BMB
Reports

BMB Rep. 2015; 48(1): 54-59
www.bmbreports.org

Autocrine stimulation of IL-10 is critical to the enrichment of IL-10-producing CD40^{hi}CD5^{+} regulatory B cells *in vitro* and *in vivo*

Hyuk Soon Kim[1,#], Jun Ho Lee[2,#], Hee Dong Han[1,#], A-Ram Kim[1], Seung Taek Nam[1], Hyun Woo Kim[1], Young Hwan Park[1], Dajeong Lee[1], Min Bum Lee[1], Yeong Min Park[1], Hyung Sik Kim[3], Young Mi Kim[4], Ji Chang You[5,]& Wahn Soo Choi[1,*]*

[1]School of Medicine, Konkuk University, Chungju 380-701, [2]College of Medicine, Korea University, Seoul 136-701, [3]College of Pharmacy, Sungkyunkwan University, Suwon 440-746, [4]College of Pharmacy, Duksung Women's University, Seoul 132-714, [5]National Research Laboratory of Molecular Virology, Department of Pathology, School of Medicine, The Catholic University of Korea, Seoul 137-701, Korea

IL-10-producing B (Breg) cells regulate various immune responses. However, their phenotype remains unclear. CD40 expression was significantly increased in B cells by LPS, and the Breg cells were also enriched in CD40^{hi}CD5^{+} B cells. Furthermore, CD40 expression on Breg cells was increased by IL-10, CD40 ligand, and B cell-activating factor, suggesting that CD40^{hi} is a common phenotype of Breg cells. LPS-induced CD40 expression was largely suppressed by an anti-IL-10 receptor antibody and in IL-10$^{-/-}$CD5^{+}CD19^{+} B cells. The autocrine effect of IL-10 on the CD40 expression was largely suppressed by an inhibitor of JAK/STAT3. *In vivo*, the LPS treatment increased the population of CD40^{hi}CD5^{+} Breg cells in mice. However, the population of CD40^{hi}CD5^{+} B cells was minimal in IL-10$^{-/-}$ mice by LPS. Altogether, our findings show that Breg cells are largely enriched in CD40^{hi}CD5^{+} B cells and the autocrine effect of IL-10 is critical to the formation of CD40^{hi}CD5^{+} Breg cells. [BMB Reports 2015; 48(1): 54-59]

INTRODUCTION

B cells have been mainly described for their capacity to produce antibodies, promote naïve T cell differentiation into helper T cells, and antigen presentation (1). However, recent data has shown that some B cell subsets play a protective role in various inflammatory animal models such as contact hypersensitivity, experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), inflammatory bowel disease (IBD), and lupus, through the production of IL-10 (2-7).

A number of regulatory B (Breg) cell phenotypes have been reported as CD1d^{hi}, CD1d^{hi}CD5^{+}, transitional 2 marginal zone precursor (T2-MZP), marginal zone (MZ) (CD21^{hi}CD23^{-}), and T cell Ig domain and mucin domain protein-1 (Tim-1)-positive in various inflammatory disease models. CD1d^{hi} B cells, but not CD1d$^{-/-}$ B cells, suppressed the development of chronic colitis in mice (2). IL-10-producing T2-MZP and MZ B cells were induced by the stimulation of CD40 signaling pathways to produce IL-10 in a collagen-induced arthritis model (3, 4). Tedder and coworkers also showed that CD1d^{hi}CD5^{+} B cells regulate contact hypersensitivity responses in mice (5). TIM-1 was also suggested as a unique marker on IL-10-producing Breg cells (8). However, characterization of the phenotypes of Breg cells remains far from complete.

Recently, Tedder and coworkers suggested that IL-10-producing Breg (B10) cells originate from a progenitor population (B10pro). They reported that the activation of CD40 signaling and LPS treatment can induce B10 pro (CD1d^{-}CD5^{-}) cells to become competent for IL-10 expression (9). Following a transient period of IL-10 expression by CD40 and Toll-like receptor (TLR) signaling, Breg cells can differentiate into antibody- secreting plasma cells and memory Breg cells (10). Although the differentiation phase of Breg cells has been recognized, the phenotypic modulation and cellular mechanisms are not yet fully understood.

