B and T Lymphocyte Attenuator is a Target of miR-155 during Naive CD4$^+$ T Cell Activation

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ABSTRACT

Background: MicroRNA-155 (miR-155) is upregulated during T cell activation, but the exact mechanisms by which it influences CD4$^+$ T cell activation remain unclear. **Objective:** To examine whether the B and T lymphocyte attenuator (BTLA) is a target of miR-155 during naïve CD4+ T cell activation. **Methods:** Firefly luciferase reporter plasmids pEZX-MT01-wild-type-BTLA and pEZX-MT01-mutant-BTLA were constructed. Lymphocytes were nucleofected with miR-155 inhibitor or negative control (NC). Then, naïve CD4+ CD62L+ helper T cells purified from lymphocytes were stimulated with immobilized antibody to CD3 and soluble antibody to CD28. miR-155 and BTLA expression were examined by real-time RT-PCR. Cell surface CD69 expression and IL-2 secretion were measured by ELISA and flowcytometry, respectively. **Results:** Luciferase reporter assay showed that miR-155 targeted the BTLA 3'UTR region. Compared with non-stimulated condition, both miR-155 and BTLA mRNA expression were upregulated after T cell activation. Similar results were observed for BLTA protein expression. Compared with NC, the miR-155 inhibitor decreased miR-155 by about 45%, but did not influence BTLA mRNA expression. Compared with NC, the miR-155 inhibitor decreased the surface BTLA expression by about 60%. Upregulation of BTLA in miR-155 knockdown CD4$^+$ T cells did not influence the cell surface expression of CD69, an early activation marker (p=0.523). Similarly, IL-2 production was not changed. **Conclusion:** miR-155 is involved in the inhibition of BTLA during CD4$^+$ T cell activation. These results might serve as a basis for an eventual therapeutic manipulation of this pathway to treat inflammatory and autoimmune diseases.

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INTRODUCTION

MicroRNAs have recently been identified as important post-transcriptional regulators of gene expression by repressing translation or inducing mRNA degradation in mammalian cells (1,2). As a typical multifunctional microRNA, miR-155 is involved in a number of immunological processes including immune cell activation, differentiation, and immune homeostasis (3). Upon activation, miR-155 is expressed in several types of immune cells including B cells (4), T-cells (5,6), macrophages (7,8), and dendritic cells (4,9), indicating that it plays an important role in the activation of these cells. The crucial role of miR-155 in both T- and B-cell responses has been demonstrated by transgenic mice deficient in bic/miR-155 (4,10). Of interest, the splenocytes from these mice fail to produce significant amounts of IL-2 and IFN-γ upon T cell stimulation (4); IL-2 is mainly secreted by T cells upon activation, indicating that T cell activation was inhibited in bic/mir-155-deficient mice (4). Knockout of miR-155 might reinforce some inhibitory signals or molecules, which subsequently inhibit T cell activation. However, the detailed mechanisms by which miR-155 influences CD4+ T cell activation remain unclear.

In this study, the prediction programs TargetScan, miRanda, and Pictar were used to identify the potential targets of miR-155. According to miRanda and TargetScan, among the potential targets of miR-155, B and T lymphocyte attenuator (BTLA) has putative miR-155-binding elements in the 3' untranslated regions (UTRs). These binding elements were attractive because they have been identified to play a negative role during T cell activation (11-13). These data strongly suggest that BTLA might be a target of miR-155.

BTLA is a protein that is induced during the activation of T cells (13). It has been reported that the cross-linking of BTLA and the T cell receptor (TCR) on murine T cells leads to T cell proliferation and IL-2 production, and T cell activation is enhanced in BTLA-deficient mice (13-15). Moreover, T cell activation through TCR activation leads to the upregulation of BTLA (16,17). T cell proliferation is inhibited after the activation of BTLA (18). BTLA activation inhibits the function of human CD8+ cancer-specific T cells (19). Therefore, the aim of this study was to validate if BTLA was a target of miR-155, and to observe the effects of modulating BTLA by miR-155 on the activation of CD4+ T cells.

