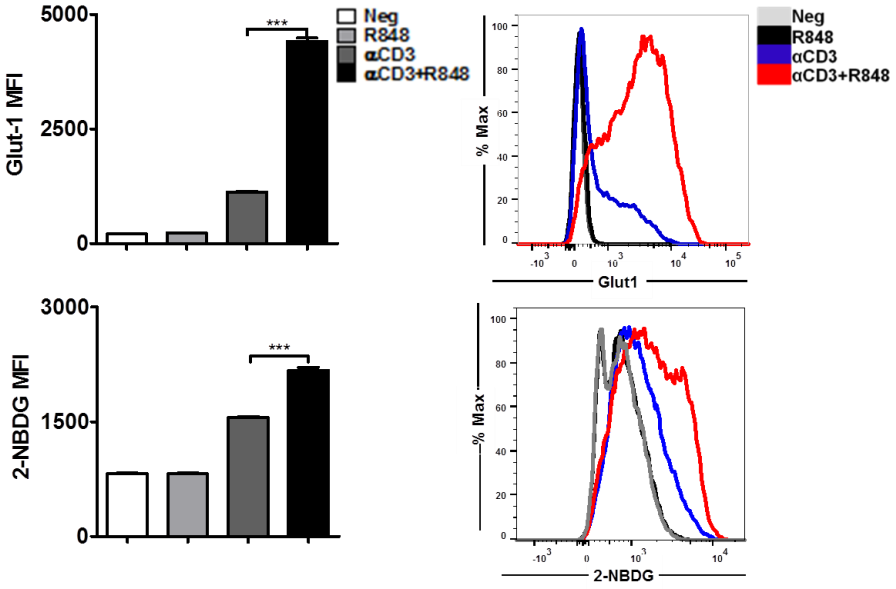



**Figure 4.19 TLR7 agonists improve glucose uptake via mTOR signaling in CD8+ T cells.** Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL) in the presence of Rapamycin (2 μM) or Akti-1/2 (1 μM). The Glut-1 expression was detected by flow cytometry and uptake of glucose was measured by detecting MFI of the glucose analog 2-NBDG in CD8+ T cells 48 h after stimulation. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

HK2 expression at the protein level was also significantly increased with αCD3+R848 treatment compared to that with αCD3 alone (Fig. 4.20).

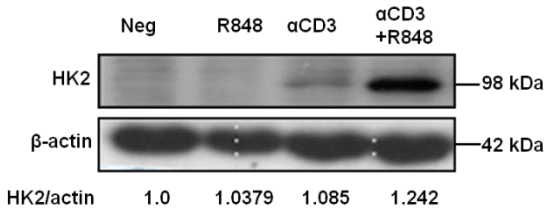



**Figure 4.20 TLR7 agonists improve HK2 expression via mTOR signaling in CD8+ T cells.** Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL). HK2 expression was detected by western blotting 24 h after stimulation. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$;*** $p<0.001$)

In addition, the production of lactate by aerobic glycolysis was measured in the culture supernatants, showing that R848 could enhance lactate production by CD8+ T cells in the presence of αCD3 (Fig. 4.21).

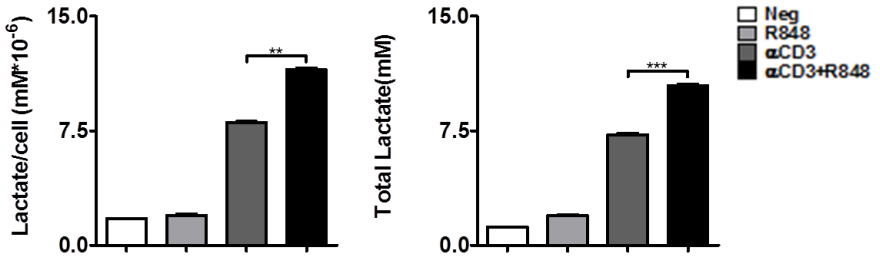



**Figure 4.21 TLR7 agonists improve lactate production in CD8+ T cells.** Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL). Glycolysis in CD8+ T cells was measured by lactate production for 24 h. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

### 4.4.2 TLR7-induced metabolic alteration is mediated by the mTOR signaling pathway

We further addressed whether TLR7-induced metabolic changes in CD8+ T cells are dependent on mTOR signaling. Inhibitors of the mTOR signaling pathway were applied to cultures of CD8+ T cells and the mean fluorescence intensity of Glut-1 and 2-NBDG in CD8+ T cells was analyzed. Glut-1 expression and 2-NBDG uptake were significantly increased with αCD3 treatment and further enhanced by R848 at 48 h; however, this was decreased or abolished by Rapamycin and Akti-1/2 respectively. (Figure. 4.22).

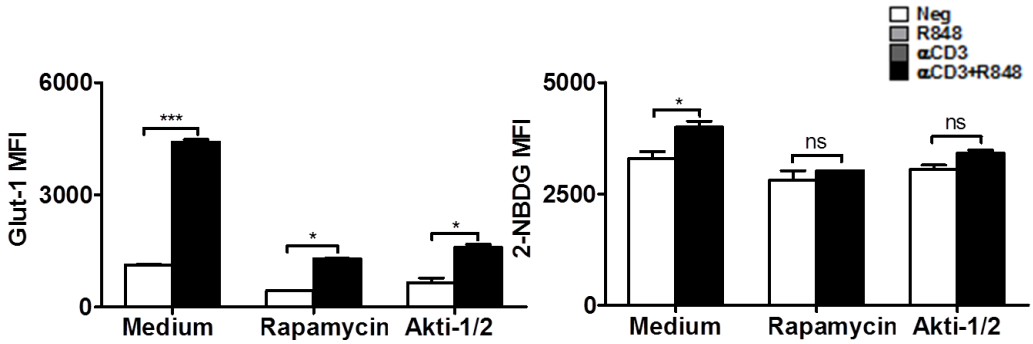



**Figure 4.22 TLR7 agonists improve glucose uptake via mTOR signaling in CD8+ T cells.** Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL) in the presence of Rapamycin (2 μM) or Akti-1/2 (1 μM). Glycolysis in CD8+ T cells was measured by detecting MFI of Glut-1 and 2-NBDG after treating the cells with Rapamycin or Akti-1/2 for 48 h. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

Lactate production was also reduced when the mTOR pathway was blocked, even in the presence of αCD3 and R848 (Figure. 4.23).

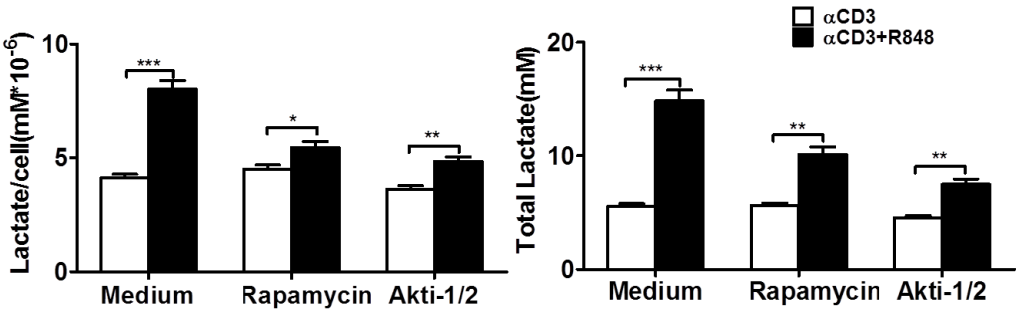



**Figure 4.23 TLR7 agonists improve lactate production via mTOR signaling in CD8+ T cells.**
Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL) in the presence of Rapamycin (2 μM) or Akti-1/2 (1 μM). Glycolysis in CD8+ T cells was measured by detecting lactate production after treating the cells with Rapamycin or Akti-1/2 for 48 h. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

By analyzing the fold change of the glycolysis-related parameters in the αCD3+R848 stimulated cells compared to the αCD3 only stimulated cells, the R848-induced increase of Glut-1 expression and lactate production was significantly decreased in the presence of Rapamycin and Akti (Figure 4.24). Thus, the mTOR signaling pathway plays an important role in mediating the TLR7-induced elevation of the glycolytic metabolism in CD8+ T cells.

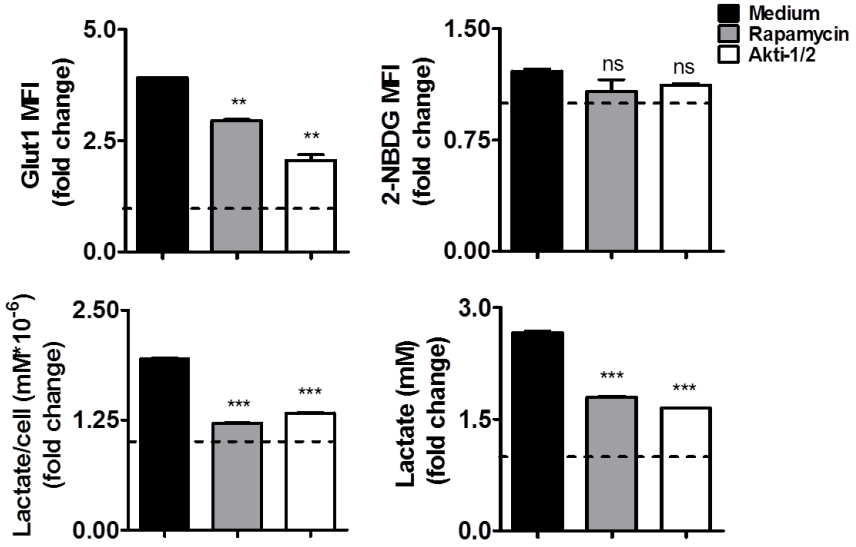



**Figure 4.24 TLR7 agonists improve glycolytic metabolism via mTOR signaling in CD8+ T cells.**
Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL) in the presence of Rapamycin (2 μM) or Akti-1/2 (1 μM). The expression of Glut-1, the uptake of 2-NBDG, and lactate production were measured as fold changes compared to the αCD3 stimulated cells in the corresponding treatment of Rapamycin/Akti-1/2. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

### 4.5 Glycolysis is essential for the TLR7-mediated enhanced effector function of CD8+ T cells

Glucose is an essential energy supply in cell culture media, and glucose deprivation can lead to impaired T cell activation.[172] To confirm that glycolysis drives the activation and functionality of CD8+ T cells, it was blocked with either the glucose analog 2-DG, which inhibits cellular hexokinase, or the removal of glucose from culture media. To maintain the energy supply, 1 mM sodium pyruvate and 2 mM L-glutamine were added to the glucose-free medium. Annexin V+7AAD staining was then used to exclude apoptotic cells (Fig. 4.25).

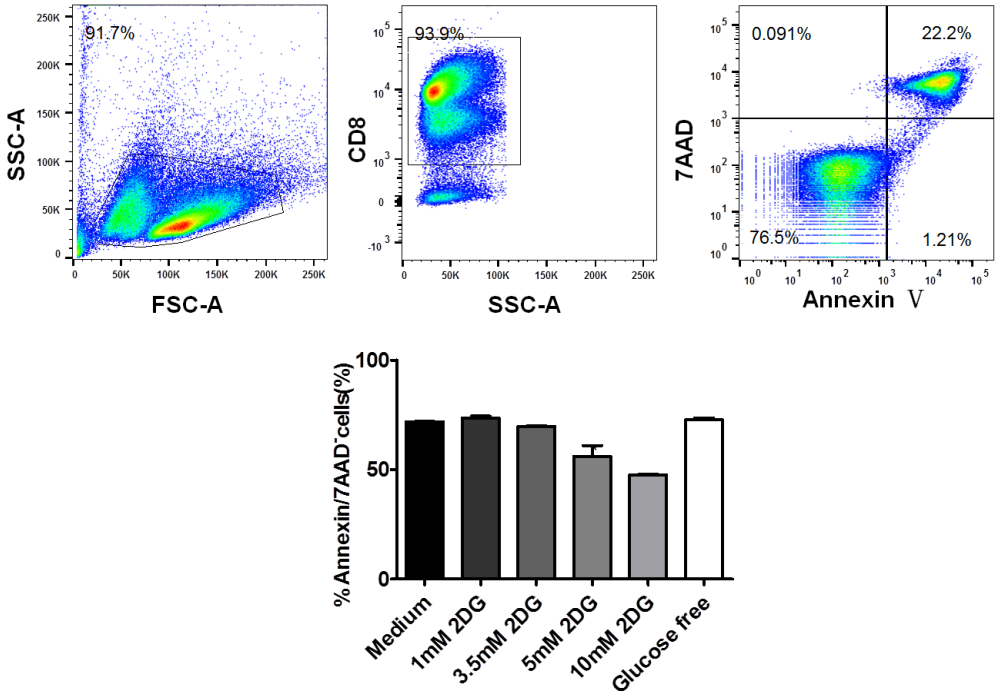



**Figure 4.25 Apoptosis in CD8+ T cells after treatment with 2DG or cultured in the glucose-free medium.** Purified CD8+ T cells were stimulated with αCD3 antibody (5 μg/mL) with R848 (10 μg/mL) in the presence of 2DG (1–10 mM) or glucose-deprived medium for 24 h. Apoptotic CD8+ T cells were stained with Annexin V and 7AAD. Dead cells were labeled with 7AAD.

The results showed that blocking glycolysis with 2-DG or glucose deprivation reduced CD44 expression and IFN-γ secretion during CD8+ T cell activation mediated by αCD3 and R848 (Fig. 4.26).

