Immunosuppression of T_H2 responses in *Trichinella spiralis* infection by *Helicobacter pylori* Neutrophil Activating Protein

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Background The Neutrophil Activating Protein of *Helicobacter pylori* (HP-NAP), is able to induce IL-12 expression by cells of the innate immunity and to shift to T_H1 human allergen-specific T_H2 cells *in vitro*.

Objective *In vivo* investigation of the ability of HP-NAP to down-modulate the T_H2 response induced in mice by *Trichinella spiralis* (Ts) infection.

Methods Groups of Ts-infected BALB/c mice received intraperitoneal (i.p.) PBS/rat IgG2b (control animals), or 10 μg HP-NAP, with or without anti-TLR2 antibody, on day 10 and 28 post infection. Blood eosinophils, total and Ts-specific IgE, and cytokine levels were measured in the plasma up to day 42, when splenocytes were cultured for cytokine production.

Results While control animals showed a significant eosinophilia and increase of total and Ts-specific IgE, IL-4 and IL-5 from day 10-14, HP-NAP-treated animals showed lower eosinophilia, total and Ts excretory/secretory antigen (TsE/S)-specific IgE in the blood. HP-NAP-treated animals also had higher IL-12 and IFN-γ plasma levels and lower IL-4, IL-5. Addition of anti-TLR2 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 parasite infection.

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

Capsule summary

Induction of endogenous IL-12 by *Helicobacter pylori* Neutrophil Activating Protein redirected to T_H1 the *Trichinella spiralis*-induced T_H2 response in mice. For its anti-T_H2 activity, the protein is proposed as adjuvant of immunotherapy for allergic diseases.

Keywords: T_H2 response, T_H2 immunosuppression, Allergy, Parasite infection, *Trichinella spiralis*, *Helicobacter pylori* Neutrophil Activating Protein (HP-NAP), endogenous IL-12, mouse model, T_H1/T_H2 redirection.

Abbreviations used in this paper: Ts, *Trichinella spiralis*; HP-NAP, Neutrophil Activating Protein of *Helicobacter pylori*; TsE/S, Excretory/Secretory antigens of *Trichinella spiralis*; Pam3, Lipohexapeptide tripalmitoyl-S-glyceryl-Cys-Ser-4(Lys); Toll-like Receptor 2, TLR2; SEB, Staphylococcal Enterotoxin B.

Introduction

T helper type 2 (T_H2) cells characterized by the production of interleukin (IL)-4, IL-5, IL-9, and IL-13,[1,2] are involved in the immune response to helminth infections,[3-5] 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* (Ts) infection was susceptible to down-regulation by IL-12 promoting signals. To address this question, we took advantage of a virulence factor of *Helicobacter pylori*: the Neutrophil Activating Protein (HP-NAP).[12,13]

HP-NAP, an oligomeric protein of 150 kDa, is a TLR-2 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 interferon (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 Ts. Ten and 28 days later, infected animals were treated with intraperitoneal (i.p.) 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 antigen of Ts (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 in control infected animals. Co-injection of HP-NAP and anti-TLR2 antibody abrogated the anti-T_H2 activity of HP-NAP.

METHODS

Animals

Female BALB/c mice (Harlan, Italy), 6 weeks old were acclimated to the University of Pisa animal care facility for one week before the 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 in order to detect anti-Th2 effects were assessed (see the E Results section of the Online Repository). 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 i.p. with HP-NAP (10 μg in 0.5 mL PBS) on day 10 and 28 p.i Three of such experiments were done, involving a total of 45 PBS- and 45 HP-NAP treated animals. In further experiments, Ts-infected animals were divided into groups of 10, that were injected on day 10 and 28 p.i. with: a) control protein (rat IgG2b isotype control, 30 μg), b) HP-NAP (10 μg) plus IgG2b (30 μg), c) HP-NAP (10 μg) plus anti-TLR2 rat IgG2bk monoclonal antibody (30 μg), or d) anti-TLR2 antibody alone (30 μg). In parallel, two other groups of 10 animals were injected on day 10 and 28 p.i. with Pam3Cys, referred to herein as Pam3, (10 μg) plus IgG2b (30 μg), or Pam3 (10 μg) plus anti-TLR2 antibody (30 μg). Peripheral blood was collected in Na-Heparine tubes from the retroorbital venous plexus before infection and at various time points (day 0, 7, 14, 28 and 42) after infection. In other experiments, blood was collected on day 0, 10, 13, 28, 31 and 42 p.i. During the experiments, both control- and HP-NAP-treated animals equally gained weight and were in good conditions. On day 42 p.i., animals were sacrificed and tissue samples were fixed and processed for the histological assessment of infection.

Parasites, *T. spiralis* Excretory/Secretory (TsE/S) antigen preparation and biotin labelling

Trichinella 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 giving orally 350 muscle larvae to each mouse.

Ts muscle larvae were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing streptomycin (500 μg/mL) at 37° C in a 5% CO_2 atmosphere. After 18 hours, surpernatant was collected and desalted into the appropriate buffer using a PD-10 column (Amersham Biosciences Europe, Freiburg, Germany). E/S antigen protein concentration was estimated by absorbance at 280 nm using a Varian Cary Bio 50 spectrophotometer.

Biotin labelling of TsE/S antigen was performed as described.[16] Briefly, TsE/S antigen (4 mg/mL) in sodium bicarbonate buffer pH 8.5 was reacted with biotin *N*-hydroxysuccinimide ester-water soluble (Vector Laboratories, Burlingame, CA) 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 lipopolysaccharide contamination.[14] The recombinant protein was pure as judged from overloaded gels composed of different percentages of polyacrylamide. Mass spectrometry analysis, performed with a Maldi Reflex (Brucker Analytik), confirmed that the protein consisted of a single molecule of 16,875 ± 20 Da. Immune-depleted preparation of HP-NAP

was obtained with a purified anti-HP-NAP antibody, using a G-Sepharose matrix, as reported.[14] Pam3 was purchased from EMC microcollection GmbH (Tuebingen, Germany), purified anti-mouse TLR2 rat IgG2bk (clone 6C2) and rat IgG2b isotype control were purchased from eBioscience. In separate experiments, the possible inhibitory effect of anti-HP-NAP antibodies induced by repeated HP-NAP administration was investigated (see E Methods, E Results and E Table II of the Online Repository).

