CagA+ *H pylori* infection is associated with polarization of T helper cell immune responses in gastric carcinogenesis

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Abstract

AIM: To characterize the immune responses including local and systemic immunity induced by infection with *H pylori*, especially with CagA+ *H pylori* strains and the underlying immunopathogenesis.

METHODS: A total of 711 patients with different gastric lesions were recruited to determine the presence of *H pylori* infection and cytotoxin associated protein A (CagA), the presence of T helper (Th) cells and regulatory T (Treg) cells in peripheral blood mononuclear cells (PBMCs), expression of plasma cytokines, and RNA and protein expression of IFN-γ and IL-4 in gastric biopsies and PBMCs were determined by rapid urease test, urea [^{14}C] breath test, immunoblotting test, flow cytometry , real time RT-PCR and immunohistochemistry.

RESULTS: Of the patients, 629 (88.47%) were infected with *H pylori*; 506 (71.16%) with CagA+ and 123 (17.30%) with CagA- strains. Among patients infected with CagA+ *H pylori* strains, Th1-mediated cellular immunity was associated with earlier stages of gastric carcinogenesis, while Th2-mediated humoral immunity dominated the advanced stages and was negatively associated with an abundance of Treg cells. However, there was no such tendency in Th1/Th2 polarization in patients infected with CagA- *H pylori* strains and those without *H pylori* infection.

CONCLUSION: Polarization of Th cell immune responses occurs in patients with CagA+ *H pylori*

infection, which is associated with the stage and severity of gastric pathology during the progression of gastric carcinogenesis. This finding provides further evidence for a causal role of CagA+ *H pylori* infection in the immunopathogenesis of gastric cancer.

INTRODUCTION

In 1994, epidemiological surveys by WHO showed that more than half of the world's population was infected with *H pylori* and *H pylori* infection was closely related to chronic gastritis, gastric ulcer and gastric adenocarcinoma. These results led to the conclusion that *H pylori* is a first class risk factor (a definite carcinogen) for gastric adenocarcinomas[1]. It has been generally accepted that *H pylori* infection is involved in all stages of gastric carcinogenesis which progresses from chronic gastritis (CG) to gastric atrophy (GA), intestinal metaplasia (IM), dysplasia(DP) and ultimately gastric cancer (GC)[2-4]. However, the functional interaction of *H pylori* infection with distinct members of the immune compartment, especially T cell immune responses, in gastric carcinogenesis has not been fully elucidated. T helper (Th) cells can be divided into two subsets, Th1 cells and Th2 cells. Th1 cells mediate cellular immunity mainly by producing interferon (IFN)-γ, interleukin (IL)-2, IL-12, and tumor necrosis factor (TNF)-β, while Th2 cells primarily mediate humoral immunity by secreting IL-4, IL-5, IL-6, IL-10 and IL-13. Previous studies have reported the differential expression of cytokines between *H pylori* positive and *H pylori* negative patients[5-7] or between gastritis and gastric cancer patients[8]. However, these studies did not examine the cellular role of *H pylori* infection in the immune responses during the progression of gastric pathology. Under normal homeostasis, the cytokines produced by one Th subset reciprocally inhibit the development of the other to keep

the balance of Th1 and Th2. Moreover, regulatory T (Treg) cells, which are a low abundance cell subset, help mediate the balance of Th1 and Th2, Treg cells inhibit the proliferation of CD4+ CD25-T lymphocytes, CD8+ T lymphocytes, immune memory cells, and antigen presenting cells (APCs) by recognizing inner and outer antigens. These immune responses result in the decrease of various cytokine secretion and the weakening of cellular immune function *in vitro*. The aim of the present study was to characterize the immune responses including local and systemic immunity induced by infection with *H pylori*, especially with CagA+ *H pylori* strains and the underlying immunopathogenesis, by analyzing the populations of T cells present in a range of progressive gastric pathologies during gastric carcinogenesis.

MATERIALS AND METHODS

Patients

Candidates with gastric discomfort, who had an endoscopy during October 2004 to May 2006, were recruited into this study based upon three clinical screening tests for *H pylori* infection. These tests were rapid urease test (RUT) (Lizhu Company, Zhuhai, China) of gastric tissue, urea [14C] breath test (UBT) (Syncor Medicine Company Ltd., Shanghai, China) and the immunoblotting test (Yuangu company Ltd., Shanghai, China). The RUT and UBT tests have been reported to have sensitivity of 80%-99% and 95%-99% and specificity of 92%-100% and 77%-99%, respectively[9,10]. The immunoblotting test was designed to detect four major antigens including vacuolating cytotoxin (VacA, 95K), the cytotoxin associated protein A (CagA, 128K), urease A, urease B, and has been shown to be high sensitive and specific[11]. Patients were considered to be *H pylori*-positive when more than two tests were positive and to be negative when all three tests were negative, while those with two negative results were excluded from this study. In addition, two gastric biopsies taken from the antrum during the upper endoscopy were used for histological examination, real-time reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry. Histological examination was performed by experienced pathologists who were blinded to the patients' clinical diagnosis according to the updated Sydney system[11]. Patients with serious diseases or immune diseases were excluded from the study. None of the current study participants received surgery, radiotherapy, chemotherapy, or any other medical interventions before this study and all provided written informed consent after consultation. All the protocols and patient inclusion and exclusion criteria were approved by the Committee for Human Use and Institutional Review Board of Nanjing Medical University affiliated Nanjing first hospital for human subject studies.

Detection of IFN-γ, IL-4 expression and Treg cells

Heparinized venous blood (5 mL) taken from each patient was used to detect the expression of IFN-γ and IL-4. Treg cells were identified with a FACSCalibur flow cytometer (FCM, BDIS Biosciences, Franklin Lakes, USA). All reagents for FCM were provided by Caltag laboratories

