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Circulating $CD14^+CD163^+CD209^+$ M2-like monocytes are associated with the severity of infection in *Helicobacter pylori*-positive patients

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ABSTRACT

Helicobacter pylori (H. pylori) initiates a robust host immune response and subsequently results in chronic inflammation in the gastric mucosa. This study monitored the circulating monocyte subsets and measured the plasma levels of IL-10 and IL-12 in response to *H. pylori* infection in 35 *H. pylori*-associated gastritis patients and 14 healthy controls. We found that the numbers of $CD14^+CD163^-CD64^+$ M1-like monocytes as well as $CD14^+CD163^+CD206^+$, $CD14^+CD163^+CD209^+$, and $CD14^+CD163^+IL\text{-}10^+$ M2-like monocytes were significantly increased in *H. pylori*-infected patients in comparison with the controls, accompanied by higher levels of plasma IL-10. In addition, IL-10 production was significantly higher in the stimulated M2-like cells from patients with *H. pylori* infection compared with controls. Moreover, the *H. pylori*-infected patients with CagA- or VacA-positive strains had a significantly higher number of $CD14^+CD163^+CD206^+$, $CD14^+CD163^+CD209^+$, $CD14^+CD163^+IL\text{-}10^+$ monocytes compared to those with CagA- or VacA-negative strains. Furthermore, patients suffering from *H. pylori*-positive peptic ulcers had a greater number of $CD14^+CD163^+CD209^+$ monocytes than *H. pylori*-positive nonatrophic or atrophic gastritis patients, with the numbers of $CD14^+CD163^+CD209^+$ monocytes positively correlated with the extent of *H. pylori* infection. Notably, the triple anti-*H. pylori* therapy significantly reduced the numbers of $CD14^+CD163^+CD206^+$ and $CD14^+CD163^+CD209^+$ monocyte subsets. In conclusion, $CD14^+CD163^+CD209^+$ M2-like monocyte subsets are increased in *H. pylori* infection, especially in patients with peptic ulcers. $CD14^+CD163^+CD209^+$ M2-like monocytes are positively associated with the severity of *H. pylori* infection.

1. Introduction

Helicobacter pylori (H. pylori) is a Gram-negative bacterium, affecting approximately 50% of the population worldwide (Bures et al., 2011). Although a proportion of individuals infected with *H. pylori* can be asymptomatic, persistent and prolonged infection with this bacterium is directly associated with the development of chronic gastritis and peptic ulcers (PU), which may progress into gastric carcinoma. In fact, *H. pylori* infection has been demonstrated to play an important role in bleeding and the recurrence of PU (Chang and Hu, 2015; Nagata et al., 2015). The standard triple therapy with lansoprazole, amoxicillin, and clarithromycin has been the standard treatment for the eradication of *H. pylori* infection; however, successful eradication of the infection has been challenging, mainly due to drug resistance (Milani et al., 2012) and the lack of insight into the pathogenesis of *H. pylori*-related gastritis.

Extensive studies have demonstrated that the mucosal immune system plays a critical role in the chronic inflammation triggered by *H. pylori* infection, resulting in damage to epithelial cells, and the innate immune response is a leading contributing factor. A number of previous studies have reported that neutrophils (Whitmore et al., 2017; Uberti et al., 2013), natural killer cells (Rudnicka et al., 2013, 2015), and

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dendritic cells (DCs) (Shiu and Blanchard, 2013; Rizzuti et al., 2015) are involved in *H. pylori* infection. It is also well-recognized that monocytes represent the first line of defense in combating pathogens in the innate immune system by differentiating into macrophages or DCs and migrating into inflamed tissues (Randolph et al., 2008). However, the exact roles of monocytes in the pathogenesis of *H. pylori* infection and its related chronic gastric diseases remain largely unknown. It is well-accepted that the macrophages in a tissue are derived from peripheral blood monocytes and can be divided into two subtypes: classically activated M1 macrophages and alternatively activated M2 macrophages (Wynn et al., 2013), which are based on the function and phenotypic plasticity in different microenvironments. Previous studies have found that both M1 and M2 markers are expressed and detected in circulating peripheral blood mononuclear cells (PBMCs) from patients with various diseases (Tang et al., 2014; Zhang et al., 2017; Medeiros et al., 2014). CD14, a monocyte-expressed coreceptor of the toll-like receptor, has been commonly used to identify monocyte populations. CD64, the human high-affinity Fc gamma receptor, is highly expressed on monocytes and generally serves as an M1-specific cell surface marker (Daeron, 1997). CD163 and CD206 are typical markers for M2-like monocytes (Etzerodt and Moestrup, 2013). Furthermore, M1-like monocytes secrete proinflammatory cytokines, including interleukin (IL)-12, which triggers the Th1 immune response; while M2-like monocytes produce anti-inflammatory cytokines, such as IL-10, with a role in tissue repair and immune escape (Wynn et al., 2013; Gordon and Martinez, 2010). CD209 is a C-type lectin receptor, also referred to as DC-SIGN, which is highly expressed on monocyte-derived DCs and M2 macrophages (Park et al., 2016; Soilleux et al., 2002). It has been shown that CD209 contains a specific domain that recognizes pathogens (Gringhuis et al., 2009). Tanne and colleagues have suggested that CD209 plays an important role in the recognition by DCs and macrophages in those with tuberculosis (Tanne and Neyrolles, 2010). In addition, it has been reported that CD209 is likely to rely on DCs and monocytes/macrophages to facilitate HIV-1 transmission to CD4 T cells (Trumpfheller et al., 2003; Preza et al., 2014). Recent studies also have shown that upregulation of $CD209^+$ cells contributes to the pathogenesis of leprosy (Teles et al., 2010) and dengue virus infection (Schaeffer et al., 2015). Until now, however, the circulating $CD209^+$ monocyte subsets in *H. pylori* infection and their relationship with further pathogenesis have not been investigated. Recently, Th17 has been found to be significantly associated with the *H. pylori* burden and inflammation in the stomach (Larussa et al., 2015). However, whether the numbers of different monocyte subsets are correlated with the bacterial load has not yet been explored.

In this prospective study, we aimed to determine the numbers of circulating monocyte subsets in patients with and without *H. pylori* infection, and to assess the relationship between different subsets of monocytes and the clinical parameters of *H. pylori* before and after triple therapy for eradiation of the bacterium. The findings gained through this study may help to understand the role of monocytes in the pathogenesis of *H. pylori*-associated chronic gastric diseases.

2. Materials and methods

2.1. Study subjects

A total of 35 *H. pylori*-positive (+) patients, including 13 chronic nonatrophic gastritis (NAG) patients, 7 chronic atrophic gastritis (CAG) patients, 6 duodenal ulcer patients, and 9 gastric ulcer patients, as well as 13 patients negative for *H. pylori* (-) were prospectively enrolled through both the inpatient and outpatient services in the Department of Gastroenterology at The First Hospital affiliated with Jilin University (Changchun, Jilin, China) between October 2017 and May 2018. In parallel, 14 healthy volunteers who were gender-, age-, and ethnicity-matched with the above experimental groups but without a medical history of any chronic diseases or recent *H. pylori* infection were recruited at the Physical Examination Center in the same hospital and served as controls. The patients who had the following conditions were eventually excluded from this study: (1) the use of medications, including corticosteroids and nonsteroidal anti-inflammatory drugs, proton-pump inhibitors, H2 receptor antagonists, and antibiotics of any type, within 4 weeks prior to enrollment; (2) medical history of autoimmune or oncological disease; (3) age less than 18 years or older than 80 years; (3) chronic diseases or conditions such as diabetes mellitus, chronic heart, lung, liver, or kidney diseases; or (4) medical history of systemic infection within 4 weeks prior to enrolment. The demographic and clinical characteristics of the study subjects are summarized in Tables 1 and 2, and the data were subsequently analyzed.

