

Cutting Edge: *Helicobacter pylori* Induces Nuclear Hypersegmentation and Subtype Differentiation of Human Neutrophils In Vitro

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Helicobacter pylori infects the human stomach and causes a spectrum of disease that includes gastritis, peptic ulcers, and gastric adenocarcinoma. A chronic, neutrophil-rich inflammatory response characterizes this infection. It is established that *H. pylori* stimulates neutrophil chemotaxis and a robust respiratory burst, but other aspects of this interaction are incompletely defined. We demonstrate here that *H. pylori* induces N1-like subtype differentiation of human neutrophils as indicated by profound nuclear hypersegmentation, a CD62L^{dim}, CD16^{bright}, CD11b^{bright}, CD66b^{bright}, CD63^{bright} surface phenotype, proinflammatory cytokine secretion, and cytotoxicity. Hypersegmentation requires direct neutrophil–*H. pylori* contact as well as transcription and both host and bacterial protein synthesis, but not urease, NapA, VacA, CagA, or CagT. The concept of neutrophil plasticity is new and, to our knowledge, these data are the first evidence that neutrophils can undergo subtype differentiation in vitro in response to bacterial pathogen infection. We hypothesize that these changes favor *H. pylori* persistence and disease. *The Journal of Immunology*, 2017, 198: 1793–1797.

Polymorphonuclear leukocytes (or neutrophils) (PMNs) are the most abundant leukocyte in humans, and their ability to be rapidly recruited to sites of infection and kill ingested microbes is unequivocal (1). Though traditionally thought of as a homogenous, terminally differentiated population of cells, this view has been radically altered by the recent discovery of neutrophil subsets, which revealed their potential to exhibit phenotypic plasticity and undergo subtype differentiation in tissue microenvironments in vivo (2, 3). Although this field is in its infancy, PMNs with distinct properties have been identified in the circulation of humans with systemic inflammation and following trauma, in the joints of persons with rheumatoid arthritis, in the lungs, and in the murine tumor microenvironment (4–12). Attempts to

recapitulate these phenotypes in vitro have not been successful, suggesting that in vivo cues may be essential, and leaving researchers to question whether PMN subsets arise during neutrophil development, or whether mature neutrophils have the capacity to differentiate into subtypes upon encountering specific stimuli.

Helicobacter pylori is a Gram-negative bacterial pathogen of humans that resides in the mucus layer over the gastric epithelium and causes gastritis, peptic ulcers, and gastric cancer (13). A chronic neutrophil-dominant inflammatory response characterizes this infection, and PMN density correlates with disease severity and tissue destruction (14). *H. pylori* uses multiple mechanisms to recruit and activate PMNs, but bacterial killing is inefficient (14). In particular, NADPH oxidase targeting is manipulated such that toxic reactive oxygen species (ROS) are released into the extracellular space rather than into bacterial phagosomes, which contributes to epithelial damage and sustains infection (14, 15). Although PMN chemotaxis and activation have been studied in detail, other aspects of *H. pylori*–neutrophil interactions are understudied. Herein, we advance understanding of neutrophil plasticity by demonstrating that subtype differentiation can be induced in mature human neutrophils by in vitro infection with an important human pathogen, and provide insight into the underlying mechanisms.

Materials and Methods

Isolation of human neutrophils

Human neutrophils were isolated from heparinized venous blood drawn from healthy adult volunteers in accordance with a protocol approved by the Institutional Review Board for human subjects at the University of Iowa. Neutrophils were purified using sequential dextran sedimentation, density gradient separation, and hypotonic lysis of erythrocytes (16). Neutrophil purity was routinely 95–98%, with eosinophils as the major contaminant. Replicate experiments used PMNs from different donors.

H. pylori strains and infection of neutrophils

H. pylori strains NCTC11637 (hereafter 11637) and 60190 were grown under microaerophilic conditions, washed, and quantified by measurement of the absorbance at 600 nm (15, 17). Neutrophils were diluted in

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Abbreviations used in this article: hpi, hour postinfection; PMN, polymorphonuclear leukocyte (or neutrophil); ROS, reactive oxygen species; TAN, tumor-associated neutrophil.

