

Immunosuppression of T_H2 responses in *Trichinella spiralis* infection by *Helicobacter pylori* neutrophil-activating protein

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Background: The *Helicobacter pylori* neutrophil-activating protein (HP-NAP) is able to induce IL-12 expression by cells of innate immunity and to shift to T_H1 human allergen-specific T_H2 cells *in vitro*.

Objective: We performed an *in vivo* investigation of the ability of HP-NAP to downmodulate the T_H2 response induced in mice by *Trichinella spiralis* infection.

Methods: Groups of *T spiralis*-infected BALB/c mice received intraperitoneal PBS/rat IgG2b (control animals) or 10 µg of HP-NAP with or without anti-Toll-like receptor 2 antibody on days 10 and 28 after infection. Blood eosinophils, total and *T spiralis*-specific IgE levels, and cytokine levels were measured in the plasma up to day 42, when splenocytes were cultured for cytokine production.

Results: Although control animals showed significant eosinophilia and increase of total and *T spiralis*-specific IgE, IL-4, and IL-5 levels from days 10 to 14, HP-NAP-treated animals showed less eosinophilia and total and excretory/secretory antigens of *T spiralis*-specific IgE in the blood. HP-NAP-treated animals also had higher IL-12 and IFN-γ plasma levels and lower IL-4 and IL-5 levels. The addition of anti-Toll-like receptor 2 antibody abrogated the anti-T_H2/pro-T_H1 activity of HP-NAP.

Conclusion: This study provides evidence that HP-NAP enhances endogenous IL-12 and IFN-γ response and exerts a powerful anti-T_H2 activity *in vivo*, targeting both IL-5-induced eosinophilia and IL-4-mediated hyper-IgE responses induced by parasitic infection. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: T_H2 response, T_H2 immunosuppression, allergy, parasitic infection, *Trichinella spiralis*, *Helicobacter pylori* neutrophil-activating protein, endogenous IL-12, mouse model, T_H1/T_H2 redirection

Abbreviations used

HP-NAP: *Helicobacter pylori* neutrophil-activating protein
Pam3: Lipohexapeptide tripalmitoyl-S-glyceryl-Cys-Ser-4(Lys)
SEB: Staphylococcal enterotoxin B
TLR2: Toll-like receptor 2
TsE/S: Excretory/secretory antigens of *Trichinella spiralis*

T_H2 cells characterized by the production of IL-4, IL-5, IL-9, and IL-13^{1,2} are involved in the immune response to helminth infections³⁻⁵ and in the development of disorders such as atopy and asthma.⁶⁻⁹ Like IL-4, IL-13 produced by T cells, eosinophils, and mast cells is an important factor during T_H2 responses, mediating mechanisms similar to those induced by IL-4, such as stimulation of B-cell proliferation, antibody class switching to IgE, and, like IL-5, induction of eosinophilia.^{10,11}

The reciprocal antagonism of T_H1 and T_H2 responses led us to ask whether the T_H2-dominated response to *Trichinella spiralis* infection was susceptible to downregulation by IL-12-promoting signals. To address this question, we took advantage of a virulence factor of *Helicobacter pylori*: the *H pylori* neutrophil-activating protein (HP-NAP).^{12,13}

HP-NAP, an oligomeric protein of 150 kd, is a Toll-like receptor 2 (TLR2) agonist able to induce the *in vitro* the expression of IL-12 and IL-23 by human neutrophils and monocytes.¹⁴ Moreover, addition in culture of HP-NAP to allergen-induced human T-cell lines resulted in a remarkable increase of IFN-γ-producing T cells and decrease of IL-4-secreting cells, thus shifting the cytokine profile of allergen-activated T cells from the T_H2 to the T_H1 cytotoxic phenotype.¹⁴

In this study BALB/c mice were orally infected with *T spiralis*. Ten and 28 days later, infected animals were treated with intraperitoneal injections of control protein (rat IgG2b) or HP-NAP, with or without anti-TLR2 antibody, and the effects on blood leukocytes and plasma levels of cytokines, total IgE, and IgE specific for the excretory/secretory antigens of *T spiralis* (TsE/S) were evaluated. At the end of the experiments, spleen cells were tested for cytokine production in response to medium or TsE/S antigen. HP-NAP-treated animals consistently showed reduced T_H2 and increased T_H1 activity, with lower production of IL-4, IL-5, and IL-13 and much higher production of IFN-γ than seen in control-infected animals. Coinjection of HP-NAP and anti-TLR2 antibody abrogated the anti-T_H2 activity of HP-NAP.