Here we show that LPS treatment stimulated formation of Breg cells and these were significantly enriched in a population of CD40^{hi}CD5^{+} B cells. Furthermore, we show that IL-10 autocrine signaling is critical to enhancing the population of CD40^{hi} CD5^{+}CD19^{+} Breg cells.

RESULTS

LPS-stimulated IL-10 production is associated with CD40 expression on B cells

We initially determined whether splenic B cells produce IL-10 upon LPS stimulation. The LPS/TLR4 axis is a well-known trigger for stimulation of intracellular IL-10 expression and secretion from mouse B cells (11). We found that the frequency of Breg cells was increased by LPS stimulation in CD19^{+} B cells in a

*Corresponding authors. Wahn Soo Choi, Tel: +82-2-2030-7843; Fax: +82-2-2049-6192; E-mail: wahnchoi@kku.ac.kr, Ji Chang You, Tel: +82-2-2258-7312; Fax: +82-2-532-6537; E-mail: jiyou@catholic.ac.kr
#These authors contributed equally to this work.

http://dx.doi.org/10.5483/BMBRep.2015.48.1.213

Received 17 October 2014, Revised 23 October 2014, Accepted 23 October 2014

Keywords: Autocrine, CD5, CD40, Interleukin-10, Regulatory B cells

ISSN: 1976-670X (electronic edition)

time-dependent manner (Fig. 1A and 1B). Accordingly, secretion of IL-10 from Breg cells was strongly elevated in LPS-stimulated B cells (Fig. 1C). Notably, CD40 expression on B cells was significantly increased by stimulating with LPS for 48 h, but not 5 h (Fig. 1D and 1E). This result was further confirmed that the expression of CD40 was increased by LPS for 48 h in these cells (Fig. 1F). Based on these results, we expected that the activation of Breg cells is closely associated with upregulation of CD40 expression.

Next, we investigated whether upregulation of CD40 is associated with IL-10 production in Breg cells. The increase in CD40 expression was apparent by stimulating with LPS for 48 h, and the CD40 expression on IL-10^{+} B cells was higher than that on IL-10^{-} B cells (Fig. 1G). The production of IL-10 by CD40^{hi} B cells was consistently robust (Fig. 1G). Next, we phenotypically characterized LPS-induced CD40^{hi} Breg cells. We could not find any phenotypic differences between CD40^{hi} and CD40^{lo} B cells in the expression of IgD, CD11b, CD23, and B220. However, expression of CD1d, CD19, CD21, and CD5 was enhanced by LPS (Fig. 1H). It was also evident that the production of IL-10 was enhanced in the population of CD40^{hi} B cells from spleen, mesenteric lymph nodes, inflamed lymph nodes, and blood, but not the peritoneal cavity, compared to CD40^{lo} B cells (Fig. 1I). These results suggest that LPS-triggered expansion of the CD40^{hi} population is closely correlated with the production of IL-10 in Breg cells.