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HEPES-buffered RPMI 1640 (Lonza, Walkersville, MD) containing 10% heat-inactivated FBS (HyClone Laboratories, Pittsburgh, PA) to 1×10^6 cells per ml. *H. pylori* were added at a ratio of five bacteria per PMN, and cell suspensions were incubated at 37°C under microaerophilic conditions for up to 48 h. Some experiments used 0.4 μ m Transwell inserts (Corning, Oneonta, NY), or included neutrophils that were pretreated with 5 μ g/ml actinomycin D or 10 μ g/ml cycloheximide, or *H. pylori* that were pretreated with 50 μ g/ml chloramphenicol for 30 min, and drugs were maintained in the medium for the duration of the experiment.

Quantitation of bacterial load and nuclear morphology

Neutrophils were cytocentrifuged onto coverslips, and fixed and stained using a Hema 3 Stat Pack (Fisher Scientific, Kalamazoo, MI) (18). Nuclear morphology and bacterial load were examined by light microscopy, and at least 200 neutrophils were analyzed per coverslip and condition. Each experiment was performed at least three times.

Cell viability and cytokine secretion

Neutrophil apoptosis was quantified by flow cytometry after Annexin V-FITC (Invitrogen, Camarillo, CA) and propidium iodide staining (18). AGS cell viability was quantified by exclusion of 0.2% trypan blue. Cytokines in PMN supernatants were detected using a human cytokine array kit (R&D Systems, Minneapolis, MN) and IL-8 was quantified by ELISA (18).

Cell surface marker flow cytometry

Control and *H. pylori*-infected neutrophils, and neutrophils treated with 10 ng/ml ultrapure *Escherichia coli* LPS (InvivoGen, San Diego, CA) were pelleted, resuspended in HBSS with divalent cations containing 0.2% BSA and 0.2% sodium azide, and then stained with either Alexa Fluor 647, Alexa Fluor 488, FITC, or PE-conjugated isotype control Abs, or conjugated primary Abs specific for CD16, CD62L, CD11b, active CD11b, CD66b, CD35, CD63, or CD54 (from BD, Franklin Lakes, NJ, or from BioLegend, San Diego, CA) for 30 min in the dark on ice. Following washing and resuspension, cells were analyzed on an Accuri C6 Cytometer.

Statistical analysis

Control and single experimental groups were compared using two-tailed Student *t* tests. For multiple comparisons, data were analyzed by one-way ANOVA followed by a Tukey post hoc test. GraphPad Prism version 6.0 software was used, and $p < 0.05$ was considered statistically significant.

Results and Discussion

H. pylori infects human neutrophils and induces nuclear hypersegmentation

Human neutrophils were incubated in the presence and absence of two *H. pylori* strains, 60190 and 11637 (15, 17, 19), at a ratio of five bacteria per PMN under microaerophilic conditions for up to 48 h. Our data demonstrate that the majority of neutrophils were infected by 6 h postinfection (hpi) and that bacterial load continued to increase out to 48 hpi (Fig. 1A, 1B, Supplemental Fig. 1A). Notably, the bacterial load we observed resembles infected PMNs in patient biopsy samples (20), and is therefore physiologically relevant. Moreover, in our system and in vivo, uptake occurs by lectinophagocytosis, and the majority of intracellular *H. pylori* retained their characteristic morphology, which is a hallmark of bacterial health as these organisms undergo rapid conversion from spiral bacilli to coccoid forms in response to stress (Fig. 1A) (14, 20, 21). Infected neutrophils also contained small cytoplasmic vacuoles (Fig. 1A, see arrowheads) that were often in close proximity to *H. pylori* phagosomes. The nature of these vacuoles and the composition of *H. pylori* phagosomes in PMNs are unknown. Nevertheless, *H. pylori* phagosomes exclude flavocytochrome *b*₅₅₈ and lactoferrin (15), which suggests a defect in phagosome–secondary granule fusion.

