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Cytokine BAFF Released by *Helicobacter pylori*–Infected Macrophages Triggers the Th17 Response in Human Chronic Gastritis

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BAFF is a crucial cytokine that affects the activity of both innate and adaptive immune cells. It promotes the expansion of Th17 cells in autoimmune disorders. With this study, we investigated the BAFF/Th17 responses in *Helicobacter pylori*–induced gastritis in humans. Our results show that the mucosa from *Helicobacter*$^+$ patients with chronic gastritis is enriched in IL-17 and BAFF, whereas the two cytokines are weakly expressed in *Helicobacter*$^-$ patients with chronic gastritis; moreover, the expression of both BAFF and IL-17 decreases after bacteria eradication. We demonstrate that BAFF accumulates in macrophages in vivo and that it is produced by monocyte-derived macrophages in vitro, after *Helicobacter* stimulation. Application of BAFF on monocytes triggers the accumulation of reactive oxygen species that are crucial for the release of pro-Th17 cytokines, such as IL-23, IL-1β, and TGF-β. Moreover, BAFF directly promotes the differentiation of Th17 cells. In conclusion, our results support the notion that an axis BAFF/Th17 exists in chronic gastritis of *Helicobacter*$^+$ patients and that its presence strictly depends on the bacterium. Moreover, we demonstrated that BAFF is able to drive Th17 responses both indirectly, by creating a pro-Th17 cytokine milieu through the involvement of innate immune cells, and directly, via the differentiation of T cells toward the specific profile. The results obtained in this study are of great interest for *Helicobacter*-related diseases and the development of novel therapeutic strategies based on the inhibition of the BAFF/IL-17 response. ***The Journal of Immunology*****, 2014, 193: 5584–5594.**

Helicobacter pylori is a Gram-negative bacterium that establishes chronic infection in the stomach. Colonization often remains asymptomatic but, in some cases, it can progress into gastritis, gastric ulcer, and gastric malignancies. It is known that the infection by the bacterium is accompanied by a Th1 immune response that, instead of being useful for clearing the bacterium, is probably responsible for the mucosa damage (1). The paradigm that the Th1 profile is the only one that sustains *H. pylori*–associated diseases has been recently revised, because it has been demonstrated that other proinflammatory cytokines, besides IFN-γ, such as IL-17 and TNF-α, are also involved (2).

It has been proposed that Th17 cells precede and may regulate the Th1 response and contribute to the pathology in mice (3), but the real contribution of the IL-17 activity in *H. pylori*–induced diseases remains a controversial issue. Many data support the idea that the Th17 response favors the bacterial growth in mice and contributes to the inflammation both in mice and in humans (3–5), although its role is probably not essential because IFN-γ has the largest effect (2). Along with this evidence, other data suggest that this response may exert a regulatory effect rather than a proinflammatory one (6). Moreover, conflicting data are also available on the role of Th17 cells in tumors: some findings support a promoting activity, whereas others suggest an inhibitory action. However, these conflicts are more apparent than real, because the biological impact of Th17 cells depends critically on the type of cancer (7). In the case of gastric cancer, a recent study demonstrated that Th17 cells infiltrate the tumor and that the secreted IL-17 leads to tumor progression (8).

Macrophages have been suggested to play a central role in driving the differentiation of Th17 cells in *H. pylori*–infected mice; indeed, upon the application of the bacterium or of its virulence factor urease, macrophages release pro-Th17 cytokines (9, 10). Moreover, two recent studies conducted on two different mouse models of autoimmune disorders identified in the BAFF (also named BLyS) of the TNF-α family a promoting factor for the Th17 response (11, 12). One study suggests a direct role of the cytokine in the generation of this immune profile, whereas the other one leans toward an indirect role for BAFF as modulator for the cytokine milieu, which, in turn, would affect the generation and function of Th17 cells.

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Abbreviations used in this article: DPI, diphenyleneiodonium; ET, eradication therapy; H_2DCF-DA, 2′,7′-dichlorofluorescein diacetate; MDM, monocyte-derived macrophage; MOI, multiplicity of infection; PKA, protein kinase A; rBAFF, recombinant human BAFF; ROS, reactive oxygen species; wt, wild type.

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Despite this growing amount of knowledge in mice, little is known about the mechanism of $CD4^+$ T cell differentiation into Th17 cells in *H. pylori*–infected humans, both in terms of cells involved and in terms of the contribution of both bacterial and host factors.

In this study, we demonstrate that the mucosa of *H. pylori*–infected subjects, affected by chronic gastritis, is enriched in IL-17 and BAFF, and that the accumulation of the two cytokines is strictly dependent on the presence of the bacterium. We found that BAFF is mainly secreted by macrophages, which are abundant in the gastric mucosa of patients, and by acting on monocytes, it triggers the release of pro-Th17 cytokines in a reactive oxygen species (ROS)–dependent manner. Interestingly, BAFF is capable of promoting the differentiation of Th17 cells also by acting directly on proliferating human $CD4^+$ T cells.

Collectively, our results suggest that BAFF, which accumulates in the mucosa of *H. pylori*–infected patients with chronic gastritis, might be one of the host factors that participate in generating Th17 cells.

Materials and Methods

Reagents

PBS, RPMI 1640, and FBS were from Euroclone (Siziano, Italy). Gentamicin, HEPES, HBSS, TRIzol solution, Hoechst stain, SuperScript II, anti-rat Alexa 488, anti-mouse Alexa 488, and anti-mouse Alexa 594 secondary Abs were from Life Technologies (Glasgow, U.K.); diphenyleneiodonium (DPI) chloride, Triton X-100, Tween 20, sodium citrate, BSA, forskolin, the Jak inhibitor AG490, and the protein kinase A (PKA) inhibitor H89 were from Sigma-Aldrich (St. Louis, MO); 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) was from Cayman Chemical (Ann Harbor, MI). Recombinant human BAFF (rBAFF) and M-CSF were from Immunological Science (Rome, Italy). Rat anti-human BAFF blocking Ab was from R&D Systems (Minneapolis, MN). Rat anti-human BAFF polyclonal Ab was from Alexis Biochemicals (San Diego, CA); rabbit anti-human IL-17 and mouse anti-human CD68 Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). mAbs anti-human CD3 and anti-human CD28 were from BD Biosciences (Saint Jose, CA). mAb anti-human CD4 was from Abcam (Cambridge, U.K.). Monoclonal blocking Ab anti-human TLR2 (clone TL2.1) was from eBioscience (High Wycombe, U.K.); monoclonal blocking Ab anti-human TLR4 (W7C11) was from Invivogen (Toulouse, France). mAb anti-human phospho-CREB (Ser^{133}) and rabbit polyclonal Ab against the phosphorylated PKA consensus phosphorylation site, R-X-X-pT-X-X/R-R-X-pS-X-X, were from Cell Signaling Technology (Danvers, MA). Monoclonal anti-actin Ab was from Merck-Millipore (Darmstadt, Germany). Ficoll-Paque solution and Percoll were from GE Healthcare Life Science (Buckinghamshire, U.K.). Human IL-23, IL-1β, and IL-6 were from eBioscience (San Diego, CA). Neutralizing anti–IL-4 Ab was from Biolegend (San Diego, CA), and neutralizing anti–IL-12 Ab was from Serotec (Oxford, U.K.). StemSep Human T-cell enrichment kit was from Voden Medical Instruments (Milan, Italy).

Patients

The cases considered in this study were retrospectively collected from the files of the Surgical Pathology and Cytopathology Unit at the University of Padua (January 2003 to December 2011). All patients were white and native of the Veneto region, and they underwent endoscopy at Padua University Hospital. The institute's ethical regulations on research conducted on human tissues were followed.

The study was conducted on a total of 40 endoscopic biopsy samples obtained from different biopsy sets and all collected from oxyntic proximal mucosa (i.e., gastric corpus). The series included: 1) normal gastric oxyntic mucosa obtained from dyspeptic patients, as control (n = 10); 2) mucosa from *H. pylori*$^-$ nonatrophic chronic gastritis (n = 10); 3) mucosa from *H. pylori*$^+$ nonatrophic chronic gastritis, before *H. pylori* eradication therapy (ET; n = 10); and 4) mucosa from the same patients as in 3), who underwent clinical and histological remission, after *H. pylori* ET. *H. pylori* was assessed by histology (modified Giemsa staining) and confirmed by clinical history, rapid urease testing, and/or ELISA (*H. pylori*–specific IgG Abs; GastroPanel, Biohit HealthCare, Milan, Italy) (13). Two trained gastrointestinal pathologists, blinded to any of the patients' endoscopic or clinical information, jointly assessed the original slides (H&E, Alcian blue–periodic acid schiff, and Giemsa for *H. pylori*). The semiquantitative assessment of the severity of mononuclear inflammation was graded in a three-tier scale (Supplemental Table I): grading is defined as the measure of the severity of the mononuclear inflammatory lesions and was evaluated according to Dixon and colleagues (14).

Immunofluorescence on tissue slices

Tissues were obtained from the archive of the Surgical Pathology and Cytopathology Unit at the University of Padua. Four-micrometer, formalin-fixed, paraffin-embedded tissue sections were deparaffinized with xylene washes and rehydrated with decreasing ethanol concentration. Heat-mediated Ag retrieval was obtained with repeated heat cycles in microwave for 10 min in retrieval buffer (10 mM sodium citrate, pH 6, 0.05% Tween 20). After permeabilization (45 min in 0.2% Triton X-100 in PBS), sections were blocked in 10% goat serum, 1% BSA in PBS for 1 h.

