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Complete List of Authors:	de Bernard, Marina; University of Padua, Department of Biology; Venetian Institute of Molecular Medicine, Padua, VIMM Cappon, Andrea; Venetian Institute of Molecular Medicine, Padua, VIMM Babolin, Chiara; Venetian Institute of Molecular Medicine, Padua, Dept of Biomedical Sciences, University of Padua Segat, Daniela; University of Padua, Department of Biomedical Sciences Cancian, Laila; Imperial College, Department of Virology Amedei, Amedeo; Azienda Ospedaliera Universitaria Careggi, Department of Biomedicine; University of Florence, Department of Internal Medicine Calzetti, Federica; University of Verona, Department of Pathology Cassatella, Marco; University of Verona, Department of Pathology D'Elios, Mario; Azienda Ospedaliera Universitaria Careggi, Department of Biomedicine
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Helicobacter pylori derived Neutrophil Activating Protein (HP-NAP) increases the life span of monocytes and neutrophils and activates B cells

Andrea Cappon[1*], Chiara Babolin[1*], Daniela Segat[2], Laila Cancian[3], Amedeo Amedei[4,5], Federica Calzetti[6], Marco A. Cassatella[6], Mario M. D'Elios[4,5], and Marina de Bernard[1,7]

[1] Venetian Institute of Molecular Medicine, Padua, Italy

[2] Department of Biomedical Sciences, University of Padua, Padua, Italy

[3] Department of Virology, Faculty of Medicine, Imperial College London Imperial College, London, UK

[4] Department of Internal Medicine, University of Florence, Florence, Italy

[5] Department of Biomedicine, Azienda Ospedaliera Universitaria Careggi, Florence, Italy

[6] Department of Pathology, Division of General Pathology, University of Verona, Strada Le Grazie 4, Verona

[7] Department of Biology, University of Padua, Padua, Italy

*These authors equally contributed to the work

Running Title: HP-NAP promotes monocyte and neutrophil survival

Address correspondence to:

Marina de Bernard, Venetian Institute of Molecular Medicine, Via Orus 2, 35129 Padua Italy

e-mail address: marina.debernard@unipd.it, Phone: +39.049.7923223, Fax: +39.049.7923250

Summary

An invariable feature of *Helicobacter pylori*-infected gastric mucosa is the persistent infiltration of inflammatory cells. The neutrophil activating protein (HP-NAP) has a pivotal role in triggering and orchestrating the phlogistic process associated with *H. pylori* infection. Aim of this study was to address whether HP-NAP might further contribute to the inflammation by increasing the life span of inflammatory cells. We report that HP-NAP is able to prolong the life span of monocytes, in parallel with the induction of the anti-apoptotic proteins A1 and Mcl-1. This effect does not result from a direct action on the apoptotic machinery, but rather it requires the release of pro-survival factors. Accordingly, HP-NAP stimulates monocytes to secrete TNF-α, IL-1β and GM-CSF. We also report that the bacterial protein promotes the survival of Ficoll-purified neutrophils. However, using mononuclear cell depletion by negative selection, we found that neutrophil survival induced by HP-NAP was actually monocyte-dependent. Finally, we demonstrate that T cells, activated by HP-NAP-treated monocytes, trigger the activation of B cells to produce antibodies. In conclusion, our data not only reinforce the notion that HP-NAP has a pivotal role in sustaining a prolonged activation of myeloid cells but also in triggering the production of antibodies.

Introduction

Helicobacter pylori (Hp) infection is worldwide known as the leading cause of gastritis, peptic ulcer and ultimately of gastric cancer. Colonization of stomach by *Helicobacter pylori* is followed by a mucosa infiltration of polymorphonuclear leukocytes (PMNs), monocytes/macrophages and T helper (Th) 1 lymphocytes, with active production of interleukin (IL)-12 and interferon (IFN)-γ (D'Elios *et al.*, 1997). The host immune response to the infection is largely ineffective, as the bacterium persists and the inflammation continues for decades. A prolonged inflammation can predispose to neoplastic transformation (Mantovani *et al.*, 2008) and an increase in life-span of innate immune cells is postulated to contribute to the exacerbation of inflammation. Indeed, the activation of monocytes and neutrophils must be tightly regulated in order to avoid damage to surrounding healthy tissue and to help resolving inflammation (Savill, 1997; Serhan and Savill, 2005). Accordingly, there is compelling evidence that cell death by apoptosis plays a major role in promoting resolution of the acute inflammatory response. Neutrophils, for instance, are constitutively programmed to undergo apoptosis, and such a process limits their pro-inflammatory potential and leads to rapid and non-phlogistic recognition by macrophages. Similar mechanisms have been implicated also in the clearance of monocytes and lymphocytes (Ramsdell *et al.*, 1994). However, the possibility that the bacterium increases the life-span of these cells, by preventing the natural occurring apoptosis, thus causing the prolongation of the phlogistic process, is an issue almost fully unexplored. It has been reported that *Helicobacter pylor*i water extracts inhibited neutrophil apoptosis (Kim *et al.*, 2001; Kim *et al.*, 2001), but the bacterial factor(s) responsible for such an effect remained unidentified.

The neutrophil-activating protein (HP-NAP) produced by *H. pylori* is a dodecameric protein of 150 kDa, with a structure similar to that of bacterioferritin, including a central cavity for iron accumulation (Tonello *et al.*, 1999; Zanotti *et al.*, 2002). It was initially identified as a promoter of

endothelial adhesion of neutrophils, and was defined as neutrophil-activating protein because it stimulates a remarkable production of oxygen radicals from PMNs (Evans *et al.*, 1995). In addition, HP-NAP increases the synthesis of tissue factor (TF) and the secretion of type-2 plasminogen activator inhibitor in monocytes (Montemurro *et al.*, 2001; Montecucco and de Bernard, 2003). HP-NAP is chemotactic for neutrophils, and it participates *in vivo* in creating a peculiar cytokine milieu at the site of infection (Amedei *et al.*, 2006; Polenghi *et al.*, 2007). In virtue of the latter activity, we demonstrated that HP-NAP is crucial for driving the differentiation of Th cells into the Th1 phenotype, a polarized response that occurs in the stomach of infected individuals and that is associated with more serious diseases (D'Elios *et al.*, 1997; D'Elios *et al.*, 2007). Furthermore, HP-NAP activity on monocytes resulted in sustaining a progressive maturation into mature dendritic cells (Amedei *et al.*, 2006).

