A THESIS FOR THE DEGREE OF MASTER

Immunomodulatory effect of canine peripheral blood mononuclear cell-derived B lymphocytes pretreated with lipopolysaccharide through macrophage polarization

Lipopolysaccharide로 전처리한 개 말초혈액 단핵세포 유래 B 림프구에 의한 대식세포 분극화 유도를 통한 면역 조절 효과

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지도교수 윤 화 영

이 논문을 수의학 석사 학위논문으로 제출함

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Abstract

Immunomodulatory effect of canine peripheral blood mononuclear cell-derived B lymphocytes pretreated with lipopolysaccharide through macrophage polarization

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Regulatory B cells (Bregs), the specific subset of B cells, downregulate inflammation and autoimmunity. It has been studied secreting interleukin (IL)-10 is the key contributor of Bregs and preconditioning B cells with lipopolysaccharide (LPS) increases IL-10 protein production and secretion. Therefore, various studies are conducted with the goal of applying IL-10 overexpressing B cells as therapeutic agents to patients with immune-related inflammatory diseases, but there are only a few studies on canine B cells. This study aimed to investigate the immune regulatory gene expression of canine peripheral blood mononuclear cell (PBMC)-derived B cells pretreated with LPS, and evaluate the anti-inflammatory and immunomodulatory capacity of primed B cell by identifying anti-inflammatory macrophage M2

polarization when cocultured with primed B cells.

Canine B cells were isolated from canine PBMCs, which were obtained from three healthy canine donors. The B cells were treated with 5 ng/mL, 10 ng/mL of LPS to evaluate the cytotoxicity of LPS on B cells by CCK-8 assay. Then expression marker of Bregs such as IL-10 was investigated by flow cytometry, and determined the immunomodulatory capacity of primed B cells, by investigating the gene expression of immunoregulatory factors such as *IL-10*, *programmed death-ligand 1 (PD-L1)*, and *transforming growth factor beta (TGF-β)* using real-time quantitative PCR (RT-qPCR). Moreover, LPS pretreated macrophage cell lines (RAW 264.7 and DH82 cell line) were co-cultured with primed B cells to assess immunomodulatory effect of primed B cell on macrophages in inflammatory condition. Macrophages were pretreated with LPS to induce inflammatory condition. After RNA extraction from macrophages, it was investigated for immune condition, and M1, M2 macrophage markers, and to investigate M2 polarization in protein level, immunofluorescence analysis was performed, using CD206 as M2 marker protein.

From cell viability assay, confirmed that LPS have no cytotoxicity on B cells. Bregs expression marker IL-10 was increased significantly by 2.32-fold in LPS 5 ng/mL group and increased significantly by 2.64-fold in LPS 10 ng/mL group, in comparison with control group. The gene expression of immunoregulatory factors in LPS 5 ng/mL group, only *IL-10* showed 3.07-fold significant increase ($P < 0.05$) compared to control group and in LPS 10 ng/mL group, *IL-10*, *PD-L1*, and *TGF-β*, respectively, showed 8.75-fold ($P < 0.001$), 2.46-fold ($P < 0.05$), 2.47-fold ($P < 0.05$) significant increase compared to control group. Since the effect of LPS treatment was higher in LPS 10 ng/mL group, it was chosen for further experiment.

The immune condition of macrophages when co-cultured with primed B cells compared to the LPS-treated group, in RAW 264.7 cell line, showed 0.04-fold decrease in proinflammatory cytokine *TNF-α* and 8.21-fold increase in anti-inflammatory cytokine *IL-10* and in DH82 cell line, showed 0.26-fold decrease in *TNF-α* and 12.39-fold increase *IL-10*, respectively ($P < 0.001$). Additionally, the changes in M1 macrophage marker *iNOS* and M2 macrophage marker *CD206* compared to the LPS-treated group, in RAW 264.7 cell line, showed 0.03-fold decrease in *iNOS* ($P < 0.01$) and 7.97-fold increase in *CD206* ($P < 0.001$) and in DH82 cell line, showed 0.11-fold decrease in *iNOS* ($P < 0.01$) and 8.72-fold increase in *CD206* ($P < 0.05$), respectively. In protein level, macrophages expressing CD 206 were increased 6.5-fold in RAW 264.7 cell line and 14.16-fold in DH82 cell line in comparison with LPS-stimulated macrophages, respectively ($P < 0.001$).

This study revealed that pretreatment of LPS on canine PBMC-derived B cells induced IL-10 overexpressing B cells, and that LPS-primed canine B cells have an anti-inflammatory and immune modulation effect by polarizing macrophages to M2 phenotype, suggesting the possibility of using LPS-primed B cells as a therapeutic agent for its capacity in canine immune related inflammatory diseases.

Keywords: regulatory B cell, macrophage, anti-inflammatory agent, inflammatory disease, M2 macrophage

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1. Introduction

B cells perform several immunological functions, and because of their antigen presenting and antibody producing capacity, they have been primarily regarded as positive regulators of the immune response and central contributors to the development of immune-related diseases. However, specific regulatory B cells (Bregs) subset, reversely, downregulate adaptive and innate immunity, inflammation, and autoimmunity. Multiple mechanisms are involved, it has been revealed by various studies that the anti-inflammatory effect of B cells is achieved by the expression of interleukin (IL)-10 [1, 2]. Moreover, there are multiple studies about relationship between presence of B cells and reduced disease severity in autoimmune diseases [3, 4].

IL-10 is an anti-inflammatory cytokine that plays an important role in controlling the inflammatory response that causes tissue damage [5]. In addition, IL-10 plays an inhibitory role primarily by limiting the innate effector function of macrophages and dendritic cells and subsequent activation of T cells [6]. Therefore, B cells producing IL-10 are attracting attention as a pioneering cell therapy, and various studies are being conducted to apply IL-10-producing B cells as therapeutic agent for immunomodulatory treatments [7]. Therefore, to develop more effective cell therapy products, various studies are in progress to overexpress IL-10 in B cells [8, 9]. According to Parekh et al., when pre-stimulating B cells with lipopolysaccharide (LPS), resulted in transcriptional activation of the IL-10 gene, and IL-10 protein production and secretion increased [10]. However, in order for

LPS-primed B cells to be applied as a therapeutic agent to patients with inflammatory diseases and immune diseases, additional studies are needed to evaluate the mechanism by which IL-10-overexpressed B cells have anti-inflammatory effects.

Macrophage, which belongs to the innate immune system, is recognized to be involved in chronic inflammation, and it plays central roles in inflammatory diseases [11]. Many studies have been conducted to understand the mechanisms involved in activation of macrophage and to relate them to macrophage function. The well-known bipolar model [12] distinguishes macrophage into two main types of polarization: the classically activated type 1 macrophage (M1, pro-inflammatory type) and the alternatively activated type 2 macrophage (M2, anti-inflammatory type) [13]. Thus, in inflammatory disorders, down-regulating M1, or re-polarizing M1 to M2 are the two major approaches to relieve inflammation [14].

Preconditioning with LPS is used to improve the secretion of anti-inflammatory agents in B cells and the anti-inflammatory effects of B cells are mainly involved with IL-10. Therefore, various studies are being conducted with the goal of applying IL-10 overexpressing B cells as therapeutic agents to patients with immune-related inflammatory diseases, but there are only a few studies on canine B cells. Therefore, this study aimed to investigate the immune regulatory gene expression, especially Il-10, of canine peripheral blood mononuclear cell-derived B cells pretreated with LPS, and evaluate the anti-inflammatory and immunomodulatory capacity of primed B cell by identifying anti-inflammatory macrophage M2 polarization when cocultured with primed B cells.