A distinctive feature of mature human neutrophils is their nuclei. These structures contain three to four interconnected lobes that merge and condense as cells age and undergo ap-

optosis (18, 22) (Fig. 1A, 1C). In sharp contrast, we found that *H. pylori*-infected PMNs acquired a hypersegmented morphology (Fig. 1A, marked as HS), which is defined conservatively as six or more nuclear lobes per cell (4). We analyzed nuclear morphology over 48 h (Fig. 1A, 1C, Supplemental Fig. 1B), and confirmed that although control PMNs acquired an apoptotic nuclear morphology over this time course, the infected PMNs did not. Rather, these cells exhibited marked nuclear hypersegmentation that was apparent by 3 hpi, and increased thereafter, yet was absent in the controls. Each hypersegmented nucleus exhibited a cloverleaf arrangement of interconnected segments, but individual cells differed with respect to total lobe number (range of 6–17 per cell) and size (homogeneous or heterogeneous) (Fig. 1A). This phenotype appears to be neutrophil specific, as the few eosinophils in our samples were not affected (data not shown), and hypersegmentation of PMNs in the infected gastric mucosa demonstrates in vivo relevance (23).

Because fewer than 4% of infected PMNs contained condensed nuclei, we hypothesized that these cells may not undergo

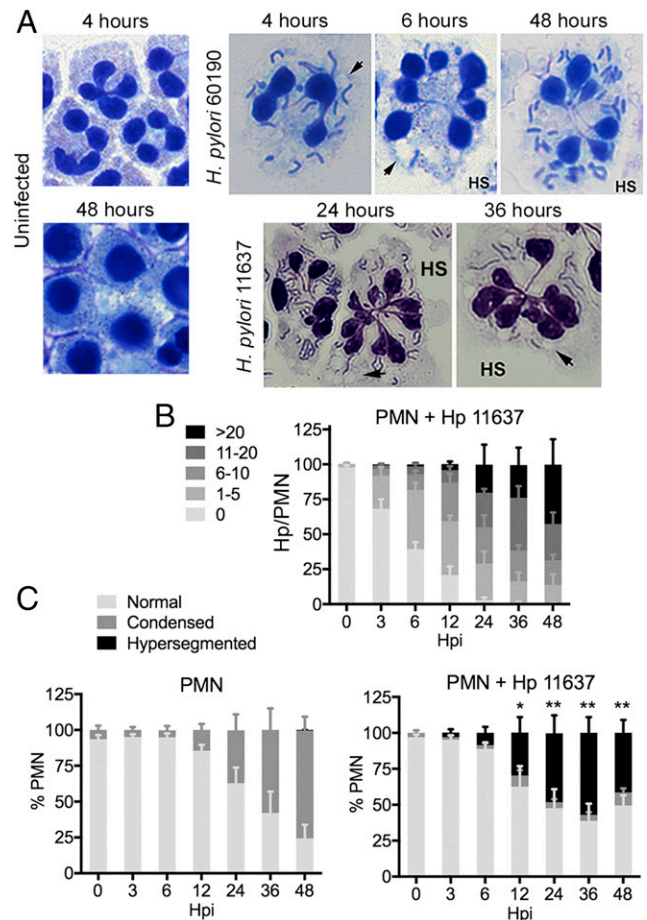


FIGURE 1. *H. pylori* accumulates in human neutrophils and induces nuclear hypersegmentation. (A) Microscopy images of control PMNs and cells that were infected with *H. pylori* strains 11637 or 60190 for the indicated amounts of time. Data shown are representative of eight or more independent experiments. Original magnifications $\times 63$ or $\times 100$. Arrowheads indicate vacuoles. HS indicates hypersegmented cells. (B) Bacterial load in infected PMNs. Data are the mean \pm SEM ($n = 4$). (C) PMN nuclear morphology was scored as normal (three to five lobes), condensed (one to two lobes), or hypersegmented (six or more lobes). Data are the mean \pm SEM ($n = 4$). * $p < 0.05$, ** $p < 0.01$ versus 0 h hypersegmentation.

apoptosis over 48 hpi. Indeed, Annexin V-FITC/propidium iodide staining and flow cytometry demonstrated that $84 \pm 5\%$ of control PMNs were apoptotic at 48 h versus only $20 \pm 5\%$ and $26 \pm 5\%$ of cells infected with *H. pylori* 11637 and 60190, respectively, ($n = 4$, $p < 0.001$, not illustrated), and DNA release was not detected (data not shown). Thus, human neutrophils support robust *H. pylori* infection that is characterized by profound nuclear hypersegmentation and extended lifespan.

