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Helicobacter pylori-derived neutrophil-activating protein increases the lifespan of monocytes and neutrophils

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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 lifespan of inflammatory cells. We report that HP-NAP is able to prolong the lifespan of monocytes, in parallel with the induction of the anti-apoptotic proteins A1, Mcl-1, Bcl-2 and Bcl-X_L. This effect does not result from a direct action on the apoptotic machinery, but rather it requires the release of endogenous pro-survival factors, such as interleukin-1β, which probably acts in synergy with other unidentified mediators. We also report that HP-NAP promotes the survival of Ficoll-purified neutrophils in a monocyte-dependent fashion: indeed, mononuclear cell depletion of Ficoll-purified neutrophils completely abolished the pro-survival effect by HP-NAP. In conclusion, our data reinforce the notion that HP-NAP has a pivotal role in sustaining a prolonged activation of myeloid cells.

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Introduction

Helicobacter pylori infection is worldwide known as the leading cause of gastritis, peptic ulcer and ultimately of gastric cancer. Colonization of stomach by *H. 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 lifespan 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 *H. pylori* increases the lifespan 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 *H. pylori* water extracts inhibited neutrophil apoptosis (Kim *et al.*, 2001a,b), 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 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 via endogenous mediators, including IL-1β, (ii) the regulation of monocyte lifespan by HP-NAP occurs following the induction of the anti-apoptotic genes Mcl-1, A1, Bcl-2 and Bcl-X_L and (iii) the bacterial protein indirectly promotes the survival of neutrophils, as such an effect requires the presence of monocytes. Collectively, our data suggest that HP-NAP has a pivotal role in sustaining a prolonged activation of monocytes and neutrophils.

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 dead between 3 and 5 days, the addition of HP-NAP to the culture medium totally protected the cells from apoptosis (Fig. 1A). 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 a 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 to date. Because caspase-3 is an executioner caspase for the apoptotic process, originating from the cleavage of the inactive precursor pro-caspase-3 (Salvesen and Dixit, 1997), we evaluated the levels of both the precursor and its active form. Figure 2 shows that