2. Materials and Methods

2.1. B cell isolation

The canine B cells were isolated from canine peripheral blood mononuclear cells (cPBMCs). To isolate cPBMCs, all experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (protocol no. SNU-201228-4). cPBMCs were collected using Ficoll-Paque PREMIUM (GE Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer's instruction. With the owners' consent, whole blood samples were obtained from three healthy canine donors. The blood samples were blended with an equal volume of Dulbecco phosphate buffered salt (DPBS) solution DPBS (Pan Biotech, Aidenbach, Germany) and placed on Ficoll-Paque PREMIUM according to the manufacturer's guidelines.

B cells were isolated using mouse CD79a antibody (dilution, 1:200; Invitrogen, Carlsbad, CA), anti-mouse immunoglobulin (Ig)-G microbeads (Miltenyi Biotec, Audurn, CA) with MACS (Miltenyi Biotec, Audurn, CA) according to the manufacturer's instruction. A mixture of 10% fetal bovine serum (FBS) (Pan Biotech) and phosphate buffered saline (PBS) were used for the MACS separation buffer. The CD79a antibody was added to the cPBMCs, which were then incubated for 2 hours at 37℃ in a humidified atmosphere with 5% carbon dioxide. The CD79a+ B cells were collected after centrifugation at 780 × *g* for 10 minutes. For magnetic labeling, 10 μl of anti-mouse IgG microbead and 40 μl of the MACS separation buffer were

added to the CD79a+ B cells, which were incubated for 2 hours. The CD79a+ B cells were collected after centrifugation at 780 × *g* for 10 minutes. After washing the LS column of MACS with 3 mL of MACS separation buffer, the CD79a+ B cells were added to the LS column for isolation. After another washing with 3 mL of MACS separation buffer, the CD79a+ B cells were collected in 5 mL of MACS separation buffer. The B cell population obtained out of total cPBMCs were at the mean of 5%.

2.2. Preconditioning B cells with Lipopolysaccharide (LPS)

To stimulate the B cells with LPS (Sigma-Aldrich, St. Louis, MO), the cells were plated at a density of 1×10^5 cells/mL in six-well plates. The medium used was the control, which did not contain LPS, and 5 ng/mL of LPS and 10 ng/mL of LPS were added to the experimental B cell groups. The cells were cultured for 24 hours for stimulation. Since the effects of LPS occurred in a dose-dependent manner, 10 ng/mL LPS-primed B cells were chosen for further study.

2.3. Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay (D-Plus™ CCK cell viability assay kit; Dong-in Biotech, Seoul, Republic of Korea) to confirm that the concentrations of LPS were not cytotoxic for the B cells. The cells were seeded at a density of 5×10^5 cells/well in a 96-well plate. The medium used was the control, which did not contain LPS, and 5 ng/mL of LPS and 10 ng/mL of LPS were added to the experimental B cell groups. After stimulating the cells for

24 hours, 10 μL of the Cell CCK-8 solution was added, and the cells were incubated in the dark at 37°C for 1 hour. The absorbance at a 450-nm wavelength was determined with a spectrophotometer (Epoch Microplate Spectrophotometer; BioTek Instruments, Winooski, VT).

2.4. Flow cytometry analysis

LPS-primed B cells were obtained as described above, and then used the IL-10 antibody (dilution, 1:100; ABclonal, Woburn, MA) to evaluate the expression of the regulatory B cell marker. After incubation for 1 hour, the cells were washed with DPBS. Indirect immunofluorescence was performed using mouse anti-rabbit IgG-PE (dilution, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Unstained cells were used as the negative control. Cell fluorescence was analyzed with a flow cytometer (FACS Aria II; BD Biosciences, Franklin Lakes, NJ). The results were analyzed using FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR).

2.5. Co-culture system

RAW 264.7 cells, the murine macrophage cell line, and DH82 cells, the canine macrophage cell line, were purchased from the Korean Cell Line Bank (Seoul, Korea). Both cells were seeded in six-well plates at a density of 1×10^6 cells/well and incubated overnight. After adherence to the plates was confirmed, 200 ng/mL of LPS was added for 24 hours. Next, the medium was removed, the cells were washed three times with DPBS and then replaced with the control medium. Using 0.4-μm

pore size inserts, naïve and primed B cells were plated onto the macrophage cells at a density of 1×10^5 cells/well at the ratio of 10:1. The total number of cells were divided into four groups: macrophage cells at the bottom and no insert, macrophage in the inflammatory condition at the bottom and no inserts, macrophage in the inflammatory condition and naïve B cells in the upper chamber, and macrophage in the inflammatory condition and LPS-primed B cells in the upper chamber. All cells were incubated for 48 hours and then harvested for RNA extraction and immunofluorescence analysis.

2.6. RNA extraction, cDNA synthesis, and real-time quantitative PCR (RT-qPCR)

The Easy-BLUE Total RNA Extraction Kit (Intron Biotechnology, Seongnam, Korea) was used to isolate RNA according to the manufacturer's instructions. For each sample, total RNA concentration was measured at 260-nm absorbance using a nanophotometer (IMPLEN, Munich, Germany). Complementary (c)-DNA was synthesized using Cell Script All-in-One 5× 1st cDNA Strand Synthesis Master Mix (Cell Safe, Seoul, Korea), and the samples were detected using AMPIGENE qPCR Green Mix Hi-ROX with SYBR Green dye (Enzo Life Sciences, Farmingdale, NY) and forward and reverse primers (Bionics, Seoul, Korea). The expression levels of each gene were normalized to that of glyceraldehyde 3-phosphate dehydrogenase. The primer sequences used in this study are listed in Tables 1 and 2. The mRNA expression levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.7. Immunofluorescence analysis

RAW 264.7 and DH82 cells were washed three times with DPBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature. After washing with DPBS, the cells were permeabilized for 1 hour with 0.2% Triton X-100 (Sigma-Aldrich) and then blocked for 1 hour at room temperature with 2% FBS. The cells were incubated sequentially with FITC-conjugated CD206 (1:200; Santa Cruz Biotechnology) and PE-conjugated CD11b antibodies (1:200; Abcam, Cambridge, MA) at 4°C overnight in the dark. Finally, the cells were washed three times with PBS and mounted. The samples were observed using an EVOS FL microscope (Life Technologies, Darmstadt, Germany). Immunoreactive cells were counted in 20 random fields per group, and the percentage of CD206+ positive cells was recorded.

2.8. Statistical analysis

GraphPad Prism version 6.01 software (GraphPad Software, La Jolla, CA) was used to perform the statistical analysis. The Student t-test and one-way analysis of variance were used to analyze the data, followed by the Bonferroni multiple comparison test. The data are presented as mean value ± standard deviation. Differences with a P-value < 0.05 were considered statistically significant.

3. Results

3.1. LPS-stimulated B cells

CCK-8 assay was performed to evaluate the cytotoxicity of 5 ng/mL LPS and 10 ng/mL LPS to B cells. The result showed there was no significant difference between the control and experimental groups (Figure 1A). After stimulation with LPS, both 5 ng/mL LPS and 10 ng/mL LPS-stimulated B cell groups were determined to express IL-10, which is known as a regulatory B cell expression marker, based on flow cytometry analysis (Figure 1B). In addition, the 10 ng/mL LPS-stimulated group expressed significantly increased immunomodulatory factors, such as *IL-10*, *programmed death-ligand 1 (PD-L1)*, and *transforming growth factor beta (TGF-β)* and the 5 ng/mL LPS-stimulated group showed significant increase in only *IL-10* (Figure 2).

3.2. Anti-inflammatory effect of the primed B cells

The result of RT-qPCR showed that after LPS stimulation of macrophages, proinflammatory cytokine *TNF-α* expression levels were significantly increased, and they were decreased after co-culture in both the naïve and LPS-primed B cell groups. This result was more significant in the co-cultured with LPS-primed B cell group than in the naïve B cell group. Moreover, the level of anti-inflammatory cytokine *IL-10* expression was increased after co-culture in both B cell groups, and this result

was more significant after co-culture in the LPS-primed B cell group than in the naïve B cell group. All these results were observed in the RAW 264.7 and DH82 cell lines (Figure 3A, 3B). Therefore, these results indicated that primed B cells have anti-inflammatory effects on macrophages in inflammatory condition.