MATERIALS AND METHODS

Animals. Male BALB/c mice (6-8 weeks) were obtained from the experimental animal center of the Second Military Medical University (Shanghai, China). All animals were subjected to a 12-h dark-light cycle at 23°C and provided with water and food ad libitum. All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Second Military Medical University.

Plasmid Construction. Full-length 3'UTRs of the mouse BTLA gene (Gene Accession: NM_001037719.2) were amplified from mouse genomic DNA. The primers are shown in Table 1. Then, they were cloned downstream of the firefly luciferase gene in the pEZX-MT01 expression vector system (GeneCopoeia, Rockville, MD, USA),

which contains both the firefly luciferase and Rennila luciferase genes. The region of the mouse BTLA mRNA 3'UTR was predicted to be the target of miR-155 seeds region using the prediction programs miRanda and TargetScan. Then, the wild-type (WT) BTLA 3'UTR luciferase vector was mutated in the putative miR-155 binding site using the QuikChange Site-Directed Mutagenesis kit (Stratagene, Wilmington, DE, USA), with the primers presented in Table 1. PCR products were sequenced to validate the mutation.

Table 1. Primers used in the present study.

Primer	Forward (5'->3')	Reverse (5'->3')
Plasmid construction		
3'UTRs of mourse BTLA	aac ggt acc GGA ATC CAA GAC AGG AAA ACA ACA T	aag ctc tcg agG TGA CAA TCT TAA AGT TCA AGG AAA TCA T
Mutation primers		
miR-155 binding site	AAA TAA ATT TGT TCC TCG TAA TTT TTC ATG GTT ACC T	ATT ACG AGG AAC AAA TTT ATT TGA GGA GCT
Real-time PCR		
miR-155	CTC GTG GTT AAT GCT AAT TGT GA	GTG CAG GGT CCG AGG T
U6	CTC GCT TCG GCA GCA CA	AAC GCT TCA CGA ATT TGC GT
BTLA	TTC AGT AAC CAT CCA TGT GAC AG	AAG CAA GGT GTA AAG CAG CCA
GAPDH	GTG CAG TGC CAG CCT CGT CC	CGC AGC ACA GTC ACC CGG AC

3'UTR Luciferase Reporter Assays. Reporter assays were performed using HEK293 T cells, as previously described (20). In brief, HEK293T cells were cotransfected with 200 ng of the empty plasmid (pEZX-MT01), pEZX-MT01-WT BTLA 3'UTR, or pEZX-MT01-mutant BTLA 3'UTR, and 20 nM of miR-155 mimics (dsRNA oligonucleotides) or negative control (NC) (GenePharma, Shanghai, China) with Fugen HD transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 hours, luciferase activities were

measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The transfection efficiency was normalized based on Renilla luciferase activity.

Transfection of Mouse Lymphocytes. Mice were sacrificed by cervical dislocation. The spleen was obtained and disaggregated into single cell suspension. Lymphocytes were obtained by EZ-Sep^{TM} Mouse Lymphocyte Separation Medium (Dakewe Biotech Co., Ltd., Beijing, China). Lymphocytes (1×10^7 cells/well) were transfected with 2 μM of miR-155 inhibitor or 2 μM of miR-155 inhibitor negative control (NC) (GenePharma, Ltd, Shanghai, China) using the VPA-1006 Mouse T Cell Nucleofector Kit (X-001, AMAXA, Cologne, Germany). The miR-155 inhibitor NC Fam was used to assess transfection efficiency.

CD4+ CD62L+ Helper T Cell Purification and Activation. Mice were sacrificed by cervical dislocation. The spleen was obtained and disaggregated into single cell suspension. Lymphocytes were obtained by EZ-Sep^{TM} Mouse Lymphocyte Separation Medium (Dakewe Biotech Co., Ltd., Beijing, China). Naïve CD4+ CD62L+ helper T cells were purified by magnetic cell sorting (MACS) Using a mouse CD4+CD62L+ T Cell Isolation Kit II (Miltenyi Biotec, 130-093-227) from lymphocytes after being transfected with miR-155 inhibitor or miR-155 inhibitor negative control (NC) for 16 h, according to the manufacturer`s instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the CD4+ CD62L+ helper T cells was >95% according to flowcytometry.

