Biomolecules & Therapeutics, **18(4)**, 463-468 (2010)
www.biomolther.org

ISSN: 1976-9148(print)/2005-4483(online)
DOI: 10.4062/biomolther.2010.18.4.463

Effects of Anti-B7.1/B7.2 Antibodies on LPS-Stimulated Macrophages

Tae Joon WON[a], Yoon Joo HUH[a], Young Tae LIM, Dong Sup SONG, and Kwang Woo HWANG*

Cellular and Molecular Immunology Lab., College of Pharmacy, Chung-Ang University, Seoul 156-756, Republic of Korea

(Received July 23, 2010; Revised September 17, 2010; Accepted October 4, 2010)

Abstract − T-cell activation depends on signals received by the T-cell receptor and CD28 co-stimulatory receptor. Since B7.1 and B7.2 molecules expressed on the surface of antigen presenting cells provide co-stimulatory signals through CD28 to T-cells, an inhibitor of CD28-B7.1/B7.2 binding has been proposed as a therapeutic agent for suppression of excessive T-cell activity. Although anti-B7.1/B7.2 antibodies are known to block B7.1 and B7.2 molecules, their effects on intracellular events in antigen presenting cells remain unclear. In this study, anti-B7.1/B7.2 antibodies decreased secretion of nitric oxide and pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-12 in LPS-activated RAW264.7 macrophage-like cells and peritoneal macrophages. Moreover, anti-B7.1/B7.2 antibodies inhibited IκBα phosphorylation and down-regulated expression of co-stimulatory molecules including B7.1, B7.2, and PD-L1 in LPS-stimulated peritoneal macrophages. These findings suggest that CTLA4-Ig and anti-B7.1/B7.2 antibodies may be candidates to treat chronic inflammatory diseases and autoimmune responses caused by excessive activation of both T-cells and macrophages.

Keywords: Anti-B7.1/B7.2 antibodies, RAW264.7, Peritoneal macrophage, Co-stimulatory molecule, Pro-inflammatory cytokine

INTRODUCTION

The incidence of autoimmune diseases such as type 1 diabetes (T1D) is dramatically increasing in the developed world (Cooke, 2009). In most autoimmune disorders, activation of auto-reactive T-cells is induced by self peptide-displaying antigen presenting cells (APCs) (Howard *et al.*, 2005). T-cell activation depends on signals received by the T-cell receptor and co-stimulatory molecules. The most prominent co-stimulatory molecule on the T-cell surface is CD28, which induces T-cell activation. B7.1 and B7.2 (CD80 and CD86, respectively) expressed on the surface of professional APCs provide co-stimulatory signals to T-cells through CD28. On the other hand, the interaction of B7.1/B7.2 molecules with CTLA-4 expressed on T-cells induced an inhibitory signal for down-regulation of T-cell functions. CTLA-4, which is one of the CD28 homologues, does not exist on resting T-cells, however, it is expressed following T-cell activation (Ostrov *et al.*, 2000). Thus, the balance between opposing signals elicited by CD28 and CTLA-4 is very important for regulation of T-cell responsiveness. CTLA-4 has received attention as a therapeutic agent due to its modulating role in T-cell activity. CTLA4-Ig has been used as an inhibitor of CD28-B7.1/ B7.2 binding for the treatment of autoimmune diseases, transplant rejection, and hypersensitiveness in animal models (Ostrov *et al.*, 2000; Park *et al.*, 2003). Although CTLA4-Ig blocks co-stimulatory receptors on the surface of APCs, the molecular events inside APCs induced by their binding are not fully understood. Recently, it was demonstrated that CTLA4-Ig induced indoleamine-2,3-dioxygenase (IDO) expression on dendritic cells but not on macrophages or lymphoid cells (Mellor *et al.*, 2003). IDO catalyzes the initial and rate-limiting step of tryptophan degradation, which leads the suppression of T-cell function and the production of pro-apoptotic metabolites (Fallarino *et al.*, 2003). This result suggests that ligation of B7.1/B7.2 molecules on APCs to their ligand could trigger the modulation of immune response and the suppression of APC activation.

