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**The CagA protein of *Helicobacter pylori* suppresses the functions of dendritic cell in mice.**

Hiroshi Tanaka <sup>1)</sup>, Masaru Yoshida <sup>1,2)</sup>, Shin Nishiumi <sup>1,2)</sup>, Naomi Ohnishi <sup>3)</sup>,  
Kazuki Kobayashi <sup>1)</sup>, Koji Yamamoto <sup>1)</sup>, Tsuyoshi Fujita <sup>1)</sup>, Masanori  
Hatakeyama <sup>4)</sup>, Takeshi Azuma <sup>1)</sup>

<sup>1)</sup> Division of Gastroenterology, Department of Internal Medicine, Graduate  
School of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chu-o-ku, Kobe,  
Hyogo 650-0017, Japan

<sup>2)</sup> The Integrated Center for Mass Spectrometry, Graduate School of Medicine,  
Kobe University, 7-5-1 Kusunoki-Cho, Chu-o-ku, Kobe, Hyogo 650-0017, Japan

<sup>3)</sup> Division of Molecular Oncology, Institute for Genetic Medicine, Hokkaido  
University, Kita-15, Nishi-7, Kita-ku, Sapporo 060-0815, Japan

<sup>4)</sup> Division of Microbiology, Graduate School of Medicine, Tokyo University, 7-3-1  
hongo, bunkyo-ku, Tokyo 113-0033, Japan

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1 Address correspondence to:

2 Masaru Yoshida M.D., Ph.D.

3 Division of Gastroenterology, Department of Internal Medicine, Graduate School  
4 of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chu-o-ku, Kobe, Hyogo  
5 650-0017, Japan

6 Tel: +81-78-382-6305

7 FAX: +81-78-382-6309

8 E-Mail: [myoshida@med.kobe-u.ac.jp](mailto:myoshida@med.kobe-u.ac.jp)

## Abstract

CagA protein is the most assessed effector molecule of *H. pylori*. In this report, we demonstrate how CagA protein regulates the functions of dendritic cells (DC) against *H. pylori* infection. In addition, we found that CagA protein was tyrosine-phosphorylated in DC. The responses to *cagA*-positive *H. pylori* in DC were reduced in comparison to those induced by *cagA*-negative *H. pylori*. CagA-overexpressing DC also exhibited a decline in the responses against LPS stimulation and the differentiation of CD4<sup>+</sup> T cells toward Th1 type cells compared to wild type DC. In addition, the level of phosphorylated IRF3 decreased in CagA-overexpressing DC stimulated with LPS, indicating that activated SHP-2 suppressed the enzymatic activity of TBK1 and consequently IRF3 phosphorylation. These data suggest that CagA protein negatively regulates the functions of DC via CagA phosphorylation and that *cagA*-positive *H. pylori* strains suppress host immune responses resulting in their chronic colonization of the stomach.

## 1 Introduction

2  
3 *Helicobacter pylori* (*H. pylori*), a gram-negative microaerophilic bacterium,  
4 chronically colonizes the gastric epithelium of more than half of all people  
5 worldwide. It is well known that *H. pylori* infection induces chronic gastritis,  
6 peptic ulcers, gastric cancer, and gastric MALT lymphoma and that the  
7 interactions among host immune responses, bacterial virulence factors and  
8 environmental factors are deeply involved in the pathogenesis of these diseases  
9 [1-4]. Although *H. pylori* infection evokes host immune responses, for example  
10 the infiltration of neutrophils, monocytes and lymphocytes into the gastric  
11 mucosa and the induction of pro-inflammatory cytokines, *H. pylori* can not be  
12 eliminated from the stomach in many cases [5]. However, it is unknown how *H.*  
13 *pylori* manages to persist in the stomach for decades.

14  
15 *H. pylori* infection initially activates the host innate immune system. Although *H.*  
16 *pylori* are not able to invade into the gastric mucosa, antigen-presenting cells  
17 (APC), such as dendritic cells (DC) and macrophages, recognize the antigens  
18 from *H. pylori*, and then acquired immunity is activated. Many studies have  
19 revealed that Peyer's patches (PP) play important roles in the induction of innate  
20 immunity and acquired immunity during *H. pylori* infection [6, 7]. *H. pylori* is  
21 transformed to its coccoid form, which is able to access the intestinal lumen, and  
22 then is captured by DC in PP [7]. CD4<sup>+</sup> T cells activated in PP migrate to the  
23 gastric mucosa, resulting in the development of gastritis [6, 7]. *H. pylori* infection  
24 predominantly induces cell-mediated immune responses. The T cells from *H.*

1 *pylori*-infected gastric mucosa are Th1 cells, which secrete IFN- $\gamma$  [8-10]. In  
2 addition, studies using IFN- $\gamma$  *-/-* and IL-4 *-/-* mice have demonstrated the roles of  
3 Th1 cells in the development of gastritis caused by *H. pylori* infection [11]. Thus,  
4 *H. pylori* induces innate and acquired immunity.

5  
6 CagA protein is one of the most studied bacterial pathogen factors of *H. pylori*.  
7 The *cagA* gene is encoded in a pathogenicity island known as 'cag PAI' and is  
8 deeply correlated with the severity of *H. pylori*-related diseases. The injury of  
9 epithelial cells is well studied with regard to the roles of CagA in diseases  
10 evoked by *H. pylori*. After the attachment of *H. pylori* to gastric epithelial cells,  
11 CagA is translocated into gastric epithelial cells via a type IV secretion system  
12 consisting of various factors encoded in *cagPAI* [12-14]. In gastric epithelial cells,  
13 tyrosine residues on CagA are phosphorylated by src-family kinase [15, 16], and  
14 the tyrosine-phosphorylated CagA forms a complex with the src homology 2  
15 domain-containing tyrosine phosphatase SHP-2, which positively regulates  
16 mitogenic signal transduction [17]. *H. pylori* is subdivided into *cagA*-positive and  
17 *cagA*-negative strains, and the induction of mucosal damage and severe  
18 atrophic gastritis by the *cagA*-positive strains are much more potent compared to  
19 those of the *cagA*-negative strains [18]. Furthermore, epidemiological studies  
20 have suggested a critical role of the *cagA*-positive *H. pylori* in the development  
21 of gastric adenocarcinoma [19, 20]. In contrast to the effects of CagA in gastric  
22 epithelial cells, the roles of CagA in host immune systems remain to be  
23 elucidated. Clinically, the grade of atrophic gastritis is more severe in  
24 *cagA*-positive strains than in *cagA*-negative strains [18], and the incidence rate