Sorted cells were stimulated with anti-CD3 (10 μg/mL; 145-2C11, immobilized; eBioscience, San Diego, CA, USA) and anti-CD28 (2 μg/mL; 37.51, soluble; eBioscience, San Diego, CA, USA) antibodies for 24 hours.

Flowcytometry. Surface staining was done in activated CD4+ T cells by anti-CD3 and anti-CD28 stimulation for 24 h with the corresponding fluorescence-labeled surface antibodies. The following antibodies were used for immunolabeling: CD4-FITC, BTLA(8F4)-PE, CD69-PE, CD62L-(PE) (Biolegend, San Diego, CA, USA). Stained cells were analyzed using a FACSCalibur flowcytometer with the Cellquest software (BD Biosciences, Franklin Lake, NJ, USA). Isotype controls were used. Dead cells were excluded from analysis based on low forward-light scatter by gating on cells within the lymphocyte scatter.

Real-Time RT-PCR. Total RNA was extracted from activated CD4+ T cells by anti-CD3 and anti-CD28 antibodies for 24 h using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA purity and concentration was determined by spectrophotometry. The RNA was used to prepare cDNA using the ReverTra Ace qPCR RT Kit (FSQ-101; TOYOBO Co., Ltd, Tokyo, Japan). Real-time PCR analysis was performed using the SYBR Green Realtime PCR Master Mix (QPK-201; TOYOBO Co., Ltd, Tokyo, Japan) in a LightCycler Real-Time PCR System (Roche Applied Science, Penzberg, Germany).

The primers for miRNA analysis are presented in Table 1. The relative expression level of miRNAs and mRNA was normalized to that of internal control U6 and GAPDH, respectively, using the $2^{-\Delta\Delta Ct}$ cycle threshold method.

ELISA. CD4+ CD62L+ helper T cells were seeded onto 24-well plates (0.5 mL of 5×10^5 cells/mL), and activated by anti-CD3 and anti-CD28 antibodies for 24 h. Mouse IL-2 in culture supernatants of CD4+ CD62L+ helper T cells was quantified by ELISA (eBioscience, San Diego, CA, USA). The assay was standardized with recombinant murine IL-2, and detection ranged from 2 to 200 pg/mL.

Statistical Analysis. Statistical analysis was performed using STATA 11.0 (Stata Corporation, College Station, TX, USA). Data are expressed as mean ± standard deviation (SD) from the three independent experiments. Statistical significance was evaluated by student's *t*-test. Two-sided p values <0.05 were considered statistically significant.

RESULTS

miR-155 Targets BTLA through 3'UTR Interaction. The prediction programs miRanda and TargetScan showed that there were putative miR-155-binding elements (7 mer-m8) in BTLA 3'UTRs (Figure 1A). The empty, WT, or mutant reporter plasmids containing BTLA 3'UTRs with miR-155 mimics or NC were transfected into HEK293T cells. Results showed that miR-155 mimics markedly decreased the luciferase activity of the WT BTLA 3'UTR vector compared with miR-155 mimic control ($p<0.01$), while it failed to repress the BTLA 3'UTR containing a mutated miR-155 seed sequence (Figure 1B), indicating that miR-155 may directly target the BTLA 3'UTR and inhibit expression.

