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Lactoferrin Induces Tolerogenic Bone Marrow-Derived Dendritic Cells

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ABSTRACT

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that initiate both T-cell responses and tolerance. Tolerogenic DCs (tDCs) are regulatory DCs that suppress immune responses through the induction of T-cell anergy and Tregs. Because lactoferrin (LF) was demonstrated to induce functional Tregs and has a protective effect against inflammatory bowel disease, we explored the tolerogenic effects of LF on mouse bone marrow-derived DCs (BMDCs). The expression of CD80/86 and MHC class II was diminished in LF-treated BMDCs (LF-BMDCs). LF facilitated BMDCs to suppress proliferation and elevate Foxp3$^+$ induced Treg (iTreg) differentiation in ovalbumin-specific CD4$^+$ T-cell culture. Foxp3 expression was further increased by blockade of the B7 molecule using CTLA4-Ig but was diminished by additional CD28 stimulation using anti-CD28 Ab. On the other hand, the levels of arginase-1 and indoleamine 2,3-dioxygenase-1 (known as key T-cell suppressive molecules) were increased in LF-BMDCs. Consistently, the suppressive activity of LF-BMDCs was partially restored by inhibitors of these molecules. Collectively, these results suggest that LF effectively causes DCs to be tolerogenic by both the suppression of T-cell proliferation and enhancement of iTreg differentiation. This tolerogenic effect of LF is due to the reduction of costimulatory molecules and enhancement of suppressive molecules.

Keywords: Lactoferrin; Dendritic cells; B7 antigens; Immune tolerance; Regulatory T cells; Suppressive factor

INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that play an important role in immune defenses and the maintenance of immune tolerance to self-tissues. DCs not only activate T cells but also tolerize the T-cell response, thereby maintaining immune homeostasis (1,2). Inflammatory mediators, such as CD40 ligands or LPS, induce the terminal differentiation of immature DCs into fully matured immunogenic DCs by upregulating MHC class II and costimulatory molecules (3). By contrast, immunosuppressive cytokines such as IL-10 and TGF-β as well as various pharmacological agents, including dexamethasone, rapamycin, vitamin D3, and retinoic acid, induce tolerogenic DCs (tDCs) (4). The tDCs are regulatory DCs that suppress immune responses through the induction of T-cell apoptosis/anergy and Tregs (5). Furthermore, arginase-1 (Arg-1), indoleamine

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Conflicts of Interest
The authors declare no potential conflicts of interest.

Abbreviations
Age-1, arginase-1; APC, antigen-presenting cell; BMDC, bone marrow-derived dendritic cell; CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; iTreg, induced Treg; LF, lactoferrin; LF-BMDC, lactoferrin-treated bone marrow-derived dendritic cell; MARCH1, membrane-associated RING-CH 1; MFI, mean of fluorescence intensity; OVA, ovalbumin; tDC, tolerogenic dendritic cell.

Author Contributions
Conceptualization: Jang YS, Kim PH; Data curation: Park HW, Park SH, Jang YS, Kim PH; Formal analysis: Park HW, Park SH, Jo HJ, Kim TG, Lee JH; Funding acquisition: Kim PH; Investigation: Park HW, Park SH, Jo HJ, Jang YS; Methodology: Park HW, Park SH, Kim TG, Lee JH; Project administration: Jang YS; Resources: Kim PH; Supervision: Jang YS, Kim PH; Visualization: Park HW, Park SH, Jo HJ; Writing - original draft: Park HW, Jang YS, Kim PH; Writing - review & editing: Kang SG, Jang YS, Kim PH.

2,3-dioxygenase (IDO)-1, and CD39/CD73 expression by DCs are involved in the suppression of T-cell response via the catabolism of essential amino acids (6-10). The mechanisms underlying the induction of Tregs by tDCs were reported and include low levels of antigen presentation and costimulatory molecules as well as the secretion of regulatory cytokines, such as IL-10 and TGF-β1 (11).

Lactoferrin (LF) is an 80-kDa multifunctional iron-binding glycoprotein of the transferrin family. It is widely found in most mammalian exocrine secretions and secondary granules of neutrophils (12). LF demonstrates significant physiological anti-bacterial, anti-viral, and anti-inflammatory activity (13). Concerning immune tolerance, LF prevents the release of inflammatory cytokines such as TNF-α, IL-1, and IL-6 by LPS-activated mononuclear cells (14). Furthermore, perorally delivered LF enhances the secretion of anti-inflammatory cytokine IL-10 but decreases IFN-γ production, resulting in the alleviation of colitis in rats (15). These protective effects of LF also improve the prognosis of autoimmune diseases, such as experimental autoimmune encephalomyelitis and autoimmune hepatitis (16,17). These results suggest that LF can act as a key factor that causes immune tolerance, leading to the maintenance of immune homeostasis. Nevertheless, it has not yet been elucidated whether LF directly modulates DCs to be tolerogenic.