1 of gastric cancer is also higher for *cagA*-positive strains [19, 20]. *cagA*-positive *H.*  
2 *pylori* infection elicits larger amounts of IL-8 production from epithelial cells, and  
3 then IL-8 induces cellular infiltration into the mucosa and consequently leads to  
4 the activation of anti-bacterial immune responses [21, 22]. For the development  
5 of atrophic gastritis and gastric cancer, it is required that *H. pylori* colonizes the  
6 stomach for long periods and continues to damage gastric epithelial cells. CagA  
7 must be recognized as an antigen by APC and affect the host immune response  
8 because the serum from *H. pylori*-infected patients contains anti-CagA antibody  
9 [23]. However, it is unclear how CagA regulates innate and acquired immunity  
10 against *H. pylori* infection.

11  
12 In this study, we investigated the influence of CagA on the function of DC. Bone  
13 Marrow derived DC (BMDC) were infected with *cagA*-positive or *cagA*-negative  
14 *H. pylori* strain, and then pro-inflammatory cytokine production profiles and the  
15 degree of DC maturation were examined. *cagA*-positive *H. pylori* infection  
16 attenuated the functions of DC compared to *cagA*-negative *H. pylori* infection via  
17 tyrosine phosphorylation of CagA. In addition, a decline in the functions of  
18 BMDC from CagA-transgenic mice was observed. Our results suggest that  
19 CagA protein suppresses host immune responses and permits *H. pylori* to  
20 colonize the stomach, resulting in continuous injury to gastric epithelial cells,  
21 which is linked to the incidence of gastric cancer.

## Methods

### Antibodies and reagents

LPS from *E. coli* serotype 055: B5 was used (Sigma-Aldrich, St. Louis, MO). For immunoprecipitation and Western blotting, anti-CagA antibody (Austral Biologicals, San Ramon, CA), anti-IRF3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), and anti-phospho-Ser-396 IRF3 antibody (Cell Signaling Technology, Danvers, MA) were purchased.

### Bacteria

*H. pylori* *cagA*-positive strain ATCC43504 (wild type) and *cagA*-negative strain ( $\Delta cagA$ ) were cultured on blood agar plates (Nippon Becton Dickinson, Tokyo, Japan) under microaerobic conditions (5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>) at 37°C. Before each experiment, *H. pylori* were grown in Brucella Broth (BD Biosciences, Franklin Lakes, NJ) supplemented with 5% equine serum for 48 hr.

### Preparation of DC from Bone Marrow

The preparation of DC from bone marrow was performed according to the method of a previous report [24]. Briefly, bone marrow cells from CagA transgenic mice [25] or wild type C57BL/6 mice were cultured with 10 ng/ml GM-CSF and 10 ng/ml IL-4 [26]. On day 10, nonadherent cells were harvested by pipetting and then separated by density gradient centrifugation using OptiPrep (Axis-Shield, Oslo, Norway) density gradient solutions according to



manufacturer's instructions. The separated cells were washed twice with PBS and then resuspended with RPMI-1640 before each experiment was performed.

#### **Cell treatment**

BMDC were infected with the *cagA*-positive or *cagA*-negative *H. pylori* at an MOI of 50:1 in serum-free medium for the periods specified in the figures and then subjected to the following experiments. AGS human gastric carcinoma cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum. After the AGS cells had been serum-starved for 12 hr, the cells were infected with the *cagA*-positive or *cagA*-negative strain of *H. pylori* at an MOI of 50:1 for the periods specified in Figure 1.

#### **Immunoprecipitation and Western blotting**

AGS cells were lysed in lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1% Brij-35, protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 µg/ml trypsin inhibitor, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) and phosphatase inhibitors (10 mM NaF and 2 mM Na<sub>3</sub>VO<sub>4</sub>). For the detection of IRF3 and phosphorylated IRF3, BMDC were lysed in 100 µl of low stringency buffer consisting of 50 mM HEPES (pH 7.5), 100 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5% Nonident P-40, and the same protease inhibitors and phosphatase inhibitors. The homogenate was stirred for 1 hr on ice and centrifuged at 15,000 × g for 20 min at 4°C, and the supernatant obtained was used as a cell lysate. Immunoprecipitation using protein A/G-agarose beads (Santa Cruz Biotechnology Inc.) was performed according

to the manufacturer's protocols, and protein-antibody complexes were obtained. The cell lysates and protein-antibody complexes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred onto a PVDF membrane (Millipore, Molsheim, France), and this was followed by the blocking of non-specific binding sites. The membrane was probed with the primary antibody before being reacted with the corresponding horseradish peroxidase-conjugated secondary antibody. The protein/antibody complex was visualized with ChemiLumiONE (Nacalai tesque, Japan) and then was detected using an Image Reader (LAS-3000 Imaging System, Fuji Photo Film).