3.3. Macrophage polarization from M1 to M2

As target genes, *CD206* for the M2 macrophage and *iNOS* for the M1 macrophage were used for RT-qPCR in RAW 264.7 and DH82 cells (Figure 4A, 4B). The results showed that *iNOS* expressions were highly increased after LPS stimulation, and they were decreased significantly after co-culture with LPS-primed B cells. The *CD206* expressions were significantly increased after co-culture with LPS-primed B cells. For immunofluorescence assay, CD206 was used for M2 macrophage marker protein. The results showed that in both RAW 264.7 and DH82 cells, the percentage of CD206+ M2 macrophages was increased in both naïve and LPS-primed B cell co-cultured groups, and this finding was more significant in the LPS-primed B cell group than in the naïve B cell co-culture group (Figure 5A, 5B, 5C, 5D). These results suggest that LPS-primed B cells have an ability to repolarize the M1 macrophage to the M2 macrophage.

4. Discussion

Cell therapy is the most recent phase of biotechnology in medicine, and it offers the advantage of treating and altering the course of diseases, which cannot be addressed by current pharmaceutical techniques [15]. B cells are generally considered to play a pathogenic role in the development of autoimmune diseases because they produce autoantibodies that cause target tissue damage, but autoantibodies can also exert a protective effect through the elimination of apoptotic cells and reduction of autoantigen load [16]. B cells also act as antigen-presenting cells that contribute to the activation and amplification of naive and activated autoreactive T cell responses [8]. However, in order to apply B cells as cell therapy to patients with various autoimmune diseases, including rheumatoid arthritis, autoimmune diabetes, autoimmune encephalomyelitis and lupus, additional studies about the treatment mechanisms are needed [3].

LPS, which a major component of environmental microbial products, is one of the most well-studied pathogen-associated molecular patterns that can induce innate immune recognition. It signals through the toll-like receptor 4 (TLR-4) [17], and interacts with dendritic cells, macrophages, and B cells [18-21]. In B cells, activation of pattern recognition receptors, especially members of the toll-like receptor family, has been shown to be an effective stimulus to induce IL-10 production. In these cells, stimulation of TLR-4 results in transcriptional activation of the IL-10 gene, increasing IL-10 protein production and secretion. According to Xu et al., when B cells were pre-stimulated with LPS, the expression of IL-10 was

increased [22]. In this study, firstly, to evaluate the cytotoxicity of LPS on B cells, CCK-8 assay was performed, which allows sensitive colorimetric assays using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, which is reduced by dehydrogenase activities in cells to give orange-colored formazan, for the determination of cell viability, and the result showed there was no difference in control group and LPS-treated group, confirming that LPS have no cytotoxicity on B cells. Then confirmed that the number of cells expressing IL-10 increased when B cells were pretreated with LPS [9]. In addition, LPS-primed B cells expressed regulatory B cell markers, such as IL-10, PD-L1, and TGF-β [23, 24].

B cells that produce IL-10, TGF-β and express PD-L1 are known as regulatory B cells (Bregs) in the B cell subset [23, 25, 26], and Bregs have the effect of suppressing activated immunity [7]. Therefore, various studies are being conducted with the goal of applying B cells as therapeutic agents to patients with immune-related inflammatory diseases. However, there has been no research on this in veterinary medicine. Moreover, multiple mechanisms are involved, but secreting IL-10 is the key contributor of Bregs, which is considered the master negative regulator of inflammation [27]. As described above, IL-10 is an anti-inflammatory cytokine that plays an inhibitory role by limiting the innate effector function of macrophages and dendritic cells and subsequent activation of T cells [6]. However, in order to apply IL-10-overexpressing B cells as a therapeutic agent to patients with immune-related inflammatory diseases, it is necessary to determine how IL-10-overexpressing B cells regulate immune cells in inflammatory conditions. In this study, pretreating 10 ng/mL LPS showed no cytotoxicity on B cells, and induced specific B cells which expresses significantly upregulated gene expression of

immunomodulatory factor. However, in order to apply IL-10 overexpressing B cells in anti-inflammatory and immunomodulatory therapy, for its capacity of immune cell regulation, additional studies are needed.

In the inflammatory condition, M1 is responsible for active inflammation, such as the expression of high levels of pro-inflammatory cytokines, enhanced phagocytosis and assistance with T-helper type 1 cells, whereas M2 is responsible for immune modulation and wound repair functions [28]. Currently, strategies to reduce M1 or re-polarization of M1 to M2 have been studied, and re-educating M1 to M2 can be beneficial in not only decreasing pro-inflammatory effects but also inducing anti-inflammatory effects as well. Moreover, there are studies about the requirement of IL-10 for macrophage M2 polarization [29]. In the present study, RAW 264.7 and DH82 macrophages in inflammatory conditions were co-cultured LPS-stimulated B cells, which expresses higher IL-10 than control, and confirmed the effect of primed B cells on re-polarizing M1 to M2 [30, 31]. Considering that changing macrophages from M1 to M2 in various inflammatory diseases is a major therapeutic target to suppress activated immunity, these results may serve as the supporting data for future studies on the applying primed B cell as an immunotherapy.

In conclusion, it was confirmed that the expression of IL-10 increased when B cells were pretreated with 10 ng/mL of LPS for 24 hours. In addition, this data suggest that IL-10-overexpressed B cells play an important role in suppressing inflammation though macrophage polarization from M1 to M2. This study could serve as a basis for future in vivo studies on the anti-inflammatory effects of LPS-primed B cells, and clinical applications of canine B cells may become a new option of cell therapy for refractory inflammatory diseases.

5. Conclusion

It was demonstrated that LPS-stimulated B cells derived from canine peripheral blood mononuclear cells showed anti-inflammatory effect, and have immunomodulatory effect on macrophages, by re-polarizing M1 macrophages to M2 macrophages. This novel study could play a role as a basis for clinical applications of anti-inflammatory therapy.

6. References

1. Murphy K, Weaver C: **Janeway's immunobiology**: Garland Science; 2016:643-652.
2. Cassell DJ, Schwartz RH: **A quantitative analysis of antigen-presenting cell function: activated B cells stimulate naive CD4 T cells but are inferior to dendritic cells in providing costimulation**. *The Journal of Experimental Medicine* 1994, **180** (5):1829-1840.
3. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM: **B cells regulate autoimmunity by provision of IL-10**. *Nature Immunology* 2002, **3** (10):944-950.
4. Mizoguchi E, Mizoguchi A, Preffer FI, Bhan AK: **Regulatory role of mature B cells in a murine model of inflammatory bowel disease**. *International Immunology* 2000, **12** (5):597-605.
5. Oberholzer A, Oberholzer C, Moldawer LL: **Interleukin-10: a complex role in the pathogenesis of sepsis syndromes and its potential as an anti-inflammatory drug**. *Critical Care Medicine* 2002, **30** (1):S58-S63.
6. Soukou S, Brockmann L, Bedke T, Gagliani N, Flavell RA, Huber S: **Role of IL-10 receptor signaling in the function of CD4+ T-regulatory type 1 cells: T-cell therapy in patients with inflammatory bowel disease**. *Critical Reviews™ in Immunology* 2018, **38** (5):415-431.
7. Bankó Z, Pozsgay J, Szili D, Tóth M, Gáti T, Nagy G, Rojkovich B, Sármay G: **Induction and differentiation of IL-10–producing regulatory B cells**

from healthy blood donors and rheumatoid arthritis patients. *The Journal of Immunology* 2017, **198** (4):1512-1520.

8. Stanic B, Van De Veen W, Wirz OF, Rückert B, Morita H, Söllner S, Akdis CA, Akdis M: **IL-10–overexpressing B cells regulate innate and adaptive immune responses**. *Journal of Allergy and Clinical Immunology* 2015, **135** (3):771-780.