Based on these premises, we investigated in a more detailed manner whether HP-NAP might interfere with the natural apoptotic process in monocytes and neutrophils. Our results indicate that: i) HP-NAP promotes the survival of monocytes; ii) the regulation of monocyte life span by HP-NAP occurs following the induction of the anti-apoptotic genes Mcl-1 and A1; iii) the bacterial protein promotes the survival of neutrophils, but such an effect requires the presence of monocytes; iv) T cells, activated by HP-NAP-treated monocytes, trigger the activation of B cells to produce antibodies. Collectively, our data suggest that HP-NAP has a pivotal role in sustaining a prolonged activation of monocytes and neutrophils and in triggering the production of antibodies.

Results

Monocytes exposed to HP-NAP are protected from apoptosis

To investigate the rate of monocyte survival after HP-NAP exposure, we monitored the percentage of apoptotic cells in the presence or absence of 1 μM HP-NAP for several days. The evaluation, carried on by the Annexin V staining, clearly revealed that while untreated monocytes progressively underwent apoptosis and were all died between 3 and 5 days, the addition of HP-NAP to the culture medium totally protected the cells from apoptosis. Moreover, such an effect was maintained for at least 7 days, even if the bacterial protein was not re-added (Fig. 1A). We next examined monocyte viability after 72 h-culture in the absence or presence of lower concentrations of HP-NAP and we found that the protein was able to significantly sustain cell survival already at 125 nM (Fig. 1B), a concentration 10 times lower than that considered to be the threshold for any biological effect ascribed to HP-NAP. Because caspase-3 is an executioner caspase for the apoptotic process, and it originates from the cleavage of the inactive precursor pro-caspase-3 (Salvesen and Dixit, 1997), we evaluated the levels of the precursor as indicator for the generation of the active form of the enzyme. Fig. 2 shows that, while in untreated monocytes the uncleaved inactive caspase was totally absent, the treatment with HP-NAP for 72 h significantly reduced such a cleavage. Caspase-3 activates the poly-ADP-ribose polymerase (PARP) by cleaving it at the sequence Asp-Glu-Val-Asp, causing the irreversible nuclear alteration (Depraetere and Golstein, 1998). Accordingly, HP-NAP administration prevented the polymerase cleavage (Fig. 2).

HP-NAP-induced suppression of apoptosis is a transferable effect

To further clarify the mechanism of action of HP-NAP, we first determined the minimum time required to achieve its pro-survival effect: hence, monocytes were exposed to HP-NAP for 2, 4, 8, 24 h before being washed and left in culture for additional 72 h. As positive control, monocytes were treated with the bacterial protein for 96 h. Surprisingly, the Annexin-V staining revealed that a 2 h-incubation with HP-NAP was sufficient to suppress apoptosis with the same efficiency of the 96

h-incubation (Fig. 3A). Next, we examined whether the pro-survival effect of HP-NAP resulted from a direct effect on the cells rather than from an induction of trophic factor release. To address this point, monocytes were exposed to HP-NAP for 2 h; then, after extensive washings to remove the unbound protein, cells were incubated in a fresh medium for additional 2, 4, 8 and 24 h. At the end of incubations, culture supernatants were collected and transferred on naïve autologous monocytes; finally, 72 h later viability was assessed. Fig. 3B clearly shows that modulation of apoptosis exerted by HP-NAP is mostly due to the endogenous production of pro-survival factors: in fact, while the administration to naïve monocytes of medium conditioned by untreated cells did not result in an increased viability, the medium of HP-NAP-exposed monocytes was able to exert a strong pro-survival effect (Fig. 3B). To confirm our observation we blocked the exocytosis in HP-NAP-exposed monocytes with monensin, before transferring culture supernatant to naïve monocytes. Such a procedure was preferred with respect to the direct evaluation of HP-NAP/monensin-treated monocytes in order to exclude any bias due to drug toxicity. Fig. 3C shows that the pre-treatment of monocytes with monensin abrogated the ability of HP-NAP to counteract apoptosis. This latter result definitely ruled out that the pro-survival effect exerted on naïve monocytes exposed to the supernatant of HP-NAP-treated monocytes (Fig. 3B) was due to a small fraction of bacterial protein remained in the medium.

HP-NAP induces the expression of IL-β, TNF-α and GM-CSF mRNA and protein

It has been demonstrated that an improvement of monocyte survival can be achieved by the addition of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and GM-CSF (Mangan and Wahl, 1991; Moulding *et al.*, 1998; Flad *et al.*, 1999). In a previous report we demonstrated that HP-NAP stimulates monocytes to secrete TNF-α (Amedei *et al.*, 2006) but the possibility that IL-1 β and GM-CSF were also induced has not been yet investigated. To shed light upon this aspect, total RNA was extracted from monocytes incubated with HP-NAP and examined by quantitative real-time PCR. As shown in Fig. 4, treatment with HP-NAP resulted in a

rapid induction of IL-1β mRNA that reached maximum level within the first 2 hours and slowly declined thereafter. In line with the gene expression data, culture supernatant of monocytes stimulated with HP-NAP showed a remarkable time-dependent extracellular production of IL-1β. Furthermore, the transcription of TNF-α gene was rapidly induced by HP-NAP, in agreement with our previous data (Amedei et al., 2006), in addition to that of GM-CSF, although to a lesser extent. For both TNF-α and GM-CSF the kinetics of their mRNA levels were consistent with the kinetics of protein accumulation in the supernatant.