We recently found that LF strongly stimulates activated $CD4^+$ T cells to differentiate into $Foxp3^+$ Tregs (18), leading us to investigate the effect of LF on tDC generation. In the present study, LF decreased the expression of costimulatory molecules (CD80 and CD86) and increased suppressive molecules (Arg-1 and IDO-1). Furthermore, LF-treated bone marrow-derived DCs (LF-BMDCs) caused both the suppression of the T-cell proliferation and enhancement of Ag-specific induced Treg (iTreg) differentiation.

MATERIALS AND METHODS

Animals

BALB/c mice were obtained from Orient Bio Inc. (Seongnam, Korea). C57BL/6 WT and ovalbumin (OVA)-specific TCR-transgenic OT-II mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The animals were fed Purina Laboratory Rodent Chow 5001 ad libitum. Mice that were 8–12 wk of age were used in this study. Animal care was performed in accordance with the institutional guidelines set forth by Kangwon National University (approval No. KW-190515-1).

Preparation of BMDCs and phenotype analysis

BM cells obtained from mouse femurs were cultured in 24-well plates in RPMI-1640 (Gibco, Grand Island, NY, USA) containing GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) (Peprotech, Rocky Hill, NJ, USA). After 3 days, non-adherent cells were removed by gentle shaking and replacing the medium. These cells were harvested after 5 days. This procedure resulted in $CD11b^+CD11c^+$ (>85%) and $CD83^+$ (up to 5%) cells, designated immature BMDCs. Next, the cells were exposed to 2 μg/ml of LPS (*Escherichia coli* O111:B4; Sigma-Aldrich, St. Louis, MO, USA) containing GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) for 2 days to generate mature DCs (>77% CD83).

Bovine LF was kindly donated by Morinaga Milk Co., Ltd. (Zama, Japan) and contained less than 5.0 pg mg^{-g} of LPS (endotoxin). Different concentrations of LF were added from the start of BM cell culture (on day 0) or only during the generation of mature DCs (on

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day 6) with 2 μg/ml of LPS. Cultured BMDCs were harvested after 7 days and used for the studies of T-cell proliferation and Treg differentiation. Cell surface staining was performed in PBS containing 2% FBS using the following Abs: anti-CD80-PE, CD86-PE, CD73-PE, and MHC II-APC (Invitrogen Life Technologies, Carlsbad, CA, USA), as well as CD39-APC (eBioscience, San Diego, CA, USA). Intracellular staining was performed using the Fixation and Permeabilization Buffer kits (eBioscience) with anti-Arg-1, anti-IDO-1, and IgG-APC Abs (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions.

Allogeneic and Ag-specific T-cell proliferation assay

Naïve $CD4^+CD25^-$ T cells from the spleens of 8- to 12-wk-old mice were purified by selection using naïve $CD4^+$ T-cell isolation kits and magnetic cell sorting (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions.

Allogeneic T-cell proliferation was assessed using the mixed lymphocyte reaction. Carboxyfluorescein succinimidyl ester (CFSE)-labeled responder T cells (1×10^5) from BALB/c mice were co-cultured with irradiated/violet-labeled-BMDCs/LF-BMDCs (5×10^4) derived from C57BL/6 as an allogeneic stimulator. Irradiation (2,000 rad) was performed using a Gammacell 40 Exactor (Best Theratronics Ltd., Ottawa, Ontario, Canada). The CFSE and Violet kits were obtained from Invitrogen Life Technologies, Carlsbad, CA, USA). Dilution of CFSE was measured by counting 10,000 viable cells using FACSverse (BD Biosciences, San Diego, CA, USA). To inhibit enzyme the activities of IDO-1 and Arg-1, the IDO1 inhibitor (MedChemExpress, Monmouth Junction, NJ, USA) and Arg-1 inhibitor (Calbiochem, San Diego, CA, USA) were added to the cells 24 h before co-culture.

To assess Ag-specific T-cell proliferation, irradiated BMDCs/LF-BMDCs (5×10^4) from C57BL/6 mice were pulsed with OVA peptide ($OVA_{323-339}$; Peptron, Daejeon, Korea) and then co-cultured with CFSE-labeled naïve $CD4^+$ responder T cells (1×10^5) from OT-II transgenic mice.