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METHODS

Animals

Female 6-week-old BALB/c mice (Harlan, Italy) were acclimated to the University of Pisa animal care facility for 1 week before experimental infection. Animals were used in accordance with local and national regulations and approved by the University Ethical Review Committee of the Pisa University School of Medicine. In a preliminary experiment the schedule of blood sampling and the dose of HP-NAP to be injected to detect anti- T_H2 effects were assessed (see the Results section in this article's Online Repository at www.jacionline.org). In subsequent experiments a group of infected animals received PBS alone (0.5 mL; control animals), and a second group of infected animals was injected intraperitoneally with HP-NAP (10 μ g in 0.5 mL of PBS) on days 10 and 28 postinfection. Three of such experiments were done, involving a total of 45 PBS- and 45 HP-NAP-treated animals. In further experiments *T. spiralis*-infected animals were divided into groups of 10 that were injected on days 10 and 28 pi with (1) control protein (rat IgG2b isotype control, 30 μ g), (2) HP-NAP (10 μ g) plus IgG2b (30 μ g), (3) HP-NAP (10 μ g) plus anti-TLR2 rat IgG2bk mAb (30 μ g), or (4) anti-TLR2 antibody alone (30 μ g). In parallel, 2 other groups of 10 animals were injected on days 10 and 28 pi with Pam3Cys, referred to herein as lipohexapeptide tripalmitoyl-S-glycerol-Cys-Ser-4(Lys) (Pam3; 10 μ g) plus IgG2b (30 μ g) or Pam3 (10 μ g) plus anti-TLR2 antibody (30 μ g). Peripheral blood was collected in Na-heparin tubes from the retro-orbital venous plexus before infection and at various time points (days 0, 7, 14, 28, and 42) after infection. In other experiments blood was collected on days 0, 10, 13, 28, 31, and 42 pi. During the experiments, both control- and HP-NAP-treated animals equally gained weight and were in good condition. On day 42 pi, animals were killed, and tissue samples were fixed and processed for the histologic assessment of infection.

Parasites, TsE/S antigen preparation, and biotin labeling

T. spiralis (code ISS003) muscle larvae (the International Trichinella Reference Centre, www.iss.it/site/Trichinella/index.asp) were recovered from infected mice after artificial digestion according to standard procedures and suspended in PBS.¹⁵ Experimental infection was performed by orally administering 350 muscle larvae to each mouse.

T. spiralis muscle larvae were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, Mo) containing streptomycin (500 μ g/mL) at 37°C in a 5% CO₂ atmosphere. After 18 hours, the supernatant was collected and desalted into the appropriate buffer with a PD-10 column (Amersham Biosciences Europe, Freiburg, Germany). TsE/S antigen protein concentration was estimated by means of absorbance at 280 nm by using a Varian Cary Bio 50 spectrophotometer.

Biotin labeling of TsE/S antigen was performed as previously described.¹⁶ Briefly, TsE/S antigen (4 mg/mL) in sodium bicarbonate buffer, pH 8.5, was reacted with water-soluble biotin N-hydroxysuccinimide ester (Vector Laboratories, Burlingame, Calif) for 2 hours at room temperature. Glycine (10 mg) was then added to stop the reaction, and biotinylated TsE/S antigen was extensively dialyzed against PBS.

Reagents and HP-NAP protein preparation

HP-NAP was cloned, expressed, and purified from *Bacillus subtilis* to avoid LPS contamination.¹⁴ The recombinant protein was pure, as judged from overloaded gels composed of different percentages of polyacrylamide. Mass spectrometric analysis, performed with a Maldi Reflex (Brucker Analytik, Karlsruhe, Germany), confirmed that the protein consisted of a single molecule of 16,875 $\pm$ 20 d. Immune-depleted preparation of HP-NAP was obtained with a purified anti-HP-NAP antibody by using a G-Sepharose matrix, as previously reported.¹⁴ Pam3 was purchased from EMC microcollection GmbH (Tuebingen, Germany), and purified anti-mouse TLR2 rat IgG2bk (clone 6C2) and rat IgG2b isotype control were purchased from eBioscience (San Diego, Calif). In separate experiments the possible inhibitory effect of anti-HP-NAP antibodies induced by repeated HP-NAP administration was investigated (see the Methods and Results sections and Table E2 in this article's Online Repository at www.jacionline.org).

Measurement of total and TsE/S-specific IgE in plasma

Total mouse IgE levels in the plasma were assessed by using a specific ELISA (Alpha Diagnostic, San Antonio, Tex), according to the manufacturer's instruction. TsE/S-specific IgE in plasma samples was determined by means of a modification of the ELISA assay for total IgE. Plasma samples diluted 1:2 in PBS were seeded in the microplates coated with the anti-mouse IgE of the total IgE assay (0.1 mL/well) and incubated for 4 hours at room temperature. Microplates were washed with PBS–0.05% Tween 20, and biotinylated TsE/S antigen (10 μ g/mL) was added. After 4 hours at room temperature, plates were washed, and avidin-peroxidase (Cappel Research Products, Durham, NC) was added to each well. Finally, orthophenylenediamine was reacted for 30 minutes with H₂O₂ and then stopped with H₂SO₄. OD at 490 nm was assessed by using a microplate spectrophotometer.

