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RESEARCH ARTICLE

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The lipoprotein HP1454 of *Helicobacter pylori* regulates T-cell response by shaping T-cell receptor signalling

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Abstract

Helicobacter pylori (HP) is a Gram-negative bacterium that chronically infects the stomach of more than 50% of human population and represents a major cause of gastric cancer, gastric lymphoma, gastric autoimmunity, and peptic ulcer. It still remains to be elucidated, which HP virulence factors are important in the development of gastric disorders. Here, we analysed the role of the HP protein HP1454 in the host–pathogen interaction. We found that a significant proportion of T cells isolated from HP patients with chronic gastritis and gastric adenocarcinoma proliferated in response to HP1454. Moreover, we demonstrated in vivo that HP1454 protein drives Th1/Th17 inflammatory responses. We further analysed the in vitro response of human T cells exposed either to an HP wild-type strain or to a strain with a deletion of the *hp1454* gene, and we revealed that HP1454 triggers the T-cell antigen receptor-dependent signalling and lymphocyte proliferation, as well as the CXCL12-dependent cell adhesion and migration. Our study findings prove that HP1454 is a crucial bacterial factor that exerts its proinflammatory activity by directly modulating the T-cell response. The relevance of these results can be appreciated by considering that compelling evidence suggest that chronic gastric inflammation, a condition that paves the way to HP-associated diseases, is dependent on T cells.

KEYWORDS

H. pylori, HP1454 protein, migration, TCR signalling, Th subsets

1 | INTRODUCTION

Helicobacter pylori (HP) is a Gram-negative bacterium able to colonise the human stomach and responsible for the infection of 50% of the worldwide population (Hooi et al., 2017; Zamani et al., 2018). It represents a major cause of chronic, active gastritis and contributes to the development of more severe gastric pathologies, including peptic ulcer, gastric cancer, and MALT lymphoma (D'Elios, Appelmelk, Amedei, Bergman, & Del Prete, 2004; Goodwin, 1997; Montecucco & Rappuoli, 2001). The type of the host immune/inflammatory response against HP is crucial for the outcome of the infection.

T lymphocytes, particularly interferon (IFN)-γ-secreting Th1 cells and Th17 cells, have been proposed to play a central role in HP persistent infection and in sustaining chronic inflammation (Bamford et al., 1998; D'Elios et al., 1997; Luzza et al., 2000; Sommer et al., 1998). The neutrophil activating protein HP-NAP is a major antigen of HP able to promote Th1 immune responses, by means of neutrophils and monocytes that release pro-Th1 cytokines upon the exposure to the protein (Amedei et al., 2006; D'Elios, Amedei, Cappon, Del Prete, & de Bernard, 2007; Dundon, de Bernard, & Montecucco, 2001; Polenghi et al., 2007). However, the possibility that HP might

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elicit such an immune response also by directly acting on T cells has never been explored. HP proteins exposed on the external surface of the bacterium or secreted by the bacterium are the candidates for interacting with the immune system. Among these, HP1454, a protein of 303 amino acids, whose structure has been recently determined (Quarantini, Cendron, & Zanotti, 2014), is particularly interesting, and its function remains unknown. HP1454 is a secreted protein characterized by a predicted cleavable N-terminal signal sequence. It has been also identified as a component of the outer membrane vesicles (OMV), which were shown to contain mainly, but not exclusively, outer membrane proteins (Olofsson et al., 2010). The *hp1454* gene belongs to an operon containing three further genes (*hp1455–hp1457*) that encode proteins that, similar to HP1454, are predicted to be in the OMV. Expression of HP1454 is pH dependent (Wen, Feng, Scott, Marcus, & Sachs, 2006).

In this study, we evaluated whether a T-cell response towards HP1454 develops in the gastric mucosa of HP-positive patients with chronic gastritis and gastric adenocarcinoma.

Moreover, in order to elucidate whether HP1454 has a role in orchestrating the adaptive immune response induced by HP infection, we assessed the impact of the protein on human T lymphocytes, comparing bacteria or vesicles from a wild-type (WT) strain with bacteria or vesicles from a strain with a deletion of the *hp1454* gene.

The results provide evidence that a significant proportion of T cells in chronic gastritis and in gastric adenocarcinoma is specific for HP1454 and that the protein is important for a full activation of T-cell antigen receptor (TCR) signalling, cell proliferation, and cell motility, as well as for the differentiation of T cells towards the Th1/Th17 profiles. Finally, we revealed that HP1454 possesses an ITIM motif that has a key role in TCR signalling and in T-cell activation and proliferation.

2 | RESULTS

2.1 | In vivo relevance of T cells specific for HP1454 in patients with chronic gastritis and gastric adenocarcinoma

Because the only evidence on the production of HP1454 by HP derives from a secretome analysis performed on the bacterium in vitro (Bumann et al., 2002), we first verified whether HP actually releases HP1454 in infected hosts, by active secretion or following bacteria lysis, considering the presence of protein-specific T cells in the gastric mucosa of HP-infected patients, as a proxy. The in vivo activated T cells infiltrating the gastric mucosa of eight HP-infected patients with uncomplicated chronic gastritis and of eight HP-infected patients with distal gastric adenocarcinoma were expanded by culture in interleukin (IL)-2-conditioned medium for 10 days. T-cell blasts were recovered and cloned by limiting dilution, as reported previously (D'Elios et al., 1997). All $CD4^+$ and $CD8^+$ T-cell clones obtained from the gastric biopsies of HP-infected patients were tested for proliferation to a recombinant form of HP1454 in the presence of irradiated autologous antigen-presenting cells. Fifteen (2.09%) of the 715 $CD4^+$ T-cell clones obtained from uncomplicated chronic gastritis were specific for HP1454, whereas 53 (7.78%) of the 681 $CD4^+$ T-cell clones obtained from the gastric mucosa of patients with gastric adenocarcinoma showed significant proliferation to HP1454 (Figure 1a). None of $CD8^+$ clones significantly proliferated to HP1454. Of the 15 $CD4^+$ T-cell clones obtained from uncomplicated chronic gastritis, five produced IFN-γ alone (33.3%), six produced IL-17 alone (40.0%), and four produced IFN-γ together with IL-17 (26.7%; Figure 1b). On the other side, upon stimulation with HP1454, 16 out of 53 HP1454-specific T-cell clones, derived from gastric adenocarcinoma T cells, produced IFN-γ alone (30.19%), 12 clones produced IL-17 alone (22.64%), and 25 produced IFN-γ in combination with IL-17 (47.17%; Figure 1b). Notably, HP1454-specific clones from the gastric mucosa of patients with gastric adenocarcinoma produced more tumour necrosis factor α (TNF-α; 3.22 ± 1.42 ng ml^{-1}) and IL-17 (1.93 ± 0.85 ng ml^{-1}), compared with the 15 HP1454-specific clones from the gastric mucosa of patients with uncomplicated chronic gastritis (TNF-α 1.1 ± 0.4 ng ml^{-1} and IL-17 1.55 ± 0.61 ng ml^{-1}; Figure 1c).