HP-NAP induces the anti-apoptotic genes A1 and Mcl-1

The Bcl-2 family of proteins is known to play a role in regulating apoptosis (Yang and Korsmeyer, 1996; Lagasse and Weissman, 1997). Therefore, we examined whether expression of the anti-apoptotic members of the Bcl-2 family in monocytes was affected by HP-NAP. In monocytes exposed to the bacterial protein, the anti-apoptotic protein A1 was significantly induced at 24 h and increased further at 48-72 h; by contrast, unstimulated monocytes showed a transient and slight accumulation of A1 at 24 h which thereafter progressively decreased returning to the basal level after 72 h (Fig. 5). Even though to a lesser extent, we found that also the expression of Mcl-1 was affected. In fact HP-NAP exposure for 24 h maintained Mcl-1 protein accumulation at a level similar to that of freshly isolated monocytes and, although the amount progressively reduced along the incubation period, it was still appreciable after 72 h. By contrast, the same evaluation carried on monocytes cultured in normal medium, revealed a partial decrease in the protein amount already after 24 h and its complete disappearance after 72 h (Fig. 5). Mcl-1 was maintained also in naive monocytes once exposed to the supernatant from HP-NAP-treated monocytes; as expected, blockage of exocytosis in donor monocytes resulted in a complete abrogation of Mcl-1 induction in the recipients (data not shown). Finally, no change in Bcl-X_L/X_S and Bcl-2 was found either in donor or in recipient monocytes after HP-NAP administration (data not shown).

HP-NAP modulates neutrophils survival through the intervention of monocytes

It has been demonstrated that *H. pylori* water-soluble surface proteins suppress neutrophil apoptosis (Kim *et al.*, 2001). Considering that HP-NAP is released by the bacterium other than weakly bound to the surface, we addressed the possibility that HP-NAP might be one of the factors capable of modulating the survival of neutrophils. In a first set of experiment we used polymorphonuclear cells (PMNs) purified by dextran sedimentation and density gradient (Ficoll-Paque) separation, a protocol commonly used, which guarantees a cell purity around 95%. The administration of HP-NAP to these cells significantly delayed the naturally occurring apoptosis: indeed, while in the absence of stimuli viable cells were 47% ± 2% (mean ± SD) and 20% ± 10% of the original input, after 18 and 48 h, respectively, in the presence of HP-NAP the percentage of survival was 85% ± 9% after 18 h and 46% ± 10% after 48 h. To exclude that the effect on PMN survival was biased by the presence of contaminant monocytes, we repeated the experiments by adding HP-NAP to neutrophil populations isolated by magnetic negative selection - thus highly purified (> 99.7 %) and devoid of any lymphocytes ($CD3^+$ $CD16^-$ $CCR3^-$), monocytes or eosinophils ($CD16^{low}$ $CCR3^+$) that often contaminate Ficoll-Paque isolated granulocytes (Tamassia *et al.*, 2008). Surprisingly, under these conditions, the administration of HP-NAP resulted in a PMN survival totally superimposable to that of control (untreated cells), while G-CSF remained highly effective in promoting PMN survival, as expected (Brach *et al.*, 1992; Calotta *et al.*, 1992), (Fig. 6, upper panels). However, if ultra pure PMNs were co-cultured with 0.5 or 2% of monocytes, then HP-NAP was able to promote PMN survival already at the lower percentage of monocytes (Fig. 6, middle and lower panels). Strikingly, 2% of monocytes maintained neutrophils vital to an extent comparable to that achieved by G-CSF. Collectively, these results demonstrate that HP-NAP has little role in the direct regulation of neutrophil apoptosis, but that in the presence of monocytes, it may become a potent neutrophil survival factor.

Immunoglobulin production induced in B cells by HP-NAP activation of antigen-specific Th cells

Finally, we tested whether HP-NAP might activate T cell-dependent B cell activation. In fact, T cell-dependent B cell activation is a multistep process involving contact help and the action of T cell-derived cytokines (Kehry and Hodgkin, 1993; Parker, 1993). Engagement of T cell receptor molecules with MHC-antigen complexes presented by B cells ascertains antigen specificity in T cell-dependent help. Ligation of MHC molecules on the surface of B cells, however, has not only been implicated in antigen-specific T-B cell interaction, but has also been linked to the induction of B cell apoptosis. Differences in the outcome of T-B cell interaction resulting in either immunoglobulin production or B cell depletion have been described in *H. pylori* infection (D'Elios et al., 2005). Accordingly, we investigated the ability of HP-NAP-specific T cell clones to provide B cell help for immunoglobulin (Ig) production. To this purpose, T cell blasts were co-cultured at 1/1 ratio with autologous B cells in the absence or presence of HP-NAP and then IgM, IgG, and IgA levels were measured in cell-free culture supernatants at day 10. In the absence of antigen, no increase in IgM, IgG, and IgA production above the spontaneous levels measured in cultures containing B cells alone was observed. However, In the presence of HP-NAP, all 30 HP-NAP-specific clones provided substantial help for antibody production (Fig. 7).

Taken together, these results, which show that HP-NAP-specific Th clones generated from the gastric antrum of *H. pylori*-infected patients display powerful helper function for Ig production, suggest that HP-NAP contributes to maintain gastric inflammation not only by increasing the life span of neutrophils and monocytes, but also by activating B cells.

Discussion

It has been demonstrated that the neutrophil activating protein (HP-NAP) exerts a pivotal role in the orchestration of the innate and adaptive immune response occurring in the gastric mucosa of *Helicobacter pylori*-infected patients (D'Elios *et al.*, 2007). Among the multiple activities ascribed to HP-NAP, the activation of neutrophils and monocytes to release IL-12 and IL-23 responsible of the differentiation of T helper cells towards the Th1 phenotype, is recognized. Interestingly, Th1 lymphocytes are the most abundant lymphocytes subset in the gastric mucosa of *H. pylori* positive patients and a consistent percentage of them are HP-NAP specific (Amedei *et al.*, 2006).

Human monocytes undergo programmed cell death when cultured for more than 8 h in the absence of appropriate stimuli. However, when exposed to LPS or to cytokines such as IL-1β, TNF-α or GM-CSF, apoptosis is reduced and monocytes remain viable and functionally active (Mangan and Wahl, 1991). In the present study, we addressed the ability of HP-NAP in exerting a pro-survival effect on monocytes, the cell type which, together with neutrophils, mostly accumulate in the gastric mucosa of *H. pylori* infected patients. We demonstrated that HP-NAP exerts an anti-apoptotic effect on monocytes which results in the maintenance of their survival for several days even if the exposure time is limited (2 h) and the concentration of the protein as low as 125 nM; accordingly, we found that HP-NAP-exposed monocytes do not undergo activation of both caspase 3 and poly-ADP-ribose polymerase (PARP), an executioner caspase and one of its substrates, respectively. Concomitantly, monocytes acquire a dendritic cell-like phenotype with high expression of MHC class II and co-stimulatory molecules, as we previously reported (Amedei *et al.*, 2006).

