

Do Th1 or Th2 sequence motifs exist in proteins? Identification of amphipatic immunomodulatory domains in *Helicobacter pylori* catalase

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Abstract

The reasons why some proteins induce a particular type of T helper (Th) response are of fundamental importance but only partially understood. In the present study, amphipatic sequence motifs were identified in N- and C-terminal domains of *Helicobacter pylori* (Hp) catalase, which are linked to the induction of Th1 or Th2 immune responses, respectively. Alignment of these motifs with other proteins known to induce either Th1 or Th2 responses has lead to the identification of Th1 and Th2 consensus motifs, termed modulotopes. Their immunomodulatory potential was demonstrated by immunisation experiments using recombinant proteins comprising the C-terminal domain of catalase fused with one or several modulotopes and by co-immunisations of C- or N-terminal catalase domains with peptides containing these motifs. In addition to these in vivo data, in vitro assays using Limulus extracts suggested that modulotopes might interfere with responses triggered by danger signals such as LPS. Th1 and Th2 modulotopes are characterised by a specific hydrophobic/hydrophilic pattern, which might be the structural determinant for their activity. Our data suggest that Th1 and/or Th2 motifs may generally exist on proteins, thus offering the possibility of a rational modulation of the immune response.

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1. Introduction

CD4⁺ T lymphocytes can polarize toward T helper 1 (Th1) or Th2 cells, which are preferentially involved in cell-mediated immunity or antibody production, respectively. Various factors have been linked to the orientation of the T helper response [1], including the existence of a “third signal” acting in addition to TCR stimulation and T cell co-stimulation [2]. Several reports have shown that some protein antigens specifically trigger either a Th1 or Th2 biased immune response [3–9]. The molecular mechanism of this behaviour

is not fully understood. We hypothesised that proteins contain intrinsic properties able to participate in the induction of one or the other type of immune response. These properties were assumed to be linked to structural motifs, which might possibly be reflected in the protein sequence.

We addressed this question using *Helicobacter pylori* (Hp) catalase, a vaccine candidate inducing in mice a protective balanced Th response [10].

2. Materials and methods

2.1. Mice and immunization

Outbred OF1 and inbred C57BL/6 or BALB/c female mice 6–8-weeks-old were purchased from IFFA Credo-Charles River (France). They were housed according to European

Abbreviations: Hp, *Helicobacter pylori*; Nter, recombinant N-terminal domain of *H. pylori* catalase; Cter, C-terminal domain of *H. pylori* catalase; ITC, isothermal titration calorimetry

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guidelines. Protocols were approved by Aventis Pasteur Internal Ethic Committee. Cages were covered with Isocaps, mice were given filtered water and irradiated food and autoclaved material was used.

Mice were immunised on days 0 and 21 by subcutaneous (SC) route (200 μ l under the skin of the left part of the lumbar region). Five micrograms of antigen (alone or in combination with various doses of peptides) were administered in presence of 200 μ g of DC Chol adjuvant (Targeted Genetics, Seattle) [15].

2.2. Measurement of cytokines-secreting spleen cells by ELISPOT and serum antibodies by ELISA

ELISPOT and ELISA were performed according to standard protocols as previously described [10].

2.3. Generation of recombinant antigens

2.3.1. Cloning procedures

Full-length *Hp* catalase had previously been cloned and expressed in *E. coli* from *H. pylori* X43-2AN [11]. For the other constructs, DNA fragments obtained by PCR or synthetic genes were cloned into the pET28c vector (Novagen) to add a hexa-histidine tag to the N-terminal end of the inserts allowing the purification of the recombinant protein by affinity chromatography on Ni-agarose columns. Positive clones were selected after transformation of *E. coli* XL1blue (Stratagene). *E. coli* BL21 λ DE3 (Novagen) were transformed with purified plasmids and protein expression was induced after the addition of IPTG. All constructs were verified by DNA sequencing of both strands of the insert and flanking regions.

2.3.1.1. N- and C-terminal domains of *Hp* catalase. The DNA fragments corresponding to the N-terminal domain (Nter, amino acids V²NK–DTH³⁴³) and C-terminal domain (Cter, amino acids P³⁶²FH–KKK⁵⁰⁶) of *H. pylori* catalase were amplified by PCR from a plasmid containing the full-length catalase sequence. PCR fragments were cloned into *Bam*H1 and *Xho*1 sites of pET28c. DNA sequencing of the plasmid containing Nter resulted in the detection of a frameshift error giving rise to a premature stop codon after amino acid 343, which resulted in a truncation of the last 17 amino acids of the selected Nter domain. In view of this relatively small deletion it was decided to use this protein for experimentation. One can notice that premature truncation generated an *H. pylori* Nter domain lacking the tyrosine residue corresponding to Tyr 370 in human catalase, involved in the catalytic site [12]. Cter domain lacks critical His, Asn, Ser and Tyr residues similarly involved in enzymatic activity.

2.3.1.2. N4-Cter and N3-Cter fusion proteins. The DNA fragments corresponding to the N3-(HTMQGVKHLTNE-EAAEVRKYDPDS) and the N4 motifs (PEEDAKKYRFH-PFDVTKIWYLQ) of *Hp* catalase were cloned into *Nhe*1 and *Bam*H1 sites of pET28c containing the sequence of Cter.

This resulted in the fusion of the N3 and N4 motif to the N-terminus of Cter.

2.3.1.3. N1234-Cter fusion. A synthetic DNA fragment corresponding to the fused [N1][N2][N3][N4] motifs was generated with synthetic oligonucleotides and cloned between *Nhe*1 and *Bam*H1 sites pET28c containing the sequence of Cter in a similar fashion as reported above, giving the following fragment fused to the N-terminus of Cter: [VN-KDVKQTAFGAPVWDDN][LLQSTWFLEKLAA][RFW-VKFHFHTMQGVKHLTNEEAAEVRKYDPDSNQ][PEE-DAKKYRFHFPFDVTKIWYLQ]. None of N1–N4 domains contains amino acids involved in enzymatic activity [12].

2.3.2. Protein expression, purification and characterisation

Cultures of transformed *E. coli* BL21 λ DE3 were carried out at 37 °C under standard conditions. Bacteria were harvested 4 h after induction with IPTG. Cells were lysed by sonication, and after solubilization with urea from the pellet; proteins were purified by affinity on a Hi-trap chelating sepharose column, (AmershamBiosciences) using standard protocols. After elution, proteins were dialyzed against 0.5M arginine/Urea1M/PBS (Nter) or 0.5M arginine/PBS (all other antigens), filtrated and stored at 2–4 mg/ml at –70 °C. The identity of the proteins was checked by SDS-PAGE and western-blots using anti His-Tag and anti-catalase antibodies. Purity was estimated to be ranging from 85 to 90% by a densitometric analysis of Coomassie Blue stained SDS gels. The residual LPS content was found to be similar for all recombinant antigens used in this study and were in a range between 0.0125 and 0.05 endotoxin units (EU)/ μ g of protein. Protein concentration was determined using the MicroBCA kit (Pierce).

2.4. Synthetic peptides

Synthetic peptides shown in Fig. 4b were purchased from Neosystem (Strasbourg) and were of immunograde quality.

2.5. Determination of endotoxin activity by the *Limulus* test

Endotoxin activity of LPS- or Cter-peptide mixtures was determined by a quantitative assay using a test kit from Cambrex in the Quality Control Department of Aventis Pasteur. Detection threshold was 0.5 EU/ml of solution. Assays were done in duplicates, and differences between duplicates did not exceed 10%. LPS was from *E. coli* 026:B6 (ref. L2654 Sigma, St. Louis, USA).

2.6. Circular dichroism

Measurements were made at 20 °C using a Jasco J-810 spectropolarimeter (Tokyo, Japan) using cuvettes with a path-

length of 0.1 mm. Peptides analysed were at concentrations between 1.4 and 2 mg/ml. Reference spectra, obtained with samples containing all components except the peptide, were recorded and subtracted from the peptide spectra. Estimates of the helical fraction of the peptide were calculated using the value of $[\theta]_{\text{obs}}$ at 222 nm and the formula: percent helix = $(([\theta]_{\text{obs}}^{222} - [\theta]_{\text{coil}}^{222}) - ([\theta]_{\text{helix}}^{222} - [\theta]_{\text{coil}}^{222})) \times 100\%$ as described in Taylor and Kaiser [13].

2.7. Statistical analysis

Differences between groups were estimated using the Cochran test (variance check) and ANOVA. Multiple comparisons were done using Bonferroni test.