(Burlingame CA, USA). Cells in the blood were stimulated as described by Morita *et al*[12]. Briefly, heparinized venous blood was incubated with a combination of 25 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma, Saint Louis MO, USA) and 1 μg/mL of the calcium ionophore, ionomycin (Sigma), for 5 h. After cells were cultured in RPMI 1640 for 1 h, 10 μg/mL Brefeldin A (Sigma) was added to enhance flow cytometric analysis of intracellular cytokine staining. After stimulation, the cells were incubated with peridinin chlorophyll (PerCP) mouse anti-human CD3 (CD3-PC) and fluorescein isothiocyanate (FITC) mouse anti-human CD8 (CD8-FITC) for 15-20 min. After fixation and permeabilization, corresponding antibodies [mouse IgG1-phycoerythrin (PE), PE conjugated mouse anti-human IFN-γ (IFN-γ-PE), Rat IgG1-PE, PE-conjugated Rat anti-human IL-4 monoclonal antibody (IL-4-PE)] were added and incubated for 15 min. The cells were then detected by FCM. Data from at least 50 000 cells in one sample were acquired and analyzed by Cell Quest software (BDIS Biosciences). As CD4 expression is known to be down-regulated after stimulation with PMA[13], the CD4+ lymphocytes were analyzed indirectly by gating the CD3+ CD8-lymphocytes. For the detection of Treg cells, whole blood (100 μL) that was incubated with 5 μL CD3-PC, 5 μL CD4-FITC, and 5 μL mouse IgG1-PE for 15-20 min and was used as a control tube, while whole blood (100 μL) that was incubated with 5 μL CD3-TC, 5 μL CD4-FITC and 5 μL CD25-PE,and lysed with 2 mL red blood cell (RBC)-lysis buffer for 5-10 min was used as the detection tube. The precipitates were analyzed by Cell Quest software of FCM.

Detection of cytokines in plasma by ELISA

Enzyme-linked immunosorbent assay (ELISA) kits for quantitative detection of soluble human IL-4 and IFN-γ were purchased from Bender MedsystemsTM (Vienna, Austria), and of IL-2, 6, 10, and 12 from R&D Systems Inc. (Minneapolis, USA). The assays were performed in accordance with the manufacturers' instructions.

Quantification of IFN-γ and IL-4 mRNA in PBMCs and gastric biopsies by real-time RT-PCR

To analyze IFN-γ and IL-4 mRNA, total RNA was extracted with TRIZOL Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) from PBMCs and gastric biopsy specimens according to the manufacturer's recommendations and then reverse transcribed into cDNA by PTC-200 DNA Engine (Bio-Rad, Hercules, CA,USA).

PCR primers for human IFN-γ, IL-4 and β-action were designed by Takara Biotechnology Company (Dalian, China, Table 1). The amount of the PCR product was monitored by the SYBR Premix Ex Taq™ (Takara Biotechnology Company) on a Lightcycler system (Roche Molecular Biochemicals, Indianapolis, USA). The PCR mixture contained 20 μL reaction solution, 2.0 μL cDNA, 10 μL 2 × SYBR Premix Ex Taq™ and 250 nM of the primer. PCR amplification was performed according to the temperature profile: 95℃ for 10 s, followed by 45 cycles of 95℃ for 5 s, annealing and extension at 62℃ for 25 s. Data analysis was performed by the Light cycler software. All data were normalized by β-actin. The up-or down-

Target	Sequences (5'→3')	Length of amplicon (bp)
IFN-γ	Forward:	189
	CTTTAAAGATGACCAGAGCATCCAA	(372-560 NM000619.2)
	Reverse:	
	GGCGACAGTTCAGCCATCAC	
IL-4	Forward:	182
	GACTGTGCTCCGGCAGTTCTA	(589-770 NM000589)
	Reverse:	
	CCAACGTACTCTGGTTGGCTTC	
β-actin	Forward:	89
	ATTGCCGACAGGATGCAGA	(991-1079 CR609136.1)
	Reverse:	
	GAGTACTTGCGCTCAGGAGGA	

regulation (F) of cytokines were calculated by the formula $F = 2^{-\Delta\Delta Ct[14]}$.

Immunohistochemistry for the detection of IFN-γ and IL-4 in antral biopsies

Immunohistochemical staining was performed on 8-μm-thick frozen sections mounted on glass slides as described previously[15]. Briefly, sections were fixed in 2% paraformaldehyde, air dried, and frozen at -20℃ for at least 1 h. After permeabilization and blocking, the sections were incubated with the cytokine-specific monoclonal antibodies (MAbs) (Caltag Laboratories, South San Francisco, CA, USA) at 4℃ overnight. They were then treated with 1% normal goat serum and subsequently incubated with 1:300 biotinylated goat anti-mouse IgG1 (Caltag Laboratories) and avidin-biotin horseradish peroxidase complex (Vector Laboratories Inc. Burlingame, CA, USA). The sections were developed with 3, 3-diaminobenzidine (Vector Laboratories Inc.) and counterstained with Mayer's hematoxylin (Histolab, Goteborg, Sweden). After dehydration, they were mounted with Mountex (Histolab). The tissue sections were analyzed by PAS-9000 Pathological Report system (Logene- I Biotech, Wuxi, China). The positively stained mononuclear cells (MNCs) and polymorphonuclear cells (PMNs) per high power were counted. Only cells with a distinct cytoplasmic staining were included. For each sample, numbers of IL-4 and IFN-γ positive cells from 200 PMNs and MNCs were counted from five randomly selected fields and averaged. The positive rate was used to grade the expression levels: negative: 0%; +: 1%-25%; ++: 26%-50%; +++: 51%-100%.

Statistical analysis

One-way analysis of variance (ANOVA) and Pearson Correlation was performed to determine the difference and association by using Statistical Product and Service Solutions (SPSS, version 11.5, Chicago, IL, USA). *P* values of less than 0.05 were considered statistically significant.

RESULTS

The prevalence of H pylori infection and CagA classification

Among 711 patients, 61 had normal gastric mucosa (normal mucosa, NM), 268 suffered from CG, 114 from GA, 104 from IM, 71 from dysplasia and 93 from GC

(Table 2**).** Overall, 629 (88.47%) were infected with *H pylori*; 506 (71.16%) with CagA+ *H pylori* and 123 with CagA-*H pylori* (Table 2)**.** Following the progression of gastric lesions, the prevalence of CagA+ *H pylori* infection in NM, CG, GA, IM, DP and GC groups increased and the rate of CagA+ *H pylori* was positively associated with the severity of gastric pathologies ($r = 0.896$, $P = 0.016$), while the prevalence of CagA-*H pylori* infection was not significantly associated with the severity of gastric pathologies ($r = -0.794$, $P = 0.059$).

