Inflammatory cytokine and microRNA responses of primary human dendritic cells cultured with *Helicobacter pylori* strains

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Primary human dendritic cells (DC) were used to explore the inflammatory effectors, including cytokines and microRNAs, regulated by *Helicobacter pylori*. In a 48 h ex-vivo co-culture system, both *H. pylori* B38 and B45 strains activated human DCs and promoted a strong inflammatory response characterized by the early production of pro-inflammatory TNFα and IL-6 cytokines, followed by IL-10, IL-1β, and IL-23 secretion. IL-23 was the only cytokine dependent on the cag pathogenicity island status of the bacterial strains. DC activation and cytokine production were accompanied by an early miR-146a upregulation followed by a strong miR-155 induction, which mainly controlled TNFα production. These results pave the way for further investigations into the nature of *H. pylori* antigens and the subsequently activated signaling pathways involved in the inflammatory response to *H. pylori* infection, the deregulation of which may likely contribute to gastric lymphomagenesis.

**Keywords:** *Helicobacter pylori*, dendritic cell, inflammation, TNFα, microRNA

**INTRODUCTION**

*Helicobacter pylori* infection causes a chronic gastric mucosal inflammation, leading to peptic ulcer disease in 5–10% of the infected people, and to gastric adenocarcinoma and low-grade mucosa-associated lymphoid tissue (MALT) lymphoma in 1% of the cases. The pathogenesis of *H. pylori* infection has been linked to the strength of inflammation it promotes, which is correlated to the functionality of the cag pathogenicity island (PAI) in the bacteria (Censini et al., 1996). Gastric epithelial cells constitute the first line of defense against *H. pylori*; they produce interleukin (IL)-8, which promotes the recruitment of polymuclear cells. Immune cells including macrophages, dendritic cells (DC) and mucosa infiltrating lymphocytes take part in the innate and adaptive immune responses to the bacteria.

The pro-inflammatory properties of *H. pylori* strains have been mainly evaluated by measuring IL-8 production of the AGS gastric epithelial cell line, but they deserve to be investigated on immune cells due to their orchestrated pro- and anti-inflammatory cytokine production in response to pathogens. In vivo the professional antigen-presenting DC were found to be recruited into the gastric mucosa and, due to the emission of dendrites inserting themselves between epithelial cell tight junctions, they may be able to interact with *H. pylori* (Rescigno et al., 2008; Shiu and Blanchard, 2013). Their role in *H. pylori*-induced inflammatory response needs to be clarified.

Host defense against pathogens requires the induction of appropriate innate immune responses, as excessive or inappropriate activation of the immune system can be deleterious to the organism. Therefore, various immune regulators, including microRNAs (miRNA), also take part in the immune responses (Baltimore et al., 2008). MiRNA received considerable attention because of their implication in maintaining homeostasis in fundamental biological processes in non-pathological states, and their deregulation in pathological states (Sonkoly and Pivarcsi, 2009). Changes in miRNA expression in response to bacterial infection have been reported, including *H. pylori* infection in the gastric mucosa, in gastric epithelial cells and in immune cells (Zhang et al., 2008; Fassi Fehri et al., 2010; Belair et al., 2011; Matsushima et al., 2011).

In the present study, primary human DCs were used to explore the inflammatory effectors induced by *H. pylori* strains. Two fully sequenced *H. pylori* strains were included for this purpose, i.e., strains B38 (Thiberge et al., 2010) and B45 (Lehours et al., 2011) which are cagPAI negative and cagPAI positive, respectively. In parallel to cytokine production, miRNA changes induced by *H. pylori* were analyzed; they included measuring miR-146 and miR-155, to which specific immunomodulatory functions in *H. pylori* infection were assigned after performing loss of function experiments.
MATERIALS AND METHODS

ETHICS STATEMENT
Written consent of hemochromatosis patients was obtained and approved by the French Ministry of Research and the French Aquitaine Limousin Blood Bank’s (Bordeaux, France) ethics committee (approval number DC-2012-1648).

In vitro GENERATION OF DCs
All tissue culture reagents were purchased at Invitrogen (Marly Le Roi, France). Peripheral blood mononuclear cells (PBMC) from hemochromatosis patients were isolated by centrifugation on a Ficoll gradient and captured using magnetic CD14 microbeads (Miltenyi Biotec, Paris, France), according to the manufacturer’s protocol. To generate immature DCs, monocytes were grown for 7 days in the presence of GM-CSF (50 ng/ml) and IL-4 (25 ng/ml) in RPMI-1640 medium supplemented with 10% heat-decomplemented fetal calf serum, 2 mM of L-glutamine, and 50 μg/ml of vancomycin (Sandoz, Levallois Perret, France) at 37°C in a 5% CO₂ atmosphere.

CULTURE OF H. pylori STRAINS
B38 is a cagPAI negative strain, harboring the vacA s2(i2)m2 allele. B45 is a cagPAI positive strain, harboring the vacA s1(i1)m1 allele (Lehours et al., 2004b; Thiberge et al., 2010; Lehours et al., 2011). The P12 H. pylori strain or its ΔcagPAI isogenic deletion mutant (kindly provided by R. Peek, Vanderbilt University, Nashville, TN, USA) as well as 6 cagPAI positive and 6 cagPAI negative ulcer-associated strains (Lehours et al., 2004a) were also included. All strains were cultivated for 48 h at 37°C under microaerobic conditions (5% O₂) on selective agar consisting of 21.5 g of Wilkins Chalgren agar, 50 ml of human blood, 10 μg/ml of amphotericin, 10 μg/ml of vancomycin, 10 μg/ml of cefsulodin, 5 μg/ml of trimethoprim, and 10 μg/ml of amphotericin B.