Taken together, these results suggest that the protein HP1454 is released by HP in infected patients, and it drives Th1 and Th17 inflammatory responses during chronic HP infection and in patients with distal adenocarcinoma.

2.2 | In silico analysis suggests the involvement of HP1454 in the host–pathogen interaction

HP1454 has no significant sequence similarities with other proteins, but it is conserved among other *Helicobacter* strains (Quarantini et al., 2014), suggesting that this protein may play *Helicobacter*-specific functions. Analysis of HP1454 by INGA (Interaction Network GO Annotator), a web server for the sequence-based prediction of protein function (Piovesan, Giollo, Leonardi, Ferrari, & Tosatto, 2015), predicts that this protein is a lipid-binding protein that localizes in the outer membrane (Figure S1A). Accordingly, HP1454 includes an N-terminal LPP20 lipoprotein motif (Pfam: PF02169) that is characteristic of a non-essential class of lipoproteins thought to act as inflammatory mediators (Vallese et al., 2017). A further analysis with FELLS, a server for the prediction of secondary structure, disorder, aggregation, and sequence complexity of a protein, revealed an extended hydrophobic cluster between residues 96 and 201. Additionally, two short disordered segments between residues 75–90 and 282–303 are predicted. A large hydrophobic cluster is expected in a lipid binding protein, whereas short disordered segments, typically of five to eight residues, are efficient antigens (Xie et al., 2007). Indeed, it was demonstrated that specific linear motifs residing in short intrinsically disordered segments are able to modulate the immune response (Uversky, Oldfield, & Dunker, 2005). Inspection of the database for the identification of eukaryotic linear motif ELM (Dinkel et al., 2016) shows the presence of a putative immunoreceptor tyrosine-based motif (ITAM/ITIM) within the disordered region at the HP1454 C-terminus (LIG_TYR_ITIM residues 284–289). A second ITAM/ITIM motif was also found in positions 112–117, but it localizes in a β-sheet forming region, thus suggesting that it may be a false positive (Figure S1B). An example of immunogenic modulation mediated

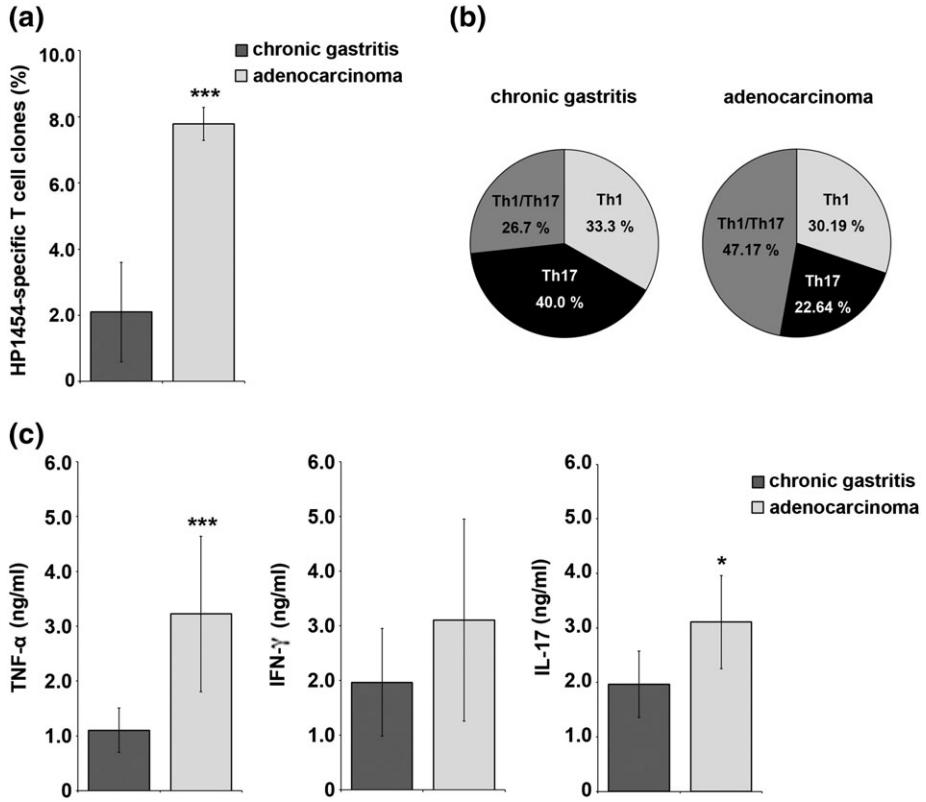

FIGURE 1 HP1454 drives Th1 and Th17 inflammatory responses during chronic *Helicobacter pylori* (HP) infection in vivo. (a) Percentage of proliferating $CD4^+$ T-cell clones, obtained from the gastric mucosa of patients with gastric adenocarcinoma (n = 53) or from patients with uncomplicated chronic gastritis (n = 15), in response to HP1454. (b,c) HP1454-specific gastric $CD4^+$ T-cell clones derived from patients with distal gastric adenocarcinoma or with chronic gastritis were tested for cytokine production. HP1454-specific $CD4^+$ T-cell clones were stimulated with HP1454 and autologous antigen-presenting cells for 48 hr, and cytokine production was measured in culture supernatants. Clones producing interferon (IFN)-γ/tumour necrosis factor (TNF)-α are named Th1, those producing interleukin (IL)-17 as Th17, and those producing IFN-γ/TNF-α and IL-17 are named Th17/Th17. In unstimulated cultures, levels of TNF-α, IFN-γ, and IL-17 were consistently <20 pg ml^{-1}. Error bars, *SD*. $^{***}p \leq 0.001$; $^{*}p \leq 0.05$

by an ITIM-containing protein was recently demonstrated for the enteropathogenic *Escherichia coli* proteins Tir, which was found to inhibit toll-like receptor (TLR) signalling by recruiting the phosphatase SHP-1 to the activated receptor (Yan, Wang, Luo, Cao, & Ge, 2012). This finding, taken together with the presence of a LPP20 lipoprotein motif, suggests that HP1454 can be an immune modulator in the context of the interaction between human and HP.

2.3 | HP1454 localization in the OMVs

To confirm the localization of HP1454 in OMV, ultrathin sections of purified OMV were incubated with a polyclonal antibody against HP1454 followed by a gold conjugated anti-rabbit IgG. Immunogold images show the presence of HP1454 protein associated with the membrane of vesicles purified from an HP broth culture at the early stationary phase (Figure S2A).

