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CTLA-4 blockade inhibits induction of *Helicobacter pylori*-associated gastritis in mice

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SUMMARY

The balance between Th1 and Th2 response determines the outcome of *Helicobacter pylori* infection. Interferon (IFN)-γ plays an inductive role in gastric inflammation, whereas interleukin (IL)-4 counterbalances Th1 response and suppresses the development of gastritis. Th cell response is regulated by co-stimulatory factors. A co-stimulatory molecule, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), plays an inhibitory role in IL-2-dependent cell growth and mediates an optimal inhibitory signal to Th1 and Th2 cells. We administered anti-CTLA-4 monoclonal antibody (MoAb), which blocks CTLA-4 signalling, to examine the relative role for this signalling during maturation of Th1 and Th2 cells in *H. pylori* infection in mice. Mice treated by anti-CTLA-4 MoAb within the first week of infection showed an inhibition of gastric inflammation, accompanied by an increasing ratio of *H. pylori*-specific IgG1/IgG2a in serum following infection. Furthermore, the treatment resulted in the higher ratio of IL-4/IFN-γ by splenocytes in response to *H. pylori* antigen at 6 weeks after infection, compared with untreated mice. These results suggest that the predominance of Th2 response by CTLA-4 blockade leads to an inhibition of the development of gastric inflammation. CTLA-4 signalling could contribute to the regulation of Th subsets and the development of gastric inflammation in *H. pylori* infection.

Keywords CTLA-4 *Helicobacter pylori* IFN-γ IL-4

INTRODUCTION

Helicobacter pylori, a spiral microaerophilic bacterium, causes active gastritis [1]. Once *H. pylori* infection is established, the organisms colonize and inflame the gastric mucosa. Previous studies have demonstrated that long-lasting gastric inflammation may predispose toward low-grade gastric lymphoma and gastric carcinoma [2,3]. With respect to host immune response, induction of gastric inflammation is associated with the up-regulation of Th1 cytokine response (e.g. interferon (IFN)-γ), whereas anti-inflammatory immune response is associated with the predominance of Th2 cytokine response (e.g. interleukin (IL)-4). Neutralization of IFN-γ during an infection results in a significant reduction of gastric inflammation [4]. IFN-$\gamma^{-/-}$ animals have no mucosal inflammation when infected with *H. pylori* [5,6]. On the other hand, IL-$4^{-/-}$ mice infected with *Helicobacter* spp. show severe mucosal inflammation [5,7]. When Th2-directed immunity is stimulated by oral and systemic administration of whole bacteria preparations or purified antigens (e.g. urease), co-administered with strong adjuvants (e.g. cholera toxin), gastritis is inhibited [8–10]. Regulation of Th subsets determines whether or not inflammation occurs.

The co-stimulatory factors play an essential role in the regulation of T cell immune response [11,12]. CD28 is expressed on naive and activated T cells and engages its ligands, B7-1 (CD80) and B7-2 (CD86). The co-stimulatory signal mediated by CD28 is required for T cell activation and increases the production of cytokines [13]. On the other hand, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4, CD152), which is homologous to CD28 and also engages B7, is not expressed on resting T cells, but is induced after the initial steps of T cell activation. CTLA-4, expressing on activated T cells, plays an inhibitory role in T cell proliferation in murine and human lymphocytes [14,15]. Furthermore, CTLA-4 regulates the production of cytokines by Th1 and Th2 cells and has diverse effects on the progress of infectious diseases, which are dependent on pathogen and host factors. Administration of anti-CTLA-4 MoAb that blocks interaction between CTLA-4 and B7 enhances their susceptibility to *Leishmania major* and exacerbates tissue inflammation in BALB/c mice [16]. This response is attributed to an increased Th2 response by CTLA-4 blockade. However, there is no effect when the MoAb is

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administered to C57BL/6 mice that are Th1-biased during the parasite infection. In pulmonary mycobacterial infection, administration of anti-CTLA-4 MoAb enhances the antigen-specific expansion and differentiation of lymphocytes in the draining lymph node. Nevertheless, CTLA-4 blockade has no effect on IFN-γ mRNA expression of lymphocytes at the primary site of infection and fails to enhance protection and affect grauloma formation [17].