Abs specific for BAFF, IL-17, CD4, and CD68 were added to sections and revealed with Alexa-labeled secondary Abs. Nuclei were detected by the Hoechst stain. Slices were visualized with an oil immersion objective on a laser-scanning confocal microscope, and images were acquired using the LAS-AF software (Leica TCS-SP5; Leica Microsystems, Wetzlar, Germany). Quantification of the expression level of IL-17 and BAFF was performed using ImageJ software.

Bacterial strain and growth conditions

$CagA^+/VacA^+$ *H. pylori* strain 342, $CagA^+/VacA^+$ *H. pylori* strain G27, and their isogenic mutants Δ*hp-nap* (15, 16) and Δ*cagPAI* (17) were provided by A. Covacci, Novartis Vaccines, Siena, Italy; $Cag^+/VacA^+$ *H. pylori* strain SPM326 and its isogenic mutant Δ*vacA* (15, 18) were provided by J.L. Telford, Novartis Vaccines. *H. pylori* strains were maintained in 5% CO_2 at 37°C on Columbia agar plates supplemented with 5% horse blood. Colonies were inoculated into brain heart infusion broth containing 5% FBS and were cultured for 2 d in rotary shaking at 180 rpm at 37°C under microaerophilic conditions.

Monocyte isolation, macrophage differentiation, and cell treatment

Monocytes from healthy donors were prepared as described previously (19). For macrophage differentiation, 5×10^5 monocytes, seeded in 24-well plates, were cultured in RPMI 20% FBS in the presence of 100 ng/ml M-CSF for a 6-d differentiation. Monocyte-derived macrophages (MDMs) were cultured for 1 d in culture medium without M-CSF, before stimuli application. Cells were cultured in RPMI 1640 containing 10% FCS in the presence of BAFF (50 ng/ml), bacteria (multiplicity of infection [MOI] = 0.5), or PBS (control). When required, cells were preincubated for 1 h with 20 μM DPI, 10 μM H89, 25 μM AG490, 20 μg/ml anti-TLR2 Ab, or 10 μg/ml anti-TLR4 Ab.

For the experiments aimed to address the signaling pathway triggered by *H. pylori*, macrophages were incubated or not with bacteria (strain SPM326 wild type [wt], MOI = 0.5) for 2 h at 37°C. When required, cells were preincubated 1 h with 10 μM H89. Cells were recovered by centrifugation, washed twice in PBS, and lysed in 1% Triton X-100 in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl in the presence of protease inhibitors. Samples were centrifuged at 16,000 × g for 20 min at 4°C, and the postnuclear supernatants were collected and processed for immunoblot analysis.

For the experiment with BAFF blocking Ab, the latter, used at the final concentration of 1 μg/ml, was preincubated for 1 h with BAFF in culture medium, before adding to the cells. For the experiment with the *H. pylori*–conditioned medium of MDMs, the latter were infected with bacteria (MOI = 0.5) for 72 h. The medium was collected, clarified by centrifugation, and applied on freshly isolated monocytes in presence or absence of anti-BAFF blocking Ab. After 96 h, supernatants were collected and the amount of extracellular cytokines was determined by ELISA.

Real-time PCR analysis

Total mRNA was isolated from paraffin-embedded biopsies using the RecoverAll Total Nucleic Acid Isolation Kit (Life Technologies). mRNA extraction from monocytes was performed using TRIzol Reagent, according to the manufacturer's specifications. mRNA was reverse-transcribed using SuperScript II, and cDNA was amplified with the following primers: GAPDH, 5′-AGCAACAGGGTGGTGGAC-3′ and 5′-GTGTGGTGGGGGACTGAG-3′; IL-23p19, 5′-TCCACCAGGGTCTGATTTTT-3′ and 5′-TTGAAGCGGAGAAGGAGACG-3′; IL-12p40, 5′-ACAAAGGAGGCGAGGTTCTAA-3′ and 5′-CCCTTGGGGGTCAGAAGAG-3′; IL-6, 5′-AACCTGAACCTTCCAAAGATGG-3′ and 5′-TCTGGCTTGTTCCTCACTACT-3′; TGF-β, 5′-AGTGGTTGAGCCGTGGAG-3′ and 5′-CCAT-

GAGAAGCAGGAAAGG-3′; IL-1β, 5′-CTGTCCTGCGTGTTGAAAG-A-3′ and 5′-TTGGGTAATTTTTGGGATCTACA-3′; IL-17, 5′-TGGG-AAGACCTCATTGGTGT-3′ and 5′-TGGGAAGACCTCATTGGTGT-3′; BAFF, 5′-CGGGACTGAAAATCTTTGAACC-3′ and 5′-TGAGTGAC-TGTTTCTTCTGGAC-3′.

After amplification, data analysis was performed using the second derivative method algorithm. For each sample, data were normalized to the endogenous reference gene GAPDH and expressed as arbitrary units.

cAMP assay

Intracellular cAMP was quantified using an enzyme-linked immunoassay kit (GE Healthcare, Life Sciences) according to the manufacturer's instructions. Cells (1×10^6, plated in 96-well plates in RMPI 1640 supplemented with 20% FCS) were treated with *H. pylori* strain SPM326 wt as described earlier. Alternatively, cells were incubated with 25 μM forskolin for 30 min in the same conditions. At the end of the treatment, cells were washed twice in PBS and lysed in the lysis reagent included in the kit.

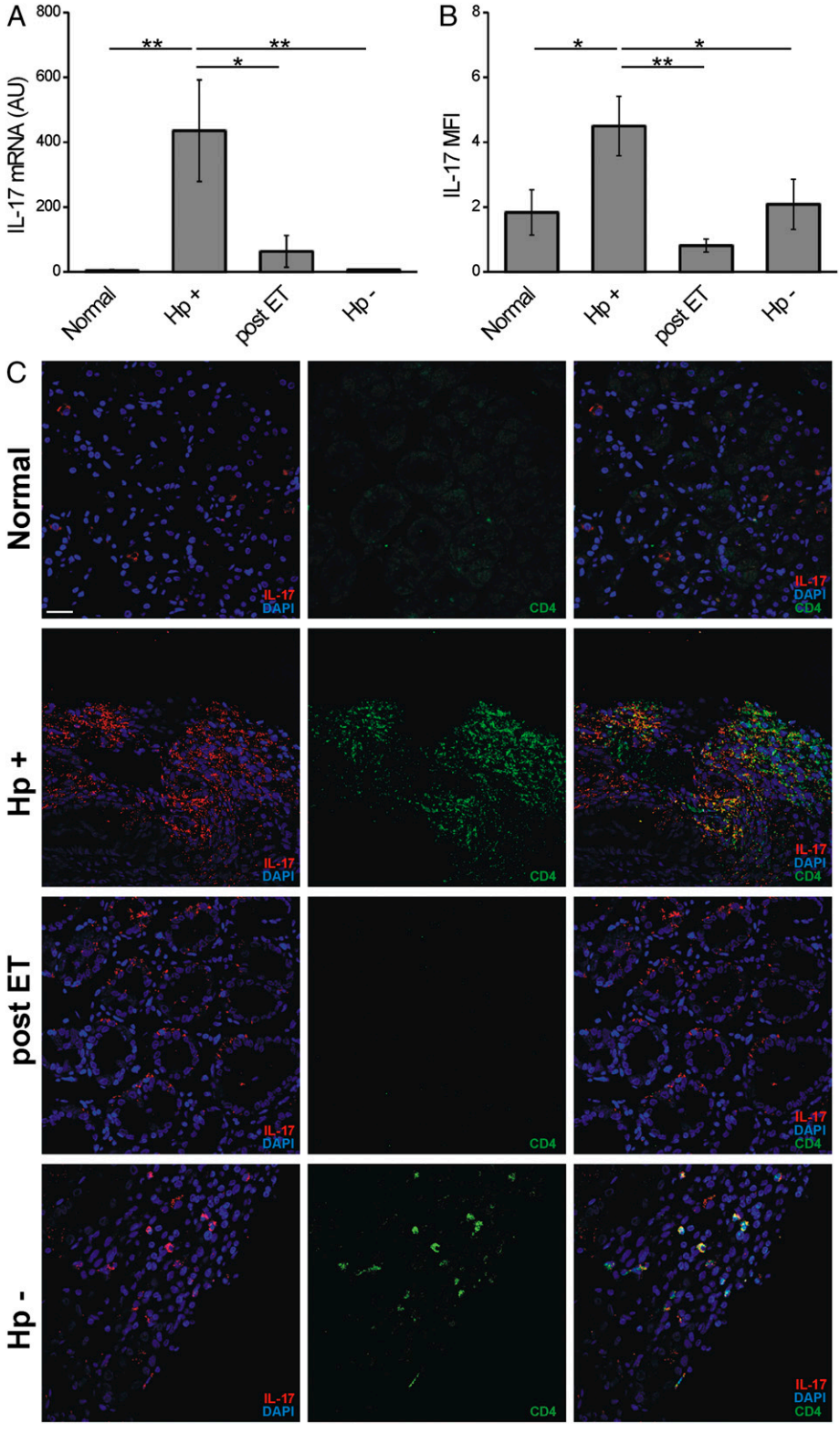

FIGURE 1. IL-17 expression in dyspeptic patients with normal mucosa (normal), *H. pylori*$^+$ patients (Hp$^+$) with chronic gastritis pre-ET and post-ET, and *H. pylori*$^-$ patients (Hp$^-$) with chronic gastritis. (**A**) mRNA was extracted from gastric biopsies and retro-transcribed. Expression of IL-17 gene was quantified as detailed in *Materials and Methods*. $n = 10$ for each group of patients. (**B**) A double-immunofluorescence staining was performed on paraffin-embedded sections of gastric mucosa using anti-CD4 (green) and anti–IL-17 (red) Abs followed by fluorophore-conjugated secondary Abs; fluorescence intensity associated to IL-17 expression was quantified using ImageJ software and expressed as mean fluorescence intensity ($n = 10$ for each group of patients). (**C**) Representative immunofluorescence staining of a single patient per group. Nuclei were revealed with Hoechst 33342 stain (blue). Scale bars, 25 μm. Error bars represent SD. *$p < 0.05$, **$p < 0.01$. AU, arbitrary units; MFI, mean fluorescence intensity.