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

Total mouse IgE levels in the plasma were assessed by a specific ELISA (Alpha Diagnostic, San Antonio, TX), according to the manufacturer's instruction. TsE/S specific IgE in plasma samples were determined by 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 μg/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 orthophenilendiamine was reacted for 30 minutes with H_2O_2 and then stopped with H_2SO_4. Optical density (OD) at 490 nm was assessed by a microplate spectrophotomer.

Spleen cell cultures and cytokine assays

Adherent and nonadherent splenocytes were obtained from 5 untreated Ts-infected mice sacrificed on day 14. Single spleen cell suspensions were prepared, counted and cultured at 1 X 10^6 cells/mL in RPMI 1640 medium supplemented with 5% FCS (HyClone, Logan, Utah) in

159 plastic flasks at 37°C. After 1 hour incubation, nonadherent cells were recovered. Adherent
160 cells were extensively washed with RPMI 1640 medium to remove residual nonadherent cells.
161 Adherent and nonadherent cells were then cultured for 24 hours in RPMI 1640 medium, 10%
162 FCS, 2 mM L-glutamine, sodium pyruvate, nonessential amino acids solution, and antibiotics
163 in the presence of: a) medium plus rat IgG2b isotype control (3 μg in 1.0 mL), b) anti-TLR2
164 antibody (3 μg in 1.0 mL), c) HP-NAP (0.1 and 1.0 μg in 1.0 mL) plus rat IgG2b isotype
165 control (3 μg), or d) plus anti-TLR2 monoclonal antibody (3 μg), e) Pam3 (0.1 and 1.0 μg in
166 1.0 mL) plus IgG2b control (3 μg), or f) plus anti-TLR2 antibody (3 μg). At the end of culture
167 supernatants were collected and assessed for IL-12 and IL-6 production.
168 On day 42 p.i., mice were sacrificed. Single spleen cell suspensions were prepared, counted
169 and cultured at 2 X 10^6 cells/mL in RPMI 1640 medium supplemented with 10% FCS, 2 mM
170 L-glutamine, sodium pyruvate, nonessential amino acids solution, and antibiotics. Spleen cells
171 (1 mL/tube) were cultured in duplicate tubes in the presence of medium alone, 10 μg/mL
172 TsE/S antigen or 100 ng/mL staphylococcal enterotoxin B (SEB) at 37°C with 5% CO_2. After
173 48 hours, culture supernatants were collected for cytokine assays. Mouse IL-6, IL-12, IL-4,
174 IL-5, IL-13, and IFN-γ levels in culture supernatants and plasma, were measured by specific
175 ELISA assays (R&D Systems GmbH, Wiesbaden, Germany), according to the manufacturer's
176 instructions.
177
178 **Statistical analysis**
179
180 Results of WBC counts, cytokine levels in plasma or supernatants, and total and TsE/S
181 specific IgE are presented as means (+ SD) for groups of animals undergoing uniform
182 treatment. Differences between groups were analyzed using the Student's two-tailed *t* test. A
183 probability (*P*) of less than 0.05 was considered significant.

RESULTS

Effect of Ts infection and of 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 eosinophils were remarkably increased on day 14 ($P < .0001$), 28 ($P < .0001$), and 42 ($P < .0005$) in comparison to pre-infection values (see E Fig 1 of the Online Repository). On day 42, a significant decrease of neutrophils was observed ($P < .0001$). In animals treated with HP-NAP on day 10 and 28, total WBC, lymphocyte and neutrophil counts on day 14, 28 and 42 were significantly lower in comparison to 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 to controls (Fig 1).

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

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

208 Before infection, plasma levels of TsE/S-specific IgE were not detectable (OD < 0.030).
209 However, on day 7 p.i., Ts E/S-specific IgE became detectable (mean ± SD OD 0.084 ± 0.035).
210 In control animals, TsE/S-specific IgE were remarkably increased on day 14, 28 and 42 ($P <$
211 .0001) in comparison with values on day 7 (Fig 2, *B*). Also in HP-NAP treated animals TsE/S-
212 specific IgE were increased on day 14 ($P < .0001$), but levels substantially decreased thereafter.
213 However, in comparison with control animals, HP-NAP-treated animals had significantly
214 lower levels of TsE/S-specific IgE on day 14 ($P < .0005$) and much lower on day 28 and day
215 42 ($P < .0001$) (Fig 2, *B*).
216
217 **Target cells of HP-NAP and role for HP-NAP-TLR2 interaction**
218
219 To identify the cell targets of HP-NAP, adherent and nonadherent splenocytes from 5
220 untreated Ts-infected mice were obtained on day 14. Both cell populations were cultured for 24
221 hours in the presence of different combinations and doses of rat IgG2b isotype control, anti-
222 TLR2 antibody, HP-NAP or Pam3. Culture supernatants were then collected and IL-12 and IL-
223 6 production was measured. Neither rat IgG2b nor anti-TLR2 antibody alone had any effect on
224 the spontaneous IL-12 and IL-6 production by adherent or nonadherent splenocytes. Addition
225 of 0.1 or 1 μg/mL Pam3 to adherent splenocytes resulted in a strong increase of IL-6 secretion,
226 whereas nonadherent cells failed to increase IL-6 over the background (data not shown). More
227 importantly, Pam3 failed to induce IL-12 production by adherent splenocytes (Fig. 3), as well
228 as by nonadherent cells. As expected, Pam3-induced upregulation of IL-6 in adherent cells was
229 abolished by addition of anti-TLR2 antibody. In parallel cultures of adherent splenocytes,
230 addition of HP-NAP resulted in upregulation of both IL-6 and IL-12 production, depending on
231 the dose of HP-NAP. Like Pam3, HP-NAP failed to induce IL-6 and/or IL-12 in nonadherent
232 cell cultures (data not shown). However, if anti-TLR2 antibody was added together with HP-
233 NAP, upregulation of IL-6 and IL-12 was abrogated, suggesting that the interaction with TLR2