Hypersegmentation requires neutrophil–bacteria contact and protein synthesis

We used Transwells (Fig. 2A) to determine if manipulation of PMN phenotype required *H. pylori* binding and/or phagocytosis. The data in Fig. 2B confirm the results shown in Fig. 1, and demonstrate that direct *H. pylori*–PMN contact is essential for hypersegmentation, as neutrophils that were separated from bacteria were indistinguishable from the uninfected controls.

Next, we tested a requirement for metabolic activity using inhibitors of host and bacterial transcription and translation, with analyses at 8 and 24 h. Blocking transcription with actinomycin D ablated hypersegmentation, and 100% of neutrophils had condensed nuclei by 24 h, suggesting rapid apoptosis induction (Fig. 2C). Blocking host or bacterial protein synthesis also caused significant inhibition of hypersegmentation by 24 hpi (Fig. 2C). Thus, whereas cycloheximide reduced hypersegmentation by $66.1 \pm 24.1\%$ ($n = 4$, $p < 0.01$), chloramphenicol reduced hypersegmentation by $81.3 \pm 7.3\%$ ($n = 3$, $p < 0.001$) relative to the no drug controls.

With respect to bacterial load, cycloheximide did not adversely affect infection (Fig. 2D). On the other hand, chloramphenicol markedly reduced bacterial load, as only $14 \pm 5\%$ and $10 \pm 5\%$ of cells contained *H. pylori* at 8 and 24 hpi, respectively, and the cells that were infected contained relatively few bacteria (Fig. 2D). Actinomycin D also impaired infection, as bacterial load was slightly diminished at 8 hpi, and was reduced further at 24 hpi, likely as a result of apoptosis induction (Fig. 2D). Thus, manipulation of PMN phenotype is an active process that is driven by both host and microbe and may be initiated at the earliest stages of infection.

The bacterial factors required for this phenotype remain unknown, as our studies of mutants generated in *H. pylori* 11637 (Supplemental Fig. 2A) show that hypersegmentation does not require bacterial urease ($\Delta ureAB$), the neutrophil activating protein ($\Delta napA$), or factors that disrupt epithelial cell function such as VacA, the type IV secretion system ($\Delta cagE$, $\Delta cagT$) or its effector CagA (14, 19, 24). Having excluded roles for several established virulence factors, a group of recently identified *H. pylori* proteins with functional nuclear localization sequences but unknown function merit further study (25).

H. pylori-infected neutrophils exhibit surface markers and functions indicative of subtype differentiation

Hypersegmentation is rare, and was described first in persons with folate or vitamin B12 deficiency, and more recently as a hallmark of two distinct PMN subsets (5, 8, 26, 27). As a 5-fold excess of folate and/or vitamin B12 had no effect on nuclear morphology in our system (Supplemental Fig. 1C),

