Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Protein 4.1R negatively regulates CD8$^+$ T-cell activation by modulating phosphorylation of linker for activation of T cells

Dandan Fan,[1] Jianhui Li,[2] Yi Li,[3] Yaxin Guo,[4] Xiaolin Zhang,[5] Wen Wang,[5] Xiaojie Liu,[4] Jingjing Liu,[1] Liping Dai,[1] Liguo Zhang,[1] Qiaozhen Kang[5] and Zhenyu Ji[1]

[1]*Institute of Medical and Pharmaceutical Sciences, Zhengzhou University, Zhengzhou, China,* [2]*Department of Pathology, Xuchang Central Hospital Affiliated To Henan University of Science and Technology, Xuchang, China,* [3]*Henan Key Laboratory of Medical Pathogen Biology, Center for Disease Control and Prevention of Henan Province, Zhengzhou, China,* [4]*Academy of Medical Sciences, Zhengzhou University, Zhengzhou, China and* [5]*School of Life Sciences, Zhengzhou University, Zhengzhou, China*

doi:10.1111/imm.13085
Received 18 January 2019; revised 12 April 2019; accepted 13 May 2019.
Correspondence: Zhenyu Ji, Institute of Medical and Pharmaceutical Sciences, Zhengzhou University, 40 University Road, Zhengzhou, China. Email: jizhenyu@zzu.edu.cn
Senior author: Zhenyu Ji

Summary

Protein 4.1R, an 80 000 MW membrane skeleton protein, is a vital component of the red blood cell membrane cytoskeleton that stabilizes the spectrin–actin network and regulates membrane properties of deformability and mechanical stability. It has been shown that 4.1R is expressed in T cells, including CD8$^+$ T cells, but its role in CD8$^+$ T cells remains unclear. Here, we have explored the role of 4.1R in CD8$^+$ T cells using 4.1R$^{-/-}$ mice. Our results showed that cell activation, proliferation and secretion levels of interleukin-2 and interferon-γ were significantly increased in 4.1R$^{-/-}$ CD8$^+$ T cells. Furthermore, the phosphorylation levels of linker for activation of T cells (LAT) and its downstream signaling molecule extracellular signal-regulated kinase were enhanced in the absence of 4.1R. *In vitro* co-immunoprecipitation experiments showed a direct interaction between 4.1R and LAT. Moreover, 4.1R$^{-/-}$ CD8$^+$ T cells and mice exhibited an enhanced T-cell-dependent immune response. These data enabled the identification of a negative regulation function for 4.1R in CD8$^+$ T cells by a direct association between 4.1R and LAT, possibly through inhibiting phosphorylation of LAT and then modulating intracellular signal transduction.

Keywords: activation; CD8$^+$ T cell; linker for activation of T cells; proliferation; protein 4.1R.

Introduction

CD8$^+$ T cells have been classified as a component of the adaptive immune system. T-cell receptor (TCR) -mediated signal transduction is an important process in the regulation of CD8$^+$ T-cell function.[1] The TCR signal is initiated when TCR engages relevant peptide–major histocompatibility complex ligands and leads to the activation of the Src family tyrosine kinases lymphocyte-specific protein-tyrosine kinase (Lck) and Fyn. Lck can phosphorylate TCR/CD3 complex immunoreceptor tyrosine-based activation motif signaling motifs in the cytoplasmic region.[2] Phosphorylated immunoreceptor tyrosine-based activation motifs recruit Syk family protein tyrosine kinase ζ-chain-associated protein kinase 70 (ZAP-70) to the plasma membrane, and then ZAP-70 is phosphorylated.[3,4] Linker for activation of T cells (LAT), a transmembrane adaptor protein that serves as a signaling hub and plays a key role in the early stages of TCR activation signaling, is one of the important substrates for ZAP-70.[5] Intracellular tyrosine residues of LAT are phosphorylated by ZAP-70 and activate downstream adaptor molecules phosphoinositide-specific phospholipase Cγ1 (PLCγ1), growth factor receptor-bound protein 2 (Grb2) and Grb2-related adaptor downstream of Shc (Gads),[6,7] which then result in the activation of cytosolic Ca^{2+} and multiple pathways such as the PLCγ1, phosphoinositide 3-kinase (PI3K) and mitogen-activate protein kinase (MAPK)

Abbreviations: CD28, cluster of differentiation 28; CD3, cluster of differentiation 3; CD8, cluster of differentiation 8; ERK, extracellular signal-regulated kinase; Gads, Grb2-related adaptor downstream of Shc; Grb2, growth factor receptor-bound protein 2; IFN-γ, interferon-γ; IL-2, interleukin-2; LAT, linker for activation of T cells; LCK, lymphocyte-specific protein tyrosine kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; PI3K, phosphoinositide 3-kinase; PLCγ1, phosphoinositide-specific phospholipase Cγ1; TCR, T-cell receptor; ZAP70, ζ-chain-associated protein kinase 70

pathways. These pathways control T-cell maturation, proliferation, activation and differentiation.[8,9] Hence, the phosphorylation of LAT plays a key role in converting extracellular signals recognized by TCR into intracellular signaling pathways. However, the molecular mechanisms involved in regulating LAT phosphorylation are not clear.

Protein 4.1R, first identified as a membrane skeleton protein in mature erythrocytes,[10] is a prototypical member of the protein 4.1 superfamily. The superfamily comprise 4.1R, 4.1B,[11] 4.1G,[12] 4.1N[13] and the ERM family (ezrin, radixin and meosin).[14] They all contain three highly conservative domains: N-terminal membrane-binding domain, spectrin–actin-binding domain and C-terminal domain, and the conservative regions were separated by non-conserved areas (U1, U2, U3).[15] Protein 4.1R serves as an adaptor between the membrane and the cytoskeleton and plays an important role in stabilizing the spectrin–actin network and maintaining the integrity of the erythrocytes in blood circulation. It has two major isoforms: a 135 000 MW ($4.1R^{135}$) isoform and an 80 000 MW ($4.1R^{80}$) isoform.[16] In the non-erythrocyte system, it is generated by complex alternative pre-mRNA splicing in different cells and tissues to form a subtype of 135 000 MW to 60 000 MW.[17] Protein 4.1R is associated with transmembrane proteins GPC, Band3, Rh, Duffy, Kell, XK and P55 to form a complex and plays an important role in red blood cells.[18] Data from our laboratory have shown for the first time that 4.1R can bind to membrane protein LAT directly and negatively regulates $CD4^+$ T-cell activation through suppression of the phosphorylation of LAT by ZAP-70.[15] However, the function of protein 4.1R in $CD8^+$ T cells has not been explored.

Here, we explore the influence of 4.1R deficiency on $CD8^+$ T-cell activation to demonstrate the function of protein 4.1R in $CD8^+$ T cells using 4.1R knockout mice, both *in vitro* and *in vivo*. Our results bring to light a crucial role for 4.1R in suppressing $CD8^+$ T-cell activation and show that it acts by inhibiting phosphorylation of LAT.

Materials and methods

Mice

The generation of 4.1R knockout mice has been previously described.[19] The $4.1R^{-/-}$ mice were a free gift from the Red Cell Physiology Laboratory of the New York Blood Center. $4.1R^{+/+}$ C57BL/6J mice, bought from Vital River, Charles River China (Beijing, China), served as controls and were acclimatized for 1 week before experimentation. All mice were maintained at the animal facility in Henan Academy of Medical and Pharmaceutical Sciences under pathogen-free conditions according to institutional guidelines. Animal protocols were reviewed and approved by the Henan Academy of Medical and Pharmaceutical Sciences and Use Committee. All mice used in experiments were 8–10 weeks old.