ROS detection

Monocytes (2×10^6 cells/well in a 24-well plate) or MDMs (10^6 cells/well in a 24-well plate) were exposed to bacteria (MOI = 0.5), to 50 ng/ml rBAFF, or to PBS; when required, the cytokine was preincubated with anti-BAFF blocking Ab or the cells were preincubated with DPI. Cells exposed to PBS were referred to as control. Cells were incubated with the oxidant-sensitive probe (H_2DCF-DA, 10 μM) in HBSS, glucose 10 mM, for 45 min. After three washes with HBSS, glucose 10 mM, fluorescence was measured with a microplate fluorometer with excitation at 485 nm and emission at 530 nm.

Detection of cytokines in culture supernatants

The amount of extracellular cytokines was determined using commercial ELISA kits, following the manufacturers' instructions. IL-23 heterodimer, TGF-β, and IL-6 ELISA kits were purchased from eBioscience (San Diego, CA); IL-1β ELISA kit was purchased from Biolegend, and BAFF ELISA kit was purchased from R&D Systems.

ELISPOT assay

Enriched T cells purified from buffy coat from healthy donors by density gradient centrifugation on Ficoll-Paque, and by using the StemSep Human T-cell enrichment kit, were activated by anti-CD3 and anti-CD28 mAbs (10 μg/ml) in the presence or absence of BAFF (100 ng/ml), or anti-BAFF blocking Ab (10 μg/ml), or human IL-23, IL-1β, IL-6 (10 ng/ml), neutralizing anti–IL-4 (10 μg/ml), and anti–IL-12 (10 μg/ml) Abs. After 10 d, cells (5×10^5) were washed, stimulated for 48 h using anti-CD3 mAb in ELISPOT microplates coated with anti–IL-17 Ab (eBioscience, San Diego,

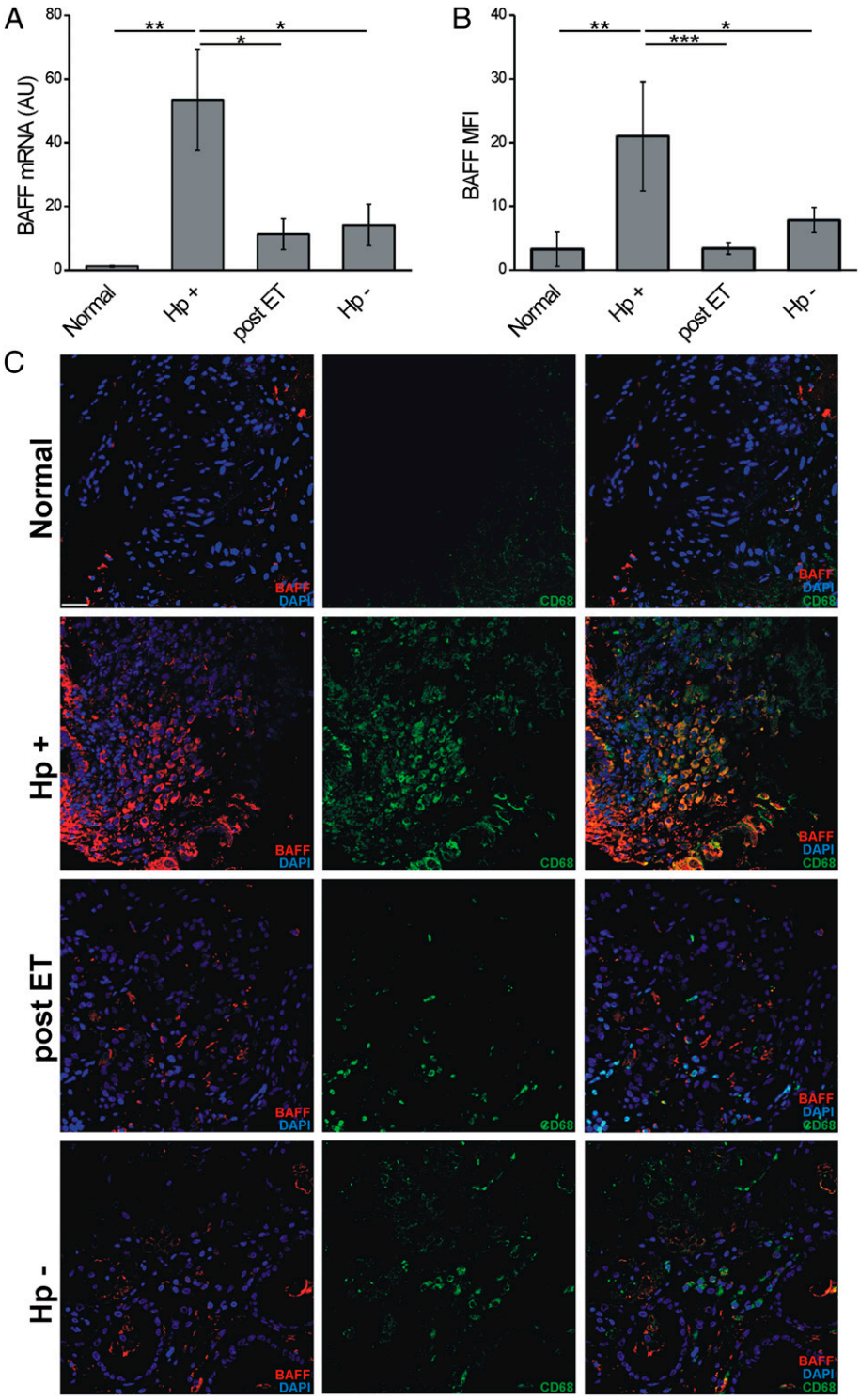

FIGURE 2. BAFF expression in dyspeptic patients with normal mucosa (normal), *H. pylori*$^+$ patients with chronic gastritis pre- and post-ET, and *H. pylori*$^-$ patients with chronic gastritis. (**A**) mRNA was extracted from gastric biopsies and retro-transcribed. Expression of BAFF gene was evaluated. $n = 10$ for each group of patients. (**B**) A double-immunofluorescence staining was performed on paraffin-embedded sections of gastric mucosa using anti-CD68 (green) and anti-BAFF (red) Abs followed by fluorophore-conjugated secondary Abs; fluorescence intensity associated to BAFF expression was quantified by using ImageJ software and expressed as mean fluorescence intensity ($n = 10$ for each group of patients). (**C**) The representative immunofluorescence staining reveals a clear-cut colocalization between CD68$^+$ cells and BAFF. Nuclei were revealed with Hoechst 33342 stain (blue). Scale bars, 25 μm. Error bars represent SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

CA), and the number of IL-17–forming cells was measured by an automated ELISPOT reader as described previously (20).

Statistical analysis

All statistical analyses were performed using Student *t* test or ANOVA when applied to patients' data; data, reported as the mean ± SD, were considered significant if p values ≤ 0.05.

Results

IL-17 and BAFF are increased in H. pylori*–induced chronic gastritis and their accumulation correlates with bacterial infection*

The infiltration of Th17 cells in the gastric mucosa of *H. pylori*–infected patients is well established (3, 21). To confirm this evidence, we evaluated the expression of IL-17, in terms of both mRNA and protein, in the mucosa of *H. pylori*–infected patients with nonatrophic chronic gastritis, a condition that represents a risk for the development of more severe gastric diseases (22). Specimens isolated from the patients were used for extracting total RNA or processed for immunofluorescence analysis. The latter was carried out by double staining for CD4 and IL-17. The same approach was adopted for samples isolated from the same subjects after bacteria ET and from *H. pylori*$^-$ patients with chronic gastritis. Normal gastric oxyntic mucosa obtained from dyspeptic patients was considered as negative control. Fig. 1A reveals a robust IL-17 mRNA expression in patients infected by *H. pylori*, and notably, it dropped after bacteria ET. This observation, which suggested a correlation between the infection and the cytokine expression, was further corroborated by the finding that in *H. pylori*$^-$ patients, the IL-17 mRNA level remained low. In accordance with these data, IL-17 staining performed on the sections of different origin revealed an abundant accumulation of the cytokine in CD4$^+$ cells only in *H. pylori* patients, whereas the signal was scanty and more similar to that of normal mucosa, both in post-ET patients and in *H. pylori*$^-$ patients; as expected, in the latter two groups, the infiltration of lymphocytes was practically absent (Fig. 1B, 1C).