Systemic immune response in patients with CagA+ H pylori infection

Because the data of Th1/Th2 did not follow a Gaussian distribution, analysis of variance (ANOVA) was performed after the data were transferred by logarithm. As shown in Figure 1, there was no significant difference in IFN-γ among the different gastric pathologies, while there was an increasing tendency of IL-4 expression ($P = 0.022$) following the progression of gastric pathologies, leading to a gradual decrease in Th1/Th2 ratio in CagA+ *H pylori* infected patients. However, there was no such a tendency both in patients with CagA- *H pylori* infection and those without *H pylori* infection for both IFN-γ and IL-4, indicating that there was no significant difference in Th1/Th2 ratios in these patients. As for Treg cells, there was an increasing trend according to the progression of gastric pathologies, which was strongly correlated with CagA+ *H pylori* infection, but not with CagA- *H pylori* infection. There was a significant negative association between Th1/Th2 and the expression of Treg cells in gastric patients' PBMCs in patients with CagA+ *H pylori* infection ($r = -0.321$, $P < 0.001$), however, no such association was found in patients with CagA- *H pylori* infection and those without *H pylori* infection.

Following the progression of gastric pathologies associated with CagA+ *H pylori* infection, the levels of IL-2 and IL-12 gradually decreased, but the levels of IL-6, IL-10 gradually increased, which indicated that there were some changes in the patterns of cellular to humoral immunity. However, there was no significant difference for the key representative cytokines of Th1 and Th2 cells in all the groups (IFN-γ and IL-4, respectively) (Table 3). The patterns of six cytokines in the patients with CagA-*H pylori* infection and those without *H pylori* infection were similar to that in CagA+ *H pylori* infected patients (Tables 4 and 5), indicating there was no correlation between *H pylori* infection and cytokine levels.

Following the progression of the CagA+ *H pylori* associated gastric pathologies, the expression of IFN-γ mRNA showed a decreasing tendency ($P = 0.006$) while the expression of IL-4 mRNA increased ($P = 0.006$) (Figure 2). To check the specificity of the experiment, the molecular weight of the amplicon was confirmed by agarose gel electrophoresis (Figure 3). The expression of IFN-γ mRNA was significantly higher in CG than in IM, DP, GC ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively), and was significantly higher in the GA than in DP and GC groups ($P < 0.001$, and $P < 0.001$), but there was no significant difference in the expression among the IM, DP and GC. On the contrary, the expression of IL-4 mRNA was significantly lower in CG than in IM, DP, and

Table 2 The prevalence of *H pylori* infection and CagA classification

| Pathological diagnosis | Gender | | Age | *H pylori* positive | | | *H pylori* negative |
	Male	Female	(yr, mean ± SD)	Overall (%)	CagA+ (%)	CagA- (%)	
Normal mucosa (*n* = 61)	31	30	44.62 ± 12.41	48 (78.69)	37 (60.65)	11 (18.03)	13 (21.31)
Chronic gastritis (*n* = 268)	121	147	44.53 ± 14.37	232 (86.57)	170 (63.43)	62 (23.13)	36 (13.43)
Gastric atrophy (*n* = 114)	73	41	51.60 ± 12.39	104 (91.23)	88 (77.19)	16 (14.03)	10 (8.77)
Intestinal metaplasia (*n* = 104)	47	57	50.69 ± 12.81	95 (91.35)	80 (76.92)	15 (14.42)	9 (8.65)
Dysplasia (*n* = 71)	45	26	54.23 ± 12.25	65 (91.55)	57 (80.28)	8 (11.26)	6 (8.45)
Gastric cancer (*n* = 93)	70	23	64.70 ± 11.33	85 (91.40)	74 (79.57)	11 (11.83)	8 (8.60)
Total (*n* = 711)	387	324	50.18 ± 14.65	629 (88.47)	506 (71.16)	123 (17.30)	82 (11.53)

The prevalence of CagA+ *H pylori* was significantly associated with the progression of gastric pathology ($r = 0.896$, $P = 0.016$).
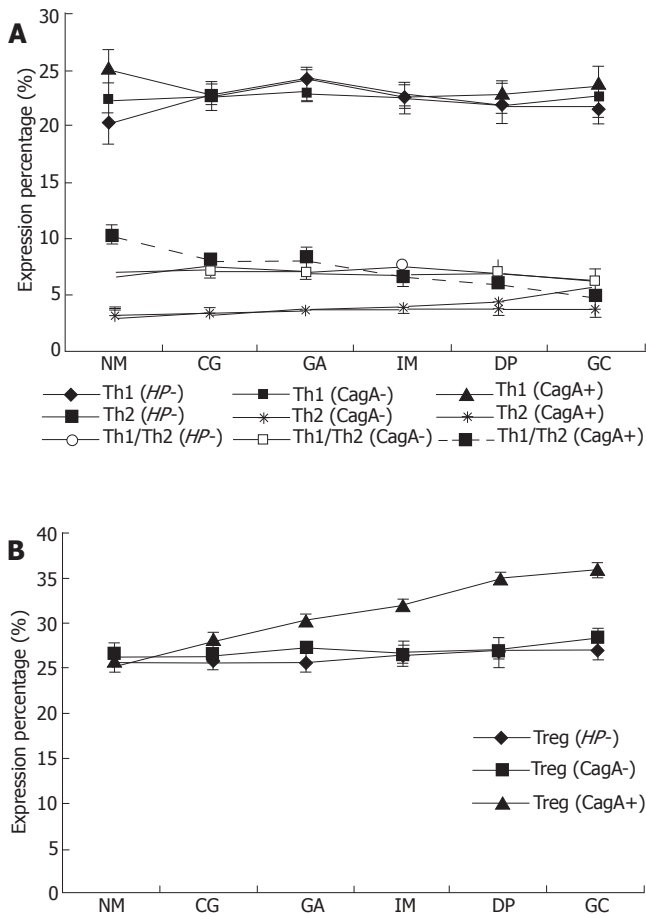
Figure 1 Expression of peripheral blood Th1, Th2 and Th1/Th2 ratio (**A**) and Treg cells (**B**) in patients with CagA+ and CagA- *H pylori* infection and in those without *H pylori* infection (Hp-) as determined by FCM. In CagA+ *H pylori* infected subjects, there is no significant difference in the percentage of Th1 cells in all groups, while the percentage of Th2 cells increases along the progression of gastric pathology, with significant difference between gastric cancer (GC) and chronic gastritis (CG) ($P < 0.001$), gastric atrophy (GA) ($P < 0.001$), and intestinal metaplasia (IM) ($P < 0.001$), respectively. There is a significant difference in the Th1/Th2 ratio between CG and dyspepsia (DP), GC ($P = 0.033$, $P < 0.001$), and between GA and GC ($P < 0.001$) (**A**). Treg cell expression increases along the progression with significant difference between CG and IM, DP, and GC ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively), between GA and DP, and GC ($P = 0.002$, and $P < 0.001$, respectively), and between IM and GC ($P = 0.012$) (**B**). There is no difference in the expression of Th1, Th2, Treg cells and Th1/Th2 ratio between the subjects with CagA- *H pylori* infection and those without *H pylori* infection among all the groups.