#### **RT-PCR and quantitative real-time PCR**

BMDC were plated at a density of  $2.0 \times 10^6$  cells/35-mm well and then subjected to each experiment. Total RNA was extracted with TRIzol Reagent (Invitrogen, Grand Island, NY) according to the manufacturer's protocols. Reverse transcription (RT) was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. The polymerase chain reaction (PCR) was carried out using the Blend Taq System (TOYOBO, Osaka, Japan), and the expression of CagA mRNA in DC was examined using primer pairs for CagA (sense: 5'-AAGCTGCTTCTGCGATTAACCG-3'; antisense: 5'-GGAGTCTTTCAGTTCGTC-3'),  $\beta$ -actin (sense: 5'-AAGGCCAACCGTGAAAAGAT-3'; antisense: 5'-GTGGTACGACCAGAGGCATAC-3'), and CD11c (sense: 5'-TGTGACGGTGTCTAATGATGG-3'; antisense:

5'-AGTTGATGCTGACTGGCACG-3'). The mRNA expression of cytokines in BMDC was evaluated by the real-time PCR system using SYBR-Green PCR reagents (Applied Biosystems) according to the manufacturer's protocols. The primer sequences used were as follows: IFN- $\gamma$  (sense: 5'-GCGTCATTGAATCACACCTG-3'; antisense: 5'-TGAGCTCATTGAA TGCTTGG-3'), TNF $\alpha$  (sense: 5'-TAGCCAGGAGGGAGAACAGA-3'; antisense: 5'-TTTTCTGGAGGGAGATGTGG-3'), IL-4 (sense: 5'-CCAAGGTGCTTCGCAT ATTT-3'; antisense: 5'-ATCGAAAAGCCCGAAAGAGT-3'), IL-10 (sense: 5'-CCA GGGAGATCCTTTGATGA-3'; antisense: 5'-AACTGGCCACAGTTTTTCAGG-3'), IL12p40(sense: 5'-AGCAGTAGCAGTTCCCCTGA-3'; antisense: 5'-AGTCCCTT TGGTCCAGTGTG-3'). The expression levels of target mRNA were normalized to that of  $\beta$ -actin.

#### **Flow cytometry**

To evaluate BMDC maturation, the expression of cell surface molecules was examined by direct immunofluorescent flow cytometric analysis using a FACS Calibur flow cytometer and Cell Quest software (BD Biosciences). BMDC were reacted with 1  $\mu$ g/100  $\mu$ L Fc Block (BD Biosciences) for 5 min, and then incubated on ice for 15 min with PE-labeled rat anti mouse CD86 antibody (BD Biosciences) at a dilution of 1:50. The cells were washed twice with ice-cold PBS containing 0.5% bovine serum albumin and sodium azide, before being resuspended in ice-cold 2% paraformaldehyde, and subjected to flow cytometric analysis.

## **ELISA**

The culture medium was centrifuged at 16,000 x g for 5 min at 4°C, and then the supernatant was collected. The TNF $\alpha$ , IL-10, and IL-12p40 levels in the culture medium were measured by commercial ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's protocols.

## **Dc-T cell interaction**

Lymphocytes isolated from the spleens of OVA-specific OTII transgenic mice [27] were separated using lymphocyte separation reagents (Wako, Osaka, Japan), and washed twice with PBS. CD4<sup>+</sup> T cells were collected from lymphocytes using the Auto-MACS system (BD Biosciences) according to the manufacturer's protocols and then were resuspended in RPMI-1640 after being washed twice with PBS. After pretreatment with 100  $\mu$ g/ml OVA-peptide<sub>323-339</sub> (Genway Biotech, San Diego, CA) for 6 hr, DC were co-cultured with CD4<sup>+</sup> T cells at a ratio of 1:5 (DC  $2.0 \times 10^5$  cells, CD4<sup>+</sup> T cells  $1.0 \times 10^6$  cells) in a round bottom 96-well plate (Nunc, Roskilde, Denmark). After 48 hr, total RNA was isolated, and real time PCR was performed.

## **Statistical analysis**

All data are expressed as means  $\pm$  S.E. of at least three independent determinations for each experiment. Statistical significance was analyzed using the Student's t-test, and a level of probability of 0.05 was used as the criterion for significance.

## Results

### Tyrosine-phosphorylation of the CagA protein in the *H. pylori*-infected DC

CagA protein is one of the most assessed bacterial pathogen factors from *H. pylori*, and the translocation of CagA into gastric epithelial cells by a type IV secretion system leads to adverse effects in the host cells [14, 17, 28-31]. In *H. pylori*-infected AGS human gastric carcinoma cells, CagA is translocated into the cells and tyrosine-phosphorylated (Figure 1a). To investigate whether CagA is translocated into DC and then has its tyrosine residues phosphorylated, BMDC from C57BL/6 mice (WT-DC) were infected with *cagA*-positive (wild type) or *cagA*-negative ( $\Delta cagA$ ) *H. pylori*. Both strains of *H. pylori* used in this study have a type IV secretion system, and the difference between both strains is only the presence or absence of CagA. As a result, the translocation of CagA protein into DC and its tyrosine-phosphorylation were observed during wild type *H. pylori* infection, but not during  $\Delta cagA$  *H. pylori* infection (Figure 1b). These results suggest that the tyrosine-phosphorylation of CagA is caused in DC as well as gastric epithelial cells.

### Cytokine production and the maturation of immature DC stimulated with *H. pylori*

The phenotypes of *H. pylori*-infected AGS cells are differ depending on the presence or absence of CagA in *H. pylori*. For example, the IL-8 production level in AGS cells infected with *cagA*-negative *H. pylori* is lower than that in *cagA*-positive *H. pylori* [21, 22]. To assess the influence of CagA on the