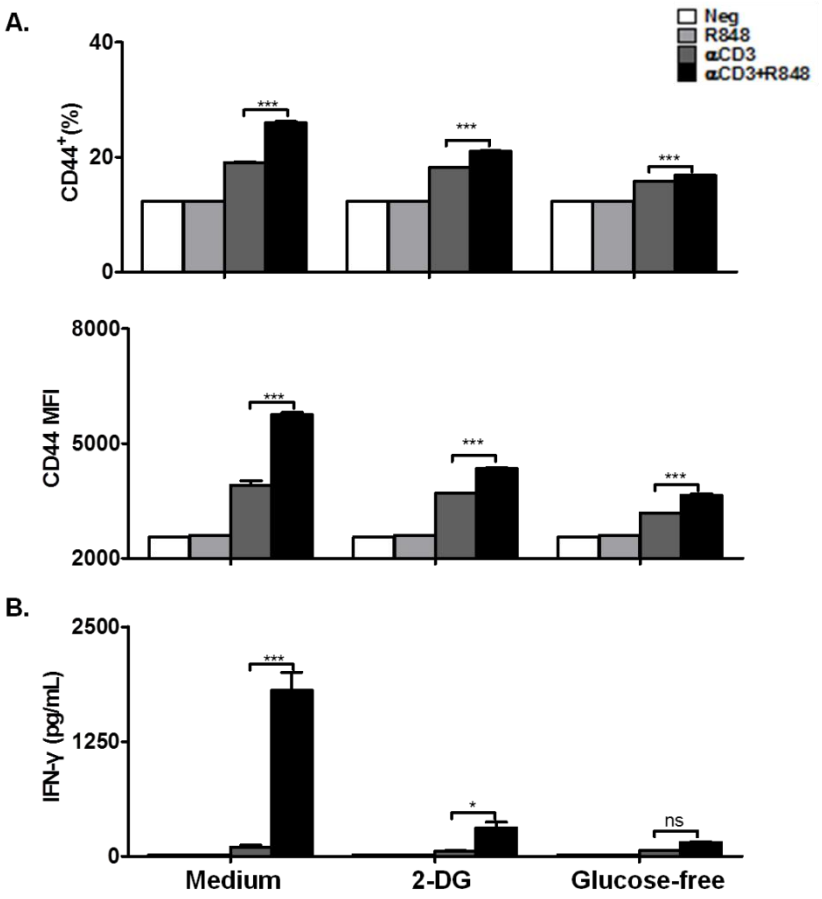



**Figure 4.26 Inhibition of glycolytic metabolism abolishes the functionality of CD8+ T cells.** Purified CD8+ T cells were stimulated with αCD3 antibody (5 μg/mL) or/and R848 (10 μg/mL) for 24 h in the presence of 2DG (2 mM) or in the glucose-free medium supplemented with pyruvate. (A) The activation of CD8+ T cells was assessed by staining with an αCD44 antibody. (B) IFN-γ secretion in CD8+ T cells was determined by a specific ELISA. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

At the same time, the expression of the transcription factors T-bet and Eomes was also downregulated in CD8+ T cells (Fig. 4.27).

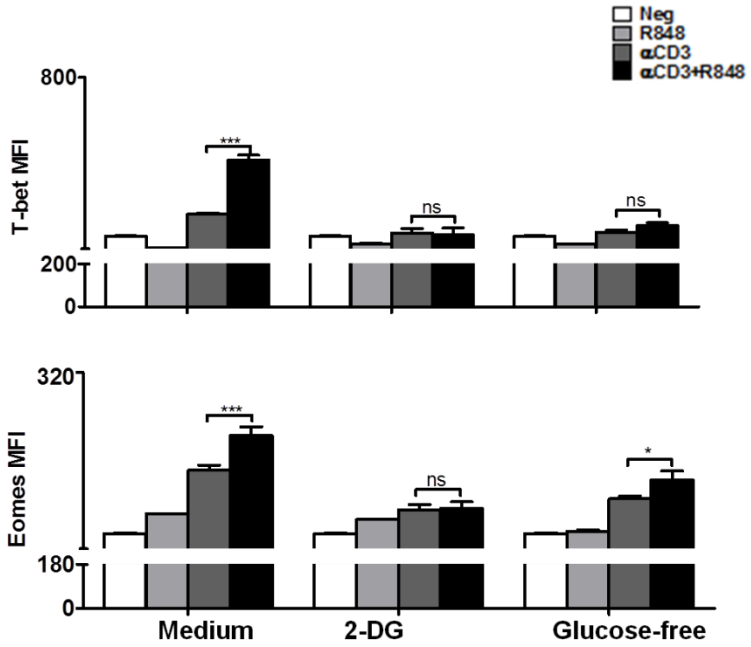



**Figure 4.27 Inhibition of glycolytic metabolism impairs the expression of transcription factors of CD8+ T cells.** Purified CD8+ T cells were stimulated with αCD3 antibody (5 μg/mL) or/and R848 (10 μg/mL) for 24 h in the presence of 2DG (2 mM) or in the glucose-free medium supplemented with pyruvate. T-bet and Eomes expression in CD8+ T cells was measured by flow cytometry and presented as MFI. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

The expression of IRF4, a transcription factor linked to both metabolism and immunity in CD8+ T cells, was significantly decreased when the glucose-free medium was used (Fig. 4.28).

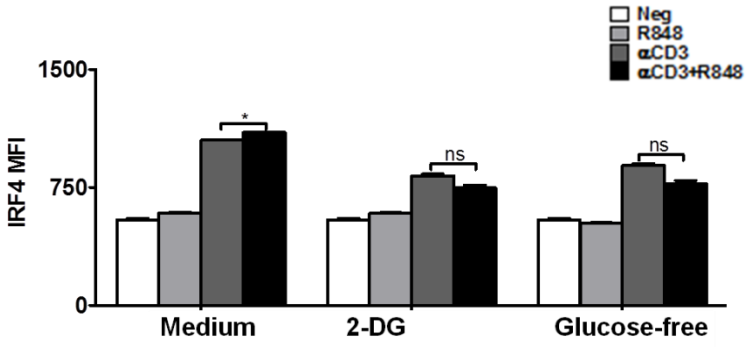



**Figure 4.28 Inhibition of glycolytic metabolism abolishes IRF4 expression of CD8+ T cells.** Purified CD8+ T cells were stimulated with αCD3 antibody (5 μg/mL) or/and R848 (10 μg/mL) for 24 h in the presence of 2DG (2 mM) or in the glucose-free medium supplemented with pyruvate. IRF4 expression in CD8+ T cells was assessed by flow cytometry. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

### 4.6 Human CD8+ T cells respond to the stimulation of R848 with enhanced expression of activation markers and glucose uptake

Consistent results were obtained using human CD8+ T cells after TLR7 stimulation and blockade of the Akt-mTOR pathway or glycolysis by inhibitors. Human CD8+ T cells were purified by MACS and cultured in plates with bound αCD3 antibody (5 μg/mL) alone or with R848 (1 μg/mL) for 24 h. CD8+ T cells were stained with αCD25 and αCD69 and analyzed by flow cytometry (Figure 4.29A). The uptake of glucose was measured by detecting MFI of the glucose analog 2-NBDG in CD8+ T cells. The stimulation with R848 enhanced both the expression of activation markers and glucose uptake in αCD3 activated human CD8+ T cells (Figure 4.29B).

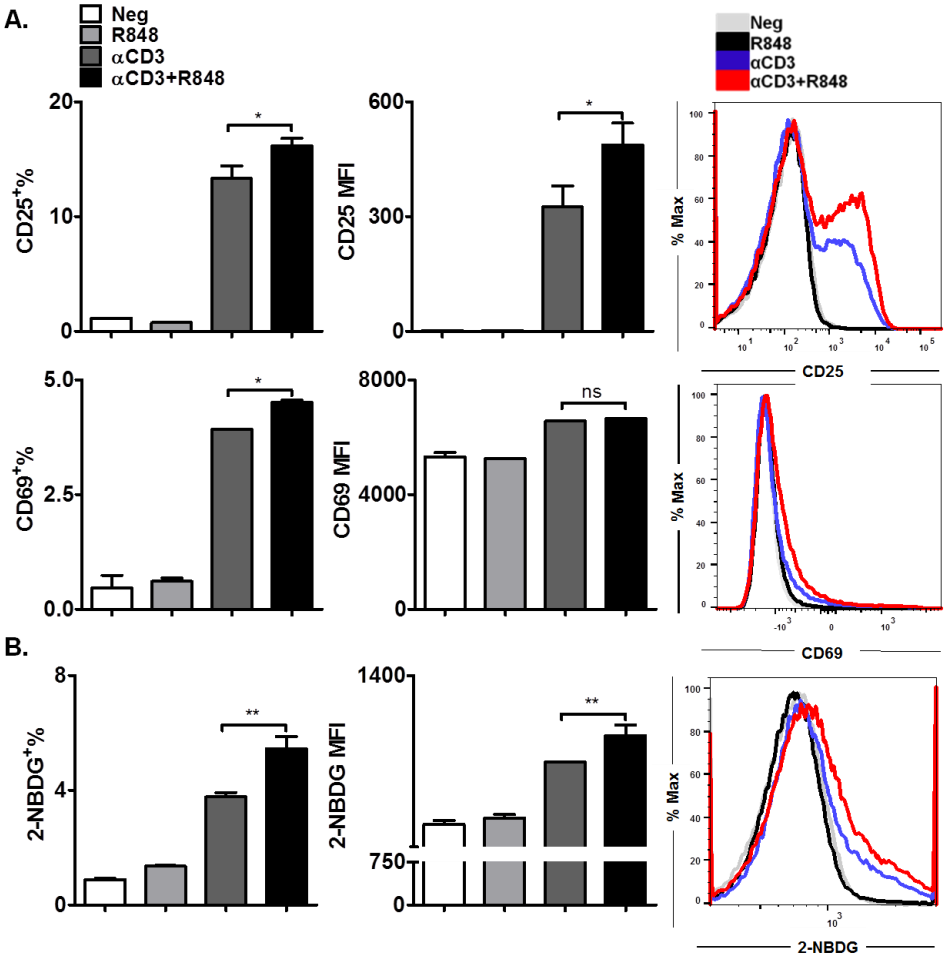



**Figure 4.29 TLR7 ligands enhance human CD8+ T cell activation and glucose up-taken.** Purified human CD8+ T cells were stimulated in plates with bound αCD3 antibody (5 μg/mL) alone or with R848 (1 μg/mL) for 24 h. (A) CD8+ T cells were stained with αCD25 and αCD69 and detected by flow cytometry. (B) The uptake of glucose was measured by detecting MFI of the glucose analog 2-NBDG in CD8+ T cells. Data are representative of three independent experiments and presented as mean±SD. (* $p<0.05$; ** $p<0.01$; ns, not significant)

Purified human CD8+ T cells were also stimulated with αCD3 antibody and R848 in the presence of either Rapamycin (10 μM), Akti-1/2 (5 μM) or 2-DG (10 mM) for 24 h. The blockade of the Akt-mTOR pathway and glycolysis significantly reduced the CD25 expression and glucose uptake in human CD8+ T cells (Fig 4.30).

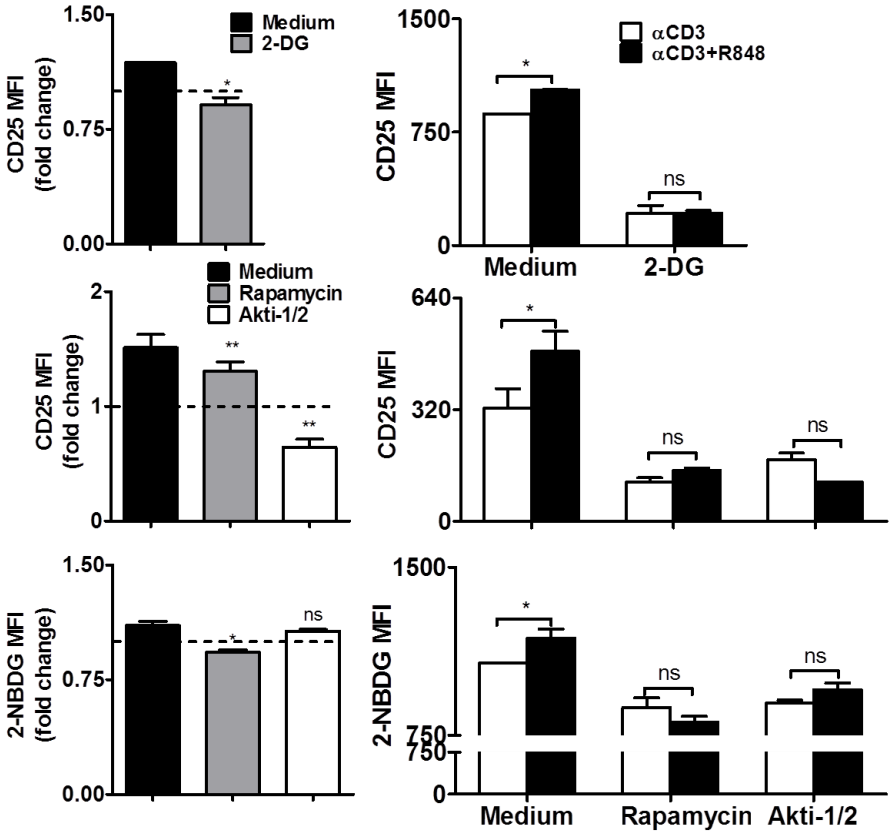



**Figure 4.30 TLR7- enhanced human CD8+ T cell activation and glucose up-taken can be blocked by 2-DG and Akt-mTOR inhibitors.** Purified human CD8+ T cells were stimulated in plates with bound αCD3 antibody (5 μg/mL) alone or with R848 (1 μg/mL) for 24 h in the presence of either 2-DG(10mM), Rapamycin (10 μM) or Akti-1/2 (5 μM) for 24 h. CD25 expression and the uptake of glucose were detected by flow cytometry and are presented as MFI (the right panel). Fold changes of CD25 and 2-NBDG expression between αCD3+R848 and αCD3 treatment in medium and 2-DG/Rapamycin/Akti-1/2 group is indicated by numbers (the left panel). Data are representative of three independent experiments and presented as mean±SD. (* $p<0.05$; ** $p<0.01$; ns, not significant). Statistical relevance was determined by Two-way ANOVA.

## 4.7 The transcription factor IRF4 plays a role in TLR7-mediated enhancement of glycolysis and effector functions in CD8+ T cells

### 4.7.1 R848 elevated functionality is impaired in IRF4$^{-/-}$ CD8+ T cells

IRF4 has been shown to be crucial for TCR affinity-induced metabolic and immune programming in T cells.[160] In the presence of TCR signaling, the expression of IRF4 was increased by TLR7 activation, whereas Rapamycin and Akti-1/2 treatment could reduce its

expression (Figure. 4.31).