Antibodies

Rabbit anti-4.1R exon18 was a generous gift from Dr Xiuli An (New York Blood Center, Melville, NY). The fluorescein isothiocyanate-conjugated CD69 antibody was from BioLegend (San Diego, CA); purified NA/LE hamster anti-mouse CD3ε and CD28 were obtained from BD Biosciences (San Jose, CA). The rabbit polyclonal antibodies to LAT, Lck, Erk1 + Erk2, Erk1 (pT202/pY204) + Erk2 (pT185/pY187) and Lck (pY505) and the rabbit monoclonal antibodies to PLCγ1, ZAP70, AKT, ZAP70 (pY292), AKT (pS473) and LAT (pY191) were from Abcam (Cambridge, UK). The goat polyclonal antibody to p-PLCγ1 (pTyr 783) was from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit monoclonal antibody to glyceraldehyde 3-phosphate dehydrogenase was from Cell Signaling Technology (Boston, MA).

Amplification of 4.1R cDNA

$CD8^+$ T cells from lymph nodes were purified by negative selection, using magnetic-activated cell sorting beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Total RNA was extracted from $CD8^+$ T cells derived from $4.1R^{-/-}$ and $4.1R^{+/+}$ mice using TRIzol reagent (Invitrogen life Technologies, Carlsbad, CA) in accordance with the manufacturer's instructions. Reverse transcription of RNA was conducted using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, Waltham, MA). Purified cDNA was used as a template for amplification containing 10 μM of each primer and 12.5 μl 2 × *Taq* MasterMix (Beijing CoWin Biotech, Beijing, China) in a 20-μl reaction mixture. Transcripts of 4.1R can initiate at two separate start sites. Therefore, the following sets of polymerase chain reaction (PCR) primers were used: ATG1 forward, ATGACAACAGAGAAGAGTTTAGTGGCTGAAGC; ATG2 forward, ATGCACTGTAAGGTCTCCTTGTTGGATGACACG; 4.1R reverse, CTCCTCAGAGATCTCTGTCTCCTGGTGGA. Thirty cycles of denaturation (30 seconds, 94°), annealing (30 seconds, 62°), and elongation (2 min, 72°) were carried out with a PCR instrument (Biometra Company, Jena, Germany). The PCR products were cloned into pGEM®-T Easy Vector (Promega, Madison, WI). All clones were sequenced at The Beijing Genomics Institute.

Western blot assay

$CD8^+$ T cells were purified as described previously and stimulated with anti-mouse CD3ε and CD28 at 37° for various times to analyze the expression of signaling molecules. Cell lysates were prepared in cold RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, 0·5% sodium deoxycholate, 0·1% sodium dodecyl sulfate) in the

presence of protease inhibitor cocktail. After centrifugation at 13680 *g* for 10 min at 4°, the supernatants were collected. Protein concentrations were determined with a bicinchoninic acid protein assay kit. Approximately 30 g protein per sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with tris buffered saline with tween 20 containing 5% skim milk at 37° for 1 hr and then incubated with the primary antibodies in blocking buffer at 4° overnight. For the loading controls, anti-glyceraldehyde 3-phosphate dehydrogenase polyclonal antibody was used. After washing, the membranes were incubated with the corresponding second antibody. Immunoreactive bands were visualized by chemiluminescence using an ECL Kit (Thermo Scientific, Waltham, MA). Protein quantification was performed using ImageJ software (NIH, Bethesda, MD).

Co-immunoprecipitation assay

$CD8^+$ T cells were purified as described previously and lyzed at 4° for 30 min in ice-cold RIPA buffer. Supernatants were separated by centrifugation (13680 *g* 10 min at 4°). Supernatant was incubated at 4° overnight with the rabbit anti-4.1R or the control IgG (pre-immune normal rabbit IgG) and then incubated with 10 μl ProteinA/G PLUS-Agarose (Santa Cruz Biotechnology) at 4° for 2 hr. Immunoprecipitation proteins were collected by centrifugation at 590 *g* at 4° for 5 min and pellets were washed four times with 1 ml RIPA buffer. Samples were boiled for 2–3 min and then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by transfer onto polyvinylidene fluoride membranes. The membranes were probed with anti-4.1R exon 18 antibody and anti-LAT antibody, respectively.

Activation assay and cytokine production analysis

$CD8^+$ T cells were purified as described previously. Flow cytometry confirmed that the purity of the isolated cells was consistently > 90%. For the activation assay, $CD8^+$ T cells were stimulated with anti-CD3ε/CD28 antibodies (2 μg/ml) for 6 hr then, cells were stained with fluorescein isothiocyanate-conjugated anti-CD69. Data were measured using FACScan flow cytometry (Beckman Coulter, Brea, CA) and analyzed using EXPo™32 ADC software (Beckman Coulter, Brea, CA). Cytokine production by cells was measured by ELISPOT. $CD8^+$ T cells were stimulated with anti-CD3ε/CD28 antibodies and cultured in 96-well ELISPOT plates (Millipore, Billerica, MA) precoated with capture antibodies to interleukin-2 (IL-2)/interferon-γ (IFN-γ) at 37° for 18 hr. Plates were washed and spots were counted using an ELISPOT plate reader and software. The IL-2 and IFN-γ in supernatants were also analyzed, $CD8^+$ T cells were cultured in 96-well plates pre-coated with anti-CD3ε/CD28 antibodies (2 μg/ml). After 24 hr of incubation, cell culture supernatants were analyzed for IL-2 and IFN-γ using a multi-analyze profiler ELISA array kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. The results were averaged from triplicates.

Proliferation assay

For labeling purified $CD8^+$ T cells with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen), cells (at a density of 10^6 cells/ml) were incubated for 10 min at 37° in 1 μl of 5 mM CFSE for 10 min. Labeling was stopped by adding five times the initial volume of ice-cold RPMI-1640/10% fetal bovine serum and incubated for 5 min on ice. Cells were washed three times with the culture medium before use and suspended in complete RPMI-1640 medium and cultured in 96-well plates (10^5/well) pre-coated with anti-CD3ε/CD28 antibodies (2 μg/ml). For the quantification of cell proliferation, flow cytometric analyses of CFSE dilution were performed at 0, 48 and 72 hr of culture. Proliferation was also assessed by Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manual of the manufacturer. Briefly, 1×10^5 cells were seeded into each well of a 96-well plate pre-coated with anti-CD3ε/CD28 antibodies. Cells were examined at 48 hr. CCK-8 (10 μl) was added to each well and incubated for 4 hr at 37°; absorbance was measured at 450 nm with a Microplate Reader (Bio-Rad, Hercules, CA). The results were averaged from triplicates.

Cell cycle analysis

$CD8^+$ T cells were activated *in vitro* for 24 and 48 hr as described above, and then cells were collected by centrifugation and fixed in ethanol for 24 hr at –20°. Samples were incubated with 1 ml PI Buffer A (Multisciences (Lianke) Biotech, Hangzhou, China) for 30 min at room temperature. DNA content was measured using FACScan flow cytometry. Cell cycle analysis was performed using Multicycle for Windows 32-bit software. The results were averaged from triplicates.