3. Results and discussion

3.1. Initial experiments

A high degree of sequence similarity (70%) exists between the N-terminal domain of *H. pylori* catalase (Nter) and human catalase whereas the C-terminal domain of *H. pylori* catalase (Cter) shares little similarity with the human enzyme. Initial experiments were therefore performed using recombinant Cter domain to minimise the induction of cross-reacting antibodies. It is to be noted that all immunizations performed in this work were in presence of the lipidic adjuvant DC Chol, which was shown in the past to increase the level of immune responses without introducing a Th1 or Th2 bias [14,15]. Furthermore, DC Chol micelles

Table 1

Immune responses induced by full-length and truncated *H. pylori* catalase

		Immunizing antigen	
		Catalase	Cter
Stimulating/coating antigen			
<i>H. pylori</i> catalase			
Cytokines	IL5	3	40
	IFN γ	23	<1
	IL5/IFN γ (%)	13	4000
Antibodies	IgG1	8000	4000
	IgG2a	6000	250
	IgG1/IgG2a	1.33	16
Human catalase			
Cytokines	IL5	<1	<1
	IFN γ	<1	<1
	IL5/IFN γ (%)	–	–
Antibodies	IgG1	300	<1
	IgG2a	125	<1
	IgG1/IgG2a	2.4	–

Outbred OF1 mice were immunised twice with 5 μg of antigen in presence of 200 μg of DC Chol adjuvant. Cytokine responses were measured by Elispots (presented as spots per million cells) and antibody responses by ELISA (presented as ELISA titers). For cytokines, spleen cells were stimulated with whole *H. pylori* catalase or human catalase; median cytokine values obtained in individual mice (five per group) are presented, subtracting mean values obtained in control wells. Antibody responses were measured on pooled sera from the same mice against the same two antigens.

were found to stabilize the native conformation of antigens [10].

As shown in Table 1, immunisation with Cter did not induce any antibody response against human catalase, which was accompanied by a dramatic shift towards a Th2 re-

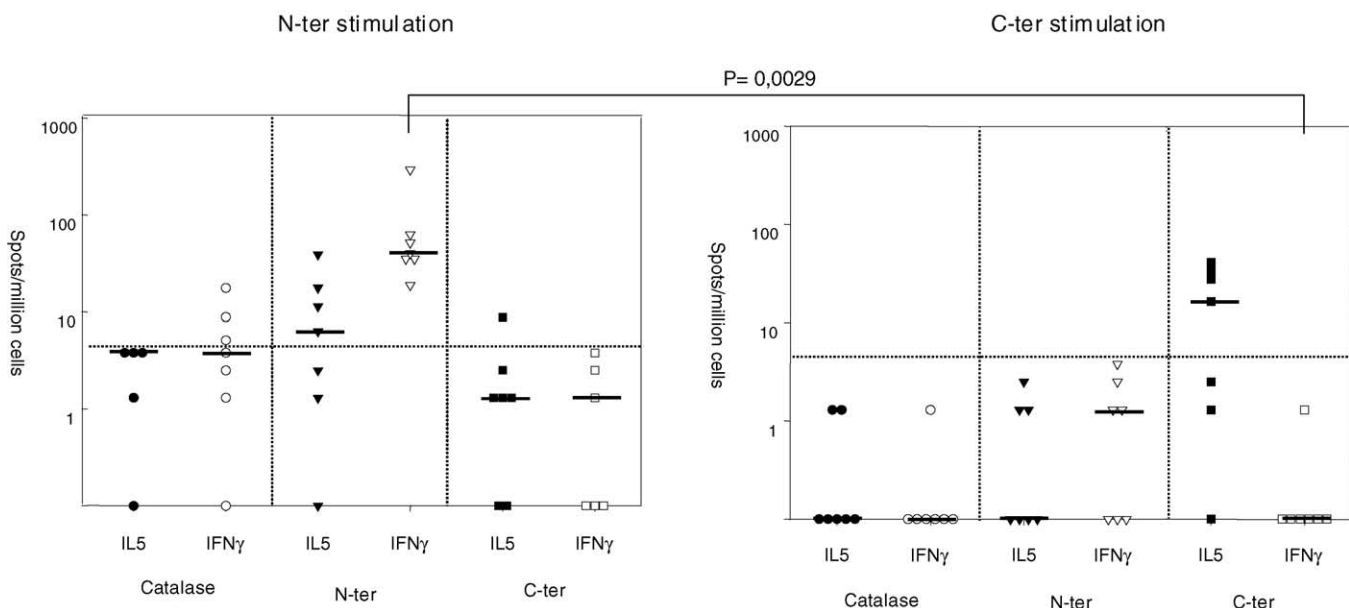


Fig. 1. Cytokine responses induced by full-length catalase, its N-terminal domain (Nter) and its C-terminal domain (Cter). Graphs are representative of two independent experiments. OF1 mice were immunised with 5 μg of the corresponding antigen as described in Section 2. Two weeks after the boost, spleen cells were stimulated by Nter (left graph) or Cter (right graph), and IFN γ or IL5 measured by Elispot. The horizontal line corresponds to 5 spots/ 10^6 cells, which is the maximal value reproducibly obtained in non-stimulated cells. Bars correspond to median values.

sponse against bacterial catalase as judged by IL5/IFN γ and IgG1/IgG2a ratios. In contrast, immunisation with recombinant Nter induced a Th1 response, whereas full-length *Hp* catalase induced very low and balanced IL5/IFN γ responses (Fig. 1). This low cytokine response observed with full-length catalase, as seen in Fig. 1 and Table 1 from two different experiments, is not due to absent immunogenicity, as full-length *Hp* catalase induced in several experiments significant IgG1 and IgG2a titers (as seen for example in Table 1). Recombinant antigens purified and solubilised from cell pellets lacked catalytic activity (not shown), which excludes the possibility that differences in Th responses were linked to differences in enzymatic activities. In addition, interactions between DC Chol adjuvant and the different antigens used here were similar (not shown), excluding a link between different immune responses and different antigen/adjuvant interactions.

3.2. Identification of Th1 or Th2 consensus sequences

Since human catalase, in contrast to *Hp* catalase, did not trigger any specific cytokine response against this antigen in mice (Table 1), *Hp*-specific sequence motifs were assumed to be present in the N-terminal half of the bacterial catalase, which are involved in the induction of IFN γ . Four short fragments (N1, N2, N3 and N4) were identified in the *Hp* sequence which had a very low degree of sequence similarity with the human enzyme and which, thus, can be considered to be specific for the *Hp* protein. Two fragments, N3 and N4, shared a common amino acid pattern, characterised by two central charged amino acids flanked by hydrophobic residues (Fig. 2a). Similar motifs were found to be present in antigens known to trigger inflammatory responses (Fig. 2a) [6–9]. It has, however, to be noted that some authors attributed this inflammatory response to LPS contamination, and it was suggested that the activity of minute amounts of LPS could be increased in the presence of some antigens (for a review see ref. [8]). Sequence alignment of these proteins with Nter allowed the identification of a Th1 consensus motif (Fig. 2a).

We then hypothesized that sequence motifs responsible for pro-Th2 orientation are present in the Cter. Three short sequence motifs were identified within Cter (denominated C1 to C3, Fig. 2b) which all contain a core of 2 hydrophobic amino acids (tyrosines, Y) flanked by charged residues. Other antigens previously described as “pro-Th2” by other authors (ES62 [3]; gp96 [16]) or in our laboratory (AlpA [10], HIV p24 and ovalbumin, unpublished) were found to contain one or several of these motifs. Sequence alignments of these proteins with Cter lead to the definition of a Th2 consensus motif (Fig. 2b). Preferred residues in Th1 and Th2 motifs are presented, but it can be anticipated that residues with similar charge properties (like phenylalanine (F) instead of Y) could be present too. We are aware that the pro-Th1 or pro-Th2 behaviour of the considered antigens might be linked to other causes.