Determination of OVA-specific inducible Tregs

Responder $CD4^+$ T cells (1×10^5) from OT- II mice were co-cultured with $OVA_{323-339}$-pulsed irradiated BMDCs/LF-BMDCs (5×10^4) in the presence of TGF-β1 (1 ng/ml), LF (100 μg/ml) or the combination for 3 days. The cells were stained with anti-mouse CD4-FITC Ab (BioLegend) for surface staining and with anti-Foxp3-PE/cy7 Ab (Invitrogen) for intracellular staining.

To perform the experiment designed to inhibit costimulatory signals, 4 μg/ml of mouse CTLA-4 Ig (R&D Systems, MN, USA) was added to the co-culture of OT- II $CD4^+$ T cells (1×10^5) and irradiated/$OVA_{323-339}$-pulsed $CD4^+$ T-cell-depleted splenocytes (APCs; 5×10^4).

Statistical analysis

Statistical differences between experimental groups were determined by ANOVA, and values of $p<0.05$ by unpaired, 2-tailed Student's t-test were considered significant.

RESULTS

LF modulates BMDCs to be tolerogenic

tDCs are characterized by the reduced expression of costimulatory molecules and decreased ability to induce T-cell proliferation and increased induction of Tregs (5). Because we previously observed that LF stimulates naïve $CD4^+$ T cells to differentiate into functional

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Tregs, we asked whether LF can also modulate DCs to be tolerogenic. BM cells under the influence of retinoic acid were demonstrated to be tolerogenic when they are differentiated into mature DCs (19). Therefore, we investigated whether LF exerts a similar effect on BM cells. We also examined the limited effect of LF during the maturation of DCs, as shown in **Fig. 1A**. We first examined the levels of costimulatory molecules and MHC class II on BMDCs under the influence of LF. The levels of CD80, CD86, and MHC class II augmented by LPS were substantially diminished by the addition of LF in a dose-dependent manner, irrespective of the exposure times of LF (**Fig. 1B**). Moreover, the levels of the surface molecules expressed on LF-BMDCs between 7 days and 2 days were quite similar, suggesting that the major effect of LF on BMDCs occurs during DC maturation. Subsequently, we explored the effects of LF-BMDCs on allogeneic $CD4^+$ T-cell proliferation. T-cell proliferation was significantly suppressed when BMDCs were treated with LF, although BMDCs treated with LF for 2 days were suppressed slightly less than those treated with LF for 7 days (**Fig. 2**). These results indicate that DCs can be tolerogenic when LF acts only during DC maturation.

Effect of LF on APCs toward the Ag-specific T-cell response

To obtain better information on the physiological role of LF on APCs, we adopted the OVA-specific T-cell response using OT-II mice (TCR^{ova} transgenic)—i.e., T-cell proliferation and iTreg differentiation (**Fig. 3A**). First, immature BMDCs were cultured with LPS and LF for 2 days. The cells were then pulsed with $OVA_{323-339}$ peptide and co-cultured with CFSE-labeled OT-II $CD4^+$ T cells (**Fig. 3B**). The proportion of proliferative $CD4^+$ T cells was substantially diminished when LF was added to BMDCs and occurred in a dose-dependent manner (**Fig. 3B**). Second, we determined whether LF-treated DCs contribute to the conversion of naïve $CD4^+$ T cells into $CD4^+$ $Foxp3^+$ Tregs. We recently found that either TGF-β1 or LF or both stimulated naïve $CD4^+$ T cells to differentiate into $Foxp3^+$ Tregs (18). The same experimental scheme was implemented using OT-II mice (**Fig. 3A**). LF-BMDCs strikingly increased Foxp3 expression by OT-II $CD4^+$ T cells conditioned with TGF-β1 or LF or both (**Fig. 3C**). These results clearly show that LF causes Ag-loaded DCs to be tolerogenic because T-cell proliferation was not only suppressed but also the frequency of $Foxp3^+$ T cells was increased in the co-culture of LF-BMDCs. Thus, we were interested in determining how LF-BMDCs drive $Foxp3^+$ T-cell differentiation. Tregs are efficiently induced under weak TCR and costimulation (20,21). Because LF reduced the expression of costimulatory molecules in BMDCs, as shown in **Fig. 1**, we explored whether the levels of the costimulatory molecule B7 affect OVA-specific $CD4^+Foxp3^+$ T-cell differentiation. An increase in the $Foxp3^+$ T-cell population by LF-BMDCs was further enhanced by CTLA4-Ig treatment; however, this increase in the $Foxp3^+$ T-cell population by LF-BMDCs was decreased by anti-CD28 Ab treatment (**Fig. 3D**). Taken together, these results indicate that the low level of costimulatory molecules in LF-BMDCs contribute to $CD4^+Foxp3^+$ T-cell differentiation.