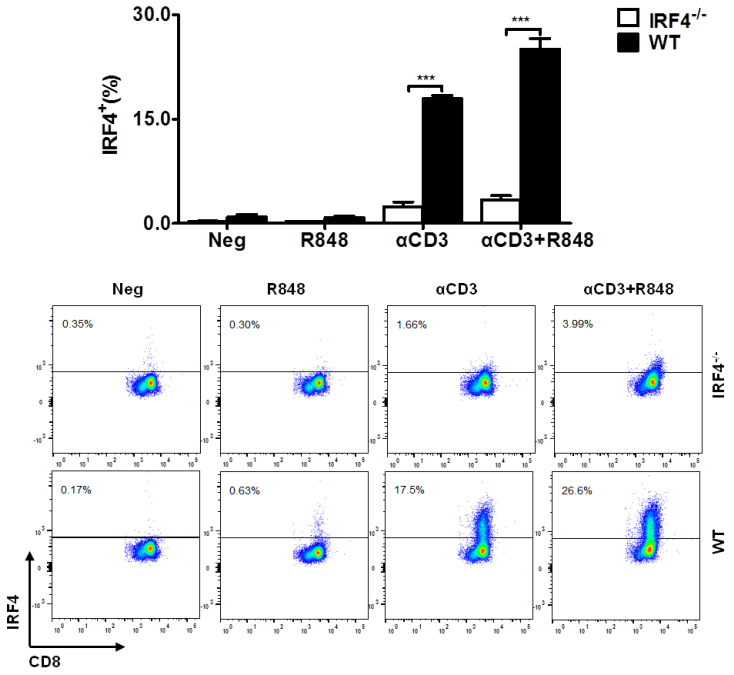



**Figure 4.31 R848-stimulated IRF4 expression in CD8+ T cells in IRF4$^{-/-}$mice.** Purified CD8+ T cells from WT or IRF4$^{-/-}$ mice were stimulated with αCD3 antibody (5 μg/mL) or/and R848 (10 μg/mL) for 24 h. Representative dot plots of IRF4 expression in CD8+ T cells as detected by flow cytometry. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

Thus, we examined the function of IRF4 in R848-stimulated CD8+ T cells derived from WT and IRF4−/− CD8+ T mice, with a focus on its role in remodeling the metabolism. Consistently, IRF4 was not expressed in these CD8+ T cells derived from IRF4−/− mice regardless of the presence of R848. Compared to that in WT CD8+ T cells, the enhanced CD25 and T-bet expression mediated by R848 treatment was impaired in IRF4−/− CD8+ T cells, whereas no decrease in Eomes expression was observed (Figure. 4.32).

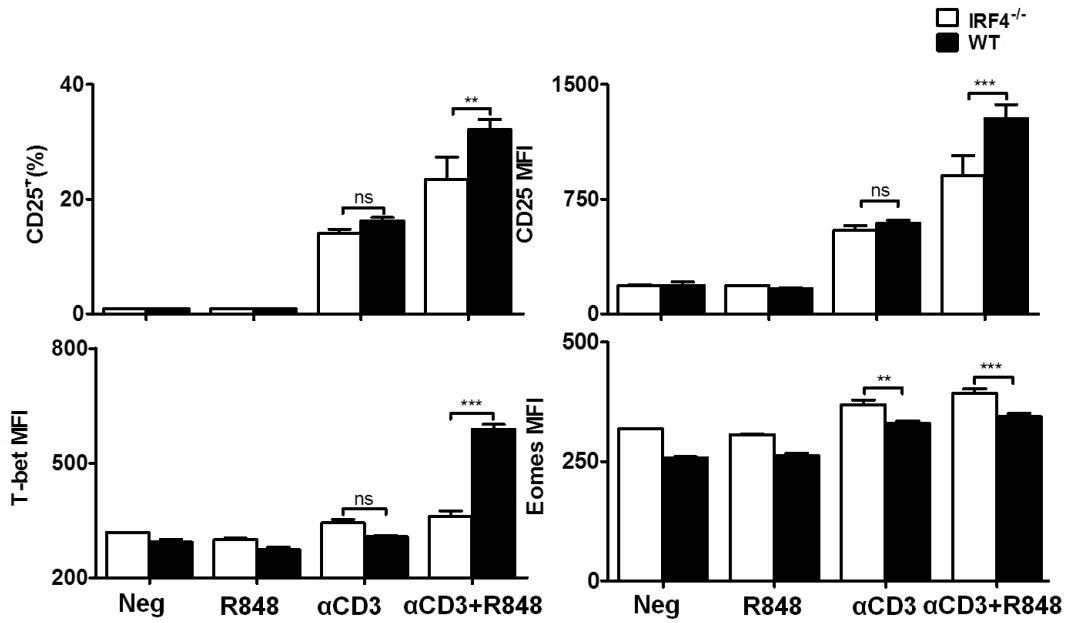



**Figure 4.32 R848-stimulated functional changes in CD8+ T cells are partly dependent on the transcription factor IRF4.** Purified CD8+ T cells from WT or IRF4$^{-/-}$ mice were stimulated with αCD3 antibody (5 μg/mL) or/and R848 (10 μg/mL) for 24 h. The expression of CD25, T-bet, and Eomes in CD8+ T cells was measured by flow cytometry. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

Moreover, enhanced IFN-γ secretion in R848-stimulated CD8+ T cells was abrogated in the absence of IRF4 (Figure. 4.33). Thus, R848-mediated increase of CD8+ T cell functions is partially dependent on IRF4 expression.

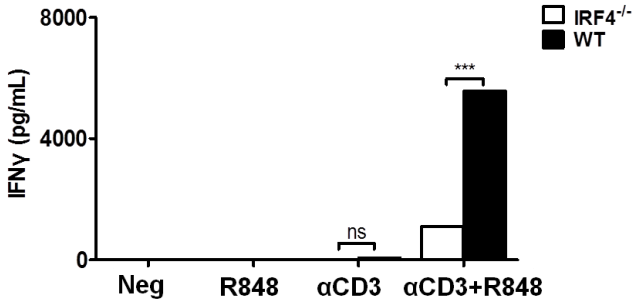



**Figure 4.33 R848-induced IFN-γ production in CD8+ T cells is partly dependent on the transcription factor IRF4.** Purified CD8+ T cells from WT or IRF4$^{-/-}$ mice were stimulated with αCD3 antibody (5 μg/mL) or/and R848 (10 μg/mL) for 24 h. IFN-γ production by CD8+ T cells from WT and IRF4$^{-/-}$ mice was detected by a specific ELISA. IFN-γ production by CD8+ T cells was measured by Elisa. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

### 4.7.2 R848 elevated glycolysis is impaired in IRF4$^{-/-}$ CD8+ T cells

We next investigated the role of IRF4 in the regulation of cellular metabolic pathways. We measured the expression of glycolysis-related genes by real-time RT-PCR and lactate production using an enzymatic assay. Whereas HK2 and LDH-α expression and lactate production were increased in both WT and IRF4$^{-/-}$ CD8+ T cells after αCD3 stimulation, R848 treatment led to a further enhancement of these parameters in WT CD8+ T cells, which was markedly diminished in IRF4$^{-/-}$ CD8+ T cells (Figure. 4.34).

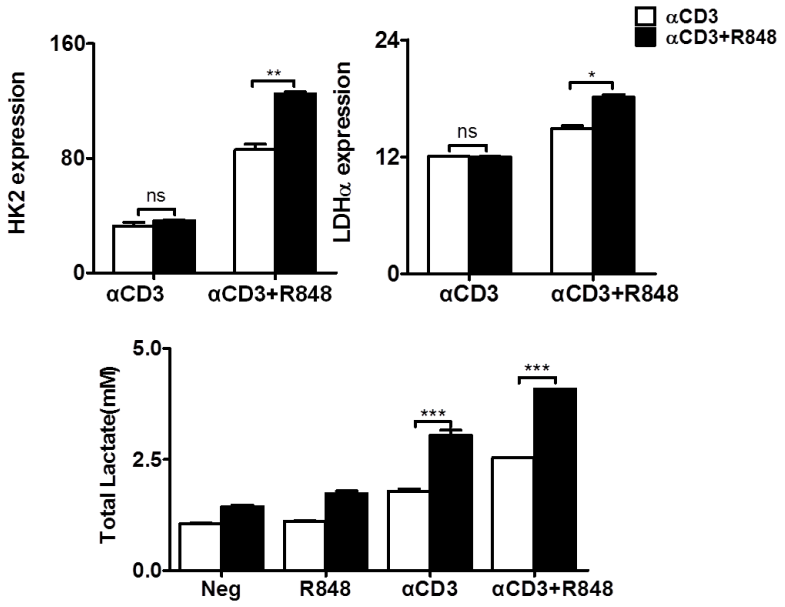



**Figure 4.34 R848-stimulated metabolic change in CD8+ T cells is partly dependent on the transcription factor IRF4.** Purified CD8+ T cells from WT or IRF4$^{-/-}$ mice were stimulated with αCD3 antibody (5 μg/mL) or/and R848 (10 μg/mL) for 24 h. The expression of HK2 and LDHα was quantified by real-time RT-PCR. Lactate production by CD8+ T cells was measured by a specific enzyme assay. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

### 4.7.3 BATF serves as a cofactor of IRF4 in regulating the function of CD8+ T

The downstream transcription factor BATF is also involved in controlling the glycolytic pathway and the regulation of CD8+ T cell functionality. This factor is critical for IRF4-mediated transcription in T cells.[173] We thus determined whether the elevated immune function after TLR7 co-stimulation was affected by BATF deficiency using CD8+ T cells isolated from WT and BATF$^{-/-}$ mice. The results demonstrated that the enhanced expression of CD25, CD44, and CD69, the production of IFN-γ, and the transcription of T-bet and Eomes were reduced in BATF$^{-/-}$ CD8+ T cells (Fig. 4.35).

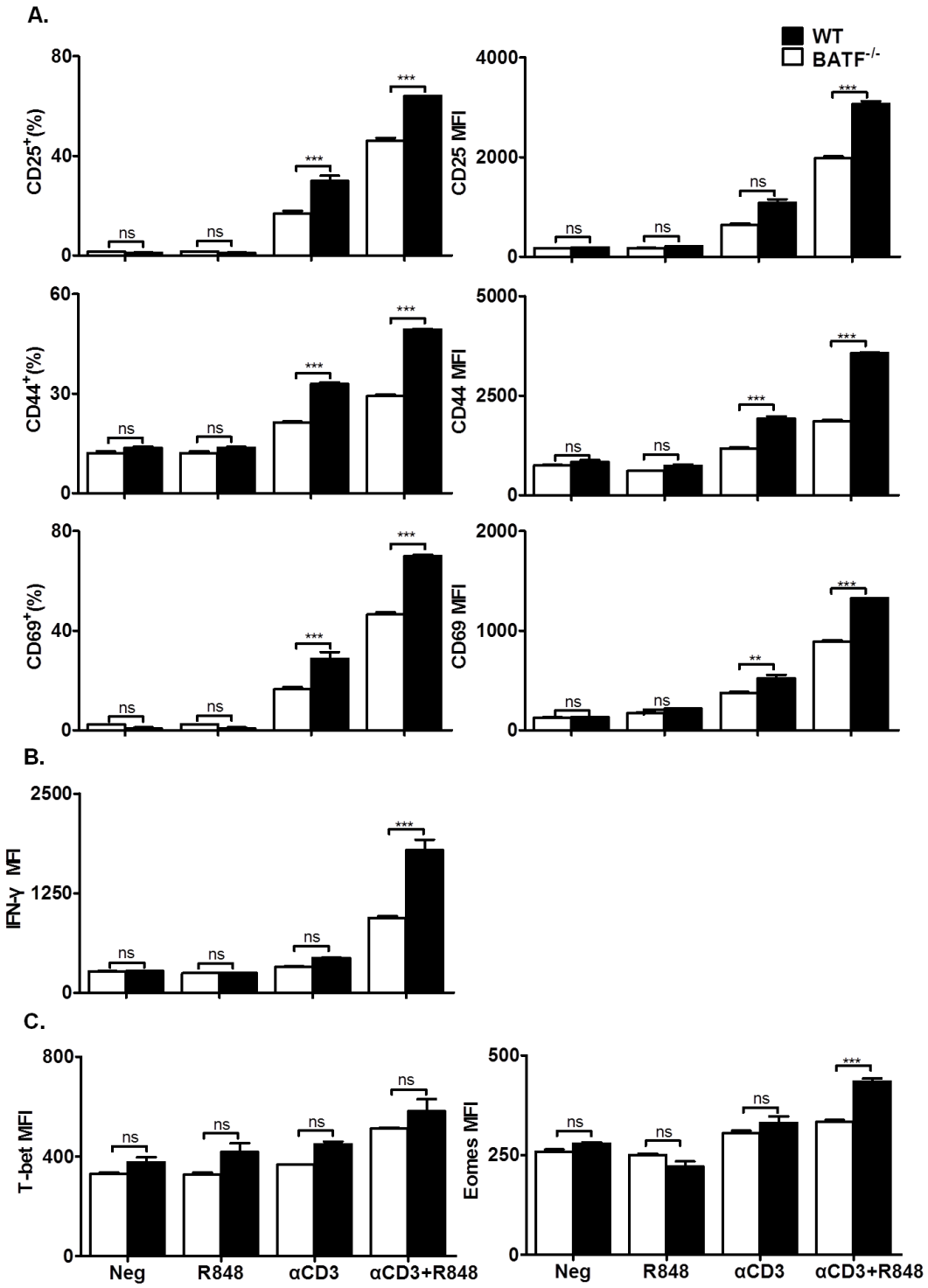



**Figure 4.35 R848-stimulated functional changes in BATF$^{-/-}$ CD8+ T cells.** Purified CD8+ T cells from WT or BATF$^{-/-}$ mice were stimulated with αCD3 or/and R848 for 24 h. (A) CD8+ T cells were stained with αCD25, αCD44, αCD69, and αIFN-γ (B) and by flow cytometry. The results are presented as dot plots and MFI. (C) T-bet and Eomes expression in CD8+ T cells was assessed by flow cytometry and is presented as MFI. Data are representative of three independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

These findings confirmed that IRF4 deficiency can partially affect the functionality and metabolism of CD8+ T cells. Therefore, IRF4 can serve as the downstream transcription

factor in the mTOR-glycolytic pathway. BATF might serve as a cofactor of IRF4 to regulate the functionality of CD8+ T cells. Thus, IRF4 and BATF are required for the full activation of CD8+ T cells via the regulation of cellular metabolism and effector functions and specifically for the TLR7-mediated signaling to enhance T cell functions.

### 4.8 Different TLRs manipulated metabolic and functional changes of CD8+ T cells

#### 4.8.1 P3C can also induce metabolic changes in CD8+ T cells, similar to R848

In our previous study, we proved that the TLR2 agonist P3C enhances the therapeutic efficiency of DNA vaccine against chronic hepadnaviral infection in the mouse model. To observe the effects of TLR2 agonists on CD8+ T cells, murine CD8+ T cells were treated with both P3C and R848 for 24/48 h. We found that both agonists promoted the functionality of CD8+ T cells. P3C is likely to be more efficient than R848, since the TLR2 agonist-P3C induced more glycolytic alteration of CD8+ T cells under the same TCR signaling than R848. Besides, a higher expression of glycolysis related genes, such as Glut-1, HK2, LDHa, BATF and IRF4, was found in P3C-costimulated CD8+ T cells than in R848-costimulated CD8+ T cells ( Fig.4.36).