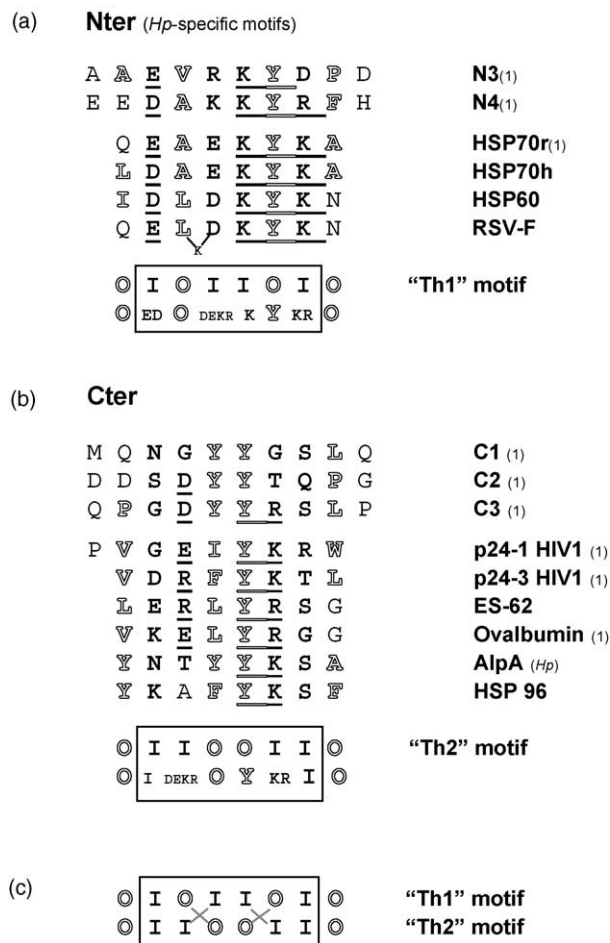


Fig. 2. Identification of specific motifs and design of experiments. (a) Alignment of the two Th1 motifs identified in *Hp* catalase (N3 and N4) with similar motifs present in proteins reported to trigger inflammatory responses such as HSP 70 (rat, NCBI 4930026; human AF134726.1), RSV-F protein (NP 056683) and human HSP60 (AAA36022). The Th1 consensus motif is boxed with O being hydrophobic residues (PALMIVFWY) and I being mainly hydrophilic/charged residue (GTSQNDEKR). The second line in the box shows in particular consensus residues identified at positions 2 and 4–7 of the motif. This motif lies in the only helix in the 3D structure of HSP70 peptide-binding domain and is located on a helix/loop junction in the 3D model of *H. pylori* catalase. In some HSP70 sequences, K residue preceding Y is Q (human, CAA36061) or R (mouse, P17979). (b) Alignment of the three Th2 motifs identified in *Hp* catalase (C1–C3) with similar motifs present in proteins known to induce Th2 responses such as ES 62 (NCBI access AAC28365), ovalbumin (230201), *Hp* AlpA (CAB 69511), HSP96 (NPO 35761), HIV p24 (12084543). The Th2 consensus motif is boxed with O being hydrophobic residues (PALMIVFWY) and I being mainly hydrophilic/charged residue (GTSQNDEKR). The second line in the box shows in particular consensus residues identified at positions 3, 5 and 6 of the motif. (1) indicates motifs that are present in proteins with known 3D structure (ovalbumin NCBI 230201, p24 NCBI 120845433D, *Hp* catalase, [17]). In all cases the motif forms a short α -helix. (c) Comparison of Th1 and Th2 consensus motifs. Illustrated is the conversion of a Th1 motif into a Th2 motif and vice versa by inverting the sequence of the central two pairs of amino acids.

3.3. Structure of the motifs

We then investigated whether the activity of the motifs, which we termed modulotopes, was reflected in their structure. The inspection of 3D structures of proteins bearing Th2 or Th1 modulotopes shows that they are surface-exposed, thus able to interact with other structures. Th2 modulotopes such as C2-3 domain were found to form a α -helix, whereas Th1 modulotopes were present on a helix-turn junction, which was equally the case for N3 and N4 in the 3D structure of *Hp* catalase [17] (see Fig. 3a). Interestingly, the modulotope in HSP70 lies within the only α -helix of the peptide-binding domain [18]. Helix-wheel projections of Th2-helices show that they are amphipathic, containing highly charged and hydrophobic faces (Fig. 3b). The action of the modulotopes might be initiated by recognition of this hydrophobicity/charge pattern. Such pattern-based interaction has been observed for peptides such as cathelicidin LPS-binding peptides [19] or VIP/PACAP neuropeptides [20], involved in the modulation of Th responses. In contrast to the Th2 motif, the helix-wheel projection of the Th1 motif (Fig. 3c) shows no clear division into a hydrophobic and hydrophilic face and a more alternating distribution of charged and hydrophobic residues is observed. Nevertheless, both types of “Th1” and “Th2” helices are mainly composed of charged or hydrophobic residues, which gives rise to two different types of amphipathic structural arrangements. These arrangements are proposed to account for the different effects observed in vivo (see below).

3.4. In vivo activity of motifs in outbred mice

Our initial observations (2.1) might indicate that the motifs are linked to specific induction of Th1 or Th2 responses and different experiments were designed to verify this hypothesis. Firstly, recombinant fusion proteins of Cter with N3, N4 or N1–N4 were generated. Secondly, 15–16 mer peptides (denominated by the extension *pep*) derived from proteins comprising the identified motifs were synthesised, in order to co-administer them with several antigens and to address their ability to act in *trans*. The Th1 consensus motif can be converted into a Th2 one by permutation of the central two pairs of amino acids (Fig. 2c). Control peptides (marked by *) containing this change have been synthesised for co-administration experiments. A summary of antigens and peptides used is given in Fig. 4a and b. A detailed description of the Cter fusion proteins with Nter motifs is given in Section 2. Purified proteins were analysed for LPS content which was similar for all protein samples (0.0125–0.05 EU/ μ g). Experiments were performed in outbred OF1 mice.

3.4.1. Cter fused with Nter motifs N3, N4 and N1234

Experiments in OF1 mice with motifs N1234 fused to Cter showed that this fusion construct did not induce an IL5 response (Fig. 5a, left panel) resulting in a dramatic difference in IL5/IFN γ ratios, which is in agreement with the proposed pro-Th1 potential of the fused sequences. In the same time, the overall specific IgG response induced by this recombinant antigen was of similar magnitude than the

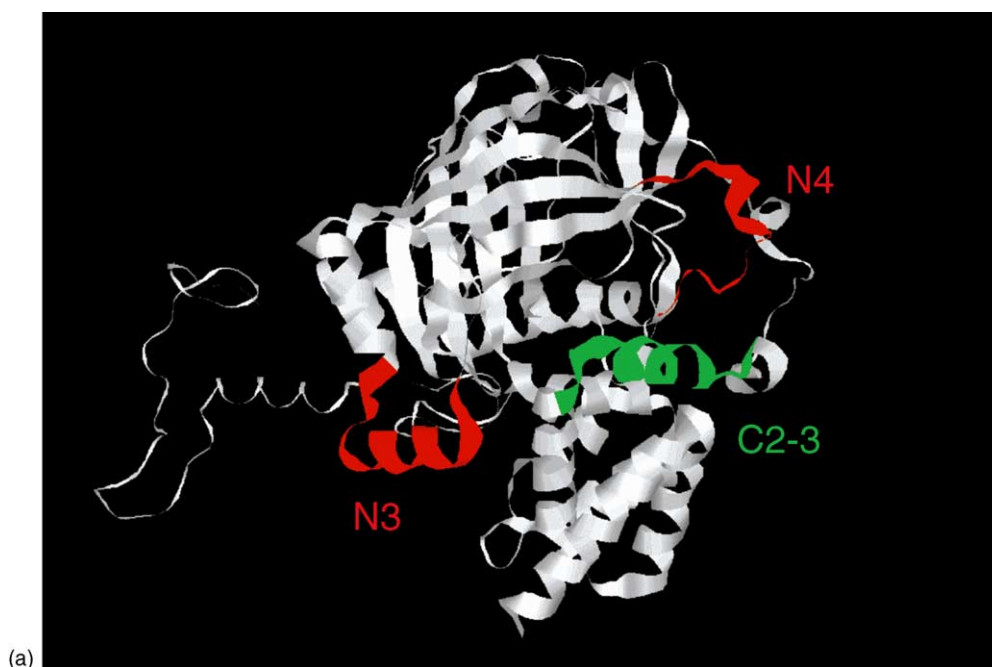


Fig. 3. Three-dimensional structure and helical wheel projection of Th2 and Th1 motifs. (a) Ribbon presentation of 3D structure of *Helicobacter pylori* catalase [17] and localisation of N3, N4 (red) and C2-3 (green) fragments. (b) Helical wheel projections of several sequences containing Th2 motifs. Basic charged residues are in black, hydrophobic residues in white. The view down the helical axis from the amino terminus is shown. (c) Comparison of wheel projections containing Th2 and Th1 motifs.