9. Burdin N, Rousset F, Banchereau J: **B-cell-derived IL-10: production and function**. *Methods* 1997, **11** (1):98-111.

10. Parekh VV, Prasad DV, Banerjee PP, Joshi BN, Kumar A, Mishra GC: **B cells activated by lipopolysaccharide, but not by anti-Ig and anti-CD40 antibody, induce anergy in CD8+ T cells: role of TGF-β1**. *The Journal of Immunology* 2003, **170** (12):5897-5911.

11. Wynn TA, Chawla A, Pollard JW: **Macrophage biology in development, homeostasis and disease**. *Nature* 2013, **496** (7446):445-455.

12. De Palma M, Lewis CE: **Macrophage regulation of tumor responses to anticancer therapies**. *Cancer Cell* 2013, **23** (3):277-286.

13. Ponzoni M, Pastorino F, Di Paolo D, Perri P, Brignole C: **Targeting macrophages as a potential therapeutic intervention: impact on inflammatory diseases and cancer**. *International Journal of Molecular Sciences* 2018, **19** (7):e1953.

14. Hu G, Guo M, Xu J, Wu F, Fan J, Huang Q, Yang G, Lv Z, Wang X, Jin Y: **Nanoparticles targeting macrophages as potential clinical therapeutic agents against cancer and inflammation**. *Frontiers in Immunology* 2019, **10**:e1998.

15. Mount NM, Ward SJ, Kefalas P, Hyllner J: **Cell-based therapy technology classifications and translational challenges**. *Philosophical Transactions of the Royal Society B: Biological Sciences* 2015, **370** (1680):e20150017.

16. Bouaziz JD, Yanaba K, Tedder TF: **Regulatory B cells as inhibitors of immune responses and inflammation**. *Immunological Reviews* 2008, **224** (1):201-214.

17. Dauphinee SM, Karsan A: **Lipopolysaccharide signaling in endothelial cells**. *Laboratory Investigation* 2006, **86** (1):9-22.

18. Lu Y-C, Yeh W-C, Ohashi PS: **LPS/TLR4 signal transduction pathway**. *Cytokine* 2008, **42** (2):145-151.

19. Minguet S, Dopfer EP, Pollmer C, Freudenberg MA, Galanos C, Reth M, Huber M, Schamel WW: **Enhanced B-cell activation mediated by TLR4 and BCR crosstalk**. *European Journal of Immunology* 2008, **38** (9):2475-2487.

20. Tripathy A, Khanna S, Padhan P, Smita S, Raghav S, Gupta B: **Direct recognition of LPS drive TLR4 expressing CD8+ T cell activation in patients with rheumatoid arthritis**. *Scientific Reports* 2017, **7** (1):1-10.

21. Mobarak E, Håversen L, Manna M, Rutberg M, Levin M, Perkins R, Rog T, Vattulainen I, Borén J: **Glucosylceramide modifies the LPS-induced inflammatory response in macrophages and the orientation of the LPS/TLR4 complex in silico**. *Scientific Reports* 2018, **8** (1):1-11.

22. Xu H, Liew LN, Kuo IC, Huang CH, Goh DLM, Chua KY: **The modulatory effects of lipopolysaccharide-stimulated B cells on differential T-cell polarization**. *Immunology* 2008, **125** (2):218-228.

23. Guan H, Wan Y, Lan J, Wang Q, Wang Z, Li Y, Zheng J, Zhang X, Wang Z, Shen Y: **PD-L1 is a critical mediator of regulatory B cells and T cells in invasive breast cancer**. *Scientific Reports* 2016, **6** (1):1-10.

24. Lee KM, Stott RT, Zhao G, SooHoo J, Xiong W, Lian MM, Fitzgerald L, Shi S, Akrawi E, Lei J: **TGF-β-producing regulatory B cells induce regulatory T cells and promote transplantation tolerance**. *European Journal of Immunology* 2014, **44** (6):1728-1736.

25. Mauri C, Menon M: **Human regulatory B cells in health and disease: therapeutic potential**. *The Journal of Clinical Investigation* 2017, **127** (3):772-779.

26. Siewe B, Stapleton JT, Martinson J, Keshavarzian A, Kazmi N, Demarais PM, French AL, Landay A: **Regulatory B cell frequency correlates with markers of HIV disease progression and attenuates anti-HIV CD8+ T cell function in vitro**. *Journal of Leukocyte Biology* 2013, **93** (5):811-818.

27. Peng B, Ming Y, Yang C: **Regulatory B cells: the cutting edge of immune tolerance in kidney transplantation**. *Cell Death & Disease* 2018, **9** (2):1-13.

28. Geng Y, Hardie J, Landis RF, Mas-Rosario JA, Chattopadhyay AN, Keshri P, Sun J, Rizzo EM, Gopalakrishnan S, Farkas ME: **High-content and high-throughput identification of macrophage polarization phenotypes**. *Chemical Science* 2020, **11** (31):8231-8239.

29. Lopes RL, Borges TJ, Zanin RF, Bonorino C: **IL-10 is required for polarization of macrophages to M2-like phenotype by mycobacterial DnaK (heat shock protein 70)**. *Cytokine* 2016, **85**:123-129.

30. Davis MJ, Tsang TM, Qiu Y, Dayrit JK, Freij JB, Huffnagle GB, Olszewski MA: **Macrophage M1/M2 polarization dynamically adapts to changes in cytokine microenvironments in *Cryptococcus neoformans* infection**. *MBio* 2013, **4** (3):e0026413.

31. Kambara K, Ohashi W, Tomita K, Takashina M, Fujisaka S, Hayashi R, Mori H, Tobe K, Hattori Y: ***In vivo* depletion of CD206+ M2 macrophages exaggerates lung injury in endotoxemic mice**. *The American Journal of Pathology* 2015, **185** (1):162-171.

32. Li Q, Han S-M, Song W-J, Park S-C, Ryu M-O, Youn H-Y: **Anti-inflammatory effects of Oct4/Sox2-overexpressing human adipose tissue-derived mesenchymal stem cells**. *In Vivo* 2017, **31** (3):349-356.

33. An J-H, Li Q, Bhang D-H, Song W-J, Youn H-Y: **TNF-α and INF-γ primed canine stem cell-derived extracellular vesicles alleviate experimental murine colitis**. *Scientific Reports* 2020, **10** (1):1-14.

34. Jung YC, Kim ME, Yoon JH, Park PR, Youn H-Y, Lee H-W, Lee JS: **Anti-inflammatory effects of galangin on lipopolysaccharide-activated macrophages via ERK and NF-κB pathway regulation**. *Immunopharmacology and Immunotoxicology* 2014, **36** (6):426-432.

35. Salih SA, Kim H-R: **Anti-inflammatory effect of an ethanolic extract of *Myagropsis yendoi* in lipopolysaccharide-stimulated BV-2 microglia cells**. *Fisheries and Aquatic Sciences* 2014, **17** (1):27-35.

36. Park S-M, Li Q, Ryu M-O, Nam A, An J-H, Yang J-I, Kim S-M, Song W-J, Youn H-Y: **Preconditioning of canine adipose tissue-derived mesenchymal stem cells with deferoxamine potentiates anti-**

inflammatory effects by directing/reprogramming M2 macrophage polarization. *Veterinary Immunology and Immunopathology* 2020, **219**:e109973.

37. Cao J, Lu X, Gao F, Zhang X, Xia X, Sun H: **Assessment of neutrophil gelatinase-associated lipocalin as an early biomarker for canine renal ischemia-reperfusion injury**. *Annals of Translational Medicine* 2020, **8** (22):e1491.

38. Kang JW, Kang K-S, Koo HC, Park JR, Choi EW, Park YH: **Soluble factors–mediated immunomodulatory effects of canine adipose tissue–derived mesenchymal stem cells**. *Stem Cells and Development* 2008, **17** (4):681-694.