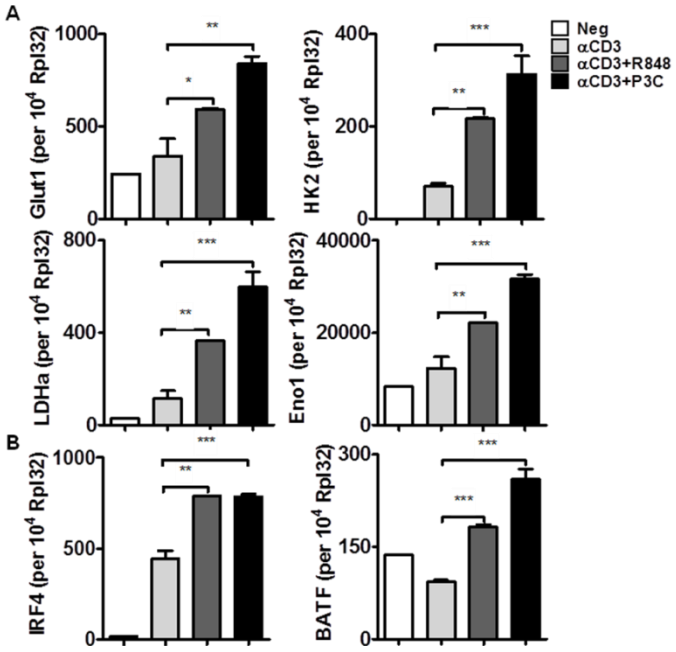



**Figure 4.36 Comparison of TLR-L-stimulated functional changes in CD8+ T cells.** Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without P3C(2μg/mL)/ R848 (10 μg/mL) for 24 h. (A) The expression of the glycolysis-related genes Glut-1, HK2, LDHα, and Eno1 was measured by real-time RT-PCR 24 h after stimulation. (B) The expression of the glycolysis-related genes IRF4 and BATF were measured by real-time RT-PCR 24 h after stimulation. Data are representative of three independent experiments.

(* p<0.05; ** p<0.01; ns, not significant)

Most importantly, the increased glycolysis rate was further verified by seahorse analysis ( Fig. 4.37).

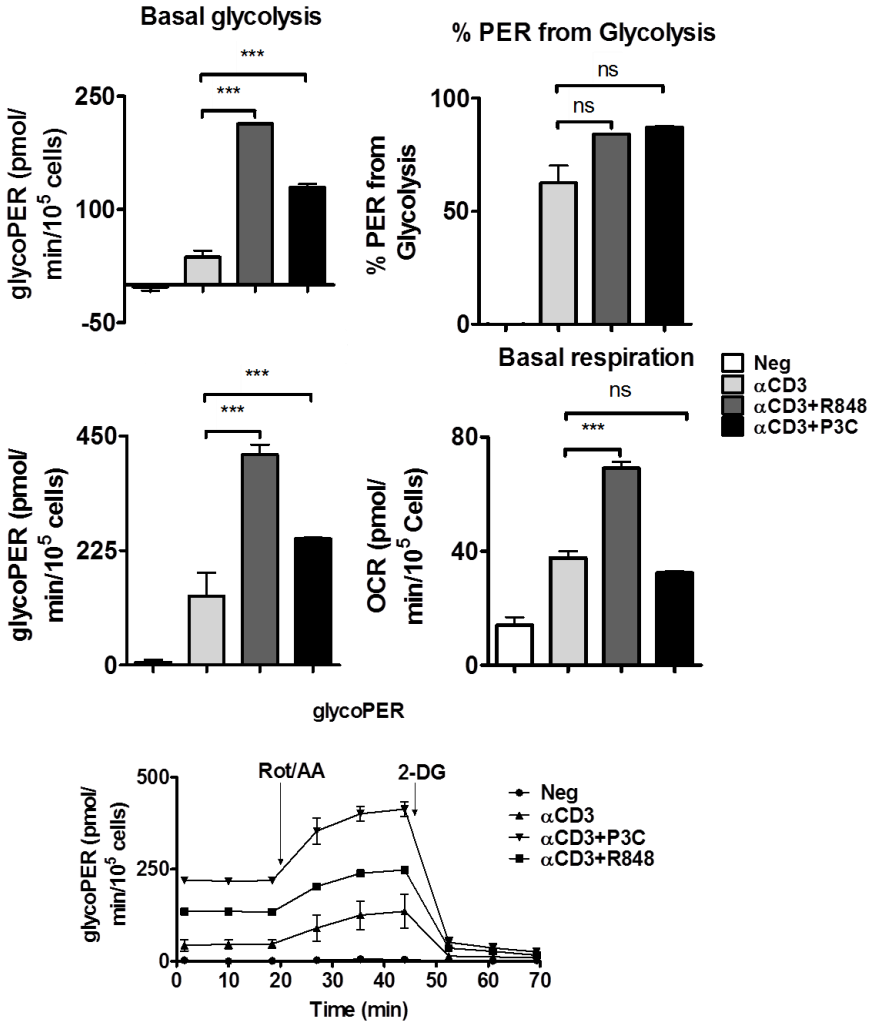



**Figure 4.37 Comparison of TLR-L-stimulated metabolic changes in CD8+ T cells by seahorse analysis.** Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL)/P3C (2 μg/mL) for 24 h. The seahorse analysis was used to detect metabolic changes in TLR2/7-activated CD8+ T cells. Data are representative of three independent experiments. (* p<0.05; ** p<0.01; ns, not significant)

Furthermore, the uptake of glucose and production of lactate is lower in R848-stimulated CD8+ T cells compared to P3C-stimulated CD8+ T cells ( Fig. 4.38).

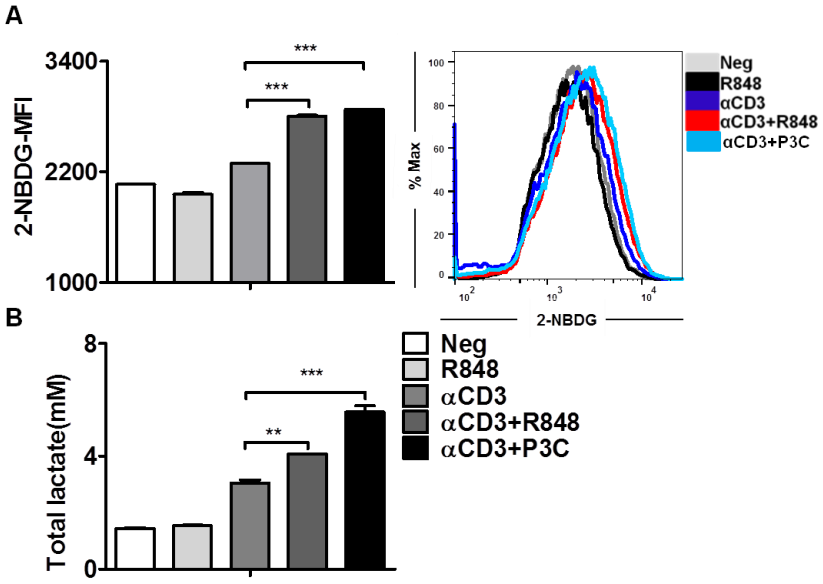



**Figure 4.38 Comparison of TLR-L-stimulated metabolic changes in CD8+ T cells.** Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL)/P3C (2 μg/mL). (A) The uptake of glucose was measured by detecting MFI of the glucose analog 2-NBDG in CD8+ T cells 24 h after stimulation. (B) Lactate production was measured by specific enzyme assay. Data are representative of two independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

### 4.8.2 P3C is a more potent costimulator of CD8+ T cells than R848

Since P3C induces more glycolytic alterations than R848 in CD8+ T cells, the functionality is also different between TLR2 and TLR7-treated CD8+ T cells. The activation markers CD25, CD44 were less expressed on R848-costimulated CD8+ T cells than P3C-costimulated CD8+ T cells. Cytokines such as IFN-γ and TNF-α were less produced in R848-costimulated CD8+T cells as well ( Fig. 4.39).

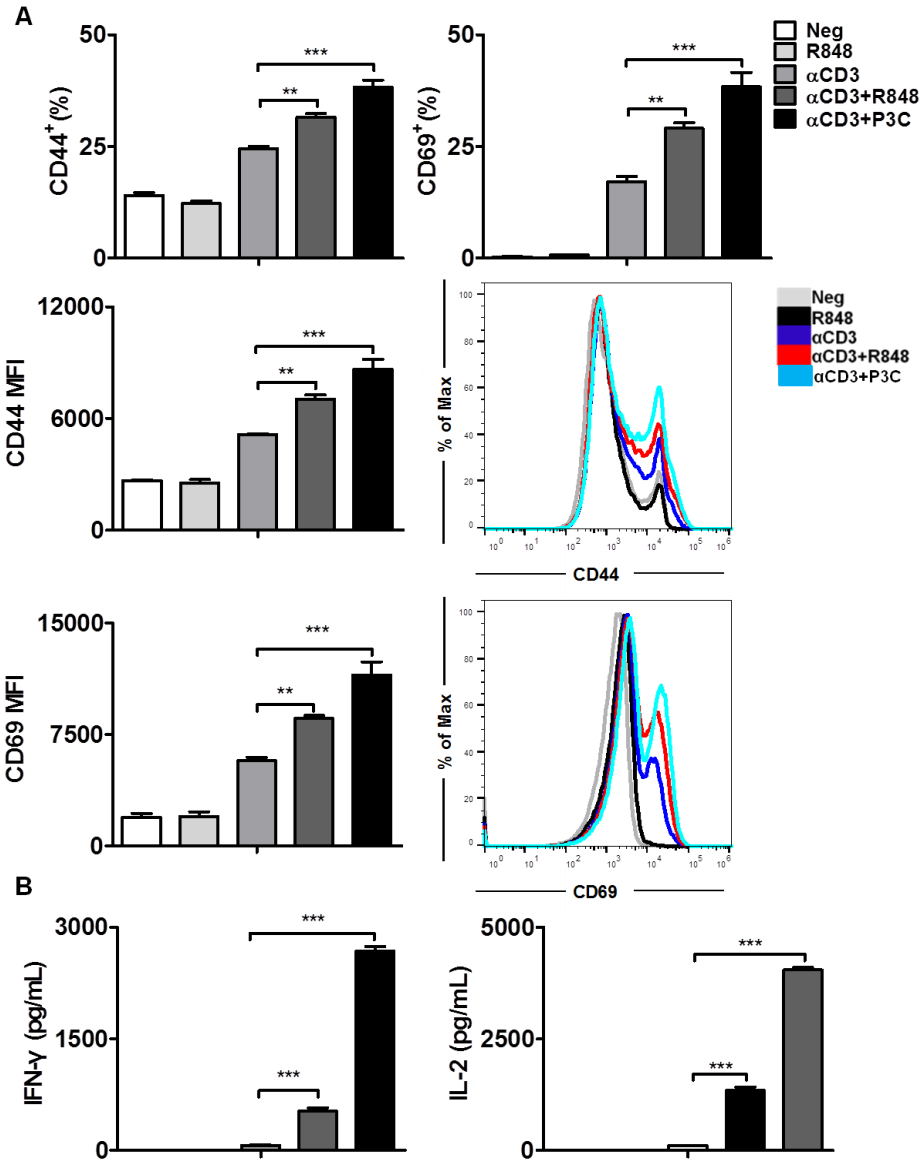



**Figure 4.39 Comparison of TLR-L-stimulated function changes in CD8+ T cells.** Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without R848 (10 μg/mL)/P3C (2 μg/mL). (A) CD8+ T cells were stained with αCD44 or αCD69 by flow cytometry. (B) The IFN-γ and IL-2 production was measured by Elisas. The results are presented as dot plots and MFI. (* $p<0.05$; ** $p<0.01$; ns, not significant)

Similar to the TLR7 agonist, P3C enhances the functionality of CD8+ T cells by up-regulating its metabolism, especially by glycolysis. The results demonstrated that the P3C-enhanced expression of CD25 was reduced by 2-DG (Fig. 4.40). These results demonstrated that TLRs induce the metabolic alterations, therefore affecting the immunity of CD8+ T cells.

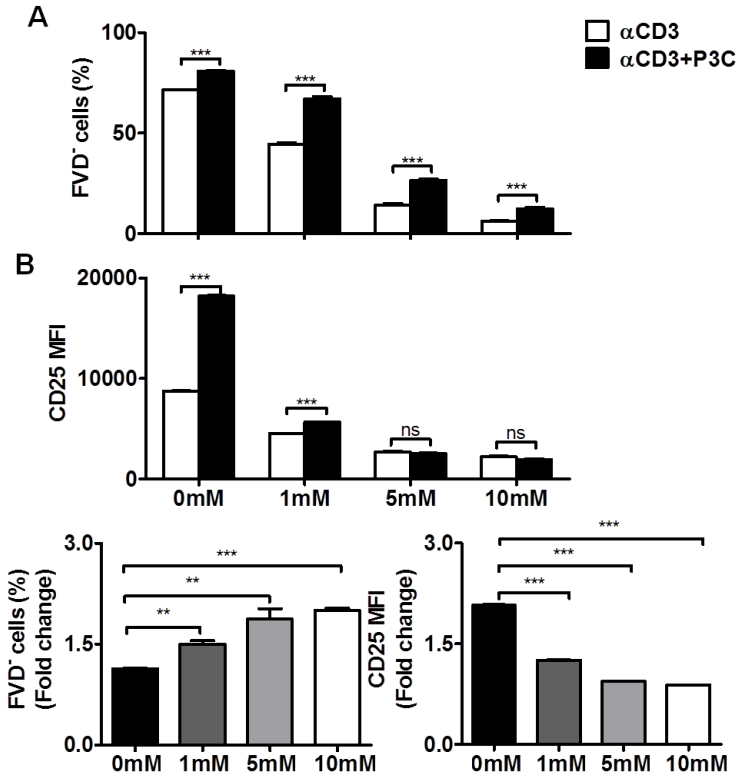



**Figure 4.40 P3C enhances the functionality of CD8+ T cells by up-regulating its glycolysis** Purified CD8+ T cells were stimulated with an αCD3 antibody (5 μg/mL) with or without P3C (2 μg/mL) in the presence of 2-DG (0mM,1Mm, 5Mm, 10mM ). (A) CD8+ T cells were stained with FVD to exclude the dead cells. (B) CD25 expression was measured by Elisas. Fold changes of $FVD^-$% cells and CD25 expression between αCD3+P3C and αCD3 treatment in different doses of 2-DG are indicated by numbers (the bottom panel). The results are presented as dot plots and MFI. (* $p<0.05$; ** $p<0.01$; ns, not significant)

## 4.9 TLR activation altered the function and metabolism of naïve B cells

### 4.9.1 TLR2 and TLR7 augment the function of B cells

To initially assess the immunomodulatory properties of TLRs on B cells, splenocytes from naïve mice were stimulated with the TLR2 ligand P3C and TLR7 ligand R848. We firstly detected that TLR2/7 agonists promote TLR2/7 expression in splenic B cells (Figure 4.41).