Spleen cell cultures and cytokine assays

Adherent and nonadherent splenocytes were obtained from 5 untreated *T. spiralis*-infected mice killed on day 14. Single spleen cell suspensions were prepared, counted, and cultured at 1×10^6 cells/mL in RPMI 1640 medium supplemented with 5% FCS (HyClone, Logan, Utah) in plastic flasks at 37°C. After 1 hour's incubation, nonadherent cells were recovered. Adherent cells were extensively washed with RPMI 1640 medium to remove residual nonadherent cells. Adherent and nonadherent cells were then cultured for 24 hours in RPMI 1640 medium, 10% FCS, 2 mmol/L L-glutamine, sodium pyruvate, nonessential amino acids solution, and antibiotics in the presence of the following: (1) medium plus rat IgG2b isotype control (3 μ g in 1.0 mL), (2) anti-TLR2 antibody (3 μ g in 1.0 mL), (3) HP-NAP (0.1 and 1.0 μ g in 1.0 mL) plus rat IgG2b isotype control (3 μ g), (4) HP-NAP (0.1 and 1.0 μ g in 1.0 mL) plus anti-TLR2 mAb (3 μ g), (5) Pam3 (0.1 and 1.0 μ g in 1.0 mL) plus IgG2b control (3 μ g), or (6) Pam3 (0.1 and 1.0 μ g in 1.0 mL) plus anti-TLR2 antibody (3 μ g). At the end of culture, supernatants were collected and assessed for IL-12 and IL-6 production.

On day 42 pi, mice were killed. Single spleen cell suspensions were prepared, counted, and cultured at 2×10^6 cells/mL in RPMI 1640 medium supplemented with 10% FCS, 2 mmol/L L-glutamine, sodium pyruvate, nonessential amino acid solution, and antibiotics. Spleen cells (1 mL per tube) were cultured in duplicate tubes in the presence of medium alone, 10 μ g/mL TsE/S antigen, or 100 ng/mL staphylococcal enterotoxin B (SEB) at 37°C with 5% CO₂. After 48 hours, culture supernatants were collected for cytokine assays. Mouse IL-6, IL-12, IL-4, IL-5, IL-13, and IFN- γ levels in culture supernatants and plasma were measured by means of specific ELISA assays (R&D Systems GmbH, Wiesbaden, Germany), according to the manufacturer's instructions.

Statistical analysis

Results of WBC counts, cytokine measurement in plasma or supernatants, and total and TsE/S-specific IgE measurement are presented as means $\pm$ SD for groups of animals undergoing uniform treatment. Differences between groups were analyzed with the Student 2-tailed *t* test. A *P* value of less than .05 was considered significant.

RESULTS

Effect of *T. spiralis* infection and HP-NAP treatment on peripheral blood leukocyte populations

In infected control animals injected with PBS or rat IgG2b, the total WBC, lymphocyte, and monocyte counts fluctuated without significant changes, whereas eosinophil counts were remarkably increased on days 14 ($P < .0001$), 28 ($P < .0001$), and 42 ($P < .0005$) in comparison with preinfection values (see Fig E1 in this article's Online Repository at www.jacionline.org). On day 42, a significant decrease of neutrophil counts was observed ($P < .0001$). In animals treated with HP-NAP on days 10 and

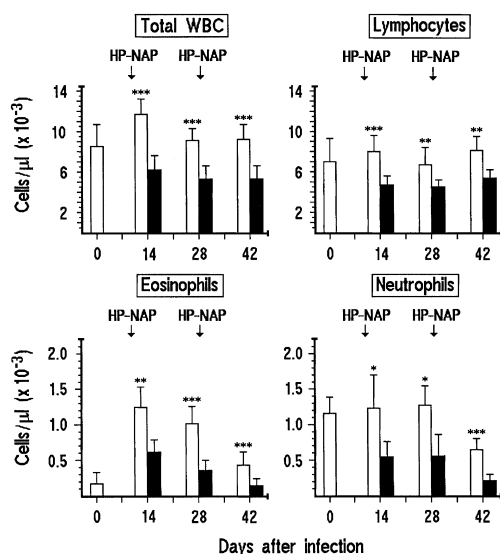


FIG 1. Effect of HP-NAP treatment on the leukocyte populations of *T. spiralis*-infected BALB/c mice. On days 10 and 28, half of the animals ($n = 45$) received intraperitoneal PBS (open columns), whereas the other half received 10 μg of HP-NAP (solid columns). Results are presented as means $\pm$ SDs. Comparisons were done with the Student 2-tailed t test. * $P < .001$, ** $P < .0005$, and *** $P < .0001$.

28, total WBC, lymphocyte, and neutrophil counts on days 14, 28, and 42 were significantly lower in comparison with those in control animals at the same time points (Fig 1). Also, HP-NAP-treated animals showed eosinophilia, but eosinophil counts were consistently and significantly lower at any sampling time compared with those seen in control animals (Fig 1).

HP-NAP treatment reduces plasma levels of total and TsE/S-specific IgE

In uninfected animals total IgE plasma levels were 72 ± 37 ng/mL. On day 7 pi, total IgE levels were increased by 2.5 times and even more on day 14 ($P < .0001$) than preinfection values in either control animals or HP-NAP-treated animals (Fig 2, A). In control animals total IgE levels remained high on days 28 and 42, whereas in HP-NAP-treated animals, after a peak on day 14, total IgE levels progressively decreased to much lower levels than seen in control animals on days 28 and 42 ($P < .0005$ and $P < .0001$, respectively).