Effect of LF on potential suppressor molecules expressed by BMDCs

DCs become tolerogenic through expressing not only a low level of costimulatory molecules but also a high level of catalytic enzymes such as Arg-1, IDO-1, and CD39/73 (6,7,10). Because LF inhibited the expression of costimulatory molecules (CD80 and CD86) in the present study, we determined the levels of these catalytic enzymes. LF significantly increased the expression of Arg-1 and IDO-1 at the levels of mean of fluorescence intensity (MFI) and population frequencies (%). However, the levels of CD39 were not altered. In addition, MFI of CD73 was not changed either though its population frequency (%) looks significantly increased (**Fig. 4A**). Subsequently, we examined the role of these molecules in allogeneic

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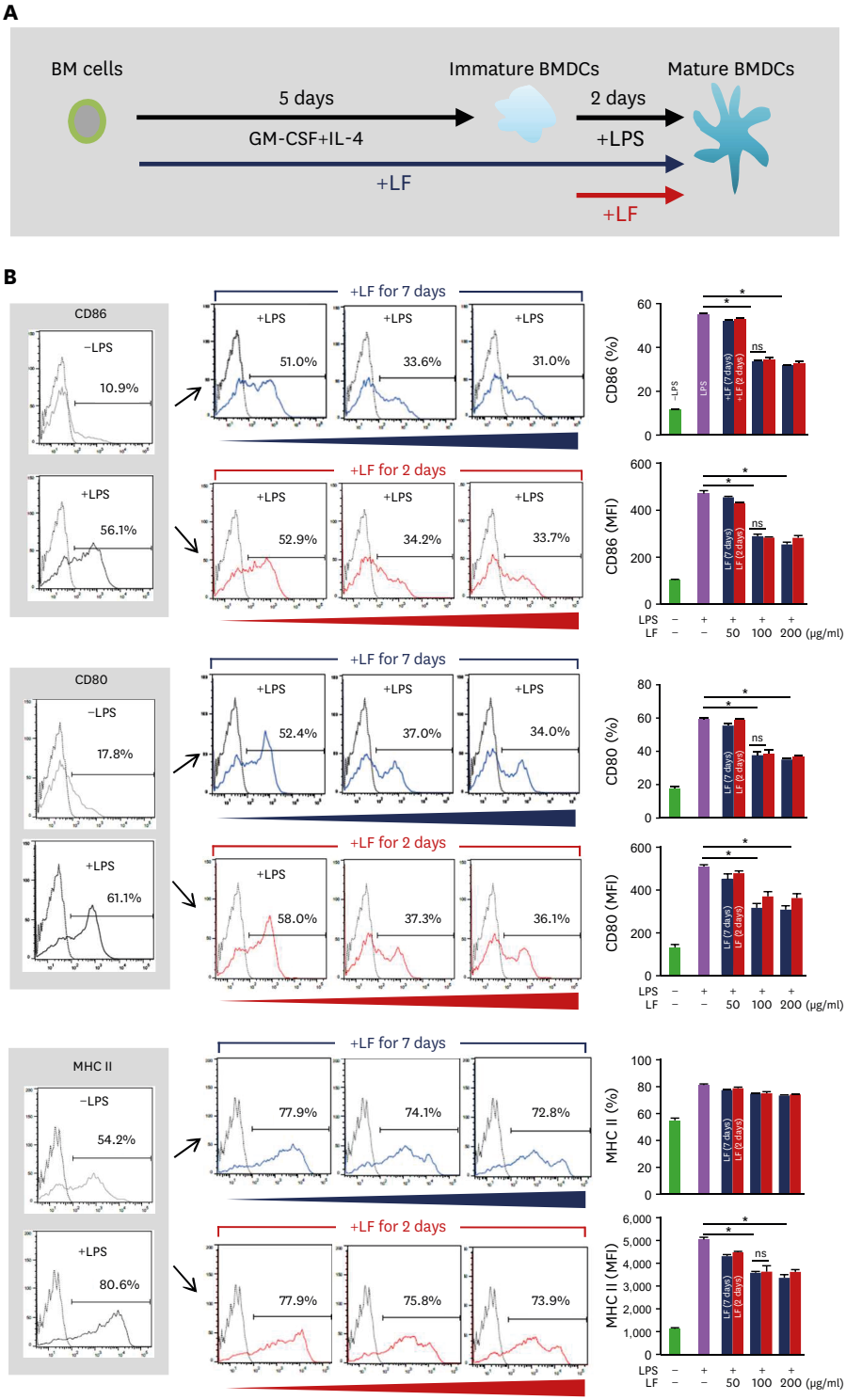

Figure 1. Effect of LF on CD86, CD80, and MHC class II on the surface of mature BMDCs.
(A) C57BL/6 BM cells were cultured with 20 ng/ml of GM-CSF and 20 ng/ml IL-4 for 7 days. LF (50, 100, and 200 μg/ml) was added for 7 days and 2 days as indicated. LPS (2 μg/ml) was added to immature BMDCs for 2 days. (B) Mature BMDCs were analyzed for the surface expression of CD86, CD80, and MHC class II molecules. The data represent the average percent and fluorescent intensity of triplicate samples with SEM (bars).
ns, not significant.
*$p<0.05$.