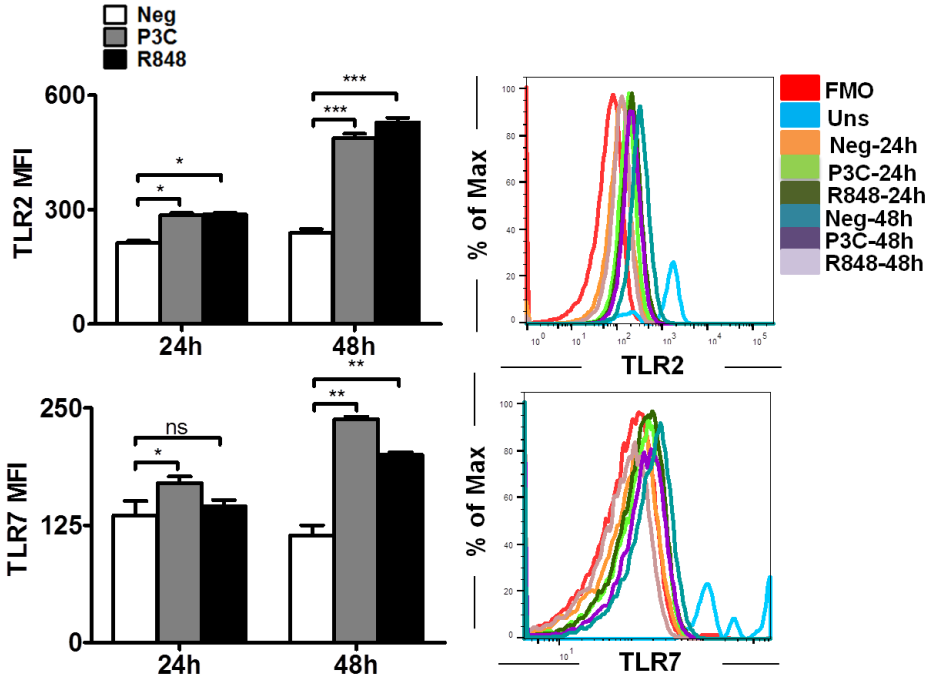



**Figure 4.41 TLR2/7 agonists promote corresponding receptors expression in splenic B cells.** Purified B cells were stimulated with P3C (2 μg/mL) or R848 (10 μg/mL) for 24-48 h. The expression TLR2 and TLR7 were detected by flow cytometry. (* $p<0.05$; ** $p<0.01$; ns, not significant)

TLR2/7 agonists also increase the expression of molecules that are related to antigen presentation in splenic B cells, such as MHC-Ⅱ and CD86 (Figure 4.42A). Furthermore, TLR2/7 agonists promote the expression of membrane-bound IgM and IgD in splenic B cells (Figure 4.42B).

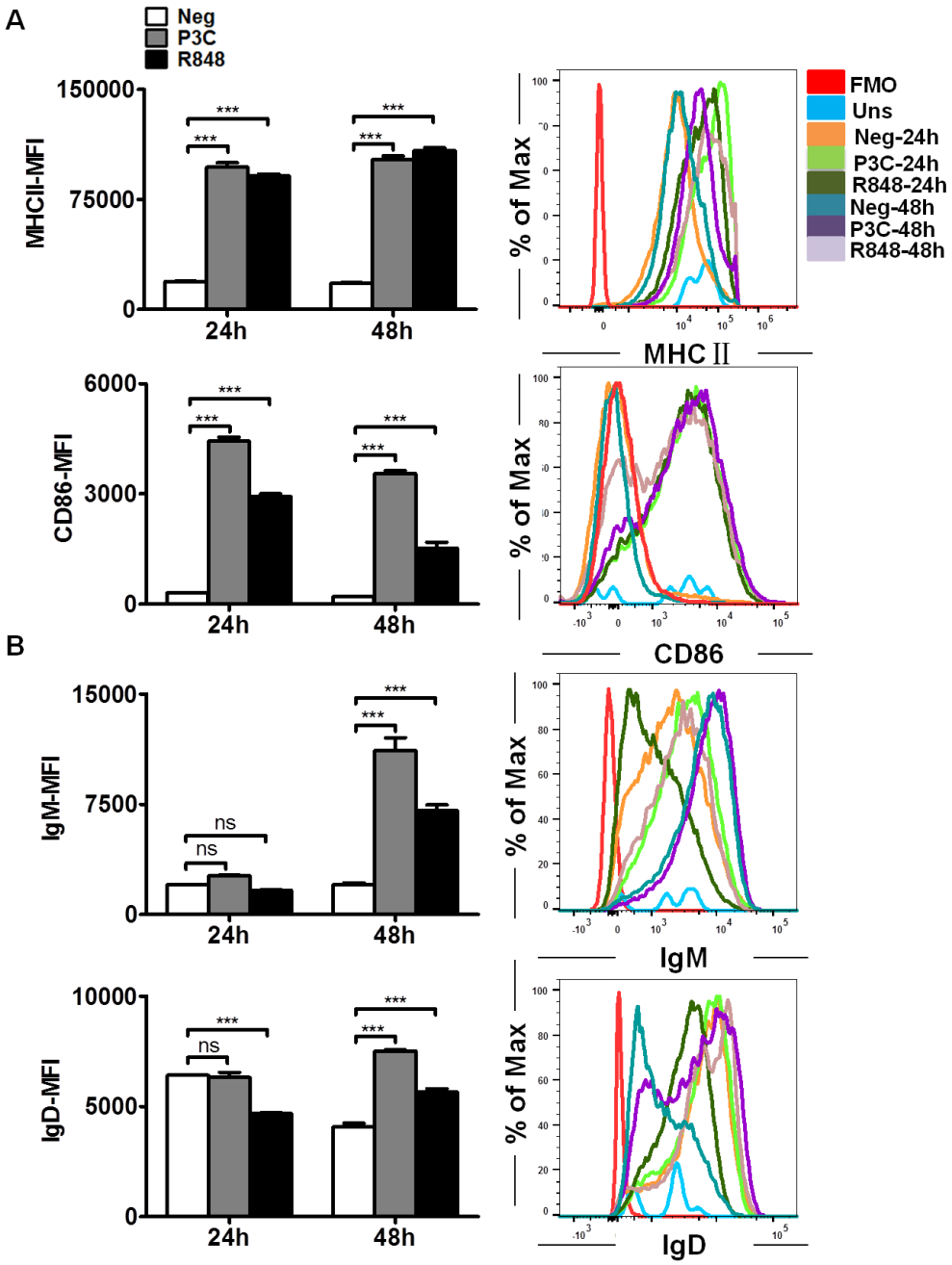



**Figure 4.42 TLR2/7 agonists promote the function of splenic B cells.** Purified B cells were stimulated with P3C (2 μg/mL) or R848 (10 μg/mL) for 24-48 h. (A)The expression of MHC-Ⅱ and CD86 was analyzed by MFI. (B) The membrane-bound IgM and IgD were detected by flow cytometry. (* $p<0.05$; ** $p<0.01$; ns, not significant)

Since there are other types of immune cells in the splenocytes, to investigate whether TLR2/7 ligands directly enhance the function of B cells, naïve splenic B cells were highly purified (> 99.0%) from WT mice by using magnet bead separation. (Figure 4.43 ).

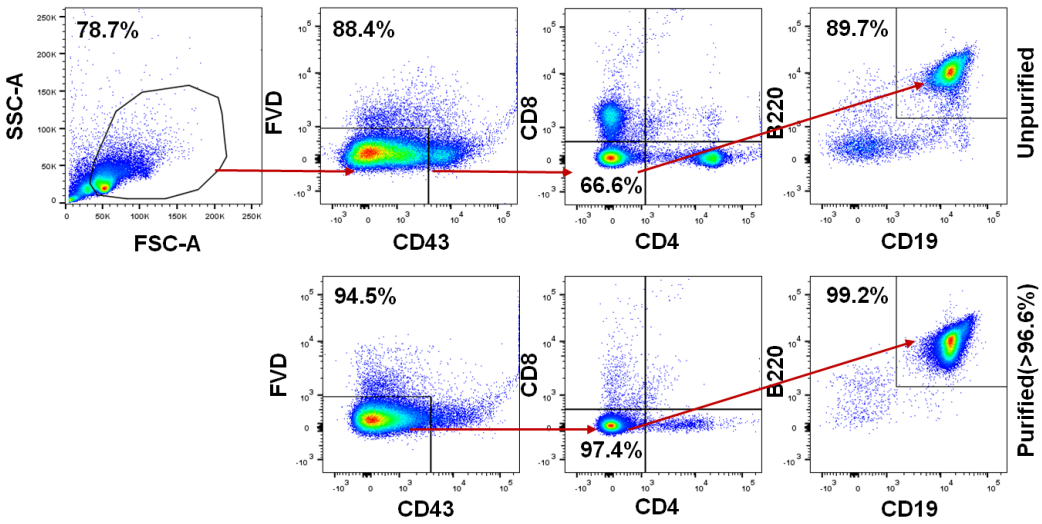



**Figure 4.43 Purification of B cells by micro-beads and verification by flow cytometry.** B cells were isolated from the spleens of WT mice using Miltenyi micro-beads. The purity of B cells was verified by flow cytometry.

The highly purified cells were then stimulated with R848 or P3C for 24 h and 48 h. We next investigated the optimal concentrations of TLR2/7 agonists for in vitro stimulation of purified B cells. The TLR2 ligand P3C induced the maximum cell activation at the dose of 0.01-2 μg/mL (Figure 4.44A), whereas R848 appeared to improve cell activation at the dose of 1–10 μg/mL (Figure 4.44B).

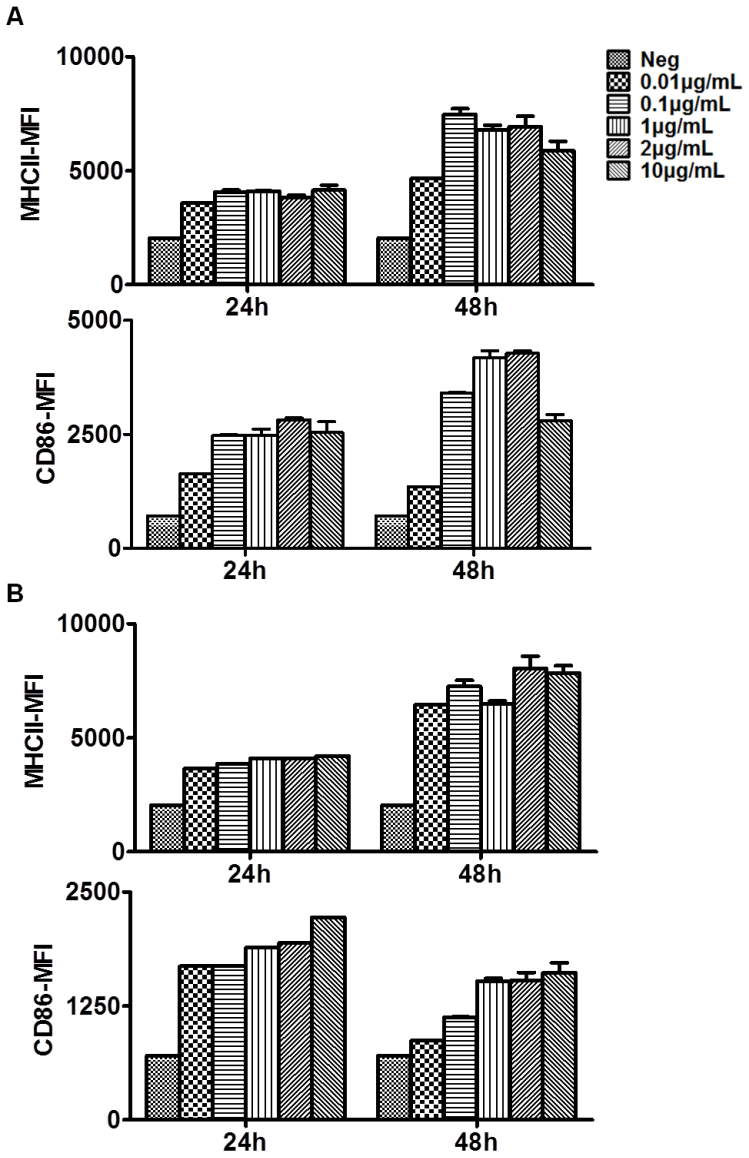



**Figure 4.44 optimal concentrations of TLR2/7 agonists for in vitro stimulation.** Purified B cells were stimulated with P3C (2 μg/mL) (A) or R848 (10 μg/mL) (B) for 24/48 h. The expression of MHC-Ⅱ and CD86 were analyzed by MFI. (* $p<0.05$; ** $p<0.01$;*** $p<0.001$)

At the optimal concentration of P3C (2μg/mL) and R848 (10 μg/mL), the two TLR ligands could enhance TLR2/7 expression (Figure 4.45A) as well as MHCⅡand CD86 expression (Figure 4.45B), and the membrane antibody mIgD and mIgM secretion (Figure 4.45C).

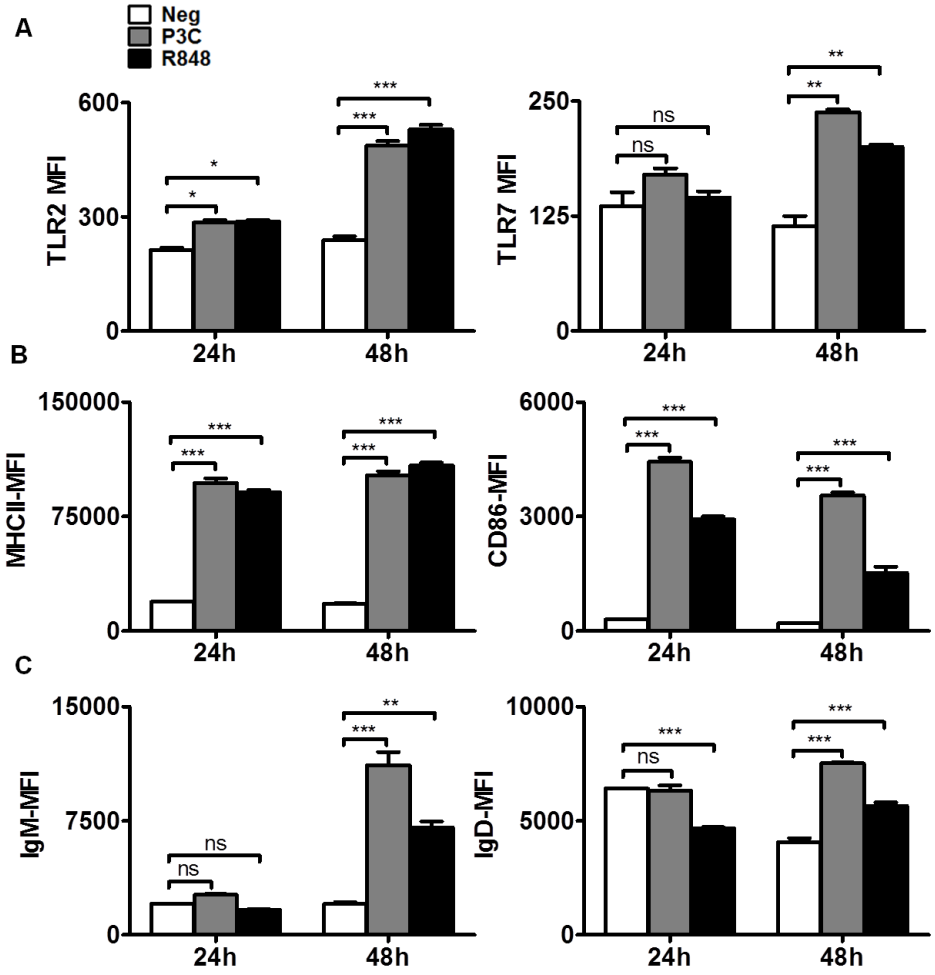



**Figure 4.45 TLR2/7 agonists promote the function of purified B cells.** Purified B cells were stimulated with P3C (2 μg/mL) or R848 (10 μg/mL) for 24-48 h. (A) The expression of TLR2 and TLR7 was analyzed by MFI. (B) The antigen presentation molecules MHC-Ⅱ and CD86 were analyzed by MFI. (C) The membrane-bound IgM and IgD were detected by flow cytometry. (* $p<0.05$; ** $p<0.01$; ns, not significant)

### 4.9.2 TLR2 and TLR7 activation in B cells induce different metabolic changes

To investigate whether TLR2/7 activation regulates mTOR signaling, we determined Akt and mTOR expression by flow cytometry. The results showed that TLR2/7 stimulation led to a high level of phosphorylated mTOR in B cells (Figure 4.46).