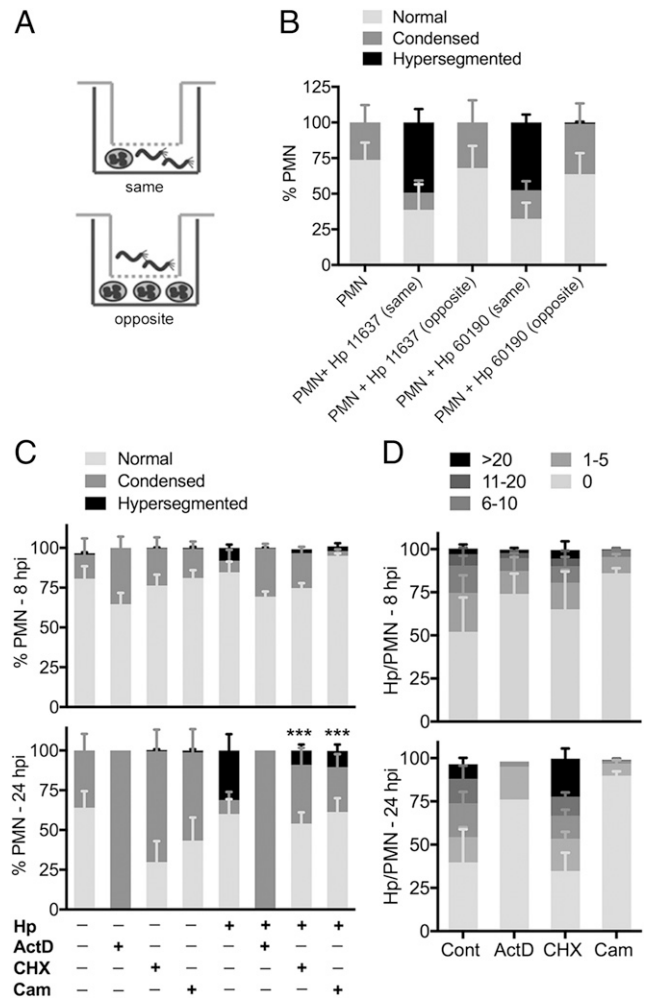


FIGURE 2. Direct contact and metabolic activity are required for *H. pylori*-induced neutrophil hypersegmentation. (A and B) Transwell infections. PMNs were left untreated or were incubated in the presence of *H. pylori* that were added to the same or opposite chamber of a Transwell for 24 h. PMN nuclear morphology was assessed by microscopy. Data are the mean + SEM, $n = 3$. (C and D) The effects of actinomycin D, cycloheximide, and chloramphenicol on nuclear morphology (C) and bacterial load (D) were assessed after 8 and 24 h, as indicated. Data are the mean + SEM, $n = 3-4$. *** $p < 0.001$. ActD, actinomycin D; Cam, chloramphenicol; CHX, cycloheximide; Hp, *Helicobacter pylori* 11637.

we hypothesized that *H. pylori* may induce PMN subtype differentiation.

The first subset of interest was described by Pillay et al. (5), is defined by hypersegmentation and a CD16^{bright}/CD62L^{dim} surface phenotype, and is present at low frequency (15% of neutrophils) in the circulation of persons with endotoxemia along with CD16^{bright}/CD62L^{bright} mature (segmented) PMNs and CD16^{dim}/CD62L^{bright} immature (banded) cells. We analyzed CD16 (FcγRIII) and CD62L (L-selectin) by flow cytometry, and as expected the vast majority of freshly isolated PMNs were CD16^{bright}/CD62L^{bright} (Fig. 3A, gray bars). However, this pattern changed markedly in response to *H. pylori*, as 61–68% of cells acquired a CD16^{bright}/CD62L^{dim} phenotype by 6 hpi (Fig. 3B, Supplemental Fig. 1D, 1E, red bars) that resembles the atypical PMN subset described above (5). This phenotype was nearly absent in the uninfected controls (Fig. 3A, red bars) ($n = 3$, $p < 0.0001$), and remained abundant in the infected PMNs until at least 24 hpi (Fig. 3B, Supplemental Fig. 1D, 1E). Certain

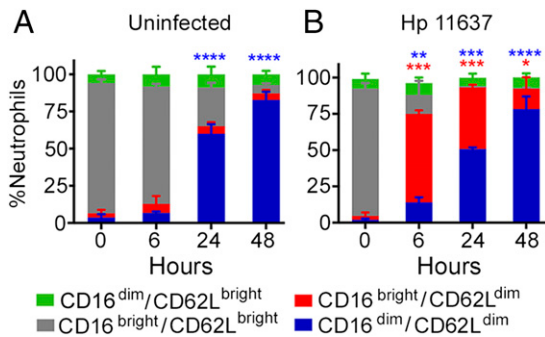


FIGURE 3. *H. pylori*-infected neutrophils have high surface CD16 and low surface CD62L. Control, uninfected (A), and *H. pylori* 11637-infected (B) PMNs were stained for CD16 and CD62L at the noted time points and analyzed by flow cytometry. The percentage of neutrophils in each category is shown as the mean \pm SEM, $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p \leq 0.0001$ versus 0 h.

activating stimuli, including some bacterial pathogens, induce CD62L shedding via ADAM17 activation (28). Although effects of *H. pylori* on ADAM17 are unknown, our data are strong evidence that a distinct PMN subset has been induced.