Expression of IL-10 is dominant in the population of CD40^{hi}CD5^{+} B cells

CD5-expressing B-1a B cells are generally recognized as one of

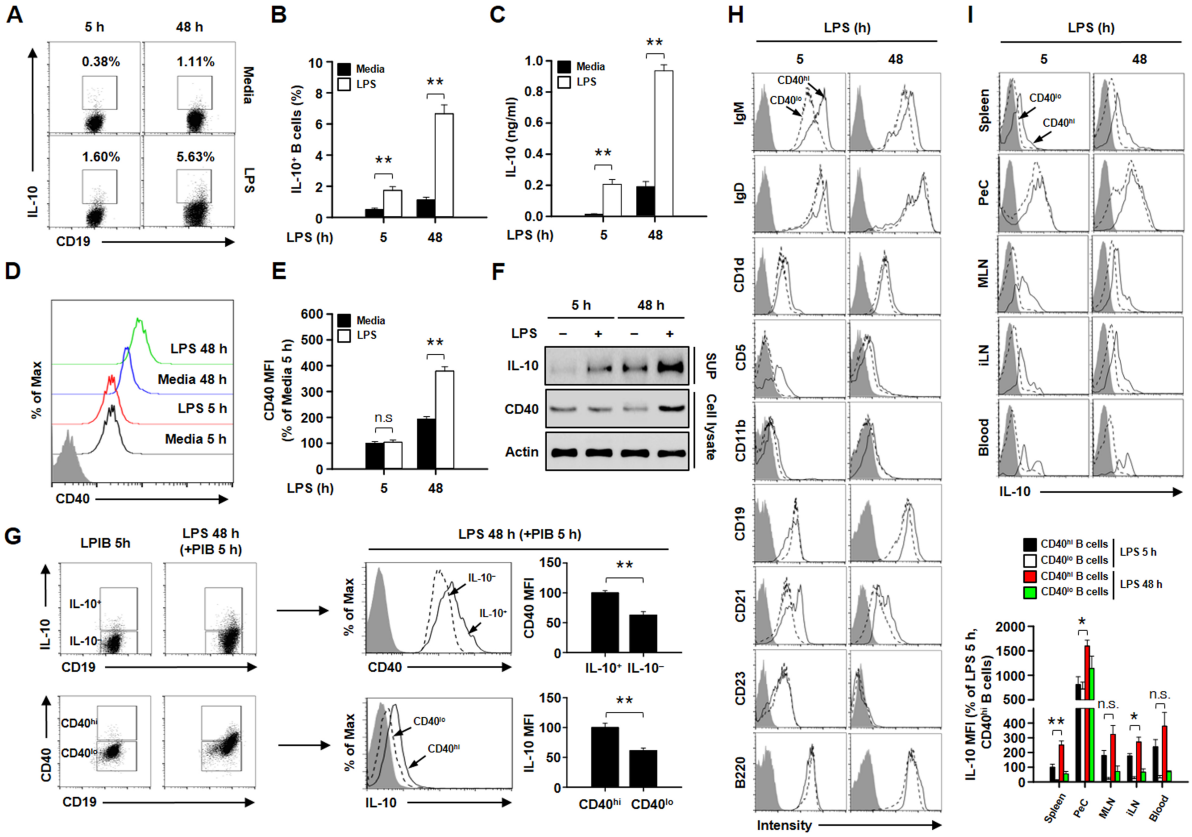

Fig. 1. LPS-induced CD40 high expression is dependent on IL-10-producing B cell differentiation. (A) Representative images of LPS-induced IL-10^{+} B cell activation in a time-dependent manner. (B) Frequencies of IL-10 in mouse splenic B cells stimulated with LPS. (C) Quantitative analysis of secreted IL-10 from LPS-stimulated B cells. Cells were stimulated with LPS for the indicated times and then single cells or supernatant were harvested. Data are the means ± SEM from three independent experiments. *P < 0.05; **P < 0.01. n.s., not significant. (D) Representative images and (E) mean fluorescence intensity (MFI) analysis of CD40 expression on LPS-stimulated B cells. (F) Levels of LPS-stimulated B-cell secretion of IL-10 and surface expression of CD40. Representative images are shown from three independent experiments. (G) Comparison of CD40 expression on IL-10^{+} or IL-10^{-} B cells and IL-10 expression in CD40^{hi} or CD40^{lo} B cells. (H) Phenotypic analysis of CD40^{hi} or CD40^{lo} B cells by LPS stimulation for 5 or 48 h. (I) Representative image and MFI of IL-10 expression in CD40^{hi} or CD40^{lo} B cells from indicated tissue-derived cells. MFI values are the means ± SEM from three independent experiments: *P < 0.05; **P < 0.01. n.s., not significant.

the regulatory B cell subsets involved in various autoimmune diseases through IL-10 production (12-15). We therefore sought to determine whether IL-10 production is correlated with phenotypic features of CD40 expression in LPS-stimulated CD5^{+} Breg cells. As shown in Fig. 2A and 2B, LPS significantly enhanced the IL-10 production in CD5^{+} B cells, but not CD5^{-} B cells, in a time-dependent manner. From these results, we hypothesized that LPS-induced CD40 expression is closely correlated with the production of IL-10 in CD5^{+} B cells. Next, we compared the expression level of CD40 in CD5^{+} and CD5^{-} B cells. CD40 expression was increased in both CD5^{+} and CD5^{-} B cells by stimulation with LPS (Fig. 2C and 2D). Notably, the CD40 expression in CD5^{+} B cells was much stronger than that in CD5^{-} B cells after 48 h of LPS stimulation (Fig. 2D). The analysis of IL-10 expression by flow cytometry and RT-PCR showed that IL-10 was produced in CD40^{hi}CD5^{+} B cells, but its expression was minimal in CD40^{lo}CD5^{+} B cells (Fig. 2E-2H). These results suggest that CD40 expression is closely associated with IL-10 production in Breg cells.