Lactate dehydrogenase release assay

Purified $CD8^+$ T cells that had been pre-treated with anti-CD3ε/CD28 antibodies (2 μg/ml) and recombinant human IL-2 (PeproTech, Rocky Hill, NJ) were resuspended to a final concentration of 2×10^7 cells/ml, and 100 μl of cells was then added to a 96-well plate together with B16 cells or H22 cells (100 μl) to obtain effector to target ratios of 10 : 1, 20 : 1 and 40 : 1. The cells were incubated at 37° for 6 hr and then centrifuged at 210 *g* for 5 min and the supernatants were collected. The lactate dehydrogenase (LDH) release assay was performed

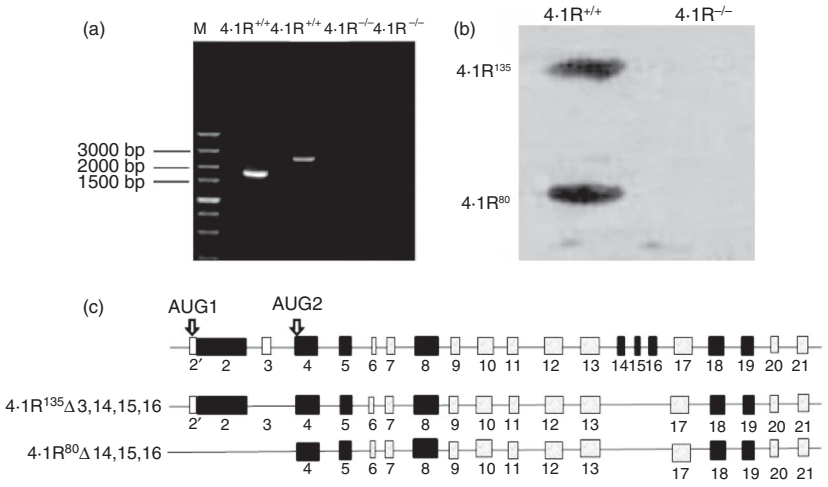

Figure 1. Expression of 4.1R in mouse CD8$^+$ T cells. (a) The cloning results of 4.1R cDNA in 4.1R$^{+/+}$ and 4.1R$^{-/-}$ CD8$^+$ T cells. RNA was extracted from 4.1R$^{+/+}$ and 4.1R$^{-/-}$ CD8$^+$ T cells, then inverse transcripted into cDNA, polymerase chain reaction amplification was used to detect 4.1R gene expression in two cell types. Two bands with the length of approximately 2500 bp and 1700 bp in 4.1R$^{+/+}$ CD8$^+$ T cells were detected. No bands were expressed in 4.1R$^{-/-}$ cells. DL5 Kb was used as the DNA maker. (b) Western blot analysis of protein 4.1R. CD8$^+$ T cells (10^6 cells) were subjected to immunoblot analysis with polyclonal rabbit antibodies against 4.1R exon 18. The positions of approximately 135 000 MW and approximately 80 000 MW 4.1R are indicated. (c) Exon composition of 4.1R isoforms. Schematic representation of the exon map of 4.1R is displayed. Two translation initiation sites are indicated. Alternatively spliced exons are shown in black, constitutive exon in gray, and non-coding exons in open boxes. Exon compositions of 4.1R 135 000 MW and 80 000 MW are shown in the middle and bottom panels, respectively.

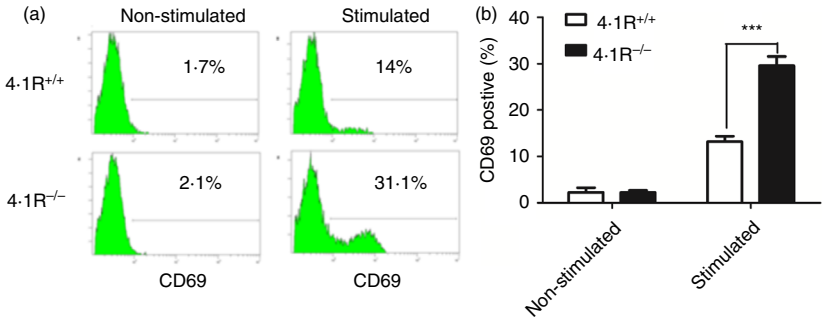

Figure 2. Increased activation of 4.1R$^{-/-}$ CD8$^+$ T cells. Both purified 4.1R$^{+/+}$ and 4.1R$^{-/-}$ CD8$^+$ T cells were stimulated with anti-CD3ε/CD28 (2 μg/ml) for 6 hr whereupon CD69 surface expression was detected by flow cytometry. (a) Representative profiles of CD69 expression of 4.1R$^{+/+}$ and 4.1R$^{-/-}$ CD8$^+$ T cells. (b) Quantitative analysis. Note that a significantly higher percentage of 4.1R$^{-/-}$ CD8$^+$ T cells express CD69. Error bars indicate SD.

according to the manufacturer's instructions (Promega). Briefly, 50 μl of culture supernatant was incubated with 50 μl LDH substrate solution. After incubation in the dark for 30 min at room temperature, the reaction was stopped by addition of 50 μl stop solution, and the optical density (OD) was measured at 490 nm. The amount of LDH released was calculated according to the following formula: % cytotoxicity = $[(E_{OD} - S_{OD})/(M_{OD} - S_{OD})] \times 100$, where E = experimental release of effector : target co-culture minus spontaneous effector cell LDH release, S = spontaneous target cell LDH release, M = total target cell LDH release and OD = optical density. The results were averaged from triplicates

Tumor xenografts in mice

The 6- to 8-week-old 4.1R$^{+/+}$ and 4.1R$^{-/-}$ C57BL/6J mice were randomly divided into two groups of six and raised at the Henan Academy of Medical and Pharmaceutical Sciences. Cells from a mouse liver cancer cell line (H22) in logarithmic phase were harvested and diluted to a concentration of 1×10^7/ml with normal saline; 200 μl of cell suspension was injected subcutaneously into the right flanks of the mice at a single site. Tumor size was measured once every day for 12 days with the use of a caliper, and tumor volume (V) was calculated according to the formula $V = ab^2/2$, where a and b were the major