234 was essential for HP-NAP to exert its ability to induce adherent cells to IL-12 expression (Fig.
235 3). In this experiment, we also observed the lack of activity on IL-6 and IL-12 production by
236 adherent cells of a preparation in which HP-NAP had been immune-depleted. Therefore, it is
237 unlikely that contaminants of our HP-NAP preparation were indeed responsible for its effects.
238 *In vitro* experiments with anti-TLR2 antibody suggested that the interaction with TLR2
239 was essential for HP-NAP to exert its ability to induce IL-12 expression. We then asked
240 whether hampering HP-NAP-TLR2 interaction *in vivo* resulted in inhibition of the anti-T_H2
241 activity of HP-NAP in Ts-infected mice. A group of 10 infected animals were injected i.p. with
242 control rat IgG2b, another group of 10 with HP-NAP plus rat IgG2b, and a third group of 10
243 with HP-NAP plus anti-TLR2 antibody. In all groups, eosinophils, total IgE, IL-12, IFN-γ, IL-
244 4 and IL-5 levels were measured before infection, on day 10 (before the first injection), day 13,
245 day 28 (before the second injection), day 31, and day 42. As summarized in **Fig. 4**, in HP-
246 NAP/IgG2b-treated animals, plasma IL-12 and IFN- γ rose on day 13 ($P < .0001$ vs day 10),
247 declining on day 28 ($P < .0005$ vs day 13), but rising up again on day 31 ($P < .0001$ vs day 28)
248 after the second delivery of HP-NAP. On day 42, plasma IL-12 was still detectable and IFN-γ
249 remained high. In the same animals, HP-NAP/IgG2b treatment resulted in strong reduction of
250 plasma IL-4 and IL-5 ($P < .0001$, day 13 vs day 10), that progressively decreased to pre-
251 infection values. The kinetics of eosinophils was similar ($P < .0005$, day 13 vs day 10),
252 whereas IgE levels were significantly reduced starting from day 28 up to day 42 in comparison
253 with IgG2b-treated controls. Co-injection of anti-TLR2 antibody with HP-NAP abrogated the
254 HP-NAP-induced reduction of T_H2 parameters (eosinophils, IgE, IL-4 and IL-5) and increase
255 of plasma IL-12 and IFN-γ. In infected animals injected with Pam3/IgG2b, no significant
256 change of T_H2 or T_H1 parameters was observed in comparison with animals treated with rat
257 IgG2b (see E Results section and Table E3 of Online Repository).
258

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

On day 42 p.i., splenocytes were isolated and cultured for 48 hours in the presence of medium alone, 10 μg/mL TsE/S antigen or 100 ng/mL staphylococcal enterotoxin B (SEB) as 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). Upon 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 four 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. Upon 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 was 16, 12, and 16 times, respectively. In conclusion, in comparison with those of controls, splenocytes from HP-NAP-treated mice showed a significantly higher ($P < .001$) IFN-γ production and 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 humans 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-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 high expression of HLA-DR, CD80, and CD86, longer survival, and a tendency to

cluster.[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 biopsies 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-polarised 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 the infection with *Trichinella spiralis*.[31]

Ts infection worldwide is sporadic in humans and common in wild animals.[32] Like against other nematodes, characteristics of the immune responses to Ts are IgE hyper-production 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 Ts infection resulted in a consistent anti-T_H2 effect, as demonstrated by reduced eosinophilia and lower levels of total IgE in comparison with control animals. Moreover, levels of TsE/S-specific IgE in HP-NAP-treated animals were already significantly lower on day 14, just only 4 days after the first HP-NAP delivery, and much lower on day 28 and day 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 Ts-induced expression of IL-4 and IL-5. Recall experiments with spleen cells stimulated with medium, 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 Ts 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 towards 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 Ts infection. In comparison to control treatment with IgG2b, injection of Pam3 on day 10 and 28 did not change the kinetics of T_H2 parameters, nor 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, and a remarkable decrease of T_H2 parameters.

In Ts-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 up-regulate IL-12. Addition in culture of an anti-TLR2 monoclonal antibody 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 up-regulate IL-12 production. *In vivo* experiments with anti-TLR2 confirmed that hampering the interaction of HP-NAP with TLR2 prevented the anti-T_H2/proT_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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Figure legends

FIG 1. Effect of HP-NAP treatment on the leukocyte populations of Ts-infected BALB/c mice. On day 10 and 28, half of the animals (n = 45) received i.p. PBS (open columns), whereas the other half received 10 μg HP-NAP (closed columns). Results represent means (± SD). Comparisons were done by the Student's two-tailed *t* test. * $P < .001$, ** $P < .0005$, *** $P < .0001$.

F IG 2. Effect of HP-NAP treatment on plasma levels of total and TsE/S-specific IgE. In controls (open columns), total IgE (A) and Ts E/S-specific IgE (B) were high up to day 42, whereas in HP-NAP-treated animals (closed columns) IgE progressively decreased after day 14. Results represent means (± SD) in two groups of 45 animals. ** $P < .0005$, *** $P < .0001$.