1 functions of DC, the profiles of cytokine production and the degree of maturation  
2 in WT-DC stimulated with wild type *H. pylori* or  $\Delta cagA$  *H. pylori* were investigated.  
3 Regarding the mRNA expression levels of  $TNF\alpha$ , IL-12p40, and IL-10, their  
4 cytokine profiles in WT-DC were different between those infected with wild type  
5 *H. pylori* and those infected with  $\Delta cagA$  *H. pylori*, although both strains of *H.*  
6 *pylori* increased their expression levels in DC (Figure 2a). The  $TNF\alpha$  and  
7 IL-12p40mRNA expression levels in DC stimulated with wild type *H. pylori* were  
8 significantly lower than those stimulated with  $\Delta cagA$  *H. pylori*. On the other hand,  
9 the IL-10 mRNA expression level in the wild type *H. pylori* infection was  
10 significantly higher than that seen in  $\Delta cagA$  *H. pylori* infection. The differences in  
11 the cytokine profiles between the cells infected with wild type *H. pylori* and  
12  $\Delta cagA$  *H. pylori* were observed measuring the levels of cytokines secreted from  
13 the stimulated WT-DC (Figure 2b), and the results of cytokine secretion levels  
14 were consistent with those of cytokine mRNA expression levels (Figure 2a),  
15 suggesting that the presence of CagA regulates cytokine production in DC  
16 infected with *H. pylori* and may lead to the suppression of the anti-bacterial  
17 immune responses. Next, it was investigated whether CagA affects the  
18 maturation of DC infected with *H. pylori*. WT-DC were infected with wild type *H.*  
19 *pylori* or  $\Delta cagA$  *H. pylori* at an MOI of 50:1 for 48 hr, and then the surface  
20 expression of the costimulatory molecule CD86 was examined by FACS (Figure  
21 2c). As a result, both strains of *H. pylori* upregulated the surface expression of  
22 CD86 in WT-DC. Interestingly, the CD86 surface expression during  $\Delta cagA$  *H.*  
23 *pylori* infection was higher compared to that observed during wild type *H. pylori*

infection. These results indicate that *cagA*-positive strain of *H. pylori* can suppress the immune response to *H. pylori* infection via CagA in DC.

#### **Cytokine production and the maturation of DC from CagA-transgenic mice**

Some bacterial virulence factors from *H. pylori* except CagA, for example VacA, are known to affect cellular functions [32-34]. To exclude the effects of other effectors than CagA on the functions of DC, BMDC derived from CagA transgenic mice (CagA Tg-DC) were used. The mRNA of CagA was expressed in CagA Tg-DC (Figure 3). The presence of DC was confirmed by expression of CD11c, which is the common surface marker of DC (Figure 3). WT-DC and CagA Tg-DC were stimulated with 200 ng/ml *E. coli* LPS, and the profiles of cytokine production and the degree of maturation in DC were examined. As shown in Figure 4a, LPS increased the expression levels of TNF $\alpha$ , IL-12p40, and IL-10 in both WT-DC and CagA Tg-DC. The TNF $\alpha$  and IL-12p40 mRNA expression levels in stimulated WT-DC were significantly higher than those in stimulated CagA Tg-DC. On the other hand, the IL-10 mRNA expression level in stimulated WT-DC was significantly lower compared to that in stimulated CagA Tg-DC. The levels of cytokines secreted from stimulated DC were consistent with the cytokine mRNA expression levels (Figure 4b). In addition, LPS increased the surface expression of CD86 on both DCs, but the expression level in stimulated CagA Tg-DC was lower than that in stimulated WT-DC (Figure 4c). These results suggest that CagA negatively regulates the functions of DC in the response to LPS.

## **The activation of CD4<sup>+</sup> T cells by CagA-Tg DC**

We next investigated how CagA regulates the functions of DC in acquired immune responses. CD4<sup>+</sup> T cells, which were isolated from the spleens of OVA-specific OT-II mice, were co-cultured with OVA-peptide-treated WT-DC or CagA Tg-DC for 24 hr, and the mRNA expression of IFN- $\gamma$ , IL-4, and IL-10 were determined (Figure 5a). The CD4<sup>+</sup> T cells activated with CagA Tg-DC exerted higher expression of IL-10 mRNA and lower expression of IFN- $\gamma$  mRNA compared to the CD4<sup>+</sup> T cells activated with WT-DC. No significant difference in IL-4 mRNA expression was observed. These results indicate that the presence of CagA in DC impairs the differentiation of CD4<sup>+</sup> T cells to Th1 type T cells because IFN- $\gamma$  plays an important role in the induction of Th1 type immune responses [35]. Therefore, the mRNA expression of T-bet and GATA-3, transcription factors essential for the development of Th1 cells and Th2 cells respectively, were examined in CD4<sup>+</sup> T cells activated with WT-DC or CagA Tg-DC (Figure 5b). As a result, the T-bet mRNA expression level in the WT-DC-activated CD4<sup>+</sup> T cells was found to be significantly higher than that in the CagA Tg-DC-activated CD4<sup>+</sup> T cells. On the other hand, the GATA-3 mRNA expression level was the same in WT-DC and CagA Tg-DC. These results suggest that CagA suppresses the differentiation of CD4<sup>+</sup> T cells to Th1 type T cells.

## **Phosphorylation of IRF3 was suppressed in CagA Tg-DC**

Many studies have revealed that LPS stimulates DC via TLR4. The TLR4-mediated signaling pathways can be divided into two groups: one is



1 Myd88-dependent and the other is Myd88-independent pathway. In the  
2 Myd88-independent signaling pathway after LPS stimulation, TRIF acts as an  
3 adapter protein instead of Myd88. IRF-3 is phosphorylated by TBK1, which is a  
4 downstream molecule of TRIF, and consequently translocates into nucleus,  
5 leading to the expression of type I IFN and IFN-inducible genes. It was reported  
6 that activated SHP-2 suppressed the enzymatic activity of TBK1 [36]. As SHP-2  
7 is activated by tyrosine-phosphorylated CagA in CagA Tg-DC, the  
8 phosphorylation levels of IRF3 were analyzed after LPS stimulation. WT-DC and  
9 CagA Tg-DC were stimulated with 200 ng/ml *E. coli* LPS. After 1 hr, total cell  
10 lysates were prepared and subjected to Western blot analysis. As a result, CagA  
11 Tg-DC had a lower level of phosphorylated IRF3 than WT-DC (Figure 6). These  
12 results suggest that phosphorylated CagA can inhibit the Myd88 independent  
13 pathway via SHP-2 activation and consequently suppress the responses of DC  
14 to LPS stimulation.

## Discussion

The infection of *H. pylori* is deeply associated with the development of cancer in the host stomach. It is well known that *H. pylori* causes gastric cancer not only by direct actions on epithelial cells but also indirect actions via inflammatory responses. Since the immune system can not completely eliminate *H. pylori* from the stomach, the inflammatory responses induced by *H. pylori* continue over the long term. This inability to eliminate *H. pylori* indicates that it may negatively regulate host immune responses. Therefore, we investigated the influence of *H. pylori* on the functions of DC in experiments using BMDC infected with *cagA*-positive *H. pylori* or *cagA*-negative *H. pylori*.