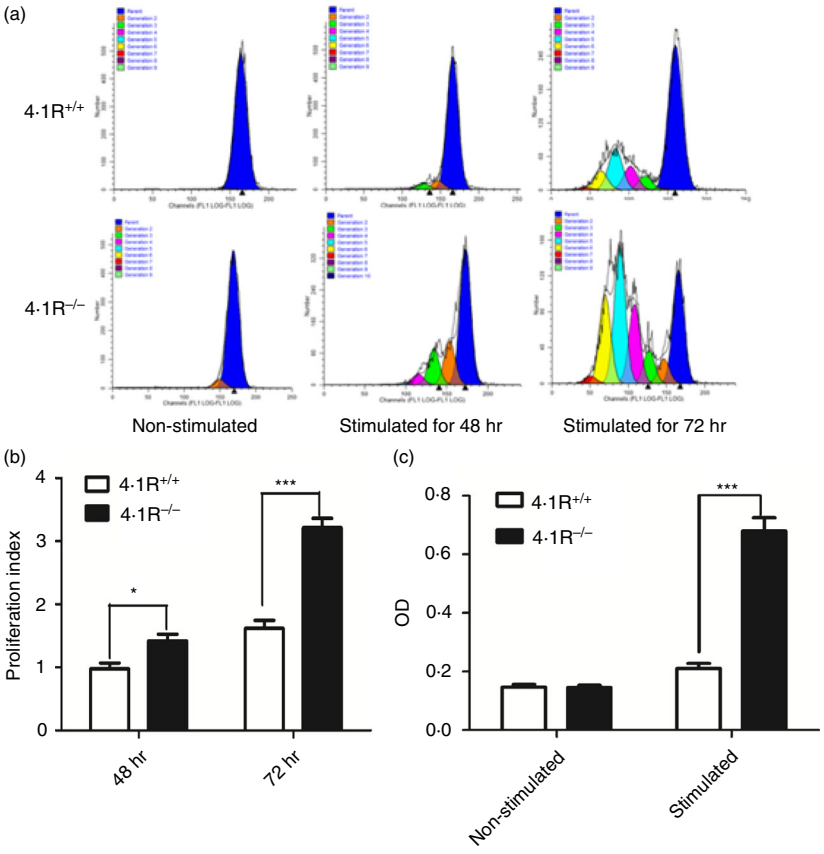

Figure 3. Increased proliferation of $4.1R^{-/-}$ $CD8^+$ T cells. CFSE-labeling experiment was used to explore proliferation of purified $4.1R^{+/+}$ and $4.1R^{-/-}$ $CD8^+$ T cells. The proliferation rates of the two cell types stimulated with anti-CD3ε/CD28 (2 μg/ml) were assessed by flow cytometry. (a) Representative profiles of proliferation rates of $4.1R^{+/+}$ and $4.1R^{-/-}$ $CD8^+$ T cells that were stimulated for 48 and 72 hr, respectively. (b) Quantitative analysis. CCK-8 experiment was also performed to detect the proliferation rate. (c) Quantitative analysis of optical density at 450 nm (OD_{450}) of $4.1R^{+/+}$ and $4.1R^{-/-}$ CD8 T cells when stimulated for 48 hr with anti-CD3/CD28. Note a significantly higher proliferation response of $4.1R^{-/-}$ $CD8^+$ T cells. Error bars indicate SD.

and minor axes of the tumor foci, respectively. Mice were killed by cervical dislocation on day 12, and the tumor tissue was weighed. The animal studies and the experimental protocols were approved by the Animal Ethics Committee of the Henan Academy of Medical and Pharmaceutical Sciences.

Statistical analysis

All experiments described were repeated at least three times. Average values were expressed as means ± SD. The difference between two groups was determined by Student's *t*-test or analysis of variance followed by the Bonferroni corrections if there were more than two groups. $P < 0{\cdot}05$ was set as the significant criterion.

Results

Expression of 4.1R in $CD8^+$ T cells

It has been shown that 4.1R is highly expressed in mouse thymus and widely present in primary human T cells and human T-cell lines. To date, the function of 4.1R in $CD4^+$ T cells has been explored. In the present study, we focused on the role of 4.1R in $CD8^+$ T cells. In the non-erythrocyte system, it is generated by complex alternative pre-mRNA splicing in different cells and tissues to form a subtype of 135 000 MW to 60 000 MW. We cloned 4.1R from $CD8^+$ T cells by reverse transcription PCR with two primer sets initiating from ATG-1 and ATG-2, and clones were all sequenced. Figure 1(a) shows two bands with the length of approximately 2500 bp and 1700 bp in $4.1R^{+/+}$ $CD8^+$ T cells. These two bands were identified as 4.1R fragments through sequence analysis. Figure 1(b) shows two bands with the molecular weights of approximately 135 000 and 80 000, corresponding to 4.1R transcripts from ATG1 and ATG2 initiation sites, respectively. In $4.1R^{-/-}$ $CD8^+$ T cells, the expression of 4.1R was not detected (Fig. 1a,b). Figure 1(c) shows the 4.1R isoforms isolated from $CD8^+$ T cells and all clones lacked exon 16. However, unlike in $CD4^+$ T cells, the clones also lacked exon 3, exon 14 and exon 15 but retained the complete exon 17.

Increased activation of $4.1R^{-/-}$ $CD8^+$ T cells

To investigate the effect of 4.1R deficiency on the activation of $CD8^+$ T cells, we used flow cytometry to examine the expression of CD69, the earliest inducible cell surface glycoprotein acquired during T-cell activation. Figure 2(a) shows that 4.1R deficiency influenced induced surface expression of CD69 on $CD8^+$ T cells. The results

revealed that expression of CD69 was almost undetectable both in $4.1R^{+/+}$ and $4.1R^{-/-}$ $CD8^+$ T cells before stimulation. However, after 6 hr of stimulation with CD3ε/CD28 antibody (2 μg/ml), the percentage of CD69 expression in $4.1R^{+/+}$ and $4.1R^{-/-}$ $CD8^+$ T cells was increased to 14% and 31·1%, respectively. Quantitative analysis (Fig. 2b) revealed a significant increase in the expression of CD69 in $4.1R^{-/-}$ $CD8^+$ T cells compared with that in $4.1R^{+/+}$ $CD8^+$ T cells (29·58 ± 1·956% versus 13·20 ± 1·169%, $n = 4$, $P < 0·001$). Hence, the deficiency of 4.1R was observed to increase activation of $CD8^+$ T cells.

Increased proliferation of $4.1R^{-/-}$ $CD8^+$ T cells

The difference in the proliferation response between $4.1R^{+/+}$ and $4.1R^{-/-}$ $CD8^+$ T cells was explored using CFSE-labeling experiments after stimulation with CD3ε/CD28 antibodies (2 μg/ml). As shown in Fig. 3(a), after 48 hr of stimulation, purified $4.1R^{-/-}$ $CD8^+$ T cells started to show a faster proliferation rate than $4.1R^{+/+}$ $CD8^+$ T cells (1·42 ± 0·104 versus 0·974 ± 0·094, $n = 4$, $P < 0·05$). When stimulated with antibodies for 72 hr, $4.1R^{-/-}$ $CD8^+$ T cells displayed significantly higher proliferation than $4.1R^{+/+}$ $CD8^+$ T cells (3·210 ± 0·15 versus 1·614 ± 0·131, $n = 4$, $P < 0·001$). A statistically significant difference was shown in Fig. 3(b), as $4.1R^{-/-}$ $CD8^+$ T cells divided faster than $4.1R^{+/+}$ $CD8^+$ T cells. To further validate this finding, we performed CCK-8 experiments. Figure 3(c) shows a significantly higher OD at 450 nm of $4.1R^{-/-}$ $CD8^+$ T cells compared with that of $4.1R^{+/+}$ $CD8^+$ T cells when stimulated for 48 hr, indicating increased proliferation response in $4.1R^{-/-}$ $CD8^+$ T cells (0·680 ± 0·044 versus 0·211 ± 0·016, $n = 4$, $P < 0·001$). These results demonstrated that the lack of 4.1R leads to an increased proliferation response of $4.1R^{-/-}$ $CD8^+$ T cells.

Altered cell cycle process of $4.1R^{-/-}$ $CD8^+$ T cells

Next, we investigated whether the cell cycle process in $4.1R^{-/-}$ $CD8^+$ T cells was changed. We detected the 24 and 48 hr cell cycle process of activated $CD8^+$ T cells using flow cytometry. As expected, in $4.1R^{-/-}$ $CD8^+$ T cells, the percentage of cells in both S phase and G2/M phase was higher than those in $4.1R^{+/+}$ $CD8^+$ T cells (Fig. 4). These findings suggested that 4.1R deficiency affects the cell cycle in $CD8^+$ T cells.