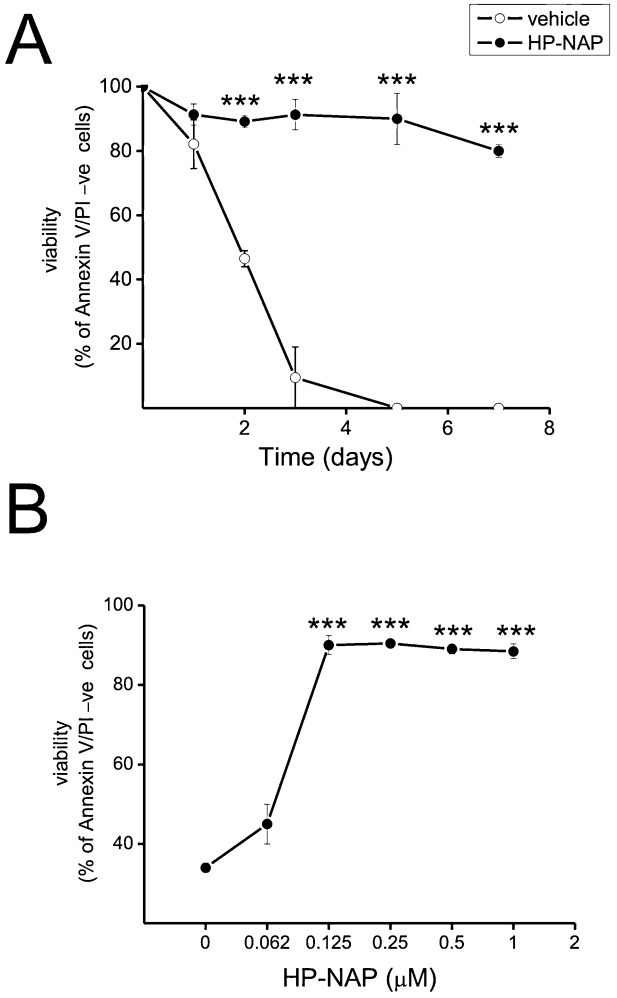

Fig. 1. HP-NAP prevents monocytes apoptosis.
A. Human monocytes (2×10^6 cells) were treated with HP-NAP (1 μM) or PBS (vehicle). At the indicated time points cells were collected and labelled with Annexin V FITC and propidium iodide. Viability was determined cytofluorimetrically and expressed as the percentage of Annexin V/PI-negative cells. Data represent mean ± SD of five independent experiments.
B. A total of 2×10^6 monocytes were stimulated 72 h 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 versus untreated cells; $^{***}P < 0.01$.

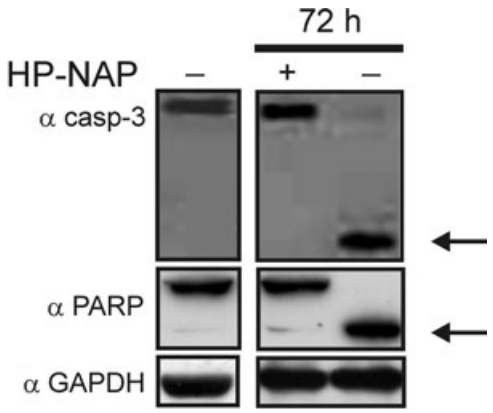

Fig. 2. HP-NAP prevents caspase-3 and PARP activation. A total of 2×10^6 monocytes incubated or not with HP-NAP (1 μM) for 72 h were lysed, and equal amounts of cell extract were separated by SDS-PAGE and immunoblotted. Caspase-3 and PARP were revealed with specific polyclonal antibodies; arrows indicate the large fragments resulting from the cleavage of caspase-3 and PARP (17 and 89 kDa) respectively; an anti-GAPDH monoclonal antibody was used as control for equal loading. A time 0 cell lysate was considered as reference.

the full-length inactive caspase, while totally converted into its active form in untreated monocytes after 72 h, remained uncleaved in the presence of HP-NAP. 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). In order to rule out that the trophic effect attributed to HP-NAP was because of a contaminating TLR2 ligand such as peptidoglycan or lipoteichoic acid, we tested a HP-NAP immune-depleted preparation. As shown in Fig. 3, immune depletion with an anti-HP-NAP antibody totally abrogated the pro-survival effect of our HP-NAP.

HP-NAP-induced suppression of monocyte 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 and 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. 4A). Next, we examined whether the pro-survival effect of HP-NAP resulted from a direct effect on the cells or not. 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. Figure 4B clearly shows that modulation of apoptosis exerted by HP-NAP is mostly due to the endogenous production of pro-survival factors rather than from its direct effect: 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. 4B). 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 because of drug toxicity. Figure 4C shows that the pretreatment of monocytes with monensin abrogated the ability of HP-NAP to counteract apoptosis. This latter result definitely ruled out that the pro-survival effect