Before infection, plasma levels of TsE/S-specific IgE were not detectable (OD, <0.030). However, on day 7 pi, TsE/S-specific IgE became detectable (mean $\pm$ SD OD, 0.084 ± 0.035). In control animals TsE/S-specific IgE levels were remarkably increased on days 14, 28, and 42 ($P < .0001$) in comparison with values on day 7 (Fig 2, B). Also, in HP-NAP-treated animals TsE/S-specific IgE levels were increased on day 14 ($P < .0001$), but levels substantially decreased thereafter. However, in comparison with control animals, HP-NAP-treated animals had significantly lower levels of TsE/S-specific IgE on day 14 ($P < .0005$) and much lower levels on days 28 and 42 ($P < .0001$; Fig 2, B).

Target cells of HP-NAP and role for HP-NAP-TLR2 interaction

Adherent and nonadherent splenocytes from 5 untreated *T. spiralis*-infected mice were obtained on day 14 to identify the cell

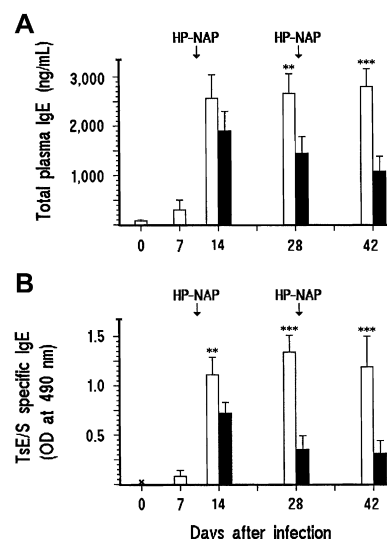


FIG 2. Effect of HP-NAP treatment on plasma levels of total and TsE/S-specific IgE. In control animals (open columns) total IgE (A) and TsE/S-specific IgE (B) levels were high up to day 42, whereas in HP-NAP-treated animals (solid columns) IgE levels progressively decreased after day 14. Results are presented as means $\pm$ SDs in 2 groups of 45 animals. ** $P < .0005$ and *** $P < .0001$.

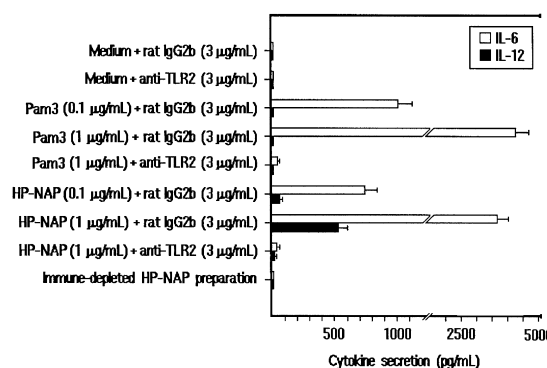


FIG 3. *In vitro* induction of cytokine secretion by the TLR2 agonists Pam3 and HP-NAP and its abrogation by anti-TLR2 antibody. Results are presented as means $\pm$ SDs of IL-6 or IL-12 levels measured in supernatants of triplicate cultures for each condition.

targets of HP-NAP. Both cell populations were cultured for 24 hours in the presence of different combinations and doses of rat IgG2b isotype control, anti-TLR2 antibody, HP-NAP, or Pam3. Culture supernatants were then collected, and IL-12 and IL-6 production was measured. Neither rat IgG2b nor anti-TLR2 antibody alone had any effect on the spontaneous IL-12 and IL-6 production by adherent or nonadherent splenocytes. Addition of 0.1 or 1 $\mu\text{g}/\text{mL}$ Pam3 to adherent splenocytes resulted in a strong increase of IL-6 secretion, whereas nonadherent cells failed to increase IL-6 levels to more than the background value (data not shown). More importantly, Pam3 failed to induce IL-12 production by adherent splenocytes (Fig 3) and nonadherent cells. As expected, Pam3-induced upregulation of IL-6 in adherent cells was abolished by addition of anti-TLR2 antibody. In parallel cultures of adherent splenocytes, addition of HP-NAP resulted in upregulation of both IL-6 and IL-12 production, depending on the dose of HP-NAP. Like Pam3, HP-NAP failed to induce IL-6, IL-12, or

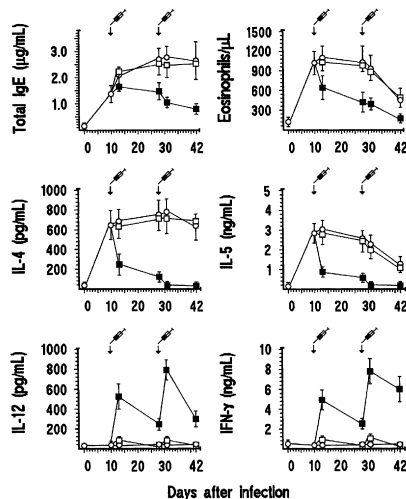


FIG 4. Effect of HP-NAP treatment on blood T_H2 and T_H1 parameters. Eosinophils and total IgE, IL-4, IL-5, IFN- γ , and IL-12 levels were measured in the blood of IgG2b-treated control animals (open circles) and compared with values in animals treated on days 10 and 28 with HP-NAP/IgG2b (solid squares) or HP-NAP plus anti-TLR2 antibody (open squares). Results are presented as means $\pm$ SDs in 3 groups of 10 animals.

both in nonadherent cell cultures (data not shown). However, if anti-TLR2 antibody was added together with HP-NAP, upregulation of IL-6 and IL-12 was abrogated, suggesting that the interaction with TLR2 was essential for HP-NAP to exert its ability to induce adherent cells to IL-12 expression (Fig 3). In this experiment we also observed the lack of activity on IL-6 and IL-12 production by adherent cells of a preparation in which HP-NAP had been immune depleted. Therefore it is unlikely that contaminants of our HP-NAP preparation were indeed responsible for its effects.