Increased secretion of IL-2 and IFN-γ in $4.1R^{-/-}$ $CD8^+$ T cells

Having demonstrated the effect of 4.1R deficiency on the activation and proliferation of $CD8^+$ T cells, we examined

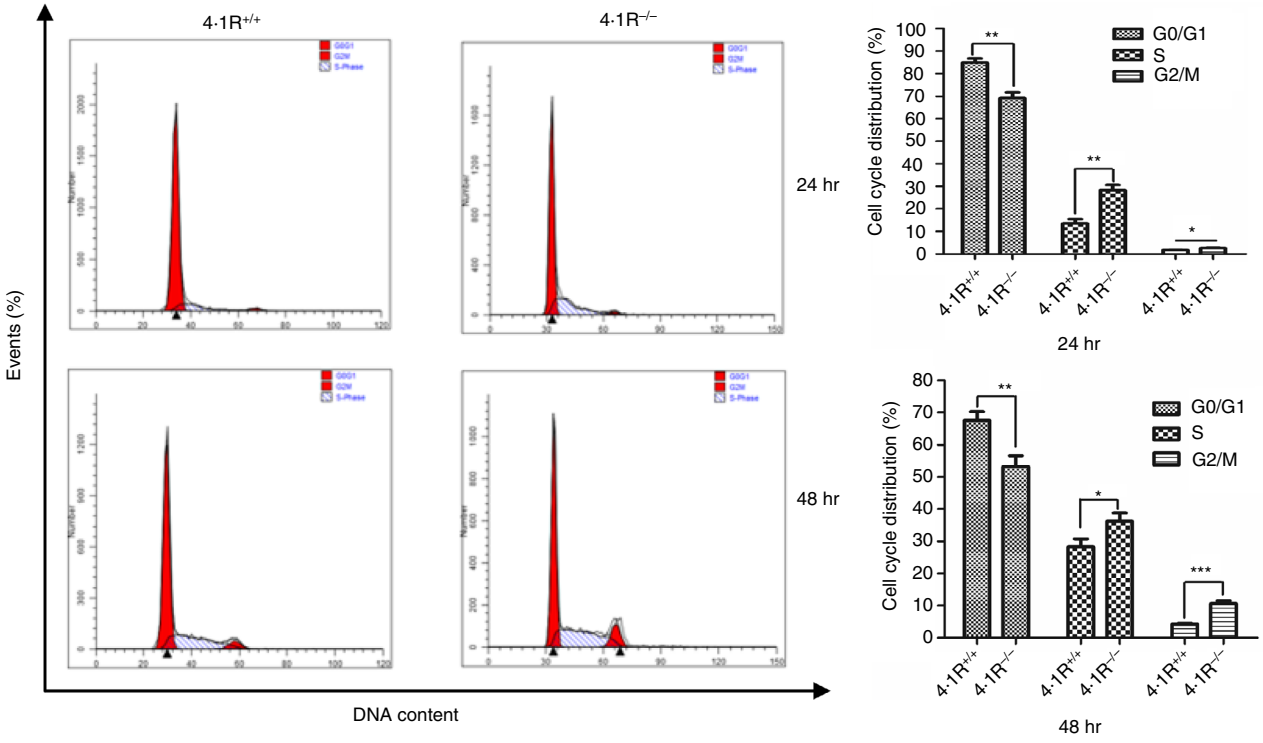

Figure 4. Altered cell cycling of $4.1R^{-/-}$ $CD8^+$ T cells. $CD8^+$ T-cell expansion was detected after both $4.1R^{+/+}$ and $4.1R^{-/-}$ $CD8^+$ T cells were stimulated with anti-CD3ε/CD28 (2 μg/ml) for 24 and 48 hr. Note that there were fewer cells in G0/G1 and more cells in S and G2/M with $4.1R^{-/-}$ $CD8^+$ T cells compared with $4.1R^{+/+}$ $CD8^+$ T cells showing an altered cell cycle in $4.1R^{-/-}$ $CD8^+$ T cells.

the secretion of IL-2 and IFN-γ in both 4.1R$^{+/+}$ and 4.1R$^{-/-}$ CD8$^+$ T cells using ELISPOT. The expression of IL-2 in 4.1R$^{-/-}$ CD8$^+$ T cells was significantly higher than that in 4.1R$^{+/+}$ CD8$^+$ T cells (134·8 ± 8·966 versus 56·75 ± 4·956, $n = 4$, $P < 0·001$). The expression of IFN-γ in 4.1R$^{-/-}$ CD8$^+$ T cells was significantly higher than that in 4.1R$^{+/+}$ CD8$^+$ T cells (682·8 ± 47·99 versus 372·8 ± 35·77, $n = 5$, $P < 0·001$) (Fig. 5a). We also determined the concentrations of IL-2 and IFN-γ in the supernatant of CD8$^+$ T cells after 24 hr incubation. In accordance with the increased secretion of IL-2 and IFN-γ in 4.1R$^{-/-}$ CD8$^+$ T cells, the concentrations of IL-2 and IFN-γ in the supernatant of 4.1R$^{-/-}$ CD8$^+$ T cells were also significantly increased (137·3 ± 8·625 pg/ml versus 90·79 ± 6·331 pg/ml, $n = 5$, $P < 0·01$, 2650 ± 229·4 pg/ml versus 746·8 ± 81·79 pg/ml, $n = 5$, $P < 0·001$, respectively) (Fig. 5b,c).

Enhanced phosphorylation levels of LAT and extracellular signal-regulated kinase in CD8$^+$ T cells from 4.1R$^{-/-}$ mice

Activation of TCR-mediated signal transduction is required for activation, proliferation and cytokine