In this study, we administered anti-CTLA-4 MoAb to mice infected with *H. pylori* to assess the role of CTLA-4 costimulatory pathway in the regulation of Th subset expression during development of the immune response to this organism. The induction of *H. pylori*-associated gastritis was inhibited by blocking CTLA-4 signalling, even though the bacterial colonization was not affected. CTLA-4 blockade at the early stage of infection gradually increased the serum levels of *H. pylori*-specific IgG1 relative to IgG2a and enhanced the production of IL-4 relative to IFN-γ by splenic cells at 6 weeks after infection. These indicated that Th2-directed response by CTLA-4 blockade appeared to be the major factor responsible for the suppressed inflammatory state.

MATERIALS AND METHODS

Animals and bacteria

Pathogen-free 6-week-old female C57BL/6 mice were obtained from Seac Yoshitomi (Fukuoka, Japan). Mice were housed in a specific-pathogen-free environment and provided with food and water *ad libitum*. Experiments were performed according to the guidelines of the Ethical Committee for Animal Experiments at Oita Medical University, Oita, Japan. Sydney strain (SS1) of *H. pylori* (kindly provided by Dr A. Lee; School of Microbiology and Immunology, University of New South Wales, Sydney, NSW, Australia) was grown in brucella broth containing 10% horse serum under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C. Mice were inoculated by gastric intubation with 0·5 ml of live SS1 [5×10^7 colony-forming units (CFU)/ml] on day 1.

In vivo *treatment with anti-CTLA-4 MoAb*

Hamster antimurine CTLA-4 [UC10–4F10-11] MoAb was kindly provided by Dr Jeffrey A. Bluestone (University of Chicago, Chicago, IL, USA). The mice were injected intraperitoneally with either 100 μg of anti-CTLA-4 MoAb or hamster IgG (Cappel, West Chester, PA, USA) on day 0, and then daily until day 7 post-infection. Spleen and stomach samples were obtained at 0, 1, 2, 4 and 6 weeks after infection. Previous studies have shown that this dose of anti-CTLA-4 MoAb will block CTLA-4 function [17,18].

Evaluation of H. pylori *infection and mucosal inflammation*

Half the stomach, divided longitudinally along the greater and lesser curvatures, was fixed with neutral buffered 15% formalin and embedded in paraffin for haematoxylin–eosin (H&E) and Giemsa staining. A quarter of the anal section of the stomach was sampled to evaluate bacterial colonization and urease activity. The specimen was homogenized with a sterile Dounce tissue grinder containing 500 μl of saline. The homogenate was diluted serially for culture and was used directly for urease activity analysis. Urease activity was assessed 4-h and 24-h postmortem in duplicate by measuring the absorbance at 550 nm [19]. The fixed sections were examined blindly by two independent examiners. The intensity of inflammatory cells was classified into four grades according to the updated Sydney system (0: no infiltration, 1: rare, 2: moderate and 3: marked) [20]. The extent of inflammation was scored using a five-point scale (0: none, 1: <25%, 2: 25–50%, 3: 50–75% and 4: >75% over the length of the entire section) [4]. The inflammatory cells included mononuclear cells and neutrophils.

Serum levels of IgG1 and IgG2a

Determination of IgG1 and IgG2a in blood samples was performed by enzyme-linked immunosorbent assay (ELISA) as described previously [9,10]. Briefly, microtitre plates (Nunc, Roskilde, Denmark) were coated with *H. pylori* whole cell sonicated antigen (HpAg) and sera at a dilution of 1 : 25 were added. After washing the wells were incubated with peroxidase-conjugated goat-antimouse IgG1 or goat-antimouse IgG2a (Cappel). 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt (ABTS) was used for colour development, and the absorbance was measured with a microplate reader (Thermo max, Molecular Devices) set at 414 nm. Each sample was tested in duplicate. The concentrations of *H. pylori*-specific serum IgG1 and IgG2a expressed as U/ml were calculated using standard curves generated by titrating pooled high-titre sera. Data were analysed using SOFTmax (Molecular Devices, Sunnyvale, CA, USA).