Table 1
Demographical characteristics and blood test results of the study subjects.

Parameter	HC (n = 14)	*H. pylori*(−) (n = 13)	*H. pylori*(+) (n = 35)
Sex (male/female)	6/8	7/6	19/16
Age (years)	42 (26–70)	45 (23–67)	58 (21–71)
Hemoglobin (g/L)	138 (116–160)	130 (110–150)	125 (84–170)
WBC (10^9/L)	5.9 (4–9.17)	6.0 (4.0–8.0)	5.4 (3–9.6)
Neutrophils (10^9/L)	3.87 (2.7–5.0)	3.9 (2.8–5.0)	3.5 (1.97–5.5)
Monocytes (10^9/L)	0.3 (0.15–0.49)	0.25 (0.12–0.58)	0.38 (0.13–0.6)
Lymphocytes (10^9/L)	2.1 (1.30–3.2)	2.3 (1.30–3.0)	1.96 (0.98–3.5)
Platelets (10^9/L)	220 (180–320)	242 (194–320)	245 (120–330)

Note: HC, healthy controls. Data are presented as median (range) or real case values. Normal range of the blood tests: hemoglobin, 130–175 (g/L); WBC (white blood count); 3.50–9.50 (10^9/L); neutrophils, 1.80–6.30(10^9/L); monocytes, 0.10–0.60 (10^9/L); lymphocytes, 1.10–3.20 (10^9/L); and platelets, 125–350 (10^9/L).

A written informed consent was obtained from all of the participants. The study protocol was in accordance with the guidelines of the Declaration of Helsinki and was reviewed and approved by the Human Ethics Committee of The First Hospital affiliated with Jilin University.

2.2. Treatment and follow-up

H. pylori(+) patients were treated orally with standard triple therapy, which included lansoprazole (30 mg twice daily), amoxicillin (750 mg twice daily), and clarithromycin (200 mg twice daily), for eradiation of *H. pylori* infection and were followed up for 8 weeks after the end of the treatment. Of the 35 patients who completed the entire course of the standard triple therapy, 8 patients returned for follow-up,

Table 2
Demographical characteristics and blood test results of the *H. pylori*-infected patients in the NAG, CAG, and PU subgroups.

Parameter	NAG (n = 13)	CAG (n = 7)	PU (n = 15)
Sex (male/female)	6/7	3/4	10/5
Age (years)	54 (26–65)	64 (50–68)	56 (21–71)
Hemoglobin (g/L)	133 (90–152)	130 (125–144)	101 (84–170)[*,§]
WBC (10^9/L)	6.1 (3–7.2)	4.3 (4–7)	5.07 (3.5–9.6)
Neutrophils (10^9/L)	3.2 (1.97–4.3)	3 (2.5–4.1)	3.8 (2.37–5.5)
Monocytes (10^9/L)	0.3 (0.16–0.48)	0.35 (0.2–0.6)	0.4 (0.13–0.5)
Lymphocytes (10^9/L)	2 (1.6–2.5)	2 (1.1–2.5)	1.8(0.98–3.5)
Platelets (10^9/L)	240 (120–300)	230 (154–280)	221 (219–330)

Note: NAG, nonatrophic gastritis; CAG, chronic atrophic gastritis. Data are displayed as median (range) or real case values. Normal range of blood parameters: hemoglobin, 130–175 (g/L); WBC (white blood count), 3.50–9.50 (10^9/L); neutrophils, 1.80–6.30 (10^9/L); monocytes, 0.10–0.60 (10^9/L); lymphocytes, 1.10–3.20 (10^9L); and platelets, 125–350 (10^9/L).

[*] $p < 0.05$ vs.NAG.

[§] $p < 0.05$ vs.CAG.

whereas the remaining 27 failed to do so. Blood samples were taken and collected in the study subjects before the start of the standard triple therapy and at the follow-up time point.

2.3. Examination of the status of H. pylori infection by a ^{14}C-urea breath test (UBT) and western blot analysis

The ^{14}C-UBT was performed to determine the status of *H. pylori* infection in the study subjects using a commercially available kit from Shenzhen Zhonghe Headway Bio-Sci & Tech (Shenzhen, Guangdong, China). The exhaled breath samples were obtained from the study subjects at 25 min after a ^{14}C-labeled urea-containing capsule was taken orally. The ^{14}C enrichment of each sample was examined in the breath on a HUBT-20 instrument from Shenzhen Zhonghe Headway Bio-Sci & Tech (Shenzhen, Guangdong, China). The exhaled breath sample was considered *H. pylori*(+) if the value of ^{14}C enrichment (mmol CO_2/min) was > 100.

In addition to the ^{14}C-UBT test, western blot analysis was conducted to further determine the status of *H. pylori* infection in the study subjects using anti-*H. pylori* IgG antibodies (Shenzhen Blot Biotech, Shenzhen, Guangdong, China), according to the manufacturer's instructions. In brief, the serum samples were subsequently added to the reaction wells containing *H. pylori* antigen. *H. pylori* antibodies of cytotoxin-associated gene A (CagA; molecular weight (MW), 116 kDa), vacuolating cytotoxin (VacA; MW, 87 kDa), and urease enzyme subunit (MW, 66 kDa) were measured using an assay kit for *Helicobacter Pylori* Testing from Shenzhen Blot Biotech (Shenzhen, Guangdong, China) on a microplate reader. The samples seropositive for CagA and/or VacA were defined as infected with the type I strain of *H. pylori*, while those positive for urease but negative for CagA and VacA were considered as infected with the type II strain of *H. pylori*, whereas the samples negative for the three antibodies were uninfected with *H. pylori*.

2.4. Flow cytometric analysis of the monocyte subsets

Venous blood samples (8 mL) were collected from the healthy controls and the patients, and peripheral blood mononuclear cells (PBMCs) were subsequently isolated by density-gradient centrifugation using Ficoll-Paque Plus from Amersham Biosciences (Little Chalfont, United Kingdom). To determine the percentages of the monocyte subsets, PBMCs at a density of 1×10^6/tube were used and stained in duplicate with the following antibodies: APC-Cy7-anti-CD14, FITC-anti-CD163, PerCP-Cy5.5-anti-CD209, PE-Cy7-anti-CD64 (BD Pharmingen, USA), and PE-CF594-anti-CD206 (BD, Horizon, USA) in the dark at 4 °C for 30 min, during which the fluorescence and isotype-matched antibodies served as negative controls. Meanwhile, we also examined the function of the monocyte subsets. Briefly, PBMCs at a density of 10^6 cells/well were stimulated by exposure to 50 ng/mL lipopolysaccharide, 50 ng/mL phorbol myristate acetate, and 1.0 μg/mL ionomycin (Sigma-Aldrich, St. Louis, MO, USA) in RPMI 1640 culture medium supplemented with 10% fetal bovine serum for 2 h at 37 °C in 5% CO_2, and subsequently treated with Brefeldin A (GolgiPlug, BD Biosciences, USA) for 4 h. The PBMCs were carefully washed, fixed, and permeabilized, followed by staining with APC/Cy™7-anti-CD14 and FITC-anti-CD163 as well as intracellular staining with BV421-anti-IL-10 (BD Pharmingen, USA) and PE-anti-IL-12p70 (BD Horizon, USA). The positive and negative populations of monocyte subsets were separated by fluorescence minus one, after which they were subjected to staining under the conditions of all of the fluorochromes used. The proportions of distinct monocyte subpopulations were eventually characterized on a FACSAria II from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). The resulting data were examined using FlowJo software.