Interestingly, the anti-apoptotic effect exerted by HP-NAP does not reflect a direct action on the apoptotic machinery, rather it results from the activity of trophic factors released by monocytes following their contact with HP-NAP; accordingly, the effect on cell viability was transferable and

was abrogated once exocytosis was blocked. Since we also show that HP-NAP is able to trigger the release of a number of well known pro-survival cytokines, including IL-1β, TNF-α and GM-CSF by monocytes (Mangan and Wahl, 1991; Flad *et al.*, 1999), the latter represent putative candidate in mediating the HP-NAP effects. In parallel, HP-NAP-exposed monocytes undergo to a strong up-regulation of A1, and maintain high the expression of Mcl-1 for 48 h before slightly declining. The intracellular high levels of these two anti-apoptotic proteins rely on the induction of trophic factors, since the blockage of exocytosis in HP-NAP-stimulated monocytes impaired their expression and, as expected, the cell survival.

Considering that *H. pylori* colonization is typically followed by infiltration of the gastric mucosa by polymorphonuclear leukocytes, besides macrophages and lymphocytes (Dixon *et al.*, 1996; D'Elios *et al.* 1997) and a strong correlation exists between gastric infiltration by neutrophils, mucosal damage, and development of duodenal ulcer disease in *H. pylori* infections (Davies *et al.*, 1994; Hamlet *et al*., 1999), we moved to evaluate whether HP-NAP could also modulate the life-span of neutrophils. The interest in addressing this issue was reinforced by the previous finding of Kim and colleagues (2001), which highlighted the ability of *Helicobacter pylori* water-soluble surface proteins in preventing neutrophil apoptosis. HP-NAP, abundantly released by the bacterium, is also weakly bound to the surface; thus we considered the possibility that it might be one of the factors responsible for the modulation of the neutrophil viability. In a first set of experiments performed on neutrophils purified by density gradient separation, HP-NAP significantly delayed the constitutive apoptosis; however, when we carried on the experiment on highly purified neutrophils, in order to exclude any bias due to the contaminant monocytes, we could not appreciate any protection from the natural occurring apoptosis. This observation suggested that HP-NAP ability in sustaining neutrophil survival could be monocyte-dependent. Accordingly, when ultra pure neutrophils were exposed to the bacterial protein in the presence of 0.5% of monocytes their viability increased two times with respect to ultrapure cells; notably, in the presence of 2% of monocytes. cells remained viable at the same extent than after the exposure to G-CSF.

Although we cannot exclude that other water-soluble surface proteins, different from HP-NAP, directly act on neutrophils sustaining their survival, as suggested (Kim *et al.*, 2001), the possibility exists that the reported results were biased by the presence of monocytes in the neutrophil preparation.

Finally, the present results which show that HP-NAP-specific T helper clones, generated from the gastric tissues of *H. pylori*-infected patients, stimulate immunoglobulin production by B cells, permit to speculate that the chronic B-cell activation induced by *H. pylori* and helped by T cells is responsible for the significant levels of HP-NAP specific antibodies found in the serum of infected patients, which decrease only after *H. pylori* eradication.

In summary, our data reinforce the notion that HP-NAP has a pivotal role in sustaining a prolonged activation of myeloid cells and as such it exerts an essential contribution in triggering and maintaining inflammation. On the other hand the ability of HP-NAP to promote antibody production reinforces the notion that HP-NAP represents an optimal vaccine candidate against *H. pylori* infection (Malfertheiner et al., 2008).

Experimental procedures

Reagents

HP-NAP was cloned, expressed, and purified from *Bacillus subtilis* to avoid contamination with LPS, as described previously (Tonello *et al.*, 1999). Annexin V FITC assay was from Bender Medsystem (Vienna, Austria). Monensin and monoclonal anti-GAPDH antibody were purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal antibody against Mcl-1 was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Polyclonal anti-PARP and anti-Caspase-3 antibodies were from Cell Signalling (Beverly, MA). Polyclonal antibody anti-A1 was from Lifespan Biosciences (Seattle, WA). G-CSF was from Chugai Pharmaceutical (Tokio, Japan). Other reagents were obtained from Sigma-Aldrich unless otherwise specified. Phosphate buffered saline (PBS), RPMI 1640 and gentamicin were from Invitrogen (Carlsbad, CA). FCS was from Euroclone (Siziano, Italy). Solutions used throughout the experiments were prepared with endotoxin-free water (Sigma-Aldrich).

Purification of monocytes and neutrophils

Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by centrifugation on Ficoll-Paque solution (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). PBMC were then laid on a cushion of Percoll 46% v/v solution (Amersham) in RPMI 1640 supplemented with 10% FCS, 50 μg/ml gentamicin and 4 mM Hepes (Gibco, United Kingdom). Monocytes were harvested, resuspended in medium 2% FCS and further separated from contaminating lymphocytes by adherence (1 h at 37°C) to plastic wells. Adherent monocytes were extensively washed with medium to remove residual non-adherent cells. The percentages of $CD14^+$ cells were >98%. Monocytes were then cultured in RPMI 1640 10% FCS, 50 μg/ml gentamicin. Human neutrophils were prepared from healthy donors as previously described using dextran sedimentation, centrifugation through Ficoll-Paque and hypotonic lysis of contaminating erythrocytes (Rossi *et al.*, 1989). The percentage of contaminating cells were <5%. Neutrophils

were then cultured in RPMI 10% FCS. In some experiments, Ficoll-Paque-isolated neutrophils were further enriched to reach a 99.7% purity by positively removing eventual contaminating cells (T cells, NK cells, B cells, monocytes, DCs, platelets, eosinophils, or erythrocytes) with antibodies against CD3, CD56, CD19, CD36, CD49d, and Gly-A using a custom-made EasySep kit (StemCell Technologies).

