Molecular Immunology 103 (2018) 133–143

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

CD8 + iTregs attenuate glomerular endothelial cell injury in lupus-prone mice through blocking the activation of p38 MAPK and NF-κB

Ya Liu[1], Weijuan Deng[1], Qiaoyun Meng, Xiaonan Qiu, Dong Sun*, Chun Dai*

Department of Nephrology, Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu, China

ARTICLE INFO

Keywords:
CD8 + iTregs
Glomerular endothelial cell
Lupus nephritis
Inflammatory mediators
Monocyte adhesion

ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic inflammatory disease. Endothelial cell injury plays an important role in the inflammatory processes associated with SLE. CD4 + Foxp3 + regulatory T cells (Tregs) reduce the injury to endothelial cells induced by inflammatory factors. As a newly identified regulatory T cell, we previously reported that CD8 + CD103 + iTregs had similar effects to those of CD4 + iTregs in the process of immunoregulation. In this paper, we further explored the effect and mechanism of CD8 + iTregs on endothelial cell injury. The expressions of vascular cellular adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in MRL/lpr mouse glomerular endothelial cells (lupus-MGECs) were estimated by quantitative real-time polymerase chain reaction, enzyme-linked immunosorbent assay and Western blotting. The lupus-MGEC apoptosis rate was detected by flow cytometry and the adhesion of monocyte-like cells to lupus-MGECs exposed to lipopolysaccharide (LPS) was determined by the adhesion assay. Additionally, the expressions of P-p38, P-NF-κB and P-IκBα were detected by Western blotting. The results showed that LPS increased the expressions of VCAM-1, ICAM-1, IFN-γ, TNF-α, IL-6 and MCP-1 in lupus-MGECs, while CD8 + iTregs significantly decreased the levels of these adhesion molecules and inflammatory mediators. Furthermore, CD8 + iTregs alleviated lupus-MGEC apoptosis and inhibited the adhesion of monocyte-like cells to lupus-MGECs. Both nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinase (MAPK), activated by LPS, were suppressed by CD8 + iTregs. These findings suggest that CD8 + iTregs attenuate LPS-induced glomerular endothelial cell injury through blocking the activation of p38 MAPK and NF-κB in lupus-MGECs. The protective effect of CD8 + iTregs indicates their possible therapeutic application in Lupus nephritis.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by widespread microvascular injury to kidneys, skin, brain, joints and other organs (Isenberg et al., 1998; Belmont et al., 1996). Lupus nephritis (LN) may occur in as many as 60% of patients with SLE (Saxena et al., 2011). Although the therapeutic approaches have been improved in recent years, many LN patients eventually progress to end-stage renal disease. A common pathological feature of LN is glomerular injury accompanied by aberrant inflammatory responses (Saxena et al., 2011). As part of these inflammatory responses, large numbers of leukocytes are recruited to affected tissues. Compelling evidence suggests that leukocytes in the circulation must undergo a sequence of interactions with the endothelial surface to enter the sites of inflammation, first tethering and rolling on the endothelial surface, then undergoing arrest on the endothelium, and finally migrating into the affected site (Norman and Hickey, 2005). Leukocyte migration into glomeruli is a typical feature of glomerulonephritis. Following the release of inflammatory mediators, leukocytes are key effector cells in glomerular injury, especially in immune-mediated glomerulonephritis (Brady, 1994; Kumar et al., 2013). These mechanisms may also be critical for the histogenesis of glomerular lesions in LN. Endothelial cell adhesion molecules (ECAMs), including vascular cellular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), are transmembrane proteins belonging to the immunoglobulin superfamily (Collins et al., 1995). ECAMs are important for the transmigration of immune cells from the circulation to tissues undergoing inflammatory processes. Previous studies show increased serum and urine levels of VCAM-1 and ICAM-1 in patients with active SLE, particularly in those with diffuse proliferative LN (Lewis et al., 2016; Molad et al.,

* Corresponding authors.
E-mail addresses: 767946775@qq.com (D. Sun), 13615133736@163.com (C. Dai).
[1] These two authors contributed equally to this work.

https://doi.org/10.1016/j.molimm.2018.09.006
Received 26 April 2018; Received in revised form 1 September 2018; Accepted 9 September 2018

2002; Singh et al., 2012; Spronk et al., 1994). Moreover, the expression of vascular endothelial adhesion molecules in glomeruli is a typical finding on the histopathology of glomerular lesions in MRL/*lpr* mice (Nakatani et al., 2004).