CO-CULTURES OF IMMATURE DCs AND H. pylori STRAINS
Immature DCs were washed once in PBS and plated onto 24-well plastic plates at a density of 5.10⁵ cells per well in 1 ml of RPMI-1640 growth medium. Bacteria were recovered from the agar plates using a swab and resuspended in RPMI-1640 growth medium at an optical density of 0.6 at 600 nm, which corresponds to 3.10⁷ CFU/ml. The bacteria were added to the DCs at the indicated multiplicity of infection (MOI) 1 and the cocultures were further incubated at 37°C in a 5% CO₂ atmosphere for 48 h.

DENDRITIC CELL ACTIVATION AND CYTOKINE ANALYSIS
The DC surface activation markers CD40, CD80, CD83, CD86, and HLA-DR, as well as the following secreted cytokines, IL-2, IL-4, IL-6, IL-10, and TNFα, were evaluated by flow cytometry using the Human Cytokine Bead Array Th1/Th2/Th17 Kit (Becton Dickinson, Le Pont de Claix, France). IL-8, IL-12, IL-1β, and IL-23 production was assessed by ELISA (ReadySET-Go, eBioscience, San Diego, CA, USA). DCs alone or stimulated with Escherichia coli lipopolysaccharide (LPS) (100 ng/ml) (Sigma Aldrich, St Quentin Fallavier, France) were used as negative or positive controls for DC activation, respectively.

DENDRITIC CELL APOPTOSIS
The occurrence of DC apoptosis was assessed using annexin V staining and propidium iodide incorporation. DCs were centrifuged at 2000 rpm for 10 min at 4°C, washed once in cold PBS and resuspended in 25 μL of 0.01 M HEPES (pH 7.4), 0.14 M NaCl, and 0.25 mM CaCl₂. The cells were labeled with 2.5 μL of anti-annexin V antibody (eBioscience) and 1 μL of propidium iodide (1 mg/mL) (Sigma Aldrich) for 15 min at 4°C and analyzed by flow cytometry.

OLIGONUCLEOTIDE TRANSFECTION
Transfections were performed in 24-well plates using lipofectamin 2000 (Invitrogen) according to manufacturer’s instructions, except that 5 μl/well were used. DCs were transfected twice (at day −2 and then 2 h before infection) with 100 nM of antisense miRNA (as155 or as146) or scrambled locked-nucleic-acid (LNA)-modified oligonucleotides (Table A1).

DATA EXTRACTION AND miRNA QUANTITATIVE RT-PCR
DCs were collected in Eppendorf tubes and pelleted by centrifugation at 2000 rpm for 5 min at room temperature. Total RNA was extracted from the DC pellet using a Trizol reagent (Invitrogen), according to manufacturer’s instructions. RNA concentrations were determined by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For miRNA quantitations, 250 ng of total RNA were retrotranscribed using the miScript Reverse Transcription kit (Qiagen, Courtaboeuf, France) and qPCR was performed using a SYBr Green PCR kit and hsa-miR-155, hsa-miR-146a and SNOR25 specific primers (Qiagen), according to the manufacturer’s instructions. The amplification profiles were measured on a Stratagene Mx3005P instrument (Life Technologies, Saint Aubin, France).

STATISTICAL ANALYSIS
Data were expressed as a ratio of fold induction compared to non-activated DCs. For each experiment, all experimental conditions were tested three times and each experiment was conducted at least three times for each strain. Statistical analyses were done using the non-parametric Mann-Whitney test. A p-value of less than 0.05 was considered significant. All statistics were performed using SPSS 16.0F for Windows software (SPSS Inc., Chicago, IL, USA).

RESULTS
DENDRITIC CELL ACTIVATION BY H. pylori
The nature, the amount and the duration of the antigenic stimulation are important factors that can influence the inflammatory and molecular responses of DCs. Before infection, the immature DC phenotype (CD1a⁺, HLA-DR⁺, CD80⁻, CD86⁻, CD14⁻, CD83⁻) was verified by flow cytometry (Figure A1). Our preliminary experiments showed that H. pylori at MOI 1 significantly activated DCs (Figure A2), with minimal apoptosis (less than 10%) (Figure A3).
CYTOKINE PRODUCTION IN *H. pylori*-ACTIVATED DCs

DCs activated by either the B38 or B45 strain at MOI 1 or by *E. coli* LPS secreted significant amounts of TNFα, IL-1β, IL-6, IL-8, IL-10, IL-12, and IL-23, but not IL-2, IL-4, IL-17, TGFβ, or IFNγ compared to non-activated DCs. *H. pylori*-activated DCs produced mainly TNFα (1500–2000 fold stimulated production over basal level), and in a decreasing order of stimulation, IL-6 (1000-fold), IL-10 (200–400 fold), IL-1β (150-fold), IL-23 (50–100 fold), IL-8 (10-fold) and IL-12 (5-fold) after 48 h of co-culture. The cytokine levels were low or nil in non-activated DC supernatants, except for IL-8 and IL-12 which were already secreted under basal conditions.