The most interesting finding of this study is that CagA negatively regulates the functions of DC. In experiments using BMDC stimulated with the *cagA*-positive and *cagA*-negative *H. pylori*, it was found that CagA was able to suppress the expression of TNF- $\alpha$  and IL-12p40; i.e., inflammatory cytokines, and promote the expression of IL-10; i.e., a suppressive cytokine (Figure 2). CagA also inhibited the maturation of DC (Figure 2). The same results were revealed in experiments using BMDC derived from CagA Tg mice (Figure 4). In gastric epithelial cells, bacterial virulence factors are very important for cellular injury, which is linked to the development of gastric cancer. For example, VacA, one of the virulence factors from *H. pylori*, is an 87kDa protein that directly induces the vacuolation of gastric epithelial cells [32-34]. VacA also has a high potency for negatively regulating the proliferation of lymphocytes *in vitro* [37-41], indicating

1 that the virulence factors of *H. pylori* may lead to the suppression of immune  
2 responses, thereby allowing the infection to persist. CagA is another important  
3 virulence factor from *H. pylori* and is closely associated with the development of  
4 gastric cancer. After the attachment of *H. pylori* to epithelial cells, CagA is  
5 directly translocated into the cells and then tyrosine-phosphorylated, resulting in  
6 carcinogenesis [19, 20]. However, it remains unclear how CagA modulates the  
7 immune responses against *H. pylori* and moreover indirectly injures epithelial  
8 cells through inflammatory responses. It is highly possible that the negative  
9 regulation of immune responses by CagA is induced in DC. DC plays major roles  
10 in the immune pathways connecting innate and acquired immunity. In a previous  
11 study, it was reported that antigens of *H. pylori* are recognized by DC in PP [6, 7].  
12 After this recognition, *H. pylori*-specific lymphocytes are activated by DC and  
13 migrate to the site of infection; i.e., the gastric mucosa [6, 7]. Although *H. pylori*  
14 are able to activate DC causing Th1 type immune responses in an *in vitro*  
15 experiment [42, 43], the *cagA*-positive *H. pylori*-secreted factors inhibited the  
16 secretion of IL-12 from DC [44]. In addition, IL-12 secretion and the maturation of  
17 DC were suppressed by long-term infection with *cagA*-positive *H. pylori* [45].  
18 These results indicate that *H. pylori* negatively regulates the functions of DC via  
19 CagA.

20  
21 Phosphorylation of CagA may be important for the *H. pylori*-caused negative  
22 regulation of DC functions. In contrast to our results in this study, it was reported  
23 that the maturation and activation of DC, as well as DC-mediated T-cell  
24 activation were independent of *cag* PAI of *H. pylori* [46]. Kranzer *et al.* used the

1 strain *H. pylori* possessing ABC- type CagA, while *H. pylori* possessing ABCCC-  
2 type CagA was selected in our study. ABCCC- type CagA is more easily  
3 phosphorylated than ABC- type CagA, suggesting that the CagA protein  
4 containing many phosphorylated tyrosine residues may lead to the dysfunction  
5 of DC. Actually, the tyrosine residues on CagA were phosphorylated in DC as  
6 well as in AGS gastric epithelial cells (Figure 1). To the best of our knowledge,  
7 this is the first report to confirm the tyrosine-phosphorylation of CagA in DC,  
8 although it was reported that CagA is tyrosine-phosphorylated in macrophages  
9 [47]. In epithelial cells, tyrosine-phosphorylated CagA binds to SHP-2 [28]. In  
10 CagA transgenic mice, the binding of CagA to SHP-2 was confirmed [25],  
11 suggesting that SHP-2 is activated through its binding to CagA in DC as well as  
12 epithelial cells. SHP-2 negatively regulates the JAK/STAT (STAT1 and STAT3)  
13 pathways [48, 49]. Signal transducers and activators of transcription (STAT) play  
14 important roles downstream of receptors for several cytokines. Therefore,  
15 CagA-activated SHP-2 might suppress the functions of DC. SHP-2 also  
16 negatively regulates TLR signaling [50]. In a previous report, C-terminal of  
17 SHP-2 directly bound to the kinase domain of TBK1 leading to suppression of  
18 the TBK activity and the consequent nuclear translocation of IRF3, which occur  
19 downstream of TLR3 and TLR4 [36] and lead to production of interferons [51]. In  
20 this study, it was revealed that the phosphorylation of IRF3 was reduced in  
21 LPS-stimulated CagA Tg-DC compared to WT-DC (Figure 6). From these results  
22 and those of previous reports, we propose the following putative mechanisms: 1)  
23 The phosphorylated CagA binds to SHP-2 in DC. 2) The CagA-bound SHP-2  
24 inhibits the enhanced kinase activity of TBK1. 3) Downregulation of the TBK

1 activity reduces phosphorylation of IRF3 and the following nuclear translocation.

2 4) The production of interferons is suppressed in DC. Thus, CagA would  
3 negatively regulate the functions of DC.

4  
5 In conclusion, our data suggest that *cagA*-positive *H. pylori* infection can  
6 suppress host immune reactions via CagA phosphorylation and create more  
7 favorable environments for the colonization of the gastric mucosa. Through the  
8 chronic colonization and continuous epithelial injury caused by the negative  
9 regulation of DC, the development of gastric cancer by *H. pylori* infection may be  
10 increased.

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## Figure Legends

**Figure 1. The tyrosine-phosphorylation of the CagA protein in AGS cells and DC infected with *H. pylori*.** AGS cells (a) and BMDC (b) were infected with the *cagA*-positive or *cagA*-negative strain of *H. pylori* at MOI of 50:1 for 5 hr. Cell lysates were subjected to immunoprecipitation with anti-CagA antibody followed by Western blotting with anti-phosphotyrosine antibody. Lane1, No infection; Lane2, Infection with *cagA*-positive *H. pylori* (wild type); Lane3, infection with *cagA*-negative *H. pylori* ( $\Delta cagA$ ). Typical images are shown from at least triplicate determinations.