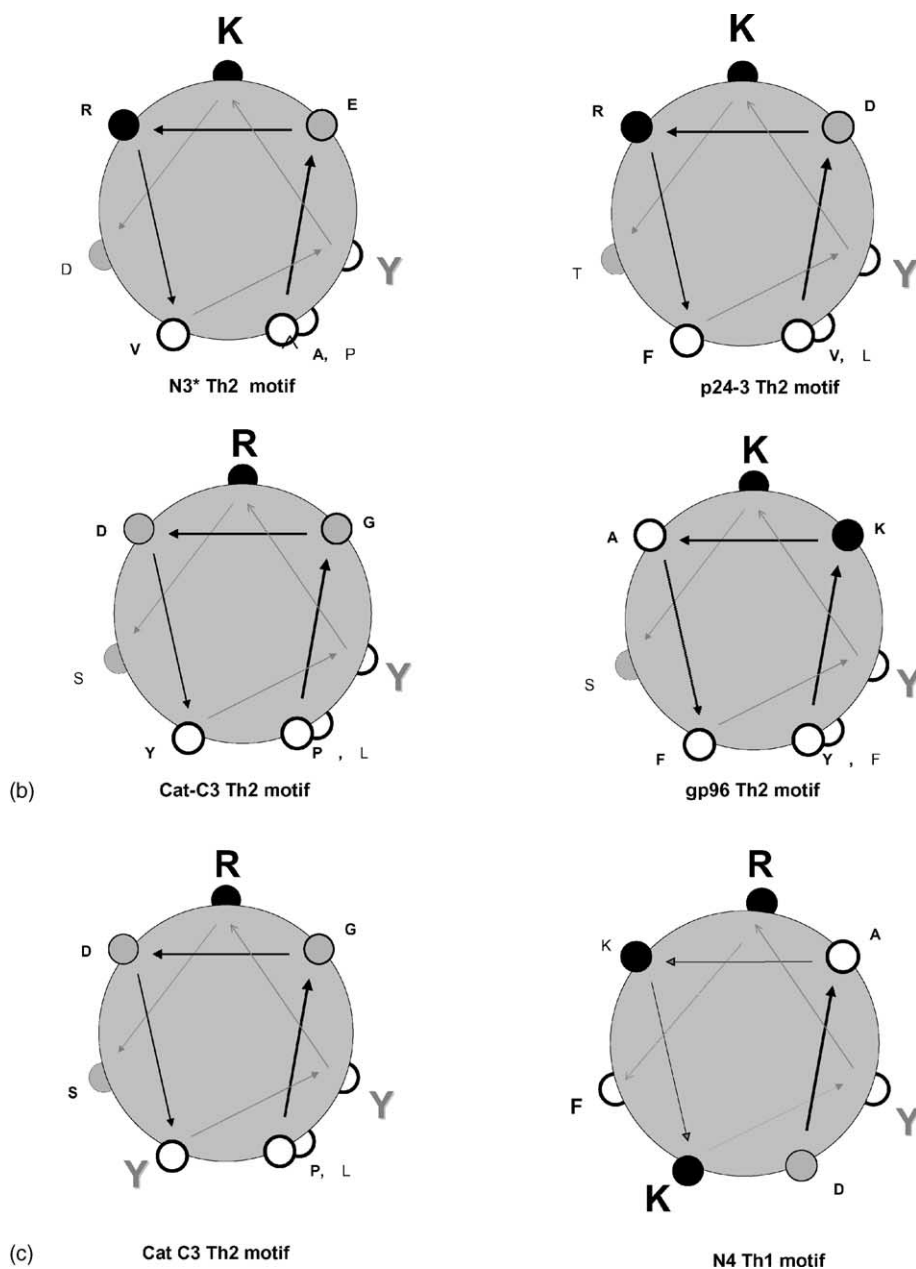


Fig. 3. (Continued).

one induced by Cter alone (although low and heterogeneous, see below), indicating that it still retained similar immunogenicity. Cter fused with individual N3 or N4 motifs showed that only the addition of the N4 motif induced a comparable undetectable IL5 response while fusion of the N3 motif did not induce significant changes (Fig. 5a, right panel). One might conclude that the effect seen with the fusion construct Cter/N1234 is mostly due to the effect of N4. The major difference between the sequence of the N4 motif (“active”) and the N3 motif (“inactive”) is the presence of either an acidic or basic residue following the core tyrosines (Fig. 2a).

3.4.2. Cter co-administered with peptides containing motifs N3 and N4 (Th1-peptides)

We tested the ability of peptides to act in *trans*, when co-injected with Cter. Fig. 5b shows that 5 μ g of N4 $_{\text{pep}}$ in combination with 5 μ g of Cter reversed the Th-balance, while the co-administration of control peptide N4* $_{\text{pep}}$ (Th2) had effect in this respect. This underlines the critical importance of the six amino acid cores of both consensus motifs. These experiments confirmed the Th1 potential of the N4 motif, as shown above with the Cter-N4 fusion construct. N3 $_{\text{pep}}$ co-injected with Cter did not change immune responses, which furthermore confirmed above results using the Cter-N3 fu-

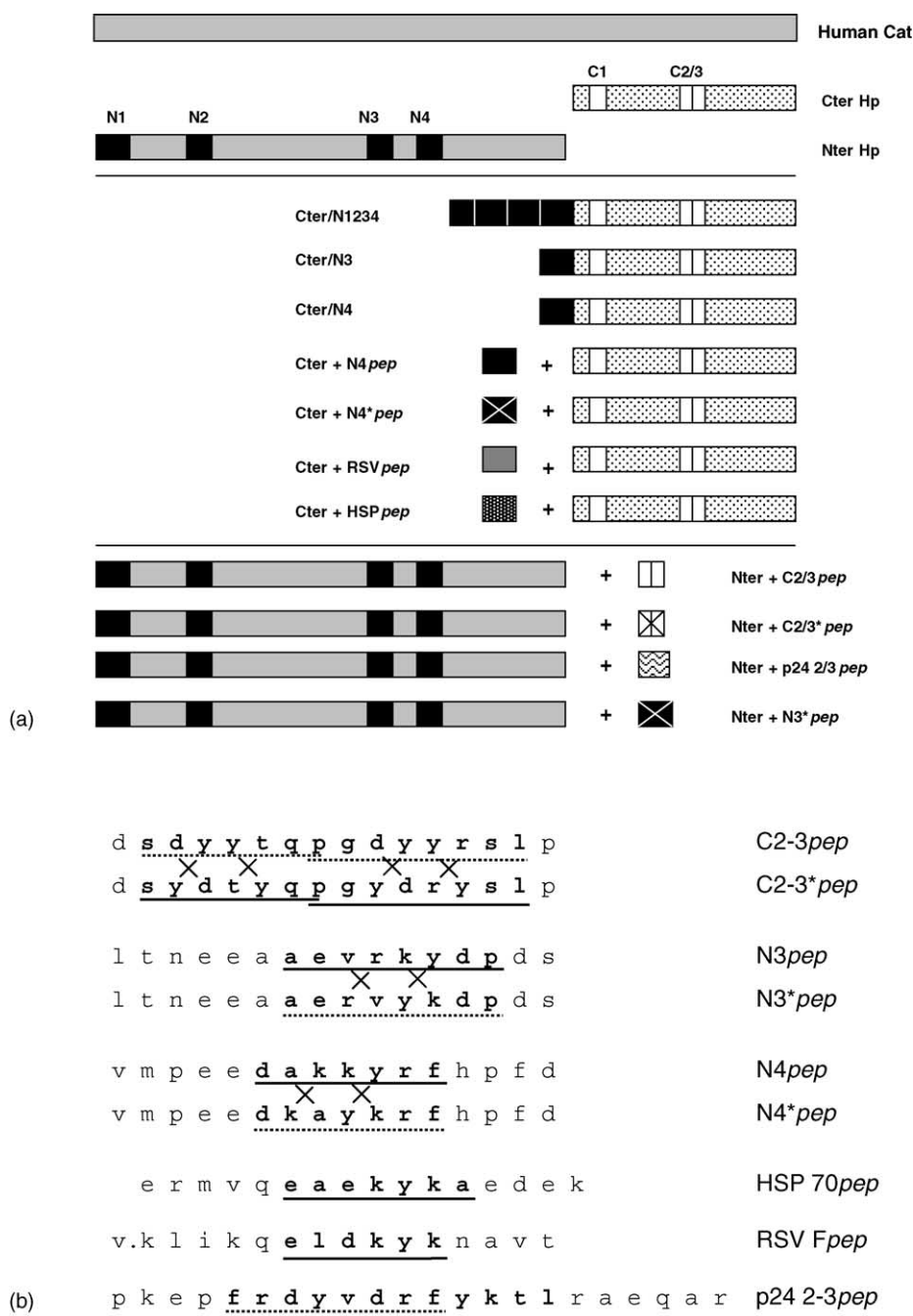


Fig. 4. Constructions and peptides. (a) Recombinant antigens and antigen/peptide combinations used in animal experiments. From the top to the bottom: human catalase; Cter Hp, C-terminal domain of Hp catalase; Nter Hp, N-terminal domain of Hp catalase; Cter/N1234, fusion protein of Cter with sequences N1-N4 (see Section 2); Cter/N3 and Cter/N4, fusion protein of Cter with sequence N3 and N4 (see Section 2); the rest, co-injection of antigens Cter and Nter with the peptides indicated (peptide sequences are in Fig. 4b). The Th1 motifs of Nter are boxed in black and the Th2 motifs in Cter are in white. The Nter regions homologous to human catalase are in grey. (b) Sequences of synthetic peptides used for experiments. The asterisks indicates peptides in which the sequence of the central two pairs of amino acids has been inverted (as indicated in Fig. 2c leading to the conversion of a Th1 motif (underlined, dotted line) and vice versa).