GC ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively), and in GA than in DP and GC ($P < 0.001$ and $P < 0.001$),

but there was no significant difference in the expression between DP and GC. In patients with CagA- *H pylori* infection and those without *H pylori* infection, there was no significant difference in the expression of IFN-γ and IL-4 mRNAs along the progression of gastric pathologies (Figure 2).

Local immune response in patients with CagA+ H pylori infection

As shown in Figure 2, the expression of IFN-γ mRNA in gastric biopsies of patients with CagA+ *H pylori* infection decreased gradually along the progression of gastric pathologies from CG to GC; there was a significant difference in the expression of IFN-γ mRNA between CG and DP, GC ($P = 0.013$, $P = 0.001$), between GA and DP, GC ($P = 0.043$, $P = 0.004$). However, the gastric expression of IFN-γ mRNA was constant from CG to GC in patients with CagA- *H pylori* infection and those without *H pylori* infection. On the contrary, there was an increase in gastric IL-4 mRNA expression in patients with CagA+ *H pylori* infection along the progression of gastric pathologies from CG to GC ($P = 0.004$). The expression of IL-4 mRNA was significantly lower in CG and GA than in IM, DP and GC. However, the gastric IL-4 mRNA in patients with CagA- *H pylori* infection and those without *H pylori* infection was expressed at low levels and remained constant in all the gastric pathologies.

Those results indicated that IFN-γ mRNA expression showed a decreasing tendency while the IL-4 mRNA expression showed an increasing tendency following the progression of the CagA+ *H pylori* associated pathologies. Pearson correlation analysis indicated that there was a significant positive correlation in IFN-γ mRNA expression ($r = 0.201$, $P < 0.001$), and IL-4 mRNA expression ($r = 0.212$, $P < 0.001$) between gastric biopsy and PBMCs. Compared with the expression of IFN-γ mRNA and IL-4 mRNA in PBMCs, changes in the biopsy specimens were more conspicuous, especially in IM, DP and GC (Figure 2).

As shown in Figure 4A, gastric IFN-γ expression from CG to GC in patients with CagA+ *H pylori* infection showed a decreasing tendency with the percentage of cases with IFN-γ expression of "+++" decreasing from 45.9% in CG patients to 18.9% in GC patients. IFN-γ expression with "+++" predominated in CG, while the percentage of cases with IFN-γ expression with "+" increased significantly in GC. On the other hand, the percentage of cases with IL-4 expression with "+"

Table 3 Plasma cytokine contents in patients with CagA+ *H pylori* infection (mean ± SE, pg/mL)

Pathological diagnosis	IFN-γ	IL-4	IL-12	IL-10	IL-6	IL-2
Normal mucosa (NM, *n* = 37)	27.46 ± 1.05	10.86 ± 0.62	81.42 ± 1.89	66.92 ± 2.15	47.96 ± 1.99	54.17 ± 1.09
Chronic gastritis (CG, *n* = 170)	28.94 ± 0.59	11.22 ± 0.45	72.49 ± 0.93	80.65 ± 0.99	63.40 ± 1.57	45.95 ± 0.49
Gastric atrophy (GA, *n* = 88)	30.48 ± 0.75	11.23 ± 0.33	53.29 ± 0.79	100.80 ± 1.69	94.10 ± 2.57	39.61 ± 0.69
Intestinal metaplasia (IM, *n* = 80)	31.71 ± 1.02	12.08 ± 0.59	52.18 ± 0.94	104.18 ± 1.90	112.03 ± 2.64	34.07 ± 0.54
Dysplasia (DP, *n* = 57)	30.14 ± 1.04	10.92 ± 0.39	43.30 ± 1.10	157.72 ± 2.64	156.74 ± 3.66	32.71 ± 0.81
Gastric cancer (GC, *n* = 74)	31.00 ± 0.84	11.03 ± 0.29	39.10 ± 0.89	196.65 ± 1.83	159.32 ± 2.57	28.39 ± 0.65

There was no difference in the expression of IFN-γ and IL-4 in the plasma of CagA+ *H pylori* subjects among all the groups. There were significant differences for IL-12 among all the groups except between GA and IM (P = 1.000), DP and GC (P = 0.237). There were significant differences for IL-10 between all groups except between GA and IM (P = 1.000). For IL-6, there were significant differences among all the groups except between DP and GC (P = 1.000). For IL-2, there were significant differences among all the groups except between IM and GA, DP (P = 1.000, P = 1.000).

Table 4 Plasma cytokine contents in patients with CagA- *H pylori* infection (mean ± SE, pg/mL)

Pathological diagnosis	IFN-γ	IL-4	IL-12	IL-10	IL-6	IL-2
Normal mucosa (NM, *n* = 11)	29.28 ± 0.54	11.07 ± 0.89	82.17 ± 4.59	67.29 ± 3.26	48.37 ± 4.01	54.11 ± 2.87
Chronic gastritis (CG, *n* = 62)	29.19 ± 1.18	10.89 ± 0.27	64.59 ± 3.10	80.25 ± 1.87	57.99 ± 2.61	53.14 ± 1.25
Gastric atrophy (GA, *n* = 16)	30.25 ± 2.01	10.67 ± 0.67	49.67 ± 3.28	91.28 ± 3.19	92.56 ± 5.79	42.33 ± 2.32
Intestinal metaplasia (IM, *n* = 15)	28.91 ± 2.23	11.08 ± 0.74	48.19 ± 2.99	98.11 ± 4.08	98.20 ± 5.92	40.98 ± 2.38
Dysplasia (DP, *n* = 8)	31.08 ± 2.19	11.01 ± 1.27	42.64 ± 5.49	157.09 ± 6.81	147.65 ± 6.89	34.79 ± 3.05
Gastric cancer (GC, *n* = 11)	28.06 ± 2.45	12.07 ± 0.91	38.97 ± 3.29	185.29 ± 9.38	154.68 ± 6.78	30.29 ± 2.11

There was no difference in the expression of IFN-γ and IL-4 in plasma of subjects with CagA- *H pylori* infection among all the groups (P_{all} > 0.05). For IL-12, there were significant differences between CG and GA, IM, DP, and GC (P < 0.05, P < 0.05, P < 0.05, P < 0.001). For IL-10, there were significant differences between all the groups except between GA and CG and IM (P_{all} > 0.05). For IL-6, there were significant differences among all the groups except between GA and IM (P = 1.000) and DP and GC (P = 1.000). For IL-2, there were significant differences between CG and GA, IM, DP, and GC (P_{all} < 0.001) and between IM and GC (P < 0.05).