Macrophages are important players in innate immunity and are members of the professional APC family. Activated macrophages secrete nitric oxide and pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, and TNF-α (Trinchieri, 2003; Martinez *et al.*, 2008). It is known that IL-12 directly

*Corresponding author
Tel: +82-2-820-5597 Fax: +82-2-823-5597
E-mail: khwang@cau.ac.kr
[a]These authors equally contributed to this work.

induces activation of T-cells. B7.2 molecules are constitutively expressed on macrophages, whereas B7.1 has a low expression level on the resting cells and a high expression level on macrophages after stimulation by antigens (McAdam *et al.*, 1998; Kim *et al.*, 2001). Recently, novel B7 family molecules, B7-H1(PD-L1) and B7-DC (PD-L2), which are expressed on activated T-cells, B-cells, and myeloid cells have been identified as ligands for programmed death-1 (Ishida *et al.*, 1992). PD-L1 molecules are ubiquitously expressed on many immunological cells, including macrophages. Although a number of studies suggest an inhibitory role of PD-L1 on activating T-cells, less is known about the expression and function of PD-L2 (Menke *et al.*, 2007). In the present study, we examined the effects of anti-B7.1/B7.2 antibodies on the production of pro-inflammatory cytokines and nitric oxide, and the expression of co-stimulatory molecules in LPS-stimulated macrophages.

MATERIALS AND METHODS

Mice and cell-lines

Male C57BL/6 mice, 8-10 weeks old, were obtained from the Orient Bio Inc. (Gyeonggi-do, Korea). Animals were housed in an environmentally controlled, pathogen-free animal facility for the duration of experiments. All experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Institutional Animal Care and Use of the Laboratory Animal Research Center. RAW264.7 macrophage cell- lines were obtained from the American Type Culture Collection (Manassas, USA) and maintained in DMEM medium (Cellgro, Manassas, USA) supplemented with 10% heat-inactivated FBS (fetal bovine serum, Cellgro), 2 mM L-glutamine, 100 U/ml penicillin and streptomycin (Cellgro) at 37^{o}C in a 5% CO_2 humidified incubator.

Isolation of peritoneal macrophages

The thioglycollate-elicited peritoneal macrophages were harvested by peritoneal lavage from C57BL/6 male mice 72 hr after i.p. injection with 1.5 ml of sterile 4% brewer thioglycollate (Sigma, St. Louis, USA). Red blood cells were eliminated by ACK buffer and the cells were washed and resuspended in RPMI1640 (Cellgro) supplemented with 10% inactivated FBS, 10 mM HEPES, 2 mM glutamine, and 100 U/ml penicillin-100 mg/ml streptomycin. The cells were examined after achieving an adherent state.

Antibodies and reagents

Cells were cultured in the presence of lipopolysaccharide (Sigma, 100 ng/ml), IL-4 (BD Biosciences, San Jose, USA, 10 ng/ml), anti-B7.1/B7.2 antibodies (eBioscience, San Diego, USA, 10 μg/ml each), or control IgG (Santa Cruz Biotechnology, Santa Cruz, USA, 10 μg/ml). Surface molecules were detected by flow cytometry using anti-mouse-CD80-PE, anti-mouse-CD86-PE, anti-mouse-CD273-PE, anti-mouse-CD274-PE, and anti-mouse-F4/80-FITC (BD Biosciences). Capture and detection antibodies used in ELISA for TNF-α and IL-12 were purchased from BD Biosciences and antibodies for IL-1β were purchased from eBioscience.

Nitric oxide assay

Supernatants from cell cultures were assessed for nitric oxide production. Each supernatant was mixed with an equal volume of Griess reagent (Sigma). The absorbance of the mixture at 540 nm was determined using a microplate reader, and nitrite concentration was determined using a dilution of standard nitrite.

Enzyme-linked immunosorbent assay (ELISA)

The 96-well plates were coated overnight at 4^{o}C with the capture antibody. The wells were washed three times with PBS-T, incubated for 1 hr with blocking solution at room temperature, and then washed four times with PBS-T. Samples and diluted standards were added to the plate and incubated overnight at 4^{o}C. Following 4 washing cycles, the detection antibody was added. After 45 min incubation at room temperature, the wells were washed and avidin-conjugated alkaline phosphatase was added at room temperature and incubated for 30 min. The substrate solution was added and the plates were maintained at room temperature for 5-30 min before addition of a stop buffer. Absorbance of the colored product was read at 405 nm.