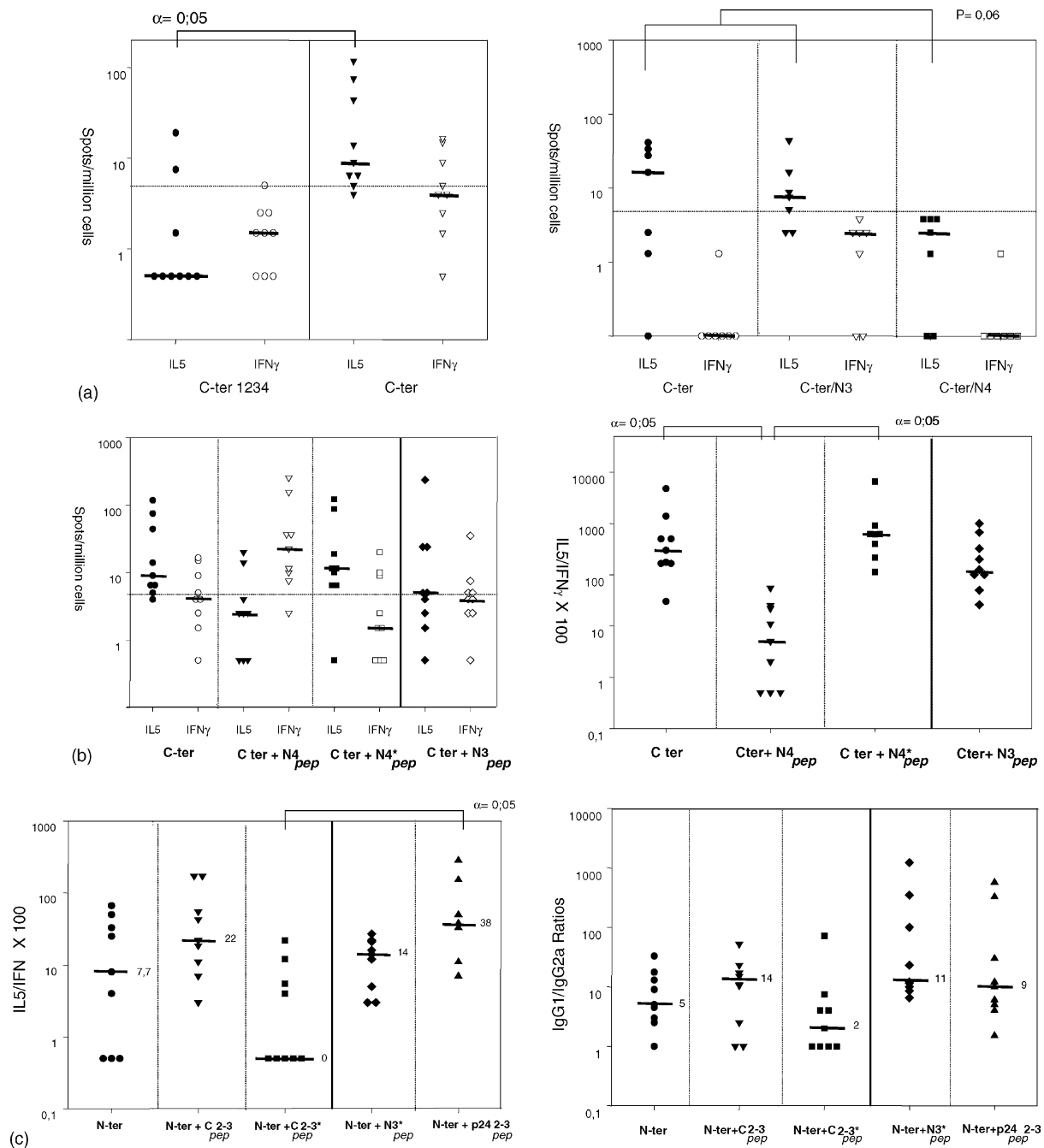


Fig. 5. Cytokine and antibody responses induced by the different catalase-derived antigens in outbred OF1 mice. Graphs are representative of two independent experiments. (a) Left graph: cytokine responses induced by C-ter/N1234 compared to C-ter. Spleen cells were stimulated with C-ter (or N-ter, giving no significant response over background, not shown). Right graph: cytokine responses induced by C-ter/N3 and C-ter/N4 compared to C-ter. Spleen cells were stimulated with C-ter or N-ter (giving no significant response over background, not shown). The horizontal line corresponds to 5 spots/ 10^6 cells, which is the maximal value reproducibly obtained in non-stimulated cells. Bars correspond to median values. (b) Left graph: cytokine responses induced by C-ter in presence of different peptides. Mice were immunised with 5 μ g of C-ter in presence of 5 μ g of N3 pep , N4 pep or N4* pep peptides. Two weeks after the boost, spleen cells were stimulated by C-ter (or N-ter, giving no significant response over background, not shown), and IFN γ or IL5 measured by Elispot. The horizontal line corresponds to 5 spots/ 10^6 cells, which is the maximal value reproducibly obtained in non-stimulated cells. Bars correspond to median values. Differences were significant: IFN γ : C < C + N4, $\alpha = 0,05$; C + N4* < C + N4, $\alpha = 0,05$; IL5: C > C + N4, $\alpha = 0,08$; C + N4* > C + N4, $\alpha = 0,12$. Right graph: Individual cytokine ratios. (c) Cytokine and antibody ratios measured in mice immunized with N-ter. Left graph: individual cytokine ratios induced after immunization with 5 μ g of N-ter in presence of 5 μ g of C2-3 pep , C2-3* pep , N3* or p24-2-3 pep . Two weeks after the boost, spleen cells were stimulated by N-ter (or C-ter, giving no significant response over background, not shown), and IFN γ or IL5 measured by Elispot. Bars correspond to median values. Right graph: individual ratios of anti-Nter antibody isotypes. Mice were immunised with N-ter as described above, and 2 weeks after the boost, IgG1 and IgG2a specific responses were measured by Elisa against N-ter (or C-ter, giving no significant response over background, not shown). Bars correspond to median values. (d) Individual IgG1 and IgG2a antibody levels in the same mice as in Fig. 5c. Mice were immunised with N-ter as described above, and 2 weeks after the boost, IgG1 and IgG2a specific responses were measured by Elisa against N-ter (or C-ter, giving no significant response over background, not shown). Bars correspond to median values.

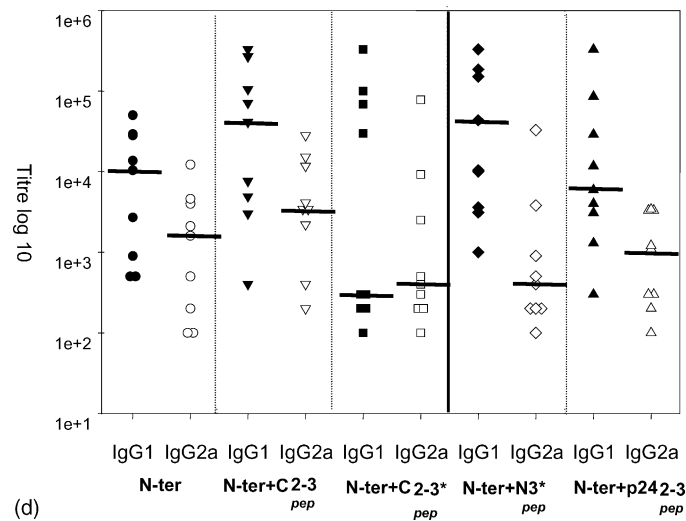


Fig. 5. (Continued).

sion construct. N4 and N3 pep were not strongly immunogenic in our assays, since they induced no significant response against Nter, which contains these motifs. In order to establish whether the observed immunomodulation was specific for *Hp* catalase or whether it is a general phenomenon, experiments were performed using peptides derived from other antigens, such as Th1 motif-containing peptides from RSV and HSP70 (Fig. 4b). Co-administration of Cter with RSV pep increased the IFN γ level as seen with N4 pep while the effect of HSP pep was less dramatic (not shown).

3.4.3. Nter co-administered with Th2-peptides

The ability of Th2-peptides (5 μ g) to modulate responses induced by Nter (Th1-antigen) was also tested. As shown in Fig. 5c, left panel, C2-3 pep co-injected with Nter increased the IL5/IFN γ ratio, while co-administration of control peptide C2-3* pep (Th1) decreased this ratio. This is in agreement with their proposed Th1 or Th2 potential. Other Th2 motifs such as control N3* pep or p242-3 pep also increased the IL5/IFN γ ratio, confirming the ability of peptides containing the Th2 consensus pattern to modulate Th1 responses. As for Th1 sequences, C2-3 pep was not strongly immunogenic, since no significant response was induced against Cter containing this motif (not shown).

3.4.4. Antibody responses

Antibody responses have also been assessed in immunised animals. Mice were immunised with N-ter alone or in presence of different peptides. Individual IgG1 and IgG2a responses were measured and their respective ratios were calculated. Data presented as IgG1/IgG2a ratios in Fig. 5c, right panel, were indicative of Th2/Th1 profiles similar to those observed in Fig. 5c, left panel, based on IL5/IFN γ levels. As shown in Fig. 5d, not only ratios but also absolute IgG1 and IgG2a levels in the same mice indicate that Th2 motifs (C 2-

3 pep , N3* pep and p242-3 pep) favour high IgG1 responses, thus increasing IgG1/IgG2a ratios, while immunisation with Th1 motifs decreased this ratio. In this respect, it is to be noted that mice immunized with Nter in presence of C 2-3* pep (Th1 motif) presented a dichotomic response, half of the animals having still high IgG1 values while the other half had dramatically depressed values. Nevertheless, mice presenting high IgG1 levels had also high IgG2a levels, generating moderate IgG1/IgG2a ratios (Fig. 5c, right panel).

Cter appeared to be a weak immunogen at the antibody level in OF1 mice: responses were very heterogeneous, and only a minority of mice presented significant Ig1 and IgG2a responses (one third to one half according to the experiments). However, results obtained from seven independent series of experiments, using some 300 mice, showed that high IgG1 responders (titers > 100,000) were mostly seen in “Th2” conditions, i.e. in presence of active doses of peptides such as N4* pep or N3* pep , acting then like as co-adjuvants for this response. Under these conditions, 50/197 mice showed high IgG1 titers as compared to 9/118 mice when equivalent doses of N4 pep peptide were co-administered. For instance, IgG1 titers up to several millions were observed in presence of 5 μ g N4* pep while in the same experiment, maximum IgG1 levels were found to be 10–100 times lower in presence of N4 pep . As similar reduction was also seen for the IgG1/IgG2a ratios (not shown).