*H. pylori*-activated DCs produced the same cytokines as LPS-activated DCs, both qualitatively and quantitatively, except for IL-10 (Figure 1). This potent anti-inflammatory molecule was found at a significantly higher level upon stimulation by both *H. pylori* B38 and B45 than by LPS. Moreover, compared to *E. coli* LPS, IL-10 production was significantly induced by *H. pylori* B38 strain as early as 6 h.

Analysis of cytokine production kinetics in *H. pylori*-activated DCs revealed the early, massive production of TNFα (Figure 1). TNFα reached its maximal level as early as 6 h in the presence of LPS or the bacteria, and remained quite stable thereafter. IL-6 was maximally stimulated by *E. coli* LPS as early as 6 h, and progressively with *H. pylori* (Figure 1).

There was generally no significant difference in the cytokine production between the two *H. pylori* strains, except for IL-23, a major cytokine involved in Th17 response, which was higher with B45 than with B38 (Figure 1). This difference could be attributed to the presence of the capPAI in the B45 strain. To test this hypothesis, DCs were activated for 48 h by either *H. pylori* P12 strain or its isogenic capPAI deletion mutant (Figure 1), and by 12 more *H. pylori* strains tested individually (6 capPAI positive and 6 capPAI negative) from our collection (Lehours et al., 2004a) (Figure 1). IL-23 secretion was significantly lower with the P12 mutant and the capPAI negative strains than with the P12 wild type and the capPAI positive strains confirming the positive influence of the capPAI gene products on the secretion of this cytokine. IL-23 secretion started rather late following *H. pylori* infection, at 48 h.

**FIGURE 1** | Cytokine production in *H. pylori*-activated dendritic cells. (A) Kinetics of TNFα, IL-6, and IL-10 secretion by *E. coli* LPS- or *H. pylori*-activated dendritic cells. DCs were either stimulated with *E. coli* LPS (100 ng/ml) or cocultivated with B38 *H. pylori* strain at MOI 1 for 6, 24, or 48 h. TNFα, IL-6, and IL-10 secretion in the culture supernatants was evaluated by flow cytometry (as described in Materials and Methods). The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values of fold induction ratio vs. DCs alone obtained for 6 independent experiments corresponding to 6 different donors. These results are representative of the results obtained for *H. pylori* strain B45 for the same experiments. *for p < 0.05 and NS for p > 0.05 compared to *E. coli* LPS at the corresponding time-point. (B) Influence of the *H. pylori* capPAI on the production of IL23 by dendritic cells after *H. pylori* infection. (1) DCs were cocultured with B38 or B45 *H. pylori* strain at MOI 1 for 6, 24, or 48 h. IL-23 levels in culture supernatants were measured by ELISA. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values of fold induction ratio vs. non-activated DCs, for 6 independent experiments for each strain and for 6 different donors. *for p < 0.05 between the *H. pylori* strains B38 and B45 at the corresponding time-point. (2) DCs were cocultured for 48 h with *H. pylori* strain P12 and its isogenic capPAI mutant (KO-P12) at MOI 1 or (3) with 12 ulcer-associated *H. pylori* strains tested individually (6 capPAI positive and 6 capPAI negative). IL-23 levels in culture supernatants were measured by ELISA. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values of fold induction ratio vs. non-activated DCs, for 3 independent experiments for each group of strains and for three different donors (*if p < 0.05).
for B38 and 24 h for B45 (Figure 1). Finally, both LPS- and \textit{H. pylori}-mediated activation displayed similar patterns of progressive production of IL-1\(\beta\), IL-8, and IL-12 over the time course (Figure A4).

**EXPRESSION OF miRNA IN \textit{H. pylori}-ACTIVATED DCs**

MiRNAs, differentially regulated in \textit{H. pylori}-activated DCs as compared to immature DCs, were investigated using a qPCR array of 88 miRNA involved in human immunopathology. Among the screened miRNAs, 12 showed the highest induction levels (3–21 fold) and 13 the strongest repression (3–6 fold) (Table A1). Nevertheless, taking into account only those that were expressed the most upon induction (over 1/100 of average housekeeping genes), we identified miR-187, miR-155, miR-146a, let-7e, miR-29b, miR-34, miR-214, and miR-147 as being substantially upregulated following \textit{H. pylori} infection. The relatively abundant miR-152, miR-195, miR-16, miR-30c, miR-223, miR-126, miR-574-3p, miR-21, and miR125b were found to be the most repressed by the bacteria (Table 1).

Kinetic analyses of miR-146 and miR-155 expression in DCs co-cultured with either the B38 or the B45 strain at MOI 1 showed that both miRNAs were upregulated as early as 6 h post-infection and increased progressively thereafter (Figure 2A). Whereas miR-146a reached 50% of its maximal stimulation as early as 6 h post-infection, miR-155 was dramatically upregulated between 6 and 24 h. Thus, at 48 h post-infection, miR-146 levels were increased by 5–10 fold over the basal level, and miR-155 by 100–200 fold. The statistical analysis of 13 independent experiments showed no significant difference between the B38 and B45 strains in their ability to upregulate the miRNAs, since miR146a expression was enhanced by 4.06 ± 0.47 and 4.8 ± 0.78 fold (\(p = 0.927\)) and miR-155 by 84.03 ± 15.87 and 99.17 ± 26.94 fold (\(p = 0.224\)), respectively. This suggests that the miRNA response of \textit{H. pylori}-activated DC was independent of the strain's cagPAI status. miR-146a was upregulated more by the \textit{H. pylori} strains than by LPS, contrary to miR-155, which reached the same intensity after LPS stimulation as after co-culture with either of the \textit{H. pylori} strains (Figure 2A). At 48 h post-infection, miR-146a upregulation was positively correlated with a MOI up to 40, whereas miR-155 upregulation peaked at MOI 1–5 and decreased at higher ones (Figure 2B). The global results indicate that miR-146a and miR-155 upregulations take part in the DC immune response to \textit{H. pylori}, independently of their cagPAI status, with miR-146a upregulation preceding the massive miR-155 biosynthesis.