Finally, in accordance with the fact that the Th17 profile is well represented in *H. pylori* patients, the same mucosa specimens enriched in IL-17 showed also an increased expression of IL-6, IL-1β, IL-23, and TGF-β, the cytokines that play a central role in the generation of Th17 cells (23). No significant expression of pro-Th17 cytokines was found in the other groups of subjects (Supplemental Fig. 1). Therefore, the Th17 profile does not accompany indiscriminately any chronic gastric inflammation; on the contrary, it appears to be strictly dependent on the presence of *H. pylori*.

Based on the knowledge that the expansion of Th17 cells requires BAFF, a trophic factor for B cells, released by various cell types (11, 12), we next evaluated the expression of BAFF in the same specimens analyzed for IL-17. We found that the expression pattern of BAFF was superimposable to that of IL-17, in terms of both mRNA and protein, being highly represented only in *H. pylori*$^+$ patients (Fig. 2A, 2B).

BAFF is expressed by monocytes/macrophages in gastric mucosa

H. pylori–associated chronic gastritis is characterized by infiltration of the mucosa by chronic inflammatory cells (monocytes/macrophages, lymphocytes, plasma cells, and occasional eosinophils) and a variable presence of neutrophils (24). Because BAFF is expressed by a wide variety of cell types, including monocytes, macrophages, and neutrophils (25, 26), we sought to define the source of BAFF among the mucosa-infiltrating cells in *H. pylori* patients. An immunofluorescence double staining was performed using Abs specific for either monocytes/macrophages or neutrophils together with a mAb anti-BAFF. Neutrophils that were identified because of their abundant expression of myeloperoxidase were negative in terms of expression of BAFF (data not shown). On the contrary, we found an undeniable localization of the cytokine in monocytes/macrophages, identified as CD68$^+$ cells (Fig. 2C).

In accordance with the previous data, we found that the exposure of MDMs to *H. pylori* induced a time-dependent extracellular accumulation of BAFF. Notably, monocytes, which also express CD68, release significantly less BAFF than MDMs upon *H. pylori* stimulation (Fig. 3A). Because LPS is capable of inducing BAFF expression in APCs (26), we addressed the role of the endotoxin in the induction of BAFF by *H. pylori*. The receptor involved in the detection of *H. pylori* LPS remains a matter of debate: indeed, although several studies have implicated the classical LPS sensor TLR4, others support the idea that TLR2 is the main receptor of

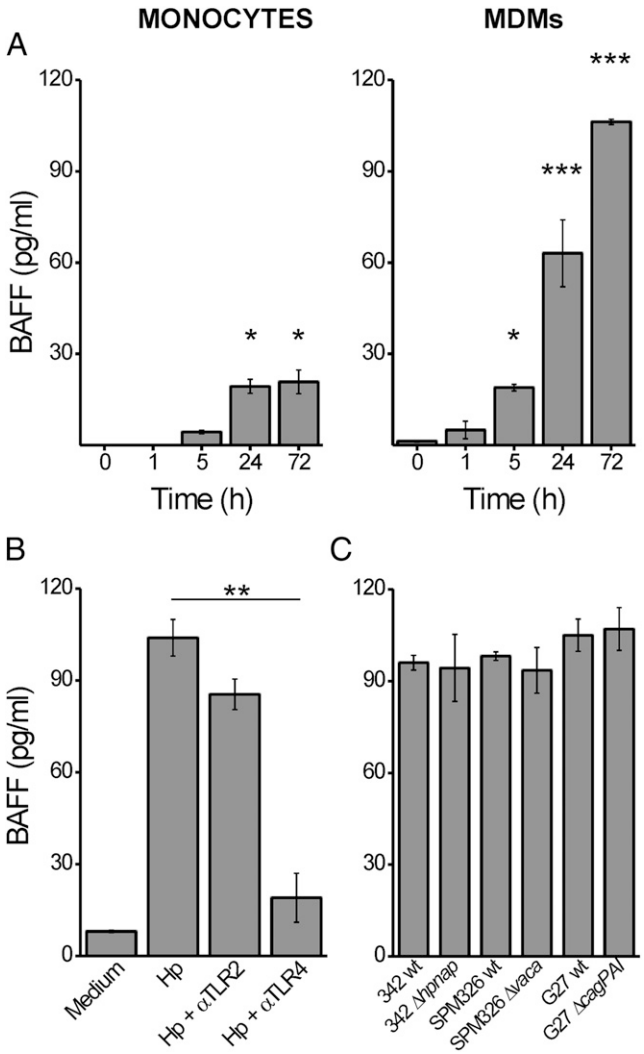

FIGURE 3. BAFF production by monocytes and MDMs exposed to *H. pylori*. (**A**) Cells were infected with *H. pylori*, 10^6 CFU/ml (MOI = 0.5), and at the indicated time points culture supernatants were collected for BAFF content determination. *$p < 0.05$, ***$p < 0.001$ versus time 0. (**B**) MDMs were preincubated with the indicated Abs before the exposure to *H. pylori*; after 72 h, supernatants were collected for BAFF content determination. **$p < 0.01$. (**C**) MDMs were infected with wt or mutant strains of *H. pylori*; after 72 h, supernatants were collected for BAFF content determination. Values are expressed as means ± SD of duplicate determinations of four separate experiments.

H. pylori LPS (27). Therefore, we evaluated the release of BAFF by macrophages exposed to *H. pylori* in the presence of either a TLR2 or a TLR4 blocking Ab. Results shown in Fig. 3B imply an almost exclusive contribution by a ligand of TLR4; however, because TLR4 (as well as TLR2) participate in the detection of other non–LPS-related PAMPs of *H. pylori* (28), we cannot exclude that ligands other than LPS can be involved. Seeking for other virulence factor(s) responsible for BAFF secretion, we finally compared the prosecretory activity of wt bacteria with that of isogenic strains deficient for the toxin VacA, for the immune modulant Ag HP-NAP (29), or lacking the entire Cag pathogenicity island. Data depicted in Fig. 3C do not reveal any consistent difference in the secretion of BAFF among the strains.

H. pylori *increase the cAMP level and activate PKA and CREB signaling in MDMs*

The signaling pathways that lead to BAFF expression involve either the cAMP/PKA/CREB or the Jak/Stat axis, depending on the stimuli (30, 31). Seeking for the pathway triggered by *H. pylori*, we measured the intracellular cAMP levels in macrophages incubated with bacteria. The infection of the cells led to an increase of the cAMP level (Fig. 4A). cAMP regulates many physiological processes via the activation of PKA that phosphorylates CREB on serine 133. We examined the PKA activity in macrophages infected with *H. pylori*, and we revealed the phosphorylation of numerous proteins at a consensus PKA phosphorylation site, reduced when cells were pretreated with the PKA inhibitor H89 (Fig. 4B). In accordance with these data, the infection with bacteria induced the phosphorylation of CREB, and this event was prevented by H89 (Fig. 4B). To finally demonstrate that the cAMP/PKA/CREB axis activated by *H. pylori* was determinant in the BAFF production, we quantified the cytokine release by infected MDMs in presence or absence of H89 and of the Jak inhibitor AG490. Results shown in Fig. 4C indicate that the *H. pylori*–induced release of BAFF relies on the cAMP-triggered pathway without any involvement of the Jak/Stat pathway.

BAFF promotes the release of pro-Th17 cytokines by monocytes

Although BAFF receptors are mainly expressed by B cells (32), it is emerging that the role of the cytokine is not strictly limited to the B cell biology. For example, the expression of one of the receptors, BAFF-R, is upregulated by T cells after activation (33). Moreover, the administration of BAFF to human monocytes enhances their survival, promotes their activation, in terms of release of proinflammatory cytokines, and leads to their differentiation into macrophage-like cells with a progressive expression of the other BAFF receptor transmembrane activator and CAML interactor (TACI) (34). Because chronic *H. pylori*–associated gastritis is mainly accompanied by monocyte infiltration, in addition to lymphocyte infiltration, we were interested to know whether the exposure of monocytes to BAFF could lead to the release of pro-Th17 cytokines. The release of IL-1β and IL-6 by monocytes, after BAFF administration, was already shown (34), but the possibility that BAFF promotes also the secretion of the other two cytokines, which are crucial in generating Th17 cells, namely, IL-23 and TGF-β, has never been considered. Freshly isolated human monocytes were exposed to BAFF, and the supernatant was harvested at different time intervals for determining the concentration of the single cytokines by ELISA. We found that BAFF triggered the secretion and the time-dependent accumulation of IL-1β and IL-6 in the culture supernatant of monocytes (Fig. 5), even at a concentration of BAFF lower than that applied in the previous study. In addition, we found that BAFF triggered the secretion of IL-23 and TGF-β (Fig. 5). We also verified that BAFF induced the expression of all the cytokines at the mRNA level (data not shown).

We did not exclude the possibility that BAFF, secreted by macrophages, could act in an autocrine way on the same cells. However, data obtained by exposing MDMs to BAFF suggested that in vivo the contribution of macrophages in creating a pro-Th17–enriched milieu is minimal. Moreover, the significant reduction of cytokine secretion by monocytes, in the presence of the BAFF neutralizing Ab, enables the exclusion of any putative contamination of the commercial cytokine by LPS (Fig. 5).