2.4 | HP1454 is required for the TCR-dependent signalling in T cells exposed to HP

To assess the role of HP1454 protein in the activation of T lymphocytes, primary T cells, purified from peripheral blood of healthy donors, were cultured in vitro with either HP WT or HP KO or exposed to either OMV WT or OMV KO. Control cells were not exposed to bacteria or to OMV. In addition, to assess whether the effect of HP1454 on T lymphocytes could rely on the ITIM motif, we produced a mutant in the conserved tyrosine (Tyr286 ➔ Phe; Verbrugge, Ruiter Td, de Clevers, & Meyaard, 2003; Vuyyuru, Shen, & Manser, 2015) and compared the activation of T lymphocytes exposed to the recombinant protein HP1454 WT with that elicited by the mutated protein (HP1454 mut).

After a 24-hr incubation with HP, OMV, or recombinant protein HP1454, either WT or mutated, cells were stimulated with an agonistic anti-CD3 monoclonal antibody and the expression level of the activation marker CD69 was determined by flow cytometry. As shown in Figure 2a, infection of T lymphocytes reduced by 50% the expression of CD69 compared with control cells, in accordance with previous evidence (Boncristiano et al., 2003). However, it is noteworthy that the expression of the activation marker was further downregulated in lymphocytes cultured with HP KO or exposed to OMV KO compared with WT counterparts. Similar to WT HP, treatment with the recombinant protein HP1454 WT induced high levels of CD69 surface expression, whereas a significant reduction in CD69 surface levels was observed when cells were treated with the

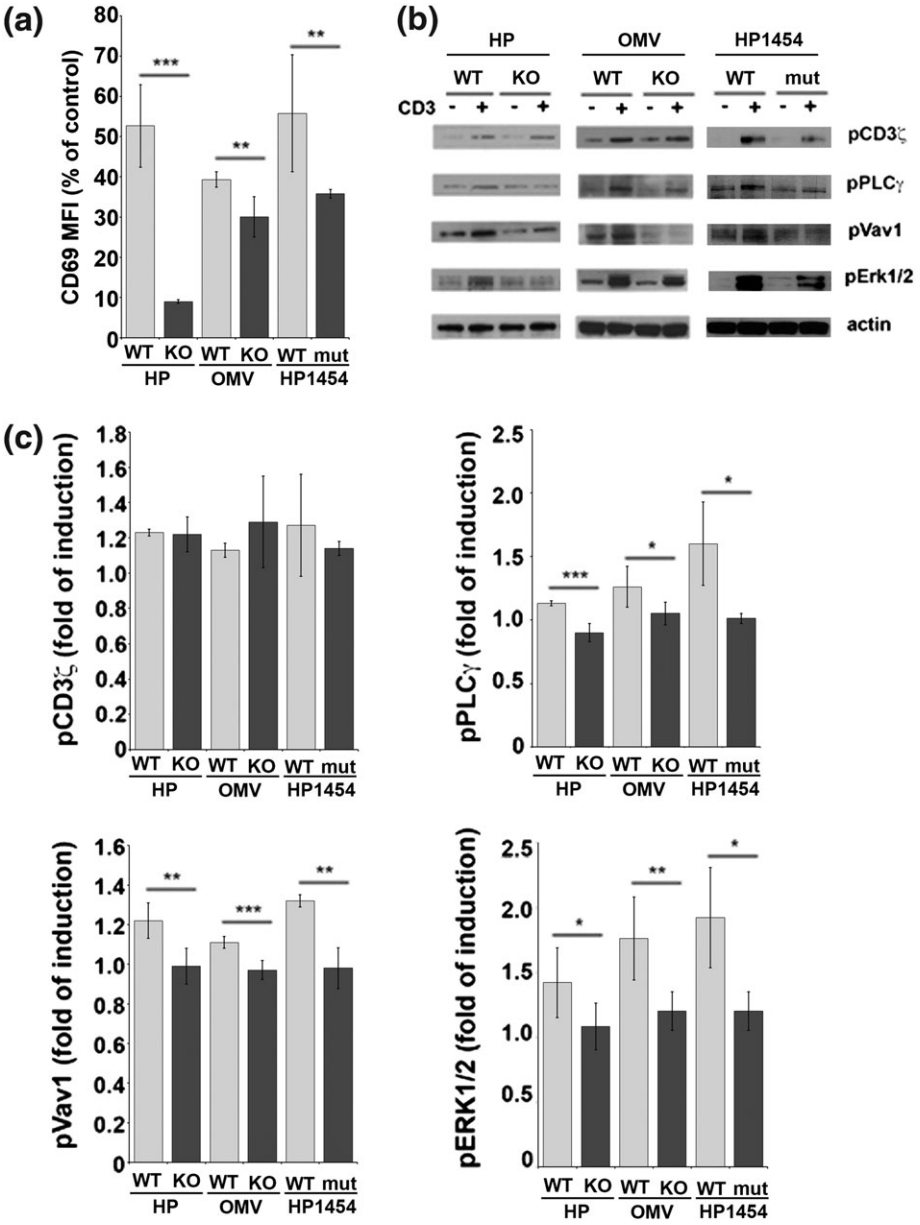

FIGURE 2 HP1454 positively regulates T-cell activation. (a) Expression of CD69 on purified peripheral T cells from six healthy donors cultured with *Helicobacter pylori* (HP), outer membrane vesicles (OMV), and HP1454, either wild-type (WT) or mutant, for 24 hr, and then stimulated with anti-CD3 mAb. Data are expressed as percentage of mean fluorescence intensity (MFI) relative to untreated cells ± *SD*. (b) Post-nuclear supernatants of T cells incubated as in (a) for 24 hr and then activated by TCR cross-linking were processed for immunoblot, and the phosphorylated forms of CD3ζ, PLCγ, Vav1, and Erk1/2 were revealed. A representative experiment is shown ($n = 3$). (c) Quantification by laser densitometry of the protein bands. Each sample was normalised to the relative actin, and data are expressed as *n*-fold between phosphorylated proteins in anti-CD3 stimulated samples and those in unstimulated ones ($n = 3$). Error bars, *SD*. $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$

mutated form of HP1454, indicating a key role of the ITIM motif in T-cell activation.

We then moved to dissect the signalling cascade upon TCR stimulation. Primary T cells were cultured as above, and after the 24-hr incubation, they were either left unstimulated or stimulated with anti-CD3 to trigger TCR activation. The phosphorylation of downstream signalling molecules, namely, the ζ chain of CD3 receptor, the phospholipase C-γ (PLCγ), the guanine nucleotide exchange factor Vav1, and the MAP Kinase Erk1/2, was assessed by immunoblot assay. Cells cultured with HP WT strain or with OMV WT, following TCR stimulation, showed phosphorylation of all molecules analysed following TCR stimulation. Conversely, although CD3ζ phosphorylation was maintained in T cells cultured with HP KO or OMV KO, upon TCR stimulation, in the same cells, the downstream molecules were not phosphorylated (Figure 2b,c). The exposure of T cells to HP1454 led to the full activation of the signalling cascade, whereas the latter was strongly affected in cells exposed to the mutant form of the protein (Figure 2b,c), as observed for HP KO and OMV KO.