In order to investigate the importance of these miRNAs in the regulation of cytokine production upon infection, their mature forms were functionally inhibited using antisense oligonucleotides (or their scrambled controls), which were introduced into immature DCs using lipofectamin prior to the LPS or \textit{H. pylori} challenge. Both miR-146a and miR-155 knockdown had little influence on the LPS- or \textit{H. pylori}-mediated upregulation of DC surface activation markers, suggesting that neither one was required for this process (data not shown). Anti-miR-146 antisense oligonucleotides totally prevented LPS- or \textit{H. pylori}-triggered miR-146 upregulation (Figure 3A) and had no influence on the level of miR-155 induction, and vice versa, showing the efficiency of antisenses to inhibit miRNA expression, most likely by sequestering them. This also suggests that miR-146a and miR-155 expression may not be correlated in this cell system. Nevertheless, in response to \textit{H. pylori}, two cytokines were clearly affected by this treatment (Figure 3B). First, in comparison to scrambled oligonucleotide-treated cells, TNF\(\alpha\) production at 48 h was induced to a 50% greater extent in anti-miR-155-treated cells.

### Table 1 | miRNA differentially regulated in \textit{H. pylori} B38-activated dendritic cells.

| miRNA       | Fold change upon infection | Relative level in infected cells |
|-------------|---------------------------|---------------------------------|
| hsa-miR-187 | 21.0756                   | 0.0515                          |
| hsa-miR-155 | 13.4310                   | 2.5491                          |
| hsa-miR-198 | 76.079                    | 0.0023                          |
| hsa-miR-135b| 6.5319                    | 0.0049                          |
| hsa-miR-299-3p| 5.5693                  | 0.0020                          |
| hsa-miR-451 | 4.8821                    | 0.0052                          |
| hsa-miR-146a| 4.8149                    | 21.8566                         |
| hsa-let-7e  | 4.7816                    | 2.4284                          |
| hsa-miR-184 | 4.3094                    | 0.0016                          |
| hsa-miR-29b | 4.2797                    | 0.1216                          |
| hsa-miR-34a | 4.1339                    | 0.7738                          |
| hsa-miR-214 | 4.1626                    | 0.0110                          |
| hsa-miR-206 | 3.1987                    | 0.0046                          |
| hsa-miR-147 | 3.0898                    | 0.3276                          |
| hsa-miR-302a| 3.0262                    | 0.0015                          |

| miRNA       | Fold change upon infection | Relative level in infected cells |
|-------------|---------------------------|---------------------------------|
| hsa-miR-409-3p| 0.1728                  | 0.0904                          |
| hsa-miR-152  | 0.2070                    | 0.2897                          |
| hsa-miR-150  | 0.2113                    | 0.0333                          |
| hsa-miR-195  | 0.2584                    | 145.2606                        |
| hsa-miR-16   | 0.2675                    | 202.6014                        |
| hsa-miR-30c  | 0.3051                    | 12.6626                         |
| hsa-miR-223  | 0.3115                    | 143.2608                        |
| hsa-miR-126  | 0.3248                    | 1.6386                          |
| hsa-miR-574-3p| 0.3270                  | 1.3218                          |
| hsa-miR-21   | 0.3293                    | 13074137                        |
| hsa-miR-203  | 0.3362                    | 0.0168                          |
| hsa-miR-125b | 0.3529                    | 1.9219                          |

One \(\mu\)g of total RNA from DCs cultured for 48 h in the absence or presence of \textit{H. pylori} B38 at MOI 1 was retro-transcribed using the RT2 miRNA First Strand Kit (Qiagen, Courtaboeuf, France). The first strand reaction was mixed with RT2 SYBR Green qPCR mix and distributed onto PCR array plates containing primers for 88 miRNA involved in human immunopathology (SA Bioscience, Qiagen) according to manufacturer’s protocol. The amplification profiles were measured on a Stratagene Mx3005P instrument (Life Technologies, Saint Aubin, France). Data were analyzed by the \(C_\text{t}\) method (Livak and Schmittgen, 2001), using the average \(C_\text{t}\) of the housekeeping RNA for normalization. Data are expressed as fold induction compared to non-activated DCs in the medium lane. For each miRNA, the level relative to the mean expression of housekeeping genes is shown in the right lane, in order to assess which miRNA may be expressed at levels high enough to be relevant (highlighted).