Interestingly, we found that *H. pylori* was able per se to promote the release of pro-Th17 cytokines by monocytes, whereas the effect on MDMs was scanty; to some degree, BAFF contributes to this effect, as documented by the partial inhibition observed in presence of the blocking Ab (Fig. 6). It is notable that the amount of IL-1β released by monocytes exposed to BAFF is higher than that released upon the infection of the same cells with *H. pylori* (compare Fig. 5 with Fig. 6); considering the importance of IL-1β

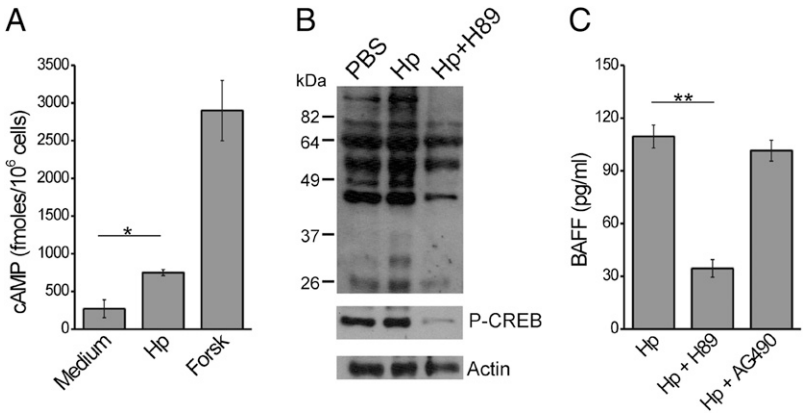

FIGURE 4. cAMP production and PKA/CREB activation in MDMs exposed to *H. pylori*. (**A**) MDMs were infected 2 h with *H. pylori*, exposed 30 min to 20 μM forskolin, or left unstimulated before proceeding with the quantification of cAMP. (**B**) Immunoblot analysis of the phosphorylation state of PKA substrates and of CREB in postnuclear supernatants of MDMs cultured 2 h with *H. pylori*. Where indicated, macrophages were pretreated for 1 h with 10 μM H89. Filter was stripped and reprobed with anti-actin Ab. (**C**) MDMs were treated for 1 h with 10 μM H89 or 25 μM AG490 before being infected with *H. pylori*. After 72 h, supernatants were collected for BAFF content determination. Values are expressed as means ± SD of duplicate determinations of three separate experiments. $*p < 0.05$, $**p < 0.01$.

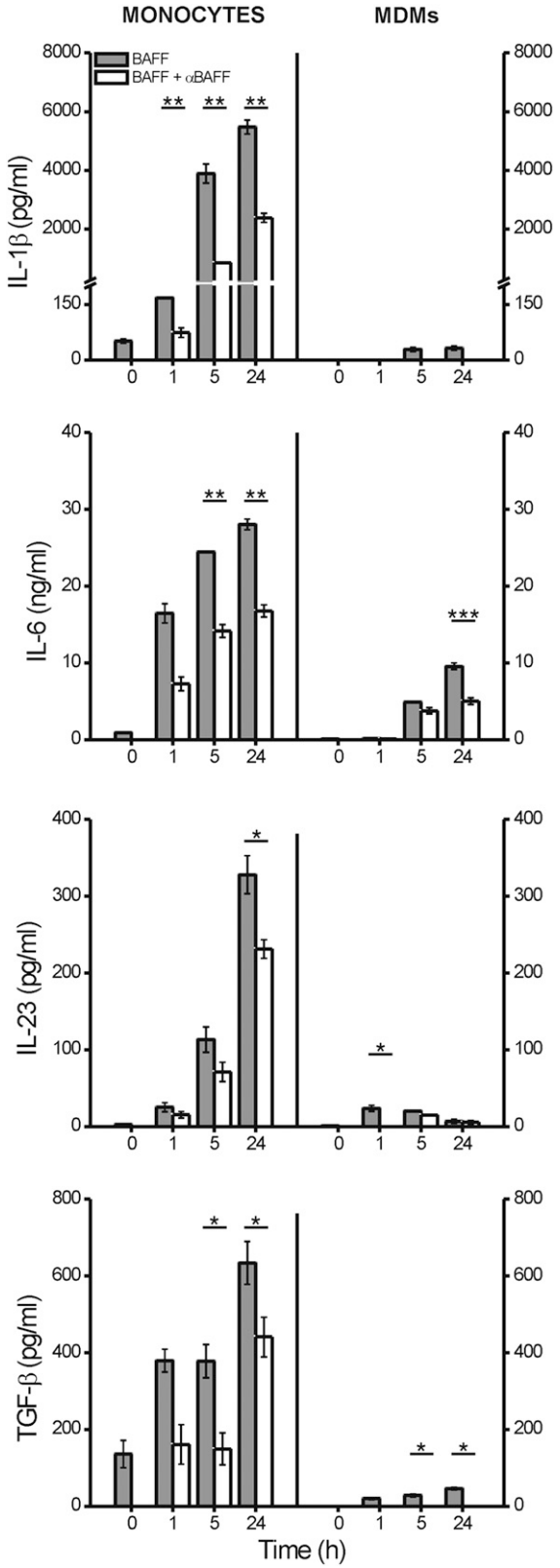

FIGURE 5. Administration of BAFF to monocytes promotes the secretion of pro-Th17 cytokines. Monocytes and MDMs were exposed to rBAFF (50 ng/ml) in the presence or absence of BAFF blocking Ab (1 μg/ml). At the indicated time points, culture supernatants were collected for cytokine content determination. Values are expressed as means ± SD of duplicate determinations of four separate experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

in the human Th17 polarization (35), it is plausible that the role of BAFF in creating a pro-Th17 cytokine milieu especially pertains to IL-1β.

Notably, once monocytes were incubated with the conditioned medium of MDMs infected with *H. pylori*, they release pro-Th17 cytokines, whereas in the presence of the anti-BAFF Ab, the secretion dropped (Supplemental Fig. 2). Collectively, these results add support to the conclusion that *H. pylori* and the cytokine BAFF, released by *H. pylori*–infected macrophages, cooperate in activating monocytes to generate the cytokine mixture required for the differentiation of Th17 cells.

BAFF induces monocytes to produce ROS, which are essential for the production of pro-Th17 cytokines

ROS is a collective term to describe the chemical species formed by incomplete reduction of oxygen, such as superoxide anion, hydrogen peroxide, and the hydroxyl radical. ROS are involved in many signaling pathways leading to many different functions including inflammation and immune response. Accordingly, expression of several genes encoding for cytokines are under the control of transcription factors activated by ROS. We started by verifying whether BAFF triggered ROS formation in monocytes. To this aim, we took advantage of the well-known tracer H_2DCF-DA (36). Cells loaded with the tracer were exposed to BAFF, and the fluorescence of the intracellularly cleaved and oxidized form of the tracer, DCF, was monitored. Considering the fluorescence of DCF proportional to the amount of ROS inside the cells, we found that the administration of BAFF led to ROS accumulation in monocytes (Fig. 7A, 7C). The kinetic of ROS production revealed that this peaked after 2- to 3-h incubation with BAFF and, notably, it was consistent with the kinetics of the pro-Th17 cytokine gene expression (data not shown). If BAFF was preincubated with the blocking Ab before being administrated to monocytes, the effect was significantly reduced. Similarly, the treatment of monocytes with DPI, a competitive inhibitor of flavin-containing cofactors that prevents ROS production by NAD(P)H oxidase, NO synthase, and mitochondrial complex I (37–39), strongly impaired BAFF-induced ROS accumulation (Fig. 7A, 7C). Exposure of MDMs to BAFF resulted in a small production of ROS, supporting once more the idea that BAFF does not act in an autocrine way on the same cells that produce the cytokine (Fig. 7B, 7D).

ROS production was observed also when monocytes, but not MDMs, were infected with *H. pylori* (Supplemental Fig. 3). However, in this case, although the effect on monocytes was abrogated by DPI, it remained unaffected by the anti-BAFF Ab. These results suggest that *H. pylori* leads to ROS production in monocytes per se, independently of BAFF.

Interestingly, when monocytes and MDMs, independently of the stimulus, were preincubated with DPI, the secretion of pro-Th17 cytokines was almost completely abrogated (Fig. 8 and data not shown), in accordance with the impact that ROS have on cytokine expression (40). The activation of the ROS-independent nonclassical pathway of NF-κB, by BAFF, may account for the residual cytokine secretion measured in the presence of DPI.

