

The *Mycoplasma*-derived lipopeptide MALP-2 is a potent mucosal adjuvant

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The adjuvanticity of MALP-2, a 2-kDa synthetic lipopeptide with macrophage-stimulatory activity, was evaluated in BALB/c mice using β -galactosidase (β -gal) as model antigen. When co-administered with β -gal by either the intranasal (i.n.) or i.p. route, MALP-2 (0.5 μ g) was capable of increasing β -gal-specific serum IgG titers by 675–3,560-fold (i.n.) and 64–128-fold (i.p.), respectively, as compared to immunization with β -gal alone. Using MALP-2, almost maximal IgG responses were already stimulated following the first immunization, and the IgG titers were similar to those observed using 10 μ g of cholera toxin B subunit (CTB) as adjuvant. The mucosal immune system was also effectively stimulated ($p < 0.05$) when MALP-2 was administered by the i.n. route (36% and 23% of β -gal-specific IgA in lung and vaginal lavages, respectively). The i.n. co-administration of MALP-2 stimulated a stronger cellular immune response than CTB, both in submandibular lymph nodes and spleen ($p < 0.05$). The analysis of β -gal-specific IgG isotypes and the profiles of cytokines secreted by *in vitro* re-stimulated cells showed that co-administration of MALP-2 triggered a dominant Th2-response pattern. A recruitment of B220⁺ and MAC-1⁺ cells with an up-regulated expression of MHC class I, CD80 (B7.1) and CD54 (ICAM-1) was observed in nasal associated lymphoid tissues from MALP-2 treated mice. Taken together, our results demonstrated that the synthetic lipopeptide MALP-2 represents a very promising adjuvant for the mucosal delivery of vaccine antigens.

Key words: Mucosal adjuvant / Vaccine / Intranasal vaccination / Monocyte/macrophage

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1 Introduction

Most pathogenic organisms are either restricted to or need to transit through the mucosa during the course of infection. Mucosal immunization has emerged as one of the most effective strategies for the induction of both systemic and mucosal immune responses against pathogens transmitted by this route. In addition, it may represent the most cost-effective approach for vaccination campaigns due to the easy administration logistics. However, soluble antigens administered by this route are poorly immunogenic. Therefore, recent work has been

focused on the development of mucosal adjuvants capable of enhancing antigen-specific immune responses [1–4]. A limited number of molecules exhibiting this property have been so far identified. In addition, the intrinsic toxicity and/or side effects associated with some of them hampers their application in humans. Thus, there is a critical need for the identification of new compounds with adjuvant activity by the mucosal route.

In spite of their chemical heterogeneity, most adjuvants, ranging from the classical Freund's complete adjuvant to more modern adjuvants such as subunits of bacterial toxins or oligonucleotides are acting on APC, in particular on macrophages [5, 6]. The characterization of products able to enhance the biological activities of APC constitutes a reasonable starting point for the screening of novel molecules with adjuvant activity. The biochemical nature of the compound may also represent a selection criterion. Indeed, lipidated molecules constitute interest-

[1 23105]

Abbreviations: β -gal: β -Galactosidase **CTB:** Cholera toxin B subunit **i.n.:** Intranasal **MALP-2:** Macrophage-activating lipopeptide-2 **NALT:** Nasal-associated lymphoid tissue **TLR:** Toll-like receptor

ing targets, since they can easily traverse cellular and basement membranes and/or can be absorbed by the mucosa.