Detection of apoptosis

Monocytes and neutrophils (2×10^6) cultured in presence or absence of HP-NAP in RPMI 1640 10% FCS, 50 μg/ml gentamicin at 37°C were stained with Annexin V/propidium iodide. Viability was measured by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA) as the percentage of Annexin-V/PI-negative events in the gated population.

Real-time PCR analysis

Total RNA was isolated from 2×10^6 monocytes using TRIzol solution (Invitrogen, San Diego, CA) according to the manufacturer's instructions. RNA was reverse-transcribed and amplified with the following primers: for GAPDH, 5'-AGCAACAGGGTGGTGGAC-3' and 5'-GTGTGGTGGGGGACTGAG-3'; for IL-1β, 5'-CTGTCCTGCGTGTTGAAAGA-3' and 5'-TTGGGTAATTTTTGGGATCTACA-3'; for TNF-a, 5'-ATGAGCACTGAAAGCATGATCC-3' and 5'-GAGGGCTGATTAGAGAGAGGTC-3'; for GM-CSF, 5'-TCTCAGAAATGTTTGACCTCCA-3' and 5'- GCCCTTGAGCTTGGTGAG-3'. After the amplification, data analysis was performed using the second derivative method algorithm. For each sample, the amount of messenger RNA (mRNA) of the single cytokine was expressed as the *n*-fold of the normalized amount of mRNA in untreated cells (1 arbitrary unit = cytokine mRNA concentration/GAPDH mRNA concentration [both in fmoles/μl]).

Detection of IL-1β, TNF-α and GM-CSF in culture supernatants

Culture supernatants of monocytes harvested for mRNA quantification were collected at the same time points, and the amounts of IL-1β, TNF-α and GM-CSF protein were quantified by commercial ELISA (kits from BioSource International, Camarillo, CA).

Immunoblot analysis

Monocytes, stimulated with or without HP-NAP, were collected at the indicated times (0, 24, 48 and 72 h), washed in ice-cold PBS and lysed with ice-cold lysis buffer (20 mM Hepes, pH 7.4, 50 mM NaCl, 1% Triton X-100 supplemented with protease inhibitors, Roche) for 30 min at 4°C. Lysates were centrifuged at 12000 g for 20 min at 4°C, and equal amount of each cell extract was applied on a 4-12% SDS-PAGE and analyzed by immunoblotting. Immunoreactive bands were revealed with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) combined with the enhanced chemiluminescence system (Millipore).

Generation of HP-NAP specific T cell clones and assays for helper function to B cells by HP-NAP activated T cell clones

30 HP-NAP specific T-cell clones were derived form the antral gastric mucosa of six *H. pylori*-infected patients, as previously described (Amedei *et al*. 2006). Mononuclear cell suspensions were obtained from peripheral blood by centrifugation on Ficoll-Hypaque gradient. Isolation of B cells from PBMC was performed by using the B cell isolation kit II (Miltenyi Biotec.). The ability of HP-NAP-stimulated T cell clones to induce B cell activation and Ig production was investigated by co-culturing B cells (5×10^4) alone or with autologous clonal T cell blasts (5×10^4) in the absence or presence of HP-NAP. The induction of Ig synthesis was performed in a cell culture system consisting of duplicate tubes by using complete medium supplemented with 10% FCS, as described

(D'Elios *et al*, 1997). After 10 days, culture supernatants were collected and assayed for their Ig content, as previously described (Romagnani *et al.*, 1983).

Statistical analysis

Data were expressed as mean values ± standard deviation. Student's *t*-test was used for statistical analysis. A *p*-value equal or below 0.05 was considered as significant.

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Figure Legends

Fig. 1. HP-NAP prevents monocytes apoptosis. (A) Human monocytes (2×10^6 cells) were treated with HP-NAP 1 μM or PBS (vehicle). At the indicated time points cells were collected and labeled with Annexin V FITC and Propidium iodide. Viability was determined cytofluorimetrically and expressed as the percentage of Annexin-V/PI-negative cells. Data represent mean ± SD of five independent experiments. (B) 2×10^6 monocytes were stimulated 72h with different HP-NAP concentrations (from 62 nM to 1 μM). Viability was determined cytofluorimetrically and expressed as the percentage of Annexin-V/PI-negative cells as in (A). Significance was determined by Student's *t* test for paired data of HP-NAP-treated cells *vs* untreated cells; ***, $p<0.01$.

Fig. 2. HP-NAP prevents caspase-3 and PARP activation. 2×10^6 monocytes incubated or not with HP-NAP 1 μM for 72 h, were lysed and equal amounts of cell extract were separated by SDS-PAGE and immunoblotted. Pro-caspase 3 and PARP were revealed with specific polyclonal antibodies; an anti-GAPDH monoclonal antibody was used as control for equal loading. A time 0 cell lysate was considered as reference.

Fig. 3. The pro-survival effect of HP-NAP is rapid and mediated by secreted factors. (A) Human monocytes (2×10^6 cells) were exposed to 1 μM HP-NAP for 2, 4, 8 and 24 h; afterward cells were extensively washed, and incubated in fresh medium for 72 h. As positive control, cells were maintained in HP-NAP-conditioned medium for 96 h. Monocytes exposed to PBS (vehicle) represented the negative control. Cells were processed for the Annexin V-FITC assay, as reported in legend of Fig. 1. (B) 2×10^6 monocytes were exposed to 1 μM HP-NAP for 2 h. Cells were extensively washed, incubated with fresh medium for additional 2, 4, 8 and 24 h before the cell supernatant (Sup) being transferred on naïve autologous monocytes. After 72 h recipient cells were collected and labelled with Annexin V-FITC and Propidium iodide to evaluate cell viability.

Monocytes recipient of supernatant from PBS (vehicle)-exposed monocytes were considered as negative control. (C) 2×10^6 monocytes pre-incubated or not with 10 μM monensin for 1 h were treated with 1 μM HP-NAP for 2 h. After extensive washing, cells were incubated in fresh medium for additional 4 and 24 h before transferring supernatants on naive autologous monocytes. After 72 h, recipient cells were collected and labelled with Annexin V-FITC and Propidium iodide to evaluate cell viability. Significance was determined by Student's *t* test; ***, $p<0.01$.