Flow cytometry

Peritoneal macrophages were incubated with anti-B7.1/B7.2 antibodies in the presence of LPS for 15 hr. The cells were harvested, resuspended in PBS, and incubated on ice with 20 μg/ml antibodies for 30 min. The cells were then washed and resuspended in PBS. Fluorescence acquisition and analysis were performed using FACScan and Cellquest Pro (BD Biosciences).

Western blot analysis

Peritoneal macrophages were incubated for 15 min with anti-B7.1/B7.2 antibodies in the presence of LPS. The

whole cell proteins were extracted in RIPA buffer (Elpis Biotech, Daejeon, Korea). The extracts were separated by SDS-PAGE and transferred to a PVDF membrane. Blots were blocked and incubated at 4°C overnight with anti-IκBα, anti-phosphorylated IκBα, or anti-β-actin antibodies (Cell Signaling, Danvers, USA). The membranes were then incubated with anti-mouse IgG or anti-rabbit IgG HRP-linked antibodies (Cell Signaling). The signals were detected using the ECL detection system and visualized by X-ray film.

Statistical analysis

Experiments were repeated at least three times. Data are expressed as mean ± SD, and statistical analyses were performed using the Student's t-test.

RESULTS

Anti-B7.1/B7.2 antibodies decrease secretion of nitric oxide and pro-inflammatory cytokines in macrophages

To investigate whether the ligation of B7.1 and B7.2 molecules to their counter receptors suppresses the activation of macrophages, RAW264.7 cell-lines and peritoneal macrophages were stimulated by LPS in the presence or absence of anti-B7.1/B7.2 antibodies. As shown in Fig. 1A and 1D, anti-B7.1/B7.2 antibodies significantly reduced LPS-induced nitric oxide production in both of RAW264.7 and peritoneal macrophages. The secretions of TNF-α and IL-1β, which are representative pro-inflammatory cytokines produced by macrophages, were also lower in cells treated with anti-B7.1/B7.2 antibodies than in either LPS-stimulated RAW264.7 (Fig. 1B and 1C) or peritoneal macrophages (Fig. 1E and 1F). These data suggest that anti-B7.1/B7.2 antibodies do not only block the binding of CD28 receptor expressed on T-cells, but may also suppress the activation of macrophages.