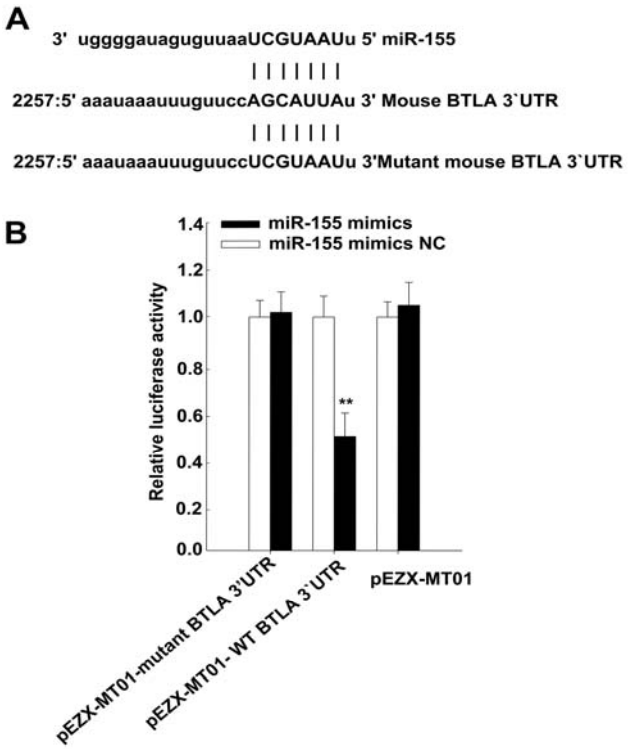

Figure 1. The luciferase reporter assay identified BTLA as a direct target of miR-155. (A) The 3'UTR region of the mouse BTLA mRNA was predicted to be the target of miR-155 using the prediction programs miRanda and TargetScan. (B) The luciferase reporter assays were performed in HEK-293T cells cotransfected with 200 ng of the empty plasmid (pEZX-MT01), pEZX-MT01-wild-type (WT) BTLA 3'UTR, or pEZX-MT01-mutant BTLA 3'UTR, and 20 nM of either miR-155 mimics or miR-155 mimics negative control (NC) for 24 h. Firefly luciferase activity was normalized to the activity of Renilla luciferase. Data are shown as mean ± standard deviation (SD) from three independent experiments. ***P*<0.01 *vs.* miR-155 mimics NC.