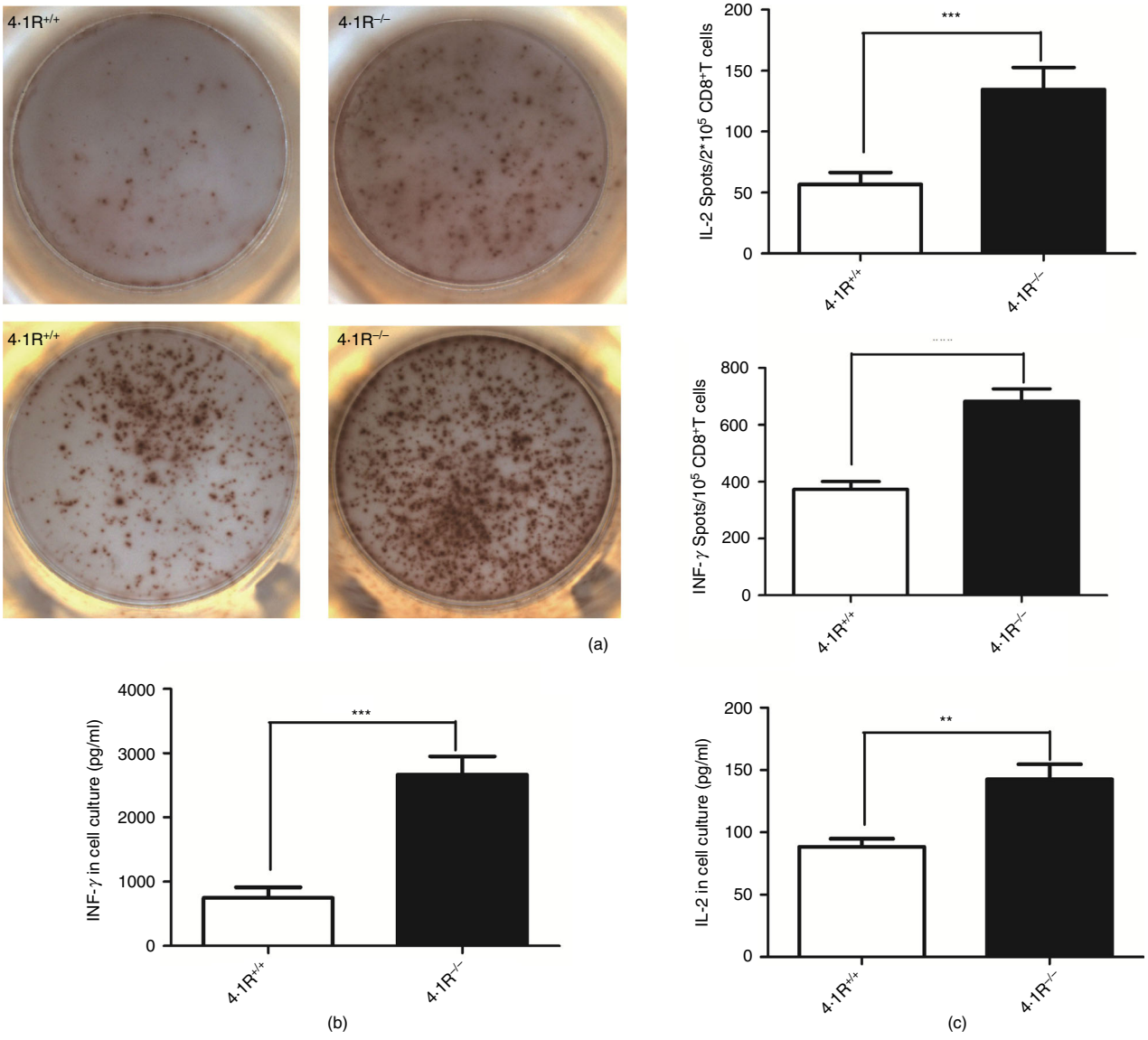

Figure 5. Increased secretion of interleukin-2 (IL-2) and interferon-γ (IFN-γ) in 4.1R$^{-/-}$ CD8$^+$ T cells. Purified 4.1R$^{+/+}$ and 4.1R$^{-/-}$ CD8$^+$ T cells were stimulated with anti-CD3/CD28 (2 μg/ml). ELISPOT assays were performed to measure secretion of IL-2 and IFN-γ. The ELISPOT results are shown, spots were counted using ELISPOT plate reader and software. The quantitative analysis is shown (a). Note the significantly higher IL-2 and IFN-γ production in 4.1R$^{-/-}$ CD8$^+$ T cells. Measurements of IL-2 and IFN-γ were performed in cell supernatant of both 4.1R$^{+/+}$ and 4.1R$^{-/-}$ CD8$^+$ T cells (b, c). The quantitative analysis also shows a significantly higher concentration of IL-2 and IFN-γ in 4.1R$^{-/-}$ CD8$^+$ T cells. Error bars indicate SD.

secretion of $CD8^+$ T cells. To explore whether 4.1R deficiency affects TCR signal transduction, Western blot was used to analyze the phosphorylation levels of major proteins in TCR-mediated signal transduction in $4.1R^{+/+}$ and $4.1R^{-/-}$ $CD8^+$ T cells after stimulation by CD3ε/CD28 (2 μg/ml). The results showed that there was no discernible difference between phosphorylation level of Lck or ZAP-70 but enhanced phosphorylation level of LAT (Y191) in $4.1R^{-/-}$ $CD8^+$ T cells (Fig. 6a,b). Because the phosphorylated LAT activates three important downstream pathways, including the PLCγ1, PI3K and MAPK pathways, we next explored the phosphorylation of PLCγ1, Akt, extracellular signal-regulated kinase (ERK). Our findings demonstrated a great increase in ERK phosphorylation in $4.1R^{-/-}$ $CD8^+$ T cells (Fig. 6) but no difference in PLCγ1 and Akt phosphorylation (Fig. 6). Our findings confirmed that 4.1R inhibits phosphorylation levels of LAT and ERK in the MAPK pathway, implying a negative function of 4.1R in regulating TCR signal transduction.

Co-immunoprecipitation of 4.1R with protein LAT

We sought direct evidence for the association of 4.1R with LAT in $CD8^+$ T cells by performing co-immunoprecipitation studies. As shown in Fig. 7, immunoprecipitation of 4.1R by its specific antibody in $4.1R^{+/+}$ $CD8^+$ T cells followed by Western blot using an anti-4.1R antibody revealed the presence of protein 4.1R (Fig. 7a), and using an anti-LAT antibody revealed the presence of protein LAT (Fig. 7b). No signal was detected when this experiment was performed with $4.1R^{-/-}$ $CD8^+$ T cells. These results imply an association between 4.1R and LAT *in situ*.

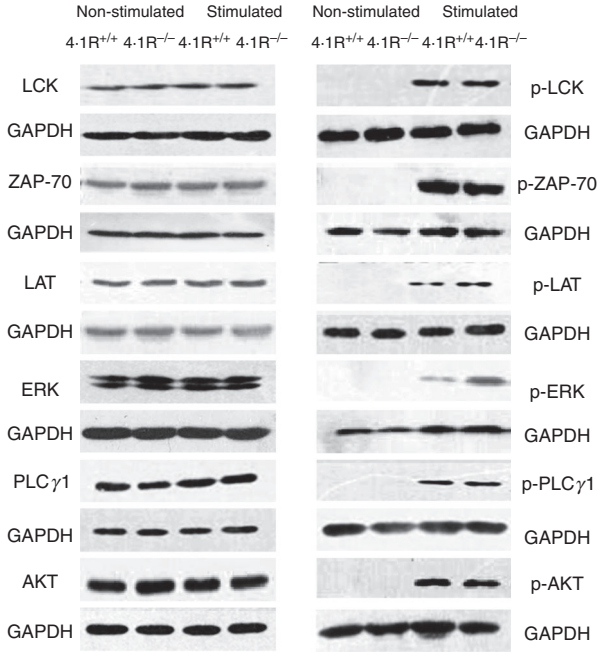

Figure 6. Enhanced phosphorylation levels of linker for activation of T cells (LAT) and extracellular signal-regulated kinase (ERK) in $CD8^+$ T cells from $4.1R^{-/-}$ mice. The phosphorylation levels of signaling molecules were detected using Western blot analysis. The $4.1R^{+/+}$ and $4.1R^{-/-}$ $CD8^+$ T cells were stimulated with anti-CD3ε/CD28 (2 μg/ml), and the levels of protein expression and phosphorylation were investigated. Note the significantly enhanced phosphorylation of LAT and ERK in $4.1R^{-/-}$ $CD8^+$ T cells. GADPH was used as the control.

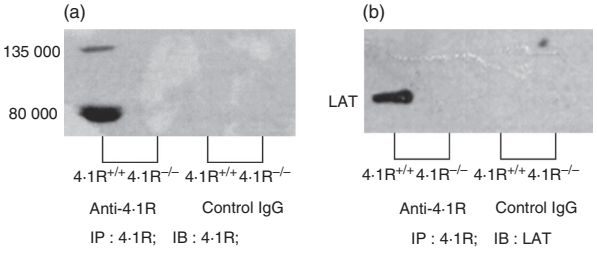

Figure 7. Co-immunoprecipitation of 4.1R and LAT. $4.1R^{+/+}$ and $4.1R^{-/-}$ $CD8^+$ T cells were lyzed, and extracts were subjected to immunoprecipitation with anti-4.1R antibody (IP) or pre-immune IgG. Presence of protein 4.1R or LAT was determined by Western blot in IP eluates using anti-4.1R antibodies (a) or anti-LAT antibodies (b), respectively.