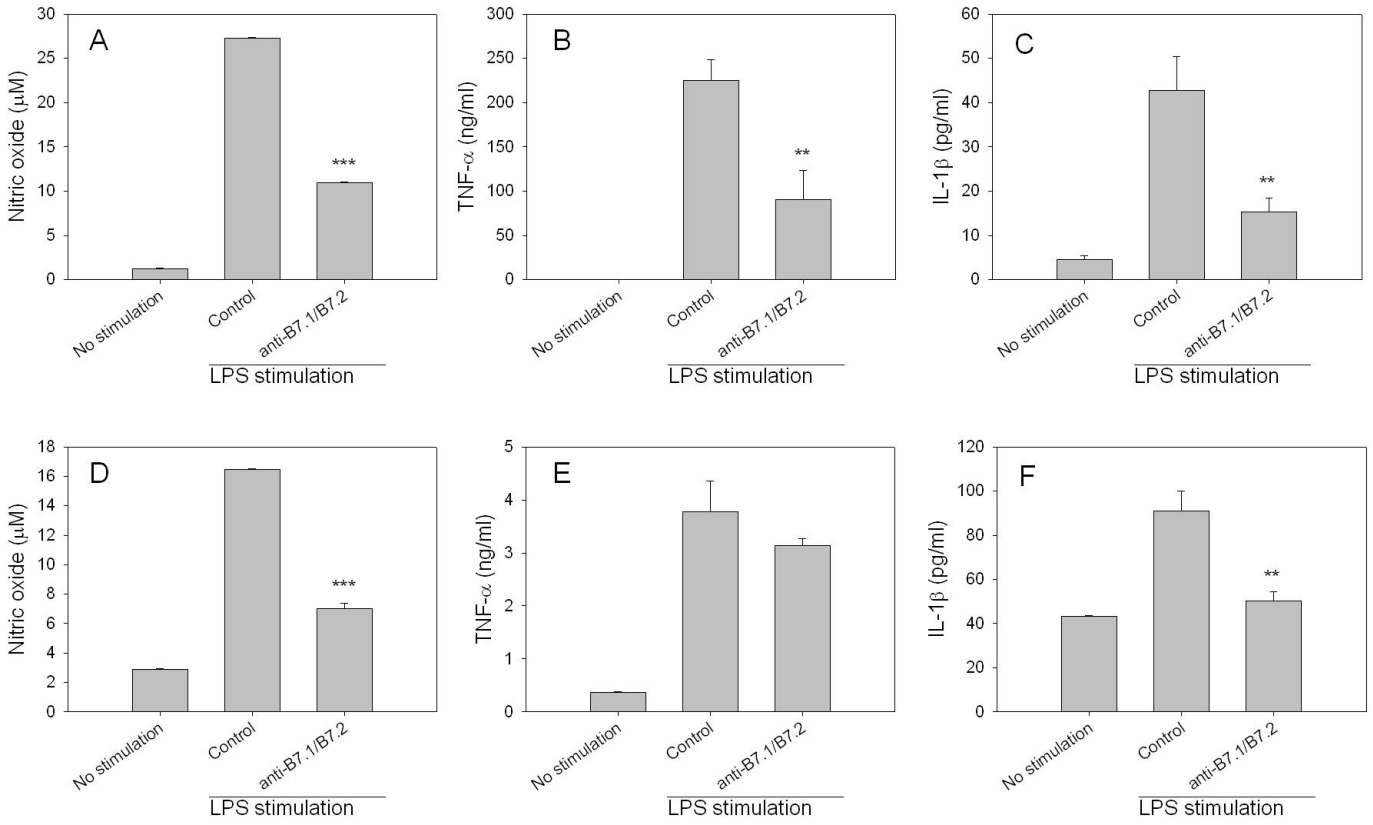

Fig. 1. Decreased production of nitric oxide, TNF-α, and IL-1β by anti-B7.1/B7.2 antibodies on LPS-activated RAW264.7 cell-lines (A-C) and peritoneal macrophages (D-F). RAW264.7 or peritoneal macrophages were cultured with LPS (100 ng/ml) in the presence or absence of anti-B7.1/B7.2 antibodies (10 μg/ml each) for 24 hr. Values represent the mean ± SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Control in LPS stimulation).

Anti-B7.1/B7.2 antibodies reduce IL-12 production in macrophages

IL-12, which is principally produced by macrophages and dendritic cells, is a major inducer of T-cell activation, which is a central event in the cellular immune response. To test whether anti-B7.1/B7.2 antibodies diminish production of the immunomodulatory cytokine IL-12, the two agents were added to the culture of LPS-activated peritoneal macrophages. IL-4 was used as a positive control for suppression of IL-12 secretion in macrophages. As shown in Fig. 2, the addition of anti-B7.1/B7.2 antibodies resulted in significantly lower production of IL-12 by LPS-stimulated peritoneal macrophages. These results illustrate that anti-B7.1/B7.2 antibodies can suppress the activation of T-cells through reduction of IL-12 production in macrophages as well as by blocking CD28-B7 ligation.