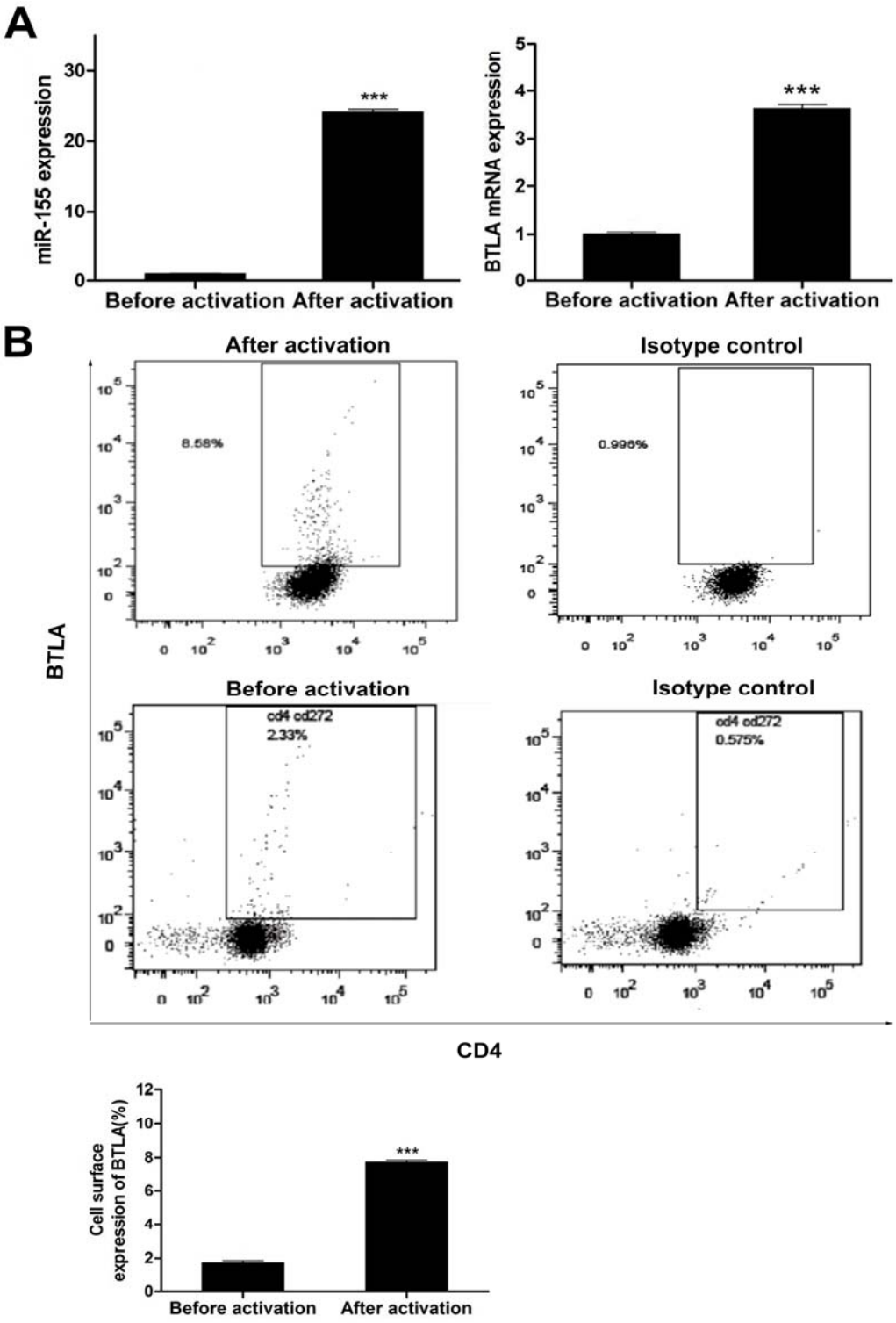

Figure 2. miR-155 and BTLA expression in activated CD4+CD62L+ helper T cells. Naïve CD4+ CD62L+ helper T cells were stimulated with immobilized antibody to CD3 (10 µg/ml) and soluble antibody to CD28 (2 µg/ml) for 24 h. (A) miR-155 and BTLA mRNA expressions were determined by real-time RT-PCR. U6 and GAPDH were used as controls. (B) Cell surface expression of BTLA was determined by flowcytometry. ***P<0.001 *vs.* before activation.

Enhanced Expression of BTLA on Naïve CD4+ T Cells before and after anti-CD3 and anti-CD28 Stimulation. Whether BTLA is regulated by miR-155 under physiological conditions was evaluated. miR-155 and BTLA mRNA and protein expression in transfected mouse CD4+ T cells was measured by real-time quantitative RT-PCR and flowcytometry before and after activation. Compared with before activation, both miR-155 and BTLA mRNA expression were upregulated after activation (both $p<0.001$) (Figure 2A). Similar results were observed for BLTA protein expression (Figure 2B).

Enhanced Expression of BTLA on miR-155 knockdown Naïve CD4+ T Cells Following anti-CD3 and anti-CD28 Stimulation. Flowcytometry showed that the transfection was successful (Figure 3A). Compared with the NC control, the miR-155 inhibitor decreased miR-155 by about 45% ($p<0.05$), but did not influence the mRNA expression of BTLA (Figure 3B). Compared with the NC control, the miR-155 inhibitor decreased the surface BTLA expression by about 60% ($p<0.01$) (Figure 3C).