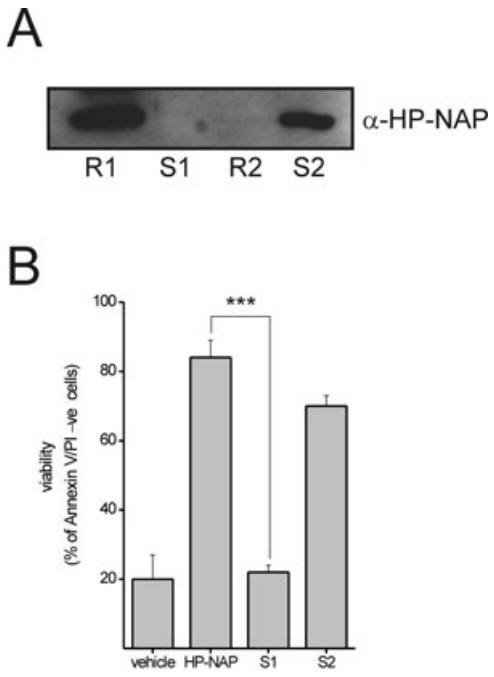

Fig. 3. Immuno-depleted preparation of HP-NAP does not protect monocytes from apoptosis. An immuno-depleted preparation of HP-NAP was obtained with a purified rabbit anti-HP-NAP antibody using an A-Sepharose matrix. The efficacy of HP-NAP pull-down was confirmed by using a specific polyclonal antibody in a Western blot analysis (A); R1 and R2 refer to matrix loaded or not with anti-HP-NAP antibody respectively; S1 and S2 refer to the supernatant of R1 and R2 respectively. (B) A total of 2×10^6 monocytes were stimulated with HP-NAP (0.5 μM), S1 or S2. Viability was determined cytofluorimetrically after 72 h and expressed as the percentage of Annexin V/PI-negative cells as in Fig. 1. Significance was determined by Student's *t*-test for paired data of HP-NAP-treated cells versus cells treated with immuno-depleted preparation of HP-NAP (S1); $^{***}P < 0.01$.

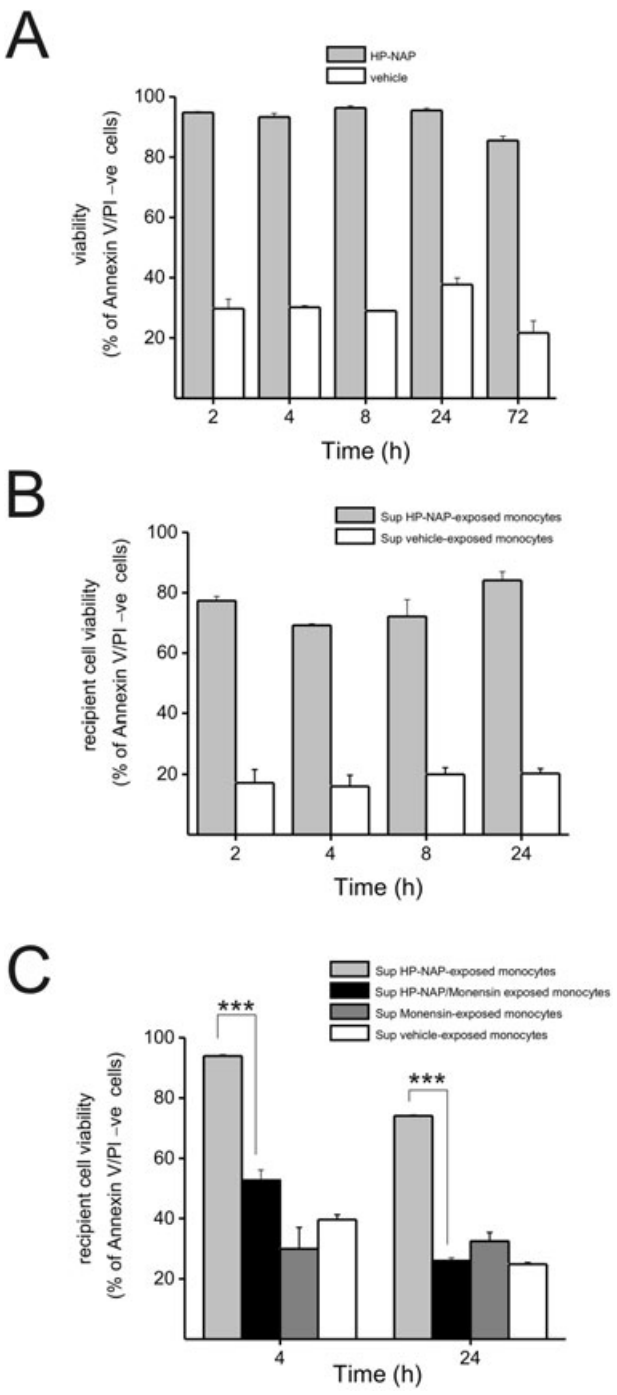

Fig. 4. The pro-survival effect of HP-NAP is rapid and mediated by secreted factors.
A. Human monocytes (2×10^6 cells) were exposed to 1 μM HP-NAP for 2, 4, 8 and 24 h; afterward cells were extensively washed and incubated in fresh medium for 72 h. As positive control, cells were maintained in HP-NAP-conditioned 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. A total of 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. A total of 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 naïve 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; $^{***}P < 0.01$.

exerted on naïve monocytes exposed to the supernatant of HP-NAP-treated monocytes (Fig. 4B) was due to a residual fraction of bacterial protein in the medium.