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cells notably upon *H. pylori* infection, whereas it was weakly impaired (≤20%) by anti-miR-146. Second, anti-miR-146 treatment clearly impaired IL-10 production upon infection, whereas anti-miR-155 had no significant effect. miR-146a or miR-155 knockdown did not affect the cytokine response to LPS. This treatment did not alter IL-1β or IL-8 production and only weakly affected that of other cytokines after *H. pylori* challenge (Figure A5).

**DISCUSSION**

In this manuscript, we aimed at analyzing the initial steps of the immune response to *H. pylori* strains. We therefore developed an *ex vivo* co-culture cell system, in which we showed that *H. pylori* strains were able to activate primary human DCs and promote strong proinflammatory cytokine and miRNA responses. Although most of the observed effects were independent of the *H. pylori* cagPAI status, the cagPAI positive strain specifically upregulated IL-23, most likely to orientate DCs to promote a Th17 response.

Numerous articles have been published on the interaction between *H. pylori* and DCs, however, with a huge heterogeneity in the bacterial strains, MOI and experimental conditions (viable/sonicated/paraformaldehyde-treated bacteria) (Guiney et al., 2003; Hafsi et al., 2004; Kranzer et al., 2004, 2005; Kao et al., 2006; Mitchell et al., 2007; Rad et al., 2009; Andres et al., 2010; Kao et al., 2010; Fehlings et al., 2012). Our data were obtained using primary human DCs from several donors. All experiments were repeated with at least three different donors in order to ensure the reliability of the results. Significant productions of several cytokines (TNFα, IL-1β, IL-6, IL-8, IL-10, IL-12, and IL-23) were observed in *H. pylori*-activated DCs, TNFα and IL-6 being produced at the highest levels. *In vivo*, TNFα contributes to monocyte maturation after recruitment, whereas IL-6 supports the transition between the early stages of the infection and the sustained mononuclear influx into the infected gastric mucosa. Early production of TNFα and IL-6 upon LPS stimulation reflects the intrinsic property of *E. coli* LPS to trigger TLR4 signaling pathways, a property that is not shared by *H. pylori* LPS (Rad et al., 2009). Delayed secretion of TNFα and IL-6 by *H. pylori*-activated DCs as compared to LPS-activated ones may depend on bacterial DNA and RNA recognition by endosomal TLR9 and TLR8, respectively (Rad et al., 2009), a later process depend on bacterial DNA and RNA recognition by endosomal TLR9 and TLR8, respectively (Rad et al., 2009), a later process depend on bacterial DNA and RNA recognition by endosomal TLR9 and TLR8, respectively (Rad et al., 2009), a later process.
The role of miR-146 and miR-155 in the innate immune responses was deduced from the identification of their respective targets. As miR-146a targets and silences TNF-α receptor-associated factor and IL-1β receptor-associated kinase mRNAs, which are key adapter molecules in the TLR/ NFκB pathway, the role of miR-146a upregulation in response to pathogens was shown to moderate TLR-triggering of the NFκB pathway via a negative feedback loop, thus avoiding overproduction of pro-inflammatory IL-1β and TNFα cytokines (Taganov et al., 2006; Nahid et al., 2009; Lu et al., 2010). A similar immune regulatory function was assigned to miR-155 which targets several gene transcripts of the NF-κB pathway (FADD, Ripk1, IKK, and NIK), as well as the immune cell transcription factor PU. Src homology 2 domain-containing inositol-5-phosphatase (SHIP) and CCAAT enhancer-binding protein beta (C/EBPβ) (Rodriguez et al., 2007; Tili et al., 2007; Costinean et al., 2011), suggesting that simultaneous anti- and pro-inflammatory effects of miR-155 most likely contribute to the fine-tuning of the TNF-mediated inflammation. We assessed the role of miR-146 and miR-155 in the DC cytokine response to H. pylori using specific antisenses to each miRNA, which inhibit their silencing function by competition with their binding sites on mRNA targets. Thus, the most noticeable effect of miR-155 loss of function in our cell system was an enhanced TNFα production by H. pylori, an effect consistent with the immunomodulatory role assigned to miR-155 upregulation in response to microbial stimuli (O’Connell et al., 2007; Rodriguez et al., 2007; Ceppi et al., 2009; Martinez-Nunez et al., 2009). Enhanced TNFα response to H. pylori in the absence of miR-155 may also be facilitated by the downregulation of miR-125b which we observed in our microarray (Table 1), and which directly targets TNFα (Tili et al., 2007). In our miR-146 loss of function experiment, no cytokine response was markedly altered with the exception of IL-10. The impaired production of this anti-inflammatory cytokine in response to H. pylori is concordant with the previously established immunomodulatory role of miR-146 upon infection, but it was not accompanied by an enhanced production of inflammatory cytokines in anti-miR-146-treated cells as expected. A possible explanation for the decreased IL-10 response to H. pylori in the absence of miR-146 could be that miR-146 loss of function unMASKS the regulatory effects of other miRNAs: for instance, let-7e, which directly targets IL-10 (Schulte et al., 2011) was upregulated in H. pylori-activated DCs (Table 1) and may subsequently negatively regulate IL-10. The differences in the effects of miR-146 and miR-155 knockdown, which stress the prominent role of miR-155 in H. pylori-activated DCs, could be related to different functional specializations of these two seemingly co-induced miRNAs, as recently reported in LPS-stimulated murine macrophages (Schulte et al., 2013); miR-146 responded to sub-inflammatory stimuli to prevent TLR activation, and miR-155 responded to pro-inflammatory stimuli as a global limiter of the inflammatory response. Our experimental conditions established a TNFα-filled, pro-inflammatory environment in cultured DCs, and thus preferentially elicited miR-155 functions. miR-146a and miR-155 expressions may be of special interest in H. pylori-mediated immune pathologies, because they are associated with NFκB activation, a pathway strongly activated during H. pylori infection and in H. pylori-driven MALT lymphoma. High expression of miR-146a was associated with...
chronic inflammatory diseases (Sonkoly and Pivarczi, 2009) and miR-155 represents an important element in B cell lymphoma development (Costinean et al., 2006), indicating that alterations in the fine-tuning of innate immune responses by miRNAs may contribute to inflammatory disorders. Other miRNAs, which are differentially regulated in *H. pylori*-activated DCs, deserve further investigation; among them, miR-187, which was strongly up-regulated in the *H. pylori*-activated DCs, could control IL-10-driven anti-inflammatory responses (Rossato et al., 2012).