2.5. PBMC isolation and purification

PBMCs were freshly isolated from peripheral blood samples of the study subjects by Ficoll density gradient separation. Monocytes were subsequently purified by flow cytometry using APC/Cy™7-anti-CD14 (BD Pharmingen, USA). The purity of the $CD14^+$ cells was greater than 95%.

2.6. Cell activation and cytokine detection

Human monocytes were induced to differentiate into M2 macrophages as previously described (Furudate et al., 2014; Kittan et al., 2013). Briefly, purified monocytes were induced by macrophage colony-stimulating factor (M-CSF; 50 ng/mL) for 6 days in RPMI 1640 medium supplemented with 10% fetal calf serum and 100 IU/mL penicillin and streptomycin at 37 °C with 5% CO_2. For the M2-like macrophage polarization experiments, the macrophages were further exposed to IL-4 (25 ng/mL)/IL-13 (25 ng/mL) for an additional 24 h. Supernatants were collected for the measurements of IL-10, while the remaining cells were collected on the sixth and seventh days for identification by staining with PE-anti CD163 and APC-anti CD206 (BD, USA).

2.7. Analysis of plasma cytokines with a cytometric bead array assay

The levels of the selected plasma cytokines (IL-10 and IL-12) were quantified by a cytometric bead array, according to the manufacturer's protocol (BD Biosciences, USA). In Brief, 50 μL of plasma samples was taken from the study subjects and tested for levels of IL-10 and IL-12 on a FACSAria II from Beckton, Dickinson and Company (Franklin Lakes, NJ, USA). The concentrations of IL-10 and IL-12 were calculated using Cell Quest Pro software, followed by analysis using the cytometric bead array software from Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

2.8. Statistical analysis

All data in this study were presented as a median and range. The difference between two groups was compared by statistical analysis using the Mann-Whitney U nonparametric test. The correlation between the studied variables was then examined and determined using the Spearman rank correlation test. All statistical analyses were conducted using the SPSS version 19.0 software. A two-sided P value less than 0.05 was considered statistically significant.

3. Results

3.1. Demographic and clinical characteristics of the study subjects

To analyze the frequency of the monocyte subsets in relation to *H. pylori* pathogenesis, 49 patients were prospectively enrolled in this study, of which 35 were positive and 14 were negative for *H. pylori* infection. The demographic and clinical features are presented in Tables 1 and 2. There were no significant differences in the distributions of age or gender in the study groups ($p > 0.05$). Furthermore, no significant differences in the numbers of white blood cells, neutrophils, monocytes, and lymphocytes were observed among the groups ($p > 0.05$) (Tables 1 and 2). Significant differences in the serum levels of hemoglobin between the NAG and PU groups ($p < 0.05$) as well as the CAG and PU groups ($p < 0.05$) were detected, which were likely due to bleeding in the PU group.

3.2. Increase in numbers of M1-like and M2-like monocytes in H. pylori infection

We initially examined the numbers of peripheral blood $CD14^+CD163^-CD64^+$ M1-like monocytes in the study subjects by flow cytometry. As shown in Fig. 1, the numbers of $CD14^+CD163^-CD64^+$ M1-like monocytes were significantly greater in the *H. pylori*(+) patients than in the *H. pylori*(-) and healthy control individuals ($p < 0.05$), whereas no significant differences were observed in the numbers of peripheral blood $CD14^+CD163^-CD64^+$ M1-like monocyte subsets between the *H. pylori*(-) gastritis patients and healthy controls.

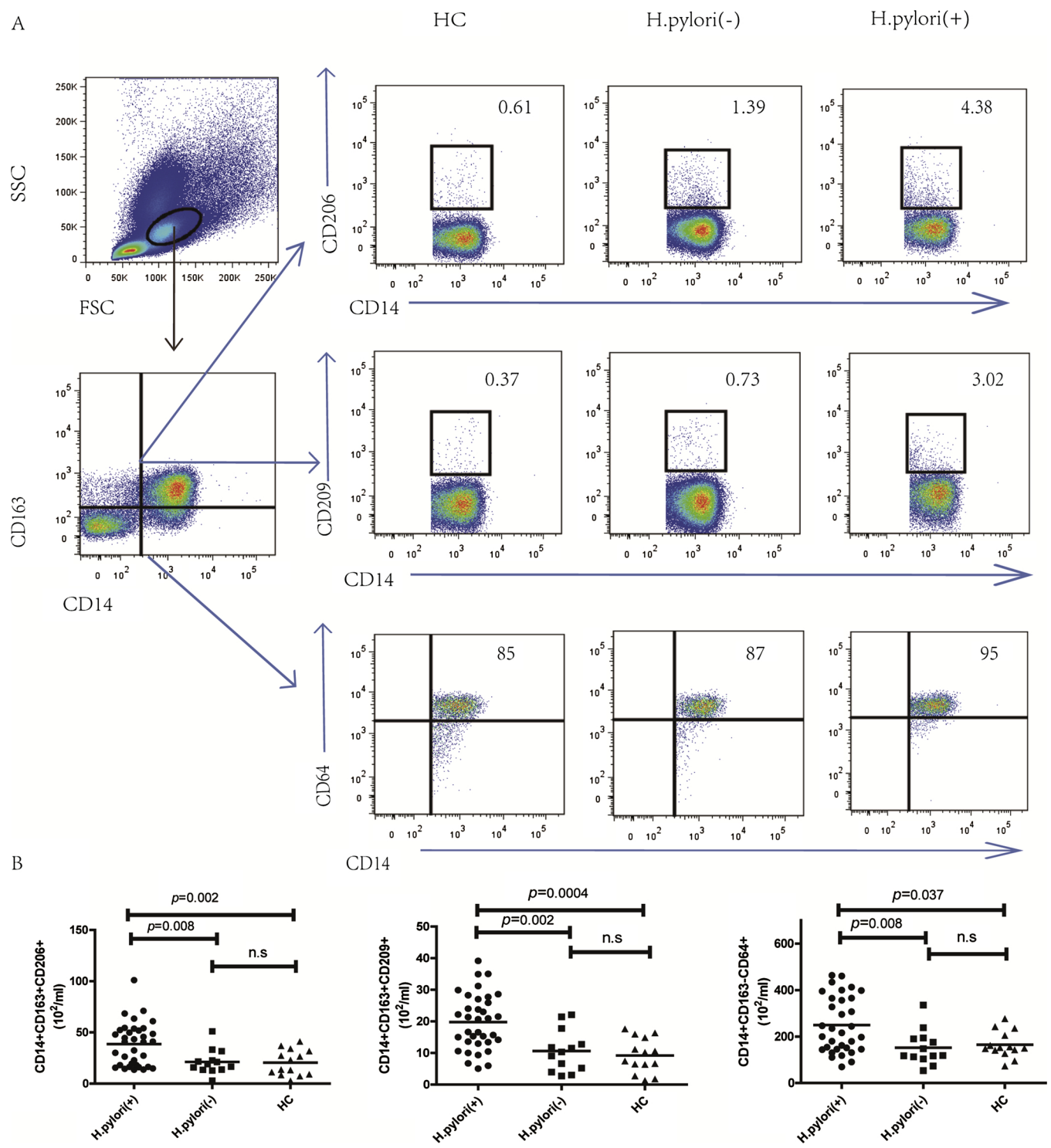

Fig. 1. Analysis of peripheral blood monocytes by flow cytometry. The cells were initially gated on living mononuclear cells, and the ratios of $CD14^+CD163^-CD64^+$ M1-like, $CD14^+CD163^+CD206^+$ M2-like monocytes, as well as $CD14^+CD163^+CD209^+$ M2-like monocytes were examined by flow cytometry. The numbers of the monocyte subsets were subsequently calculated based on the total numbers of monocytes. Data are expressed as the mean values of individual subjects. The horizontal lines indicate the median numbers for the monocyte subsets. (A) Flow cytometric analysis of the peripheral blood monocytes. (B) Quantitative analysis of the monocyte subsets in the study subjects. FSC: Forward scatter; SSC: Side scatter; HC: Healthy control.