Cytokine response to H. pylori

Specimens of spleen were pressed gently and ground in a coarse glass grinder with chilled RPMI-1640 medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS). Cell suspensions were depleted of erythrocytes by TRIS-ammonium chloride lysis then washed twice in medium. After filtration through 150-μm and 50-μm nylon meshes, the cells were centrifuged at 400 ***g*** for 10 min at 4°C and the supernatant was discarded. The cells were resuspended in complete medium (sterile RPMI-1640 containing 10% FCS with 200 U/ml penicillin and 100 μg/ml streptomycin) and then counted in a haemocytometer by the trypan blue dye exclusion method. Single cell suspensions of splenic cells ($2{\cdot}0 \times 10^6$ cell/ml) in six-well plates (Nunc-Immuno Plate, Roskilde, Denmark) were incubated for 48 h at 37°C in the presence of 10 μg/ml of HpAg in complete medium. Immunoassay kits for IL-4 and IFN-γwere purchased from BioSource International, Inc. (Camarillo, CA, USA) and cytokine analysis was performed using the instructions provided by the supplier. After colour development, cytokine concentrations were determined with a microplate reader.

Statistical analysis

Differences between groups were examined for statistical significance using the Mann–Whitney *U*-test. A $P < 0{\cdot}05$ denotes a statistically significant difference.

RESULTS

Treatment with anti-CTLA-4 MoAb suppresses the development of gastric inflammation

To investigate the role of CTLA-4 during an acute *H. pylori* infection, we administered purified anti-CTLA-4 MoAb to infected mice between day 0 and day 7. *H. pylori*-infected mice without the MoAb treatment (control mice) developed moderate to severe and extensive gastric inflammation, dominated by mononuclear cells, infiltrating into the lamina propria and the

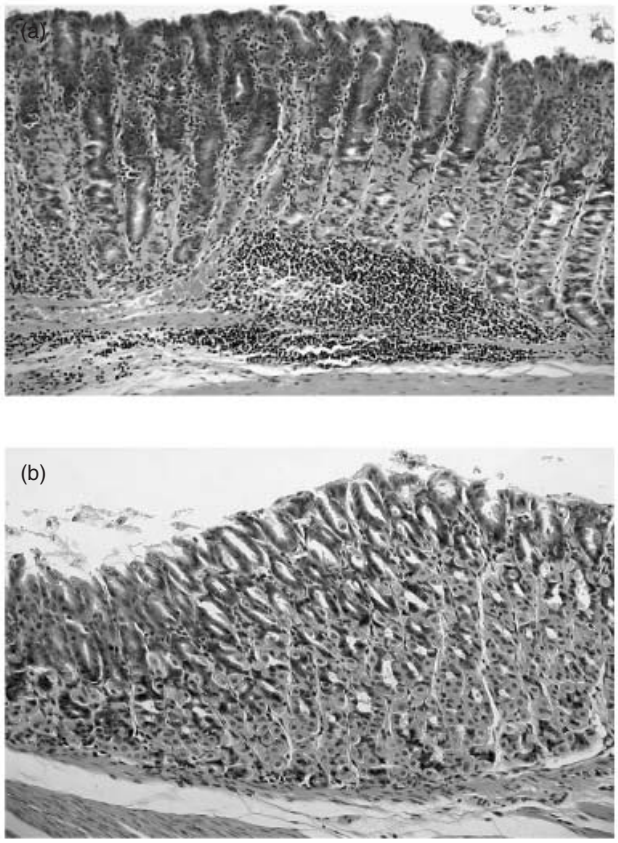

Fig. 1. Histological findings. (a) Section of the stomach from a representative control mouse (*H. pylori*-infected mouse without anti-CTLA-4 MoAb treatment) at 6 weeks after the challenge. (b) Section of the stomach from a representative treated mouse (*H. pylori*-infected mouse with anti-CTLA-4 MoAb treatment) at 6 weeks after the challenge. (a) Control mice developed moderate to severe and extensive gastric inflammation, dominated by mononuclear cells, infiltrating into the lamina propria and the submucosal layer and penetrating the lamina muscularis. Magnification × 160. (b) Anti-CTLA-4 MoAb-treated mice did not show more than mild focal inflammation. Magnification × 200.