Regulatory T cells (Tregs) play important roles in the homeostasis of immune tolerance, and mainly consist of naturally occurring Tregs (nTregs) and induced Tregs (iTregs) (Zheng et al., 2013; Sharafieh et al., 2011). Studies have shown that Tregs exhibit inhibitory functions through a variety of mechanisms, including the secretion of inhibitory cytokines (Choi et al., 2012; Zheng et al., 2008a, b), cytotoxic effects (Chen et al., 2003), and their participation in the genesis and development of autoimmune diseases by modulating the maturation and function of antigen presenting cells (Tarella et al., 2013). Therefore, the role of Tregs in SLE or LN has attracted increasing attention, with multiple studies showing that nTregs isolated from the peripheral blood of SLE patients are reduced and dysfunctional (Liu et al., 2004; Valencia et al., 2007; Huehn et al., 2004; Miyara et al., 2005). Similar results have been observed in lupus mice models (Tarella et al., 2013; Monk et al., 2005). Furthermore, the glomerulonephritis in lupus mice can be prevented by *in vitro* expanded nTregs, and the development of LN is alleviated (Scalapino et al., 2006a). Significantly, our previous research reported that the infusion of CD8 + CD103 + iTregs to lupus mice has a potent therapeutic effect on LN (Zhong et al., 2018). However, the exact mechanism of CD8 + iTregs in LN is still unclear. Further investigations are needed to determine how iTregs exert their protective role in LN.

Studies have shown that Tregs protect against lipopolysaccharides (LPS), OX-LDL, and fine particulate matter-induced inflammatory responses in human umbilical vein endothelial cells (HUVEC) (He et al., 2010; Zhang et al., 2014), which reveals a critical role of Tregs in endothelial atheroprotective effects. To determine whether CD8 + iTregs could delay the progression of LN by preventing the injury of glomerular endothelial cells, the adhesion molecules and inflammatory mediators of MRL/lpr mice glomerular endothelial cells (lupus-MGECs) were detected with or without CD8 + iTreg treatment.

In this paper, we report that the stimulation of LPS led to the increased expressions of VCAM-1, ICAM-1 and inflammatory mediators in lupus-MGECs, which was due to increased phosphorylation of p38 MAPK and NF-κB. In contrast, CD8 + iTregs attenuated the expressions of adhesion molecules and inflammatory mediators in lupus-MGECs following LPS induction by inhibiting the phosphorylation of NF-κB and p38 MAPK. In short, CD8 + iTregs prevented lupus-MGEC injury through blocking the activation of p38 MAPK and NF-κB.

2. Methods

2.1. Mice

We purchased four to six week-old female MRL/*lpr* mice from the SLRC Laboratory (Shanghai, China). All mice were housed under specific pathogen-free conditions and kept under a 12 h light/dark cycle with controlled humidity (60%–80%) and temperature (22 ± 1°C). Experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Xuzhou Medical University.

2.2. Flow cytometry

Fluorescent mouse antibodies (Abs), such as CD4, CD8, CD11c, CD45R/B220, CD103 and CD62 L from Biolegend (San Diego, CA, USA), Foxp3, from eBioscience (San Diego, CA, USA) and CD3 from BD (Biosciences, San Diego, CA, USA), were used for flow cytometry analysis. Cell subsets were stained with mAbs and isotype control as indicated above and analyzed on the BD LSRFortossa™ flow cytometer (BD, Biosciences) using the FACSDiva Software (BD, Biosciences). For intracellular Foxp3 staining, cells were fixed and permeabilized using the Fixation and Permeabilization Solution (eBioscience). Finally, pseudo-color images were obtained by using the FlowJo Software (Tree Star, Ashland, OR, USA).