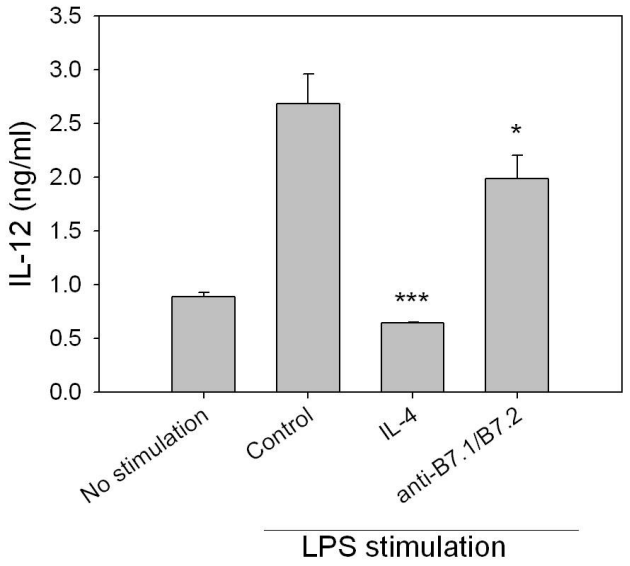

Fig. 2. Reduced secretion of IL-12 by anti-B7.1/B7.2 antibodies on LPS-activated peritoneal macrophages. Peritoneal macrophages were cultured with LPS (100 ng/ml) in the presence or absence of IL-4 (10 ng/ml) or anti-B7.1/B7.2 antibodies (10 μg/ml each) for 24 hr. Values represent the mean ± SD (*$p<$ 0.05, ***$p<0.001$ vs. Control in LPS stimulation).

Anti-B7.1/B7.2 antibodies down-regulate expression of co-stimulatory molecules in macrophages

To assess whether the treatment with anti-B7.1/B7.2 antibodies reduced the expression of co-stimulatory molecules on the macrophage surface, the expression levels of B7.1, B7.2, PD-L1, and PD-L2 on peritoneal macrophages were examined using flow cytometry. The stimulation by LPS increased the expression of B7.1, B7.2, and PD-L1 molecules on peritoneal macrophages (Fig. 3), while anti-B7.1/B7.2 antibodies decreased their expression levels. However, neither LPS-stimulation nor other treatments altered the expression of PD-L2 molecules on peritoneal macrophages. These data indicate that activated macrophages showed the increment of co-stimulatory receptor expression and that the suppression of macrophage activation by anti-B7.1/B7.2 down-regulated the expression of co-stimulatory molecules.

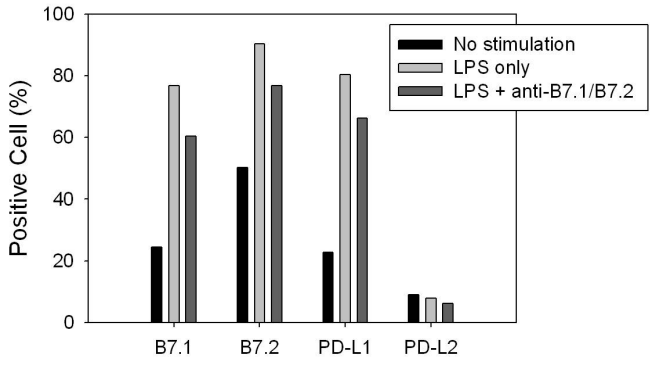

Fig. 3. Down-regulation of B7.1, B7.2, and PD-L1 expression by anti-B7.1/B7.2 antibodies on LPS-activated peritoneal macrophages. Peritoneal macrophages were cultured with LPS (100 ng/ml) in the presenceor absence of anti-B7.1/B7.2 antibodies (10 μg/ml each) for 15 hr. Data shown are representative of three independent experiments.

Anti-B7.1/B7.2 antibodies inhibit phosphorylation of IκBα in macrophages

To determine the induced down-stream mechanism through which the ligation of B7.1 and B7.2 receptors to

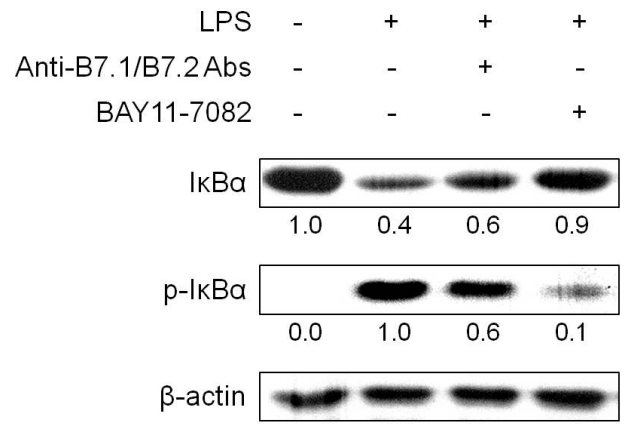

Fig. 4. Inhibition of IκBα phosphorylation by anti-B7.1/B7.2 antibodies on LPS-activated peritoneal macrophages. Peritoneal macrophages were cultured with LPS (100 ng/ml) in the presence or absence of anti-B7.1/B7.2 antibodies (10 μg/ml each) or BAY11-7082 (10 μM) for 30 min.