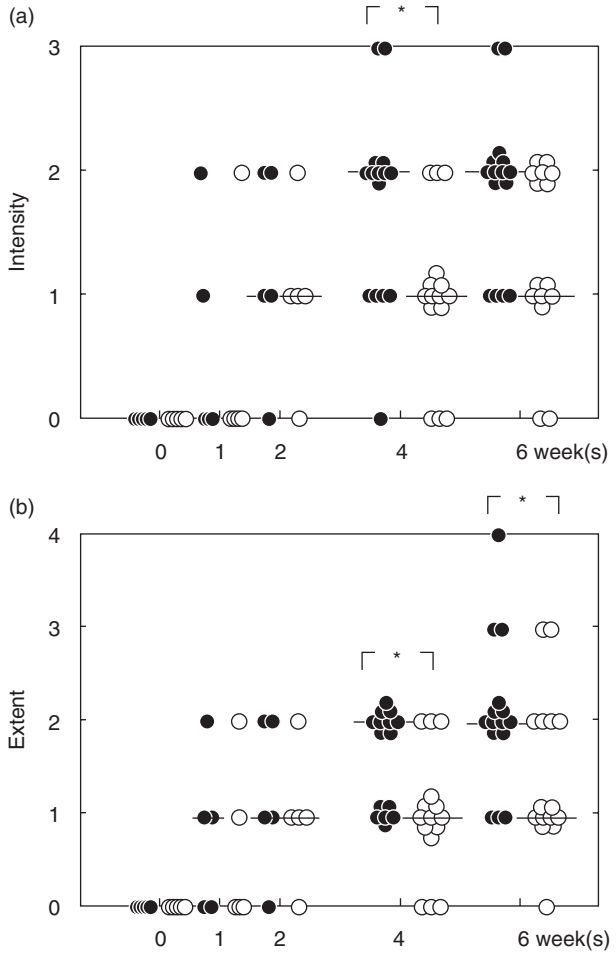

Fig. 2. Intensity score (a) and extent score (b). Gastric inflammation is evaluated as the intensity and extent score. Closed circles: control mice; open circles: treated mice. Data points are given in blots and a horizontal line shows the median for each group each point. *$P < 0{\cdot}05$ control *versus* treated mice. Anti-CTLA-4 MoAb was administered within the first week of infection. The treatment led to the state of controlled inflammation in *H. pylori*-infected mice.

submucosal layer and penetrating the lamina muscularis (Fig. 1a). On the other hand, anti-CTLA-4 MoAb-treated mice showed mild and focal inflammation following infection (Fig. 1b). Figure 2 shows significant differences between groups of mice for the intensity of inflammation at 4 weeks and for the extent of inflammation on and after 4 weeks after infection. The presence of *H. pylori* was confirmed in all mice used. Treatment with anti-CTLA-4 MoAb had no significant effect on bacterial dosage and urease activity (Fig. 3). The bacterial number reached a steady state within the first week of infection, irrespective of treatment.

In vivo *CTLA-4 blockade enhances serum levels of* H. pylori*-specific IgG1*

To determine the phenotypes of Th cells induced by anti-CTLA-4 MoAb treatment, serum levels of *H. pylori*-specific IgG1 and IgG2a were measured by ELISA. As shown in Fig. 4, specific IgG1 in control mice was undetectable until 4 weeks post-infection, while IgG1 response in treated mice increased at the first week of infection, and was then sustained significantly above that in control mice even after discontinuation of treatment. On the other hand, IgG2a response increased gradually following infection and was not affected notably by *in vivo* treatment with anti-CTLA-4 MoAb. Consequently, a higher ratio of IgG1/IgG2a in treated mice was observed during infection, compared with control mice.

CTLA-4 blockade increased the ratio of IL-4/IFN-γ at 6 weeks after infection

Figure 5 shows cytokine response to *H. pylori* at 6 weeks after infection. Production of IL-4 and IFN-γ by splenocytes in response to HpAg was measured by ELISA. Mice treated with anti-CTLA-4 MoAb showed a significant increase of IL-4 production ($P < 0{\cdot}05$), but are impaired in the ability to produce IFN-γ, resulting in a higher ratio of IL-4/IFN-γ compared with control mice ($P < 0{\cdot}05$). The ratio of IL-4/IFN-γ was nearly twofold higher than that of control mice.