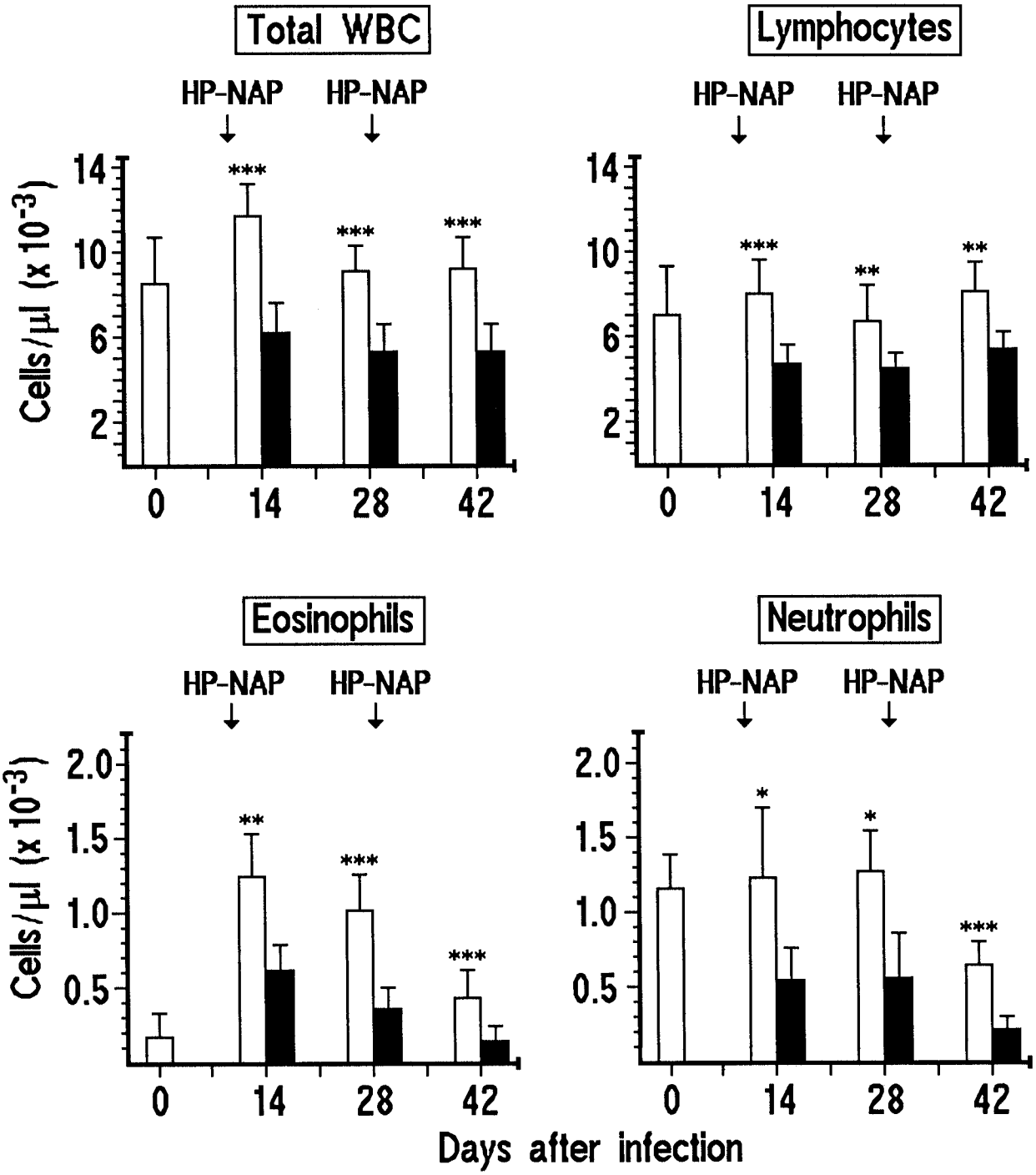
FIG 3. In vitro induction of cytokine secretion by the TLR2 agonists Pam3 and HP-NAP and its abrogation by anti-TLR2 antibody. Results represent mean levels (± SD) of IL-6 or IL-12 measured in supernatants of triplicate cultures for each condition.