**Figure 2. The regulation of cytokine expression and DC maturation by *cagA*-positive *H. pylori*.** BMDC were infected with *cagA*-positive or *cagA*-negative strain of *H. pylori*. As a vehicle control, DCs were treated with PBS alone. a) After 5 hr, total RNA was isolated, and the expression of  $\text{TNF}\alpha$ , IL-10, and IL-12p40 mRNA was determined by quantitative real-time PCR. The mRNA expression levels were normalized to those of  $\beta$ -actin as an internal control. b) After 48 hr, the  $\text{TNF}\alpha$ , IL-10, and IL-12p40 levels in the culture medium were measured by ELISA. Data are represented as the mean  $\pm$  S.E. (n=3), and asterisks indicate significant differences according to the Student's t-test ( $P < 0.05$ ). c) After 48 hr, the surface expression of CD86 was examined by FACS. Typical images are shown from at least triplicate determinations.

**Figure 3. The expression of CagA in DC from CagA transgenic mice.** The mRNA expression of CagA and CD11c, and  $\beta$ -actin as an internal control in BMDC from C57BL/6 mice or CagA transgenic mice was determined by RT-PCR.

**Figure 4. The cytokine production and maturation of DC from CagA-transgenic mice.** BMDC from C57BL/6 mice or CagA-transgenic mice were treated with 200 ng/ml LPS. a) After 5 hr, total RNA was isolated, and the expression of  $\text{TNF}\alpha$ , IL-10, and IL-12p40 mRNA was determined by quantitative real-time PCR. The mRNA expression levels were normalized to those of  $\beta$ -actin as an internal control. b) After 48 hr, the  $\text{TNF}\alpha$ , IL-10, and IL-12p40 levels in the culture medium were measured by ELISA. Data are represented as the mean  $\pm$  S.E. (n=3), and asterisks indicate significant differences according to the Student's t-test ( $P < 0.05$ ). c) After 48 hr, the surface expression of CD86 was examined by FACS. Typical images are shown from at least triplicate determinations.

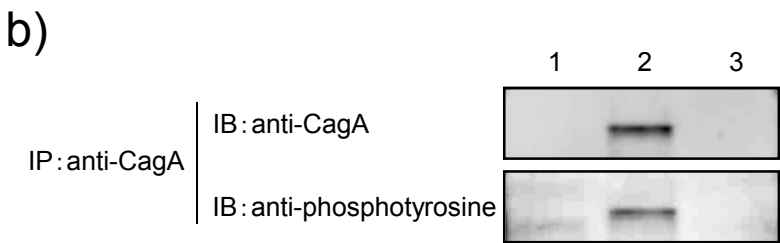
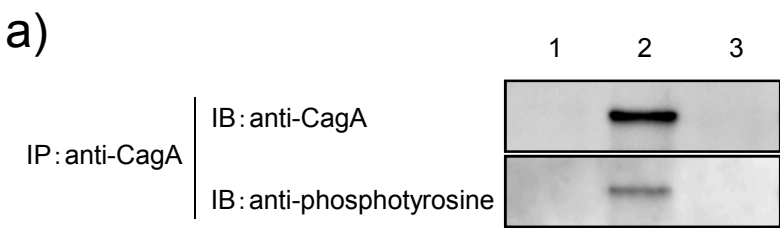
**Figure 5. The activation of  $\text{CD4}^+$  T cells by DC from CagA-transgenic mice.** Splenocytes were derived from OVA-specific OT-II mice.  $\text{CD4}^+$  T cells were prepared from the splenocytes by Auto-MACS and then co-cultured with OVA-peptide-treated BMDC from C57BL/6 mice or CagA-transgenic mice. a) After 24 hr, total RNA was isolated, and the expression of  $\text{IFN-}\gamma$ , IL-10, and IL-4 mRNA was determined by quantitative real-time PCR. The mRNA expression

1 levels were normalized to those of  $\beta$ -actin as an internal control. b) After 24 hr,  
2 total RNA was isolated and the expression of T-bet and GATA3 mRNA was  
3 determined by quantitative real-time PCR. The mRNA expression levels were  
4 normalized to those of  $\beta$ -actin as an internal control. Data are represented as the  
5 mean  $\pm$  S.E. (n=3), and asterisks indicate significant differences according to the  
6 Student's t-test ( $P < 0.05$ ).

7  
8 **Figure 6. LPS-induced phosphorylation of IRF3 in CagA Tg-DC.**

9 BMDC from C57BL/6 mice or CagA transgenic mice were treated with 200 ng/ml  
10 *E. coli* LPS for 1 hr. Cell lysates were subjected to Western blotting with  
11 anti-IRF3 antibody and anti-phospho IRF3 antibody. Lane1, No stimulation;  
12 Lane2, LPS-stimulated WT-DC; Lane3, LPS-stimulated CagA Tg-DC. Typical  
13 images are shown from at least triplicate determinations.