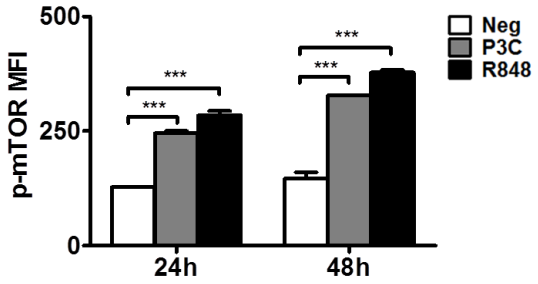



**Figure 4.46 TLR2/7 agonists promote the expression of p-mTOR in B cells.** Purified B cells were stimulated with P3C (2 μg/mL) or R848 (10 μg/mL) for 24-48 h. The expression p-mTOR was detected by flow cytometry and analyzed by MFI. (* p<0.05; ** p<0.01; ns, not significant)

It has been verified that TLR signaling induces metabolic reprogramming including glycolysis enhancement in CD8+ T cells. We investigated whether TLRs induce metabolic changes in B cells. The uptake of glucose was measured by detecting the mean fluorescence intensity of the glucose analog 2-NBDG. In addition, the production of lactate by aerobic glycolysis was measured in the culture supernatants. Results showed that both P3C and R848 were able to enhance the uptake of glucose, while only P3C enhanced lactate production in B cells (Figure 4.47 ).

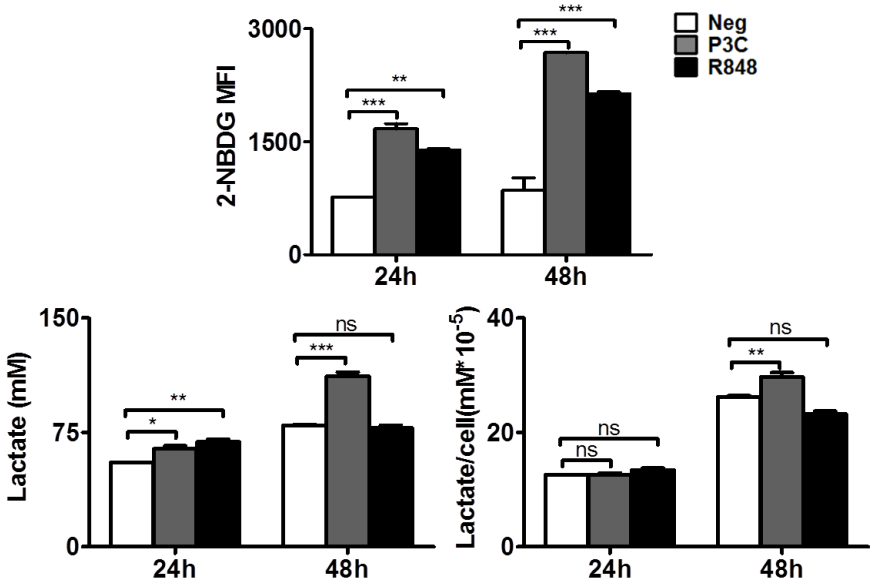



**Figure 4.47 TLR2/7 agonists induce different metabolic alterations in B cells.** Purified B cells were stimulated with P3C (2 μg/mL) or R848 (10 μg/mL) for 24-48 h. (A) The uptake of glucose was measured by detecting MFI of the glucose analog 2-NBDG in B cells. (B) Lactate production was measured by specific enzyme assay. Data are representative of two independent experiments. (* p<0.05; ** p<0.01; ns, not significant)

We further addressed whether TLR-induced metabolic pathway changes in B cells are required for the activation of B cells. To confirm that the metabolic pathway drives the activation and functionality of B cells, inhibitors of indicating metabolic pathways were used. Glycolysis was blocked with the glucose analog 2-DG, glutaminolysis was blocked with glutamine analog DON, TCA cycle was blocked with Oligomycin, PI3K-mTOR pathway was blocked with Rapamycin and Akti-1/2. Purified B cells were treated with TLR2 agonist-P3C with/without metabolic or signaling inhibitors. The indicated doses of 2-DG (2mM), Oligomycin (10nM), Rapamycin (2μM) and Akti-1/2 (1nM) did not cause additional cell death (Figure 4.48A ) in contrast to reducing the expression of Ag presentation related molecules, such as MHC-Ⅱ and CD86. The results showed that blocking either the glycolysis, TCA cycle or PI3K-mTOR pathway reduced TLR2-induced activation of B cells (Figure 4.48B ).

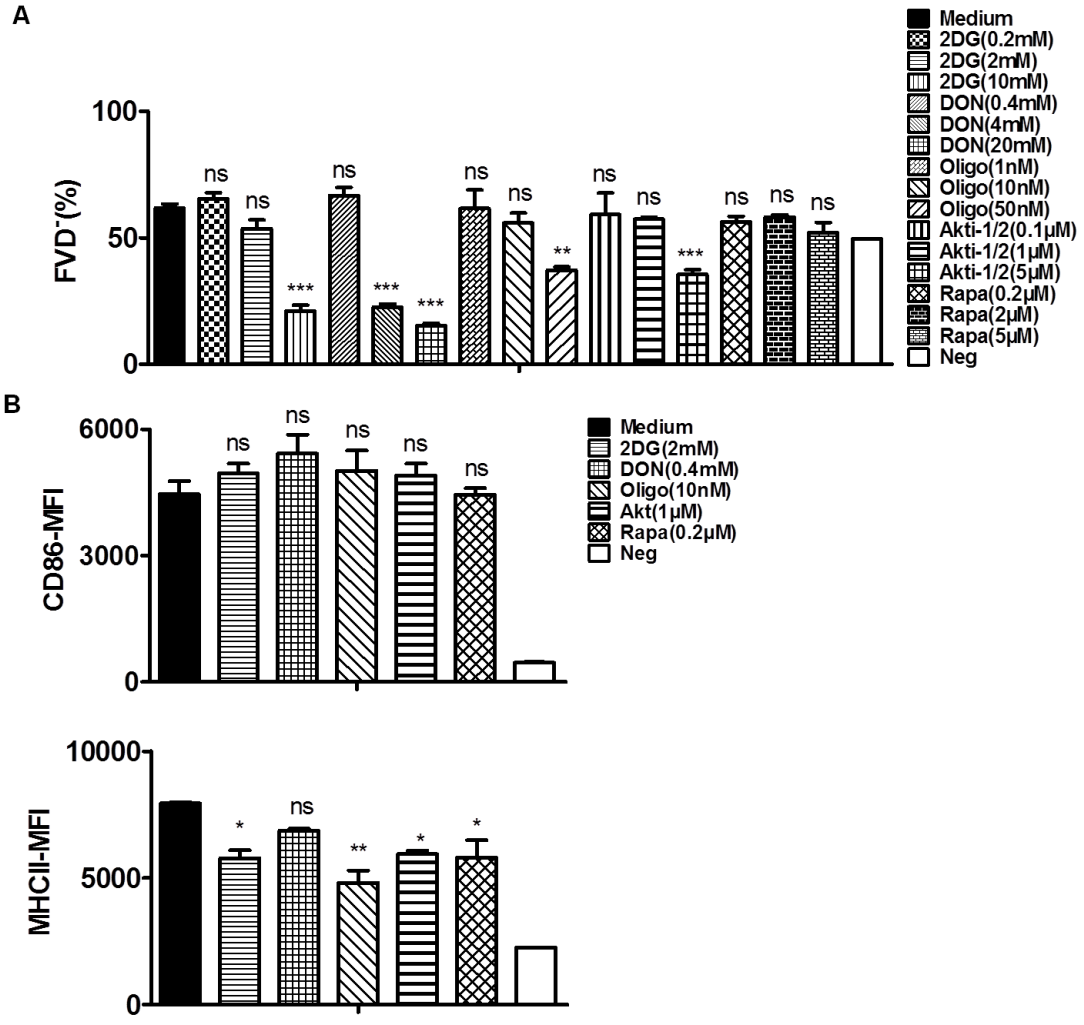


**Figure 4.48 Different metabolic pathway blocking in TLR2-activated B cells.** Purified B cells were stimulated with P3C (2 μg/mL) for 24 h in the presence of different doses of inhibitors 2-DG (0.2 mM, 2mM, 10 mM), DON (0.4 mM, 4mM, 20 mM), Oligomycin (1 nM, 10 nM, 50 nM), Akti-1/2 (0.1 μM, 1μM, 5μM) and Rapamycin (0.2 μM, 2μM, 5μM) . (A) The dead cells are excluded by FVD staining. (B) The antigen presentation molecules MHC-Ⅱ and CD86 were analyzed by MFI. Data are representative of two independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

However, only the inhibitors Oligomycin (10 nM) and Rapamycin (2 μM) can reduce the expression of MHC-Ⅱ and CD86 in TLR7-activated B cells. Oligomycin at 10 nM and Rapamycin at 2 μM did not cause additional cell death in TLR7-induced activation of B cells (Figure 4.48 A, B ). The results showed that blocking the glycolysis cannot decrease the function of B cells, whereas blocking the TCA cycle or PI3K-mTOR pathway reduced TLR7-induced activation of B cells. Thus, TLR2/7 activation in B cells induces different metabolic alterations and manipulates the function of B cells.

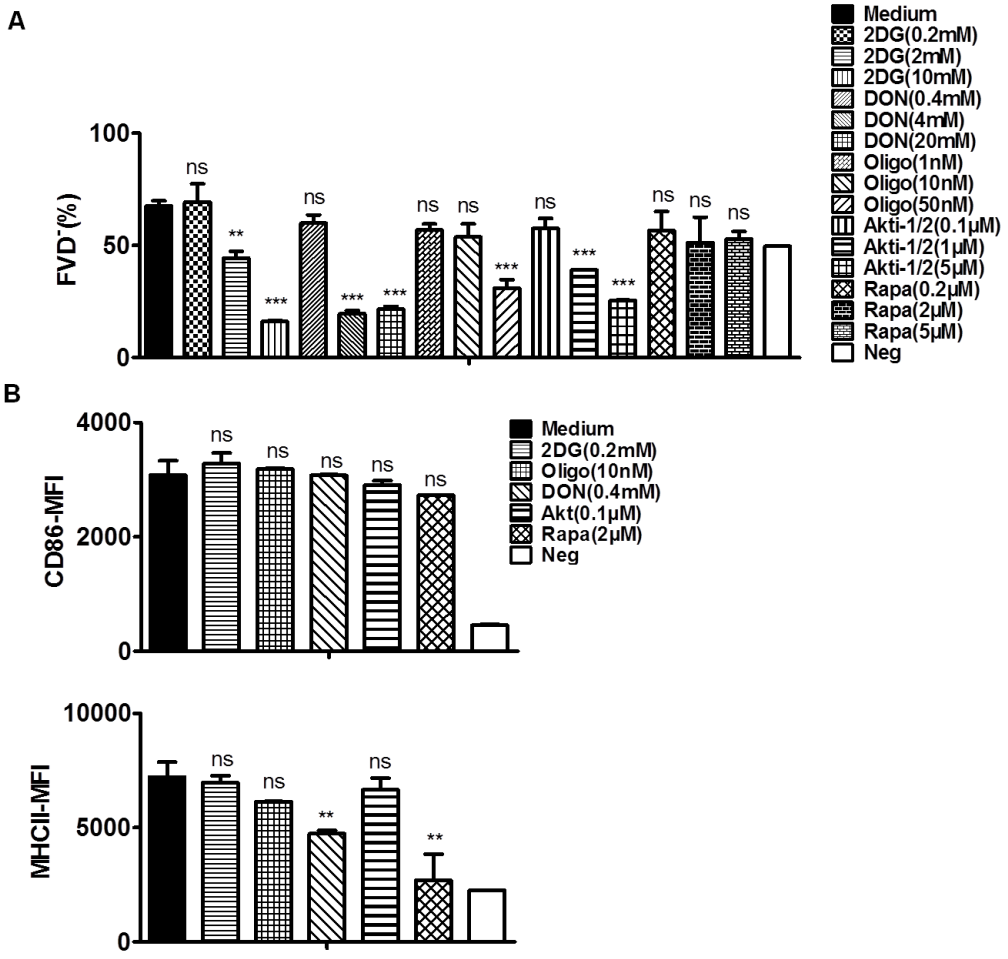



**Figure 4.49 Different metabolic pathway blocking in TLR7-activated B cells.** Purified B cells were stimulated with R848 (10 μg/mL) for 24 h in the presence of different doses of inhibitors 2-DG (0.2 mM, 2mM, 10 mM), DON (0.4 mM, 4mM, 20 mM), Oligomycin (1 nM, 10 nM, 50 nM), Akti-1/2 (0.1 μM, 1μM, 5μM) and

Rapamycin (0.2 μM, 2μM, 5μM). (A) The dead cells are excluded by FVD staining. (B) The antigen presentation molecules MHC-Ⅱ and CD86 were analyzed by MFI. Data are representative of two independent experiments. (* $p<0.05$; ** $p<0.01$; ns, not significant)

## 5.Discussion

In the present study, we investigated the activation of CD8+ T cells and B cells in response to TLR2/7 ligands. We found that TLR2/7 stimulation could promote the functionality of CD8+ T and B cells in vitro. It could be shown that the TLR7-induced activation of CD8+ T cells is MyD88-dependent. Further, AKT-mTOR signaling plays a critical role in TLR7-induced T cell activation. While investigating immune-related changes, metabolic alterations in CD8+ T cells, including the upregulation of glucose uptake and glycolysis, occurred by TLR2/7 stimulation. Glycolysis was found to be regulated by the AKT-mTOR pathway and the downstream transcription factor IRF4 in TLR7-stimulated T cells. Blocking glycolysis by either direct glucose deprivation or modulation of the mTOR pathway and IRF4 expression was found to impair T cell activation and functions. The enhanced function can also be found in TLR2/7-stimulated B cells. Whereas, the fact that TLR2 and TLR7 induce different metabolic pathway alterations is different from the findings in CD8+ T cells. Our results indicate that TLR2/7 activation enhances the function of CD8+ T cells and B cells by inducing metabolic reprogramming. Thus, targeting TLR and metabolic pathways might represent potent strategies for immune cell-based immunotherapies.