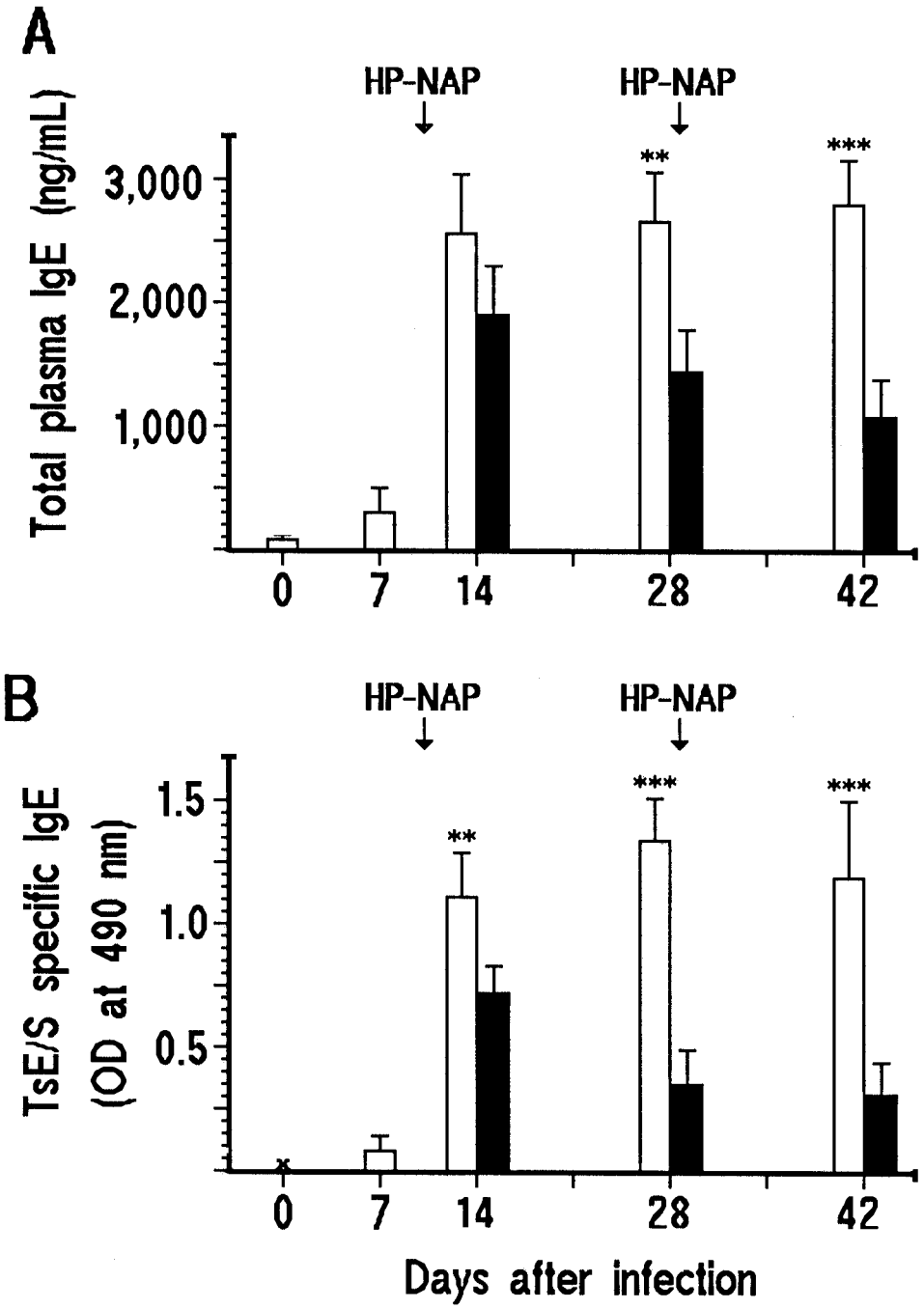
F IG 4. Effect of HP-NAP treatment on blood T_H2 and T_H1 parameters. Eosinophils, total IgE, IL-4, IL-5, IFNγ and IL-12 were measured in the blood of IgG2b-treated controls (open circles) and compared with values in animals treated on day 10 and 28 with HP-NAP/IgG2b (closed squares) or HP-NAP plus anti-TLR2 antibody (open squares). Results represent mean values (± SD) in 3 groups of 10 animals.

TABLE I. T_H1 and T_H2 cytokine production in spleen cell cultures in unteated controls or HP-NAP-treated Ts-infected animals

Ts-infected animals	Stimulant	Cytokine secretion (pg/mL)			
		IFN-γ	IL-4	IL-5	IL-13
Untreated controls (n = 30)	Medium	25 ± 9	143 ± 58	232 ± 48	326 ± 78
	TsE/S	121 ± 34	2,982 ± 304	3,645 ± 378	4,525 ± 465
	SEB	435 ± 166	3,413 ± 387	3,990 ± 416	5,520 ± 476
HP-NAP-treated (n = 34)	Medium	124 ± 51	51 ± 27	74 ± 31	64 ± 34
	TsE/S	2,315 ± 418**	487 ± 156*	687 ± 155*	812 ± 173*
	SEB	3,565 + 566**	692 ± 244*	739 ± 212*	976 + 219*

Results represent means (± SD) of duplicated determinations in duplicate cultures of spleen cells from untreated or HP-NAP-treated animals sacrificed on day 42. *P < .005 and **P < .001, compared with control group.


Total WBC
Lymphocytes
Eosinophils
Neutrophils
HP-NAP
HP-NAP
Cells/µl (x 10-3)
14
10
6
2
2.0
1.5
1.0
0.5
0
14
28
42
Days after infection