IL-1β is crucial for the improvement of monocytes survival induced by HP-NAP

It has been demonstrated that an improvement of monocyte survival can be achieved by the addition of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), 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 also IL-1β and GM-CSF could be 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. 5, the transcription of GM-CSF gene was rapidly induced by HP-NAP, in addition to that of TNF-α (Amedei *et al.*, 2006). For both TNFα and GM-CSF the kinetics of their mRNA levels were consistent with the kinetics of protein accumulation in the supernatant. Furthermore, treatment with HP-NAP resulted in a rapid induction of IL-1β mRNA that reached maximum level within the first 2 h 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. To verify whether one of these cytokines was involved in the HP-NAP-dependent trophic effect, we measured the viability of monocytes exposed to the bacterial protein in the presence of cytokine-specific blocking antibodies. The results shown in Fig. 6 clearly demonstrate that neither TNF-α nor GM-CSF is responsible for the HP-NAP-mediated pro-survival effect. As expected, both the blocking antibodies fully abrogated the improvement of monocyte survival because of the single cytokines, used as positive controls. To evaluate the contribution of IL-1β, we utilized the IL-1 receptor antagonist (IL-1Ra), specifically an anakinra preparation,

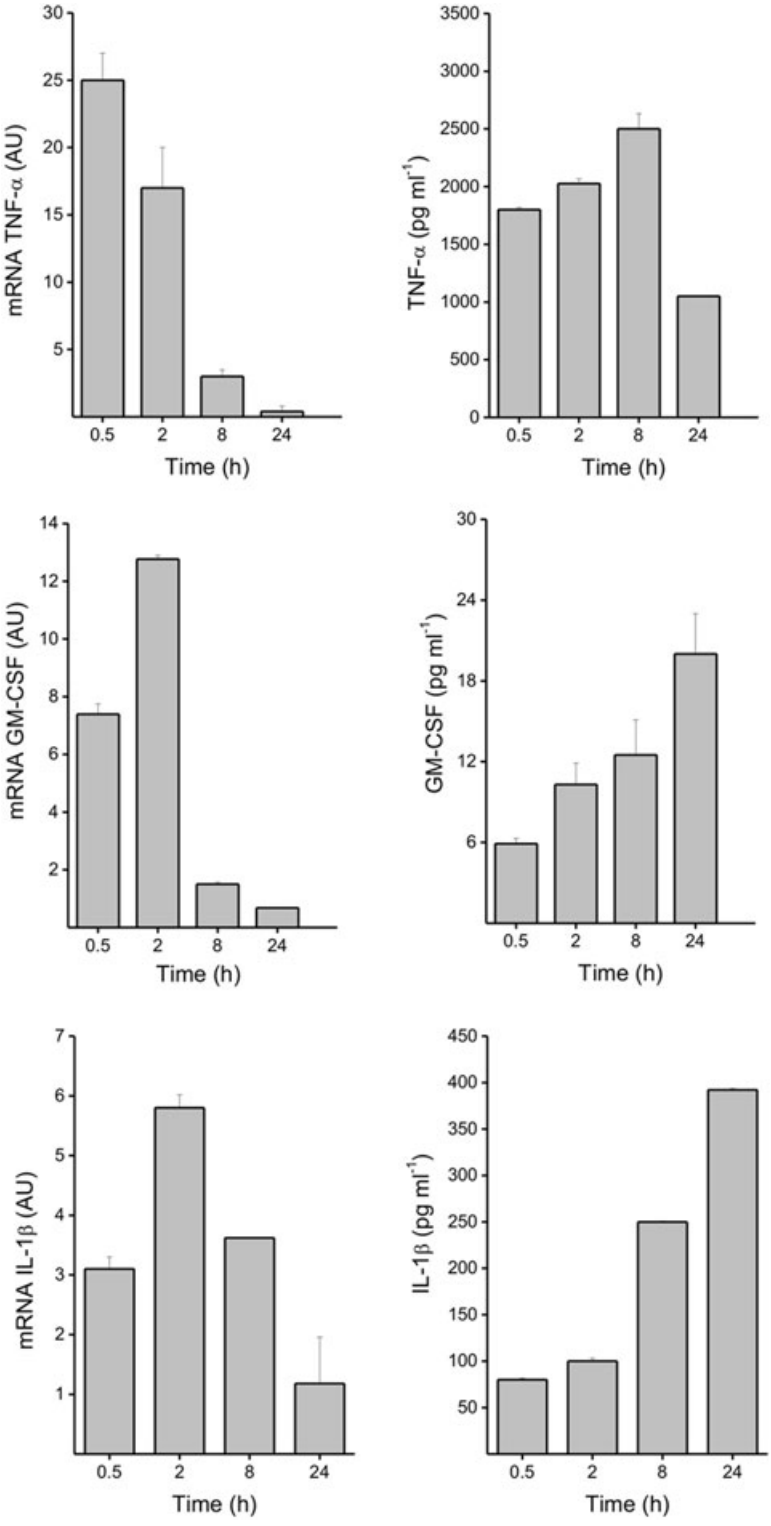

Fig. 5. Time-dependent mRNA expression and protein production for TNF-α, GM-CSF and IL-1β in HP-NAP-treated monocytes. Total RNA was extracted from monocytes cultured with HP-NAP and analysed by real-time PCR to evaluate the expression of each cytokine. Expression levels are depicted as *n*-folds of the normalized amount of mRNA from untreated cells [1 AU = mRNA cytokine concentration (fmol μl^{-1})/mRNA GAPDH (fmol μl^{-1})] of triplicate reactions for each sample. Kinetic of extracellular production of TNF-α, GM-CSF and IL-1β 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.

which is used for the treatment of patients affected by autoinflammatory diseases driven by an uncontrolled IL-1β production (Farasat *et al.*, 2008). As shown in Fig. 6, anakinra significantly decreased the percentage of viable cells observed not only upon monocyte treatment with recombinant IL-1β (by 65.8%) but also after incubation with HP-NAP, although to a lesser extent (by 37.5%).