Fig. 4. Time dependent mRNA expression and protein production for IL-1β, TNF-α and GM-CSF in HP-NAP treated-monocytes. Total RNA was extracted from monocytes cultured with HP-NAP and analyzed by real-time PCR for evaluate the expression of each cytokine. Expression levels are depicted as *n*-folds of the normalized amount of mRNA from untreated cells (1 AU = mRNA cytokine concentration [fmol/μl]/mRNA GAPDH [fmol/μl]) of triplicate reactions for each sample. Kinetic of extracellular production of IL-1β, TNF-α and GM-CSF by HP-NAP-treated monocytes is also reported. The figure shows the mean value ± SD of duplicate assays for each time point, obtained from three experiments performed under the same conditions.

Fig. 5. HP-NAP-exposed monocytes increase the production of the anti-apoptotic proteins A1 and Mcl-1. 2×10^6 monocytes, stimulated or not with 1 μM HP-NAP, were lysed at indicated time and equal amounts of cell extract were separated by SDS-PAGE and immunoblotted. A1 and Mcl-1 were revealed with specific polyclonal antibodies; an anti-GAPDH monoclonal antibody was used as control for equal loading.

Fig. 6. HP-NAP modulates netrophils survival trough the mediation of monocytes. Highly purified PMNs were incubated with or without 1000 U/ml G-CSF or 1 μM HP-NAP and in absence or presence of either 0.5% or 0.2% of monocytes.. After 18 h cells were harvested and stained with

AnnexinV-FITC and PI for cell viability evaluation. Values indicated in the left lower quadrants correspond to vital cells (Annexin V/PI negative cells). Plots are relative to PMNs only gated on the base of physical parameters (FSC vs SSC plots).

Fig. 7. In vitro synthesis of IgG, IgA, and IgM induced in autologous B cells by HP-NAP-specific Th clones stimulated with the specific antigen. T cell blasts from each clone (5×10^4) were cultured with autologous B cells (5×10^4) in the presence of HP-NAP. After 10 days, cell-free culture supernatants were assayed for their Ig content. The results represents the mean (± SD) Ig levels induced by HP-NAP-specific Th clones over the spontaneous Ig production in cultures of B cells alone.

Acknowledgements

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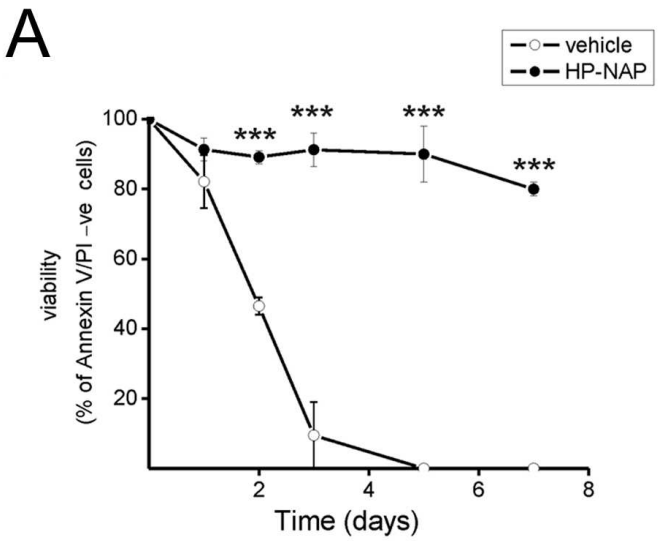

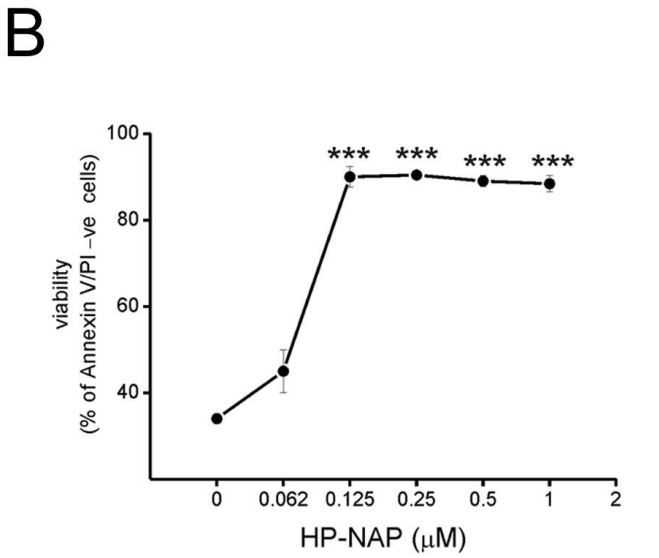

Figure 1

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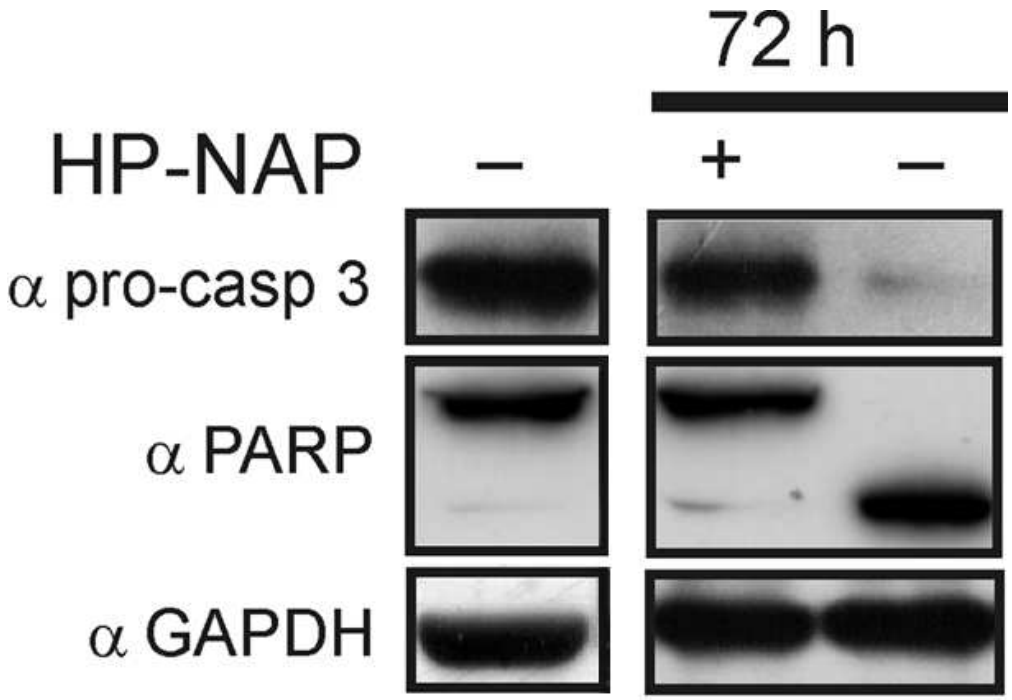

