Molecular Immunology 87 (2017) 188–199

Contents lists available at ScienceDirect

Molecular Immunology

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

TLR4 supports the expansion of FasL$^+$CD5$^+$CD1d^{hi} regulatory B cells, which decreases in contact hypersensitivity

Keng Wang[a,b,c,1], Lei Tao[a,b,1], Jianbing Su[a,b], Yueyang Zhang[a,b], Binhua Zou[a,b], Yiyuan Wang[a,b], Min Zou[b], Nana Chen[a,b], Linsheng Lei[a,b], Xiaojuan Li[a,b,*]

[a] Laboratory of Anti-inflammatory Immunomodulatory Pharmacology, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, PR China
[b] Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, PR China
[c] Department of Clinical Pharmacy, The Affiliated Nanhai Hospital of Southern Medical University, Foshan 528200, PR China

ARTICLE INFO

Keywords:
Regulatory B cell
FasL
TLR4
TLR9
Anti-CD40
Contact hypersensitivity

ABSTRACT

Certain B cells termed as "regulatory B cells" (Bregs) can suppress the ongoing immune responses and a splenic CD5$^+$CD1d^{hi} Breg subset identified earlier was shown to exert its regulatory functions through secretion of IL-10. Though FasL expression is an alternative mechanism of immune suppression used by B cells, little is known about the FasL expressing CD5$^+$CD1d^{hi} Bregs. In this study, we isolated splenocytes or splenic CD19$^+$ B cells and compared the efficiency of toll-like receptor(TLR)4 ligand (lipopolysaccharide) with TLR9 ligand (CpG), anti-CD40 and TLR9 ligand (CpG) plus anti-CD40 on the FasL expression of splenic CD5$^+$CD1d^{hi} Bregs by flow cytometry. FasL expression in CD5$^+$CD1d^{hi} B cells was rapidly increased after TLR4 ligation. Intriguingly, anti-CD40 and CpG plus anti-CD40 combinations failed to stimulate FasL expression in CD5$^+$CD1d^{hi} B cells although the IL-10 production was up-regulated in this subset. In addition, LPS and other B10-cell inducers increased the expression of surface molecules like CD86 and CD25, which are correlated to the regulatory functions of B cells. Furthermore, NF-κB and NF-AT inhibitors decreased the TLR4-activated FasL expression in CD5$^+$CD1d^{hi} B cells. Then we sorted splenic CD5$^+$CD1d^{hi} Bregs using flow cytometry and found that TLR4-activated CD5$^+$CD1d^{hi} Bregs suppressed the proliferation of CFSE-labeled CD4$^+$ T cells *in vitro*, which was partly blocked by anti-FasL antibody. In oxazolone-sensitized mice having contact hypersensitivity, FasL expression in splenic CD5$^+$CD1d^{hi} B cells was decreased compared to the control group after TLR4 ligation. Our findings suggest that the regulatory function of CD5$^+$CD1d^{hi} B cells could be partly mediated by Fas-FasL pathway and this FasL expressing CD5$^+$CD1d^{hi} Bregs might participate in the regulation of inflammatory diseases.

1. Introduction

B cells produce antibodies, which are generally considered as positive regulators of immune responses. However, a suppressive role for B cells has been identified recently (Mauri and Ehrenstein, 2008). The suppressive capacity of Breg is ascribed mainly to their secretion of IL-10 (Lykken et al., 2015). In mice, two well-characterized IL-10 producing Breg subsets are identified until now (Matsushita, 2014; Yang et al., 2013): T2-MZP (Transitional 2-marginal zone precursor) B cell subsets with the phenotype of CD19$^+$CD23$^+$CD21^{hi}CD24^{hi}CD1d^{hi} (Evans et al., 2007) and "B10 cells" with the phenotype of CD19$^+$CD5$^+$CD1d^{hi} (Yanaba et al., 2008). Both of these two Breg subsets were identified from murine splenocytes. IL-10 competent CD19$^+$CD5$^+$CD1d^{hi} Bregs from wild-type (WT) mice can suppress Th1 responses and inhibit the exaggerated contact hypersensitivity (CHS) reactions (Yanaba et al., 2008), murine lupus (Watanabe et al., 2010), DSS-induced intestinal injury (Yanaba et al., 2011) and imiquimod-induced psoriasis-like skin inflammation (Yanaba et al., 2013) in IL-10 deficient CD19$^{-/-}$ mice. In addition, transfer of BAFF-induced IL-10 producing CD5$^+$CD1d^{hi} B cells suppressed collagen induced arthritis (Yang et al., 2010) and the transfer of myelin oligodendrocyte glycoprotein(33-35)-sensitized CD5$^+$CD1d^{hi} B10 cells into WT mice reduced the initiation of experimental autoimmune encephalomyelitis (EAE) dramatically but not the ongoing EAE disease (Matsushita et al., 2010).