Thus, an increase in the numbers of $CD64^+$ M1-like cells was correlated with *H. pylori* infection.

In the measurement of the M2-like monocyte subpopulations, we found that there was no significant difference in the numbers of $CD14^+CD163^+$ M2-like monocytes between the *H. pylori*(+) patients and controls. However, the subpopulations of M2-like monocytes with different surface markers (e.g., CD206 and CD209) exert different functions. Therefore, we examined the numbers of peripheral blood $CD206^+$ and $CD209^+$ M2-like monocytes in the study subjects with or without *H. pylori* infection by flow cytometry. As shown in Fig. 1, the numbers of $CD14^+CD163^+CD206^+$ and $CD14^+CD163^+CD209^+$ M2-like monocytes in the *H. pylori*(+) patients were significantly greater than those in the *H. pylori*(-) patients, whereas there were no significant differences in the numbers of the M2-like monocyte subpopulations

$CD14^+CD163^+CD206^+$ and $CD14^+CD163^+CD209^+$ between the *H. pylori*(-) gastritis patients and the healthy controls. Apparently, increased numbers of $CD206^+$ and $CD209^+$ M2-like monocytes were significantly associated with the presence of *H. pylori*.

3.3. Higher proportion of IL-10^+ M2-like monocytes in patients with H. pylori Infection

As M1-like monocytes secrete mainly pro-inflammatory IL-12, while M2-like monocytes are capable of producing IL-10 (Zhang et al., 2017; Benoit et al., 2008; Mantovani and Locati, 2009), we next examined the percentages of IL-12^+ M1-like and IL-10^+ M2-like monocytes in the study subjects by flow cytometry; the results are presented in Fig. 2A. The numbers of IL-10^+ M2-like monocytes in the *H. pylori*(+) patients were significantly greater than in those without *H. pylori* infection (Fig. 2B), whereas no significant differences in the levels of peripheral blood $CD14^+CD163^+$IL-10^+ and $CD14^+CD163^-$IL-12^+ monocytes were detected in the *H. pylori*(-) gastritis patients compared with the healthy controls. In contrast, there was no significant difference in the numbers of $CD14^+CD163^-$IL-12^+ M1-like monocytes between the *H. pylori*(+) and *H. pylori*(-) patients (Fig. 2C). The above observations clearly indicated that there were significantly greater numbers of IL-10^+ M2-like monocytes in the pathological site of *H. pylori*(+) patients.

In the measurement of the concentrations of plasma IL-10 and IL-12 in the individual subjects by the cytometric bead array method, we found that the plasma levels of IL-10, but not IL-12, in the *H. pylori*(+) patients were significantly greater than those in the *H. pylori*(-) individuals (Fig. 2D).

3.4. Changes in the numbers of $CD14^+CD163^+CD209^+$ monocyte subsets in the H. pylori-infected patients with different clinical manifestations

According to the different clinical manifestations of *H. pylori* infection, the patients with *H. pylori* infection were divided into the following three subgroups: NAG, CAG, and PU patients; and the different monocyte subpopulations were analyzed. We found that the numbers of peripheral blood monocyte subsets, including $CD14^+CD163^+CD209^+$ and $CD14^+CD163^+$IL-10^+ M2-like monocytes, and the plasma IL-10 concentrations were significantly elevated in the *H. pylori*(+) PU patients (Fig. 3A–C) compared to the CAG and NAG patients, whereas no significant differences in the levels of peripheral blood $CD206^+$ monocyte subsets and plasma IL-12 concentrations were detected between the *H. pylori*(+) CAG and NAG patients. Thus, an increase in the circulating level of $CD209^+$ M2-like monocytes was observed in the *H. pylori*-associated PU patients, and the alterations were positively correlated with the development of gastritis.

The ^{14}C-UBT is well-accepted to predict the bacterial load of *H. pylori*; therefore, we examined whether an association between the