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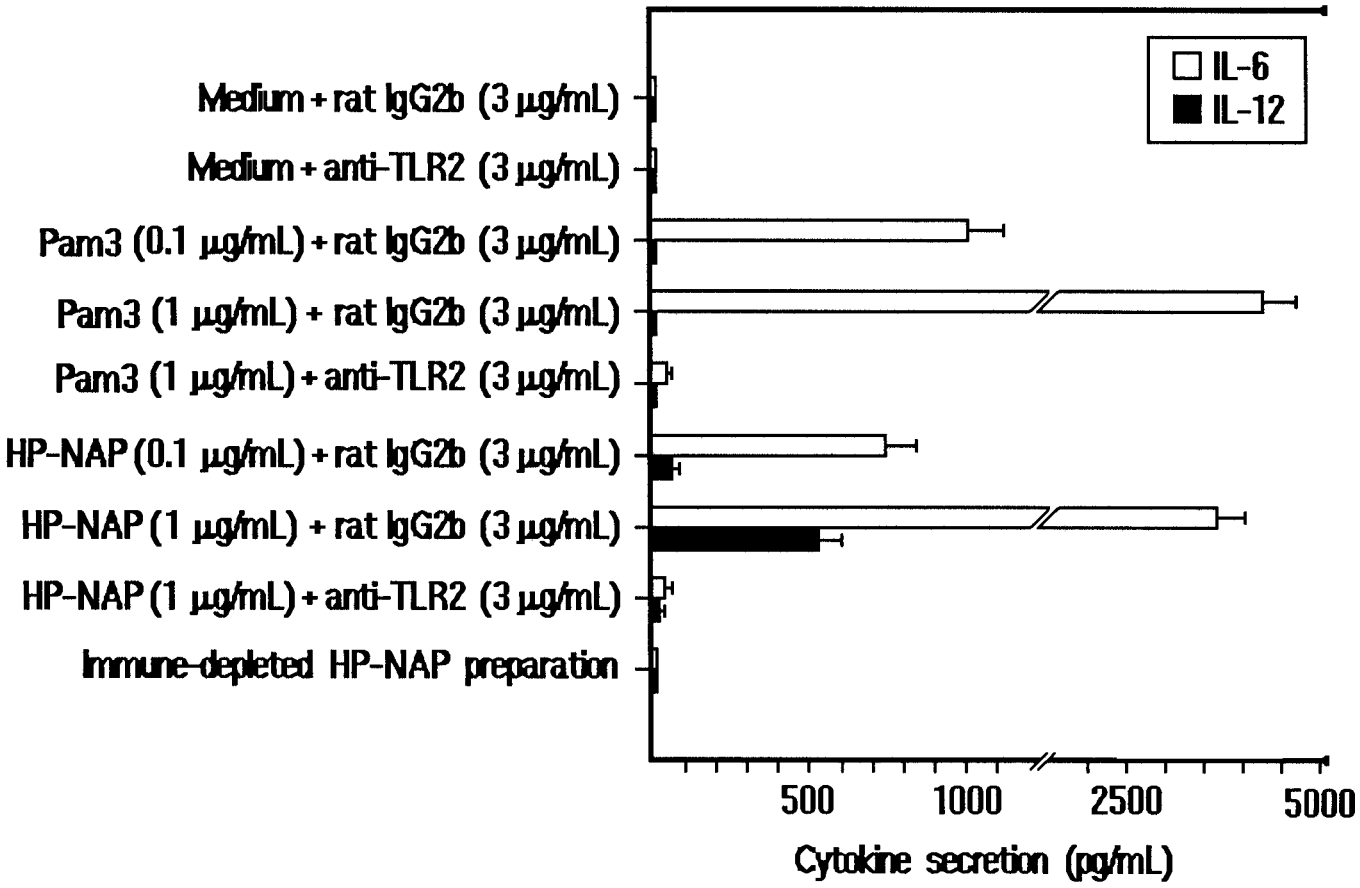
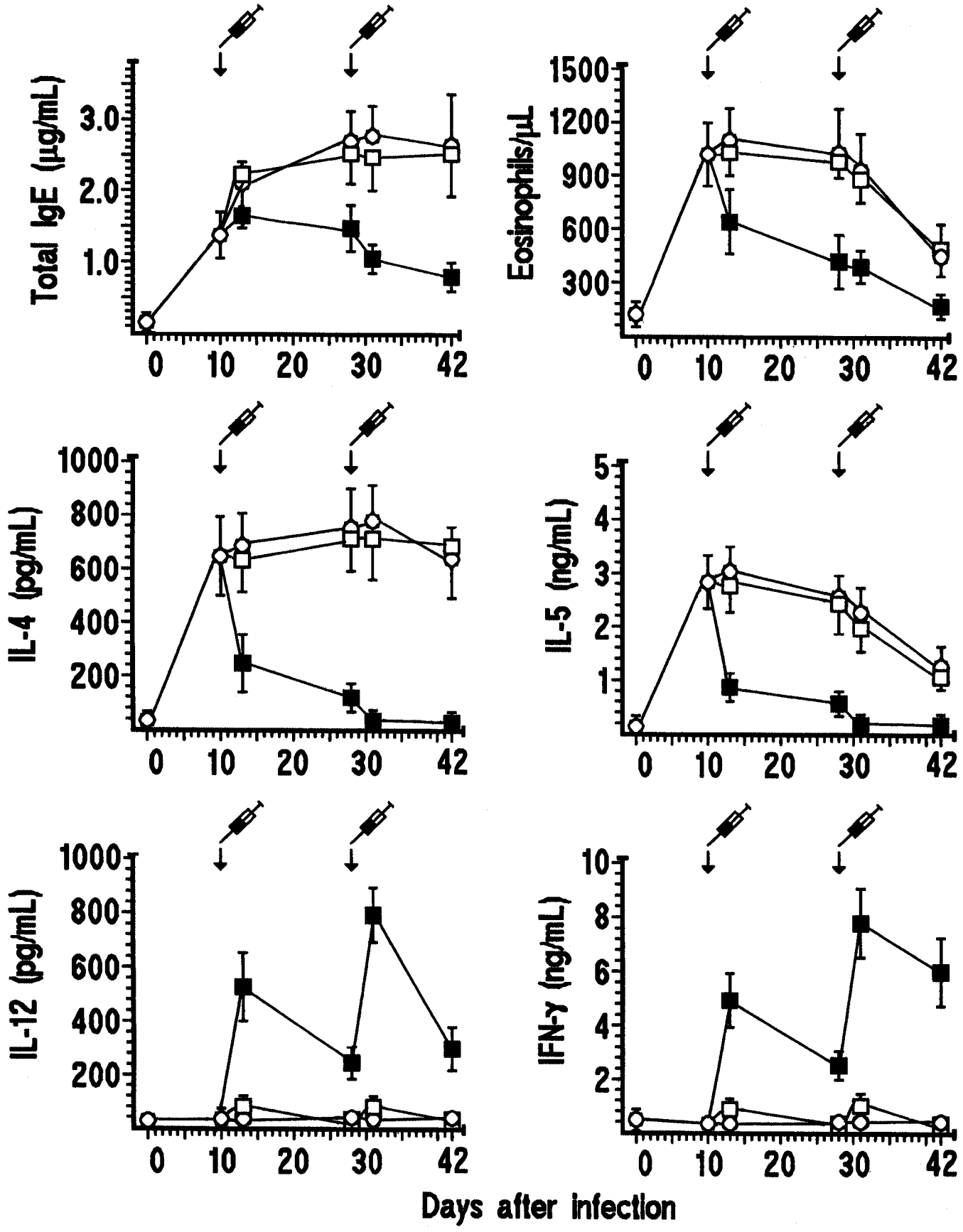