2.3. Generation of CD8 + med and CD8 + iTregs ex vivo

First, enriched and non-adherent T cells were prepared after the spleen cells of MRL/*lpr* mice were passed through a nylon wool column, and the enriched T cells were then incubated with the following antibodies in appropriate amounts: PE anti-mouse CD4, PE anti-mouse B220, and PE anti-mouse CD11c. Thereafter, T cells were treated with an appropriate amount of anti-mouse PE beads (Miltenyi Biotec, Germany), and the autoMACSPro Separator (Miltenyi Biotec) was used to collect cells in the negative selection channel, namely CD8 + T cells, whose purity was greater than 95% detected by flow cytometry. These CD8 + T cells were then mixed with anti-mouse CD62 L beads (Miltenyi Biotec) and positively selected by the auto MACS separator to obtain CD8 + CD25-CD62L + naive T cells, whose purity was greater than 97% detected by flow cytometry. Cells were subsequently cultured in 48 or 96-well plates and stimulated with plate-bound anti-mouse CD3 (1 mg/ml, Biolegend), soluble anti-mouse CD28 (1 mg/ml, Biolegend) and IL-2 (100 U/ml, R&D systems, San Diego, CA, USA) in combination with (CD8 + iTregs) or without (CD8 + med) TGF-β1 (2 ng/ml, R&D Systems) for 3 days. A total of 100 U/ml IL-2 was renewed on day 2. RPMI 1640 medium supplemented with 100 U/ml penicillin (Gibco, Carlsbad, CA, USA), 100 mg/mL streptomycin (Gibco), 10 mM HEPES (Gibco) and 10% heat-inactivated fetal bovine serum (FBS; Gibco) was used for all cultures. Foxp3 expression was determined by flow cytometry.

2.4. Isolation and culture of primary lupus-MGECs

Whole kidneys from MRL/*lpr* mice were surgically removed under sterile conditions. Then, we removed the connective tissue, peeling the renal capsule, removed the medulla with ophthalmic scissors, cut the cortex, and digested at 37 °C for 30–40 min. Immunofluorescent staining of Von Willebrand factor (vWF, Abcam, Cambridge, MA, USA), which is specifically expressed in blood vessel endothelial cells, was performed to identify lupus-MGECs. The results revealed that nearly 100% of isolated cells were vWF positive (Supplementary Figure S1A). Primary lupus-MGECs were then cultured in endothelial cell basal medium (ECM) (ScienCell, San Diego, CA, USA) with 10% FBS and 1% penicillin/streptomycin. For LPS (Sigma, St. Louis, MI, USA) treatment experiments, lupus-MGECs grown to 70–80% confluency on 6-well plates (0.5×10^6 cells per well) were washed once with phosphate-buffered saline (PBS, Suolaibao, Beijing, China) and cultured in serum-free medium for 12 h for synchronization, after which 10 ug/ml LPS were added. Finally, cells were harvested for a series of analyses. p38 MAPK inhibitor, SB203580, and NF-κB inhibitor, SN50, were purchased from MedChemExpress (New Jersey, USA). All experiments were performed using lupus-MGECs at passages 3–5. All cultures were incubated at 37°C and 5% CO_2.

2.5. Co-culture of CD8 + iTregs and lupus-MGECs

Lupus-MGECs grown to 70–80% confluency on 6-well plates (0.5×10^6 cells per well) were washed once with PBS and cultured in serum-free medium for 12 h for synchronization. Then, non-adherent cells were removed, and the culture medium was changed. For co-culture experiments, lupus-MGECs were cultured without T cells, and with CD8 + iTregs (2.5×10^5 cells per well, after 3 days *in vitro* induction) or CD8 + med (2.5×10^5 cells per well, after 3 days *in vitro* induction) for 24 h, and then with LPS (10 ug/mL) for an additional 24 h. In the control group (CTL), lupus-MGECs were grown in serum-free medium for 24 h. After the incubation period, floating T cells were aspirated, lupus-MGECs were harvested, and supernatants were collected for further experiments.