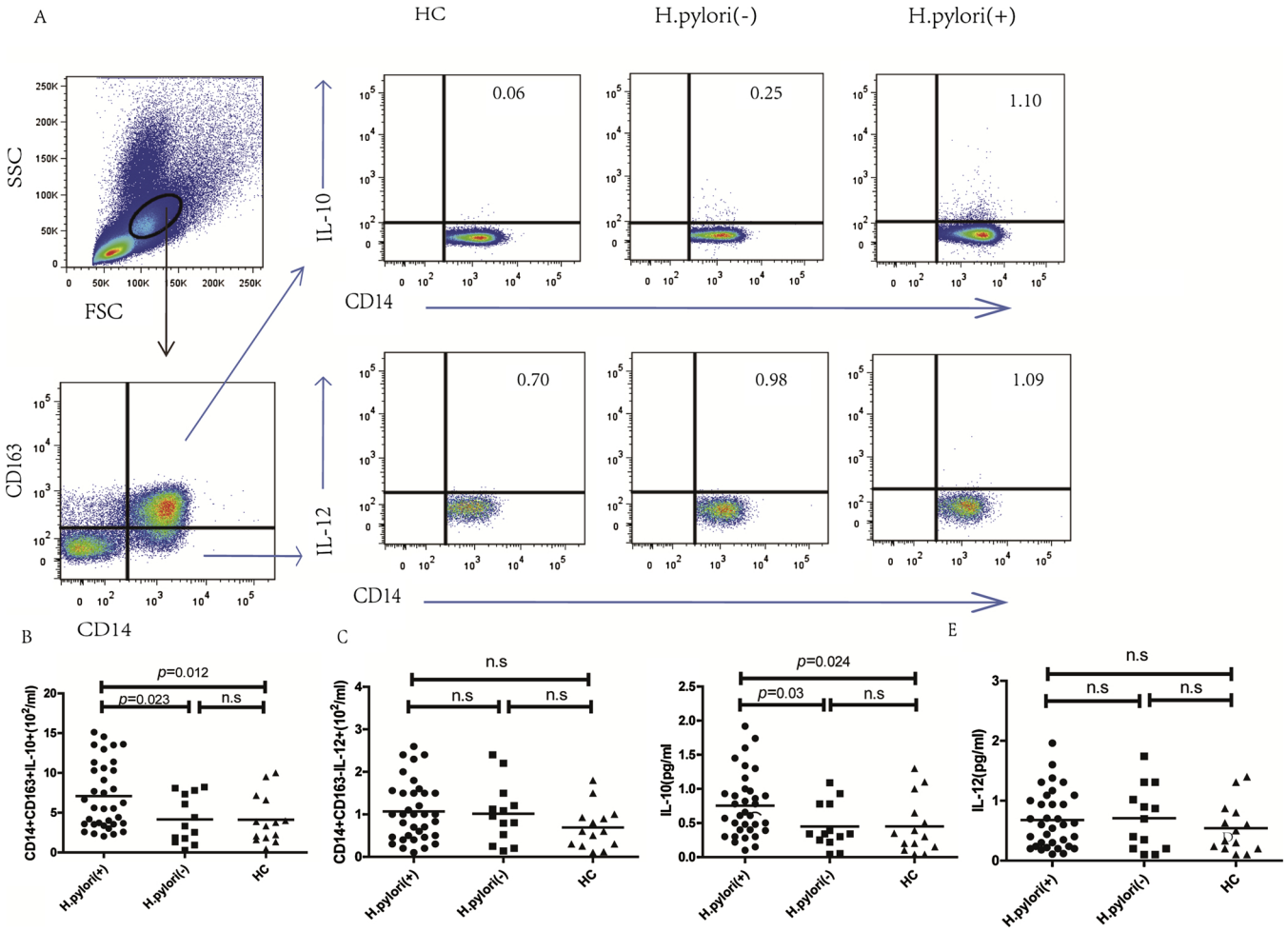

Fig. 2. Examination of IL-12^+ M1-like and IL-10^+ M2-like monocyte subpopulations and the levels of plasma IL-10 and IL-12. Peripheral blood mononuclear cells (PBMCs) from the study subjects were stained in duplicate with APC/CyTM7-anti-CD14, FITC-anti-CD163, or isotype controls. The cells were fixed, permeabilized, and stained with BV421-anti-IL-10 and PE-anti-IL-12p70. The numbers of the $CD14^+CD163^-$IL-12^+ M1-like and $CD14^+CD163^+$IL-10^+ M2-like monocytes were analyzed by flow cytometry. The levels of plasma IL-10 and IL-12 were quantified by a cytometric bead array. (A) Flow cytometric analysis. (B—C) Quantitative analysis of $CD14^+CD163^+$IL-10^+ M2-like and $CD14^+$CD163-IL-12^+ M1-like monocytes. (D—E) The levels of plasma IL-10 and IL-12. Data are expressed as the mean numbers of each monocyte subset. The horizontal lines indicate the median values for each group. FSC: Forward scatter; SSC: Side scatter; HC: Healthy control.

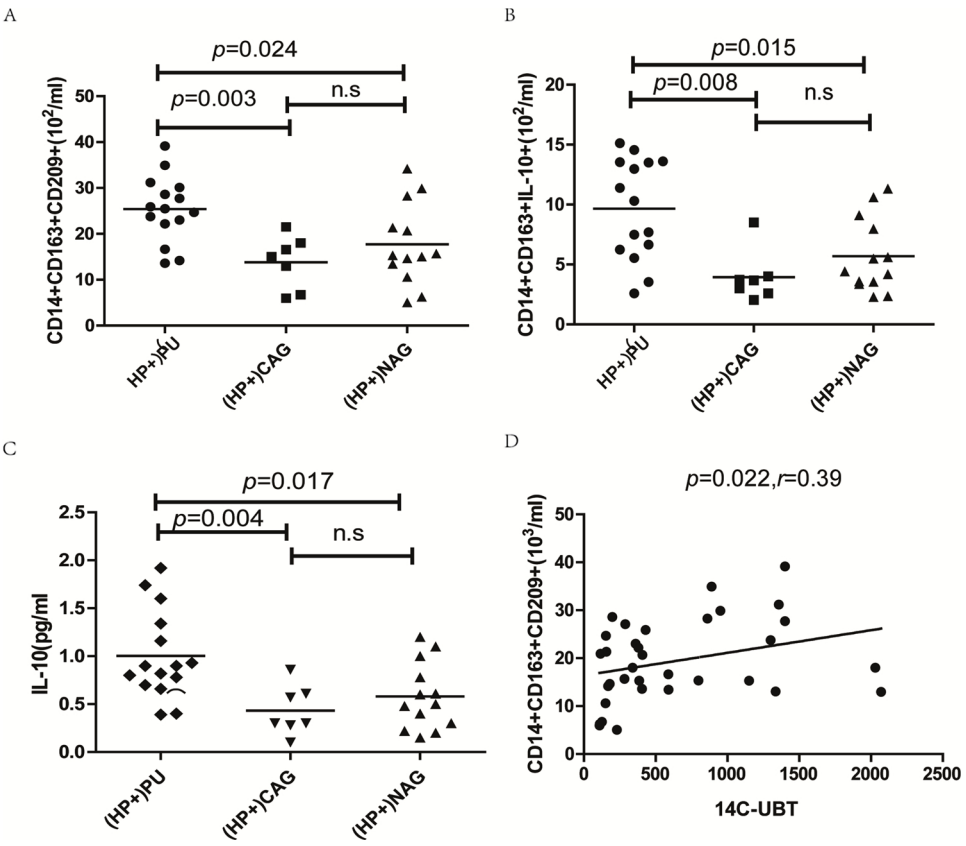

Fig. 3. Circulating CD14$^+$CD163$^+$ monocyte subsets in different subgroups of *H. pylori*-infected patients by clinical manifestations. The numbers of the selected monocyte subsets from the three subgroups of *H. pylori*-infected patients were examined by flow cytometry. The numbers of (A) CD14$^+$CD163$^+$CD209$^+$, (B) CD14$^+$CD163$^+$IL-10$^+$, as well as (C) levels of plasma IL-10 in the peptic ulcer (PU), chronic atrophic gastritis (CAG), and nonatrophic gastritis (NAG) groups. (D) The correlation between the numerical values of the ^{14}C-urea breath test (UBT) and the numbers of CD14$^+$CD163$^+$CD209$^+$ circulating monocytes in the *H. pylori*(+) patients. The corresponding rho value is denoted. Data are expressed as the mean values of participants from the various subgroups. Horizontal lines indicate the median values for each group.

numbers of different monocyte phenotypes and the severity of *H. pylori* infection as determined by ^{14}C-UBT readouts in the *H. pylori*(+) patients exists. As expected, we found a positive correlation between the number of CD14$^+$CD163$^+$CD209$^+$ monocytes and the severity of *H. pylori* infection in the study cohort (p = 0.022, r = 0.39, Fig. 3D), indicating that CD209$^+$ M2-like monocytes could reflect the degree of *H. pylori* load.

3.5. Relationship between the CD206$^+$ and CD209$^+$ M2-like monocyte subpopulations and the CagA$^+$ and VacA$^+$ H. pylori infection strains

CagA and VacA are recognized as important virulence factors of *H. pylori*. Using CagA- and VacA-specific antibodies, we analyzed changes of the monocyte subpopulations in the study patients with CagA- or VacA-specific *H. pylori* infection. Interestingly, we found that the *H. pylori*-infected patients with the CagA$^+$ strain had significantly greater numbers of CD14$^+$CD163$^+$CD209$^+$, CD14$^+$CD163$^+$CD206$^+$, and CD14$^+$CD163$^+$IL-10$^+$ M2 monocytes compared to those with CagA$^-$ strains (Fig. 4A–C). In addition, there was a significant correlation in the numbers of CD14$^+$CD163$^+$CD209$^+$, CD14$^+$CD163$^+$CD206$^+$, and CD14$^+$CD163$^+$IL-10$^+$ cells between the *H. pylori*-infected patients with VacA$^+$ strains and the *H. pylori*-infected patients with VacA$^-$ strains (Fig. 4D–F). Therefore, the above findings in the study patients implicated that the CD209$^+$ and CD206$^+$ M2-like monocytes were largely dependent on the CagA and VacA status of *H. pylori*.