BAFF promotes the differentiation of Th17 cells by acting directly on proliferating CD4$^+$ cells

To assess the impact of BAFF on human Th cell polarization, we exposed enriched human CD4$^+$ T cells from healthy donors to BAFF and primed them by TCR/CD3/CD28 cross-linking using immobilized anti-CD3 and anti-CD28 mAbs. After 10 d, cells were washed and restimulated for 48 h using anti-CD3 mAb in ELISPOT microplates coated with anti–IL-17 Ab. The number of IL-17–forming cells was determined by ELISPOT analysis of

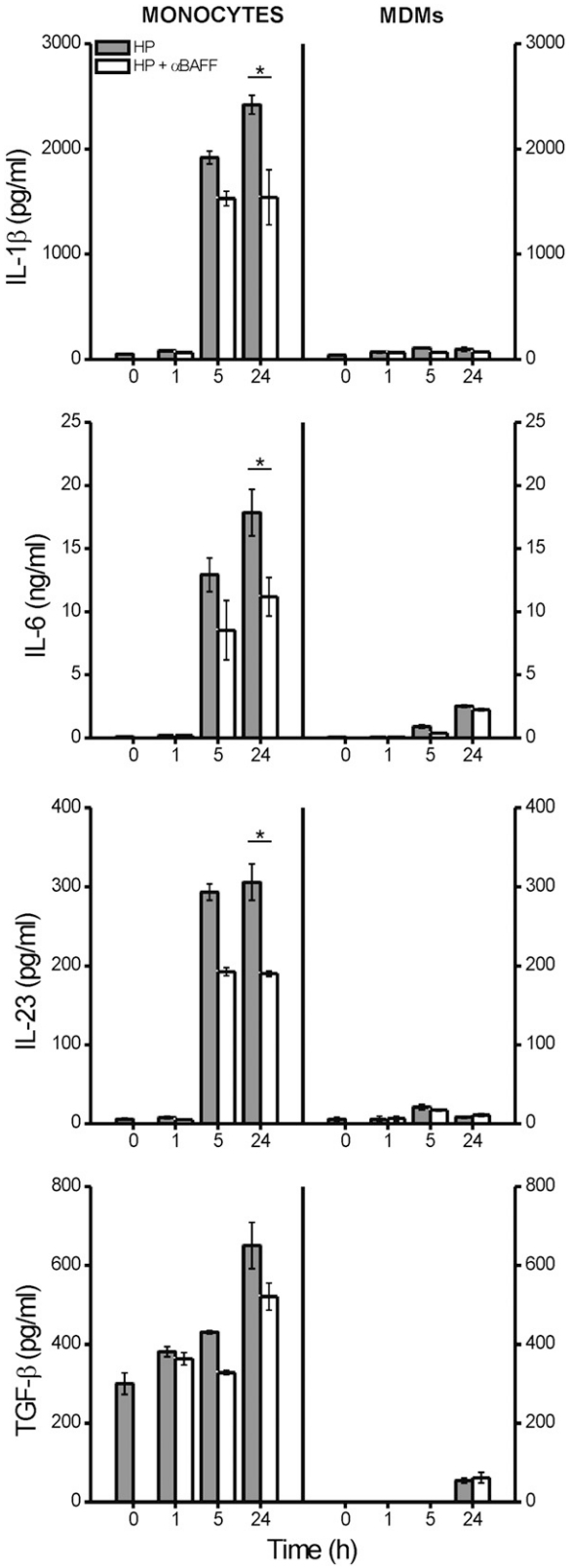

FIGURE 6. Infection of monocytes and MDMs with *H. pylori* promotes the secretion of pro-Th17 cytokines. Monocytes and MDMs were exposed to *H. pylori* (MOI = 0.5) in the presence or absence of 1 μg/ml BAFF blocking Ab. At the indicated time points, culture supernatants were collected for cytokine content determination. Values are expressed as means ± SD of duplicate determinations of two separate experiments. *$p < 0.05$.

cytokine production. As shown in Fig. 9, priming of cells that had been exposed to BAFF resulted in a remarkable production of the Th17 cytokine, IL-17, suggesting that BAFF is able to promote a polarized Th17 response. No significant enhancement in IL-17 greater than the levels produced by T cells primed in neutral conditions (TCR/CD3/CD28 cross-linking alone) was observed when cells were pretreated with the anti-BAFF Ab, thus highlighting the Th17 driving activity of BAFF.

Discussion

It is well established that the human infection by *H. pylori* results in an interplay between the bacterium and the host. *H. pylori* releases several virulence factors that per se harm the gastric mucosa; moreover, in virtue of its capacity to modulate the immune response, it exacerbates the disease because of the strong inflammation evoked. Finally, despite the robust adaptive immune response, *H. pylori* survives in the host for a long time.

The question addressed by this study was how *H. pylori* leads to the development of the Th17 immune response. Different studies have demonstrated that the activation of BAFF and APRIL signal transduction pathways may contribute to both *H. pylori*–independent and *H. pylori*–dependent growth of gastric MALT lymphomas (41, 42). Our investigation stemmed from the evidence, obtained in two different models of autoimmune disease, that BAFF is a cytokine crucial in promoting the expansion of Th17 cells (11, 12). None of these two studies, however, defined how BAFF leads to the generation of Th17 cells: whereas one study suggested a direct action of BAFF on T cells (11), the other concluded that probably BAFF is an important modulator of the cytokine milieu that affects the differentiation of the specific T cell subset (12). With the aim to investigate the involvement of BAFF in the *H. pylori*–induced disease, we started analyzing the expression of the cytokine in infected patients with chronic gastritis. We found that BAFF strongly accumulated in the gastric mucosa of patients and, notably, its expression decreased after *H. pylori* ET; the evidence that, in chronic gastritis not attributable to *H. pylori* infection, the cytokine was poorly represented further supported the conclusion that the bacterium exerts a crucial role in inducing BAFF. It is notable that, in the same specimens, IL-17 showed an expression superimposable to that of BAFF, thus leading to the first conclusion of our study that an axis BAFF/Th17 exists in *H. pylori*–infected patients. When we looked for the source of BAFF in the gastric mucosa, we found that this cytokine abundantly accumulated in monocytes/macrophages and, in accordance with this observation, the infection of MDMs with living *H. pylori*, in vitro, led to the secretion of BAFF. Moreover, we found that the main *H. pylori* inducer of BAFF is a TLR4 ligand: this could be LPS and/or another PAMP, such as the protein HP0175 that promotes the secretion of IL-23 via TLR4 signaling (43). We excluded any contribution by the vacuolating cytotoxin VacA, by the immune modulatory protein HP-NAP, and by the Cag pathogenicity island. We also demonstrated that *H. pylori*–induced BAFF secretion relies on the cAMP/PKA/CREB pathway.

The second important finding of this investigation was the role of BAFF in creating a pro-Th17 cytokine milieu. The possibility that BAFF could exert its effects not only on B and T cells, but also on innate immune cells and, in particular, on monocytes, was previously considered, and it was shown that monocytes activated by BAFF secreted a significant amount of IL-6 and IL-1β, two of the cytokines crucial for leading the differentiation of Th17 cells (34). In accordance with this evidence, we found that the administration of a recombinant form of BAFF to monocytes, cells well represented in *H. pylori*–associated gastritis, resulted in the generation of the cytokine milieu required for the differentiation of Th17

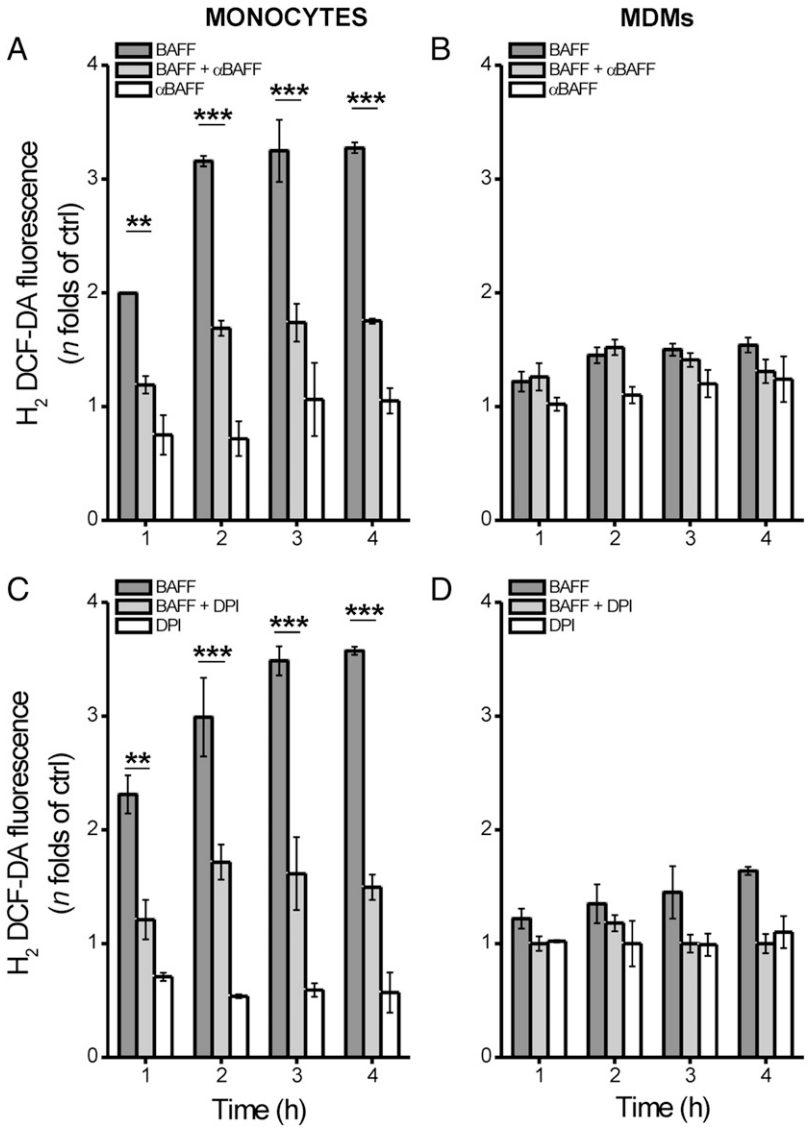

FIGURE 7. BAFF triggers ROS production in monocytes. (**A** and **B**) Monocytes and MDMs were exposed to 50 ng/ml rBAFF, in the presence or absence of 1 μg/ml BAFF blocking Ab, for different time intervals. Cells were loaded with the probe H_2DCF-DA and fluorescence was measured. (**C** and **D**) The same experiment as in (A) and (B) was performed pretreating the cells with 20 μM DPI. Data are expressed as *n*-folds relative to negative control (cells exposed to PBS). Values are expressed as means ± SD of duplicate determinations of four separate experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

cells, enriched not only in IL-6 and IL-1β, but also in TGF-β and IL-23. Similarly, the exposure of monocytes to a conditioned medium of MDMs infected with *H. pylori* also resulted in the accumulation of pro-Th17 cytokines, and this effect was significantly impaired when a neutralizing anti-BAFF Ab was present. This finding, obtained with an experimental setup, which mimics the in vivo infection, corroborates the conclusion that macrophage-secreted BAFF may lead to the development of the Th17 profile in the gastric mucosa of *H. pylori*–infected patients. Interestingly, although we found that *H. pylori* is capable per se to activate the secretion of pro-Th17 cytokines by monocytes in a BAFF-independent manner, thus suggesting a cooperation of the two mechanisms, the exposure of monocytes to BAFF resulted in a greater release of IL-1β compared with cells infected with bacteria. IL-1β is crucial in the human Th17 polarization (35); therefore, it is plausible that the role of BAFF in creating a pro-Th17 cytokine milieu especially pertains to IL-1β.