In conclusion, the co-culture cell system presented here allowed the identification of pro- and anti-inflammatory effectors in response to *H. pylori* and revealed the influence of the cagPAI on the immune responses. These important findings pave the way for further investigations on the nature of *H. pylori* antigens and the subsequently activated signaling pathways involved in the inflammatory response to *H. pylori* infection.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
APPENDIX

Table A1 | MiRNA microarray data in uninfected DC (control) and DCs infected for 48 h by *H. pylori* B38 strain at MOI 1.

| Mature miRNA ID | Ct control DC | Ct B38-infected | Rel value in control | Rel value in B38-infected | Fold change upon infection |
|-----------------|---------------|------------------|----------------------|---------------------------|---------------------------|
| hsa-let-7a      | 21.6          | 18.34            | 2.7368               | 4.3169                    | 1.5773                    |
| hsa-let-7c      | 29.79         | 26.4             | 0.0094               | 0.0162                    | 1.7261                    |
| hsa-let-7d      | 25.72         | 22.11            | 0.1574               | 0.3164                    | 2.0104                    |
| hsa-let-7e      | 24.03         | 19.17            | 0.5079               | 2.4284                    | 4.7816                    |
| hsa-let-7g      | 21.28         | 17.74            | 3.4165               | 6.5432                    | 1.9152                    |
| hsa-miR-103     | 20.51         | 17.97            | 5.8260               | 5.5790                    | 0.9576                    |
| hsa-miR-106a    | 19.89         | 18               | 8.9538               | 5.4642                    | 0.6103                    |
| hsa-miR-125a-5p | 19.32         | 17.27            | 13.2921              | 9.0631                    | 0.6818                    |
| hsa-miR-125b    | 22.11         | 21.01            | 1.9219               | 0.6783                    | 0.3529                    |
| hsa-miR-126     | 22.34         | 21.36            | 1.6386               | 0.5322                    | 0.3248                    |
| hsa-miR-128     | 23.57         | 22.36            | 0.6986               | 0.2661                    | 0.3809                    |
| hsa-miR-130a    | 30.73         | 27.14            | 0.0049               | 0.0097                    | 1.9827                    |
| hsa-miR-132     | 21.35         | 19.8             | 3.2546               | 1.5692                    | 0.4821                    |
| hsa-miR-135b    | 33.42         | 28.11            | 0.0008               | 0.0049                    | 6.5319                    |
| hsa-miR-138     | 31.96         | 28.63            | 0.0021               | 0.0034                    | 1.6558                    |
| hsa-miR-142-3p  | 18.17         | 15.83            | 29.4971              | 24.5900                   | 0.8336                    |
| hsa-miR-142-5p  | 21.05         | 19.47            | 4.0069               | 1.9725                    | 0.4923                    |
| hsa-miR-143     | 28.96         | 27.05            | 0.0167               | 0.0103                    | 0.6188                    |
| hsa-miR-145     | 29.57         | 27.39            | 0.0109               | 0.0081                    | 0.761                     |
| hsa-miR-146a    | 20.87         | 16               | 4.5394               | 21.8566                   | 4.8149                    |
| hsa-miR-147     | 26.29         | 22.06            | 0.1060               | 0.3276                    | 3.0898                    |
| hsa-miR-148a    | 23.17         | 21.27            | 0.9218               | 0.5664                    | 0.6145                    |
| hsa-miR-150     | 27.96         | 27.6             | 0.0333               | 0.0070                    | 0.2113                    |
| hsa-miR-152     | 24.84         | 24.51            | 0.2897               | 0.0600                    | 0.2070                    |
| hsa-miR-155     | 25.45         | 19.1             | 0.1898               | 2.5491                    | 13.4310                   |
| hsa-miR-15a     | 22.35         | 19.39            | 1.6273               | 2.0849                    | 1.2812                    |
| hsa-miR-15b     | 21.33         | 19.35            | 3.3001               | 2.1435                    | 0.6495                    |
| hsa-miR-16      | 15.39         | 14.69            | 202.6014             | 54.1917                   | 0.2675                    |
| hsa-miR-181a    | 25.92         | 22.12            | 0.1370               | 0.3143                    | 2.2934                    |
| hsa-miR-182     | 29.4          | 27.82            | 0.0123               | 0.0060                    | 0.4923                    |
| hsa-miR-183     | 32.22         | 29.45            | 0.0017               | 0.0020                    | 1.1231                    |
| hsa-miR-184     | 34.48         | 29.77            | 0.0004               | 0.0016                    | 4.3094                    |
| hsa-miR-185     | 23.65         | 21.63            | 0.6609               | 0.4414                    | 0.6678                    |
| hsa-miR-186     | 24.85         | 21.41            | 0.2877               | 0.5141                    | 1.7870                    |
| hsa-miR-187     | 31.73         | 24.73            | 0.0024               | 0.0515                    | 21.0756                   |
| hsa-miR-18a     | 21.9          | 20.46            | 2.2230               | 0.9931                    | 0.4467                    |
| hsa-miR-18b     | 28.4          | 25.7             | 0.0246               | 0.0263                    | 1.0699                    |
| hsa-miR-191     | 18.39         | 17.18            | 25.3252              | 9.6465                    | 0.3809                    |
| hsa-miR-194     | 25.03         | 23.52            | 0.2539               | 0.1191                    | 0.4689                    |
| hsa-miR-195     | 15.87         | 15.22            | 145.2606             | 37.5307                   | 0.2584                    |
| hsa-miR-196a    | 32.29         | 29.02            | 0.0017               | 0.0026                    | 1.5683                    |
| hsa-miR-198     | 34.73         | 29.2             | 0.0003               | 0.0023                    | 7.6079                    |
| hsa-miR-19a     | 18.9          | 16.6             | 17.7839              | 14.4200                   | 0.8108                    |
| hsa-miR-19b     | 19.13         | 16.88            | 15.1632              | 11.8762                   | 0.7832                    |
| hsa-miR-200a    | 31.24         | 29.64            | 0.0034               | 0.0017                    | 0.4991                    |
| hsa-miR-203     | 28.95         | 27.92            | 0.0168               | 0.0056                    | 0.3362                    |
| hsa-miR-205     | 31.25         | 30.96            | 0.0034               | 0.0007                    | 2.013                     |
| hsa-miR-206     | 32.49         | 28.21            | 0.0014               | 0.0046                    | 3.1987                    |
| hsa-miR-20a     | 19.34         | 17.42            | 13.1091              | 8.1681                    | 0.6231                    |
| hsa-miR-20b     | 20.8          | 19.06            | 4.7651               | 2.6208                    | 0.5500                    |