3.4.5. Dose response

When experiments were repeated using a 5-fold higher peptide concentration (25 μ g) an abolition or partial reversion of the effect on cytokine secretion or antibody production as seen with the 5 μ g dose was noted (Table 2). This demonstrates the importance of the peptide dose in the modulation of the Th balance, as reported previously for MHC-restricted peptides [21–23], proteins [24] or LPS [25].

Table 2
Dose effect of peptides in OF1 mice

Median value of individual ratios	IL5/IFN γ (%)	IgG1/IgG2a
Nter	7.7	5
Nter + C2-3 <i>pep</i> (5 μ g)	22	14.6
Nter + C2-3 <i>pep</i> (25 μ g)	4	6.4 ($p = 0.09$)
Nter + p24 2-3 <i>pep</i> (5 μ g)	38	9.6
Nter + p24 2-3 <i>pep</i> (25 μ g)	18	6.7 ($p = 0.09$)
Nter + N3* <i>pep</i> (5 μ g)	14	11.6
Nter + N3* <i>pep</i> (25 μ g)	0	2 ($p = 0.09$)
Cter	300	–
Cter + N4 <i>pep</i> (5 μ g)	5	–
Cter + N4 <i>pep</i> (25 μ g)	105 ($p = 0.05$)	–

Mice were immunised with antigens in presence of 5 or 25 μ g of peptides. Median values of individual IL5/IFN γ and IgG1/IgG2a ratios are shown. The determination of antibody isotype ratios was not possible after immunisations with Cter due to an insufficient number of responding mice.

3.4.6. Experiments in inbred mice

Animal experiments using C57BL/6 and BALB/c mice confirmed the opposite activities of N4-type 1 and N4*-type 2 motifs on Cter. However, the active “Th1” or “Th2” doses were different in inbred mice compared to OF1 mice. In C57BL/6 mice for instance (Fig. 6), 1 μ g of N4 co-administered with Cter induced a Th1 bias, whereas 5 μ g showed an opposite effect (for IL5 at one given dose, N4 versus N4*, $p = 0.0347$; for IFN γ : N4 versus N4*, $p = 0.0145$). In OF1 mice, similar opposing effects were observed at higher doses, namely 5 and 25 μ g respectively. Differences similar to those observed in C57BL/6 at 1 and 5 μ g doses were also observed in BALB/c (not shown). At the antibody level, IgG2a was not measured in C57BL/6 mice as this strain does not produce this isotype. However, N4 at 1 μ g co-administered with Cter decreased as expected the number of high IgG1 responders (0/16 mice versus 6/16 mice for experiments without N4), in agreement with a Th1 effect of N4 at this dose.

These results showed that, in addition to peptide dose and antigen nature, the background of the mouse strain used also influences the observed effect.

3.5. In vitro activity of the motifs

To elucidate whether peptides may interfere with “danger signals” such as LPS, we have evaluated the ability of the peptides to specifically interfere with the action of LPS in a Limulus assay. Firstly, increasing doses of N4*pep* and N4**pep* were shown to modulate the activity of a fixed very small amount of *E. coli* LPS (Fig. 7a). Low to medium peptide doses decreased LPS activity to undetectable levels whereas higher concentrations resulted in an increase. High doses of N4*pep* had a slightly higher capacity to increase LPS activity than N4**pep*, but the observed effects were modest.

Assays were then carried out without addition of LPS but in presence of Cter shown to contain minor LPS contaminations. In the absence of any added peptide no activity was detected under the conditions of the assay. Fig. 7b shows that peptides N4*pep* and N4**pep* at high doses dramatically enhance LPS activity, which was especially pronounced for N4*pep*. In contrast to N4*pep*, low doses of N4**pep* also enhanced LPS activity. The peptide-induced effects in presence of Cter were qualitatively similar but quantitatively superior to those obtained with low doses of purified LPS alone. This might be explained by an underestimation of the Cter LPS activity due to down regulation by C2-3 motifs (similar to N4*) present in this antigen, contributing to its Th2 profile.

Finally, the experiments reported in Fig. 7a were repeated using a 10 times higher LPS concentration (Fig. 7c) and a similar V-shaped dose-response curve was obtained. Low doses of both peptides gradually decreased LPS activity, but in contrast to experiments using low LPS doses, high doses of N4**pep* were activatory while N4*pep* remained inhibitory. Peptide solutions at the highest concentration used in these

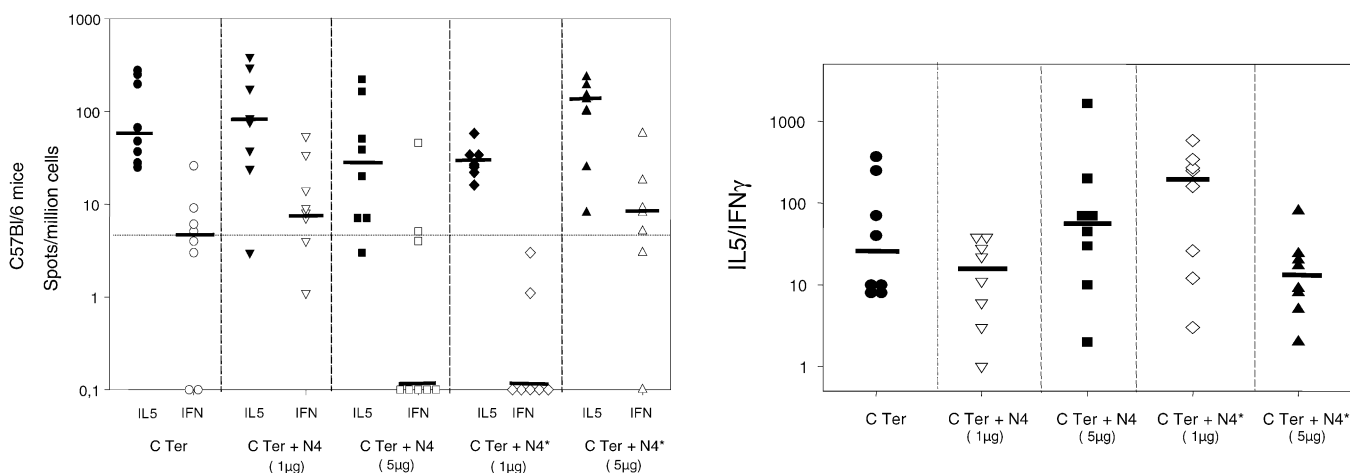


Fig. 6. Cytokine responses (left panel) and cytokine ratios (right panel) induced in C57BL/6 by Cter in presence of 1 and 5 μ g of N4 and N4*. Two weeks after the boost, spleen cells were stimulated by Cter and IFN γ or IL5 were measured by Elispot. Left panel, the horizontal line corresponds to 5 spots/106 cells, which is the maximal value reproducibly obtained in non-stimulated cells. Bars correspond to median values.