### 5.1 TLR2/7 plays an essential role in modulating the effector functions of T cells

T cells that exert optimal effector functions rely on multiple signals, specifically the involvement of TCRs, the presence of co-stimulatory molecules, and the supply of pro-inflammatory cytokines.[12,13] TCR engagement can be caused by either non-specific or antigen-specific stimulation. The activation of T cells is restricted by T cell receptor binding affinities and kinetics.[14,15] Further, co-stimulatory molecules such as CD28 are required for the full activation of T cells by decreasing the threshold of TCR binding affinity.[16,17] Our findings demonstrated that TLR2/7 can also act as a co-stimulatory molecule on CD8+ T cells. P3C/R848 co-stimulation significantly improved T cell activation, cytokine production, and the expression of relevant transcription factors such as T-bet and Eomes. These data demonstrate the contribution of TLR2/7 to induce full activation of CD8+ T cells. Insufficient TCR signaling results in T cell anergy, leading to immune tolerance or immune evasion.[18-20] Thus, our findings hint to the potential use of TLR2/7 co-stimulation to rescue the effector

functions of exhausted T cells, e.g. during chronic viral infection.

### 5.2 TLR2/7-enhanced effector function of CD8+T cells is MyD88 dependent

Toll-like receptors (TLRs) recognize distinct pathogen-associated molecular patterns and traffic adaptor proteins, such as TRIF and MyD88, to support the further accumulation of kinase-like IL-1 receptor-associated kinase-4 (IRAK-4) for signaling transduction.[174] In our study, MyD88$^{-/-}$mice had an impaired activation and cytokine production of CD8+ T cells, whereas the activation and cytokine production can be further enhanced by R848 in the presence of αCD3 antibody in TRIF$^{-/-}$mice. It is believed that MyD88 binds to transcription factors T-bet, thereby modulating the function of CD8+ T cells. Consistently, our study also verified that TLR7 activation on CD8+ T cells relies on the adaptor protein MyD88. The same findings can be also found in TLR2-activated CD8+ T cells, while the data has not been shown here. These data answered the question whether the TLR2/7-enhanced function of CD8+T cells is depending on the MyD88-dependent manner.

### 5.3 TLR2/7 plays an essential role in reprogramming the metabolism of T cells

TLR-MyD88 activated T cells have been proved to enhance the activation of the mTOR pathway. mTOR signaling has been shown to regulate important cellular processes such as cell survival, cellular metabolism, and autophagy.[175,176] In our study, we also verified that R848, together with αCD3 up-regulated the expression of mTOR and p-mTOR. Notably, blocking the mTOR signaling pathway with the inhibitor Rapamycin or Akti-1/2 abolished the up-regulated functionality of CD8+ T cells, while the costimulatory molecule R848 decreased the mortality of cells even with inhibitors of mTOR signaling. This suggested that the mTOR signaling pathway controls the functionality of R848-stimulated CD8+ T cells.

Along with transcriptional program activation, cytokine synthesis, secretion, and rapid proliferation of T cell activation require increased metabolic precursors for ATP and biomass synthesis.[177] Cellular metabolism has recently been found to remold the immunity of T cells and can serve as a new strategy for several diseases.[178] Our results suggest that TLR2/7 enhanced the uptake of glucose, the expression of glycolytic relevant genes, including Glut-1, HK2, and LDHα, the protein level of glycolytic-related kinase HK2 and the production of

lactate after stimulation with P3C/R848 and αCD3 antibodies. To further clarify the role of glycolysis in TLR2/7-stimulated CD8+ T cells, inhibitors of glycolysis were applied and found to block the functionality of TLR2/7-activated CD8+ T cells.

It's known that the mTOR signaling pathway induces complex network reprogramming, including enhanced aerobic glycolysis to facilitate rapid clonal expansion. Blocking mTOR signaling pathway by inhibitors also abolished glycolysis of TLR7-activated CD8+ T cells. We are the first to verify that the mTOR signaling pathway is responsible for regulating glycolysis on TLR7-stimulated CD8+ T cells. These findings were not found in P3C-activated CD8+ T cells. This indicates that mTOR-regulated glycolysis plays an essential role in the functionality of TLR7-activated CD8+ T cells.

Many transcription factors work synergistically at the downstream of the mTOR signaling pathway, such as c-Myc, HIF-1α and IRF4, and BATF.[159,163,179] They are closely related to the metabolism and immune reprogramming of CD8+ T cells. To investigate the downstream transcription factors of mTOR signaling, we observed that IRF4 is up-regulated on CD8+ T cells by the stimulation of R848 in an αCD3 dependent manner. Interestingly, inhibiting mTOR signaling reduced the expression of IRF4. Therefore, we compared the function of TLR7-activated CD8+ T cells from WT mice to $IRF4^{-/-}$ mice by using the same treatment. It has become clear that $IRF4^{-/-}$CD8+ T cells have a diminished activation and cytokine production compared to WT CD8+ T cells at the stimulation of R848. Besides, the metabolic changes in CD8+ T cells were also decreased. We noted a decrease in the expression of glycolytic related genes and a decrease in the production of lactate in the R848-costimulated $IRF4^{-/-}$CD8+ T cells. Furthermore, two transcriptional regulators, T-bet and Eomes, may be responsible for the phenotype and functional changes of $IRF4^{-/-}$CD8+ T cells. BATF serving as the cofactor for IRF4 may also be responsible for the enhanced function of CD8+ T cells. We found a decreased functionality but no glycolytic alterations in $BATF^{-/-}$CD8+ T cells (Figure 5.1).

Similar to the findings of the TLR7 treatment, IRF4 has also proved to play a role in P3C-induced function and metabolic changes of CD8+ T cells (data not shown here). They all suggested that IRF4 is responsible for both glycolysis and functional changes of TLR2/7-activated CD8+ T cells. The exact binding site involved in the TLR2/7 pathway, the

glycolytic pathway, and the expression of T-bet, and how they interact with IRF4 need to be clarified in future experiments.

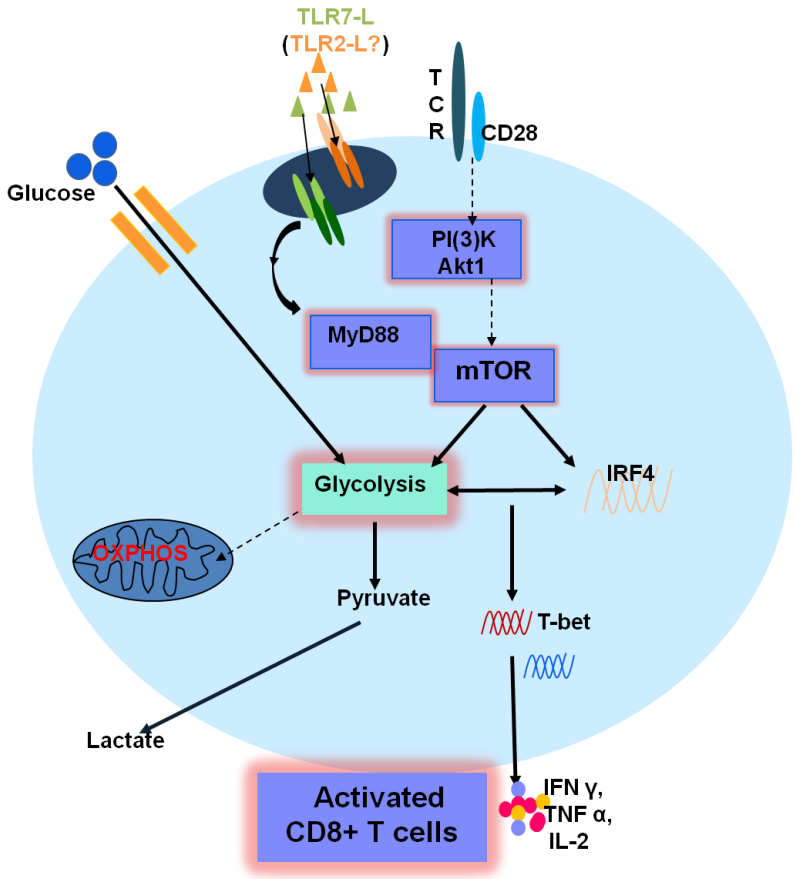



**Figure 5.1 A schematic description of TLR7-mediated enhancement of CD8+ T cell function.** TLR7 stimulation promotes the effector functions of CD8+ T cells, if CD8+ T cells are primed by αCD3 and express TLR7. TLR7-mediated T cell activation is dependent on MyD88 and AKT-mTOR signaling, leading to the expression of immune-related changes and metabolic alterations in CD8+ T cells, including the upregulation of glucose uptake and glycolysis. Glycolysis was found to be regulated by the AKT-mTOR pathway and the downstream transcription factor IRF4. Glycolysis is critical for TLR7-mediated CD8+ T cell activation and enhanced effector functions of CD8+ T cells. Further evidence for the detailed mechanism of TLR2-mediated CD8+ T cell function enhancement is needed. Solid arrows represent signaling pathways identified in this study. Broken arrows indicate potential signaling pathways.

### 5.4 TLR activation altered the function and metabolism of naïve B cells

B cells are the other arm of the adaptive immune response. Murine naïve B cells express different kinds of TLRs. TLR stimulation in B cells induces T cell-independent activation. The activation of B cells can be restricted by TLR binding affinities and kinetics. Our findings demonstrated that TLR2/7 can promote activation in B cells directly. P3C/R848 significantly improved the expression of antigen presentation molecules such as MHC-Ⅱ and CD86 as well as membrane-bonded antibodies such as mIgM in B cells. In addition, P3C/R848

significantly enhanced the uptake of glucose, while only P3C stimulation accumulates the production of lactate. Thus, either blocking the glycolysis, TCA cycle or PI3K-mTOR pathway reduced the P3C- enhanced function of B cells. Only blocking the TCA cycle and mTOR pathway reduced the R848-enhanced function of B cells. Our findings suggest that TLR2/7 induced different metabolic alterations in B cells (Figure 5.2).

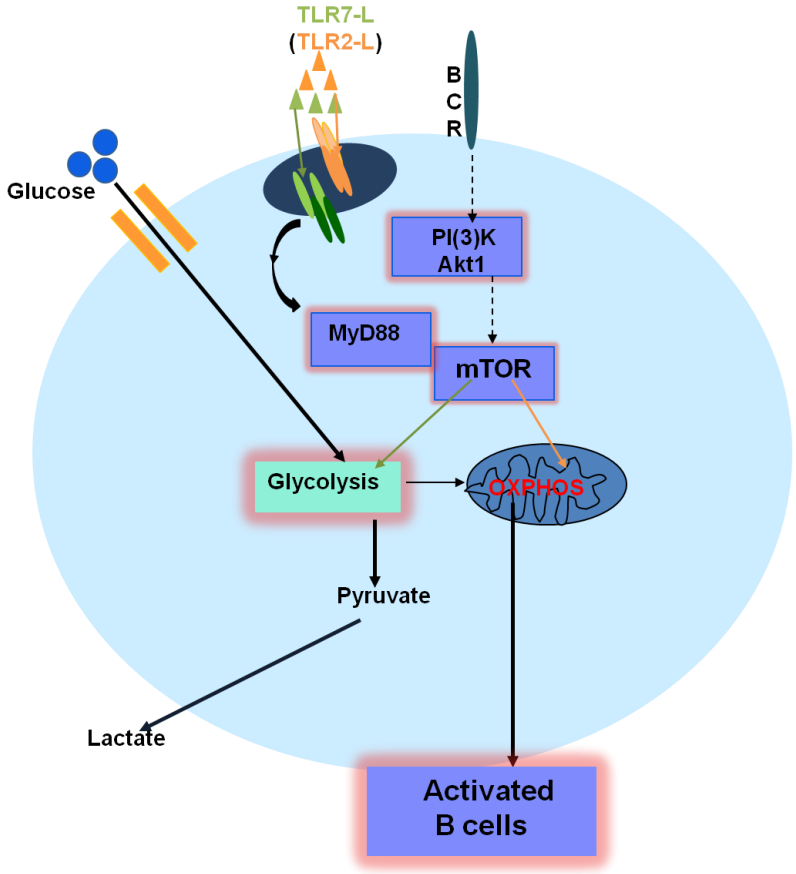



**Figure 5.2 A schematic description of TLR-mediated enhancement of B cell function.** TLR2/7 stimulation promotes the effector functions of B cells. TLR2/7-mediated T cell activation is dependent on MyD88 and AKT-mTOR signaling, leading to the expression of immune-related changes and metabolic alterations in B cells, including the upregulation of glucose uptake, glycolysis, and oxidative phosphorylation. Glycolysis is critical for TLR2-mediated rather than TLR7-mediated enhanced effector functions of B cells. Further evidence for the detailed metabolic mechanism of TLR2/7-mediated B cell function enhancement is needed. Solid arrows represent signaling pathways identified in this study. Broken arrows indicate potential signaling pathways. Arrows with different colors represent different signaling activation.