Bacterial lipopeptides have been used as adjuvants, either by admixing them to the antigen or by coupling poor immunogenic peptides to a lipid portion, which was used as a built-in adjuvant [7, 8]. For these reasons we focused our attention on a recently characterized *Mycoplasma*-derived macrophage-activating lipopeptide (MALP-2), which exhibits exceptional stimulatory properties on monocytes/macrophages [9, 10]. A synthetic derivative of MALP-2 (S-[2,3-bis-palmitoyloxypropyl] cysteinyl-GNNDENISFKEK) is, like the natural MALP-2, a powerful inducer of chemokines and cytokines [9–11]. In contrast to conventional bacterial lipopeptides, MALP-2 has a free N terminus, which contributes to its extraordinary potency (e.g. MALP-2 acts *in vitro* at $<10^{-11}$ M concentration). When administered i.p., MALP-2 was able to stimulate the *in vivo* release of chemokines MIP-1 α , MCP-1 and MIP-2, as well as the influx of leukocytes [11]. This is in contrast with the rather weak *in vivo* activity of “conventional” lipopeptide with three long-chain fatty acids [12]. MALP-2 signals via receptors of the innate immune response, the Toll-like receptors 2 (TLR-2) and TLR-6, which are expressed by monocytes/macrophages [13, 14]. This combination of receptors enable cells to discriminate between MALP-2 and other lipopeptides, which signal via TLR-2 [13, 14].

In the present study we investigated the efficacy of MALP-2 as mucosal vaccine adjuvant, using β -galactosidase (β -gal) as a model antigen. The obtained data indicate that co-administration of MALP-2 with the model antigen resulted in a potent enhancement of humoral and cellular antigen-specific immune responses at both systemic and mucosal levels.

2 Results

2.1 Intranasal co-administration of MALP-2 with a soluble antigen stimulates efficient systemic humoral responses

We first evaluated the capacity of MALP-2 to stimulate efficient humoral immune responses, by determining the serum titers of β -gal-specific antibodies in vaccinated mice. As shown in Fig. 1A, intranasal (i.n.) administration of β -gal alone (50 μ g/dose) resulted in the induction of very low antibody titers, even after the second boost (endpoint titer \approx 1,000). In contrast, in the presence of MALP-2, i.n. administration of β -gal induced very high titers ($>60,000$) of specific IgG in all mice already after a

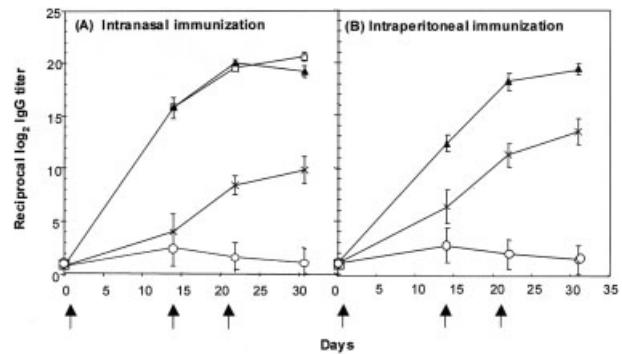


Fig. 1. Kinetics of β -gal-specific IgG responses in sera from vaccinated mice. Groups of animals ($n=5$) were immunized by either i.n. (A) or i.p. (B) route with 50 μ g of β -gal (\times), β -gal plus 10 μ g of CTB (\square), β -gal plus 0.5 μ g of MALP-2 (\blacktriangle) or buffer alone (\circ). Immunizations (days 0, 14 and 21) are indicated by arrows. Results are expressed as the reciprocal \log_2 of the geometric mean end point titer. SEM is indicated by vertical lines.

single dose, and by the end of the immunization protocol, titers were higher than 500,000 (Fig. 1). The kinetics and the overall efficacy of the antibody responses obtained using 0.5 μ g of MALP-2 per dose were similar to those observed by administering β -gal with 10 μ g cholera toxin B subunit (CTB), a well-known mucosal adjuvant that was used as a positive control.

A significant adjuvanticity was also observed when MALP-2 was administered by the i.p. route. Specifically, co-injection of MALP-2 resulted in approximately a 100-fold increase of β -gal-specific IgG titers in comparison to animals immunized with β -gal alone (Fig. 1B). This difference was already present after the first immunization and was maintained upon booster injections. Similar antibody titers were detected at day 31 in animals immunized by either the i.n. or the i.p. route.