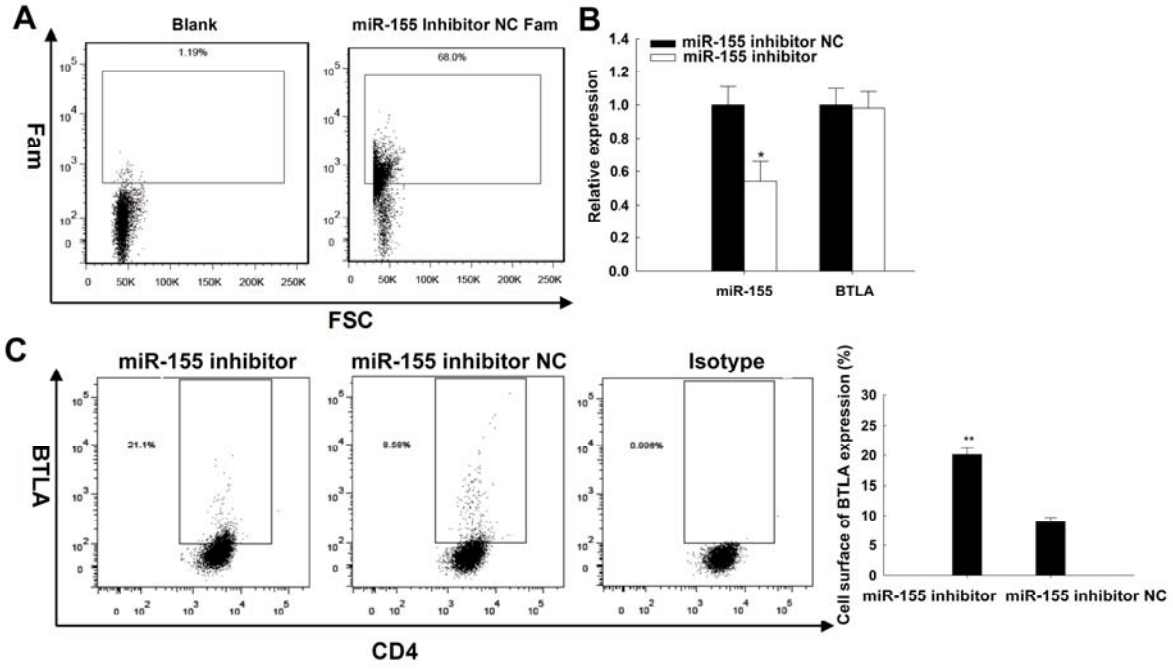

Figure 3. Knockdown of miR-155 resulted in upregulated surface BTLA expression levels in activated CD4+ CD62L+ helper T cells. Lymphocytes were nucleofected with 2 μM miR-155 inhibitor or 2 μM miR-155 inhibitor negative control (NC). Then, naïve CD4+ CD62L+ helper T cells were purified by magnetic cell sorting from lymphocytes, and stimulated with immobilized antibody to CD3 (10 μg/ml) and soluble antibody to CD28 (2 μg/ml) for 24 h. (A) Transfection efficiency for CD4+ CD62L+ T cells was measured by flowcytometry analysis for FAM expression. (B) Relative expressions of miR-155 and BTLA were measured by real-time RT-PCR. U6 and GAPDH were used as inner controls. (C) Cell surface BTLA expression was detected by flowcytometry. Dot plots represent gated $CD4^+$ T cells. Data are shown as mean ± SD. **P*<0.05, ***P*<0.01 *vs*. miR-155 inhibitor NC.

MiR-155 Knockdown in Naïve CD4$^+$ T Cells does not Affect CD69 Expression and IL-2 Level. The significance of upregulated BTLA by down-regulation of miR-155 expression was investigated. IL-2 levels were measured in the supernatant of stimulated naïve CD4+ T cells in both groups. Upregulation of BTLA in miR-155 knockdown CD4+ T cells did not influence the cell surface expression of CD69, an early activation marker (p=0.523) (Figure 4A). Similarly, IL-2 production was not changed (Figure 4B).