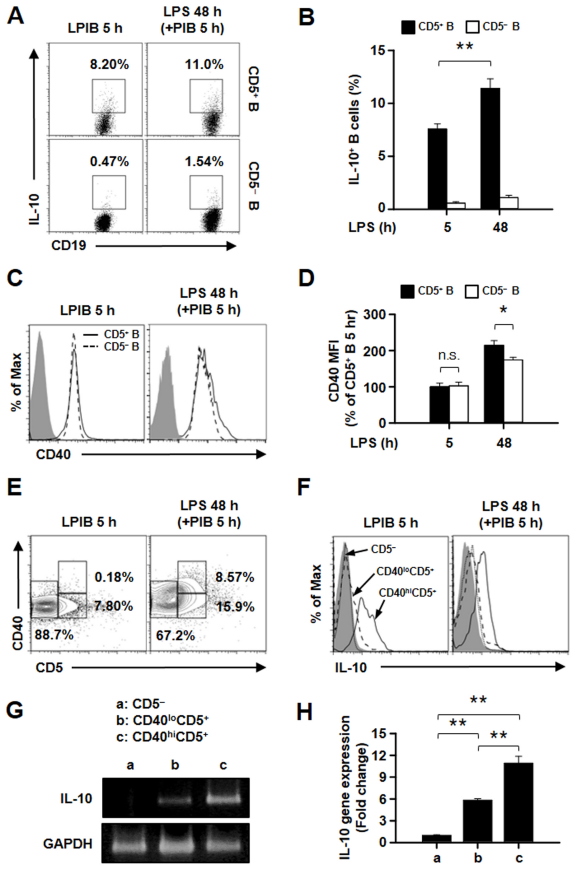

Fig. 2. The predominant IL-10-producing B cells are CD40^{hi}CD5^{+} B cells in long-term LPS stimulation. (A) Representative images and (B) frequencies of IL-10 expression in CD5^{+} or CD5^{-} B cells induced by LPS treatment for 5 or 48 h. (C) Representative histogram and (D) mean fluorescence intensity (MFI) of surface CD40 expression on CD5^{+} or CD5^{-} B cells treated with LPS for the indicated times. MFI values are the means ± SEM from three independent experiments: *P < 0.05; **P < 0.01. n.s., not significant. (E) Flow cytometric analysis based phenotypic separation of CD40^{hi}CD5^{+}, CD40^{lo}CD5^{+}, and CD5^{-} LPS-induced B cells. (F) MFI, (G) representative gene expression images, and (H) numeric values for IL-10 protein band density in lysates of CD40^{hi}CD5^{+}, CD40^{lo}CD5^{+}, and CD5^{-} B cells.

Autocrine IL-10-stimulated JAK/STAT3 signaling is critical to the induction of CD40^{hi}CD5^{+} Breg cells

Next, we investigated whether various IL-10 inducers (LPS, IL-10, CD40 ligand, and B cell-activating factor (BAFF)) enhance the population of CD40^{hi}CD5^{+} Breg cells. All inducers stimulated both the expression of IL-10 (Fig. 3A) and the formation of CD40^{hi}CD5^{+} B cells (Fig. 3B). These results suggest that the expansion of CD40^{hi}CD5^{+} Breg cells is associated with the IL-10 production capacity of Breg cells.