Notably, both *H. pylori* and BAFF had minimal effect on MDMs in terms of producing release of pro-Th17 cytokines, if compared with monocytes. This reduced reactivity of MDMs is not a new finding and reflects the evidence that macrophages are less sensitive to proinflammatory stimuli than monocytes, at least for what concerns lung macrophages (44, 45).

Accumulating evidence suggests that radical oxygen species (ROS), generated by inflammatory cells, not only help to kill pathogens but also act on the inflammatory cells themselves, altering the intracellular redox balance and functioning as signaling molecules involved in the regulation of inflammatory and immunomodulatory genes. Indeed, at the transcriptional level, ROS play a key role in the control of NF-κB, AP-1, and other transcription factors involved in gene expression of both inflammatory and immune mediators. In agreement with this notion, we found that BAFF and *H. pylori* trigger ROS production in monocytes, but not in MDMs, and the radicals are essential for the production of the pro-Th17 cytokines: indeed, the secretion of all the cytokines declined after the administration of DPI. Ranging the inhibitory effect of DPI from NADPH to mitochondrial complex I, we cannot precisely define the source of ROS induced by BAFF, but this is beyond the scope of this study and deserves further investigation.

The final question addressed in this study was the ability of BAFF in directly promoting the differentiation of Th17 cells, as suggested elsewhere (11). In this study, we have demonstrated that, besides indirect effect, involving innate immune cells and the generation of a peculiar cytokine milieu, BAFF is able to promote directly the development of Th17. This finding supports the con-

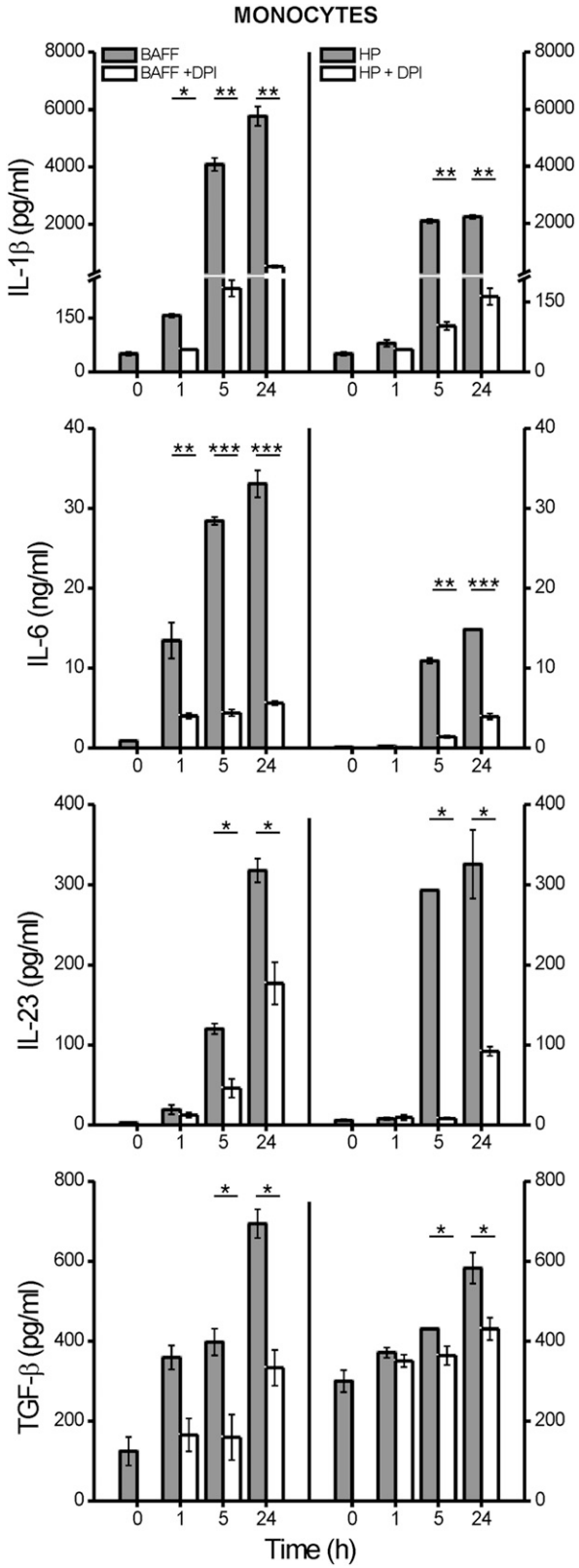

FIGURE 8. The ROS inhibitor DPI significantly impairs secretion of pro-Th17 cytokines by monocytes exposed to BAFF or *H. pylori*. Monocytes were preincubated or not with 20 μM DPI before being exposed to rBAFF (50 ng/ml) or to *H. pylori* (MOI = 0.5). At the indicated time points, culture supernatants were collected for cytokine content determination. Values are expressed as means ± SD of duplicate determinations of four separate experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

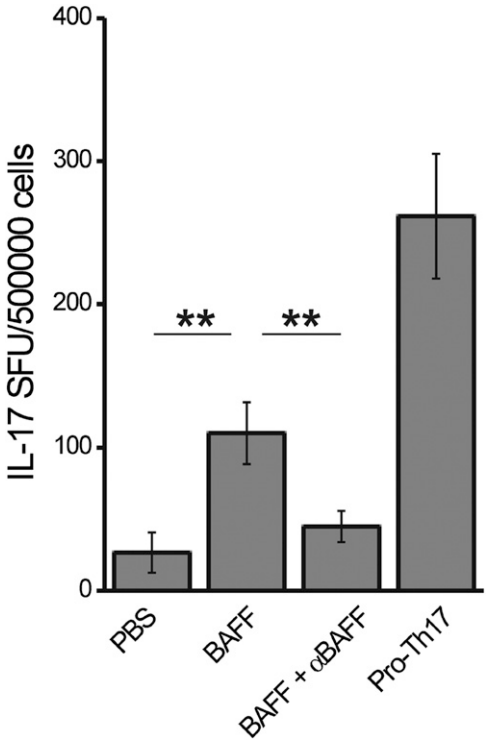

FIGURE 9. BAFF promotes Th17 cell polarization. T cells were primed by anti-CD3 and anti-CD28 mAbs (10 μg/ml) in the presence of PBS, BAFF, BAFF plus its blocking Ab or human IL-23, IL-1β, IL-6 together with neutralizing anti–IL-4 and anti–IFN-γ Abs (pro-Th17). After 10 d, cells (5×10^5) were washed, stimulated for 48 h using anti-CD3 mAb in ELISPOT microplates coated with anti-IL-17 Ab, and the number of IL-17–forming cells (SFU) were quantified by ELISPOT. Values are expressed as means ± SD of triplicate determinations of three separate experiments. $**p < 0.01$.

clusion reached by Zhou and colleagues with the study carried out in a mouse model of autoimmune encephalomyelitis (11).

In summary, our observations document the existence of a BAFF/Th17 axis in *H. pylori*–associated chronic gastritis, and they pointed out the role of the bacterium in creating this specific immune scenario. In contrast, the fact that the receptors for BAFF are expressed on other T cell subsets and the evidence that this cytokine contributes to their differentiation (46) enable us to speculate that in the gastric milieu of *H. pylori*–infected patients, BAFF might also cooperate in the differentiation of Th1 and regulatory T cells, the two lymphocyte subsets that together with Th17 are well represented in the stomach of the patients (47–49).

Disclosures

The authors have no financial conflicts of interest.

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Supplementary Table I. Gastritis grading distribution among the considered series*.