These data indicate that the absence of HP1454 negatively impacts on the TCR signalling by impinging immediately downstream of TCR ζ chain phosphorylation and that this regulatory activity of T-cell activation relies on the ITIM motif.

2.5 | HP1454 modulates the expression levels of genes involved in T helper cell differentiation

It was previously reported that a Th1/Th17 response predominates during persistent HP infection (D'Elios et al., 1997; Luzza et al., 2000). In order to assess the role of HP1454 in Th cell differentiation, primary T cells were cultured as above and analysed by real-time polymerase chain reaction (PCR) for the expression of the transcription factors c-Maf, T-bet, and RORγ that drive the Th2, Th1, and Th17 differentiation, respectively. Although the expression of c-Maf remained the same, regardless of the stimulus, the expression levels of T-bet and RORγ decreased in cells cultured with HP KO and OMV KO, compared with the WT counterparts, and increased in the presence of recombinant HP1454 compared with untreated cells (Figure 3). These results suggest that the protein HP1454 is one of the bacterial factors required for Th1/Th17 polarisation during HP infection.

2.6 | HP1454 is crucial to trigger T-cell proliferation upon exposure to HP

To test the effects of HP1454 on T-cell proliferation, primary T cells were cultured as above, loaded with the fluorescent vital dye CFSE, and proliferation was assessed by flow cytometric analysis. TCR-dependent T-cell proliferation was evaluated 72 hr after the receptor activation (Figure 4a,b). A significant proportion of T lymphocytes was found to proliferate, with up to three rounds of division in samples treated with either HP WT or OMV WT, as well as in the presence of the recombinant protein HP1454 WT, whereas incubation with HP KO or OMV KO markedly affected cell proliferation (Figure 4a,b). HP WT itself is able to inhibit T-cell proliferation, as expected from data previously obtained with purified HP components (Beigier-Bompadre et al., 2011; Boncristiano et al., 2003; Gebert, Fischer, & Haas, 2004; Gerhard et al., 2005; Schmees et al., 2007). However, the absence of HP1454 protein resulted in a further decrease in T-cell proliferation, accounting for a key role of the protein in this process. Notably, treatment of T cells with HP1454 with the mutation in the ITIM motif resulted in a reduced cell proliferation (Figure 4a,b).

2.7 | HP1454 is required for CXCL12-dependent adhesion and chemotaxis in HP-exposed T cells

Fine-tuning of immune cell migration may be of paramount importance to the outcome of an infection (Marelli-Berg, Cannella, Dazzi, & Mirenda, 2008). Because the activation of Vav1, a key molecule in the regulation of actin cytoskeleton and responsible for cell motility, seemed to be dependent on HP1454 protein expression, we analysed its effect on the two main steps of lymphocyte homing: adhesion and migration (Umemoto et al., 2011). Lymphocyte adhesion to endothelial cells mainly depends on the lymphocyte function-associated antigen 1 (LFA-1) and on the very late antigen 4 (VLA-4), which interact with the intercellular adhesion molecule 1 (ICAM-1) and with fibronectin on the vascular endothelium, respectively (Margadant, Charafeddine, & Sonnenberg, 2010). In order to assess the role of HP1454 protein in this process, we first analysed the expression levels of LFA-1 and VLA-4 integrins by real-time PCR in primary T cells purified from peripheral blood of healthy donors, exposed to the different stimuli. As shown in Figure 5a, the expression levels of LFA-1 significantly decreased in cells cultured with HP KO or OMV KO, compared with WT counterparts, whereas no significant differences were observed for VLA-4 expression levels. Treatment of T cells with the recombinant protein HP1454 resulted in an increase in both LFA-1 and VLA-4 expression (Figure 5a). T cells treated as described were plated on immobilised ICAM-1, and the proportion of cells that adhered to the substrate was determined by flow cytometry. T lymphocytes incubated with either HP KO or OMV KO showed an impaired adhesion to ICAM-1, in marked contrast with cells cultured with HP WT or OMV WT (Figure 5b). Interestingly, T cells stimulated with the recombinant HP1454 WT, as well as HP1454 mutated in the ITIM motif, showed a strong adhesion to ICAM-1 (Figure 5b). Taken together,

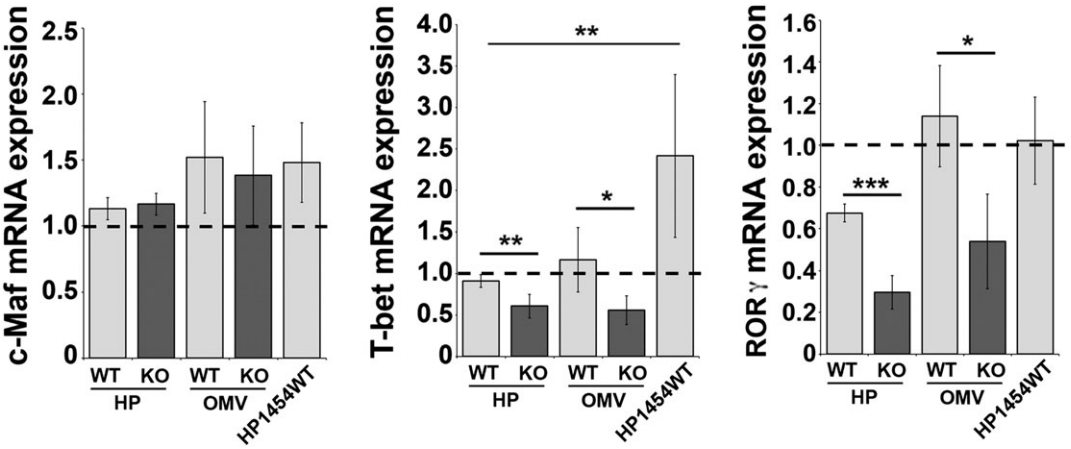

FIGURE 3 HP1454 deficiency abrogates Th1/Th17 polarisation. T cells purified from 10 healthy donors were cultured and activated as in Figure 2. After 24 hr, total RNA was extracted and the amount of mRNA encoding c-Maf, T-bet, and RORγ quantified by reverse transcription polymerase chain reaction. Transcript levels were normalised to the expression level of HPRT1. The abundance of gene transcripts was determined on triplicate samples using the ΔΔCt method and expressed as fold change of untreated T cells (dotted line; mean ± *SD*). $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$. OMV: outer membrane vesicles