The above results led us to investigate whether Breg cell-produced IL-10 enhances the formation of the CD40^{hi}CD5^{+} B cell population. We reconfirmed that the expansion of CD40^{hi}CD5^{+} B cells is increased by LPS and IL-10 (Fig. 3C). Next, we determined whether the expansion of CD40^{hi}CD5^{+} B cells is associated with the autocrine effects of IL-10. The expansion of CD40^{hi}CD5^{+} B cells was significantly suppressed by an anti- IL-10 receptor antibody (Fig. 3D). Furthermore, the expansion of CD40^{hi}CD5^{+} B cells was minimal in IL-10$^{-/-}$ CD5^{+} B cells stimulated with LPS (Fig. 3E), suggesting that an autocrine effect of IL-10 is critical for the expansion of CD40^{hi}CD5^{+} B cells and the production of IL-10. The activation of the JAK/ STAT3 signaling pathway by IL-10 is critical for the regulation of various immune cells (16). Therefore, we further assessed whether the autocrine effect of IL-10 is associated with the IL- 10-stimulated JAK/STAT3 pathway in Breg cells. Of note, the induction of IL-10 and CD40^{hi} expression by LPS was inhibited by AG490, a specific inhibitor of the JAK/STAT3 pathway (Fig. 3F and 3G), suggesting that IL-10-induced JAK/STAT3 signaling is critical to the enhancement of CD40^{hi}CD5^{+} Breg cells.

Autocrine effect of IL-10 is also critical to the formation of CD40^{hi}CD5^{+} Breg cells in LPS-stimulated mice

To assess the induction of the IL-10-producing CD40^{hi} population of CD5^{+} B cells *in vivo*, we intravenously injected LPS (1 mg/kg) into mice, and then analyzed the production of IL-10-producing splenic CD40^{hi}CD5^{+} Breg cells. Similar to the *in vitro* results, the expression of IL-10 was significantly increased in a time-dependent manner (Fig. 4A and 4B). Next, we determined whether the enhancement of CD40^{hi}CD5^{+} B cells was dependent on IL-10 in mice. The splenic population of CD40^{hi}CD5^{+} Breg cells was substantially increased in LPS-stimulated WT (IL-10$^{+/+}$) mice, but not in IL-10$^{-/-}$ mice (Fig. 4C). These results strongly suggest that the formation of CD40^{hi}CD5^{+} Breg cells was regulated by IL-10 in mice.