Table 5 Plasma cytokine contents in patients without *H pylori* infection (mean ± SE, pg/mL)

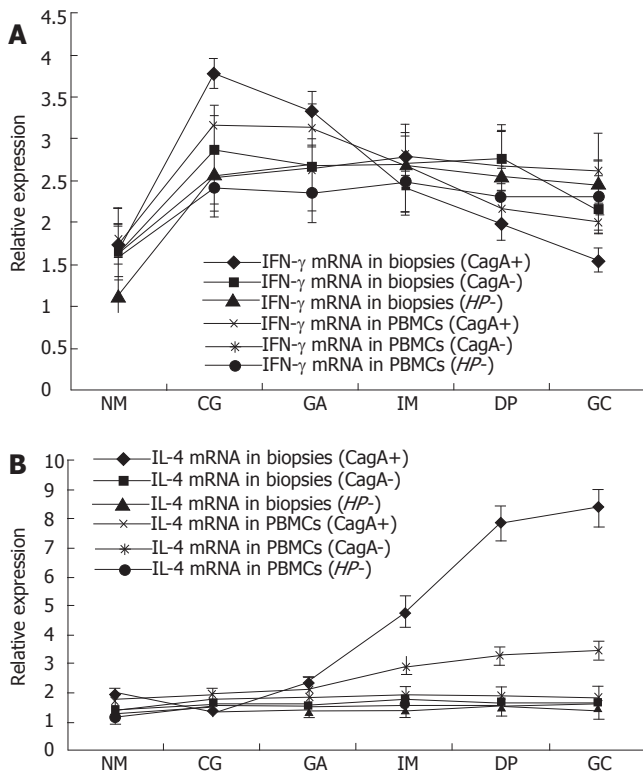| Pathological diagnosis	IFN-γ	IL-4	IL-12	IL-10	IL-6	IL-2
Normal mucosa (NM, *n* = 13)	31.83 ± 0.17	10.57 ± 0.68	80.84 ± 4.81	68.56 ± 3.87	49.53 ± 3.94	53.13 ± 1.48
Chronic gastritis (CG, *n* = 36)	29.10 ± 1.27	10.37 ± 0.30	68.57 ± 2.43	79.03 ± 1.64	57.66 ± 2.56	52.54 ± 1.41
Gastric atrophy (GA, *n* = 10)	29.06 ± 1.93	10.49 ± 0.58	51.14 ± 3.47	90.94 ± 3.63	91.08 ± 6.11	41.39 ± 2.18
Intestinal metaplasia (IM, *n* = 9)	28.57 ± 2.72	11.75 ± 0.61	50.97 ± 1.54	97.60 ± 3.53	96.35 ± 5.19	41.54 ± 0.99
Dysplasia (DP, *n* = 8)	31.59 ± 1.29	11.46 ± 0.58	43.10 ± 5.02	153.34 ± 6.12	149.92 ± 6.23	35.00 ± 2.04
Gastric cancer (GC, *n* = 6)	27.04 ± 2.30	11.91 ± 1.26	40.06 ± 2.08	181.36 ± 9.02	153.04 ± 6.63	30.95 ± 1.91

There was no difference in the expression of IFN-γ and IL-4 in plasma of subjects without *H pylori* infection among all the groups (P_{all} > 0.05). For IL-12, there were significant differences between CG and GA, IM, DP, and GC (P = 0.006, P = 0.009, P = 0.001, P < 0.001). For IL-10, there were significant differences between all the groups except between GA and CG and IM (P = 0.219, P = 1.000). For IL-6, there were significant differences among all the groups except between GA and IM (P = 1.000) and DP and GC (P = 1.000). For IL-2, there were significant differences between CG and GA, IM, DP, and GC (P_{all} < 0.001) and between IM and GC (P = 0.037).

decreased gradually from 68.2% in CG patients to 35.1% in GC patients, but the rate of IL-4 expression with "+++" increased gradually from 0% in CG patients to 28.4% in GC patients. In patients with CagA- *H pylori* infection and those without *H pylori* infection, there was no difference between IFN-γ and IL-4 expression among the different gastric pathologies; IFN-γ expression with "++" was always dominant, while IL-4 expression with "+" was always dominant (Figure 4B and C). The representative immunohistochemistry staining results in the gastric biopsies of with CagA+, CagA- *H pylori* infected patients and uninfected patients with different gastric pathologies are shown in Figure 5A-C.

DISCUSSION

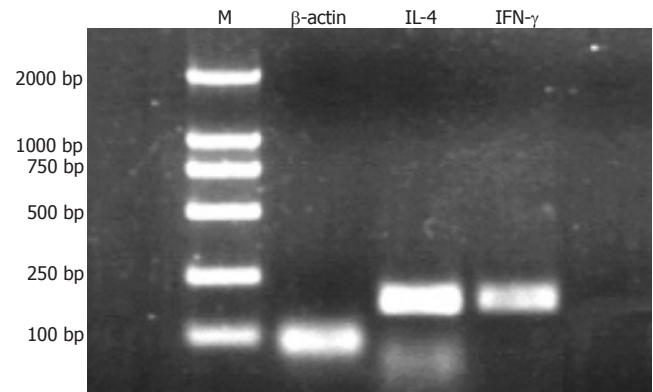*H pylori* is a small, curved, highly motile, Gram-negative bacterium that colonizes the epithelium of the human stomach. Previous studies have shown that *H pylori* infection is associated with the development of chronic gastritis and gastric cancer[3,4,16]. Several factors including virulence among different *H pylori* strains have been attributed to the diversity in clinical outcome[17]. Until now, very few papers have reported the prevalence of *H pylori* infection in China, especially the subtypes of the organism[18,19]. Our study showed that the prevalence of *H pylori* infection in our outpatients is 88.47% (629/711) and the majority (80.45%, 506/629) of the infected subjects is infected with CagA+ *H pylori*. This confirms that CagA+ *H pylori* infected subjects are predominant in the Chinese *H pylori*-infected population[19]. Moreover, we observed that the subtypes of *H pylori* infection may play different role in the progression of gastric carcinogenesis; CagA+ *H pylori* infection was positively associated with the severity of gastric pathologies, especially gastric cancer, whereas CagA- *H pylori* infection was not associated with the