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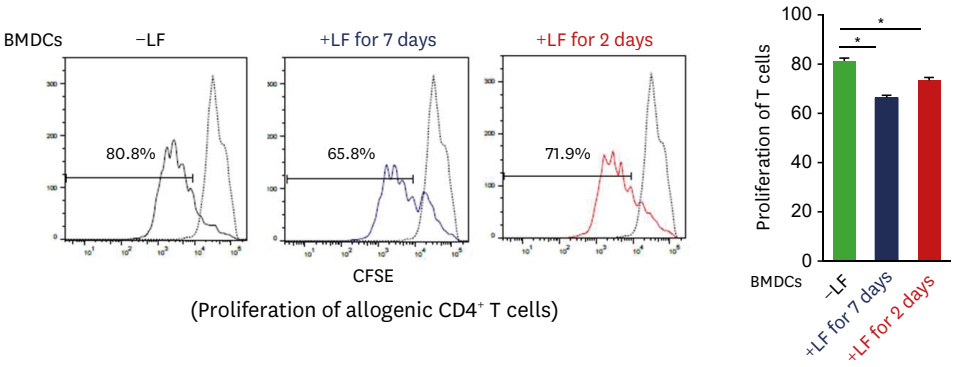

Figure 2. Effect of the suppressive activity of LF-stimulated BMDCs on the proliferation of allogenic CD4⁺ T cells. C57BL/6 BMDCs stimulated with LF (100 μg/ml) for 7 days and 2 days as indicated in **Figure 1A** were irradiated and cultured with CFSE-labeled BALB/c CD4⁺ T cells. T-cell proliferation was determined after 3 days by analyzing CFSE dilution. The data represent the means of 3 independent experiments with SEM (bars).
*$p<0.05$.

CD4⁺ T-cell proliferation. Inhibition of the activities of Arg-1 and IDO-1 substantially abrogated the suppressive ability of LF-BMDCs (**Fig. 4B**), indicating that the suppressive property of LF-BMDCs is, at least in part, attributed to the activities of Arg-1 and IDO-1.

DISCUSSION

The present study demonstrated that LF modulates BMDCs to be tolerogenic, leading to both the suppression of T-cell proliferation and enhancement of iTreg differentiation. We found that costimulatory molecules (CD80 and CD86) and MHC class II were significantly diminished in LF-BMDCs (**Fig. 1**). The reduction of these molecules is a typical property of tolerogenic DCs as shown by others (5,22-24). Similarly, the augmentation of Foxp3⁺ T-cell differentiation by LF-BMDCs is likely attributed to the diminished levels of CD80 and CD86 because treatment with CTLA-Ig further increased the frequency of the OVA-specific Foxp3⁺ T-cell population, whereas the addition of anti-CD28 Ab strongly decreased it (**Fig. 3D**). Costimulatory signals ensure T-cell proliferation via the PI3K-AKT signaling pathway, which is the predominant signaling mechanism for IL-2 production (25). Our observation agrees with the report that strong costimulatory signals inhibit Foxp3 induction and functional Treg differentiation (21,26). We considered whether LF simply suppresses the proliferation of cultured BMDCs; however, the proliferation assay excluded this possibility (data not shown). Furthermore, we found that the suppressive activity of LF-BMDCs was partially attributed to its enhancement of Arg-1 and IDO-1 expression (**Fig. 4**). Similar to our observation, retinoic acid induces Arg-1 expression in DCs, which are involved in the induction of FoxP3⁺ Treg cells (27). Additionally, IDO-1-expressing mature DCs contributed to the differentiation and expansion of Treg cells (28). Therefore, increased expression of known suppressor molecules—e.g., Arg-1 and IDO-1—in LF-BMDCs are likely to be involved in Treg differentiation.