In vitro experiments with anti-TLR2 antibody suggested that the interaction with TLR2 was essential for HP-NAP to exert its ability to induce IL-12 expression. We then asked whether hampering HP-NAP–TLR2 interaction *in vivo* resulted in inhibition of the anti- T_H2 activity of HP-NAP in *T. spiralis*-infected mice. Ten infected animals were injected intraperitoneally with control rat IgG2b, another 10 were injected with HP-NAP plus rat IgG2b, and a third group of 10 were injected with HP-NAP plus anti-TLR2 antibody. In all groups eosinophils and total IgE, IL-12, IFN- γ , IL-4, and IL-5 levels were measured before infection and on days 10 (before the first injection), 13, 28 (before the second injection), 31, and 42. As summarized in Fig 4, in HP-NAP/IgG2b-treated animals plasma IL-12 and IFN- γ levels increased on day 13 ($P < .0001$ vs day 10), decreased on day 28 ($P < .0005$ vs day 13), but increased again on day 31 ($P < .0001$ vs day 28) after the second delivery of HP-NAP. On day 42, plasma IL-12 was still detectable, and IFN- γ levels remained high. In the same animals HP-NAP/IgG2b treatment resulted in strong reduction of plasma IL-4 and IL-5 levels ($P < .0001$, day 13 vs day 10) that progressively decreased to preinfection values. The kinetics of eosinophils were similar ($P < .0005$, day 13 vs day 10), whereas IgE levels were significantly reduced starting from day 28 to day 42 in comparison with those seen in IgG2b-treated control animals. Coinjection of anti-TLR2 antibody with HP-NAP abrogated the HP-NAP-induced reduction of T_H2 parameters (eosinophils, IgE, IL-4, and IL-5) and increase of plasma IL-12 and IFN- γ

levels. In infected animals injected with Pam3/IgG2b, no significant change of T_H2 or T_H1 parameters was observed in comparison with values seen in animals treated with rat IgG2b (see the Results section and Table E3 in this article's Online Repository at www.jacionline.org).

Effect of treatment with HP-NAP on cytokine production by spleen cells

On day 42 pi, splenocytes were isolated and cultured for 48 hours in the presence of medium alone, 10 μ g/mL TsE/S antigen, or 100 ng/mL SEB as a polyclonal activator. In infected control mice ($n = 30$) the spontaneous production of IFN- γ by splenocytes was extremely low, whereas that of IL-4, IL-5, and IL-13 was easily detectable (Table I). On stimulation with TsE/S antigen, IFN- γ production increased by only 5 times, whereas that of IL-4, IL-5, and IL-13 increased by 22, 16, and 14 times, respectively. Stimulation with SEB resulted in increased secretion of all 4 cytokines. In HP-NAP-treated animals spontaneous secretion of T_H2 cytokines was consistently lower and that of IFN- γ was higher than in supernatants of splenocytes from control animals. On stimulation with TsE/S antigen, IFN- γ production increased by about 21 times, whereas that of IL-4, IL-5, and IL-13 increased by 11, 11, and 14 times, respectively (Table I). Stimulation with SEB resulted in a 32-fold increase of IFN- γ production, whereas the increase of IL-4, IL-5, and IL-13 levels was 16, 12, and 16 times, respectively. In conclusion, in comparison with those of control animals, splenocytes from HP-NAP-treated mice showed a significantly higher ($P < .001$) IFN- γ production and a much lower ($P < .005$) secretion of T_H2 cytokines.

DISCUSSION

The mechanisms responsible for successful immunotherapy in allergic subjects are still only partially understood. Both inhibition of allergen presentation and suppression of T-cell responses by regulatory cells are suggested, but most studies point at immune deviation from T_H2 cells toward a less pathogenic T_H1 phenotype.¹⁷ On the other hand, $CD4^+$ T cells from nonatopic subjects produce IFN- γ and little or no IL-4 in response to common environmental allergens.^{18,19} Even if recombinant or modified allergens are proposed for safer treatments, a promising strategy seems to be the use of novel adjuvants or immunomodulators to induce immune deviation, and several compounds were tested over the years to enhance the immune response of allergic subjects.^{20,21}

IL-12 is the major cytokine in the induction of T_H1 responses both *in vivo* and *in vitro*.²² However, its side effects and toxicity in human subjects raise major concerns.²³⁻²⁵ A safer approach might be to use an adjuvant able to induce moderate production of endogenous IL-12, resulting in efficient immune deviation to T_H1 of allergen-specific T_H2 responses.