A

Relative expression

- ◆ IFN-γ mRNA in biopsies (CagA+)
- ■ IFN-γ mRNA in biopsies (CagA-)
- ▲ IFN-γ mRNA in biopsies (HP-)
- × IFN-γ mRNA in PBMCs (CagA+)
- ✳ IFN-γ mRNA in PBMCs (CagA-)
- ● IFN-γ mRNA in PBMCs (HP-)

B

Relative expression

- ◆ IL-4 mRNA in biopsies (CagA+)
- ■ IL-4 mRNA in biopsies (CagA-)
- ▲ IL-4 mRNA in biopsies (HP-)
- × IL-4 mRNA in PBMCs (CagA+)
- ✳ IL-4 mRNA in PBMCs (CagA-)
- ● IL-4 mRNA in PBMCs (HP-)

Figure 2 The relative expression of IFN-γ (**A**) and IL-4 (**B**) mRNA in peripheral blood mononuclear cells (PBMCs) and gastric biopsies in patients with CagA+ and CagA- *H pylori* infection and those without *H pylori* infection as determined by real time RT-PCR. The relative expression of IFN-γ mRNA decreases from CG to GC, while the relative expression of IL-4 mRNA increases from chronic gastritis (CG) to gastric cancer (GC) in PBMCs and biopsies of CagA+ *H pylori* infected subjects. However, the magnitudes of relative expression of IFN-γ mRNA and IL-4 mRNA in biopsies are obviously larger than those in PBMCs. There is no difference in the expression of IFN-γ and IL-4 mRNA in PBMCs and local biopsies of subjects with CagA- *H pylori* infection and of those without *H pylori* infection.

Figure 3 A representative of real time RT-PCR for IFN-γ and IL-4 mRNA expression in PBMCs by agarose gel electrophoresis. Using the real time PCR products to run agarose gel electrophoresis is to confirm the size of every fragment. M: DNA marker, and the molecular weight of IL-4, IFN-γ and β-actin was 189 bp, 182 bp and 89 bp, respectively.

severity of gastric pathologies.

Previous epidemiological studies have demonstrated that severe clinical manifestations do not frequently occur in most individuals with *H pylori* infection and the different clinical manifestations caused by this bacterium could be a consequence of interactions among microorganism characteristics, environmental influences, and the host immune response[20-22]. In the present study, 711 patients with different gastric pathologies were recruited to explore the interaction between bacterial virulence and host immune responses including systemic immunity and local immunity. We observed that in patients with CagA+ *H pylori* infection, the expression of IFN-γ remained unchanged, while IL-4 increased gradually in PBMCs during the progression of gastric pathologies, resulting in decreases of Th1/Th2 ratios, which was negatively correlated to the increased expression of Treg cells. During the development of gastric pathologies, IFN-γ mRNA expression gradually decreased, while IL-4 mRNA expression gradually increased, which was consistent with Th1/Th2 results in PBMCs. In addition, IL-2 and IL-12 protein expression decreased gradually while IL-6 and IL-10 increased gradually following the progression of gastric pathologies, although IFN-γ and IL-4, the representative cytokines of Th1 and Th2 cells, did not

change. However, the ratios of Th1/Th2 in patients with CagA- *H pylori* infection and those without *H pylori* infection remained unchanged at both the cellular level and the mRNA level. Therefore, we postulate, based on our observations, that following the progression of the gastric pathologies, Th1 cells change slightly but Th2 cells are gradually increased, resulting in a gradual decrease in the ratio of Th1/Th2. Indeed, our systemic immunity analysis demonstrated that there was a shift from Th1 response to Th2 response during the progression of CagA+ *H pylori* infection associated gastric pathologies. However, it is noticed that IFN-γ and IL-4 expression in the plasma did not show any tendency of the Th1/Th2 shift, which may be due the fact that the levels of cytokines in plasma are affected by many factors other than *H pylori* infection[23,24], which may also explain the observation that IFN-γ and IL-4 expression were similar among patients with CagA+, or CagA- *H pylori* infection and those without *H pylori* infection.