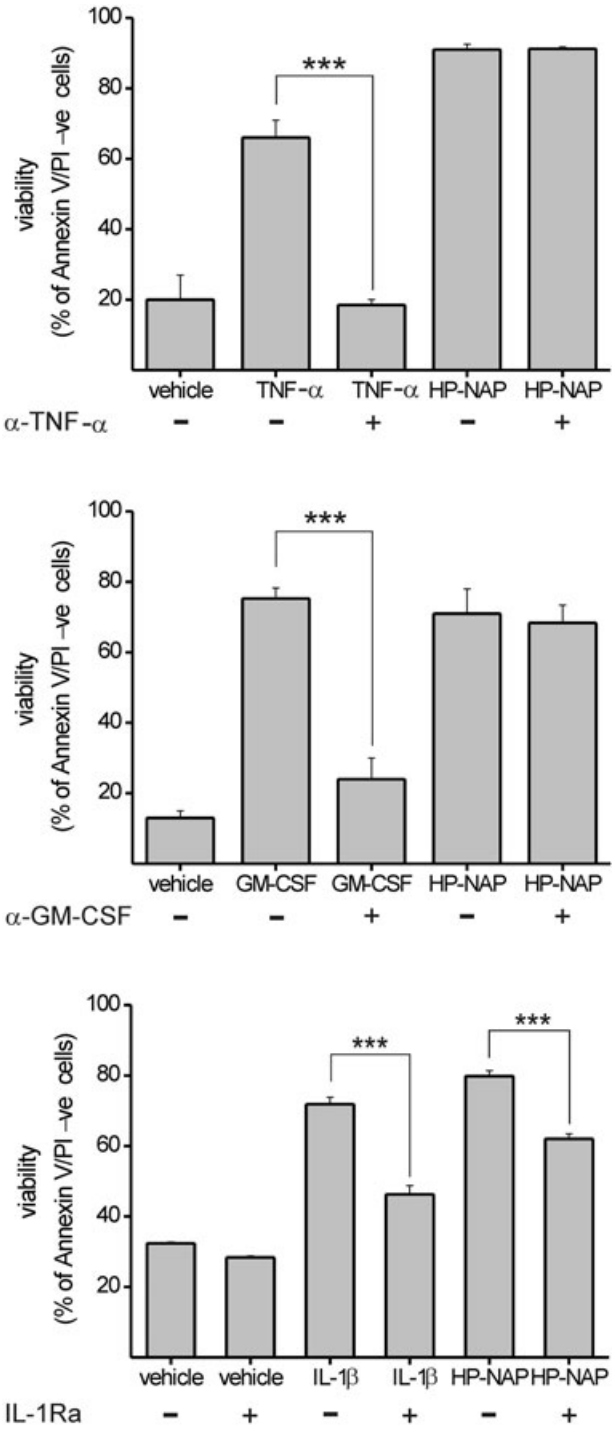

Fig. 6. IL-1β is involved in the pro-survival effect. A total of 2×10^6 monocytes were pre-incubated with blocking antibodies against either TNF-α or GM-CSF, or with anakinra, before being exposed to HP-NAP or to the corresponding cytokine. Viability was determined cytofluorimetrically after 72 h and expressed as the percentage of Annexin V/PI-negative cells as in Fig. 1. Significance was determined by Student's *t*-test; $^{***}P < 0.01$.

HP-NAP induces anti-apoptotic genes

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 levels after 72 h (Fig. 7). 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 amounts already after 24 h and its complete disappearance after 72 h (Fig. 7). A similar trend was observed for Bcl-2, although the modulation of the latter was less prominent. Finally, a remarkable accumulation of Bcl-X_L was detected until 48 h following HP-NAP administration, before declining after 72 h. In parallel, the increased level of the pro-apoptotic protein Bcl-X_S in control monocytes was prevented by the bacterial protein administration.

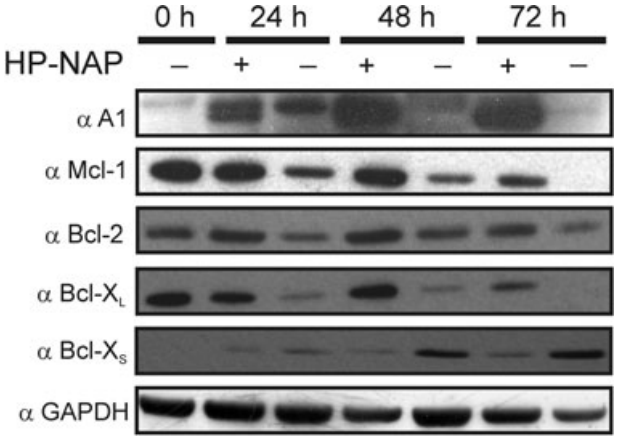

Fig. 7. HP-NAP-exposed monocytes increase the production of anti-apoptotic proteins. A total of 2×10^6 monocytes, stimulated or not with 1 μM HP-NAP, were lysed at indicated time, and equal amounts of cell extract were separated by SDS-PAGE and immunoblotted. A1, Mcl-1, Bcl-2, Bcl-X_L and Bcl-X_S were revealed with specific antibodies; an anti-GAPDH monoclonal antibody was used as control for equal loading. Panels derive from different blots of a single experiment.