#	Normal gastric mucosa from dyspeptic patients	Hp+ gastritis	Post Hp-eradication	Hp- gastritis
1	0	2	1	1
2	0	2	0	1
3	0	2	1	1
4	0	1	1	1
5	0	2	1	1
6	0	2	0	1
7	0	2	1	1
8	0	1	0	1
9	0	2	0	1
10	0	2	0	1

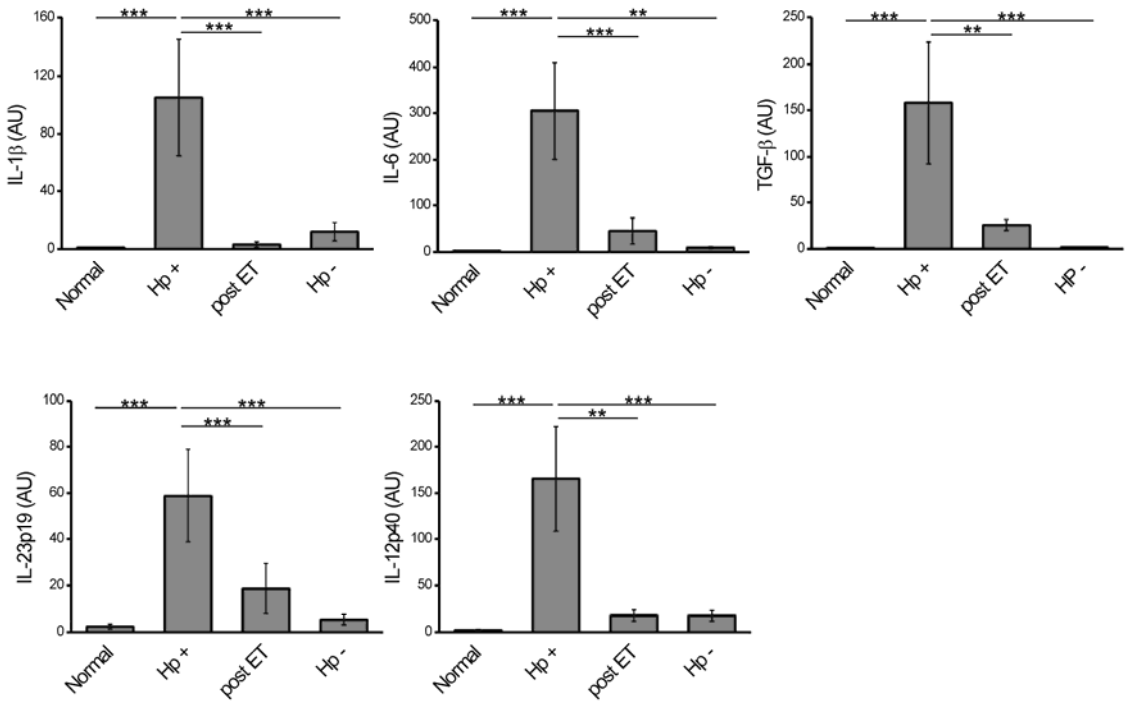
*Only samples from gastric antrum were selected and used in the study. Grading is defined as the measure of the severity of the mononuclear inflammatory lesions. Normal gastric mucosa contains only individual scattered chronic inflammatory cells in the lamina propria. Thus, any increase indicates chronic gastritis. The semi-quantitative assessment of the severity of mononuclear inflammation was graded in a three-tier scale:

Score 0= no increase in comparison to normal gastric mucosa

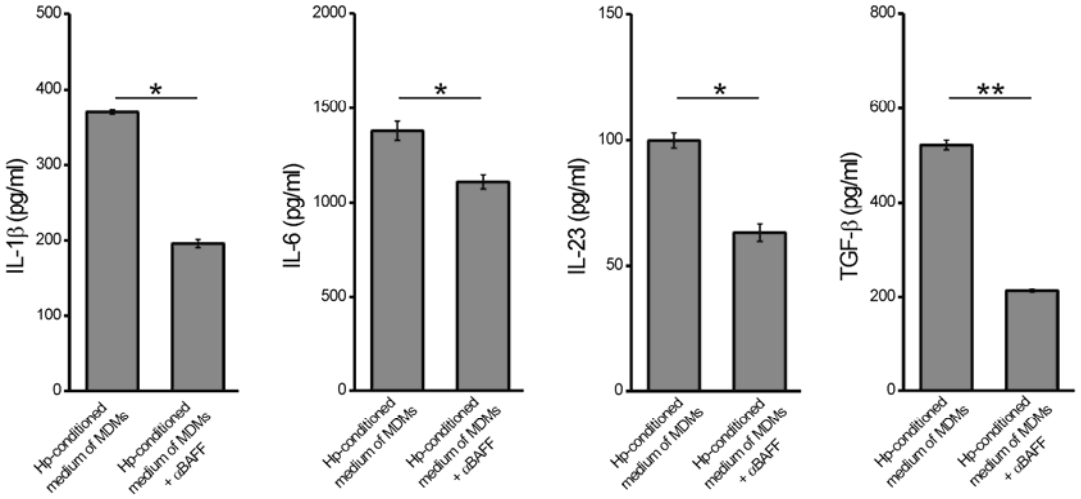
Score 1= slight increase of mononuclear cells in the lamina propria

Score 2= moderate increase without evident follicles or lymphoid nodules

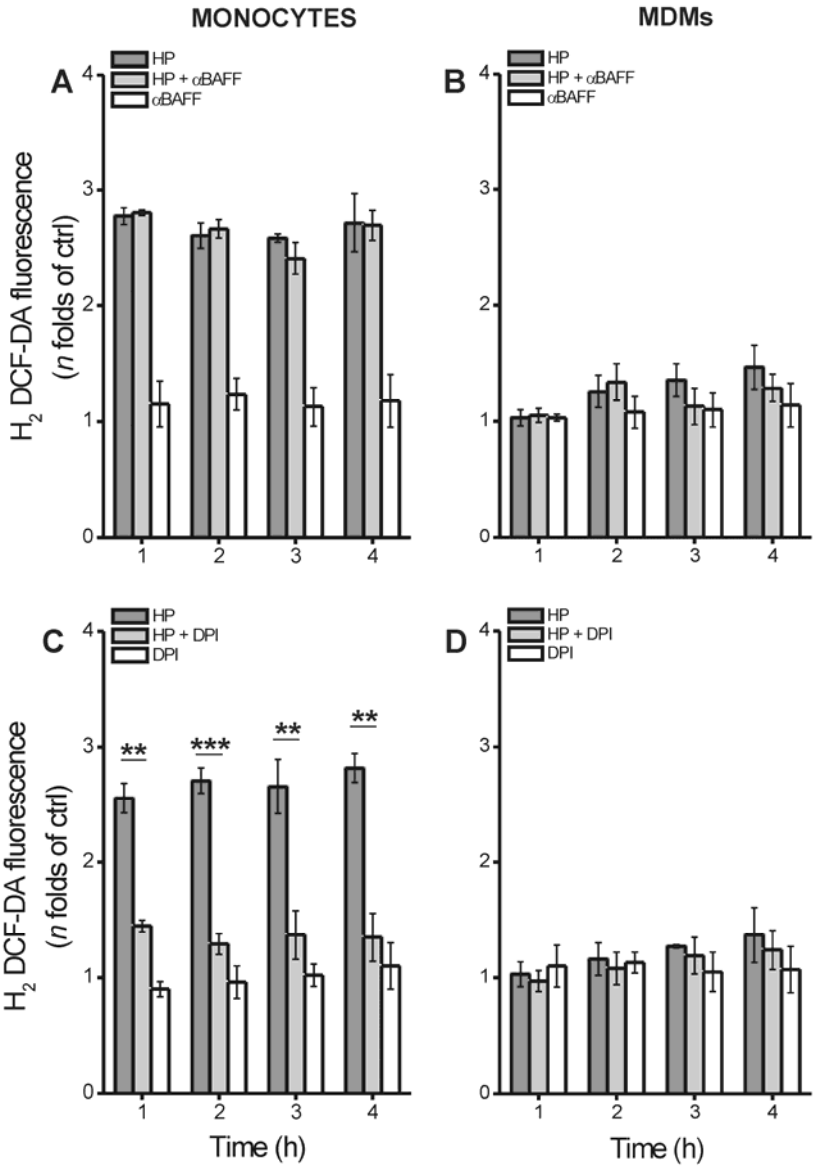
Score 3= marked increase and/or follicles or lymphoid nodules within the lamina propria.



Supplementary Figure 1. Expression of pro-Th17 cytokines in dyspeptic patients with normal mucosa, Hp+ patients with chronic gastritis pre and post eradication (post ET) and Hp- patients with chronic gastritis. mRNA was extracted from gastric biopsies and retro-transcribed. Expression of IL-1β, IL-6, TGF-β, IL-23p19 and IL-12p40 genes was evaluated as detailed in Materials and Methods section. (n = 10 for each group of patients). Error bars = S.D. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.



Supplementary Figure 2. BAFF secreted by Hp-infected MDMs promotes the release of pro-Th17 cytokines by monocytes. MDMs were infected by Hp (MOI = 0.5) for 72 h; collected supernatants were applied to freshly isolated monocytes in presence or absence of the anti-BAFF blocking antibody. After 96 h the amount of the pro-Th17 cytokines secreted was quantified by ELISA. Values are expressed as means ± SD of duplicate determinations of three separate experiments. *, $p < 0.05$, **, $p < 0.01$.



Supplementary Figure 3: Hp triggers ROS production in monocytes. (**A**, **B**) Monocytes and MDMs were exposed to Hp (MOI = 0.5), in presence or absence of 1 μg/ml BAFF blocking antibody for different time intervals. Cells were loaded with the probe H_2DCF-DA and fluorescence was measured. (**C**, **D**) The same experiment as in A and B was performed pre-treating the cells with 20 μM DPI. Data are expressed as *n*-folds relative to negative control (cells exposed to PBS). Values are expressed as means ± SD of duplicate determinations of four separate experiments. **, $p < 0.01$, *** $p<0.001$.