3.6. Alterations in monocyte subsets after the H. pylori eradication treatment

To determine alterations in the monocyte subsets in response to the *H. pylori* eradication treatment, we compared the CD64$^+$, CD206$^+$, and CD209$^+$ monocyte subpopulations in the *H. pylori*(+) patients before and after treatment with lansoprazole, amoxicillin, and clarithromycin, according to the Clinical Practice Guideline for PU (Satoh et al., 2016) and our prior clinical experience. The clinical responses and the immunological state of the study patients with *H. pylori*-associated PU were also examined. As a result, *H. pylori* infection was successfully eradicated in eight of the treated patients, from whom the monocyte subsets were further examined. As shown in Fig. 5A, the numbers of CD14$^+$CD163$^-$CD64$^+$ M1-like monocyte subpopulations were significantly decreased after successful eradication of *H. pylori* infection. More importantly, we observed that the numbers of circulating CD14$^+$CD163$^+$CD206$^+$ and CD14$^+$CD163$^+$CD209$^+$ monocytes were significantly reduced (Fig. 5B–C) after the anti-*H. pylori* treatment. Altogether, the above changes may represent a possibly curative effect of the treatment; thus, CD206$^+$ and CD209$^+$ M2 subpopulations may hold promise as molecular markers to evaluate the curative effectiveness of *H. pylori* therapies.

3.7. IL-10 production of stimulated monocytes in H. pylori-infected patients

To characterize the function of M2-like cells, monocytes were isolated from *H. pylori*-infected patients and healthy controls, and stimulated with or without IL-4/IL-13 for 24 h after induction of M-CSF. To identify the induced M2 macrophages, we evaluated the expression of M2 phenotypic surface markers (CD163 and CD206, respectively) by flow cytometry (Fig. 6A–B). Our results indicated that cells from patients or healthy controls with stimulation showed a higher mean fluorescence intensity (MFI) of CD163 or CD206 with respect to those without stimulation. The group of stimulated cells from patients displayed a significant difference in the MFI of CD163 or CD206 in comparison with the group of stimulated cells from healthy controls (Fig. 6C–D). Furthermore, the IL-10 levels in the supernatants of cultured activated M2-like macrophages from the patients and controls were significantly higher than those in the unstimulated macrophages (Fig. 6E). In comparison with the healthy controls, significantly higher levels of IL-10 were detected in the supernatants of cultured activated M2-like macrophages from the *H. pylori*-infected patients. Together,

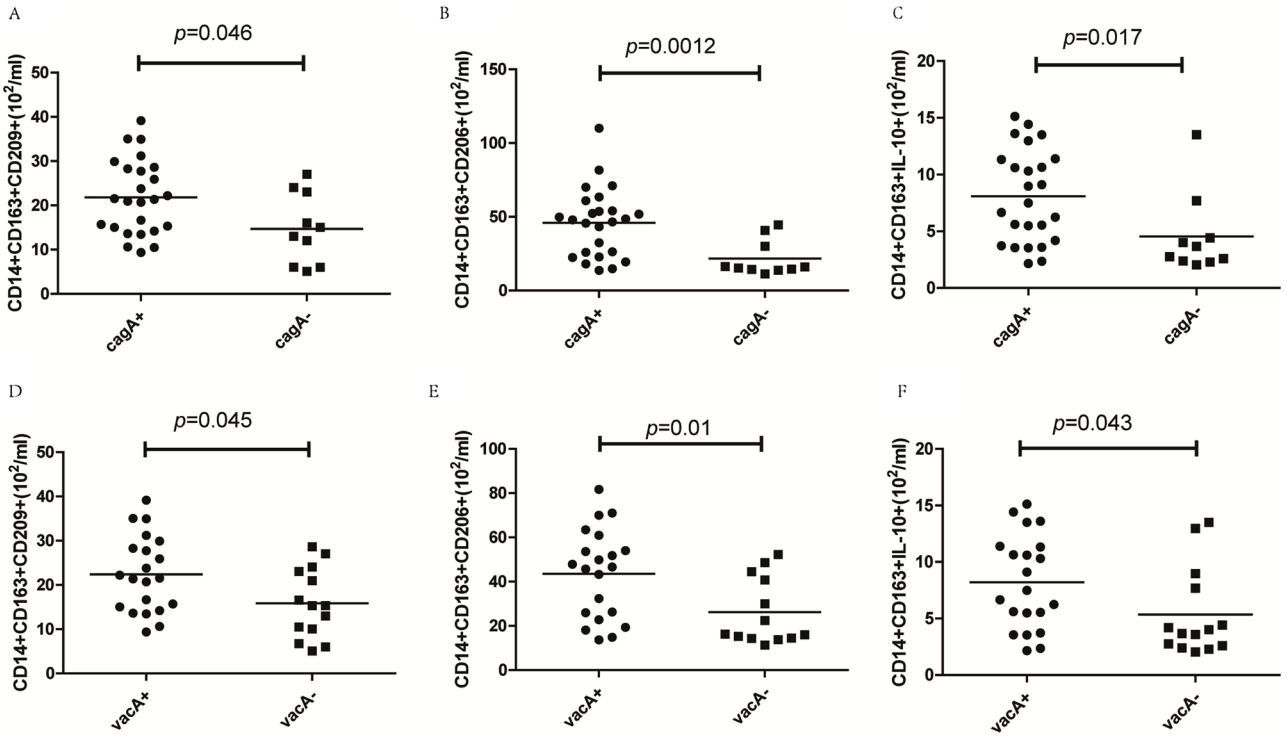

Fig. 4. Circulating $CD14^+CD163^+$ monocyte subsets in different groups of *H. pylori*-infected patients according to the virulence factor. The numbers of the circulating $CD14^+CD163^+$ monocyte subsets in the different groups of *H. pylori*-infected patients according to the virulence factor were examined by flow cytometry. The numbers of (A) $CD14^+CD163^+CD209^+$, (B) $CD14^+CD163^+CD206^+$, and (C) $CD14^+CD163^+IL\text{-}10^+$ in the $CagA^+$ group and the $CagA^-$ group are shown. The numbers of (D) $CD14^+CD163^+CD209^+$, (E) $CD14^+CD163^+CD206^+$, and (F) $CD14^+CD163^+IL\text{-}10^+$ in the $VacA^+$ group and the $VacA^-$ group. Data are expressed as the mean values of participants from the various subgroups. Horizontal lines indicate the median values for each group.

these data suggested that M2-like cells from patients with *H. pylori* infection may be activated by anti-inflammatory cytokine secretion.