LPS is a well-known stimulus for DC activation through the upregulation of MHC class II, CD80 and CD86 (29). In the present study, LF significantly reduced LPS-stimulated CD80/86 expression. Notably, the present study does not address the specific mechanisms by which LF exerts this inhibitory effect. In this regard, studies that demonstrate the inhibition of LPS binding to LBP and CD14 receptor by LF (30) suggest that LF exerts a tolerogenic effect on DCs through the direct inhibition of LPS binding. On the other hand, IL-10 has been shown

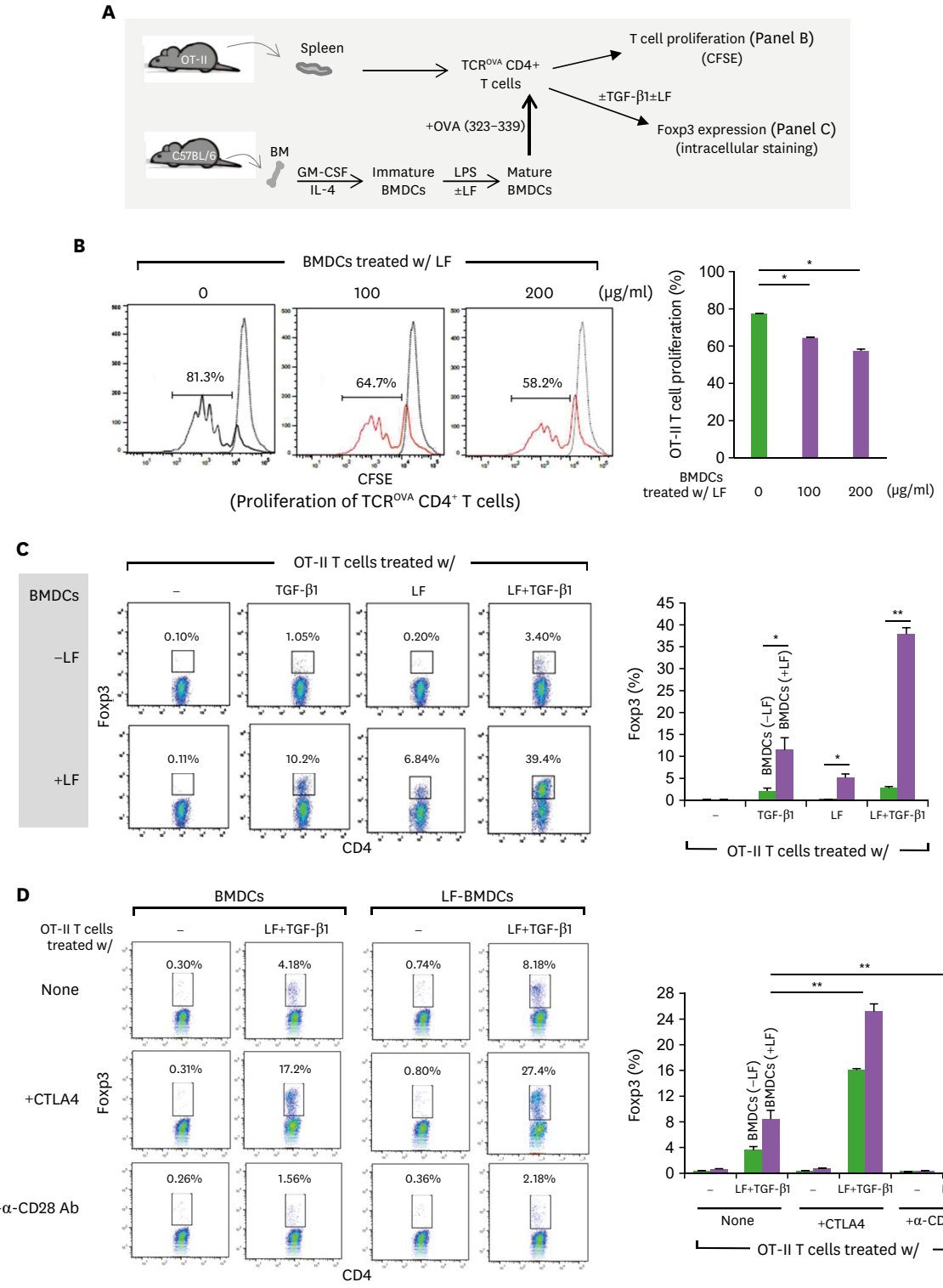

Figure 3. Effect of LF-stimulated BMDCs on the proliferation of OVA-specific CD4⁺ T cells and Treg differentiation.
(A) C57BL/6 immature BMDCs were cultured in the presence of LPS (2 μg/ml) and LF (0, 100, 200 μg/ml) for 2 days. Mature BMDCs were irradiated and pulsed with $OVA_{323-339}$ and then were co-cultured with CFSE-labeled OT-II CD4⁺ T cells for 3 days. (B) T-cell proliferation was determined by analyzing CFSE dilution. The data represent the means of three independent experiments with SEM (bars). (C, D) C57BL/6 BMDCs treated as in panel A were pulsed with $OVA_{323-339}$ and then were co-cultured with OT-II CD4⁺ T cells in the presence of TGF-β1 (1 ng/ml) and LF (100 μg/ml) for 3 days. CTLA-4 Ig (4 μg/ml) and anti-CD28 Ab (2 μg/ml) were added to the cultures as in panel C. The cells were analyzed for surface CD4 and intracellular Foxp3 by FACS. The data represent the means of triplicate samples with SEM (bars). *p<0.05; **p<0.01.