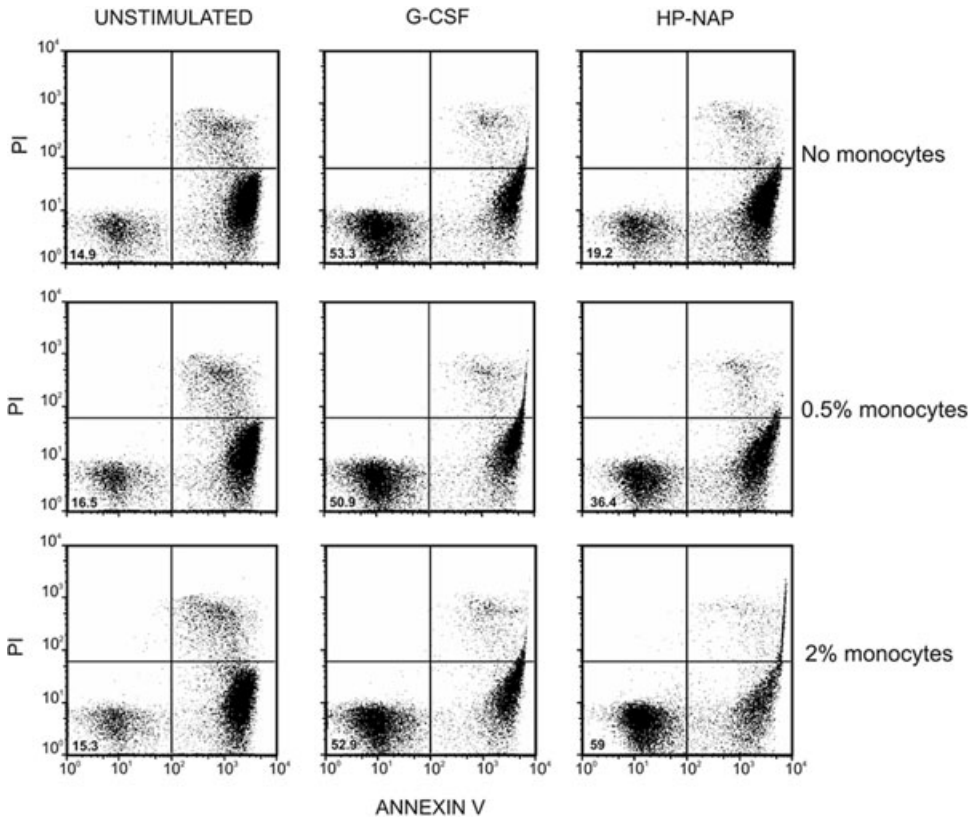

Fig. 8. HP-NAP modulates neutrophils survival trough the mediation of monocytes. Highly purified PMNs were incubated with or without 1000 U ml^{-1} G-CSF or 1 μM HP-NAP and in absence or presence of either 0.5% or 0.2% of monocytes. After 18 h cells were harvested and stained with Annexin V FITC and 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).

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*., 2001a). 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 experiments we used PMN cells 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. 8, 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. 8, 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.

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 *H. 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 Th 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 lipopolysaccharide 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 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. Experiments aimed at revealing the identity of such protective factors demonstrated that HP-NAP triggers the release of several cytokines, known to exert pro-survival actions on monocytes, including IL-1β, TNF-α and GM-CSF (Mangan and Wahl, 1991; Flad *et al*., 1999): among them, only IL-1β seems to be involved, although the not complete inhibition obtained with IL-1Ra suggests that IL-1β might be a factor crucial for the HP-NAP-induced monocytes survival but in synergy with other factors, which remain yet unidentified.

In parallel, HP-NAP-exposed monocytes undergo to a remarkable accumulation of the anti-apoptotic protein of the Bcl-2 family. The intracellular high levels of these proteins rely on the induction of trophic factors, as the blockage of exocytosis in HP-NAP-stimulated monocytes impaired their expression and, as expected, the cell survival (data not shown).

Considering that *H. pylori* colonization is typically followed by infiltration of the gastric mucosa by PMN 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 lifespan of neutrophils. The interest in addressing this issue was reinforced by the previous finding of Kim *et al.* (2001a,b), which highlighted the ability of *H. 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 because of 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*., 2001a,b), the possibility exists that the reported results were biased by the presence of monocytes in the neutrophil preparation.

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.