Real-time RT-PCR and immunohistochemistry analysis were employed in the preset study to compare the local immunity of *H pylori* infected subjects, including mRNAs and protein expression of IFN-γ and IL-4 in gastric mucosa of patients with *H pylori* infection, with that in patients without *H pylori* infection. We observed that IFN-γ mRNA expression decreased gradually, while IL-4 mRNA expression increased during the development of gastric pathologies and the changing magnitude of IFN-γ mRNA and IL-4 mRNA expression in gastric mucosa were more obvious than those in PBMCs. Moreover, during the progression of gastric pathologies associated with CagA+ *H pylori* infection, IFN-γ expression primarily decreased from grade "+++" in CG patients to grade "+" in GC patients, whereas IL-4 expression increased from "+" in CG patients to "+++" in GC patients. On the contrary, in patients with CagA- *H pylori* infection and those without *H pylori* infection, IFN-γ and IL-4 expression remained unchanged during the progression of gastric pathologies; IFN-γ expression remained with grade "++" and IL-4 expression with grade "+". These findings demonstrate that there is a shift from Th1 mediated immune response
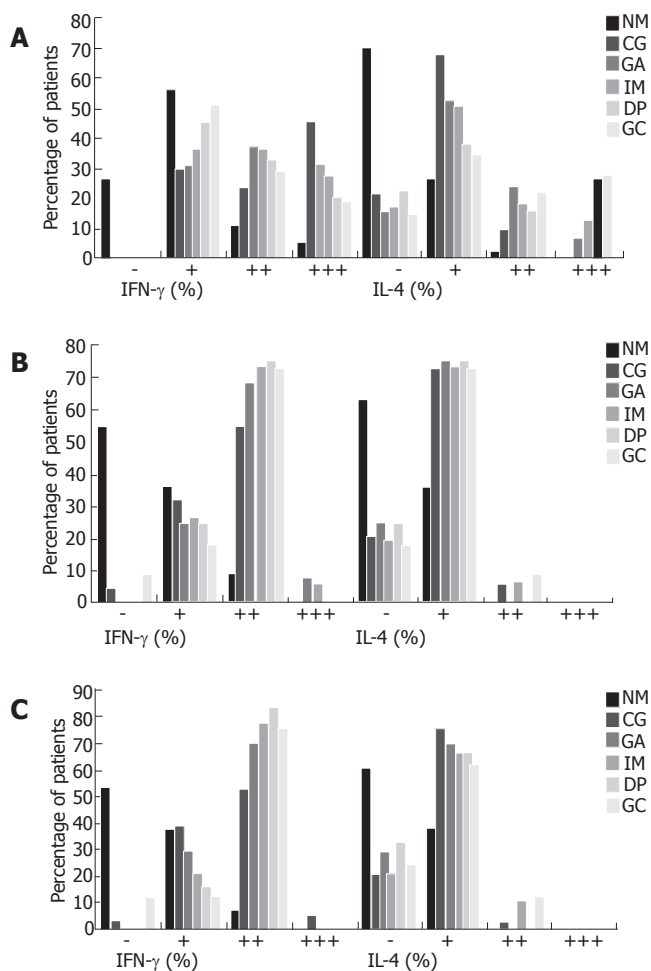
Figure 4 Local immunity responses in patients with CagA+ (**A**) , CagA- *H pylori* (**B**) infection and those without *H pylori* infection (**C**) as determined by immunohistochemistry. Numbers of IFN-γ and IL-4 positive cells from 200 polymorphonuclear cells (PMNs) and mononuclear cells (MNCs) were counted from five randomly chosen fields and averaged. The positive rate was used to grade the expression levels: negative: 0%; +: 1%-25%; ++ 26%-50%; +++, 51%-100%. **A**: IFN-γ expression with "+++" predominates in chronic gastritis (CG), while IFN-γ expression with "+" becomes the majority when the disease develops to gastric cancer (GC). On the contrary, IL-4 expression with "+" decreases gradually when the disease develops from CG to GC, but the rate of IL-4 expression with "+++" increases gradually following the progression; **B**: Following the progression of gastric lesions, there was no difference of IFN-γ and IL-4 expression in that the former was always with "++", while the latter was always with "+" in all the groups; **C**: Following the progression of gastric lesions, IFN-γ expression with "++" were at all dominant, while IL-4 expression with "+" was in the main in all the groups.
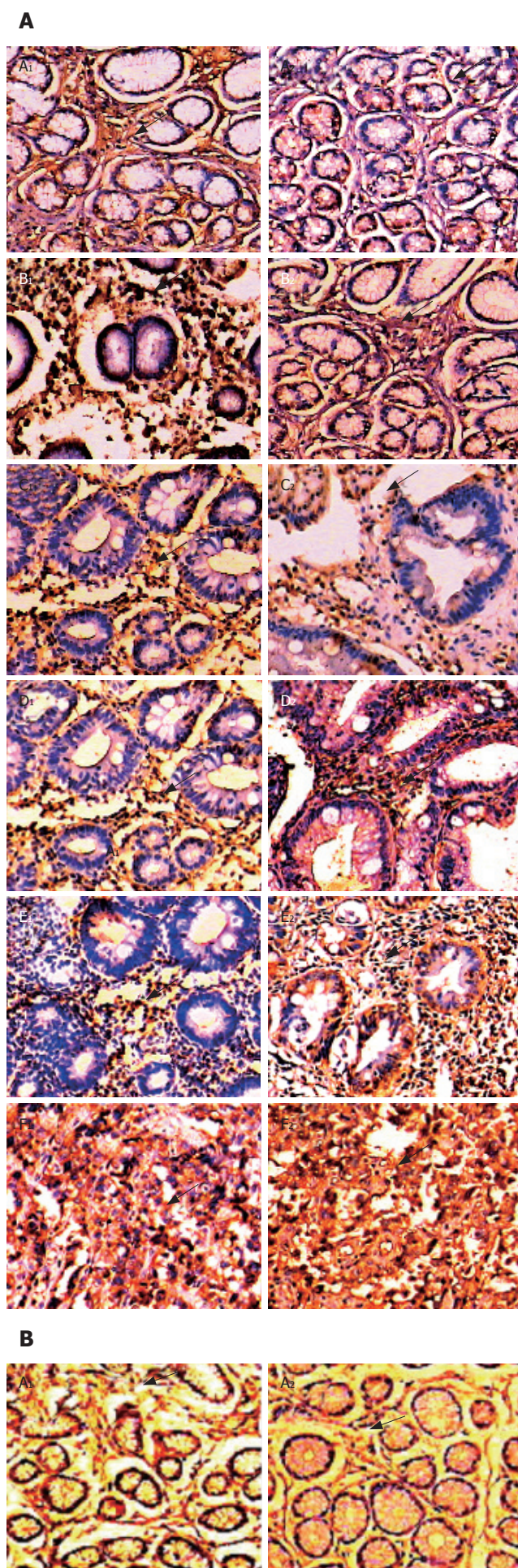
to Th2 mediated immune response within gastric mucosa during the progression of CagA+ *H pylori* infection associated gastric carcinogenesis, which is more significant than the Th1/Th2 shift in the systemic immunity.