## 5.5 Potential of manipulating metabolism in immune cells

Two kinds of TLR agonists, P3C and R848, were tested to costimulate CD8+ T cells of mice. However, under the same TCR signaling, P3C and R848 have different effects on CD8+ T cells, since TLR2/7-activated CD8+ T cells rely on the metabolic changes to exert their effector functions. This increases the likelihood that TLR2/7-induced metabolic

reprogramming led to further changes in immunity. This hypothesis was in accordance with our research on P3C/R848-stimulated CD8+ T cells. We found that less glycolytic changes of R837-costimulated CD8+ T cells induce fewer functionality changes of CD8+ T cells than R848-costimulated CD8+ T cells. Distinct biogenetic procedure mediates different effects on CD8+ T cells when the TLR2/7 pathway is activated. In order to maximize the function of T cells, we have identified the basic procedures for TLR7-stimulated CD8+ T cells. For B cells, TLR2/7 also promotes its functions by regulating different metabolic pathways. TLR2 activation in B cells relies on both glycolysis and TCA cycle, whereas blocking glycolysis with 2-DG in R848-stimulated B cell did not decrease the function of B cells. Our data suggest that manipulation of metabolic pathways in CD8+ T cells/B cells is helpful to control immunity, therefore provides a mechanism to improve the anti-viral or anti-tumor immune response.

## 6. Summary

In our present study, we first examined whether TLR7 activation on CD8+ T cells can elevate the functionality of CD8+ T cells in the presence of αCD3 in vitro. We found that the enhanced functionality of CD8+ T cells relies on a MyD88 dependent manner, since the TLR2-MyD88 axis is believed to interact with the mTOR signaling pathway. We also found activated mTOR signaling in TLR7 costimulated CD8+ T cells. However, blocking mTOR signaling with inhibitors Rapamycin and Akti-1/2 can significantly reduce the enhanced function of CD8+ T cells. Not only the enhanced functionality of CD8+ T cells was affected by mTOR pathway blocking but also the enhanced glycolysis of CD8+ T cells. Glycolysis is further verified as essential for the regulation of CD8+ T cells by R848 blocking experiments. Besides the mTOR pathway, the downstream transcription factor IRF4 also participates in upregulating the glycolysis and functionality of TLR7-stimulated CD8+ T cells.

TLR activation can be induced by different TLR agonists such as the TLR2 ligand P3C. P3C can also induce functional and metabolic alterations, especially glycolysis, in the same way as R848 in CD8+ T cells. The functional and metabolic alterations induced by TLR2/7 agonists in T cells can also be found in B cells. In summary, we observed the effects including the metabolic and functional alterations on TLR2/7 activated-CD8+ T and B cells. Our data provide a new possibility to manipulate immune functions by metabolic pathways in immune cells.

## 7. Zusammenfassung

In unserer vorliegenden Studie haben wir zunächst untersucht, ob die TLR7-Aktivierung auf CD8+ T-Zellen die Funktionalität von CD8+ T-Zellen in Gegenwart von αCD3 in vitro erhöhen kann. Wir fanden heraus, dass die verbesserte Funktionalität von CD8+ T-Zellen von MyD88 abhängt, da angenommen wird, dass die TLR2-MyD88-Achse mit dem mTOR-Signalweg interagiert. Wir fanden auch aktivierte mTOR-Signale in TLR7-costimulierten CD8+ T-Zellen. Die Blockierung der mTOR-Signalübertragung mit den Inhibitoren Rapamycin und Akti-1/2 kann jedoch die verstärkte Funktion von CD8+ T-Zellen signifikant verringern. Nicht nur die verbesserte Funktionalität von CD8+ T-Zellen wurde durch die Blockierung des mTOR-Signalwegs beeinflusst, sondern auch die verbesserte Glykolyse von CD8+ T-Zellen. Die Glykolyse ist ferner durch Blockierungsexperimente als wesentlich für die Regulation der CD8+ T-Zellen durch R848 verifiziert. Neben dem mTOR-Signalweg ist der nachgeschaltete Transkriptionsfaktor IRF4 auch an der Hochregulierung der Glykolyse und Funktionalität von TLR7-stimulierten CD8+ T-Zellen beteiligt. Die TLR-Aktivierung kann durch verschiedene TLR-Agonisten, wie dem TLR2-Liganden P3C, induziert werden. P3C kann auch funktionelle und metabolische Veränderungen induzieren, insbesondere die Glykolyse, wie dies R848 in CD8 + T-Zellen tat. Die durch TLR2/7-Agonisten in T-Zellen induzierten Funktions- und Stoffwechselveränderungen können auch in B-Zellen gefunden werden. Zusammenfassend beobachteten wir die Auswirkungen einschließlich der metabolischen und funktionellen Veränderungen auf TLR2/7-aktivierte CD8+ T- und B-Zellen. Unsere Daten bieten eine neue Möglichkeit, die Immunfunktion über Stoffwechselwege in Immunzellen zu manipulieren.

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## 9.Abbreviations

| | |
|---|---|
| IFN-γ | interferon-γ |
| TNF | tumor necrosis factor |
| IL-2 | Interleukin-2 |
| HBV | Hepatitis B virus |
| TD | T cell-dependent |
| TI | T cell-independent |
| Immunoglobulin | Ig |
| Toll-like receptors | TLRs |
| Pattern-recognition receptors | PRRs |
| NOD-like receptors | NLR |
| Retinoic acid-inducible gene I | RIG-I |
| C-type lectin receptors | CLRs |
| Pathogen-associated molecular patterns | PAMPs |
| Damage-associated molecular patterns | DAMPs |
| Pam3CSK4 | P3C |
| Resiquimod | R848 |
| Imidazoquinoline | R837 |
| double-stranded RNA | dsRNA |
| single-stranded RNA | ssRNA |
| Myeloid differentiation primary response protein 88 | MyD88 |
| TIR-domain containing adapter protein | TIRAP |
| Interleukin-1 receptor-associated kinase 4 | IRAK4 |
| Tumor necrosis factor receptor (TNFR)-associated factor | TRAF |

| | |
|---|---|
| Transforming growth factor beta-activated kinase | TAK |
| TGF-β -activated kinase | TAB |
| Mitogen-activated protein kinas | MAPK |
| TIR-domain-containing adaptor protein | TRIF |
| TRIF-related adaptor molecule | TRAM |
| Interferon regulatory factor | IRF |
| MyD88-adaptor-like protein | MAL |
| cyclic AMP-responsive element-binding protein | CREB |
| plasmacytoid DC | pDC |
| cytotoxic T-cell | CTL |
| tricarboxylic acid (TCA) | TCA |
| Antigen-presenting cell | APC |
| Major histocompatibility complex | MHC |
| MACS | Magnetic-activated cell sorting |
| MAPKs | MAP kinases |
| mammalian target of Rapamycin | mTOR |
| Mean fluorescence intensity | MFI |
| Interferon regulatory factor 4 | Irf4 |
| Hypoxia-inducible factor 1-alpha | Hif1α |
| B cell-activating transcription factor | BATF |
| Oxidative phosphorylation | OXPHOS |

## 10.List of figures

## 11.Acknowledgments

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## Curriculum vitae

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# Toll-Like Receptor 7 Activation Enhances CD8+ T Cell Effector Functions by Promoting Cellular Glycolysis

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The activation of TLR7 signaling in T cells accelerates antigen-specific responses. Such responses play an essential role in eliminating viral infections and can be anti-tumorigenic. However, the underlying mechanisms of how TLR7 can promote the optimal function of CD8+ T cells remain unclear. To investigate how TLR signaling directly contributes to CD8+ T cell functions, we examine the activation of cellular TLR7-related pathways and functional and metabolic alterations in TLR7-stimulated T cells during T cell receptor (TCR) signaling. In the present study, we investigated the activation of CD8+ T cells in response to direct stimulation by TLR7 ligands. TLR7 stimulation could promote the effector functions of purified CD8+ T cells *in vitro*. The TLR7-induced activation of CD8+ T cells occurs if CD8+ T cells were primed by αCD3 activation and increasingly expressed TLR7. MyD88 and AKT-mTOR signaling plays a critical role in TLR7-induced T cell activation. In addition to the upregulation of immune-related genes, metabolic alterations in CD8+ T cells, including the upregulation of glucose uptake and glycolysis, occurred by TLR7 stimulation. Glycolysis was found to be regulated by the AKT-mTOR pathway and a downstream transcription factor IRF4. Blocking glycolysis by either direct glucose deprivation or modulating the mTOR pathway and IRF4 expression was found to impair T cell activation and functions. Taken together, the activation of TLR7 signaling promotes the effector functions of CD8+ T cells by enhancing cellular glycolysis.

**Keywords: toll-like receptor 7, CD8+ T cells, PI3K-Akt-mTOR, glycolysis, IRF4**

## INTRODUCTION

Toll molecules comprise a class of highly conserved molecules that plays a vital role in the immune surveillance of pathogen-associated molecular patterns and host defense against many pathogenic microorganisms (1, 2). Upon activation by their ligands, Toll-like receptors (TLRs) induce various cellular anti-viral responses including the release of inflammatory cytokines and the maturation

The Journal of Immunology

# TLR2 Stimulation Increases Cellular Metabolism in CD8$^+$ T Cells and Thereby Enhances CD8$^+$ T Cell Activation, Function, and Antiviral Activity

Ejuan Zhang, Zhiyong Ma, Qian Li, Hu Yan, Jia Liu, Weimin Wu, Jiabao Guo, Xiaoyong Zhang, Carsten J. Kirschning, Haifeng Xu, Philipp A. Lang, Dongliang Yang, Ulf Dittmer, Huimin Yan and Mengji Lu

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CORRESPONDENCE

# Activation of the TLR signaling pathway in CD8+ T cells counteracts liver endothelial cell-induced T cell tolerance

Ejuan Zhang[1], Hu Yan[1], Qian Li[2], Ulf Dittmer[2], Huimin Yan[1] and Mengji Lu[2]

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The Toll-like receptor (TLR) signaling pathway, which is composed of a group of highly conserved molecules, plays a critical role in the recognition of pathogen-associated molecular patterns (PAMPs). Innate immunity activated through the TLR signaling pathway serves as a first defense against infectious diseases.[1] However, the exact function of TLR signaling in viral infections remains to be elucidated. Previously, a number of clinical reports indicated that TLR expression and function are impaired in chronic hepatitis B virus (HBV) infection.[2] A specific question was raised regarding whether deficient TLR function may contribute to chronic HBV infection, characterized by low or absent T cell responses against HBV.[3] In our previous study published in Cellular and Molecular Immunology, Ma et al. demonstrated that deficiency in TLRs or adaptor molecules (MyD88/Trif or IRAK4) resulted in not only elevated expression of HBsAg and HBV DNA but also delayed HBV clearance in the hydrodynamic injection HBV mouse model.[4] HBV-specific T cell responses, which play a key role in HBV control and clearance,[5] were detectable but functionally impaired in IL-1R/TLR deficient mice. This study highlighted the essential role of the IL-1R/TLR signaling pathway in adaptive immunity and HBV clearance in vivo. This finding suggested that TLR deficiency may be involved in the low HBV-specific T cell responses observed during chronic HBV infection in humans.

The obvious question is whether TLR signaling provides direct help for hepatic T cells to enhance their immune functions. The liver is an immune-privileged organ with potent mechanisms for tolerance induction.[6] Hepatic tolerance, though crucial for maintaining liver homeostasis, limits antiviral immune responses. Typically, liver nonparenchymal cells, including liver sinusoidal endothelial cells (LSECs) and Kupffer cells (KCs), facilitate intrahepatic T cell priming usually followed by T cell tolerance and functional impairment. Our previous studies showed that TLR stimulation may render LSECs but not KCs immune-activating instead of tolerogenic, indicating that T cell tolerance could be overcome by TLRs targeting the proper responding cell types.[7,8]

Recent studies point out that some TLRs are expressed by activated CD8+ T cells and serve as costimulatory molecules.[9] For example, TLR2 signaling in CD8+ T cells results in a reduced antigen threshold, activation of the mTOR pathway and IFN-γ production. Therefore, we presume that the TLR activation of CD8+ T cells may promote their ability to maintain functionality in the liver. To address this hypothesis, murine splenocytes were prestimulated with TLR2 or TLR7 ligands (P3C or R848, respectively) and activated by anti-CD3 antibodies in the presence of naïve LSECs after washing out free TLR ligands. The CD8+ T cell subpopulations were analyzed by mass cytometry (CyTOF)[10] (Fig. 1a). While IFN-γ production of anti-CD3 antibody-activated T cells was suppressed by LSECs, P3C-pretreated CD8+ T cells displayed reduced sensitivity to LSEC-induced suppression (Fig. 1b). Further simultaneous analysis of CD8+ T cell differentiation and function in the coculture system by using CyTOF revealed an immune landscape of the CD8+ T cell response based on 17 surface and functional markers. Thirteen major CD8+ T cell populations could be annotated based on the SPADE algorithm (Fig. 1c). Different expression patterns of the major activation and functional markers for each population are shown by heatmap (Fig. 1d).

Clearly, unstimulated CD8+ T cells did not express T cell activation or functional markers (Fig. 1c, unstimulated, without LSECs, clusters 1 and 2), whereas the anti-CD3 antibody activated CD8+ T cells (Fig. 1c, non-pretreated, without LSECs) developed into mainly prememory and memory-like $CD62^{hi}CD44^{hi}CD127^{lo}$ subsets (clusters 7, 8, and 11), with functional IFN-γ+ and TNF-α+ CD8+ T cells located in cluster 7. In the presence of LSECs, these activated CD8+ T cells differentiated into mainly effector cell populations with diminished cytokine production (clusters 5 and 6; Fig. 1c, non-pretreated, with LSECs), indicating that the presence of LSECs changed the differentiation and function of activated CD8+ T cells. Interestingly, P3C-pretreated CD8+ T cells (Fig. 1c, P3C-pretreated, with LSECs) polarized the effector populations, which expressed increased CD44, CD69, and CXCR5 and reduced CD62L markers (clusters 3 and 4, Fig. 1d). However, P3C pretreatment did not restore the number or function of prememory cells (cluster 7) but did improve effector populations with enhanced IFN-γ and TNF-α/IL-2 production (Fig. 1e, clusters 5 and 4, respectively). Similar results were obtained with R848-pretreated splenocytes. These results reveal the complexity of T cell activation and differentiation in response to LSECs and TLR stimulation. While TLR stimulation apparently compensates for the inhibitory effect of LSECs on CD8+ T cells, refined analysis demonstrated that CD8+ T cells develop additional phenotypes in the course of this complex interaction.

To investigate whether the direct TLR-activation of CD8+ T cells regulates hepatic T cell priming, TCR transgenic CD8+ T cells specific for the DbGagL Friend retrovirus (FV) epitope (FV-TCR CD8 + T cells) were activated with or without P3C costimulation during priming with peptide-loaded TLR2-deficient DCs or LSECs, which were P3C-nonresponding APCs. Antigen presentation by TLR2−/− DCs led to the expression of activation markers and cytokine production by CD8+ T cells, which was not further enhanced by

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**Erklärung**

Hiermit erkläre ich, gem. §6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema " Toll-like receptor 2/7 activation enhances the metabolism and functions of CD8+ T cells and B cells" zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von (Qian Li) befürworte.

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