2.2 Intranasal co-administration of MALP-2 with a soluble antigen stimulates efficient mucosal antibody responses

To investigate the capacity of MALP-2 to stimulate mucosal responses against antigens co-administered by the i.n. route, we analyzed the production of β -gal-specific IgA in lung and vaginal lavages from immunized animals. While i.n. immunization with β -gal alone failed to stimulate the production of detectable levels of β -gal-specific IgA in lung lavages, a significant increase ($p<0.05$) in the levels of antigen-specific IgA was detected in animals immunized with β -gal and MALP-2 (36% vs. 1% of the total IgA was β -gal specific, Fig. 2) or

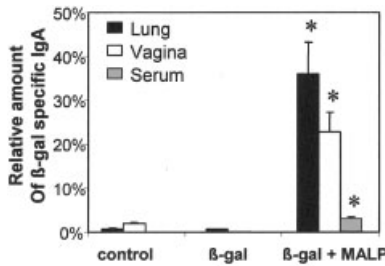


Fig. 2. β -gal-specific IgA in sera, lung washes and vaginal lavages of i.n. immunized mice. Results are expressed as the percentage of β -gal-specific IgA with respect to the total IgA present in the sample. The total mean IgA values for the groups immunized with β -gal, β -gal + CTB, β -gal + MALP-2 and controls were 74.2 μ g/ml, 102.5 μ g/ml, 96.6 μ g/ml and 92.3 μ g/ml in sera; 0.69 μ g/ml, 0.5 μ g/ml, 0.55 μ g/ml and 0.7 μ g/ml in lung washes; and 0.85 μ g/ml, 0.7 μ g/ml, 0.66 μ g/ml and 0.62 μ g/ml in vaginal lavages, respectively. The SD is indicated by vertical lines. The difference between the values obtained in the tested sample and those of the control group were statistically significant at $p < 0.05$ (*).

with β -gal and CTB (50% vs. 1%). Co-administration of MALP-2 or CTB resulted in the stimulation of efficient IgA production also at distant mucosal sites, as demonstrated by the presence of significant ($p < 0.05$) concentrations of β -gal-specific IgA in vaginal lavages (Fig. 2). No statistically significant differences were observed in the levels of mucosal β -gal-specific antibodies between animals immunized with 0.5 μ g MALP-2 or 10 μ g CTB ($p > 0.05$). The local production of the detected specific IgA from animals immunized with MALP-2 was further confirmed by the fact that the percentage of β -gal-specific IgA in serum was significantly lower than in the lung or vaginal lavages (i.e. 3%, 36% and 23% in serum, lung washes and vaginal lavages, respectively, Fig. 2).

2.3 MALP-2 stimulates efficient T cell-mediated proliferative responses when co-administered with soluble antigens

T cell-mediated immune responses were investigated at day 31 by measuring the proliferation of cells recovered from submandibular lymph nodes and spleens after *in vitro* re-stimulation with β -gal. Spleen cells from animals immunized by i.p. injection of β -gal alone, which were chosen as a positive control, exhibited a significant proliferative response ($p < 0.05$) as compared to the non-immunized group (Fig. 3A). A further increase in proliferation was noted in spleen cells from animals co-injected with MALP-2 and antigen ($p < 0.05$). While i.n. administration of β -gal alone failed to induce detectable cellular proliferation, co-administration of MALP-2 triggered the induction of an efficient proliferative response at both regional (lymph node cells) and systemic (spleen cells) levels (Fig. 3B, C). Of note, the strongest T cell proliferative response was observed with spleen cells of mice immunized with MALP-2 and β -gal by the i.n. route (Fig. 3B). A clear dose-response effect was observed in all cases by increasing β -gal concentration during re-stimulation (5, 10, 20 μ g/ml). Finally, the use of MALP-2 (0.5 μ g) as adjuvant resulted in a statistically significant ($p < 0.05$) increment of T cell proliferation with respect to i.n. immunization with CTB (10 μ g) plus β -gal (Fig. 3B).