Though IL-10 is pivotal for the regulatory function of CD5$^+$CD1d^{hi} B cells, in fact only a small portion of CD5$^+$CD1d^{hi} Breg subset secretes IL-10 (Yanaba et al., 2008). Interestingly, Bregs can also suppress immune responses in IL-10 independent manner (Floudas et al., 2016). One important alternative regulatory mechanism used by B cells is FasL

* Corresponding author at: Southern Medical University, 1838 Guangzhou Avenue North, Guangzhou 510515, PR China.
E-mail address: lixiaoj@smu.edu.cn (X. Li).
[1] Keng Wang and Lei Tao contributed equally to this work.

http://dx.doi.org/10.1016/j.molimm.2017.04.016
Received 3 November 2016; Received in revised form 18 April 2017; Accepted 23 April 2017

(CD178) (Lundy, 2009). After binding to Fas receptor (CD95), FasL could induce apoptosis in target cells such as $CD4^+$ T cells (Lundy and Fox, 2009). Splenic B cells activated by lipopolysaccharide (LPS) expressed FasL and delayed the onset of diabetes (Tian et al., 2001). Infection with *Schistosomas* induced $FasL^+$ B cells, which were enriched in $CD5^+$ B cell population (Lundy and Boros, 2002). The $FasL^+CD5^+$ B cells reduced in severe collagen-induced arthritis (Lundy and Fox, 2009). Moreover, B cells from FasL deficient mice could not transfer tolerance in a skin grafting model (Minagawa et al., 2004). In summary, $FasL^+$ B cells potentially played a role in down-regulating the immune responses and inflammatory immune diseases.

FasL expression was enriched in naïve $CD5^+CD1d^{hi}$ B cells but not $CD23^+CD21^{hi}$ T2-MZP B cells in murine splenocytes (Klinker et al., 2013). As previously described, $CD5^+CD1d^{hi}$ Bregs usually exert suppressive function after their activation. For example, adoptive transfer of $CD5^+CD1d^{hi}$ B cells purified from DNFB-sensitized mice altered DNFB but not oxazolone-induced CHS responses in $CD19^{-/-}$ recipients (Yanaba et al., 2008). TLR4 ligation (by LPS) is an innate signal required for the development and function of $CD5^+CD1d^{hi}$ Bregs producing IL-10 (Yanaba et al., 2009), while the effects of TLR4 ligand on FasL expressing $CD5^+CD1d^{hi}$ Bregs are not explored until now.

At present, we firstly observed the effects of LPS (TLR4 ligand) and other B10-cell inducers on FasL expression along with IL-10, CD86 and CD25 expressions in $CD5^+CD1d^{hi}$ Breg population. It is interesting to find that LPS can rapidly and potently up-regulate FasL expression in $CD5^+CD1d^{hi}$ Breg subset, while some other B10-cell inducers such as anti-CD40, CpG plus anti-CD40 signals fail to increase the FasL expression though the IL-10 and CD86 expressions are strongly increased in this subset. FasL expression in $CD5^+CD1d^{hi}$ Bregs activated by TLR4 ligation could be reduced by both NF-κB and NF-AT inhibitors. Furthermore, the regulatory function of $CD5^+CD1d^{hi}$ Bregs activated by TLR4 ligation was down-regulated by anti-FasL treatment *in vitro* and FasL expressing $CD5^+CD1d^{hi}$ Bregs were decreased in mice having contact hypersensitivity. Thus this study shows that FasL could partly mediate the regulatory function of $CD5^+CD1d^{hi}$ Bregs and TLR4 signals are required to generate splenic FasL expressing $CD5^+CD1d^{hi}$ Bregs, which indicates a possible role for these IL-10-independent Bregs in inflammatory diseases.

2. Materials and methods

2.1. Mice

Female C57BL/6 WT mice were purchased from Guangdong Medical Laboratory Animal Center. All the mice were bred in a specific pathogen-free barrier facility. All the animals were used between the ages of 8 and 12 weeks. Care, use and treatment of mice were in strict accordance with the Guide for the Care and Use of Laboratory Animals. Ethical approval was given by Southern Medical University Experimental Animal Ethics Committee.

2.2. Isolation of splenic lymphocytes

Spleens were dissected and mashed through nylon cell strainer under sterile conditions. Splenic lymphocytes were collected by mouse lymphocyte separation medium (DKW33-R0100, Dakewe Biotech Company). The trapped cells mainly consist of B cells and T cells. When necessary, the residual erythrocytes were eliminated using red cell lysis buffer (420301, Biolegend). $CD19^+$ B cells were purified through negative selection using a B cell isolation kit (19854, StemCell) according to the manufacturer's instructions with purity of > 95%.