(Continued)
| Mature miRNA ID | Ct control DC | Ct B38-infected | Rel value in control | Rel value in B38-infected | Fold change upon infection |
|----------------|---------------|-----------------|----------------------|---------------------------|-----------------------------|
| hsa-miR-21     | 12.7          | 11.7            | 1307.4137            | 430.5390                  | 0.3293                      |
| hsa-miR-210    | 25.13         | 21.08           | 0.2369               | 0.6462                    | 2.7274                      |
| hsa-miR-214    | 31.61         | 26.95           | 0.0027               | 0.0110                    | 4.1626                      |
| hsa-miR-223    | 15.89         | 14.97           | 143.2608             | 44.6318                   | 0.3115                      |
| hsa-miR-23b    | 22.38         | 20.89           | 1.5938               | 0.7371                    | 0.4625                      |
| hsa-miR-26a    | 18.23         | 16.05           | 28.2955              | 21.1121                   | 0.7461                      |
| hsa-miR-26b    | 18.8          | 16.95           | 19.0603              | 11.3137                   | 0.5936                      |
| hsa-miR-27a    | 17.31         | 15.27           | 53.5383              | 36.2523                   | 0.6771                      |
| hsa-miR-27b    | 22.41         | 20.95           | 1.5610               | 0.7071                    | 0.4530                      |
| hsa-miR-299-3p | 34.48         | 29.4            | 0.0004               | 0.0020                    | 5.5693                      |
| hsa-miR-29b    | 28.19         | 23.49           | 0.0284               | 0.1216                    | 4.2797                      |
| hsa-miR-29c    | 23.17         | 19.64           | 0.9218               | 1.7532                    | 1.9020                      |
| hsa-miR-302a   | 34.03         | 29.83           | 0.0005               | 0.0015                    | 3.0262                      |
| hsa-miR-30b    | 19.66         | 18.3            | 10.5013              | 4.4383                    | 0.4226                      |
| hsa-miR-30c    | 19.39         | 18.5            | 12.6626              | 3.8637                    | 0.3051                      |
| hsa-miR-30e    | 22.43         | 19.82           | 1.5395               | 1.5476                    | 1.0052                      |
| hsa-miR-335    | 28.9          | 27.56           | 0.0174               | 0.0072                    | 0.4168                      |
| hsa-miR-34a    | 25.47         | 20.82           | 0.1872               | 0.7738                    | 4.1339                      |
| hsa-miR-363    | 25.96         | 24.12           | 0.1333               | 0.0786                    | 0.5895                      |
| hsa-miR-370    | 26.25         | 24.36           | 0.1090               | 0.0665                    | 0.6103                      |
| hsa-miR-409-3p | 26.52         | 26.45           | 0.0904               | 0.0156                    | 0.1726                      |
| hsa-miR-451    | 32.92         | 28.03           | 0.0011               | 0.0052                    | 4.8821                      |
| hsa-miR-493    | 31.14         | 29.19           | 0.0037               | 0.0023                    | 0.6362                      |
| hsa-miR-574-3p | 22.65         | 21.66           | 1.3218               | 0.4323                    | 0.3270                      |
| hsa-miR-9      | 25.47         | 22.79           | 0.1872               | 0.1975                    | 1.0552                      |
| hsa-miR-98     | 24.74         | 22.01           | 0.3105               | 0.3392                    | 1.0924                      |
| hsa-miR-99b    | 21.24         | 19.79           | 3.5125               | 1.5801                    | 0.4498                      |
| SNORD48        | 26.92         | 23.15           |                      |                           |                             |
| SNORD47        | 22.99         | 20.79           |                      |                           |                             |
| SNORD44        | 19.91         | 18.54           |                      |                           |                             |
| RNU6-2         | 22.39         | 19.32           |                      |                           |                             |
| HKG mean Ct    | 23.0525       | 20.45           |                      |                           |                             |