39. Song R, Kim J, Yu D, Park C, Park J: **Kinetics of IL-6 and TNF-α changes in a canine model of sepsis induced by endotoxin**. *Veterinary Immunology and Immunopathology* 2012, **146** (2):143-149.

40. Hartley G, Faulhaber E, Caldwell A, Coy J, Kurihara J, Guth A, Regan D, Dow S: **Immune regulation of canine tumour and macrophage PD-L1 expression**. *Veterinary and Comparative Oncology* 2017, **15** (2):534-549.

41. Kim M, Kim Y, Lee S, Kuk M, Kim AY, Kim W, Kweon O-K: **Comparison of viability and antioxidant capacity between canine adipose-derived mesenchymal stem cells and heme oxygenase-1-overexpressed cells after freeze-thawing**. *Journal of Veterinary Medical Science* 2016: **78** (4):619-25.

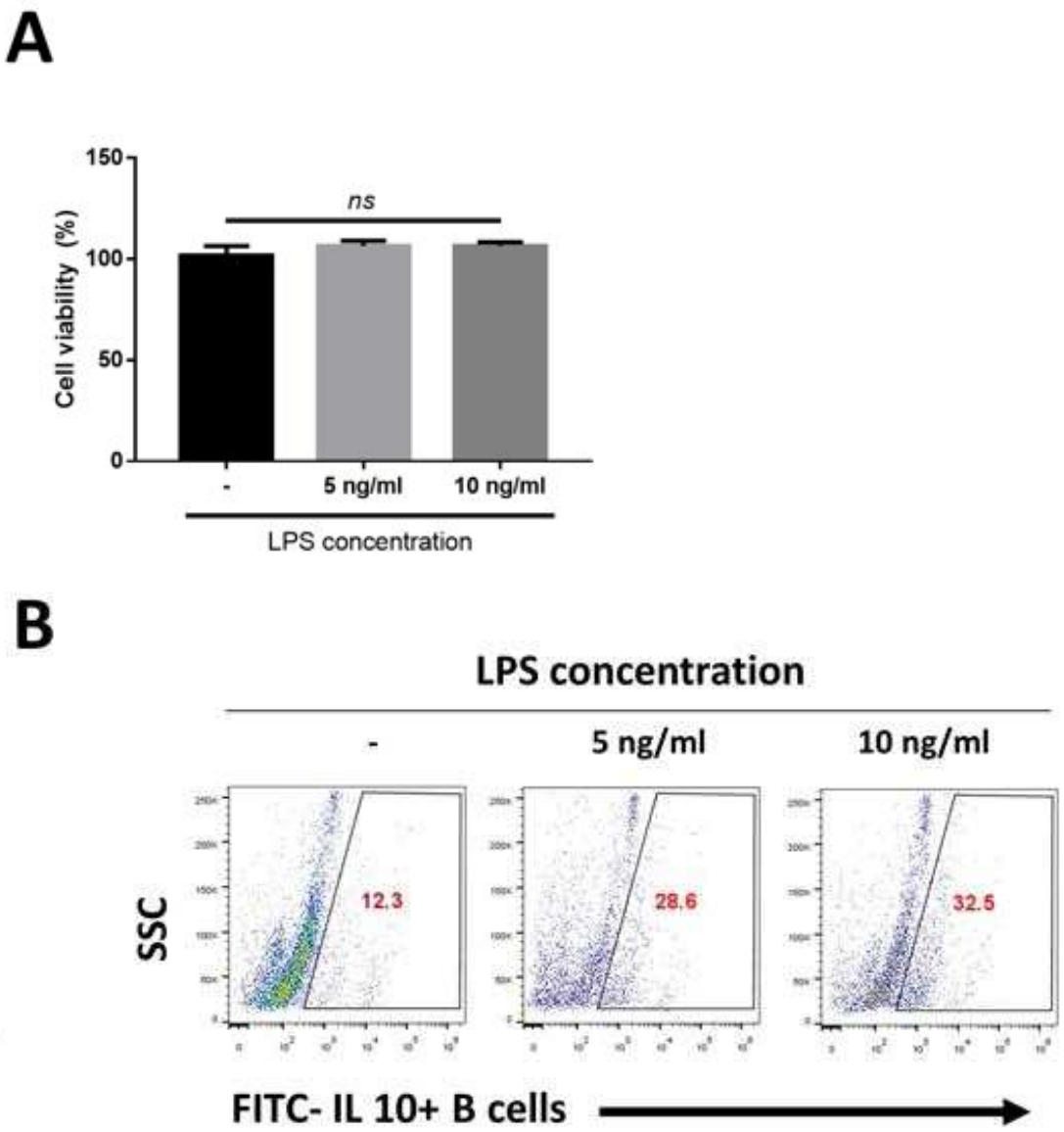

Figure 1. Characterization of B cell isolated from canine peripheral blood mononuclear cells. A. Cell viability assay. Cell viability of B cells according to the LPS concentration gradient was confirmed through CCK-8 analysis. **B**. Through flow cytometry, the degree of increase in B cells expressing IL-10 was confirmed. Representative of three independent experiments with similar results. Data are shown as mean ± standard deviation of three independent experiments (*ns* = not statistically significant by one-way ANOVA analysis).