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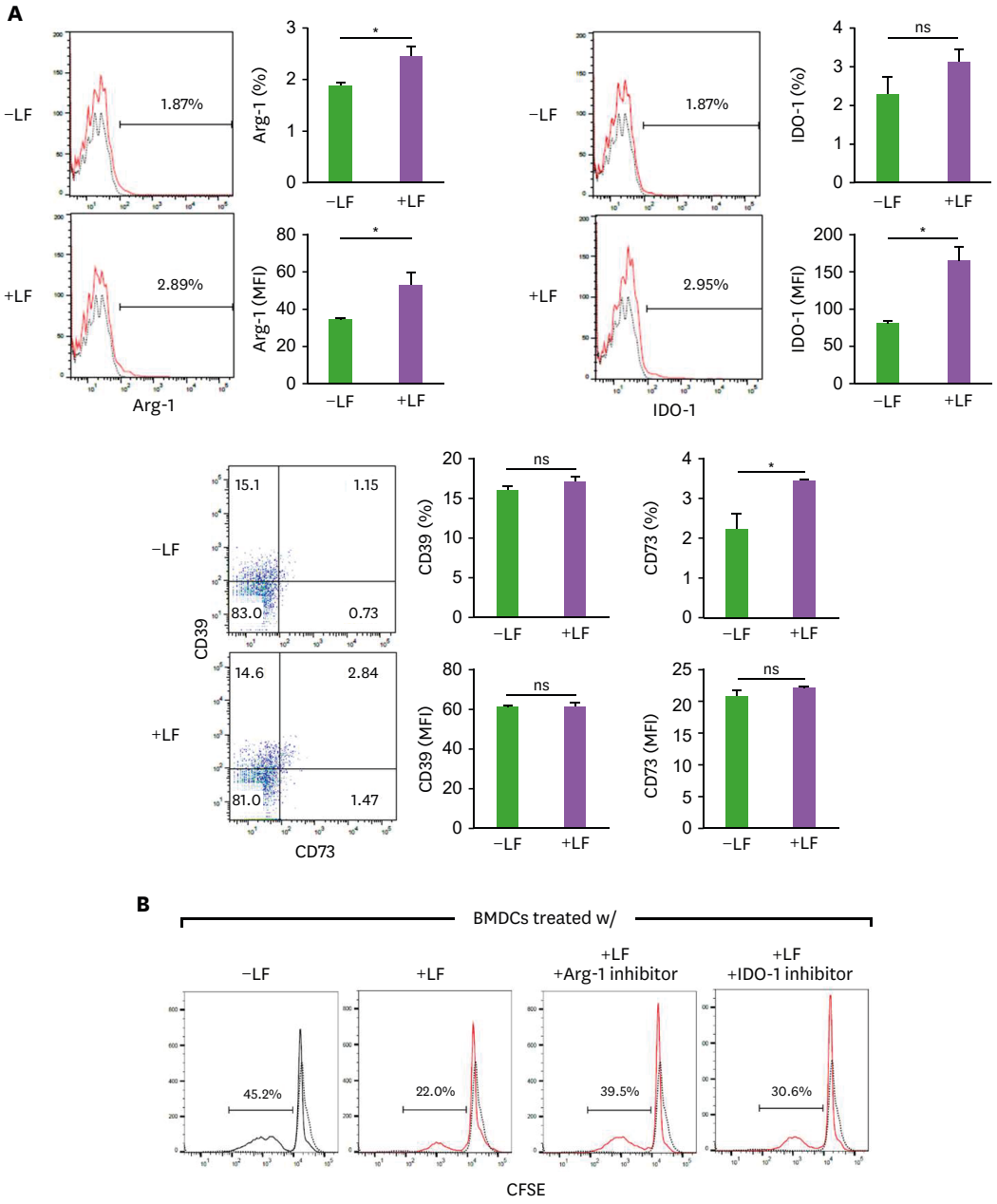