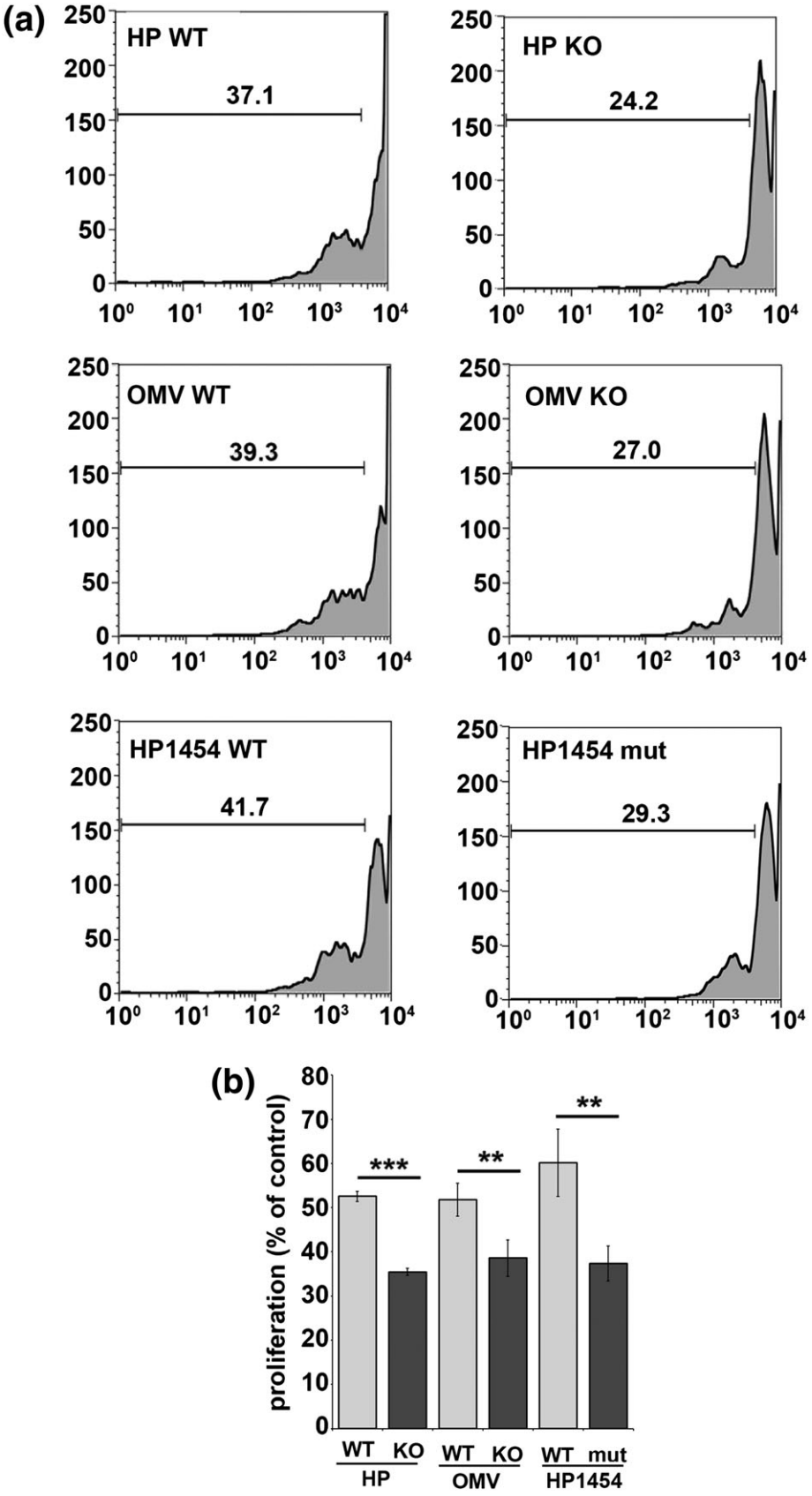

FIGURE 4 HP1454 positively regulates T-cell proliferation. (a) Representative flow cytometric profiles of primary T cells purified from the peripheral blood of healthy donors cultured and activated as in Figure 2, labelled with CFSE and activated by CD3 cross-linking. (b) Percentage of proliferating T cells was calculated in duplicate on three independent T-cell populations and showed as relative to untreated control (mean ± *SD*; $n = 3$). $^{***}p \leq 0.001$; $^{**}p \leq 0.01$. OMV: outer membrane vesicles

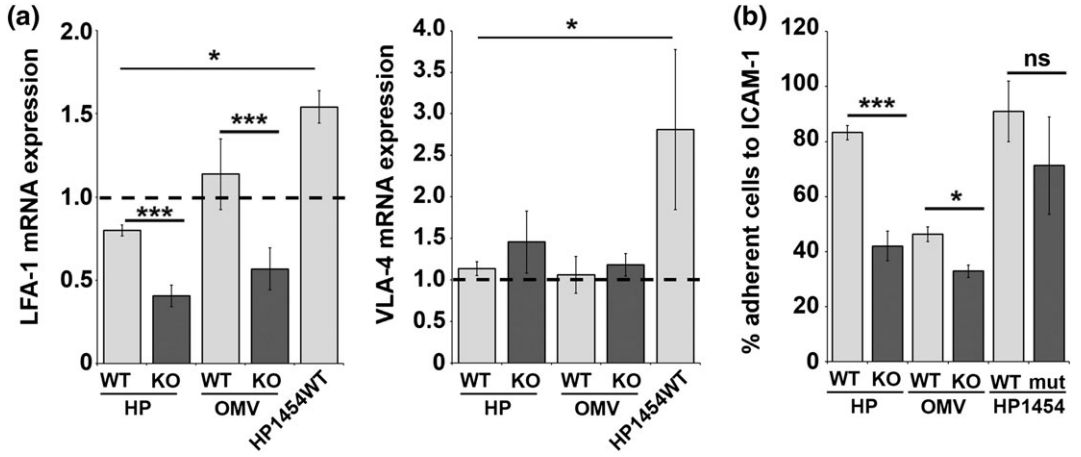

FIGURE 5 HP1454 deficiency inhibits T-cell adhesion. (a) T cells purified from 10 healthy donors were cultured as in Figure 2. After 24 hr, total RNA was extracted and the amount of mRNA encoding LFA-1 and VLA-4 quantified by reverse transcription polymerase chain reaction. Transcript levels were normalised to the expression level of HPRT1. The relative abundance of gene transcripts was determined on triplicate samples using the $\Delta\Delta$Ct method and expressed as fold change of untreated T cells (dotted line; mean ± *SD*). (b) Quantification by flow cytometry of T cells cultured as above, which adhered to rhICAM-1/Fc-coated wells upon a 10-min exposure to 100-ng ml^{-1} CXCL12. Data are expressed as percentage of adherent cells relative to untreated control (mean ± *SD*; $n = 6$). $^{***}p \leq 0.001$; $^{*}p \leq 0.05$. OMV: outer membrane vesicles

these data suggest that HP1454 exerts a fundamental role in the adhesion of T lymphocytes to endothelial cells, and such an effect is independent from the ITIM motif.