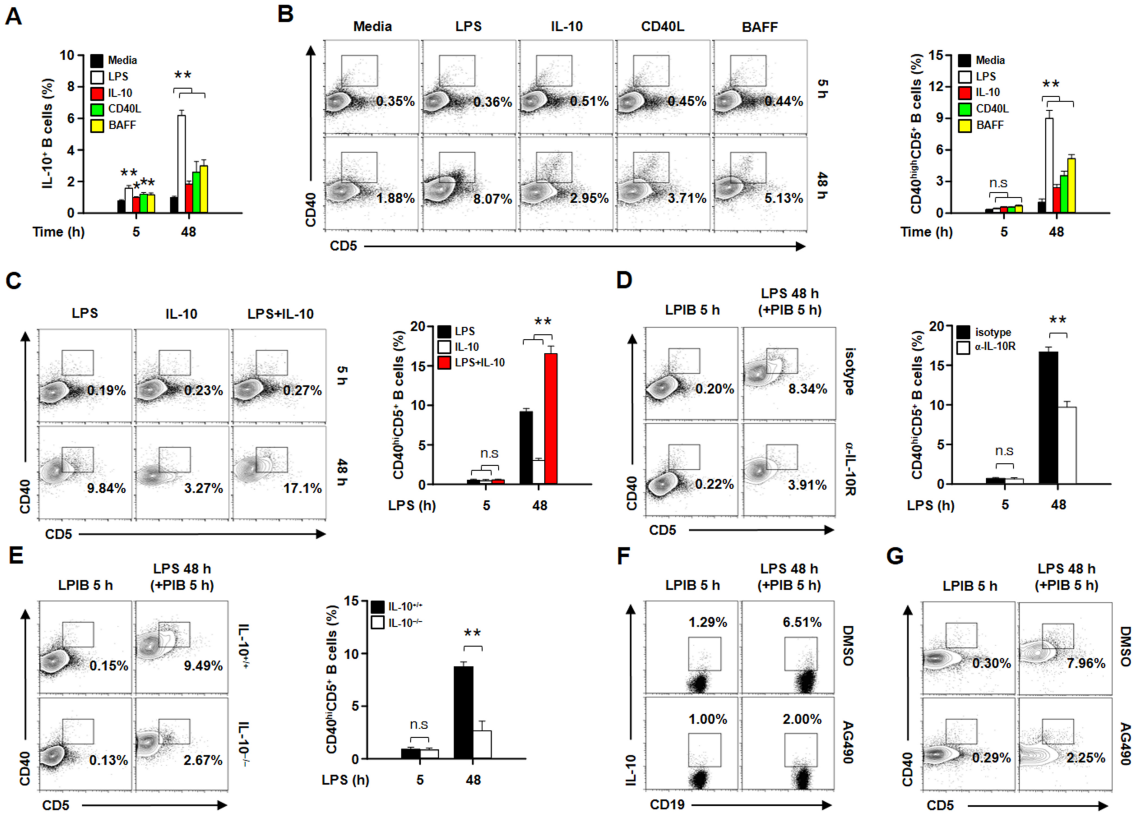

Fig. 3. The formation of CD40^{hi}CD5^{+} B cells is controlled by autocrine IL-10. (A) Frequencies of IL-10 production and (B) CD40^{hi}CD5^{+} expression in splenic B cells induced by LPS (10 μg/ml), IL-10 (10 ng/ml), CD40L (1 μg/ml), and BAFF (100 ng/ml) treatment for 5 or 48 h. Representative images for CD40^{hi}CD5^{+} expression on B cells induced by LPS, IL-10, CD40L, and BAFF treatment for the indicated times. (C) Representative images and frequency of CD40^{hi}CD5^{+} expression in B cells stimulated with LPS or IL-10 for 5 or 48 h. (D) Representative images and frequency of CD40^{hi}CD5^{+} B cells after treatment with LPS for 5 or 48 h. (E) Representative images and frequency of CD40^{hi}CD5^{+} B cells from WT (IL-10$^{+/+}$) or IL-10$^{-/-}$ mice. Data are the means ± SEM from three independent experiments. **P < 0.01. n.s., not significant. (F) Representative images of LPS-induced IL-10 production and (G) CD40^{hi}CD5^{+} expression in B cells treated with AG490 for 5 or 48 h.

DISCUSSION

Breg cells have been acknowledged to have anti-inflammatory activity in various immune responses. However, the phenotypic characterization of Breg cells remains incomplete. The goal of our study was to determine the phenotypic features of the induction mechanism of Breg cells. We found that the frequency of Breg cells was increased by LPS treatment. Interestingly, this observation was closely associated with the increased expression of CD40 on B cells. Although the increased expression of CD40 is a typical feature of activated B2 cells (11, 17), the role and induction mechanism of CD40 in B1a Breg cells are still incompletely understood. We observed that the population of Breg cells was highly enriched in CD40^{hi}CD5^{+} B cells, compared to CD40^{lo}CD5^{+} B cells (Fig. 3E and 3F). These results suggest that the high expression of CD40 on B cells is closely associated with IL-10 production in Breg cells.

We next found unexpectedly that the frequency of CD40^{hi}CD5^{+} B cells was increased by various IL-10 inducers including LPS, IL-10, CD40 ligand (CD40L), and BAFF (Fig. 3A and 3B). Although LPS, IL-10, CD40L, and BAFF stimulate different intracellular signaling pathways leading to IL-10 production in Breg cells (17, 21), the high expression of CD40 on CD5^{+} B cells was evidenced by the production of IL-10 (Fig. 3A and 3B). These results suggest that the formation of CD40^{hi}CD5^{+} Breg cells requires the activation of common signaling proteins in the cells. Here, we argue that the LPS-induced CD40^{hi} expression was critical to formation of Breg cells.

The treatment with IL-10 stimulated the formation of IL-10^{+} Breg cells (Fig. 3A and 3B), suggesting that LPS-stimulated Breg cell-induced IL-10 may enhance the formation of CD40^{hi} Breg cells as an autocrine mechanism. To further determine whether the autocrine effect of IL-10 secreted by Breg cells is critical to the formation of CD40^{hi}CD5^{+} B cell subsets, Breg cells were stimulated by LPS with or without anti-IL-10 receptor antibody and further investigated in IL-10$^{-/-}$ mice. Recombinant IL-10 showed a