2.3. Cell culture and stimulation

The splenocytes or isolated $CD19^+$ B cells were cultured with LPS (10 μg/ml, Escherichia coli serotype O111:B4, L4391, Sigma-Aldrich), CpG-ODN 1826 (50 nM, tlrl-1826, InvivoGen), anti-CD40 (2 μg/ml, 553721, BD Biosciences) or CpG plus anti-CD40 separately in 200 μl of complete medium in a 96-well flat bottom plate for 12 h, 24 h or 48 h for measuring surface FasL, CD86, CD25 and intracellular IL-10. For detection of intracellular cytokine IL-10 of B cell subsets, phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, P8139, Sigma-Aldrich), ionomycin (500 ng/ml, I0634, Sigma-Aldrich) and monensin (2 μM, 00-4505-51, eBioscience) were added to the culture for the terminal 5 h in addition to LPS or other B10-cell inducers. When treated with LPS in purified $CD19^+$ B cells, BAY11-7082 (2 μM; 1 μM, B5556, Sigma-Aldrich) and CsA (1 μM; 0.5 μM, Sigma-Aldrich) were used as NF-κB and NF-AT inhibitor respectively and added simultaneously with LPS for 24 h to detect IL-10 and FasL expression. For the detection of intracellular cytokine IFN-γ in $CD4^+$ T splenocytes in animal models, splenocytes were isolated from CHS and control mice, and stimulated with anti-CD3 and PIM (PMA, ionomycin and monensin) for 5 h.

2.4. Flow cytometric analysis

Cells were collected and washed with PBS supplemented with 0.5% (wt/vol) BSA. For the detection of cell-surface markers, cells were stained with different combinations of the following antibodies: PerCP-conjugated CD19 (551001), FITC-conjugated CD5 (553021), PE-conjugated CD1d (553846), APC-conjugated CD86 (558703), APC-conjugated CD25 (561048) and APC-conjugated CD178. For intracellular staining of IL-10 or IFN-γ, cells were fixed and permeabilized using a Cytofix/Cytoperm kit (54714, BD Biosciences) according to the manufacturer's instructions. Permeabilized cells were stained with APC-conjugated IL-10 (554468) or IFN-γ (562018). All the anti-mouse monoclonal antibodies were purchased from BD Biosciences except APC-conjugated CD178 (17-5911, eBioscience). The stained cells were acquired with a BD Canto II flow cytometer (BD Biosciences) and analyzed by FlowJo software (version 7.6, LLC, Ashland, OR, USA).

2.5. Sorting of $CD5^+CD1d^{hi}$ B cells

A B cell isolation kit was used to purify $CD19^+$ B cells as described in 2.2. The $CD19^+$ B cells were activated with LPS (10 μg/ml) in a 24 well-plate for 24 h. PE-Cy7-conjugated CD19 (552854, BD Biosciences), FITC-conjugated CD5 and PE-conjugated CD1d were used to stain the cell surface molecules, $CD5^+CD1d^{hi}$ and $CD5^•CD1d^{low}$ B cells were then sorted with a BD FACS Aria III flow cytometer (BD Biosciences) with purity of 90%–95%.

2.6. T cell proliferation assay

$CD4^+$ T cells were isolated from the splenocytes of WT mice by negative selection (19812A, StemCell) according to the instructions with purity of > 90%. The purified cells were stained with 4 μM CFSE (C34554, Invitrogen) for 10 min at 37 °C. CFSE-labeled $CD4^+$ T cells (1.5×10^5cells/well) were cultured in a 96 well-plate precoated with anti-CD3 (2 μg/ml, 16-0031-82, eBioscience) and anti-CD28 (2 μg/ml, 16-0281-82, eBioscience) antibodies. Sorted $CD5^+CD1d^{hi}$ or $CD5^•CD1d^{low}$ B cells after LPS stimulation were co-cultured with CFSE-labeled $CD4^+$ T cells at a ratio of 1:1 in the presence or absence of anti-FasL (5 μg/ml, 16-5911-81, eBioscience) (Montandon et al., 2013) for 72 h and then stained with APC-conjugated CD4. $CD4^+$ T cells were gated to analyze CFSE by flow cytometry.

2.7. Induction of contact hypersensitivity (CHS) reactions

WT mice were divided into CHS model and control groups, with 5 mice in each group. In CHS model group, mice were sensitized with 25 μl of oxazolone (100 mg/ml, E0753, Sigma-Aldrich) in 4:1 (vol/vol) acetone/olive oil applied to their shaved abdomens on days −6 and −5. Oxazolone (10 mg/ml) in 4:1 acetone/olive oil was painted on