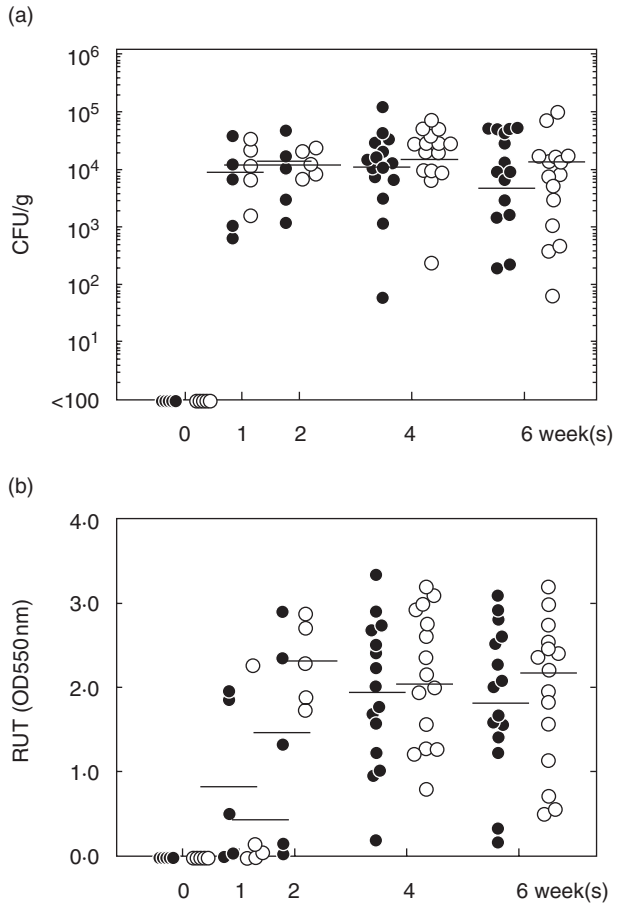

Fig. 3. Bacterial dose (a) and urease activity (b). Closed circles: control mice; open circles: treated mice. The homogenized specimen was used for culture and for urease activity analysis. Urease activity was assessed in duplicate by measuring the absorbance at 550 nm. Data points are given in blots and a horizontal line shows the mean for each group at each point. *$P < 0{\cdot}05$ control *versus* treated mice. The MoAb treatment had no significant effect on bacterial dosage and urease activity. Bacterial number reached a steady state level within the first week of infection, irrespective of treatment.