HP-NAP is a highly conserved protein of *H. pylori*¹³; it is a member of a broad superfamily of ferritin-like proteins, most of which have a DNA-protective function under starved conditions, such as oxidative or nutritional stress, including iron starvation.²⁶ Members of this family are homopolymers formed by 12 four-helix bundle subunits that assemble to provide iron ligands.²⁷ In a previous study we showed that incubation of human cells of the innate immunity with HP-NAP resulted in upregulation of cytokine mRNA expression and protein secretion, including IL-

TABLE I. T_H1 and T_H2 cytokine production in spleen cell cultures in untreated control animals or HP-NAP-treated *T. spiralis*-infected animals

T <i>spiralis</i> -infected animals	Stimulant	Cytokine secretion (pg/mL)			
		IFN- γ	IL-4	IL-5	IL-13
Untreated control animals (n = 30)	Medium	25 $\pm$ 9	143 $\pm$ 58	232 $\pm$ 48	326 $\pm$ 78
	TsE/S	121 $\pm$ 34	2982 $\pm$ 304	3645 $\pm$ 378	4525 $\pm$ 465
	SEB	435 $\pm$ 166	3413 $\pm$ 387	3990 $\pm$ 416	5520 $\pm$ 476
HP-NAP-treated animals (n = 34)	Medium	124 $\pm$ 51	51 $\pm$ 27	74 $\pm$ 31	64 $\pm$ 34
	TsE/S	2315 $\pm$ 418†	487 $\pm$ 156*	687 $\pm$ 155*	812 $\pm$ 173*
	SEB	3565 $\pm$ 566†	692 $\pm$ 244*	739 $\pm$ 212*	976 $\pm$ 219*

Results are presented as means $\pm$ SDs of duplicated determinations in duplicate cultures of spleen cells from untreated or HP-NAP-treated animals killed on day 42.

* $P < .005$.

† $P < .001$ compared with the control group.

12p35 and IL-12p40, which assemble to form the active IL-12, and IL-23p19, which pairs with the IL-12p40 chain to form IL-23.^{14,28} In addition, HP-NAP induced a progressive and consistent maturation process of monocytes into mature dendritic cells, showing longer survival, a tendency to cluster, and high expression of HLA-DR, CD80, and CD86.¹⁴ Therefore, HP-NAP activates cells of the innate immunity, and in this way it significantly contributes to induce a cytokine milieu enriched in IL-12 and IL-23, which has the potential to drive the differentiation of antigen-stimulated T_H cells toward a polarized T_H1 phenotype.^{14,29} An *in vivo* correlate of these *in vitro* effects had been offered by earlier observation of strong upregulation of IL-12p40, IL-12p35, TNF- α , and IFN- γ mRNAs in biopsy specimens of the antral mucosa of *H. pylori*-infected patients with severe gastric inflammation and peptic ulcer.³⁰

Evidence for the T_H1-promoting and T_H2-inhibiting activity *in vitro* of HP-NAP was obtained by addition of medium, HP-NAP, or IL-12 to allergen-induced T-cell lines generated from mononuclear cells of house dust mite allergen-sensitive donors. Stimulation with allergen plus medium resulted in the expansion of T_H2-polarized T-cell lines and clones, whereas conditioning with either rIL-12 or HP-NAP resulted in a shift from polarized T_H2 to predominant T_H1 allergen-specific T-cell responses.¹⁴ An obvious question was whether the same anti-T_H2 effect could be detected *in vivo* in a T_H2-dominated animal model, such as infection with *T. spiralis*.³¹

T. spiralis infection worldwide is sporadic in human subjects and common in wild animals.³² As with responses against other nematodes, characteristics of the immune responses to *T. spiralis* are IgE hyperproduction and eosinophilia. Eosinophilia is due to the selective induction and expansion of T_H2 cells that produce IL-5, and the high IgE response is due to the concomitant production of IL-4 and IL-13, which are key molecules for B-cell differentiation to IgE-producing cells.^{1,2,33,34}

In this study treatment with HP-NAP of mice with established *T. spiralis* infection resulted in a consistent anti-T_H2 effect, as demonstrated by reduced eosinophilia and lower levels of total IgE in comparison with those seen in control animals. Moreover, levels of TsE/S-specific IgE in HP-NAP-treated animals were already significantly lower on day 14, just 4 days after the first HP-NAP delivery, and much lower on days 28 and 42. In addition, evidence has been provided that HP-NAP *in vivo* results in ongoing production of endogenous IL-12 and IFN- γ , even days after its delivery, as well as in persistent inhibition of the *T. spiralis*-induced expression of IL-4 and IL-5. Recall experiments with spleen cells

stimulated with medium and the infection-related TsE/S antigen allowed us to confirm that HP-NAP-treated mice underwent a T_H2 to T_H1 shift, with high IFN- γ production and low secretion of T_H2 cytokines in spite of an ongoing T_H2-polarizing condition like *T. spiralis* infection. Interestingly, the spontaneous T_H2 cytokine production *ex vivo* and the general T_H2 profile of T-cell responses disclosed by stimulation with SEB were both shifted toward a T_H1/T_H0 profile.

An important question was whether only HP-NAP or also another TLR2 agonist, such as Pam3, was able to modify the T_H2-dominated condition induced by *T. spiralis* infection. In comparison with control treatment with IgG2b, injection of Pam3 on days 10 and 28 did not change the kinetics of T_H2 parameters or those of IL-12 and IFN- γ in the plasma. In contrast, treatment with HP-NAP resulted in a consistent and substantial increase of systemic IL-12 and IFN- γ levels and a remarkable decrease of T_H2 parameters.