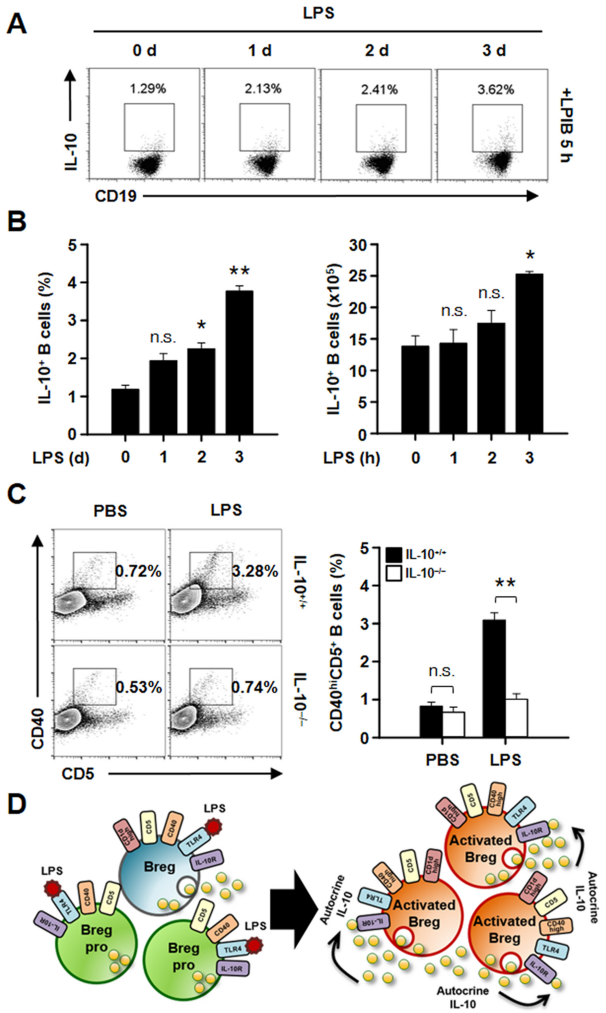

Fig. 4. The formation of LPS-induced CD40^{hi}CD5^{+} B cells is dependent on endogenous IL-10 production in mice. (A) Representative images, (B) frequency and number of IL-10-producing splenic B cells in mice treated with LPS (1 mg/kg, i.v.) for 0 to 3 days. For intracellular IL-10 detection, the cells were cultured with LPS, PMA, ionomycin, and brefeldin A for 5 h. (C) Representative images and frequency of CD40^{hi}CD5^{+} splenic B cells from WT and IL-10$^{-/-}$ mice treated with LPS for 3 days. Data are the means ± SEM from three independent experiments. *P < 0.05; **P < 0.01. n.s., not significant. (D) Schematic of the proposed mechanism for the differentiation of IL-10-producing B cells into CD40hiCD5+ B cells by autocrine IL-10. The activation of Breg and Breg progenitor cells can be initiated by LPS and autocrine IL-10 produced by Breg cells. Costimulation by LPS and autocrine IL-10 leads to the differentiation of IL-10-producing B cells to the CD40^{hi}CD5^{+} phenotype via the JAK/STAT signaling pathway.

synergistic effect with LPS in enriching CD40^{hi}CD5^{+} B cell subsets (Fig. 3C). However, the formation of CD40^{hi}CD5^{+} Breg cells was blocked by treatment of IL-10$^{-/-}$ B cells with anti-IL-10 receptor mAb (Fig. 3D and 3E). These results suggest that the autocrine effect of IL-10 is critical for the formation of the CD40^{hi} phenotype of Breg cells. Furthermore, the frequency and number of splenic Breg cells were increased by LPS in mice (Fig. 4A and 4B). The population of CD40^{hi}CD5^{+} B cells was significantly increased in LPS-treated mice, but this was not observed in IL-10$^{-/-}$ mice (Fig. 4C). Overall, our results strongly suggest that the expression of CD40^{hi} and IL-10 in Breg cells is regulated by both autocrine and paracrine mechanisms of IL-10 *in vitro* and *in vivo* (Fig. 4D). It has been well established that the JAK/STAT3 pathway plays a critical role in regulating various IL-10-mediated immune responses (16). In the present study, the expression of IL-10 and CD40^{hi} was blocked by inhibiting the JAK/STAT3 pathway with AG490 (Fig. 3F and 3H). These observations suggest that the autocrine IL-10 mechanism regulates the JAK/STAT3 pathway-induced formation of IL-10-producing CD40^{hi}CD5^{+} Breg cells.