Enhanced T-cell-dependent immune response in $4.1R^{-/-}$ $CD8^+$ T cells and $4.1R^{-/-}$ mice

To determine whether 4.1R deficiency influences T-cell function *in vitro* and *in vivo*, we first compared the cytotoxic effect between $4.1R^{+/+}$ and $4.1R^{-/-}$ $CD8^+$ T cells *in vitro*. $CD8^+$ T cells were pre-treated with anti-CD3ε/CD28 antibodies (2 μg/ml) and recombinant human IL-2, and then co-cultured with H22 and B16 tumor cells in proportion. The results showed that $4.1R^{-/-}$ $CD8^+$ T cells had a stronger cytotoxic effect than $4.1R^{+/+}$ $CD8^+$ T cells (Fig. 8a). Following this, we performed a mouse tumorigenicity assay to explore the effects of 4.1R deficiency on the anti-tumor ability of $CD8^+$ T cells *in vivo*. The results for tumors from $4.1R^{+/+}$ and $4.1R^{-/-}$ mice are shown in Fig. 8(b). The results demonstrated a significant reduction in tumor volume (Fig. 8c) and weight (Fig. 8d) in $4.1R^{-/-}$ mice compared with $4.1R^{+/+}$ mice. These findings validated that 4.1R deficiency reduces tumor formation through increasing activation of $CD8^+$ T cells.

Discussion

Protein 4.1R was first identified in the erythrocyte membrane skeleton and anchored to the plasma membrane through the band 4.1, ezrin, radixin, and moesin domain where it serves an essential role in organizing the actin skeleton and assembling transmembrane proteins.[20]

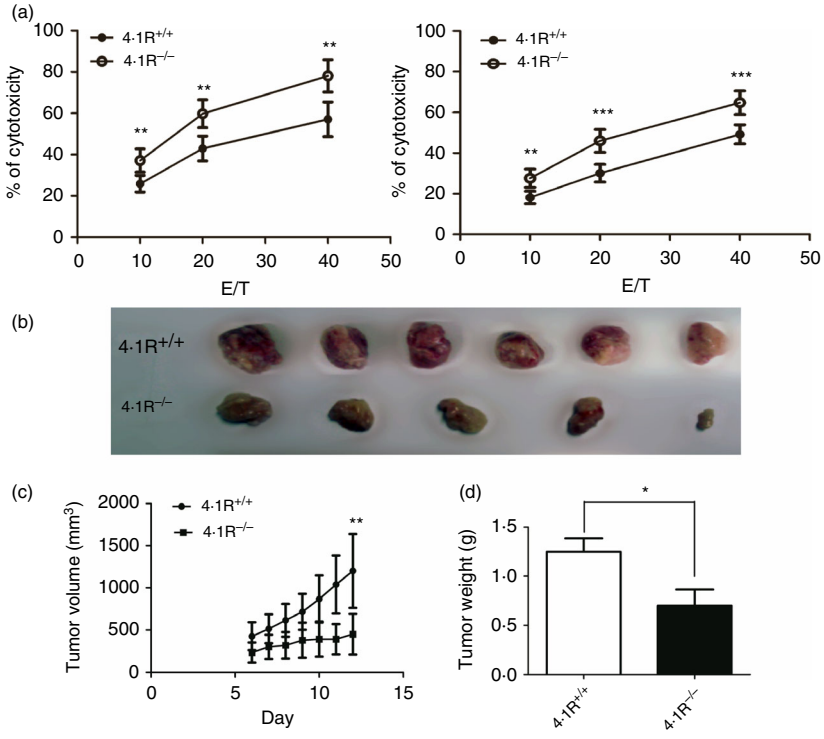

Figure 8. Enhanced T-cell-dependent immune response in $4.1R^{-/-}$ $CD8^+$ T cells and $4.1R^{-/-}$ mice. Activated $CD8^+$ T cells were cultured with H22 or B16 tumor cells in proportion. Lactate dehydrogenase (LDH) release assays were used to detect cytotoxicity. The results showed that $4.1R^{-/-}$ $CD8^+$ T cells have greater cytotoxicity than $4.1R^{+/+}$ $CD8^+$ T cells (a). Mouse liver cancer H22 cells were injected subcutaneously into the right flank of $4.1R^{+/+}$ and $4.1R^{-/-}$ C57BL/6J mice at a single site. After 12 days, tumors were taken from the mice. The results present the volume of tumors from $4.1R^{+/+}$ and $4.1R^{-/-}$ $CD8^+$ mice (b). The tumor volume was measured. The results show that tumor volume from $4.1R^{-/-}$ injected mice was smaller than that from $4.1R^{+/+}$ $CD8^+$ mice; $^{**}P < 0{\cdot}05$ (c). Quantitative analysis shows that tumors from $4.1R^{-/-}$ mice were significantly lighter (d),$^{*}P < 0{\cdot}01$. Error bars indicate SD.

Various functions for 4.1R in non-erythroid cells have also been verified. In previous studies, we have shown that 4.1R negatively regulates $CD4^+$ T-cell activation by inhibiting phosphorylation of LAT.[15] We have also demonstrated that 4.1R negatively regulates progression of experimental autoimmune encephalomyelitis by inhibiting $CD4^+$ T-cell activation signaling.[21] In the present study, a novel function for 4.1R in $CD8^+$ T cells was identified *in vitro* and *in vivo*.

In red blood cells, the expression pattern of protein 4.1R is relatively simple, but in nucleated cells, 4.1R pre-mRNA alternative splicing results in multiple mRNA and protein subtypes of different sizes from 135 000 to 60 000 MW that are expressed in virtually all tissues.[17,22] In $CD8^+$ T cells, we found two 4.1R isoforms ($4.1R^{135}$ and $4.1R^{80}$), which is consistent with the isoforms in $CD4^+$ T cells. We then cloned the full-length sequence of these two isoforms and analyzed the exon composition. We found that in $CD8^+$ T cells two isoforms of 4.1R were lacking exon 14, exon 15 and exon 16, which was different from the absence of exon 16 in $CD4^+$ T cells. Although both $CD8^+$ T cells and $CD4^+$ T cells are differentiated by progenitor T cells, their functions are not the same. In different cells the significance of the differential selective splicing of protein 4.1R exons and the effect on their function is unknown. This needs to be further explored in the post-genomics era.

One obvious purpose of the present study is that lacking 4.1R could enhance T-cell activation markers CD69 and proliferation of $CD8^+$ T cells, which implies a negative role for 4.1R in regulating activation of $CD8^+$ T cells. The secretion of IL-2 and IFN-γ in $CD8^+$ T cells has also been verified and demonstrates the importance of 4.1R in the regulation of $CD8^+$ T cells. It was interesting to note that although 4.1R deficiency affects $CD8^+$ T-cell activation, 4.1R knockout mice did not display related phenotypes of immunological disease.