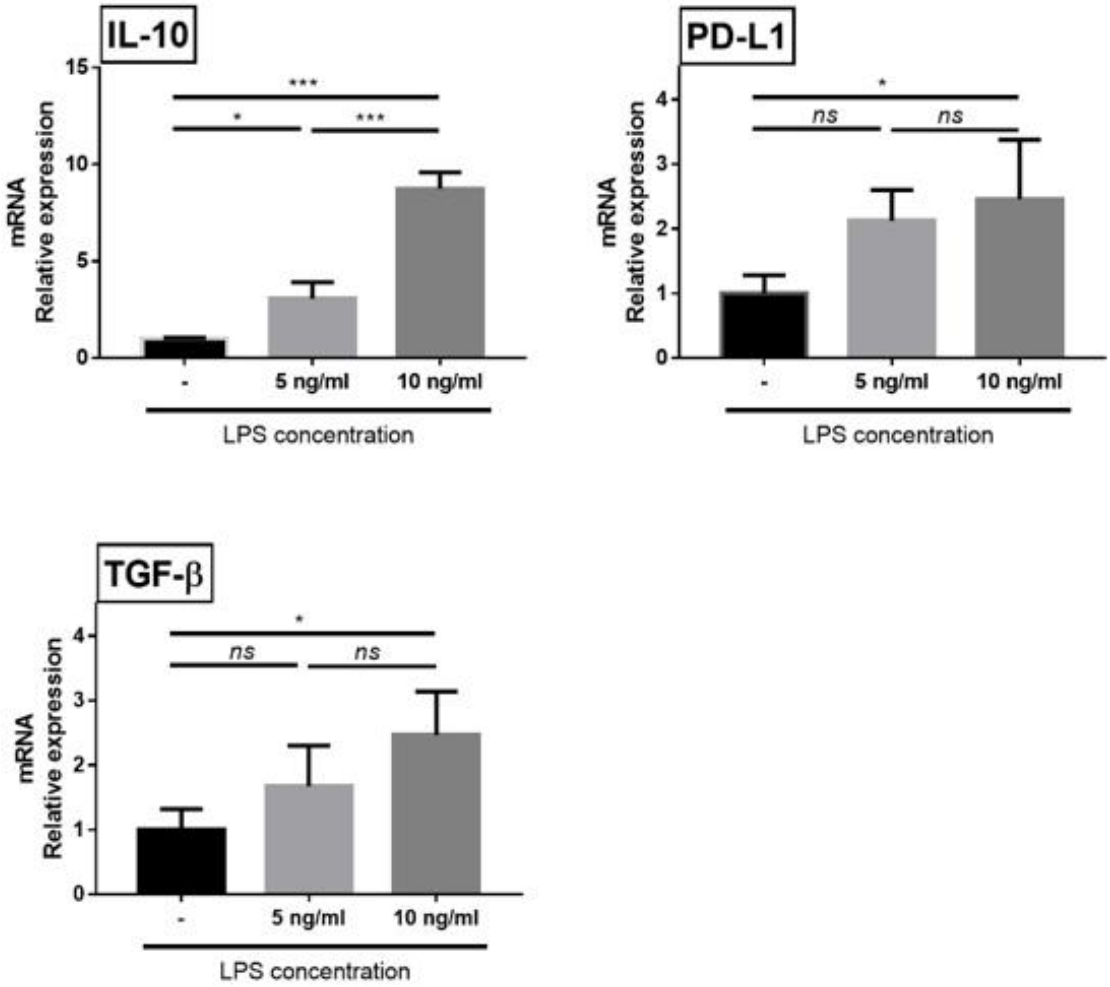

Figure 2. Increased immunoregulatory gene expression in canine B cells when treated with LPS. Increased gene expression of immunoregulatory genes such as *IL-10*, *PD-L1* and *TGF-β* in canine B cells after LPS stimulation. Results are presented as the mean ± standard deviation of three independent experiments. Data are shown as mean ± standard deviation of three independent experiments (*ns* = not statistically significant, **P* < 0.05, ****P* < 0.001 by one-way ANOVA analysis).

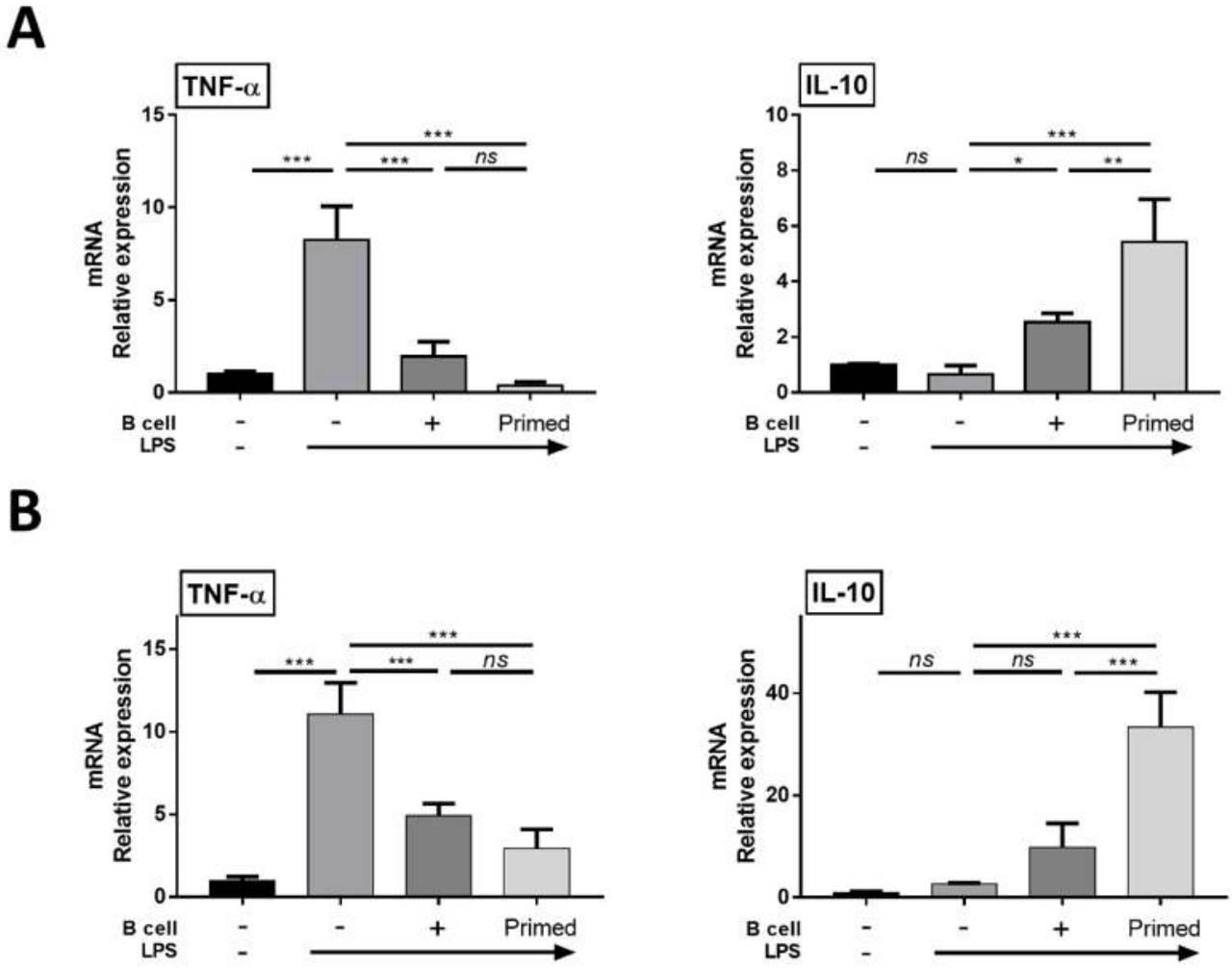

Figure 3. Increased anti-inflammatory gene expression and decreased proinflammatory gene expression in macrophage cell lines in inflammatory condition when co-cultured with LPS-primed canine B cells. LPS-stimulated RAW 264.7 and DH82 cells were co-cultured with primed B cells for 48 h. Then gene expression of inflammation in macrophage cell lines were analyzed by RT-qPCR. **A.** Relative mRNA expression levels of *TNF-α* and *IL-10* in RAW 264.7. **B.** Relative mRNA expression level of *TNF-α* and *IL-10* in DH82. Data are shown as mean ± standard deviation of three independent experiments (*ns* = not statistically significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by one-way ANOVA analysis)