In conclusion, there is a shift from Th1 mediated cellular immunity to Th2 mediated hormonal immunity in the immune response during the progression of gastric carcinogenesis in patients with CagA+ *H pylori* infection, which is negatively associated with the expression of Treg cells. Moreover, among the immune responses associated with CagA+ *H pylori* infection, local immunity is predominant over systemic immunity and the polarization of Th cells mediated immune response is associated with the chronicity and progression of gastric pathologies,
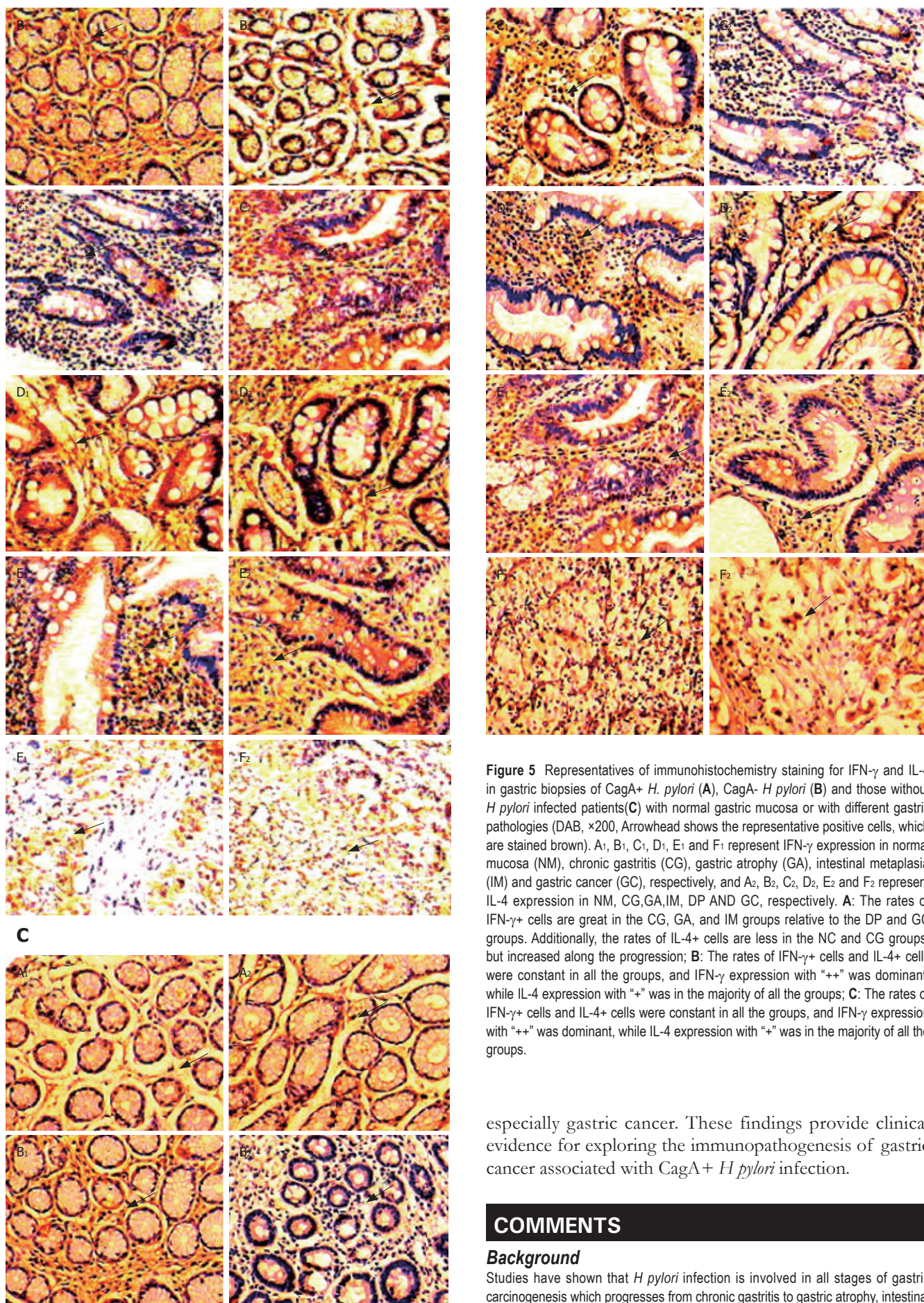
Figure 5 Representatives of immunohistochemistry staining for IFN-γ and IL-4 in gastric biopsies of CagA+ *H. pylori* (**A**), CagA- *H pylori* (**B**) and those without *H pylori* infected patients(**C**) with normal gastric mucosa or with different gastric pathologies (DAB, ×200, Arrowhead shows the representative positive cells, which are stained brown). A_1, B_1, C_1, D_1, E_1 and F_1 represent IFN-γ expression in normal mucosa (NM), chronic gastritis (CG), gastric atrophy (GA), intestinal metaplasia (IM) and gastric cancer (GC), respectively, and A_2, B_2, C_2, D_2, E_2 and F_2 represent IL-4 expression in NM, CG,GA,IM, DP AND GC, respectively. **A**: The rates of IFN-γ+ cells are great in the CG, GA, and IM groups relative to the DP and GC groups. Additionally, the rates of IL-4+ cells are less in the NC and CG groups, but increased along the progression; **B**: The rates of IFN-γ+ cells and IL-4+ cells were constant in all the groups, and IFN-γ expression with "++" was dominant, while IL-4 expression with "+" was in the majority of all the groups; **C**: The rates of IFN-γ+ cells and IL-4 cells were constant in all the groups, and IFN-γ expression with "++" was dominant, while IL-4 expression with "+" was in the majority of all the groups.

especially gastric cancer. These findings provide clinical evidence for exploring the immunopathogenesis of gastric cancer associated with CagA+ *H pylori* infection.

COMMENTS

Background

Studies have shown that *H pylori* infection is involved in all stages of gastric carcinogenesis which progresses from chronic gastritis to gastric atrophy, intestinal

metaplasia, dysplasia and gastric cancer. However, the functional interaction of *H pylori* infection with immune components, especially T cell immune responses, has not been fully elucidated.

Research frontiers

Previous studies have reported the differential expression of cytokines between *H pylori* positive and *H pylori* negative patients or between gastritis and gastric cancer patients, but these studies did not examine the cellular role of *H pylori*, especially CagA+ *H pylori*, infection in the immune responses during the progression of gastric pathology.

Innovations and breakthroughs

Among patients infected with CagA+ *H pylori* infected, Th1-mediated cellular immunity was associated with earlier stages of gastric carcinogenesis, while Th2-mediated humoral immunity dominated the advanced stages and was negatively associated with an abundance of Treg cells. However, there was no such tendency in Th1/Th2 polarization in patients infected with CagA- *H pylori* strains and those without *H pylori* infection.

Applications

Polarization of Th cell immune responses occurs in patients with CagA+ *H pylori* infection, which is associated with the stage and severity of gastric pathology during the progression of gastric carcinogenesis, will provide further evidence for a causal role of CagA+ *H pylori* infection in the immunopathogenesis of gastric cancer.

Terminology

Immune response: Actions of the body's immune system that come into play to control infection or disease; T helper cells: can be divided into two subsets, Th1 cells and Th2 cells. Th1 cells mediate cellular immunity mainly by producing interferon (IFN)-γ, interleukin (IL)-2, IL-12, and tumor necrosis factor (TNF)-β, while Th2 cells primarily mediate humoral immunity by secreting IL-4, IL-5, IL-6, IL-10 and IL-13; regulatory T cells: a low abundance cell subset, help mediate the balance of Th1 and Th2, Treg cells inhibit the proliferation of CD4 + CD25-T lymphocytes, CD8 + T lymphocytes, immune memory cells, and antigen presenting cells (APCs) by recognizing inner and outer antigens; Immunopathogenesis: the process of development of a disease in which an immune response or the products of an immune reaction are involved.

Peer review

This is an excellent work that I think has to be accepted as it is. It is well written, well done and reaches original findings.

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