Figure 2

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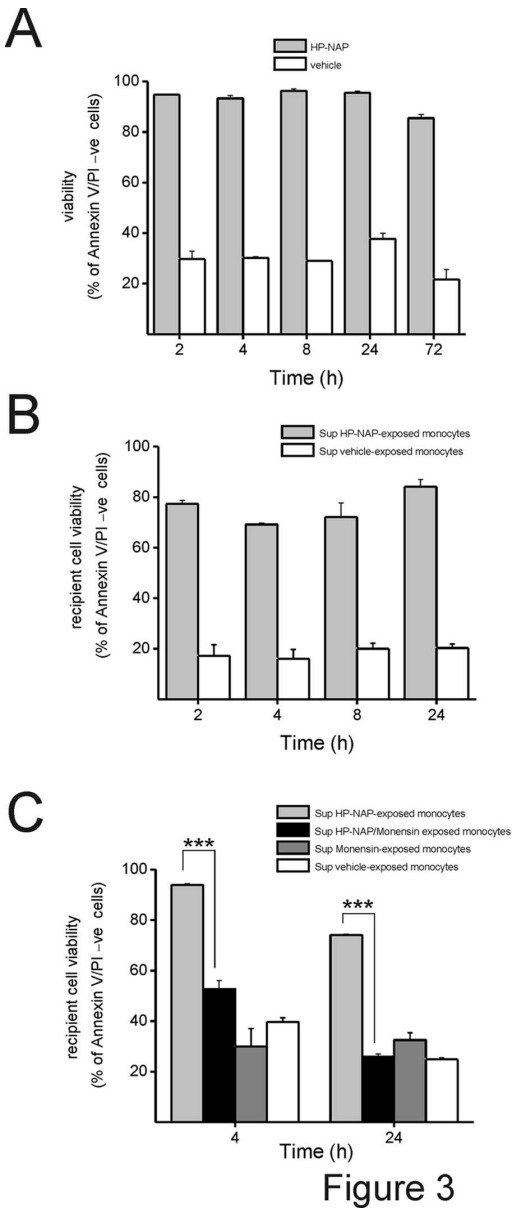

Figure 3

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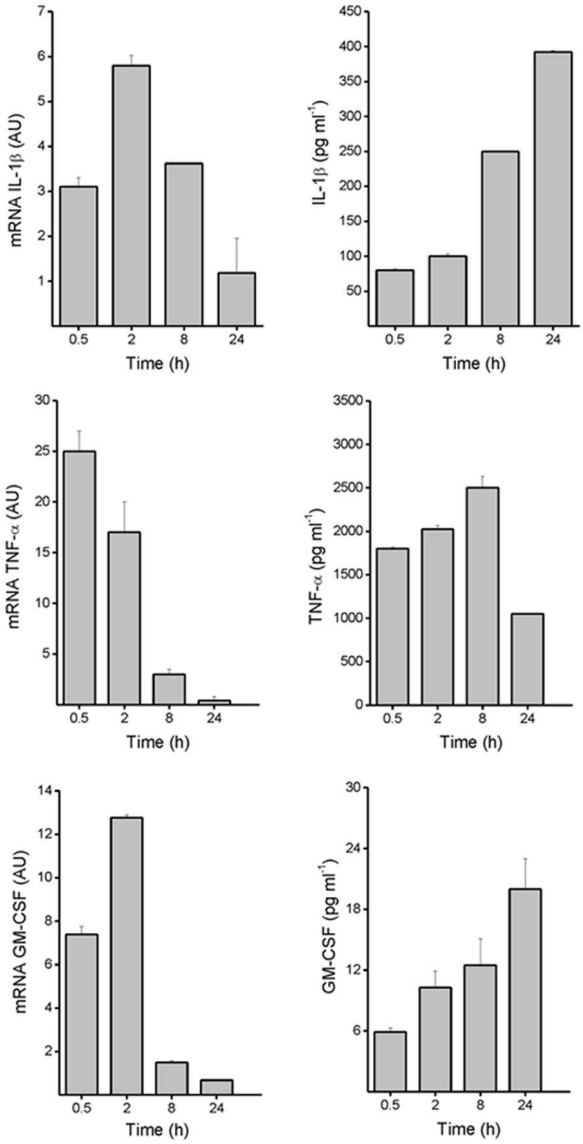