CD16 mediates opsonophagocytosis and localizes to the Golgi, intracellular vesicles, and cell surface of healthy PMNs (29). Concomitant downregulation of CD16 and CD62L on aged, uninfected neutrophils by 48 h (Fig. 3A, blue bars) indicates progression to apoptosis (18, 22) and is consistent with the nuclear condensation shown in Fig. 1. In contrast, loss of CD16 in the absence of apoptosis, as occurs in *H. pylori* infection, indicates sustained neutrophil activation, and ensues when surface shedding exceeds the capacity to replenish CD16 via new synthesis and secretion (28–30).

During neutrophil activation intracellular granules are mobilized and fuse with the plasma membrane. We analyzed the secretory vesicle marker CD35, the secondary granule marker CD66b, and the primary granule marker CD63, as well as adhesion molecules CD54 (ICAM-1) and CD11b (31) on untreated and *H. pylori*-infected PMNs, and included cells stimulated with *E. coli* LPS as an additional control. All these markers were present on *H. pylori*-infected PMNs (Fig. 4) and on the cells described by Pillay et al. (5). However, in our system, changes in CD11b, CD66b, and CD63 were statistically significant, whereas only CD11b reached significance on the Pillay et al. (5) subset. Metalloproteases and serine proteases released by mobilization of tertiary and primary granules mediate CD16 shedding (32) and likely contribute to the CD16^{dim} phenotype that arises in late *H. pylori* infection. In contrast, *E. coli* LPS elicited conventional activation, defined as moderate upregulation of all markers except CD63 (Fig. 4), and did not elicit hypersegmentation (data not shown), confirming published data (5, 33). Thus, *H. pylori*-infected PMNs are characterized by nuclear hypersegmentation and a CD62L^{dim}, CD16^{bright}, CD11b^{bright}, CD66b^{bright}, CD63^{bright} surface phenotype.

Like myeloid-derived suppressor cells, the PMN subset described by Pillay et al. (5, 6) inhibits T cell proliferation via a contact-dependent mechanism. *H. pylori* arginase (RocF) suppresses T cells directly via arginine depletion (34), and whether infected PMNs synergize with RocF to disrupt T cell function remains to be determined.

We demonstrate instead that *H. pylori*-infected PMNs share many phenotypic and functional properties with a subset of

murine tumor-associated neutrophils (TANs) that are proinflammatory and cytotoxic N1 TANs (8, 10). Both PMN populations are hypersegmented, CD11^{bright} and CD54^{positive}, and are notable for robust extracellular oxidant production (8, 11, 12, 14, 15) (Figs. 1, 4). ROS released by N1 TANs and PMA-activated PMNs are cytotoxic (8, 11, 12), and we show here that *H. pylori*-infected neutrophils killed gastric epithelial cells, whereas untreated PMNs and bacteria alone did not (Supplemental Fig. 2B). *H. pylori*-infected PMNs also resembled N1 TANs (8, 12) in their capacity to secrete multiple cytokines and chemokines including MIP-1, IL-6, IL-8, CXCL1, and IL-1 β that can reinforce neutrophil recruitment and activation (Supplemental Fig. 2C, 2D).

Our findings are noteworthy as there is convincing evidence that PMNs have multiple and potentially paradoxical roles in the development and progression of gastric disease. Sustained recruitment and activation of neutrophils is a defining feature of *H. pylori* infection, and the results presented here extend prior studies of the NADPH oxidase to include granule mobilization and PMN cytokine secretion (14, 15) (Fig. 4, Supplemental Fig. 2C, 2D). In addition to permitting bacterial survival and replication, neutrophil ROS induce DNA damage, and synergize with virulence factors such as CagA and VacA to undermine the integrity, polarity, and viability of the gastric epithelium (35) (Supplemental Fig. 2B).