IL-6
IL-12
Medium + rat IgG2b (3 µg/mL)
Medium + anti-TLR2 (3 µg/mL)
Pam3 (0.1 µg/mL) + rat IgG2b (3 µg/mL)
Pam3 (1 µg/mL) + rat IgG2b (3 µg/mL)
Pam3 (1 µg/mL) + anti-TLR2 (3 µg/mL)
HP-NAP (0.1 µg/mL) + rat IgG2b (3 µg/mL)
HP-NAP (1 µg/mL) + rat IgG2b (3 µg/mL)
HP-NAP (1 µg/mL) + anti-TLR2 (3 µg/mL)
Immune-depleted HP-NAP preparation
500
1000
2500
5000
Cytokine secretion (pg/mL)


Total IgE (µg/mL)
3.0
2.0
1.0
Eosinophils/µL
1500
1200
900
600
300
IL-4 (pg/mL)
1000
800
600
400
200
IL-5 (ng/mL)
5
4
3
2
1
IL-12 (pg/mL)
1000
800
600
400
200
IFN-γ (ng/mL)
10
8
6
4
2
0
10
20
30
42
Days after infection

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Immunosuppression of T_H2 responses in *Trichinella spiralis* infection by *Helicobacter pylori* Neutrophil Activating Protein

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E 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 μg) or with HP-NAP (100 μg) at 3-weeks intervals, without adjuvant. Three weeks after the last immunization, sera were collected and their titres of anti-OVA or anti-HP-NAP antibodies were assessed by specific ELISA assays. Anti-OVA and anti-HP-NAP sera were mixed into two pools were filtered and added at 10% final concentration to cultures of adherent splenocytes (10^6 in 1 mL) from Ts-infected mice in the presence of HP-NAP (1 μg/mL). After 24 hours, supernatants were collected and IL-12 production was measured.

E RESULTS

Effect of Ts 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 eosinophils were remarkably increased on day 14 ($P < .0001$), 28 ($P < .0001$), and 42 ($P < .0005$) in comparison to pre-infection values (E Fig 1). On day 42, a significant decrease of neutrophils was observed ($P < .0001$).

Preliminary assessment of the exprerimental protocol of HP-NAP treatment

In a preliminary experiment, 30 mice were infected with Ts and the progression of the infection was followed up with blood sampling on day 7, 14 and 28 p.i. On day 10, ten animals received i.p. injection of PBS, 10 animals had a single dose of 1 μg HP-NAP, and other 10 had a single dose of 10 μg HP-NAP. As summarized in Table E1, Ts 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 μg) HP-NAP was not effective and 10 μg HP-NAP single dose was poorly effective on eosinophilia and hyper-IgE. Therefore, we decided to double the HP-NAP treatment by delivering a first dose of 10 μg 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 Ts-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 to interact with target adherent splenocytes and to induce IL-12 secretion.

Lack of effect of Pam3 treatment on the T_H2 parameters of Ts-infected mice

A point to be addressed was whether Pam3, a classical TLR2 agonist, was able to down modulate, like HP-NAP, the *in vivo* T_H2 response induced in mice ba Ts infection. Two groups of 10 Ts-infected animals were injected with Pam3/IgG2b or Pam3/anti-TLR2 antibody on day 10 and 28, and the kinetis 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 controls, nor concomitant injection of anti-TLR2 antibody had any effect.

582 **E Figure legend**

583

584 **E FIG 1.** Effect of *Trichinella spiralis* infection on the leukocyte populations of BALB/c mice.

585 Results represent means (± SD) in 45 animals. Comparisons with pre-infection values were done by

586 the Student's two-tailed *t* test. ** $P < .0005$, *** $P < .0001$.

587

588

589 **Table E1.** Effect of a single injection or low dose of HP-NAP on eosinophilia and hyper-IgE

590 induced by *T. spiralis* infection

591

Time after infection	Treatment	Eosinophils/μl	Total plasma IgE (ng/ml)
day 0	-	100 ± 63	70 ± 32
day 7	-	304 ± 175	168 ± 204
day 14 .	PBS (day 10)	1,482 ± 206	1,563 ± 541
day 14	1 μg HP-NAP (day 10)	1,377 ± 213	1,306 ± 482
day 14	10 μg HP-NAP (day 10)	1,094 ± 278	1,154 ± 389
day 28	PBS (day 10)	932 ± 322	2,252 ± 606 [a]
day 28	1 μg HP-NAP (day 10)	1,077 ± 362	1,948 ± 512
day 28	10 μg HP-NAP (day 10)	714 ± 187	1,461 ± 418 [b]
day 42	PBS (day 10)	696 ± 204 [c]	2,680 ± 448 [e]
day 42	1 μg HP-NAP (day 10)	617 ± 265	1,818 ± 462
day 42	10 μg HP-NAP (day 10)	463 ± 107 [d]	1,461 ± 418 [f]

606

607

608 Mean values (± SD) in groups of 10 animals. [a] vs [b] $P < .05$; [c] vs [d] P .064; [e] vs [f] $P < .05$

609

610

611

Table E2. Effect of anti-HP-NAP immune rabbit serum on HP-NAP-induced IL-12 secretion by adherent splenocytes of Ts-infected animals.