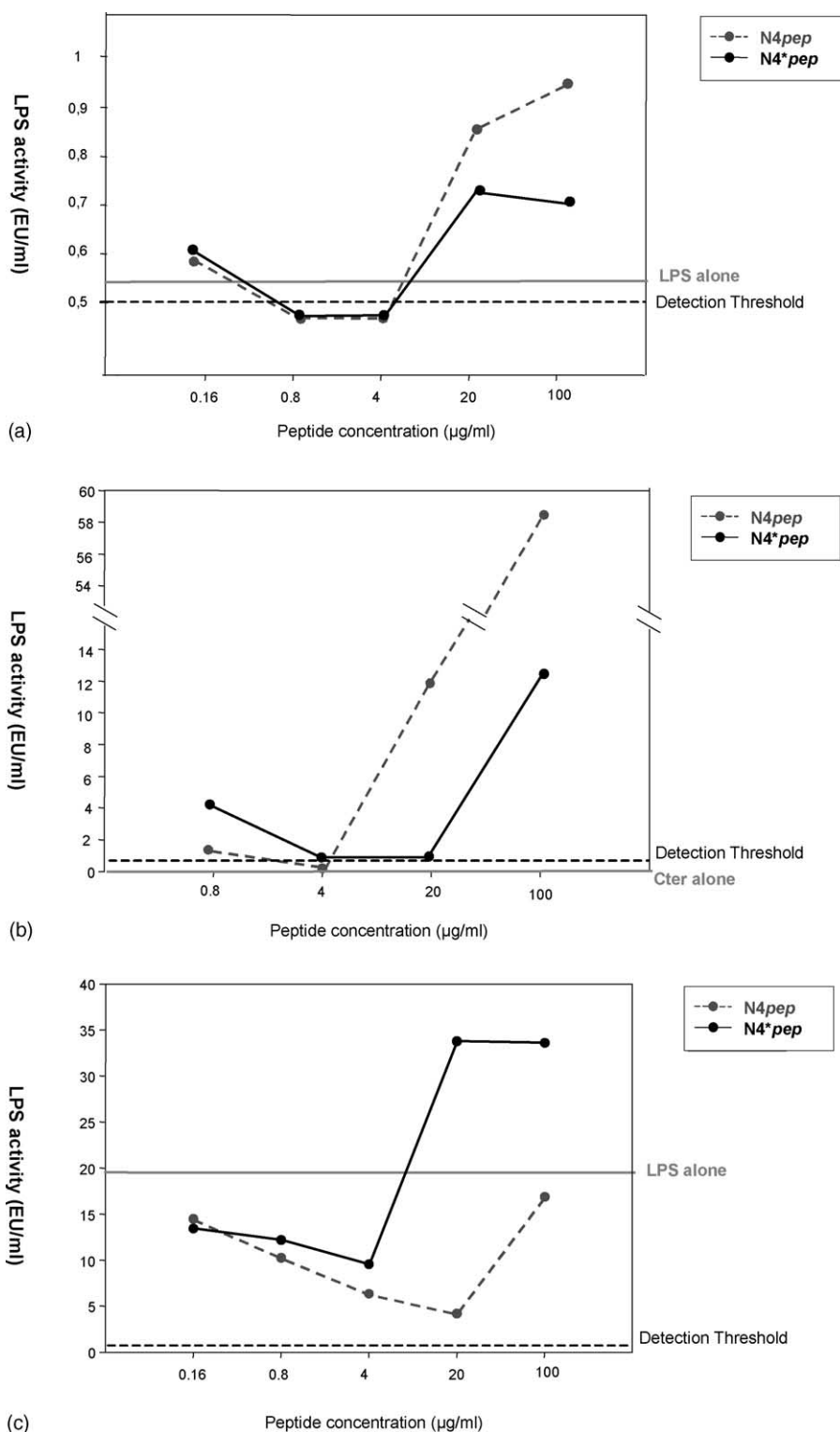


Fig. 7. Peptide-induced modulation of LPS activity in a Limulus assay. LPS activity as a function of peptide concentration is shown. Peptide solutions in absence of LPS were shown to be free of endotoxin activity (below detection level, <0.5 UI/ml). Grey line: LPS activity in absence of peptides. Dotted line: threshold detection level. Analyses were made in duplicates; the variation between the two measurements was less than 10%. (a) Peptide-induced modulation of the activity of small amounts of LPS. All analyses were carried out in the presence of 1 ng/ml LPS (Sigma). (b) Modulation of LPS activity in the absence of added LPS, but in presence of the antigen Cter. Solutions analysed contained 40 µg/ml Cter (final) and the indicated peptide concentration, but no additional LPS. (c) Repetition of the experiment shown in 6a using a fixed LPS concentration of 10 ng/ml.

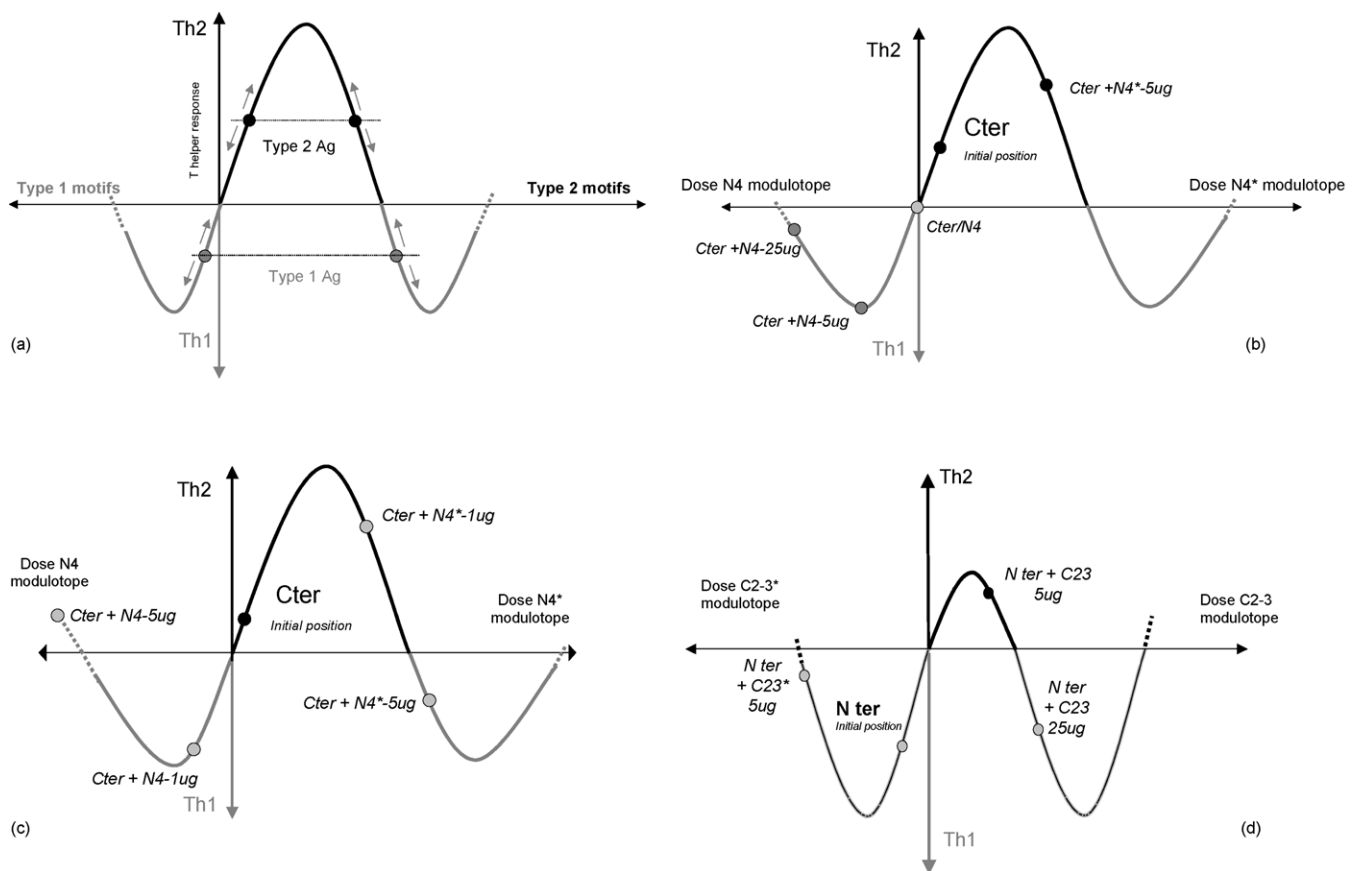


Fig. 8. "Oscillation" model proposed for the observed Th1 and Th2 responses in the presence and absence of modulator peptides. (a) General model. The horizontal line corresponds to a Th0 response. A same Th1 or Th2 antigen could have different initial positions, as presented. This would depend on a range of factors such as the mouse strain, presence of co-stimulation by adjuvants or danger signals such as LPS, sex and housing conditions of animals (including different types of microflora). (b) Model applied to the Th2 antigen Cter in OF1 mice, referring to experiments using Cter in the absence and presence of different doses of N4 and N4**pep*, as well as the Cter/N4 fusion protein (data are taken from Fig. 5). The initial position of the Cter preparation used in this experiment is indicated, and parameters determining it are considered constant in this mouse strain. The fused Cter/N4 construct was positioned at a Th0 position. (c) Model applied to the Th2 antigen Cter in C57BL/6 mice, referring to experiments using Cter in the absence and presence of different doses of N4 and N4**pep* (data are taken from Fig. 6). The initial position of the Cter preparation used in this experiment is indicated, and parameters determining it are considered constant in this mouse strain. (d) Model applied to the Th1 antigen Nter in OF1 mice, referring to experiments using Nter in the absence and presence of different doses of C2-3 and C2-3**pep* (data are taken from Fig. 5). The initial position of the Nter preparation used in this experiment is indicated, and parameters determining it are considered constant in this mouse strain.

Table 3
Far UV circular dichroism spectroscopy of peptides

	PBS		30% TFE		5 mM DPC	
	α -helix (%)	X increase/PBS	α -helix (%)	X increase/PBS	α -helix (%)	X increase/PBS
N4 <i>pep</i>	10	1	28	2.8	11	1.1
RSVF <i>pep</i>	8	1	30	3.7	11	1.4
HSP70 <i>pep</i>	3	1	11	3.7	3	1
N4* <i>pep</i>	14	1	39	2.7	52	3.7
p242-3 <i>pep</i>	31	1	91	2.9	70	2.2

Peptides were analysed in PBS, 2,2,2-trifluoroethanol (TFE) or dodecylphosphocholine (DPC) micells. TFE and DPC are compounds known to stabilise the secondary structure for which a sequence has propensity. The increase in α -helical content as compared to PBS is indicated. The spectra of N4*pep*, N4**pep*, RSVF*pep* and p24*pep* in PBS have a minima between 205 and 207 nm indicating that these peptides are not present in a random coil conformation, and a content of around 10 % α -helix has been calculated for N4*pep* and RSVF*pep*, 15% for N4**pep* and around 30 % for p24*pep*. HSV70*pep* was shown to be almost unstructured.

tests were shown to be free of endotoxin activity (below detection level, <0.5 UI/ml), indicating that the increased activity observed was not caused by the presence of LPS in peptide solutions. Our experiments demonstrate that, similar to their Th modulatory activity in vivo, N4*pep* or N4**pep* can act as activators or inhibitors of LPS activity, which in analogy to in vivo experiments was found to depend on the amount of peptide added. Jurgens et al. [26] observed a very similar dose-dependent effect with haemoglobin, which decreased or increased LPS activity as function of protein concentration.