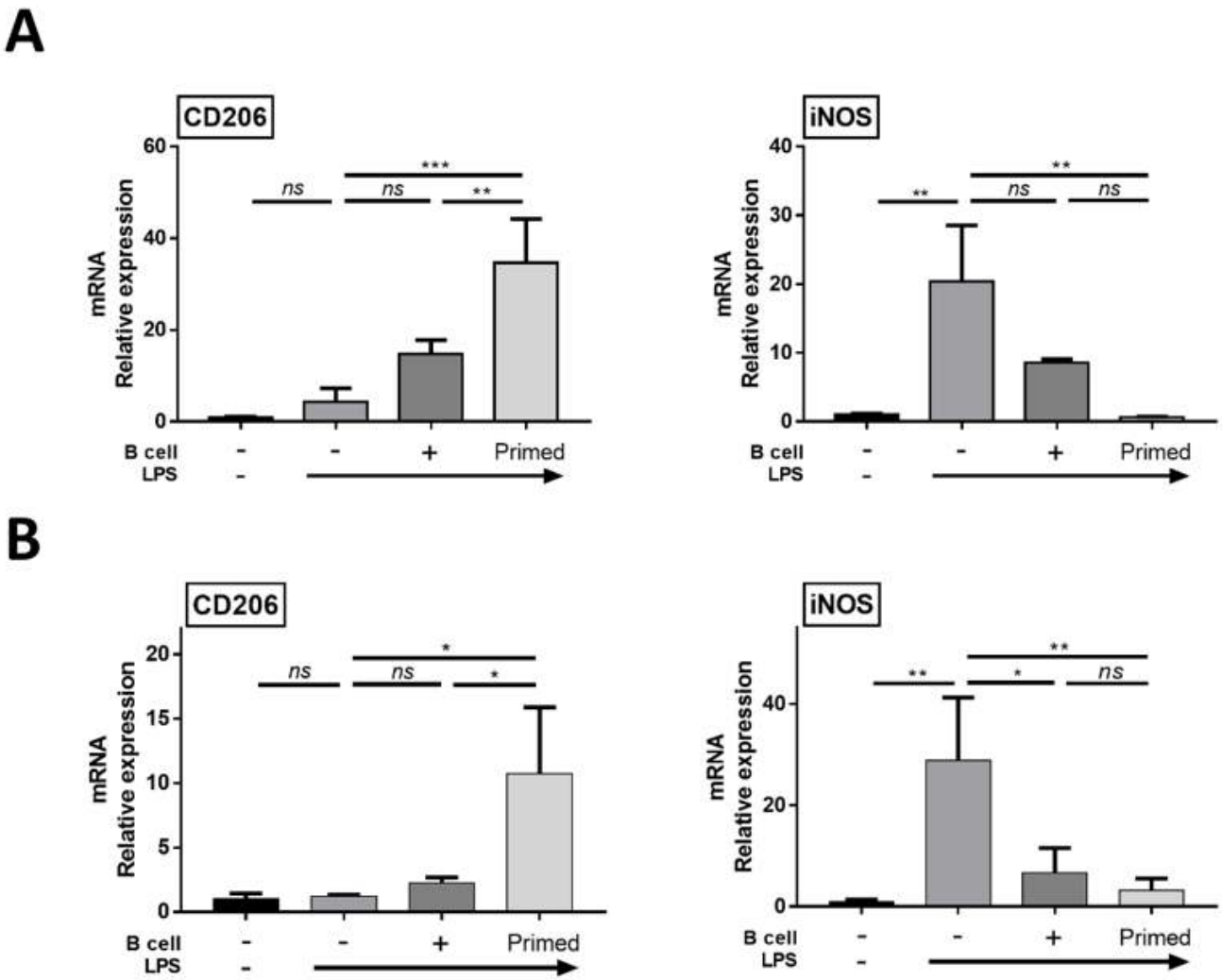

Figure 4. Increased M2 macrophage marker gene expression and decreased M1 macrophage marker gene expression in macrophage cell lines in inflammatory condition when co-cultured with LPS-primed canine B cells. LPS-stimulated RAW 264.7 and DH82 cells were co-cultured with primed B cells for 48 h. Then gene expression of macrophage target genes was analyzed by RT-qPCR. **A**. Relative mRNA expression levels of *CD206* and *iNOS* in RAW 264.7. **B**. Relative mRNA expression level of *CD206* and *iNOS* in DH82. Data are shown as mean ± standard deviation of three independent experiments (*ns* = not statistically significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by one-way ANOVA analysis).

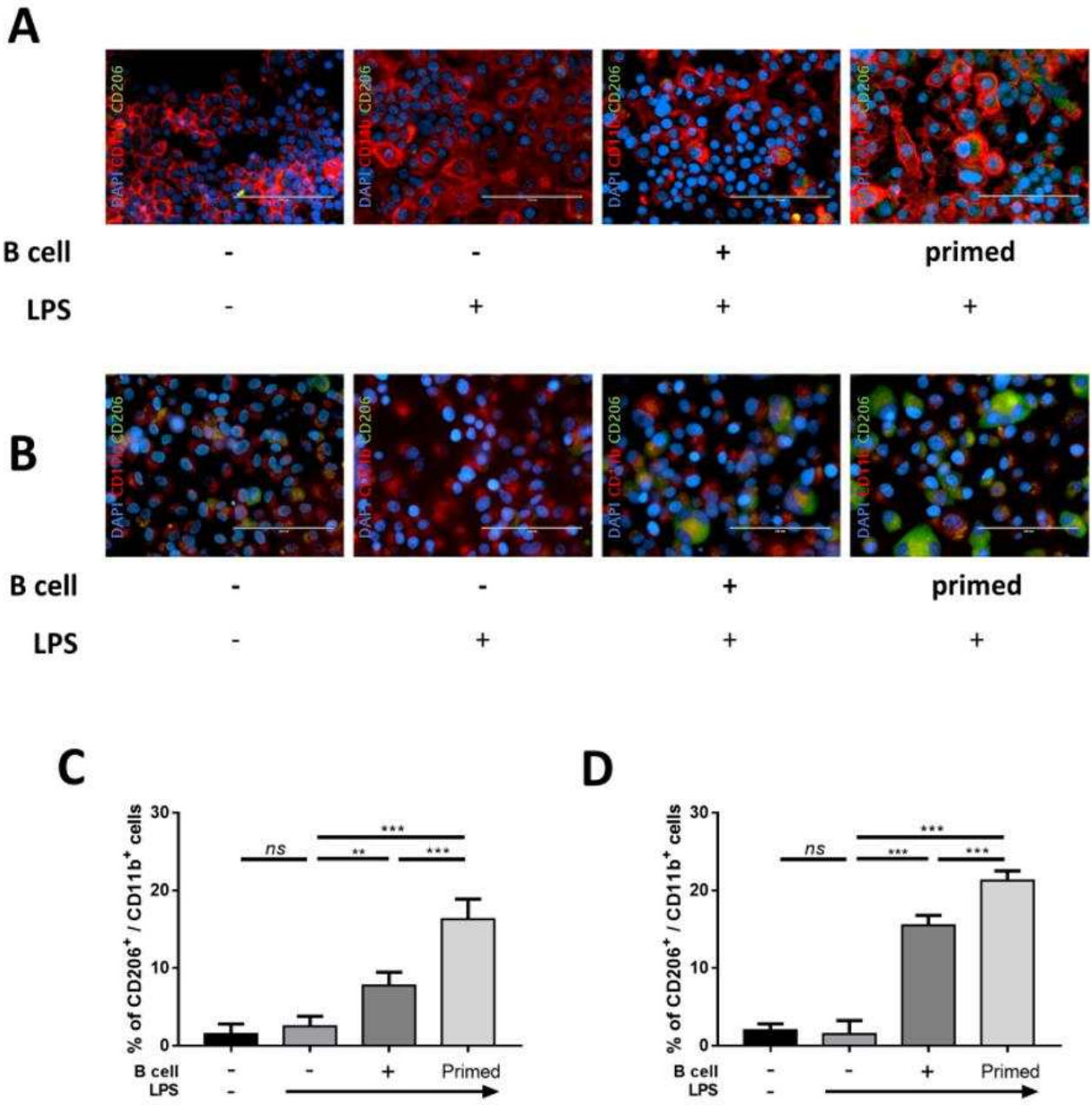

Figure 5. Increased CD206 expressing M2 macrophages in macrophage cell lines in inflammatory condition when co-cultured with LPS-primed canine B cells. LPS-stimulated RAW 264.7 and DH82 cells were co-cultured with primed B cells for 48 h. Then M2 macrophages expressing CD206 marker protein were analyzed by immunofluorescence analysis. **A**. Representative immunofluorescence

staining using anti-CD11b-PE (red) or anti-CD206-FITC (green) positive cell in RAW 264.7 cells. **B**. Representative immunofluorescence staining using anti-CD11b-PE (red) or anti-CD206-FITC (green) positive cell in DH82 cells. **C.** The calculated percentage of CD206-FITC positive cells among the CD11b-PE positive cell in RAW 264.7 are shown. **D.** The calculated percentage of CD206-FITC positive cells among the CD11b-PE positive cell in DH82 are shown. Data are shown as mean ± standard deviation of three independent experiments. Scale bars = 100μm (*ns* = not statistically significant, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA analysis).