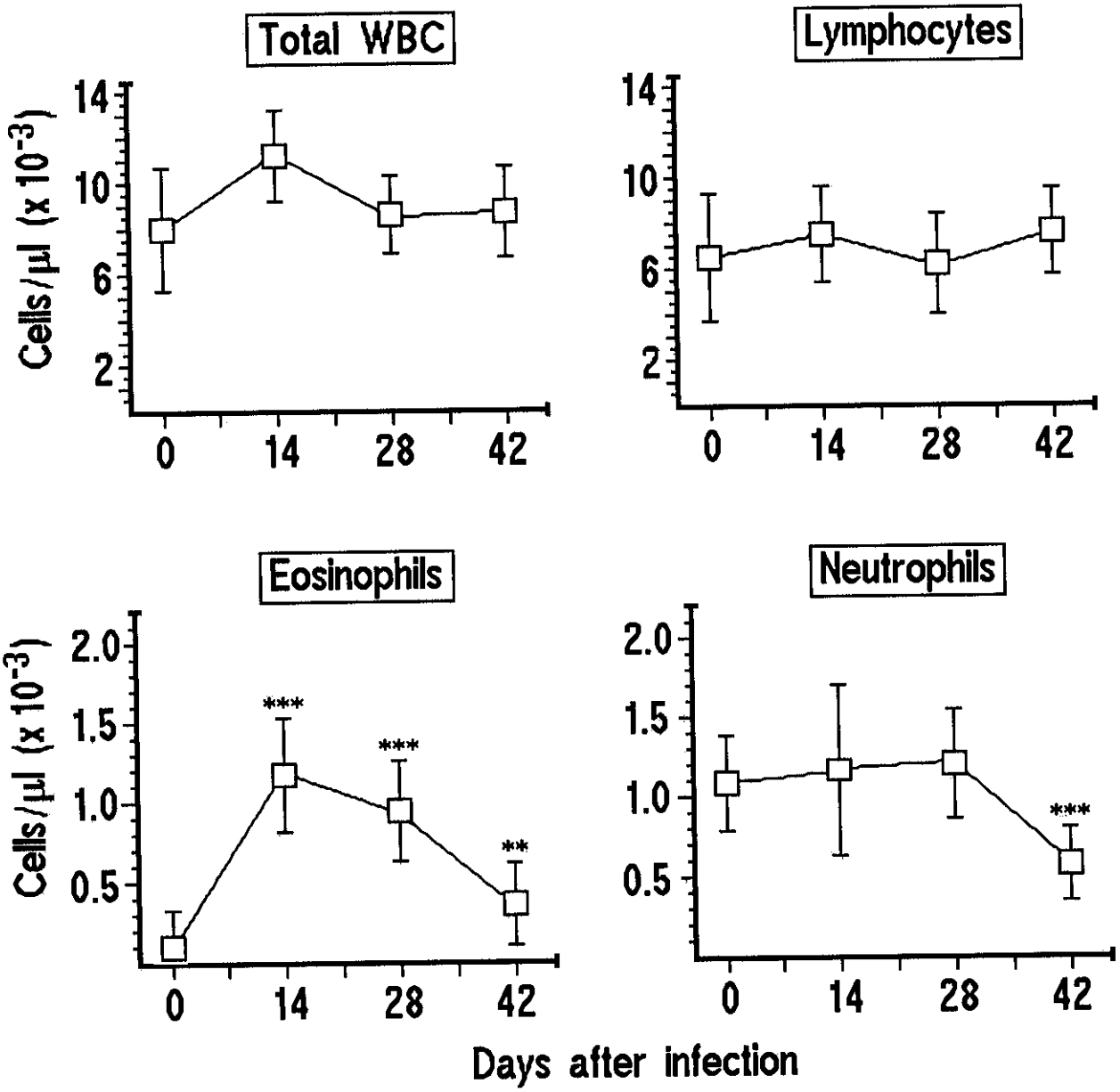
Culture conditions	IL-12 production (pg/ml)
Medium + Anti-OVA serum (10%)	< 10
Medium + Anti-HP-NAP serum (10%)	< 10
Medium + HP-NAP (1 μg/mL)	274 ± 63
Anti-OVA serum (10%) + HP-NAP (1 μg/mL)	296 ± 82
Anti-HP-NAP serum (10%) + HP-NAP (1 μg/mL)	255 ± 91

Results represent mean values (± SD) of duplicate determinations in supernatants of triplicate cultures.

Table E3. Lack of effect of Pam3 treatment on TH2 response induced by Ts infection.

Sampling time (treatment)	Eosinophils/μL	Total IgE (pg/mL)	IL-4 (pg/mL)	IFN-γ (pg/mL)	IL-12 (pg/mL)
Day 0 pre-infection	138 ± 49	43 ± 14	31 ± 15	38 ± 11	< 10
Day 10 post infection	1,017 ± 186	1,376 ± 325	642 ± 142	37 ± 9	< 10
Day 13 (day 10 IgG2b)	1,113 ± 272	2,101 ± 272	675 ± 158	38 ± 9	< 10
Day 13 (day 10 Pam3/IgG2b)	1,321 ± 288	2,532 ± 424	730 ± 192	34 ± 9	< 10
Day 13 (day 10 Pam3/anti-TLR2)	1,116 ± 205	2,088 ± 413	745 ± 226	34 ± 11	< 10
Day 28 (day 10 IgG2b)	1,031 ± 294	2,686 ± 535	753 ± 207	32 ± 7	< 10
Day 28 (day 10 Pam3/IgG2b)	1,078 ± 309	2,620 ± 425	805 ± 251	34 ± 12	< 10
Day 28 (day 10 Pam3/anti-TLR2)	1,027 ± 233	2,533 ± 753	788 ± 202	37 ± 15	< 10
Day 31 (day 10 & 28 IgG2b)	926 ± 227	2,728 ± 401	773 ± 218	32 ± 8	< 10
Day 31 (day 10 & 28 Pam3/IgG2b)	1,011 ± 243	2,663 ± 525	778 ± 223	34 ± 8	< 10
Day 31 (day 10 & 28 Pam3/anti-TLR2)	964 ± 198	2,445 ± 714	790 ± 124	35 ± 8	< 10

Mean values (± SD)


Total WBC
Lymphocytes
Eosinophils
Neutrophils
Cells/µl (x 10-3)
14
10
6
2
2.0
1.5
1.0
0.5
0
14
28
42
***
**
Days after infection

653