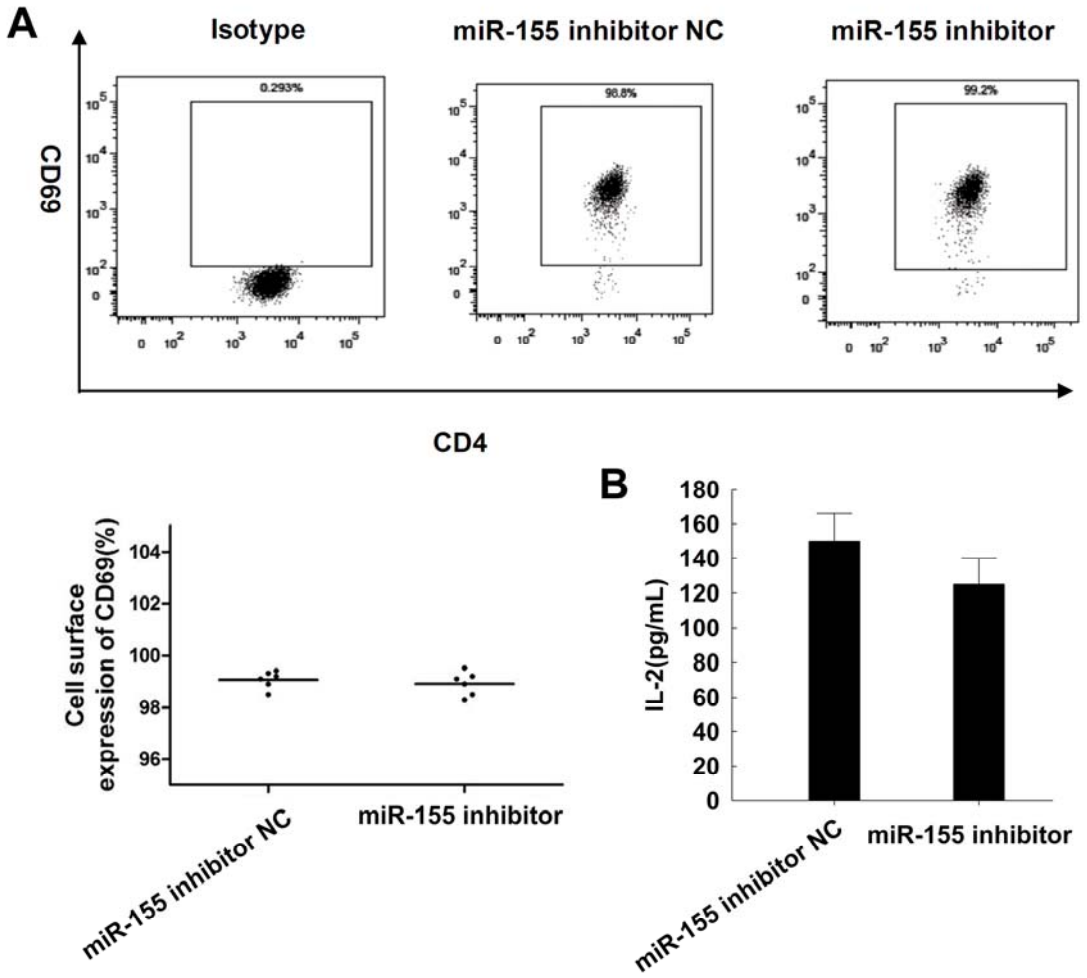

Figure 4. Knockdown of miR-155 do not influence CD69 expression and IL-2 levels in activated CD4+ CD62L+ helper T cells. Lymphocytes were nucleofected with 2 μM miR-155 inhibitor or 2 μM miR-155 inhibitor NC. Then, naïve CD4+ CD62L+ helper T cells were purified by magnetic cell sorting from lymphocytes, and stimulated with immobilized antibody against CD3 (10 μg/ml) and soluble antibody against CD28 (2 μg/ml) for 24 h. (A) Cell surface CD69 expression was assayed by flowcytometry. (B) IL-2 levels in the culture supernatant were measured by ELISA. Data are shown as mean ± SD. No significant differences were found between the miR-155 inhibitor group and miR-155 inhibitor NC group.