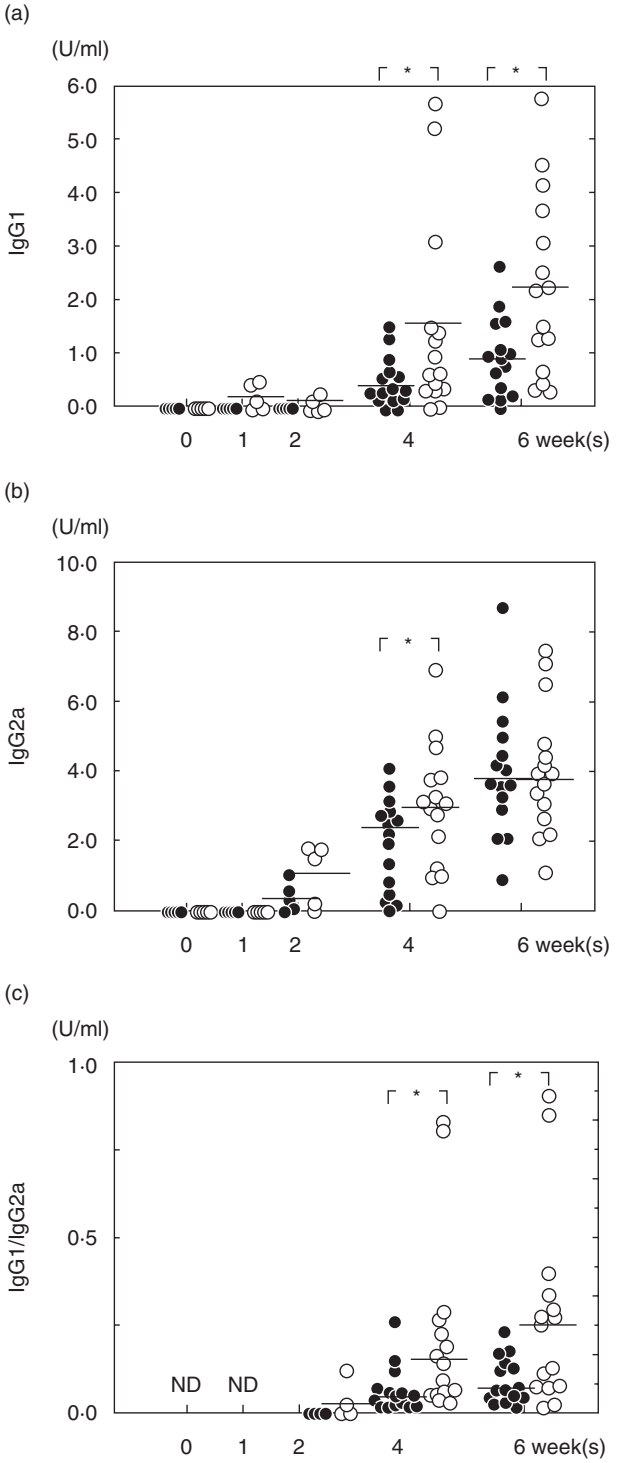

Fig. 4. Serum levels of IgG1 (a), IgG2a (b) and the ratio of IgG1/IgG2a (c). Closed circles: control mice; open circles: treated mice. Data points are given in blots and a horizontal line shows the mean for each group at each point. *$P < 0{\cdot}05$ control *versus* treated mice; ND: no data. *H. pylori*-specific IgG1 in treated mice increased at the first week of infection, and was then sustained significantly above those of control mice after discontinuation of treatment. The ratio of IgG1/IgG2a in treated mice was higher than that in control mice during infection.

DISCUSSION

CTLA-4-induced inhibition of T cell response occurs 24–72 h after initiation of T cell activation. This delay correlates with the expression of detectable CTLA-4 on the activated T cell surface following stimulation [21,22]. The inhibitory effect of CTLA-4 has been attributed to a direct inhibition of IL-2-dependent cell growth and a blockade of intracellular signals required for T cell activation, resulting in long-term unresponsiveness [23]. Therefore, the induction of CTLA-4 in early immune response can affect disease outcomes. CTLA-4 blockade at the time of immunization enhances protective immune response to cryptococcal infection [18]. A soluble form, CTLA-4Ig, administered at the time of bacterial infection, suppresses abscess formation [24]. When treatment with anti-CTLA-4 MoAb or CTLA-4Ig is delayed, disease outcome is not affected [18,24]. In murine *H. pylori* infection, transcription of CTLA-4 mRNA increases transiently to high levels on the second day after infection, returning to baseline level by day 4 (data not shown). In assessing CTLA-4

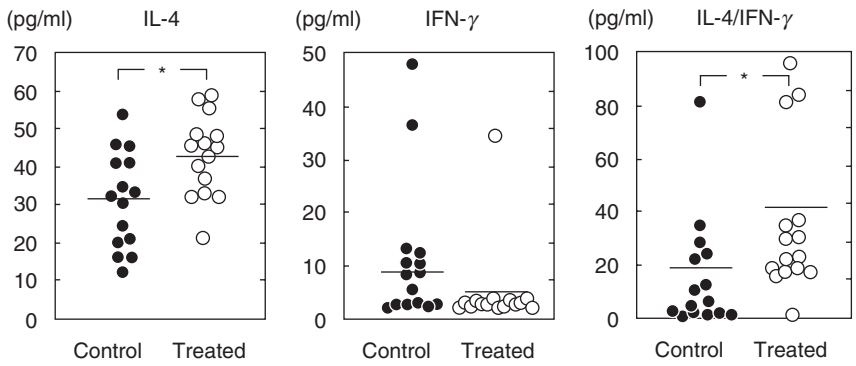

Fig. 5. Production of IL-4 and IFN-γ by splenic cells at 6 weeks after infection. Cells were obtained from control and treated mice (15 mice per group) at 6 weeks post-challenge. Closed circles: control mice; open circles: treated mice. Data points are given in blots and the horizontal line shows the mean for each group at each point. *$P < 0{\cdot}05$ control *versus* treated mice. At 6 weeks after infection, splenocytes from treated mice showed an increase of IL-4 response and an increase of IL-4/IFN-γ ratio.

activity during the acute *H. pylori* infection, it is important that anti-CTLA-4 MoAb is administered within the first week of infection.