One μg of total RNA from DCs cultured for 48 h in the absence or presence of H. pylori B38 at MOI 1 was retro-transcribed using the RT2 miRNA First Strand Kit (Qiagen, Courtaboeuf, France). The first strand reaction was mixed with RT2 SYBR Green qPCR mix and distributed onto PCR array plates containing primers for 88 miRNA involved in human immunopathology (SABioscience, Qiagen) according to the manufacturer’s protocol. The amplification profiles were measured on a Stratagene Mx3005P instrument (Life Technologies, Saint Aubin, France). The relative value for each miRNA was calculated by the ΔCt method taking account the mean Ct for housekeeping genes (HKG). The fold induction of each miRNA corresponds to the ratio of its relative value in B38-infected DCs versus its relative value in control DCs.
FIGURE A1 | Characterization of the immature state of dendritic cells after 7 days of differentiation with GM-CSF and IL-4 as determined by flow cytometry. After 7 days of culture in the presence of GM-CSF and IL-4, DCs were labeled with the following antibodies: CD14-FITC, CD1a-FITC, CD40-PE, CD80-PE, CD83-FITC, CD86-PE, and HLA-DR-PECy5. They were analyzed by flow cytometry using a Canto II cytometer (Becton Dickinson, Le Pont de Claix, France). The percentages of positively labeled cells were determined after subtraction of the isotype control values (black histograms on the top of the figure) and are presented in gray histograms for each surface marker. In mature DCs, CD40, CD80, CD83, and CD86 were low in accordance with a non-activated stage. A high proportion of DCs expressed the CD1a and HLA-DR molecules. For each activation marker data [percentage of positive cells + mean fluorescence intensity (MFI)] are indicated. One representative experiment out of 13 is shown.
FIGURE A2 | Upregulation of HLA-DR, CD86, CD40, and CD83 expression in activated dendritic cells. Dot plots on the left side of the figure correspond to DCs stained with C83-FITC and CD86-PE antibodies and dot plots on the right side to DCs stained with C40-FITC and HLA-DR-PECy5 antibodies. E. coli LPS (LPS, 100 ng/ml) was used as the positive control. For each activation marker, the percentage of positive cells and mean fluorescence intensity (MFI) are indicated on each corresponding axis and are representative of the data obtained for one experiment out of 13 performed for H. pylori strain B38 and strain B45. (PE: phycoerythrine, PECy5: phycoerythrine-cyanine 5, FITC: fluorescein isothiocyanate).

FIGURE A3 | Apoptosis rate in dendritic cell apoptosis activated by H. pylori. DCs were cocultivated for 48 h with B38 or B45 H. pylori strain at MOI 1. The cells were labeled with anti-annexin V-FITC antibody and propidium iodide and analyzed by flow cytometry. Percentages of viable DCs (double negative cells in Q3), necrotic DCs (propidium iodide positive-FITC negative in Q1), and apoptotic DCs (FITC-positive, propidium iodide ± in Q2 and Q4) are depicted in each area. The percentages are shown for one representative experiment out of 3 independent experiments (y-axis, propidium iodide; x-axis, anti-annexin V-FITC) (NI for uninfected DCs).
FIGURE A4 | Kinetics of IL-1β, IL-8 and IL-12 production by E. coli LPS- or H. pylori-activated dendritic cells. DCs were either stimulated with E. coli LPS (100 ng/mL) or cocultivated with H. pylori B38 strain at MOI 1 for 6, 24, or 48 h. IL-1β, IL-8, and IL-12 secretion levels were assessed by ELISA (as described in Material and Methods). The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values of fold induction ratio vs. DCs alone obtained for 6 independent experiments corresponding to 6 different donors. These results are representative of those obtained for H. pylori B45 strain-activated DC performed in parallel during the same experiments. NS for *p > 0.05 compared to E. coli LPS-activated DC at the corresponding time-point.

FIGURE A5 | Effect of miR-146a or miR-155 loss of function on H. pylori-induced IL-23, IL-6, IL-12, IL-1β and IL-8 production. DCs were transfected with 100 nM of miRNA-antisense (anti-miR-146a or anti-miR-155) or scrambled oligonucleotides before being challenged with B38 or B45 H. pylori strains at MOI 1 for 48 h. IL-6 secretion was evaluated by flow cytometry, whereas IL-1β, IL-8, IL-12, and IL-23 production were assessed by ELISA. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values of fold induction ratio compared to scrambled oligonucleotide-treated cells, for 4 independent experiments corresponding to 4 different donors (*if *p < 0.05).