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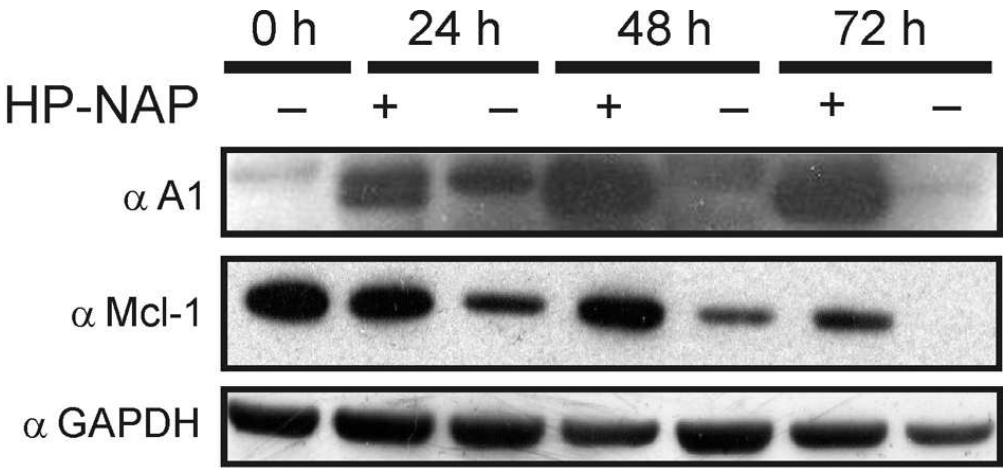

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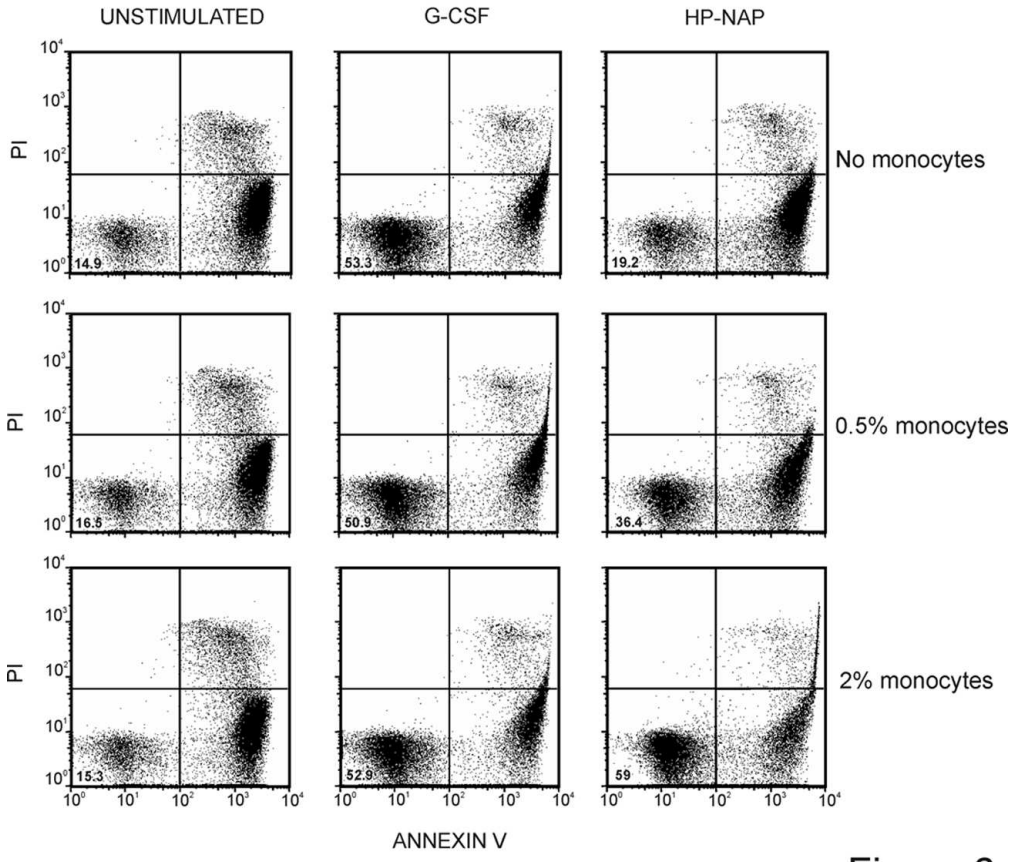

Figure 6

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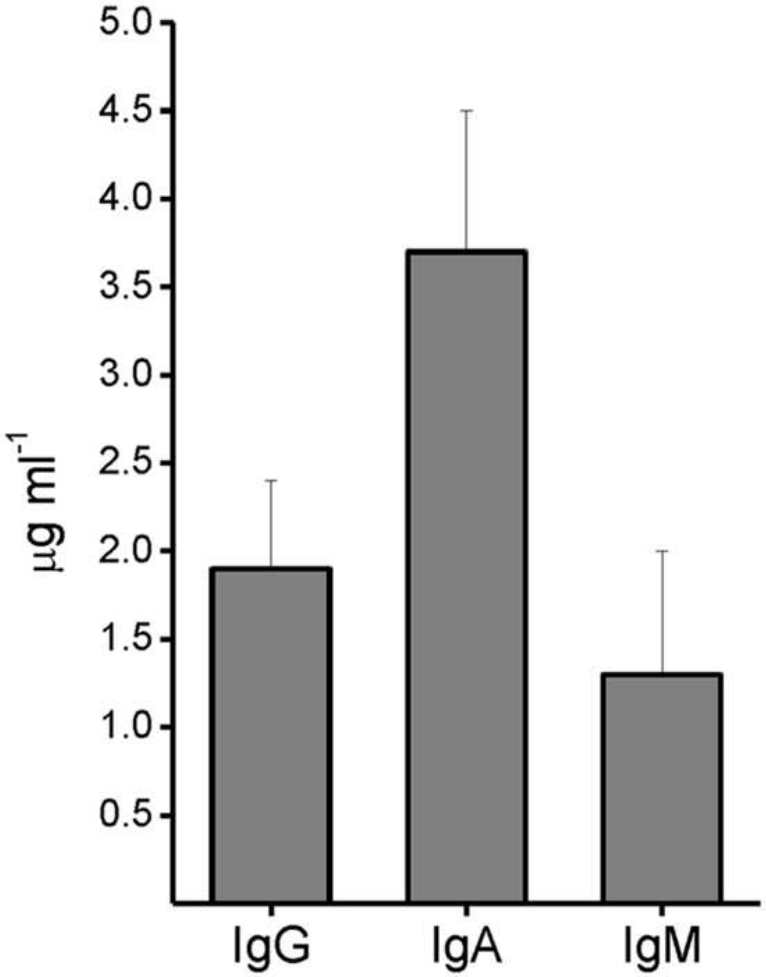

Figure 7

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