However, ITC binding experiments (data not shown) demonstrated that N4*pep* and N4**pep* bind to LPS with a relatively low K_D of around 300 μ M. This implies that both ligands are not associated at physiological concentrations. A similar affinity has been measured using control peptides indicating that binding was non-specific, which was confirmed by the non-saturable nature of interaction as seen by ITC. Therefore, it seems more likely that the observed enhancement of LPS activity is due to an indirect LPS-peptide interaction. Peptide and LPS may for instance interact with a common ligand (receptor). Some Limulus LPS binding proteins have binding motifs similar to those found in higher eukaryotes, which are potential targets for such interactions [27]. We are aware that the Limulus assay represents the consecutive action of many proteins which all have to be considered as potential targets for the action of the peptides tested. It then might be possible that the motifs exert their modulatory function through an enhancement of the biological effects of minute (sometimes undetectable) amounts of LPS or other lipidic bio-active molecules, which has been proposed by Wallin et al. [8]. This would explain the controversial activity of HSPs (bearing potential Th1 or Th2 motifs) through TLR4, which was attributed by some authors to LPS contamination [8].

Alternatively or in addition, the structure of the motifs may play a direct role in the observed immune modulation through receptors not present in the Limulus assay. To address this, preliminary experiments have been carried out on human monocytes and monocyte-derived dendritic cells, in presence of N4 versus N4*, or C 2-3 versus C 2-3* peptides. The levels of IL10, IL12 p40 and IL12 p70 cytokines have been measured 6 and 48 h after stimulation with increasing doses of peptides in presence of suboptimal doses of LPS (1 to 10 ng/ml). In some donors, a 2 to 10-fold variation was observed in IL10 levels in presence of “Th2” versus “Th1” peptides. On the other hand, IL12 p40 or p70 levels changed similarly after stimulation with different doses of N4 versus N4* and C 2-3* versus C 2-3*pep*. However, this effect was dependant on the donor, and could not be observed in other donors. This high inter-donor variation requires additional work in more subjects to optimize the conditions of the assay and investigate the potential role of other co-factors or cells before conclusions can be established from such a model. In addition, performing animal experiments with totally LPS-free antigens will help defining if modulotope effects are strictly dependant on LPS-contamination, by simply

enhancing/modulating its effect, or if other mechanisms are involved.

For instance, Seong and Matzinger [28] have recently brought forward the hypothesis in which an interaction of hydrophobic ligands (*hyppos*) with receptors such as TLR4 is proposed to stimulate the immune response. According to the authors, all hydrophobic compounds are potentially to be considered as *hyppos*. The authors suggest that *hyppos* exist on proteins and present some examples of this being of physiological relevance. Our data are consistent with this hypothesis and the Th1 and Th2 modulotopes described in this article can be regarded to some extent as protein-associated *hyppos*. This is valid for the Th2 motif, which has been shown to be present on helices having a hydrophobic side, whereas the Th1 motif can be considered as an alternative, specific arrangement of hydrophobic and charged amino acids. Finally, modulotopes could also act by molecular mimicry through binding to a more specific undefined receptor.

All in all, both Th motifs could bind differentially to the same receptor, or, alternatively, to different receptors, thus inducing different responses. As shown above the type of response differs also on the dose of peptide motif administered. Similarly, it has been shown that different doses of LPS induce either Th1 or Th2 responses through stimulation of the same TLR4 receptor [25,29]. On the other hand, it has also been shown that different types of LPS can interact with the same TLR4-dependent pathway, resulting in antagonistic effects [30]. We show that modulotopes present a similar dose- and motif-dependant behaviour in vivo and in vitro, which can be regarded as additional support for a mechanism based on indirect or direct interactions with TLR4-dependant pathways.

3.6. Model

In this respect, we tried to explain with a model the opposite immune responses induced by different doses of the same peptide, or by the same dose of peptide in different mouse strains. In other models with other ligands/receptors, it has been demonstrated that low or high doses of one given peptide triggered Th2 responses whereas medium doses induced Th1 responses, generating a “bell-shaped” response curve [23]. Our experiments can be explained using similar curves. In this “oscillation” model (Fig. 8a) the initial position of an antigen, defined as Th1 or Th2 based on its cytokine profile, would depend on factors such as its nature and dose, presence of immunomodulatory motifs and co-stimulatory (danger) signals (such as LPS or adjuvant presence for instance), mouse/human genotype, hormonal/sexual influences or other undefined environmental factors. This initial position would be displaced by co-administration of modulotopes, and the magnitude and direction of displacement would depend on the dose and nature of modulotopes, all other parameters being considered fixed. It is anticipated that a too low dose of added modulotope would not induce any variation from the initial antigen position, and that, on the other hand, no more

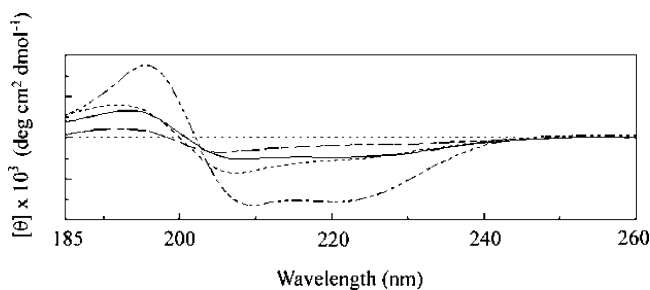


Fig. 9. Far UV circular dichroism spectra of peptides. Shown are the spectra of peptide N4pep in PBS (---) and in presence of 30 % TFE (—) and of peptide p242-3pep in PBS (···) and 30 % TFE (- - -).

variation would be observed beyond a certain position above a certain dose of modulotope. According to this model, experiments using preparations of the same antigen differing in some unidentified factor might then result in different “initial positions” (Fig. 8a). As a consequence, co-administration of the same dose of one given modulotope would sometimes induce opposite effects. This may offer an explanation why under different experimental conditions or preparations a same antigen can present a different Th behaviour. The amplitude of variation in the response to one modulotope would also depend on the individual genotype (or mouse strain) as observed in our experiments and presented in Fig. 8b versus c. Actually, this model can be used to fit the data obtained from our in vivo studies in OF1 mice (Fig. 8b and d) or in C57BL/6 mice (Fig. 8c) and is equally valid to explain the in vitro modulation of LPS activity in the Limulus assay. For instance, it may explain the heterogeneous “dichotomic” response observed in OF1 mice upon administration of C 2-3*pep with Nter (Fig. 5c and d), resulting in an intermediary Th position.

3.7. Structure of the peptides

Peptides have also been analysed by far UV circular dichroism (Fig. 9 and Table 3) to evaluate whether they preserved their α -helical structure. Peptides are relatively little structured in PBS and a dynamic equilibrium may exist between peptides in a helical and random conformation. The peptides, however, maintain a clear propensity to adopt a helical conformation as shown by an around 3-fold increase in helicity in the presence of trifluoroethanol. This might indicate that the adoption of a α -helical structure following contact with cell membranes or a putative receptor/antigen is thermodynamically possible. HSP70pep, which was found to be the less structured, was also less potent in vivo to modulate responses against Cter. DC Chol is a cationic lipid adjuvant able to form liposomes. It is therefore possible that peptide/liposomes interactions could have occurred through peptides derived from the N-terminus, which contain several acidic amino acids (Fig. 4b). The question whether the peptides form α -helical structures in the presence of DC Chol is of central interest and attempts have been made to assess this by far UV cd spectroscopy. However, due to the strong

absorbance of DC Chol in the far uv region, the determination of the secondary structure by cd is impossible. There is no realistic alternative to the use of cd spectroscopy for the determination of the secondary structure content.

3.8. Conclusion

In conclusion, our data suggest, beyond the particular case of *H. pylori* catalase, the existence of intrinsic properties of proteins that may participate to the modulation of immune responses. These properties are reflected in short sequence motifs, which we termed modulotopes. We have reported on particular Th1 and Th2 consensus motifs, which does not exclude that other motifs with similar activities exist. The presence of surface-exposed amphipatic motifs/patterns is a general phenomenon in proteins, and it is proposed that such pattern regulate qualitative immune responses by interfering with signal pathways triggered by danger signals such as LPS. This is consistent with the hypothesis of Seong and Matzinger [28], as both motifs described in our paper can be considered as particular protein-associated *hyppos* (Th2 motifs) or may constitute an alternative specific arrangement of hydrophobic with charged amino acid residues. (Th1). In addition, the data presented here offer the possibility of a rational immunomodulation by orienting responses towards the desired type.

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