Chemokine receptors such as C-X-C chemokine receptor type 4 (CXCR4), C-C chemokine receptor type 7 (CCR7), and C-X-C chemokine receptor type 5 (CXCR5) play a central role in lymphocyte migration (Umemoto et al., 2011). In particular, CCR7 and CXCR4 are responsible for lymphocyte homing to secondary lymphoid organs, whereas CXCR5 regulates B-cell trafficking within the lymphoid organ (Kehrl, Hwang, & Park, 2009; Pereira, Kelly, & Cyster, 2010). We analysed the expression levels of these chemokine receptors by real-time PCR in T cells purified from peripheral blood of healthy donors, cultured with the different stimuli. As shown in Figure 6a, the expression levels of CXCR4 significantly decreased in cells cultured with HP KO or with OMV KO compared with WT counterparts, whereas for CCR7, we observed a statistically significant decrease in expression only in cells cultured in the presence of HP KO (Figure 6a). Neither induction nor depression of CXCR5 expression was displayed by T cells, regardless of the stimulus (Figure 6a). The involvement of HP1454 in T-cell chemotaxis was further supported by a transwell migration assays, using CXCL12, ligand for CXCR4 receptor, as chemoattractant. CXCR4-dependent migration was impaired in primary T cells previously cultured with HP KO strain or with OMV KO, with respect to cells cultured with the WT counterparts. Recombinant protein HP1454 WT elicited an efficient cell migration, as well as the protein with the mutation in the ITIM domain (Figure 6b). Hence, HP1454 acts as a positive regulator of CXCR4-dependent T-cell migration, regardless of its ITIM motif.

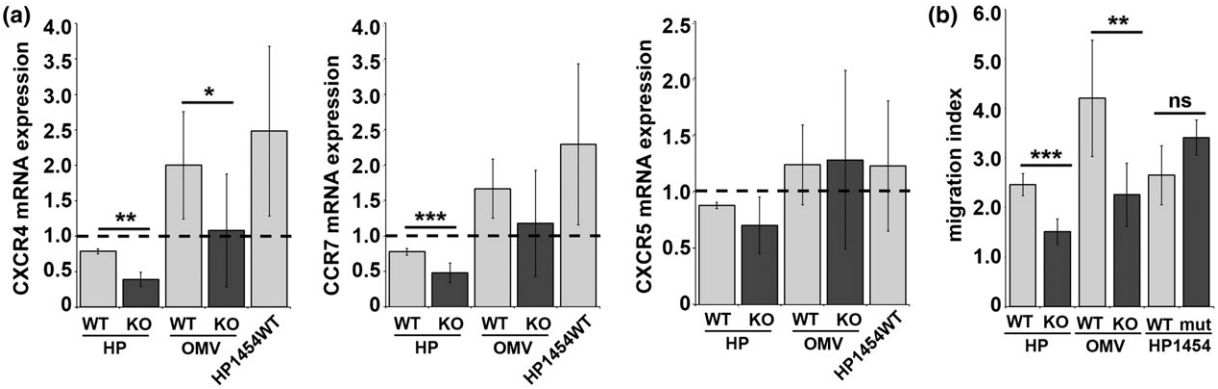

FIGURE 6 HP1454 enhances the chemotactic response to CXCL12 in T cells. (a) T cells purified from healthy donors were cultured as in Figure 2 . After 24 hr, total RNA was extracted and the amount of mRNA encoding CXCR4, CCR7, and CXCR5 were quantified by reverse transcription polymerase chain reaction. Transcript levels were normalised to the expression level of HPRT1. The relative abundance of gene transcripts was determined on triplicate samples using the $\Delta\Delta$Ct method and expressed as fold change of untreated T cells (dotted line; mean ± *SD*; $n = 10$). (b) Migration of T cells cultured as above for 24 hr, upon a 3-hr exposure to 100-ng ml^{-1} CXCL12 in a Transwell system. Data, obtained on duplicate samples from each donor, are presented as migration index (ratio between migrated cells in chemokine-exposed vs. unexposed samples; mean ± *SD*, $n = 10$). $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$. OMV: outer membrane vesicles

3 | DISCUSSION

Both innate and acquired immune responses are elicited by HP infection. One of the most intriguing aspects of HP proteins as virulence factors is their potential ability to modulate host-immune response (Amedei et al., 2006; Codolo et al., 2008; de Bernard & D'Elios, 2010). Recent studies have demonstrated the importance of HP small proteins in diagnosis, therapy, and vaccine development (Voland, Weeks, Vaira, Prinz, & Sachs, 2002). Among these proteins, HP1454 is a protein of 303 amino acids found in the extracellular HP milieu (Bumann et al., 2002), whose crystal structure was defined (Quarantini et al., 2014). Nevertheless, nothing is known about the biological functions of HP1454 as well as about its impact on cells of the innate and adaptive immunity. The present results identify T lymphocytes as a novel cellular target of HP1454 protein. It is of note that HP1454 is able to drive a Th1/Th17 response in vivo, in the gastric mucosa of patients with chronic gastritis. Interestingly, in patients with gastric adenocarcinoma, the number of HP1454-specific T cells is significantly higher than in uncomplicated gastritis and they maintain the Th1/Th17 profile. Thus, on the basis of these evidence, we can speculate that a long-lasting presence of HP in gastric mucosa might lead to a preferential Th1 and Th17 inflammation, with the contribution of HP1454, thus paving the way for gastric oncogenesis. We have demonstrated that HP1454 deficiency results in impaired T-cell activation due to the blockage of the step that couples the phosphorylation of the TCR ζ chain with PLCγ activation. Interestingly, we mapped the HP1454 ability to induce T-cell activation and proliferation within its ITIM motif, as demonstrated by the inhibitory effect on T-cell activation obtained by applying to the cells a mutant protein with the substitution of the tyrosine 286 in phenylalanine.

In infectious diseases, T helper cells orchestrate host defence against pathogens by producing different type of cytokines. In HP infection, a predominant activation of Th1 cells with production of IFN-γ occurs in vivo in the antrum (Bamford et al., 1998; D'Elios et al., 1997; Lehmann et al., 2002; Tomita et al., 2001). In vitro stimulation of T lymphocytes with the bacterial strain defective for HP1454 resulted in a prompt and remarkable downregulation of T-bet and RORγ transcription factors if compared with HP WT, thus suggesting that HP1454 has a crucial role in driving the differentiation of T lymphocytes towards the Th1/Th17 phenotype. A number of independent studies agree that Th1 and Th17 polarisation of HP-specific T-cell response rather contribute to pathology than to host protection (Amedei et al., 2006; Amedei et al., 2014; Bamford et al., 1998; Codolo et al., 2008; de Bernard & D'Elios, 2010; D'Elios et al., 1997; Sommer et al., 1998; Voland et al., 2002). Interestingly, a significant correlation was demonstrated between disease severity and IFN-γ secretion (de Jonge et al., 2004; D'Elios et al., 1997; Pereira et al., 2010).