4. Discussion

H. pylori infection is one of the major causes of gastritis, which may eventually progress to develop gastric cancer. To date, the underlying mechanisms have not yet been elucidated. This study of phenotypic alterations in the subsets of circulating monocytes in response to this pathogen and their potential roles in *H. pylori*-associated gastritis determined the following novel findings: (1) Significant increases in the numbers of $CD14^+CD163^-CD64^+$ M1-like monocytes as well as $CD14^+CD163^+CD206^+$, $CD14^+CD163^+CD209^+$, and $CD14^+CD163^+IL\text{-}10^+$ M2-like monocytes were observed in patients with *H. pylori* infection but not in the *H. pylori*(-) individuals; (2) The numbers of $CD14^+CD163^+CD206^+$ and $CD14^+CD163^+CD209^+$ monocytes were significantly reduced after *H. pylori* infection was successfully eradiated with the triple anti-*H. pylori* therapy; (3) *H. pylori*(+) patients with $CagA^+$ or $VacA^+$ strains exhibited significantly greater proportions of $CD14^+CD163^+CD206^+$, $CD14^+CD163^+CD209^+$, and $CD14^+CD163^+IL\text{-}10^+$ monocytes than those with $CagA^-$ or $VacA^-$ strains; (4) PU patients with *H. pylori* infection showed significantly elevated numbers of $CD14^+CD163^+CD209^+$ monocytes in contrast to chronic nonatrophic or atrophic gastritis patients with *H. pylori* infection, with a positive relationship between an increase in the number of monocyte subsets and the severity of *H. pylori* infection.

It is well accepted that *H. pylori* induces a Th1 response in the host (Bamford et al., 1998) and that M1-like monocytes are able to secrete proinflammatory cytokines and trigger a Th1 immune response. In the present study, our observations were in line with the finding of

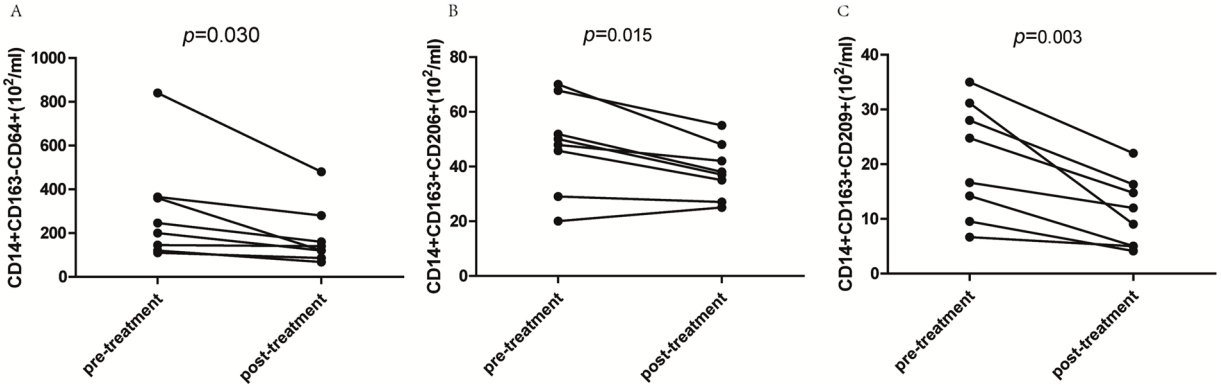

Fig. 5. Alterations in the monocyte subtypes in patients with *H. pylori* infection following the treatment. Pre- and post-treatment numbers of different subsets of monocytes are compared; (A) Pre- and post-treatment $CD14^+CD163^-CD64^+$ cell counts. (B–C) Pre- and post-treatment $CD14^+CD163^+CD206^+$ and $CD14^+CD163^+CD209^+$ cell counts.

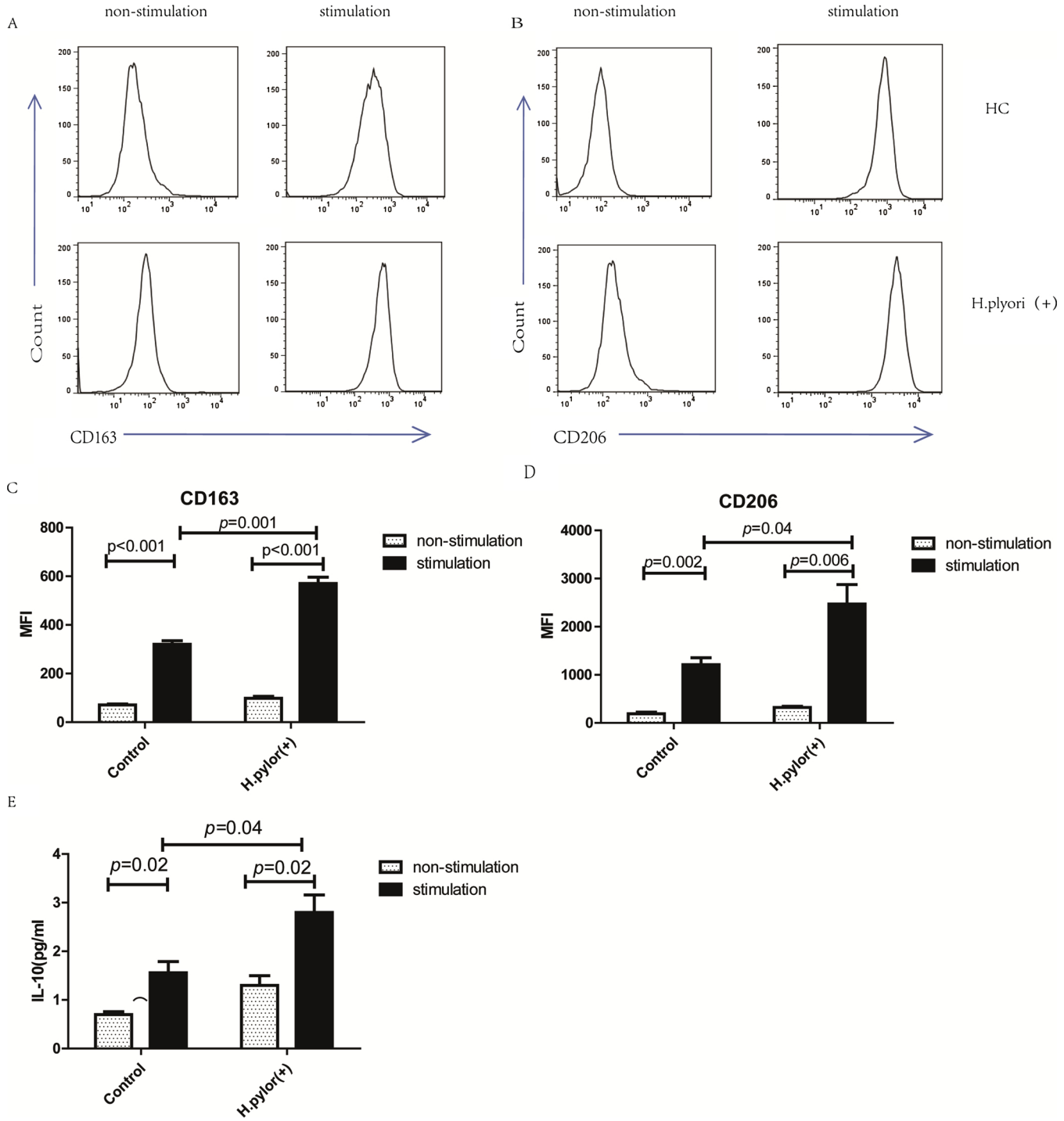

Fig. 6. Diff ;erentiation of human peripheral monocytes into M2 macrophages of *H. pylori*-infected patients. $CD14^+$ monocytes were purified from five healthy controls or *H. pylori*-infected patients and stimulated with or without, IL-4/IL-13 for 24 h. The expression of CD206 and CD163 in M2-like cells was assessed by flow cytometry. The levels of IL-10 in the supernatants of cultured cells were determined by CBA. (A–B) Flow cytometry identification of M2-like macrophages. (C–D) Quantitative analysis. (E) IL-10 production of M2-like macrophages in *H. pylori*-infected patients.