Figure1



# Figure2

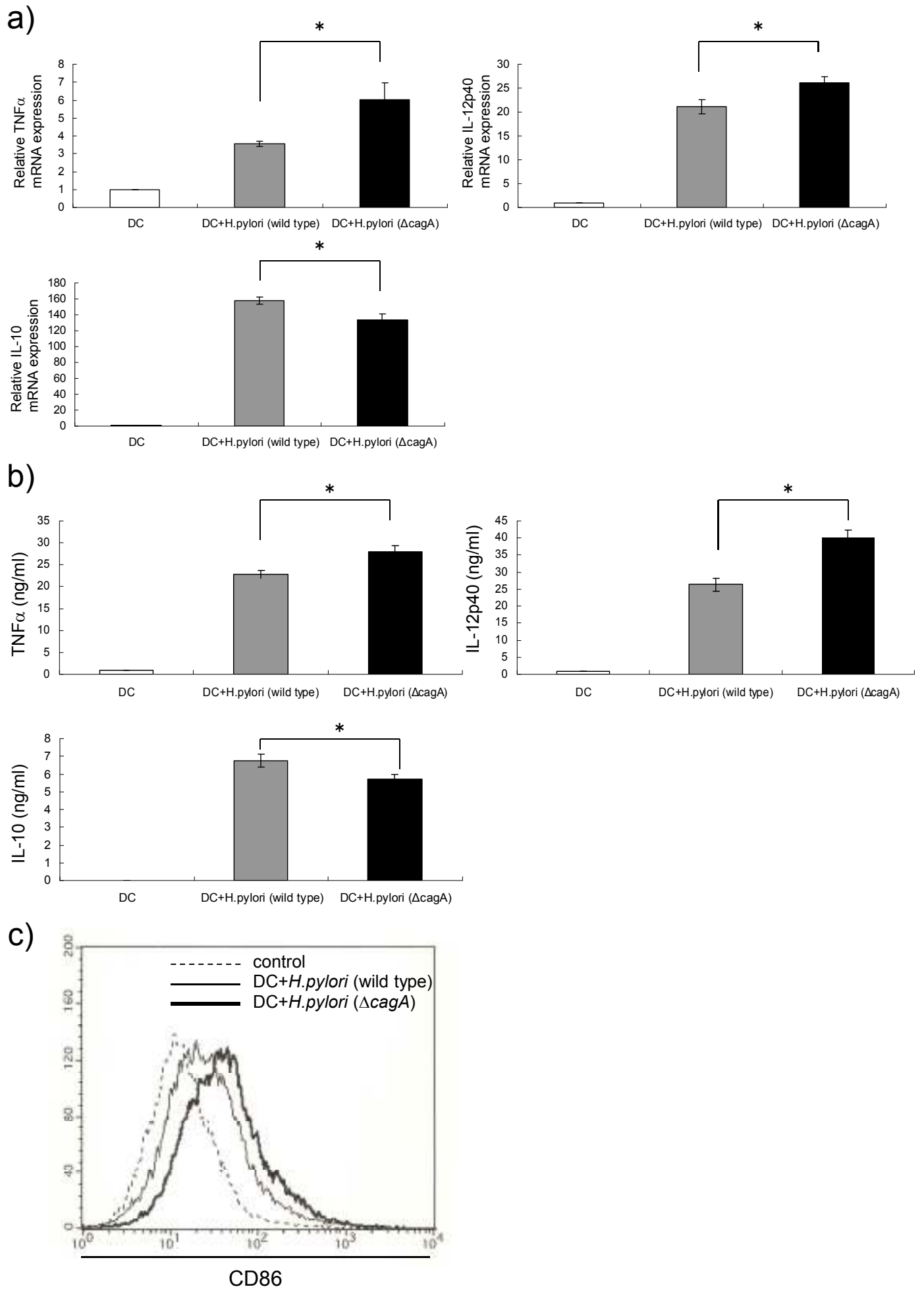


Figure3

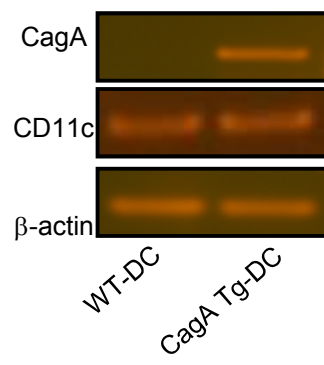
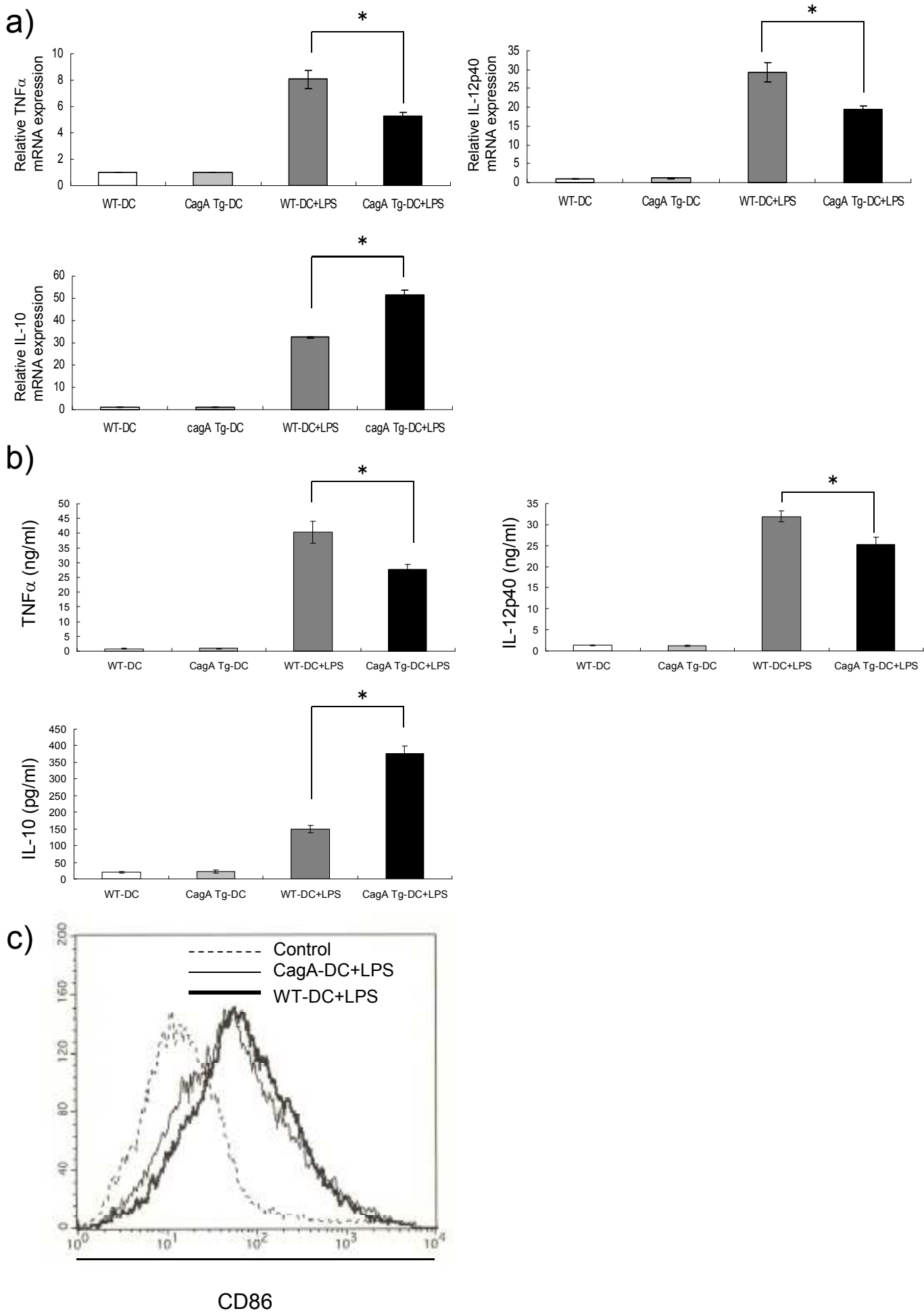