Lymphocyte trafficking is tightly controlled by chemokines and adhesion molecules present in the lymphoid micro-environment and by receptors expressed by lymphocytes themselves (Kehrl et al., 2009). In this context, we revealed the ability of HP1454 to favour the CXCL12-dependent migration of T lymphocytes and their adhesion to ICAM-1, thus suggesting a contribution by the protein to the entry of T cells into lymphoid organs. This event is expected to be followed by a robust proliferation of HP1454-specific T cells that, in turn, would infiltrate the gastric mucosa. The impaired modulation of T-cell response caused by HP is thought to sustain the ongoing inflammatory condition and immune pathology of the stomach (Larussa, Leone, Suraci, Imeneo, & Luzza, 2015).

Of note, we demonstrated that the HP1454-elicited adhesion and migration of T lymphocytes are independent from the ITIM motif, thus suggesting that the TCR signalling only partially contributes to the cell homing.

Altogether, our data highlight T lymphocytes as cellular targets of HP1454 and suggest that the protein might pursue a crucial role in the development of the severe gastric pathologies associated to the infection by HP because of its ability in amplifying the T-cell-dependent inflammation.

4 | EXPERIMENTAL PROCEDURES

4.1 | In silico investigation

The HP1454 protein sequence with accession number O25993 was retrieved from the Uniprot database. Protein function recognition was performed with the INGA (Piovesan et al., 2015), setting "database all" and "String edge confidence high" as further parameters. Sequence features were investigated with FELLS, whereas linear motifs were identified with ELM (Dinkel et al., 2016), filtering putative motifs falling inside structured regions. Conservation analysis was performed with ProViz (Jehl, Manguy, Shields, Higgins, & Davey, 2016).

4.2 | Cells, antibodies, and reagents

T cells from peripheral blood of healthy donors were purified by negative selection using the RosetteSep Human T Cell enrichment Cocktail (StemCell Technologies), followed by density gradient centrifugation on Lympholyte (Cedarlane Laboratories). The purity of T-cell population was >90%, as assessed by flow cytometry. Rabbit polyclonal antibodies recognising the phosphorylated forms of PLCγ (Y783), Vav1 (Y160), and Erk1/2 (T202/Y204) were purchased from Santa Cruz Biotechnology, Invitrogen, and Cell Signaling, respectively. Mouse monoclonal antibodies against the phosphorylated active form of CD3ζ and against actin were from Santa Cruz Biotechnology and Millipore, respectively, and secondary peroxidase-labelled antibodies from Amersham Pharmacia Biotech. FITC anti-human CD69 was from BioLegend; human CXCL12 and rhICAM-1/Fc were purchased from R&D Systems. Rabbit polyclonal custom-made antibody against the entire protein HP-1454 was purchased from Agrisera.

4.3 | Bacterial strains and growth conditions

For the construction of the isogenic *hp1454* mutant (HP KO), an inverse PCR amplification of a gene library plasmid used for genome sequencing of strain P12 (Fischer et al., 2010) was performed, using primers WS501 (5′-GAGGATCCATACGGGGCTATAAGCAAGG-3′) and WS502 (5′-ACTAGTCGACAATAATCTTTTTCATTATTAATCCTTAC-3′). The PCR product, which contained thus *hpp12_1432* (the

P12 orthologue of *hp1454*) flanking regions and the pSMART-HCKan cloning vector, was digested with BamHI and SalI and subsequently ligated with a chloramphenicol resistance cassette (to obtain plasmid pWS420). For the generation of the isogenic mutant, the corresponding plasmid was introduced by natural transformation into HP strain P12, as described (Haas, Meyer, & van Putten, 1993). Transformants were selected on serum agar plates containing 6-mg L^{-1} chloramphenicol and checked for the correct replacement of *hp1454* with the *cat* resistance gene by sequencing.

HP 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% fetal bovine serum (FBS) and were cultured for 2 days in rotary shaking at 180 rpm at 37°C under microaerophilic condition.

4.4 | Isolation of HP OMV

HP was grown in broth culture to early stationary phase and harvested by centrifugation for 30 min at 4,000 *g* at 4°C. Supernatant was filtered through a 0.22-μm cellulose acetate filter and centrifuged for 3 hr at 150,000 *g* at 4°C. Pellet containing vesicles was resuspended in 500 μl of sterile PBS, adjusted to a total protein concentration of 1 μg ml^{-1} and stored at −80°C until use.

The absence of HP1454 both in *hp1454*-deleted bacteria and in their OMV was confirmed by western blot using a HP1454-specific polyclonal antibody (Figure S2B).

4.5 | Heterologous overexpression and affinity purification of HP1454 and HP1454 Y286F mutant (HP1454 mut)

The *hp1454* gene was isolated from genomic DNA of HP (strain CCUG 17874) and cloned in pET151 plasmid as previously described (Quarantini et al., 2014). Site-directed mutagenesis of *hp1454* gene was performed with the QuickChange® II Site-Directed Mutagenesis Kit (from Agilent Technologies), using as template hp1454-pET151 recombinant plasmid and this couple of primers: 5′-GCATAATCTTT CAGTTCTTTAAAAAGCAAAGATTGCAAGCGCG-3′ and 5′-CGCGCT TGCAATCTTTGCTTTTTAAAGAACTGAAAGATTATGC-3′. HP1454 and HP1454 mut were expressed in *E. coli* BL21 (DE3) and purified, with slight modifications, as previously described (Quarantini et al., 2014). Briefly, upon transformation of bacteria with the plasmids (hp1454-pET151 and hp1454 Y286F-pET151), protein expression was induced for 4 hr at 30°C by 1-mM isopropyl-β-D-thiogalactopyranoside. Bacterial pellets were harvested by centrifugation and resuspended in 20-mM Tris, pH 7.2, and 200-mM NaCl and supplemented with protease inhibitors cocktail tablet (Sigma). Lysis was carried out by sonication. Soluble fractions were loaded onto a 1-ml His-trap Ni-NTA column (GE Healthcare) equilibrated with 20-mM Tris, pH 7.2, and 200-mM NaCl; both proteins were eluted in an imidazole gradient. Gel filtrations were performed on a Superdex 200 10/300 size-exclusion column. Purity of the two recombinant proteins was assessed (Figure S3). We also ruled out the presence of nonproteinaceous contaminants (i.e., peptidoglycans, lipoteichoic acid, and lipopolysaccharide) by testing the protein on human embryonic kidney (HEK) 293 cells constitutively expressing TLR2/CD14 (HEK293-TLR2/CD14) or TLR4/CD14/MD2 (HEK293-TLR4/CD14/MD2; Invivogen, San Diego, CA, USA).