DISCUSSION

miR-155 is a crucial microRNA that is upregulated during T cell activation, but the exact mechanisms by which it influences CD4+ T cell activation remains unclear. This study aimed to examine whether BTLA is a target of miR-155 during naïve CD4+ T cell activation. Results showed that miR-155 targeted the BTLA 3'UTR region. Compared with non-stimulated condtion, both miR-155 and BTLA mRNA expression were upregulated after T cell activation and similar results were observed for BTLA protein expression. Compared with NC controls, the miR-155 inhibitor decreased miR-155 by about 45%. Compared with the NC control, the miR-155 inhibitor decreased the surface BTLA expression by about 60%. Upregulation of BTLA in miR-155 knockdown CD4+ T cells did not influence the cell surface expression of CD69, an early activation marker. Similarly, IL-2 production was not changed. Therefore, this study suggests that miR-155 is involved in the inhibition of BTLA during CD4+ T cell activation.

As reported previously, miR-155 plays an important role in CD4+ T cell activation; knockout or knockdown of miR-155 results in impaired T cell response (4,6). With similar structure and function to CTLA-4 and PD-1, BTLA is a polymorphic molecule that can attenuate BCR- and TCR-mediated signaling, and thereby functions as a negative regulator of lymphocyte activation (12,18,21,13,22,23). This study might help explaining why bic/miR-155 knockout mice show decreased B and T cell responses upon stimulation (4,10) and understanding the critical role of miR-155 in the adaptive immune response.

This present study strongly suggests that miR-155 may enhance $CD4^+$ T cell activation through, at least partly, inhibiting BTLA expression at the post-transcriptional level. Therefore, like the miR-155-CTLA-4 axis (24), the miR-155-BTLA axis may play an important role in T cell proliferation. It can also account for decreased production of IL-2 by splenocytes from bic/miR-155 knockout mice upon T cell stimulation *in vitro*. However, in this present study, naïve CD4+ T cells, not splenocytes, were stimulated with anti-CD3 and anti-CD28 antibodies. BTLA needs to bind with its ligand and CD4+ T cells have no BTLA ligands. Therefore, no inhibitory effect of BTLA could be observed against $CD4^+$ T cell activation in this study. This is probably why miR-155 knockdown-induced BTLA increase resulted in no significant decrease of IL-2 in CD4+ T cells. To investigate the effect of BTLA on IL-2 production, BTLA interference experiment should be performed in future studies. Previous studies have shown that miR-155 deficiency in Treg cells results in increased suppressor of cytokine signaling 1 (SOCS1) expression accompanied by impaired activation of signal transducer and decreased IL-2 production (6). These results indicated that miR-155 may have many mRNA targets in different cell types in the immune system and cancer cells (25). Additional studies are necessary to examine these targets and mechanism.

miR-155 has been shown to play different roles in different physiological situations. The analysis of its specific targets such as SMAD5 in DLBCL (26), AID (27,28) in B lymphocytes, SOCS1 (6) in Treg cells, MyD88 (29) in AGS cells (human gastric cancer cell line), and SHIP1 (30,31) in macrophages, suggests that miR-155 functions in a context-dependent manner. Since BTLA is broadly expressed on hematopoietic cells including $CD4^+$ T cells, $CD8^+$ T cells, B cells, dendritic cells, macrophages, and NK cells (16,17), BTLA is probably a universal target of miR-155 in the immune system. The miR-155-BTLA axis may play an important role in both innate and adaptive immune responses. For example, miR-155 and BTLA may both play important roles in autoimmune diseases.

Mir155-/- mice are highly resistant to experimental autoimmune encephalomyelitis (EAE) (30,32,7), and BTLA-deficient mice have increased specific antibody responses and enhanced sensitivity to EAE (13).

This study is not without limitations. First, it was performed in mouse cells and additional studies are necessary in humans. Second, it was performed only in CD4+ cells and future studies should also focus on other immune cell types, including B cells and dendritic cells. Finally, this study only superficially explored the mechanisms involved in the miR-155-BTLA axis. Additional studies are necessary to address these issues.

In conclusion, this study suggests that miR-155 is involved in the inhibition of BTLA during naïve CD4+ T cell activation. These results might serve as a basis for an eventual therapeutic manipulation of this pathway to treat inflammatory and autoimmune diseases.

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