Table 1. Primer sequences of murine used to detect gene expression of immune related factors and macrophage target genes

Genes		Primer sequences (5' to 3')	References
mGAPDH	Forward	CAA AAT GGT GAA GGT CGG TG	[32]
	Reverse	CGT TGA TGG CAA CAA TCT CC	
mIL-10	Forward	TGG CCC AGA AAT CAA GGA GC	[33]
	Reverse	CAG CAG ACT CAA TAC ACA CT	
mTNF-α	Forward	GGC CTC TCA CCT TGT TGC C	[34]
	Reverse	ATG ACC CGT AGG GCG ATT AC	
mINOS	Forward	CAC CTT GGA GTT CAC CCA GT	[35]
	Reverse	AGA TGT AGG TTA TTT TCT GCC AGT G	
mCD206	Forward	AAC GGA ATG ATT GTG TAG TTC TAG C	[36]
	Reverse	TAC AGG ATC AAT AAT TTT TGG CAT T	

Table 2. Primer sequences of canine used to detect gene expression of immune related factors and macrophage target genes

Genes		Primer sequences (5' to 3')	References
cGAPDH	Forward	CCC CAA TGT ATC ACT TGT GGA TCT G	[37]
	Reverse	CCT GCT TCA CTA CCT TCT TGA TGT C	
cIL-10	Forward	AGC ACC CTA CTT GAG GAC GA	[38]
	Reverse	GAT GTC TGG GTC GTG GTT CT	
cTNF-α	Forward	CCA AAC CGA CCC TTT GAT CA	[39]
	Reverse	CCA GCC CTG AGC CCT TAA TT	
cINOS	Forward	GAG ATC AAT GTC GCT GTA CTC C	[33]
	Reverse	TGA TGG TCA CAT TTT GCT TCT G	
cCD206	Forward	GGA AAT ATG TAA ACA GGA ATG ATG C	[33]
	Reverse	TCC ATC CAA ATA AAC TTT TTA TCC A	
cPD-L1	Forward	CCG CCA GCA GGT CAC TT	[40]
	Reverse	TCC ATT GTC ACA TTG CCA CC	
cTGF-β	Forward	CTC AGT GCC CAC TGT TCC TG	[41]
	Reverse	TCC GTG GAG CTG AAG CAG TA	

7. 국문초록

Lipopolysaccharide로 전처리한 개 말초혈액 단핵세포 유래 B 림프구에 의한 대식세포 분극화 유도를 통한 면역 조절 효과

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B 림프구의 일종인 조절 B 림프구는 자가면역과 염증상황에서 면역을 억압한다는 연구결과가 있으며, 여기에는 여러가지 메커니즘이 관여하지만 그 중에서도 interleukin(IL)-10을 분비하는 것이 조절 B 림프구의 항염증 능력에 주요한 역할을 하는 것이 알려져 있다. 또한, 이전 연구에 의하면 Lipopolysaccharide(LPS)를 B 림프구에

전처리하였을 때, IL-10 분비가 증가함이 알려져 있다. 따라서 IL-10을 과발현하는 B 림프구를 면역질환에서 세포치료제로 적용하는 것에 대한 연구가 활발히 진행중이지만, 현재 개의 B 림프구에 대한 연구는 많지 않다. 따라서 이 연구의 목적은 LPS로 전처리한 개 말초혈액 단핵세포 유래 B 림프구의 면역조절 유전자 발현 정도를 조사하고, 이를 염증상황의 대식세포와 공배양한 후 항염증 M2 대식세포로 유도되는지 확인하여, LPS로 전처리한 B 림프구의 항염증 및 면역조절 능력을 평가하는 것이었다.

개의 B 림프구는 3마리의 건강한 참여견의 말초혈액 단핵세포로부터 얻었다. 먼저, B 림프구에 대한 LPS의 세포독성을 확인하기 위해 B 림프구에 LPS를 5 ng/mL, 10 ng/mL 농도로 전처리한 후 CCK-8 실험을 실시하였다. 그리고 LPS로 전처리한 B 림프구에서 유세포 분석을 통해 조절 B 림프구의 마커인 IL-10의 발현에 대해 조사하였으며, 면역조절 능력을 알아보기 위해 실시간 qPCR(RT-qPCR)을 통해 *IL-10*, *programmed death-ligand 1(PD-L1)*, *transforming growth factor beta(TGF-β)*와 같은 면역조절 유전자의 발현에 대해 조사하였다. 다음으로 염증상황에서 LPS로 전처리한 B 림프구의 대식세포에 대한 면역조절 효과를 평가하기 위해 대식세포주(RAW 264.7 cell line과 DH82 cell line)와 전처리한 B 림프구를 공배양하였다. 대식세포에 염증상황을 유도하기 위해 LPS로 자극하였고, 공배양한 대식세포에서 RNA를 추출하여 면역반응 양상과 염증성 M1 대식세포

및 항염증성 M2 대식세포의 마커에 대해 확인하였으며, 단백질 수준에서 M2 대식세포로의 분극화를 확인하기 위해 M2 대식세포 표지 단백질인 CD206을 이용하여 면역형광검사법을 실시하였다.

세포독성시험에서 대조군과 LPS 전처리군에서 세포생존율에 유의미한 차이가 없어, LPS 전처리가 B 림프구에 독성이 없음을 확인하였다. 그리고 조절 B 림프구의 마커인 IL-10의 발현이 대조군에 비해 LPS 5 ng/mL 전처리군에서 2.32배, LPS 10 ng/mL 전처리군에서 2.64배 증가됨을 확인하였다. 면역조절 유전자 발현은 LPS 5 ng/mL 전처리군에서는 대조군에 비해 *IL-10*의 유전자 발현만이 3.07배로 유의미하게 증가하였으며($P < 0.05$), LPS 10 ng/mL 전처리군에서는 대조군에 비해 *IL-10*은 8.75배($P < 0.001$), *PD-L1*은 2.46배($P < 0.05$), *TGF-β*는 2.47배($P < 0.05$) 유의미하게 증가하였다. 이와 같이, LPS 전처리에 의한 IL-10 마커 과발현 효과와 면역조절 유전자 발현 유도효과가 LPS 10 ng/mL 전처리군에서 더 크게 나타나, 이후 실험에는 LPS 10 ng/mL 전처리군만을 이용하였다.

LPS로 전처리한 B 림프구와 공배양한 대식세포의 면역반응 양상은 LPS로 자극한 대식세포군과 비교하였을 때, 각각 RAW 264.7 cell line에서는 전염증성 사이토카인인 *TNF-α*가 0.04배 유의미하게 감소하고 항염증성 사이토카인인 *IL-10*이 8.21배 유의미하게 증가하였고, DH82 cell line에서는 *TNF-α*가 0.26배 유의미하게 감소하고 *IL-10*이 12.39배 유의미하게 증가하였다($P < 0.001$). 또한

염증성 M1 대식세포 마커인 *iNOS*와 항염증성 M2 대식세포 마커인 *CD206* 유전자 발현 변화양상은, LPS로 자극한 염증상황의 대식세포와 비교하였을 때, 각각 RAW 264.7 cell line에서는 *iNOS*가 0.03배 유의미하게 감소하고($P < 0.01$) *CD206*가 7.97배 유의미하게 증가하였고($P < 0.001$), DH82 cell line에서는 *iNOS*가 0.11배 유의미하게 감소하고($P < 0.01$) *CD206*가 8.72배 유의미하게 증가하였다($P < 0.05$). 단백질 수준에서는, LPS 처리군에 비해서 LPS로 전처리한 B 림프구와 공배양하였을 때, CD206을 발현하는 세포가 RAW 264.7 cell line에서 6.5배, DH82 cell line에서 14.15배로 유의미하게 증가하였다($P < 0.001$).

이 연구를 통하여 개 말초혈액 단핵세포로부터 유래한 B 림프구를 LPS로 전처리할 경우, 면역조절 능력이 증대된 IL-10 과발현 B 림프구를 생성할 수 있음을 확인하였고, 염증상태의 대식세포가 M2 대식세포로 분극화 유도됨을 확인함으로써, LPS로 전처리한 개 B 림프구의 면역조절 효과 및 항염증 효과를 입증하였다. 이러한 결과를 통하여 수의임상에서 LPS로 전처리한 개 B 림프구의 개의 면역관련질환에 대한 선구적인 세포 치료제로서의 가능성을 확인하였다.

주요어 : 조절 B 림프구; 대식구; 항염증제; 면역 질환; M2 대식세포

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