Another striking finding of the study was the effect of 4.1R deficiency on phosphorylation of LAT and ERK in $CD8^+$ T cells. In $CD4^+$ T cells, protein 4.1R binds directly to the adaptor protein LAT and inhibits phosphorylation by ZAP-70. Therefore, we further detected phosphorylation of LAT and ERK in $4.1R^{-/-}$ $CD8^+$ T cells to identify the molecular mechanism of 4.1R in regulating $CD8^+$ T-cell function. We confirmed the enhanced phosphorylation of LAT and ERK in $CD8^+$ T cells from 4.1R-deficient mice. It has been shown in human T cells that the phosphorylation of LAT tyrosine residues Y(132) is critical for phosphorylated PLCγ1 whereas Y(171), Y(191) and Y (226) are critical for Gads binding, and that this then influences the MAPK–ERK signaling pathway.[23] Interestingly, we found Y(191) of LAT phosphorylation was increased, which results in the hyper-phosphorylation of its downstream signaling molecule ERK. Protein 4.1R specifically regulates phosphorylation intensity of LAT site Y(191) to selectively affect the downstream signaling pathway of LAT. In the PI3K–AKT signaling pathway, there was no difference in expression of AKT and

phosphorylation of protein molecules in 4.1R$^{+/+}$ and 4.1R$^{-/-}$ CD8$^+$ T cells. This gives us more reason to believe that protein 4.1R may negatively regulate CD8$^+$ T-cell signaling by inhibiting LAT molecule phosphorylation.[24] In the early stages of T-cell activation, LAT molecules enter lipid rafts and aggregate into immune synapses. It is known that the formation of the immune synapse is important and promotes signal transduction and that the immune synapse was initiated by clustering of surface receptors and rearrangement of the cytoskeleton. Interestingly, we found protein 4.1R was recruited to the immunological synapse and co-localized with LAT in CD8$^+$ T cells. We next found that 4.1R directly interacts with LAT by co-immunoprecipitation assays. However, which subtypes (4.1R^{135} and 4.1R^{80}) and domains (N-terminal membrane-binding domain, spectrin–actin-binding domain and C-terminal domain) of 4.1R interact with LAT and the detailed mechanisms involved are unknown and require further investigation.

The findings of the present study imply a negative role of 4.1R in regulating activation of CD8$^+$ T cells by modulating phosphorylation of LAT. As phosphorylation and de-phosphorylation of LAT are critical for regulating TCR-mediated signal transduction,[25,26] our findings enable us to speculate that 4.1R is a novel regulator in processes of CD8$^+$ T-cell activation and signal transduction. It will be interesting in future studies to establish detailed mechanisms involved in TCR/CD3 signal transduction.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Nos 81671542, 81373119, 81571526 and 3160067), the Major Project of Science and Technology in Henan Province (No. 161100311400) and the Key Research Projects of Henan Higher Education (No. 19B310004).

Disclosure

The authors declare no conflict of interest associated with this manuscript.

References

1 Cemerski S, Shaw A. Immune synapses in T-cell activation. *Curr Opin Immunol* 2006; **18**:298–304.

2 Courtney AH, Lo WL, Weiss A. TCR signaling: mechanisms of initiation and propagation. *Trends Biochem Sci* 2018; **43**:108–23.

3 Fu G, Chen Y, Schuman J, Wang D, Wen R. Phospholipase Cγ2 plays a role in TCR signal transduction and T cell selection. *J Immunol* 2012; **189**:2326–32.

4 Fu G, Yu M, Chen Y, Zheng Y, Zhu W, Newman DK *et al.* Phospholipase Cγ1 is required for pre-TCR signal transduction and pre-T cell development. *Eur J Immunol* 2017; **47**:74–83.

5 Zhang W, Sommers CL, Burshtyn DN, Stebbins CC, DeJarnette JB, Trible RP *et al.* Essential role of LAT in T cell development. *Immunity* 1999; **10**:323–32.

6 Seminario MC, Bunnell SC. Signal initiation in T-cell receptor microclusters. *Immunol Rev* 2008; **221**:90–106.

7 Ou-Yang CW, Zhu M, Sullivan SA, Fuller DM, Zhang W. The requirement of linker for activation of T cells in the primary and memory responses of CD8 T cells. *J Immunol* 2013; **190**:2938–47.

8 Cao Y, Li H, Liu H, Zhang M, Hua Z, Ji H *et al.* LKB1 regulates TCR-mediated PLCγ1 activation and thymocyte positive selection. *EMBO J* 2011; **30**:2083–93.

9 Hennecke J, Wiley DC. T cell receptor-MHC interactions up close. *Cell* 2001; **104**:1–4.

10 Ning S, Kang Q, Fan D, Liu J, Xue C, Zhang X *et al.* Protein 4.1R is involved in the transport of 5-aminolevulinic acid by interaction with GATs in MEF cells. *Photochem Photobiol* 2018; **94**:173–8.

11 Wang Z, Zhang J, Ye M, Zhu M, Zhang B, Roy M *et al.* Tumor suppressor role of protein 4.1B/DAL-1. *Cell Mol Life Sci* 2014; **71**:4815–30.

12 Sanuki R, Watanabe S, Sugita Y, Irie S, Kozuka T, Shimada M *et al.* Protein-4.1G-mediated membrane trafficking is essential for correct rod synaptic location in the retina and for normal visual function. *Cell Rep* 2015; **10**:796–808.

13 Ji Z, Shi X, Liu X, Shi Y, Zhou Q, Liu X *et al.* The membrane-cytoskeletal protein 4.1N is involved in the process of cell adhesion, migration and invasion of breast cancer cells. *Exp Ther Med* 2012; **4**:736–40.

14 Baines AJ. Evolution of spectrin function in cytoskeletal and membrane networks. *Biochem Soc Trans* 2009; **37**:796–803.

15 Kang Q, Yu Y, Pei X, Hughes R, Heck S, Zhang X *et al.* Cytoskeletal protein 4.1R negatively regulates T-cell activation by inhibiting the phosphorylation of LAT. *Blood* 2009; **113**:6128–37.

16 Gascard P, Lee G, Coulombel L, Auffray I, Lum M, Parra M *et al.* Characterization of multiple isoforms of protein 4.1R expressed during erythroid terminal differentiation. *Blood* 1998; **92**:4404–14.

17 Hou VC, Conboy JG. Regulation of alternative pre-mRNA splicing during erythroid differentiation. *Curr Opin Hematol* 2001; **8**:74–9.

18 Gauthier E, Guo X, Mohandas N, An X. Phosphorylation-dependent perturbations of the 4.1R-associated multiprotein complex of the erythrocyte membrane. *Biochemistry* 2011; **50**:4561–7.

19 Shi ZT, Afzal V, Coller B, Patel D, Chasis JA, Parra M *et al.* Protein 4.1R-deficient mice are viable but have erythroid membrane skeleton abnormalities. *J Clin Invest* 1999; **103**:331–40.

20 Marfatia SM, Leu RA, Branton D, Chishti AH. Identification of the protein 4.1 binding interface on glycophorin C and p55, a homologue of the *Drosophila* discs-large tumor suppressor protein. *J Biol Chem* 1995; **270**:715–9.

21 Liu X, Zhou Q, Ji Z, Fu G, Li Y, Zhang X *et al.* Protein 4.1R attenuates autoreactivity in experimental autoimmune encephalomyelitis by suppressing CD4$^+$ T cell activation. *Cell Immunol* 2014; **292**:19–24.

22 Conboy J. The role of alternative pre-mRNA splicing in regulating the structure and function of skeletal protein 4.1. *Proc Soc Exp Biol Med* 1999; **220**:73–8.

23 Perez-Villar JJ, Whitney GS, Sitnick MT, Dunn RJ, Venkatesan S, O'Day K *et al.* Phosphorylation of the linker for activation of T-cells by Itk promotes recruitment of Vav. *Biochemistry* 2002; **41**:10732–40.

24 Vivar OI, Masi G, Carpier JM, Magalhaes JG, Galgano D, Pazour GJ *et al.* IFT20 controls LAT recruitment to the immune synapse and T-cell activation *in vivo*. *Proc Natl Acad Sci U S A* 2016; **113**:386–91.

25 Zhang W, Trible RP, Zhu M, Liu SK, McGlade CJ, Samelson LE. Association of Grb2, Gads, and phospholipase Cγ1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T cell antigen receptor-mediated signaling. *J Biol Chem* 2000; **275**:23355–61.

26 Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG *et al.* SH2 domains recognize specific phosphopeptide sequences. *Cell* 1993; **72**:767–78.