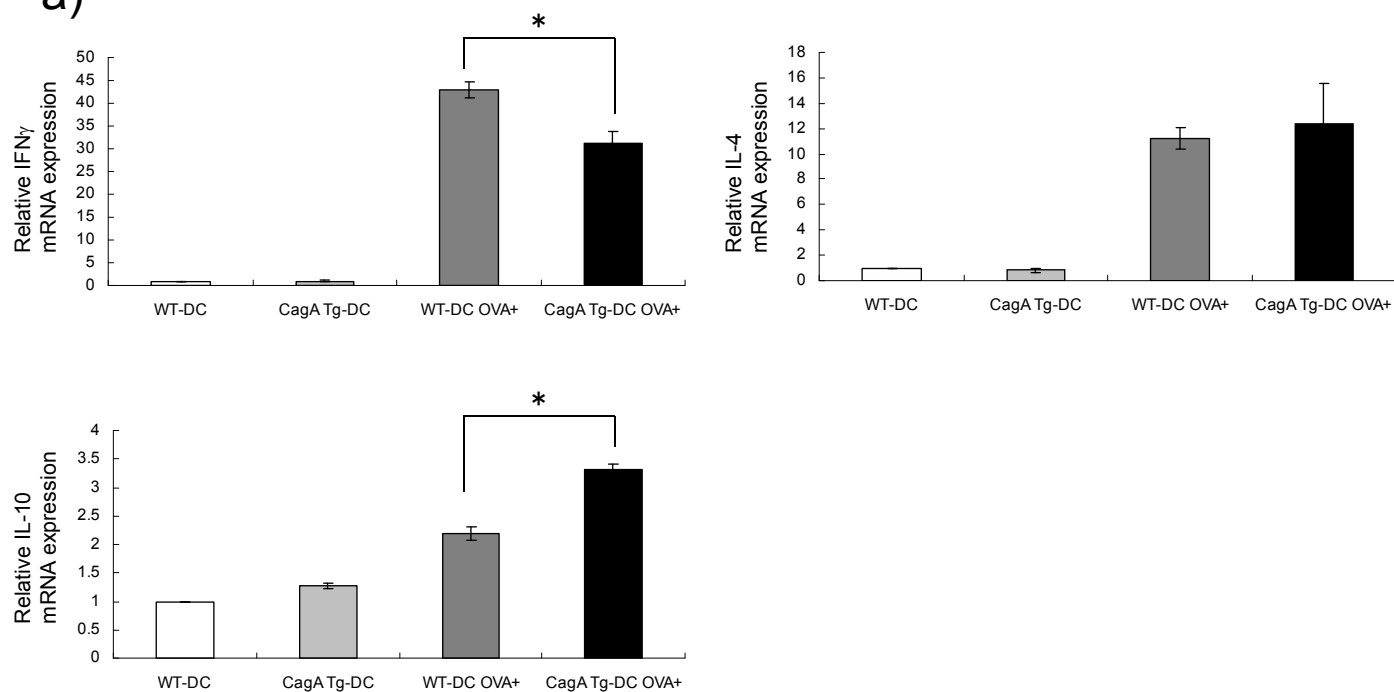
Figure4





# Figure5

a)



b)

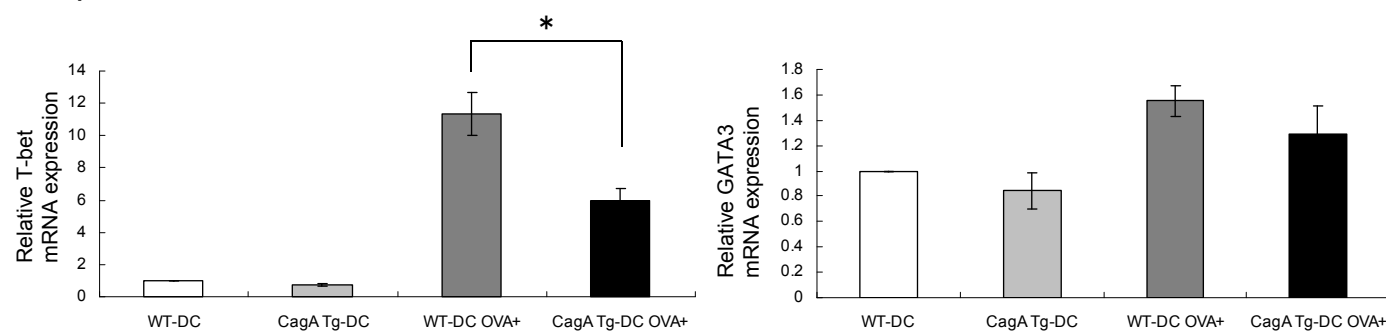


Figure6