In *T. spiralis*-infected mice adherent splenocytes were a sensitive target of HP-NAP. In agreement with previous data obtained with human monocytes *in vitro*,¹⁴ both Pam3 and HP-NAP were able to induce murine adherent splenocytes to secrete IL-6, but only HP-NAP was able to upregulate IL-12. Addition in culture of an anti-TLR2 mAb abrogated both Pam3- and HP-NAP-induced cytokine production, indicating that HP-NAP is a TLR2 agonist also in mice and that interaction with TLR2 was required for HP-NAP to upregulate IL-12 production. *In vivo* experiments with anti-TLR2 confirmed that hampering the interaction of HP-NAP with TLR2 prevented the anti-T_H2/pro-T_H1 activity of HP-NAP.

In conclusion, this study indicates that HP-NAP delivered *in vivo* confirmed its ability to inhibit T_H2 and promote T_H1 responses *in vitro*.¹⁴ Although the maintenance of the T_H1-skewing effect by HP-NAP still needs to be explored, data available suggest that NAP protein might represent an efficient adjuvant in vaccination protocols for the treatment of T_H2-mediated allergic diseases or other diseases in which stimulation of IFN- γ -associated protective responses are desired.

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Clinical implications: Administration of HP-NAP might be used as effective inducer of endogenous IL-12 and as an anti-T_H2 agent in combination with allergen immunotherapy to treat allergic disorders.

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METHODS

Induction of anti-HP-NAP immune sera in rabbits

The possible inhibitory effect of anti-HP-NAP antibodies induced by repeated HP-NAP administrations was investigated. Two groups of 5 rabbits were immunized 4 times with OVA (100 μ g) or with HP-NAP (100 μ g) at 3-week intervals without adjuvant. Three weeks after the last immunization, sera were collected, and their titers of anti-OVA or anti-HP-NAP antibodies were assessed by using specific ELISA assays. Anti-OVA and anti-HP-NAP sera were mixed into 2 pools, filtered, and added at 10% final concentration to cultures of adherent splenocytes (10^6 in 1 mL) from *T spiralis*-infected mice in the presence of HP-NAP (1 μ g/mL). After 24 hours, supernatants were collected, and IL-12 production was measured.

RESULTS

Effect of *T spiralis* infection on peripheral blood leukocyte populations

In infected control animals injected with PBS or rat IgG2b, the total WBC, lymphocyte, and monocyte counts fluctuated without significant changes, whereas eosinophil counts were remarkably increased on days 14 ($P < .0001$), 28 ($P < .0001$), and 42 ($P < .0005$) in comparison with preinfection values (see Fig E1). On day 42, a significant decrease of neutrophil counts was observed ($P < .0001$).

Preliminary assessment of the experimental protocol of HP-NAP treatment

In a preliminary experiment 30 mice were infected with *T spiralis*, and the progression of the infection was followed up with blood sampling on days 7, 14, and 28 pi. On day 10, 10 animals received intraperitoneal injection of PBS, 10 animals had a single dose of 1 μ g of HP-NAP, and another 10 animals had a single dose of 10 μ g of HP-NAP. As summarized in Table E1, *T spiralis* infection resulted in progressive eosinophilia with a peak on day 14 and increased plasma IgE levels with a peak up to day 42. Low-dose (1 μ g) HP-NAP was not effective, and a single 10- μ g dose of HP-NAP was poorly effective on eosinophilia and

hyper-IgE. Therefore we decided to double the HP-NAP treatment by delivering a first dose of 10 μ g on day 10 before the peak of eosinophils and a second dose on day 28 before the peak of IgE.

Effect of anti-HP-NAP immune rabbit serum on the HP-NAP-induced IL-12 production

To assess the possible inhibitory effect of anti-HP-NAP antibodies induced by repeated HP-NAP administrations, rabbit anti-OVA or anti-HP-NAP sera were added to cultures of adherent splenocytes from *T spiralis*-infected mice in the presence of HP-NAP, and after 24 hours, supernatants were collected and assayed for their IL-12 content. As shown in Table E2, the presence in culture of serum from rabbits immunized with HP-NAP without any adjuvant failed to inhibit the IL-12 production, suggesting that a limited concentration of anti-HP-NAP does not prevent HP-NAP from interacting with target adherent splenocytes and inducing IL-12 secretion.

Lack of effect of Pam3 treatment on the T_H2 parameters of *T spiralis*-infected mice

A point to be addressed was whether Pam3, a classical TLR2 agonist, was able to downmodulate, like HP-NAP, the *in vivo* T_H2 response induced in mice by *T spiralis* infection. Two groups of 10 *T spiralis*-infected animals were injected with Pam3/IgG2b or Pam3/anti-TLR2 antibody on days 10 and 28, and the kinetics of blood eosinophils, total IgE, IL-4, IL-5, IL-12, and IFN- γ were compared with the kinetics in control animals treated with rat IgG2b. As summarized in Table E3, no significant change of T_H2 or T_H1 parameters was observed in Pam3/IgG2b-treated animals in comparison with control animals nor did concomitant injection of anti-TLR2 antibody have any effect.