In summary, our data demonstrate that Breg cells were enriched in a population of CD40^{hi}CD5^{+} B cells by treatment with LPS or other stimulants. The signaling pathway is predominantly regulated by autocrine IL-10 (Fig. 4D). These findings suggest that the regulation of CD40 expression on Breg cells may provide an additional therapeutic application for various autoimmune and inflammatory diseases.

MATERIALS AND METHODS

Material and Methods are described in the online data supplement, available at http://www.bmbreports.org/.

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP, No. 2012R1A2A1A03670516), and in part by a National Research Foundation of Korea (NRF) grant (MSIP, NRF-2013-R1A4A1069575, NRF-2013R1A1A2058984, and 2013R1A2-A2A01068353).

REFERENCES

1. LeBien TW and Tedder TF (2008) B lymphocytes: how they develop and function. Blood 112, 1570-1580
2. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS and Bhan AK (2002) Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. Immunity 16, 219- 230
3. Gray M, Miles K, Salter D, Gray D and Savill J (2007) Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. Proc Natl Acad Sci U S A 104, 14080-14085
4. Evans JG, Chavez-Rueda KA, Eddaoudi A et al (2007) Novel suppressive function of transitional 2 B cells in experimental arthritis. J Immunol 178, 7868-7878
5. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M and Tedder TF (2008) A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell dependent in-

flammatory responses. Immunity 28, 639-650
6. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M and Tedder TF (2008) Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest 118, 3420-3430
7. Lenert P, Brummel R, Field EH and Ashman RF (2005) TLR-9 activation of marginal zone B cells in lupus mice regulates immunity through increased IL-10 production. J Clin Immunol 25, 29-40
8. Ding Q, Yeung M, Camirand G et al (2011) Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. J Clin Invest 121, 3645-3656
9. Dilillo DJ, Matsushita T and Tedder TF (2010) B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. Ann N Y Acad Sci 1183, 38-57
10. Kalampokis I, Yoshizaki A and Tedder TF (2013) IL-10- producing regulatory B cells (B10 cells) in autoimmune disease. Arthritis Res Ther 15, Suppl 1:S1. doi: 10.1186/ ar3907
11. Yanaba K, Bouaziz JD, Matsushita T, Tsubata T and Tedder TF (2009) The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. J Immunol 182, 7459- 7472
12. Murakami M, Yoshioka H, Shirai T, Tsubata T and Honjo T (1995) Prevention of autoimmune symptoms in autoimmune-prone mice by elimination of B-1 cells. Int Immunol 7, 877-882
13. Wen X, Zhang D, Kikuchi Y et al (2004) Transgene-mediated hyper-expression of IL-5 inhibits autoimmune disease but increases the risk of B cell chronic lymphocytic leukemia in a model of murine lupus. Eur J Immunol 34, 2740-2749
14. Mantovani L, Wilder RL and Casali P (1993) Human rheumatoid B-1a (CD5+ B) cells make somatically hypermutated high affinity IgM rheumatoid factors. J Immunol 151, 473-488
15. Yoshizaki A, Miyagaki T, DiLillo DJ et al (2012) Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. Nature 491, 264-268
16. Riley JK, Takeda K, Akira S and Schreiber RD (1999) Interleukin-10 receptor signaling through the JAK-STAT pathway. Requirement for two distinct receptor-derived signals for anti-inflammatory action. J Biol Chem 274, 16513-16521
17. Iwata Y, Matsushita T, Horikawa M et al (2011) Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood 117, 530-541
18. Baumgarth N (2011) The double life of a B-1 cell: Self-reactivity selects for protective effector functions. Nat Rev Immunol 11, 34-46
19. Sindhava VJ and Bondada S (2012) Multiple regulatory mechanisms control B-1 B cell activation. Front Immunol 3, 372
20. Suzuki K, Maruya M, Kawamoto S and Fagarasan S (2010) Roles of B-1 and B-2 cells in innate and acquired IgA- mediated immunity. Immunol Rev 237, 180-190
21. Yang M, Sun L, Wang S et al (2010) Novel function of B cell-activating factor in the induction of IL-10-producing regulatory B cells. J Immunol 184, 3321-3325