4.6 | Electron microscopy analysis and immunogold labelling

Isolated OMV were fixed for 1 hr in 0.05% glutaraldehyde, 4% paraformaldehyde, and 0.1-M sodium cacodylate buffer pH 7.4, before being embedded in L.R. White Resin (Polysciences) following manufacturer's instructions. Ultrathin sections were collected on nickel grids and processed for post-embedding immunogold labelling. After blocking the non-specific binding sites with 0.5% bovine serum albumin (BSA) in PBS for 10 min, sections were incubated with a polyclonal antibody against HP1454 (0.1 μg ml^{-1}) in PBS for 30 min followed by a 10-nm gold conjugated goat anti-rabbit IgG (Sigma) diluted 1:100 in PBS for 30 min. Finally, sections were counterstained with uranyl acetate and lead citrate and examined with a FEI Tecnai™ G2 transmission electron microscope operating at 120 kV. Images were captured with a Veleta (Olympus Soft Imaging System) digital camera. A negative control for staining specificity was performed by omitting the primary antibody (Griffiths & Hoppeler, 1986; Posthuma, Slot, & Geuze, 1987; Skepper, 2000).

4.7 | Real-time PCR analysis

Total RNA was extracted from T cells of healthy donors and retrotranscribed as described previously (Capitani et al., 2010). Two independent reverse transcription reactions were performed on each RNA sample. Real-time PCR was performed in triplicate on each cDNA on 96-well optical PCR plates (Sarstedt) using SSoFast EvaGreen® SuperMix (Bio-Rad) according to the manufacturer's instructions and a CFX96 Real-Time system (Bio-Rad). After an initial denaturation for 3 min at 95°C, denaturation in the subsequent 42 cycles was performed for 10 s at 95°C, followed by 30 s of primer annealing at 60°C. Results were processed and analysed using CFX Manager Version 1.5 software (Bio-Rad). Transcript levels were normalised to HPRT1, used as a housekeeping gene. Primers used for amplification are listed in Table S1.

4.8 | Generation of T-cell clones from gastric tumour-infiltrating lymphocytes of the neoplastic tissue and characterization of their cytokine profile

After approval by the local ethical committee, following informed consent, gastric mucosal specimens were obtained from eight HP-infected patients with uncomplicated chronic gastritis (four men and four women, mean age 66 years) and from eight HP-infected patients with gastric adenocarcinoma (four men and four women, mean age 68 years) and cultured for 10 days in RPMI medium 1640 supplemented with IL-2 (50 U ml^{-1}) in order to expand in vivo activated tumour-infiltrating lymphocytes. After disruption, single T-cell blasts were cloned under limiting dilution, as previously described (D'Elios

et al., 1997). To assess their cytokine production, on antigen stimulation, T-cell blasts of each HP1454-specific T-cell clone (5×10^5) were cocultured in triplicate for 48 hr in 0.5 ml of medium with 5×10^5 irradiated autologous antigen-presenting cells in the absence or presence of HP1454 (5 μg ml^{-1}; D'Elios et al., 1997). At the end of the culture period, duplicate samples of each supernatant were assayed for their IFN-γ, TNF-α, and IL-17 production by enzyme-linked immunosorbent assay (ELISA) (BioSource International).

4.9 | T-cell activation and immunoblots

Primary T cells were cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10% bovine calf serum (Hyclone) with either HP WT or KO strains at multiplicity of infection of 100:1 for the indicated time points, with OMV WT or OMV KO (10 μg ml^{-1}), or with recombinant HP1454 protein WT or HP1454 Y286F mutant (1 μg ml^{-1}) for 24 hr. Activation by TCR/CD3 cross-linking was performed by incubating T cells with the monoclonal antibody anti-CD3 (OKT3) followed by anti-mouse secondary antibody, for 2 min at 37°C. Cells (5×10^6 per sample) were lysed in 20-mM Tris-HCl, pH 8, 150-mM NaCl, and 1% Triton X-100 plus protease inhibitors. Proteins were processed for immunoblot, revealed by chemiluminescence (SuperSignal; West Pico Chemiluminescent Substrate kit; Pierce Chemical), scanned by a laser densitometer (Duoscan T2500 Agfa), and quantified with ImageQuant 5.0 software (Molecular Dynamics). Data were normalised referring to actin, taken as loading control.

4.10 | Flow cytometry

Primary T cells cultured with HP WT or HP KO, with OMV WT or OMV KO, or with recombinant protein HP1454 WT or HP1454 Y286F mutant for 24 hr were then incubated with the anti-human CD69 FITC-labelled monoclonal antibody (BioLegend). Flow cytometry was carried out using a Guava easyCyte cytometer (Millipore). Data were analysed and plotted using FlowJo Version 6.1.1 software (TreeStar). For proliferation assays, CFSE-labelled cells (2×10^5 per sample), cultured as previously described, were plated in 96-well plates and activated by CD3 cross-linking on secondary antibody-coated plates as described (Boncristiano et al., 2003). Cells were analysed 72 hr after activation.

4.11 | Adhesion and chemotaxis assays

The 48-well plates were coated o/n at 4°C with 10-mg ml^{-1} rhICAM-1/Fc, washed with PBS, and incubated for 30 min at 37°C with RPMI 1% BSA; 2×10^5 serum-starved T-cells were added to each well. After incubating 10 min at 37°C, 100-ng ml^{-1} CXCL12 was added for further 10 min. Cells that did not adhere (collected from medium and washes) were resuspended in 0.2-ml PBS. Cells that remained adherent after three washes were harvested by 1-min incubation with trypsin/EDTA and washed and resuspended in 0.2-ml PBS. Cells were counted by flow cytometry. The percentage of adherent cells was calculated as previously described (Capitani et al., 2010). Chemotaxis assay was carried out in 24-well Transwell chambers with 5-μm pore size polycarbonate membranes (Corning Life Sciences) as described previously (Patrussi et al., 2007). Filters were soaked overnight in RPMI 1% BSA. Chemotaxis medium (500-μl RPMI, 1% BSA) with or without 100-nM CXCL12 was placed in the lower chamber, and 100 μl of cell suspension (5×10^5 cells per sample) in chemotaxis medium was placed in the upper chamber. After a 3-hr incubation at 37°C in humidified air with 5% CO_2, the upper chamber was emptied, filters were removed, and cells in the lower chamber were counted by flow cytometry. The migration index was calculated by determining the ratio between migrated cells in samples exposed to CXCL12 and those in sample not exposed to the chemokine.

4.12 | Statistical analysis

Statistical analyses were performed using Student's *t* test; data, reported as the mean ± *SD*, were considered significant if p values ≥ 0.05.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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