Blocking early expression of CTLA-4 is thought to influence the fate of T cells, halting cell cycle progression and regulating proinflammatory and anti-inflammatory responses present at the time when T cells encounter an antigen [25]. Early events of the immune response stimulate the primary cytokine production that regulates the subsequent development of T cell response [26]. The polarized Th1 or Th2 pattern of cytokine production, once acquired, appears stable without a reversion from one subset to another [27,28]. In this study, despite the short treatment with anti-CTLA-4 MoAb, treated mice showed a gradual increase of IgG1 in serum following infection and higher levels of IL-4 production by splenocytes at 6 weeks after infection, compared with control mice. Cytokine response in spleens reflects not only systemic but also local response to *H. pylori*, because lymphocytes from gastric epithelia are recruited from circulation and are similar in cytokine profiles of splenocytes in *H. pylori* infection [29,30]. CTLA-4 signalling during the initial priming of T cells is required for differentiation into mature Th2 population in response to *H. pylori* infection.

$CD4^+CD25^+$ regulatory T (Treg) cells, expressing CTLA-4 constitutively, suppress the intestinal inflammation in models of inflammatory bowel disease and the injection of anti-CTLA-4 MoAb for 6 weeks abolishes the protective effect of Treg cells and leads to intestinal inflammation [31]. In *H. pylori* infection, Treg cells play an inhibitory role on the immunopathology *in vivo* and the activation of *H. pylori*-reactive T cells *in vitro* [32,33]. CTLA-4 is expressed on $CD25^+$ T cells immediately after *H. pylori* infection and then $CD25^+$ subsets increase gradually during infection [34]. The frequency of $CD25^+$ cells in duodenal ulcer patients infected with *H. pylori* and asymptomatic carriers is considerably higher, compared with uninfected individuals. Interestingly, higher expression of CTLA-4 is observed in duodenal ulcer patients than asymptomatic carriers [35]. Taken together, these reports suggest that CTLA-4 signalling reduces immune activation and pathology after *H. pylori* infection. Therefore, the treatment of anti-CTLA-4 MoAb was expected to enhance the inflammatory immune response in *H. pylori* infection.

However, our study using anti-CTLA-4 MoAb injection for 1 week after infection shows the opposite effect, resulting in a reduction of the intensity and extent of inflammation throughout the gastric mucosa. Thus, the inflammation was controlled but not abrogated. The predominance of Th2 immunity leads to controlled inflammation in *H. pylori* infection [5,7,10]. CTLA-4 is expressed at a higher level in Th2 than Th1 cells but functions similarly in both Th1 and Th2 cells [36]. The higher expression of CTLA-4 on Th2 cells means that a Th2-related immune response would be much more sensitive to CTLA-4-dependent inhibition than Th1 cells. Indeed, previous studies have demonstrated that T cells from mice treated with anti-CTLA-4 MoAb and deficient in CTLA-4 gene are Th2-biased [16,37,38]. Treatment with anti-CTLA-4 MoAb in *H. pylori* infection resulted in an up-regulation of IgG1/IgG2a ratio in serum and IL-4/IFN-γ ratio in spleens. CTLA-4 blockade induced Th2-directed immunity, which might suppress *H. pylori* gastritis.

The failure of bacterial elimination in anti-CTLA-4 MoAb-treated mice seems to contradict the notion that Th2 polarized immune response induced by vaccinations reduces gastric inflammation and bacterial burden in *H. pylori* infection [8,10]. Recent studies have shown that vaccination with complete Freund's adjuvant, a Th1-biased adjuvant, leads to clearance of *H. pylori* [39]. Mice protected against *H. pylori* infection by immunization produce high levels of IFN-γ to recall antigen [40]. IFN-$\gamma^{-/-}$ mice show higher levels of bacterial colonization with some strains of *H. pylori*, compared to wild-type infected mice [6]. In IL-$4^{-/-}$ mice infected with *Helicobacter* spp. gastric inflammation decreases, but bacterial growth is variable based on the experimental models used [5,7,40]. Th1 immune response and other host factors would interplay in the elimination of *H. pylori*. In this study, no significant difference of IFN-γ response was observed between groups of mice. Previous studies have demonstrated that severity of gastritis in *H. pylori* infection is not correlated with IFN-γ secretion by gastric mucosa [4,41]. IFN-γ plays a critical role in disease induction but is not required for the progress of inflammation after *Helicobacter* infection [42].

We have shown that CTLA-4 blockade inhibited the development of *H. pylori*-associated gastritis coinciding with the induction of Th2 immune response. CTLA-4 signalling during acute *H. pylori* infection may play an inhibitory role in Th2 response, resulting in an induction of active gastritis.

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