Figure 4. Effect of LF on the expression of known suppressor molecules in tolerogenic DCs and their possible suppressive activity on $CD4^+$ T-cell proliferation. (A) Immature BMDCs were cultured in the presence of LPS (2 μg/ml) and LF (100 μg/ml) for 2 days and then were analyzed for the expression of Arg-1, IDO-1, CD39, and CD73 by FACS. The data represent the average intensities of triplicate samples with SEM (bars). (B) BALB/c BMDCs stimulated with LF (100 μg/ml) for 2 days were irradiated and cultured with CFSE-labeled C57BL/6 $CD4^+$ T cells. Inhibitors of CD39, IDO-1, and Arg-1 were treated for 24 h before co-culture. T-cell proliferation was determined after 3 days by analyzing CFSE dilution.
ns, not significant.
*$p<0.05$.

to enhance the membrane-associated RING-CH 1 (MARCH1)-dependent ubiquitination and degradation pathway, resulting in low MHC class II and CD86 expression on LPS-activated DCs (31). Therefore, we are currently examining whether LF decreases the expression of these molecules via the MARCH1-dependent pathway. Overall, LF-BMDCs may affect the Ag-specific T-cell response via at least two mechanisms—inhibition of T-cell proliferation and increased iTreg differentiation.

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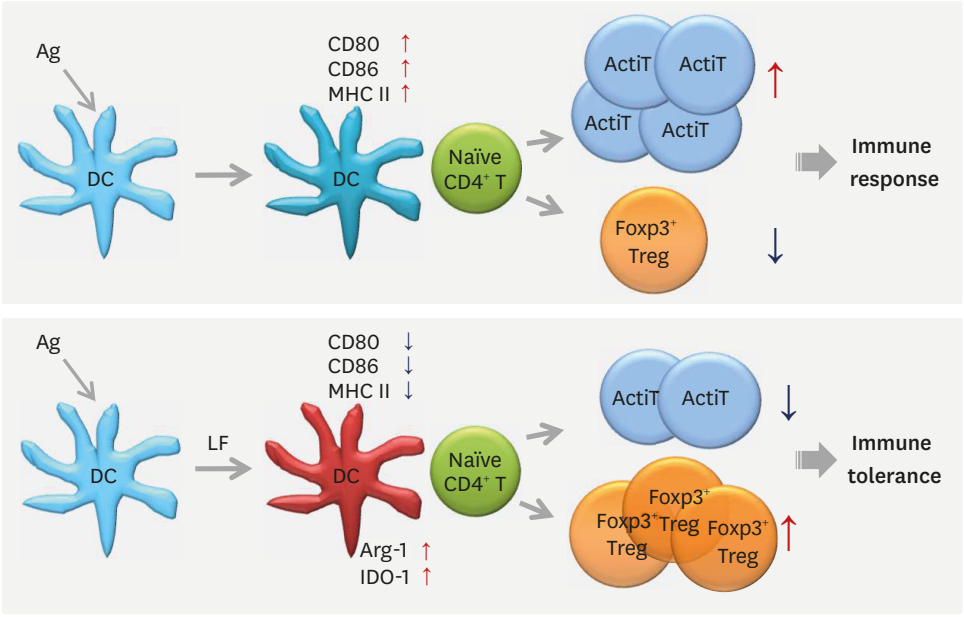

Figure 5. Possible mechanisms by which LF induces tolerogenic DCs.
LF decreases the expression of costimulatory molecules (CD80 and CD86) and MHC class II but increases Arg-1 and IDO-1 simultaneously by unknown mechanisms. In particular, a reduced level of CD86 in LF-BMDCs likely results in the enhancement of Foxp3$^+$ Treg differentiation as well as the suppression of activation/proliferation of CD4$^+$ T cells. Thus, LF likely contributes to T-cell tolerance through acting on DC maturation.
ActiT, activated T cells.

LF is abundant in maternal colostrum and milk (32). We have previously shown that LF and TGF-β1 are important IgA class-switching factors (33,34). Secretory IgA is the single most important defense component in intestinal mucosal tissues, where it acts without inflammation. This phenomenon seems to be closely related to the abundance of Tregs in the gut (35,36). Thus, LF is a key factor for both IgA production and iTreg differentiation, leading to intestinal homeostasis. Furthermore, the major LF-secreting cells are neutrophils during inflammation (37). What is the physiological meaning of this phenomenon? One of the possibilities is that, as inflammation ceases, LF derived from neutrophils may initiate iTreg differentiation for the subsequent tolerogenic state—i.e., the return to homeostasis. This notion remains to be further determined *in vivo*.

In conclusion, lactoferrin decreases the expression of costimulatory molecules (CD80 and CD86) and MHC class II and increases Arg-1 and IDO-1, leading to the diminishment of T-cell clonal expansion and enhancement of iTreg differentiation (**Fig. 5**). In addition, lactoferrin directly forces T cells to be iTregs (18,38). Taken together, our current findings as well as those of others imply that lactoferrin is a good candidate to treat autoimmune disorders.

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