domination by M1 polarization in previous reports (Quiding-Jarbrink et al., 2010; Teles et al., 2010; Moyat et al., 2015). It has been reported that the serum levels of IL-12p70 are elevated in individuals with $CagA^+$ duodenal ulcers (Eskandari-Nasab et al., 2013). However, we found no significant difference in the numbers of $CD14^+CD163^-$IL-$12p70^+$ M1-like monocytes and the IL-12p70 level of the study subjects. There is a possibility that a relatively small proportion of the duodenal ulcer patients or not all of the study subjects had the $CagA^+$ strain. M2-like monocytes are able to produce cytokines that control inflammation and help repair tissue. In this study, we demonstrated that increased numbers of $CD14^+CD163^+CD206^+$ and $CD14^+CD163^+CD209^+$ M2-like monocytes were observed in the patients with *H. pylori* infection. The arginase/ornithine decarboxylase metabolic pathway of M2-like monocytes has been found to be activated in gastric macrophages of *H. pylori*-infected mice in previous studies (Chaturvedi et al., 2010; Lewis et al., 2011). In addition, $CD14^+CD163^+CD206^+$ M2 macrophage polarization has been established in *H. pylori*(+) individuals in other studies (Quiding-Jarbrink

et al., 2010; Fehlings et al., 2012; Michalkiewicz et al., 2015). Our finding of $CD209^+$ M2-like monocytes is consistent with that of Wu et al., who have revealed that CD209 expression in the *H. pylori*(+) group was significantly greater than that in the *H. pylori*(-) group both in gastric tissue and in vitro (Wu et al., 2014). Moreover, it has been reported that the Lewis antigen and lipopolysaccharides on *H. pylori* can bind with DC-SIGN on DCs and macrophages (Miszczyk et al., 2012). Thus, we supposed that CD209 may help bacteria to escape from the proinflammatory response. In addition, we detected increases in the $CD14^+CD163^+IL\text{-}10^+$ M2 counts and the level of plasma IL-10 in *H. pylori*-infected patients; these findings were consistent with some previous observations (Rudnicka et al., 2013; Fehlings et al., 2012). Indeed, we also found that higher levels of IL-10 were derived from cytokine production by M2-like macrophages. Therefore, we proposed that the phenotypic alterations could function to respond to gastric inflammation by secreting higher levels of IL-10, thereby contributing to bacterial colonization. Further studies on how $CD209^+$ macrophages regulate IL-10 production are needed in the future. We found that the numbers of circulating $CD206^+$ and $CD209^+$ M2-like monocytes were significantly reduced compared to the pretreatment levels, and this result was accompanied by reduced numbers of $CD14^+CD163^-CD64^+$ M1-like monocytes. These findings may contribute to restoration of the M1/M2 balance with the bacterial clearance. Collectively, our data suggested that $CD14^+CD163^+CD206^+$ and $CD14^+CD163^+CD209^+$ monocyte subpopulations may participate in the pathogenesis of *H. pylori* infection.

In the analysis of the numbers of monocyte subpopulations in relation to *H. pylori* infection, we detected an increase in the number of $CD209^+$ M2-like monocytes in *H. pylori*(+) PU, compared with *H. pylori*(+) CAG and NAG. There is a possibility that PU patients suffer from more serious lesions than those with CAG or NAG. In addition, Wu and colleagues have found that the expression of DC-SIGN was greater in the moderate and severe inflammation subgroups, compared to the mild one (Wu et al., 2014). Conceivably, the detection of abnormally high levels of $CD209^+$ M2 responses may help in the diagnosis of *H. pylori*-associated PU. The ^{14}C-UBT is an appropriate test for bacterial/inflammatory load (Cho et al., 2008). The numbers of $CD209^+$ M2-like monocytes were also found to be positively associated with the extent of *H. pylori* colonization. Thus, we proposed that $CD14^+CD163^+CD209^+$ monocytes may serve as a potential biomarker for evaluating the degree of *H. pylori* infection. It is worth noting that there was no significant difference in the percentage of $CD14^+CD163^+CD209^+$ cells between the *H. pylori*(-) patients and the *H. pylori*(+) CAG patients in the present study (data not shown). We speculated that the main influencing factor might be attributed to the relatively small group or the low bacterial load of the study patients, but further study will be needed in the future.

CagA and VacA are the two main virulence factors of *H. pylori* and are closely correlated with gastric inflammation and carcinogenesis. So far, few studies have been conducted to study the relationship between CagA and VacA antibodies and monocytes in *H. pylori*-infected patients. In the present study, we found that the numbers of $CD14^+CD163^+CD209^+$, $CD14^+CD163^+CD206^+$, and $CD14^+CD163^+IL\text{-}10^+$ cells in *H. pylori*-infected patients with $CagA^+/VacA^+$ strains were significant greater than in those with $CagA^-/VacA^-$ strains. In addition, it has been reported that the levels of heme oxygenase-1 are increased in gastric macrophages of mice infected with *H. pylori* $CagA^+$ strains (Gobert et al., 2014), a functional feature of M2 macrophages (Sierra-Filardi et al., 2010; Choi et al., 2010), implicating that $CagA^+$ strains may induce M2 polarization. Furthermore, it has been documented that *H. pylori* $CagA^+$ infection causes a higher IL-10 mRNA expression compared to $CagA^-$ strains (Michalkiewicz et al., 2015). Until now, little is known about the relationship between M2-like monocytes and VacA strains. We postulated that the numbers of $CD14^+CD163^+CD209^+$ and $CD14^+CD163^+CD206^+$ M2-like monocytes could be triggered by $VacA^+$ strains, but this hypothesis has not been tested in a larger patient population. Here, we proposed that increased numbers of $CD209^+$ and $CD206^+$ monocytes participate in the pathogenesis of *H. pylori* infection through the $CagA^+$ and $VacA^+$ strains.

Despite the above strengths, our study has a few limitations. The sample size of the study population was relatively small. In addition, further investigations in vitro and in vivo are needed to elucidate the functional roles of the monocyte subpopulations in response to *H. pylori* infection as well as the signaling pathways through which *H. pylori* and host innate immunity interact to influence the pathogenesis of *H. pylori* infection. Recently, the soluble forms of CD206 and CD163 as novel biomarkers of M2a and M2c macrophage activity have been suggested (Greisen et al., 2011; Laursen et al., 2018). However, it remains unknown whether the soluble forms of CD206 and/or CD163 in serum could also be associated with the M2-like monocyte population and disease activity in *H. pylori* infection. Further studies are underway in our laboratory. Moreover, in this study, we were not able to determine which type of M2 cells were the source of IL-10, mainly due to the challenge of directly inducing the specific type of $CD14^+CD163^+CD206^+$ or $CD14^+CD163^+CD209^+$ M2 cells.

In conclusion, our results suggest that $CD14^+CD163^+CD206^+$ and $CD14^+CD163^+CD209^+$ monocytes may be valuable for the evaluation of therapeutic responses in *H. pylori*-infected patients. In addition, our finding may provide a novel approach to assess the *H. pylori*-associated PU and support a potential role for $CD14^+CD163^+CD209^+$ M2-like cells as a new biomarker to predict the severity of *H. pylori* infection.

5. Author contributions

Pujun Gao and Jiang Yanfang designed the experiments; Jie Hou performed the experiments and wrote the main manuscript text; Manli Zhang and Min Wang provided samples; Xinrui Wang analyzed data; and all authors reviewed the manuscript.

Conflict of interest

The authors declare that they have no competing financial interests.

Acknowledgments

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