Experimental procedures

Reagents

HP-NAP was cloned, expressed and purified from *Bacillus subtilis* to avoid contamination with lipopolysaccharide, 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, anti-caspase-3 and anti-Bcl-X_L antibodies were from Cell Signalling (Beverly, MA). Polyclonal antibody anti-A1

was from Lifespan Biosciences (Seattle, WA). Polyclonal antibody against Bcl-X_S and monoclonal antibody against Bcl-2 were from BD Biosciences (Erembodegem, Belgium). G-CSF was from Chugai Pharmaceutical (Tokio, Japan). GM-CSF and TNF-α were from Peprotech (Rocky Hill, NJ); neutralizing polyclonal antibodies anti-GM-CSF were from R&D System (Wiesbaden, Germany); IL-1β was from Immunological Sciences (Roma, Italy); IL-1 receptor antagonist, IL-1Ra (anakinra) was from Biovitrum (Stockholm, Sweden). Neutralizing monoclonal antibody anti-TNF-α (clone B154.2) was a kind gift from Professor G. Trinchieri (Laboratory of Experimental Immunology, National Cancer Institute at Frederick, Frederick, MD). Other reagents were obtained from Sigma-Aldrich unless otherwise specified. Phosphate-buffered saline (PBS), RPMI 1640 and gentamicin were from Invitrogen (Carlsbad, CA). Fetal calf serum (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 from healthy donors were isolated by centrifugation on Ficoll-Paque solution (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Peripheral blood mononuclear cells were then laid on a cushion of Percoll 46% v/v solution (Amersham) in RPMI 1640 supplemented with 10% FCS, 50 μg ml^{-1} 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^{-1} 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 was < 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).

HP-NAP immunoprecipitation

A total of 150 μg of a rabbit affinity-purified polyclonal antibody against HP-NAP (Amedei *et al*., 2006) was pre-adsorbed on 20 μl of protein A-sepharose (indicated as R1) for 2 h at 4°C. The same volume of the antibody-containing buffer (0.2 M Glycine-Cl pH 7.5) was used for preparing a control matrix (R2). Matrices were washed three times in PBS 0.03% BSA before adding 30 μg of HP-NAP in a final volume of 800 μl of the same buffer. After an overnight incubation at 4°C, matrix supernatants (S1 and S2) were collected and concentrated to a final volume of 70 μl. Ten microlitres of them, together with the matrix absorbed material, were analysed in a Western blot revealed with the anti-HP-NAP antibody. The remaining supernatants were frozen in liquid nitrogen and maintained at −80°C.

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^{-1} 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. For the experiment with HP-NAP immuno-depleted preparation, both the supernatants collected following immunoprecipitation (S1 and S2) were applied to monocytes. The final concentration of HP-NAP calculated for the non-depleted S2 was 0.5 μM. An equal volume of S1 was administrated and 0.5 μM HP-NAP was applied as positive control. For the experiments with cytokine-blocking antibodies, the latter were applied to the cells 20 min before stimulating either with the cytokine or with HP-NAP. In case of anakinra experiment the time of pre-incubation was extended up to 1 h.

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′-AGCAACAGGGT GGTGGAC-3′ and 5′-GTGTGGTGGGGGACTGAG-3′; for IL-1β, 5′-CTGTCCTGCGTGTTGAAAGA-3′ and 5′-TTGGGTAATTTTT GGGATCTACA-3′; for TNF-a, 5′-ATGAGCACTGAAAGCATGA TCC-3′ and 5′-GAGGGCTGATTAGAGAGAGGTC-3′; for GM-CSF, 5′-TCTCAGAAATGTTTGACCTCCA-3′ and 5′-GCCCTTG AGCTTGGTGAG-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 (AU) = cytokine mRNA concentration/GAPDH mRNA concentration (both in fmol μl^{-1})].

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

Culture supernatants of monocytes harvested for mRNA quantification were collected at the same time points, and the amounts of TNF-α, GM-CSF and IL-1β 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 12 000 *g* for 20 min at 4°C, and equal amount of each cell extract was applied on a 4–12% SDS-PAGE and analysed by immunoblotting. Immunoreactive bands were revealed with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) combined with the enhanced chemiluminescence system (Millipore).

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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