their ligands decreases macrophage activation, we assessed LPS-induced phosphorylation of IκBα, which is an inhibitory protein for the NF-κB signal pathway, in peritoneal macrophages. It is well known that phosphorylation of IκBα leads to ubiquitin-mediated degradation by proteasomes, and the subsequent translocation of NF-κB into the nucleus promotes inflammatory responses. As shown in Fig. 4, anti-B7.1/B7.2 antibodies diminished the phosphorylation and degradation of IκBα induced by LPS stimulation in peritoneal macrophages. These results explain why the binding of anti-B7.1/B72 antibodies can inhibit LPS-induced activation of macrophages via the NF-κB signal pathway.

DISCUSSION

Discovery of pro-inflammatory mediators including IL-1β, IL-6, and TNF-α has provided valuable insights into the role of macrophages in acute and chronic inflammatory processes, including autoimmune disease (Gordon, 2007). Additionally, macrophages express co-stimulatory aides for T-cell activation such as B7.1 and B7.2. In the present study, anti-B7.1/B7.2 antibodies, which have been introduced as blockers of CD28-B7.1/B7.2 ligation for inhibition of T cell activation (Goronzy and Weyand, 2008), are suggested to be inhibitory regulators of activated macrophages.

Treatment with anti-B7.1/B7.2 antibodies reduced the production of nitric oxide and pro-inflammatory cytokines, IL-1β and TNF-α, in both RAW264.7 cell-lines and peritoneal macrophages (Fig. 1). It is reported that nitric oxide contributes to tissue toxicity and vascular collapse associated with septic shock, and chronic expression of nitric oxide is involved in various carcinomas and inflammatory conditions such as juvenile diabetes, multiple sclerosis, arthritis, and ulcerative colitis (Taylor *et al.*, 1997). In a local tissue site, the excessive production of TNF-α and IL-1β by immunological cells can result in chronic inflammation and tissue destruction. Anti-B7.1/B7.2 antibodies also decreased IL-12 production by LPS-stimulated peritoneal macrophages (Fig. 2). Since IL-12 activates na?ve T-cells and induces T-cell differentiation into type 1 helper T-cells, reduction of IL-12 secretion by macrophages may down-regulate cell-mediated adaptive immunity. The up-regulation of pro-inflammatory genes including various cytokines, chemokines, and inflammatory enzymes is a result of NF-κB pathway activation in LPS-stimulated macrophages. As shown in Fig. 4, the treatment with anti-B7.1/B7.2 antibodies inhibited the phosphorylation and degradation of IκBα, thus, the two agents could reduce NF-κB activation in LPS-stimulated macrophages.

The expression of co-stimulatory molecules including B7.1, B7.2, and PD-L1 on LPS-stimulated peritoneal macrophages was down-regulated by treatment with anti-B7.1/B7.2 antibodies (Fig. 3). Since anti-B7.1/B7.2 antibodies did not alter the expression levels of co-stimulatory molecules in the absence of LPS stimulation (data not shown), it was confirmed that the binding of fluorochrome-conjugated anti-B7.1 or B7.2 antibodies to their counter molecules for flowcytometric analysis was not interrupted by treatment with anti-B7.1/B7.2 antibodies. Thus, anti-B7.1/B7.2 antibodies could simultaneously decrease the ligation of CD28 on T cells onto B7 families through the following two mechanisms: 1) anti-B7.1/B7.2 antibodies compete with CD28 molecules for binding to B7.1 and B7.2 molecules expressed on macrophages; 2) the antibodies suppress up-regulation of B7 families on activated macrophages. Anti-B7.1/B7.2 antibodies also decrease PD-L1 expression on LPS-stimulated peritoneal macrophages. Since PD-L1 is known to be a counter-molecule of PD-1, which is expressed on effector T-cells and is classified as an inhibitory receptor, the down-regulation of PD-L1 expression on APCs might reduce the interaction with PD-1 molecules and the inhibitory regulation of effector T-cells. However, a number of molecular mechanisms, including PD-1-PD-L1 interaction, are involved in the regulation of T-cell activity. Thus, reduced expression of PD-L1 in the presence of anti-B7.1/B7.2 antibodies simply reveals the suppression of macrophage activation.