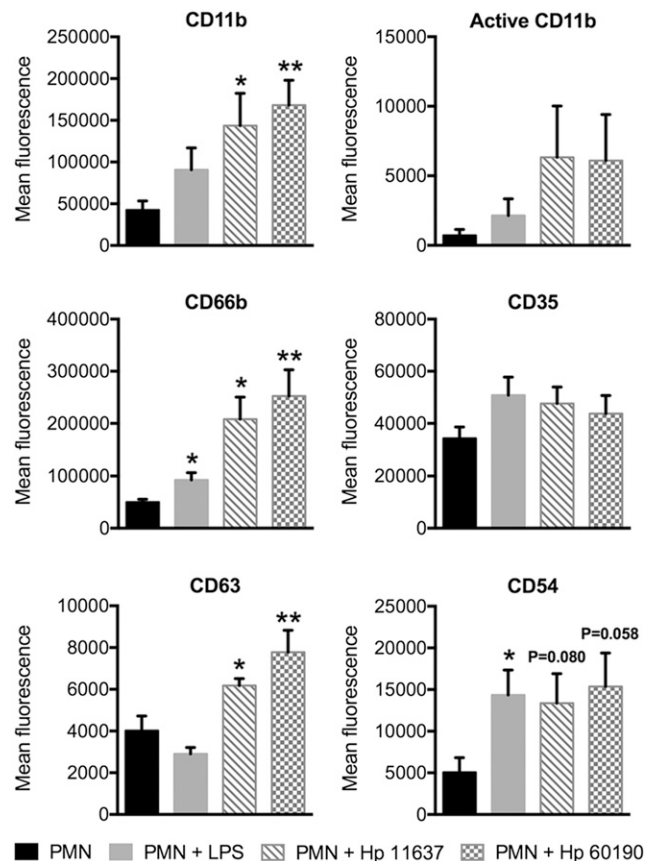


FIGURE 4. *H. pylori*-infected neutrophils display additional surface markers indicative of subtype differentiation. Mean fluorescence of CD11b, active CD11b, CD66b, CD35, CD63, and CD54 was assessed by flow cytometry at 24 h. *H. pylori*-infected neutrophils and neutrophils treated with 10 ng/ml *E. coli* LPS were compared with untreated controls. $n \geq 4$ donors. * $p \leq 0.05$, ** $p \leq 0.01$.

As N1 TANs are cytotoxic and antitumorogenic, it is tempting to speculate that *H. pylori*-infected PMNs may curtail gastric cancer progression. This hypothesis is consistent with the fact that only 1–3% of infected individuals develop gastric adenocarcinoma despite classification of *H. pylori* as a carcinogen and clear evidence that its effects on the epithelium favor cancer initiation (35). Additionally, there is evidence that abundant TANs are beneficial in human gastric cancer patients (10, 36–38). Thus, induction of PMN subtype differentiation may aid in explaining how *H. pylori* is able to chronically infect humans, inducing gastritis and peptic ulceration that rarely progresses to gastric adenocarcinoma (36, 37).

In summary, the concept of neutrophil phenotypic plasticity is new, and although recent studies have begun to identify neutrophil subsets that differ with respect to maturation state, density, nuclear morphology, surface markers, and functional properties (3, 4, 9, 39), we are only beginning to understand the range of phenotypes that these cells can exhibit. In discovering the ability of *H. pylori* to induce PMN subtype differentiation *in vitro*, we reinforce the notion that mature human neutrophils retain a capacity for phenotypic plasticity that can be evoked in response to specific stimuli in the absence of *in vivo* cues, and argue against models which propose that these cells only arise from a distinct developmental lineage (6). The PMNs we describe are hypersegmented, proinflammatory, and cytotoxic, and exhibit changes in surface markers that are indicative of strong and sustained activation. Accordingly, these N1-Hp cells share many features with, yet appear distinct from, other PMN subtypes described to date. At the same time, the requirement for *H. pylori*–PMN contact and both host and bacterial protein synthesis, but not urease, NapA, VacA, or CagA, suggests a complex underlying mechanism that merits further study and may reveal new aspects of bacterial pathogenesis. Collectively, our data advance the rapidly evolving field of neutrophil plasticity, and we hypothesize that manipulation of PMN phenotype and function is inherently linked to *H. pylori* persistence and its ability to elicit a spectrum of human disease.

Disclosures

The authors have no financial conflicts of interest.

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