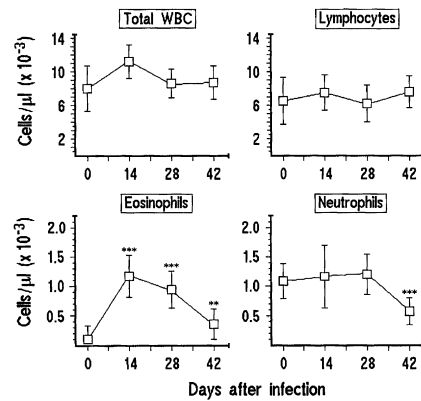


FIG E1. Effect of *T. spiralis* infection on the leukocyte populations of BALB/c mice. Results are presented as means $\pm$ SDs in 45 animals. Comparisons with preinfection values were done by using the Student 2-tailed *t* test. ** $P < .0005$ and *** $P < .0001$.

TABLE E1. Effect of a single injection or low dose of HP-NAP on eosinophilia and hyper-IgE induced by *T spiralis* infection

Time after infection	Treatment	Eosinophils/ μ L	Total plasma IgE (ng/mL)
Day 0	—	100 $\pm$ 63	70 $\pm$ 32
Day 7	—	304 $\pm$ 175	168 $\pm$ 204
Day 14	PBS (day 10)	1482 $\pm$ 206	1563 $\pm$ 541
Day 14	1 μ g HP-NAP (day 10)	1377 $\pm$ 213	1306 $\pm$ 482
Day 14	10 μ g HP-NAP (day 10)	1094 $\pm$ 278	1154 $\pm$ 389
Day 28	PBS (day 10)	932 $\pm$ 322	2252 $\pm$ 606*
Day 28	1 μ g HP-NAP (day 10)	1077 $\pm$ 362	1948 $\pm$ 512
Day 28	10 μ g HP-NAP (day 10)	714 $\pm$ 187	1461 $\pm$ 418†
Day 42	PBS (day 10)	696 $\pm$ 204‡	2680 $\pm$ 448
Day 42	1 μ g HP-NAP (day 10)	617 $\pm$ 265	1818 $\pm$ 462
Day 42	10 μ g HP-NAP (day 10)	463 $\pm$ 107§	1461 $\pm$ 418¶

Values are presented as means $\pm$ SDs in groups of 10 animals.

*Versus † P < .05.

‡Versus § P = .064.

||Versus ¶ P < .05.

TABLE E2. Effect of anti-HP-NAP immune rabbit serum on HP-NAP-induced IL-12 secretion by adherent splenocytes of *T spiralis*-infected animals

Culture conditions	IL-12 production (pg/mL)
Medium + anti-OVA serum (10%)	<10
Medium + anti-HP-NAP serum (10%)	<10
Medium + HP-NAP (1 µg/mL)	274 ± 63
Anti-OVA serum (10%) + HP-NAP (1 µg/mL)	296 ± 82
Anti-HP-NAP serum (10%) + HP-NAP (1 µg/mL)	255 ± 91

Results are presented as means ± SDs of duplicate determinations in supernatants of triplicate cultures.

TABLE E3. Lack of effect of Pam3 treatment on T_H2 response induced by *T spiralis* infection

Sampling time (treatment)	Eosinophils/ μ L	Total IgE (pg/mL)	IL-4 (pg/mL)	IFN- γ (pg/mL)	IL-12 (pg/mL)
Day 0 before infection	138 $\pm$ 49	43 $\pm$ 14	31 $\pm$ 15	38 $\pm$ 11	<10
Day 0 after infection	1017 $\pm$ 186	1376 $\pm$ 325	642 $\pm$ 142	37 $\pm$ 9	<10
Day 13 (day 10 IgG2b)	1113 $\pm$ 272	2101 $\pm$ 272	675 $\pm$ 158	38 $\pm$ 9	<10
Day 13 (day 10 Pam4/IgG2b)	1321 $\pm$ 288	2532 $\pm$ 424	730 $\pm$ 192	34 $\pm$ 9	<10
Day 13 (Pam3/anti-TLR2)	1116 $\pm$ 205	2088 $\pm$ 413	745 $\pm$ 226	34 $\pm$ 11	<10
Day 28 (day 10 IgG2b)	1031 $\pm$ 294	2686 $\pm$ 535	753 $\pm$ 207	32 $\pm$ 7	<10
Day 28 (day 10 Pam3/IgG2b)	1078 $\pm$ 309	2620 $\pm$ 425	805 $\pm$ 251	34 $\pm$ 12	<10
Day 28 (day 10 Pam3/anti-TLR2)	1027 $\pm$ 233	2533 $\pm$ 753	788 $\pm$ 202	37 $\pm$ 15	<10
Day 31 (days 10 and 28 IgG2b)	926 $\pm$ 227	2728 $\pm$ 401	773 $\pm$ 218	32 $\pm$ 8	<10
Day 31 (days 10 and 28 Pam3/IgGb2)	1011 $\pm$ 243	2663 $\pm$ 525	778 $\pm$ 223	34 $\pm$ 8	<10
Day 31 (days 10 and 28 Pam3/anti-TLR2)	964 $\pm$ 198	2445 $\pm$ 714	790 $\pm$ 124	35 $\pm$ 8	<10

Values are presented as means $\pm$ SDs.