In conclusion, anti-B7.1/B7.2 antibodies are effective modulators for control of both T-cells and macrophages. Thus, they have the potential to alleviate autoimmune diseases and chronic inflammatory disorders caused by excessive activation of T-cells and macrophages, although it remains necessary to clarify the role of several co-stimulatory molecules in the regulation of immune response.

ACKNOWLEDGMENTS

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (314-2007-1-E00067).

REFERENCES

Cooke, A. (2009). Infection and autoimmunity. *Blood Cells Mol. Dis.* **42**, 105-107.

Fallarino, F., Grohmann, U., Hwang, K. W., Orabona, C., Vacca, C., Bianchi, R., Belladonna, M. L., Fioretti, M. C., Alegre, M. L. and Puccetti, P. (2003). Modulation of tryptophan catabolism by regulatory T cells. *Nat. Immunol.* **4**, 1206-

1212.

Gordon, S. (2007). The macrophage: past, present and future. *Eur. J. Immunol.* **37(Suppl 1)**, S9-17.

Goronzy, J. J. and Weyand, C. M. (2008). T-cell co-stimulatory pathways in autoimmunity. *Arthritis. Res. Ther.* **10**, S3.

Howard, L. M., Kohm, A. P., Castaneda, C. L. and Miller, S. D. (2005). Therapeutic blockade of TCR signal transduction and co-stimulation in autoimmune disease. *Curr. Drug Targets Inflamm. Allergy* **4**, 205-216.

Ishida, Y., Agata, Y., Shibahara, K. and Honjo, T. (1992). Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* **11**, 3887-3895.

Kim, K. S., Denton, M. D., Chandraker, A., Knoflach, A., Milord, R., Waaga, A. M., Turka, L. A., Russell, M. E., Peach, R. and Sayegh, M. H. (2001). CD28-B7-mediated T cell costimulation in chronic cardiac allograft rejection: differential role of B7-1 in initiation versus progression of graft arteriosclerosis. *Am. J. Pathol.* **158**, 977-986.

Martinez, F. O., Sica, A., Mantovani, A. and Locati, M. (2008). Macrophage activation and polarization. *Front Biosci.* **13**, 453-461.

McAdam, A. J., Schweitzer, A. N. and Sharpe, A. H. (1998). The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells. *Immunol. Rev.* **165**, 231-247.

Mellor, A. L., Baban, B., Chandler, P., Marshall, B., Jhaver, K., Hansen, A., Koni, P. A., Iwashima, M. and Munn, D. H. (2003). Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. *J. Immunol.* **171**, 1652-1655.

Menke, J., Lucas, J. A., Zeller, G. C., Keir, M. E., Huang, X. R., Tsuboi, N., Mayadas, T. N., Lan, H. Y., Sharpe, A. H. and Kelley, V. R. (2007). Programmed death 1 ligand (PD-L) 1 and PD- L2 limit autoimmune kidney disease: distinct roles. *J. Immunol.* **179**, 7466-7477.

Ostrov, D. A., Shi, W., Schwartz, J. C., Almo, S. C. and Nathenson, S. G. (2000). Structure of murine CTLA-4 and its role in modulating T cell responsiveness. *Science* **290**, 816-819.

Park, C. G., Thiex, N. W., Lee, K. M., Szot, G. L., Bluestone, J. A. and Lee, K. D. (2003). Targeting and blocking B7 costimulatory molecules on antigen-presenting cells using CTLA4Ig- conjugated liposomes: in vitro characterization and in vivo factors affecting biodistribution. *Pharm. Res.* **20**, 1239-1248.

Taylor, B. S., Kim, Y. M., Wang, Q., Shapiro, R. A., Billiar, T. R. and Geller, D. A. (1997). Nitric oxide down-regulates hepatocyte-inducible nitric oxide synthase gene expression. *Arch. Surg.* **132**, 1177-1783.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* **3**, 133-146.