2.4 Analysis of the T helper patterns stimulated using MALP-2 as adjuvant

We first analyzed the subclass distribution of the β -gal-specific IgG present in the sera of immunized mice. As shown in Table 1, the main β -gal-specific IgG isotype was IgG1, irrespective of the immunization protocol. This dominant Th2 response pattern was already evident

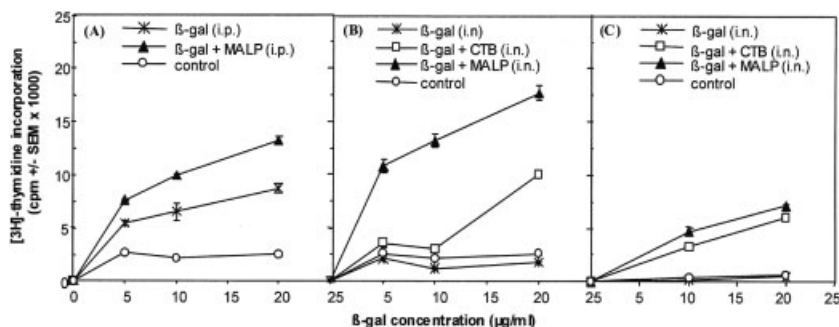


Fig. 3. β -gal-specific T cell proliferative responses of spleen (A and B) and regional lymph node (C) cells from mice immunized by the i.p. or i.n. routes. Cells were re-stimulated *in vitro* during 4 days with different concentrations of soluble β -gal. Results are expressed as the mean cpm from triplicate wells, subtracted of background values from non-stimulated cells cultured in RPMI 1640 supplemented with 10% FCS, SEM is indicated by vertical lines.

Table 1. β -gal-specific IgG isotypes in serum of immunized mice^{a)}

| Immunization group | IgG1 | IgG2a | IgG2b | IgG3 |
|------------------------------|-----------------------|----------------|-----------------|---------------|
| β -gal (i.n.) | 22.6 \pm 21.3 | 0.7 \pm 0.5 | 0.3 \pm 0.1 | 0.6 \pm 0.0 |
| β -gal + MALP-2 (i.n.) | 6,439.0 \pm 1,775.5 | 20.8 \pm 5.4 | 43.3 \pm 18.9 | 2.4 \pm 0.5 |
| β -gal + CTB (i.n.) | 4,108.3 \pm 1,437.1 | 31.9 \pm 9.5 | 49.2 \pm 17.3 | 2.4 \pm 0.6 |
| β -gal (i.p.) | 191.5 \pm 132.1 | 0.1 \pm 0.0 | 0.5 \pm 0.1 | 0.6 \pm 0.0 |
| β -gal + MALP-2 (i.p.) | 2,829.7 \pm 1,119.9 | 10.0 \pm 2.8 | 15.8 \pm 6.2 | 6.3 \pm 0.6 |
| Control | 0.4 \pm 0.0 | 0.2 \pm 0.0 | 0.1 \pm 0.0 | 0.4 \pm 0.0 |

a) Results are expressed as the mean (μ g/ml) \pm SEM (five mice per group).

after the first vaccination dose and was maintained following boosting (data not shown). IgG1 was the only isotype detected when β -gal was administered alone, whereas co-administration with CTB or MALP-2 resulted in the detection of other β -gal-specific isotypes, namely IgG2a (Th1 type), IgG2b (Th2 type) and IgG3 (Th1 type). Nevertheless, the ratio IgG1/2a (Fig. 4), IgG1/2b or IgG1/3 remained greater than 100.

To further characterize the type of Th response stimulated following immunization, the content of IFN- γ , IL-2, IL-4 and IL-10 was measured in supernatants from *in vitro* re-stimulated cells from spleen (Fig. 5) and lymph nodes. Among these four cytokines, IL-10 was found to be the most prominent cytokine in all the immunized mice, suggesting that a dominant Th2 response pattern was stimulated. The levels of IL-10 were significantly higher in mice vaccinated with MALP-2 by the i.n. route as compared to controls (2.2 ng/ml vs. 0.009 ng/ml,

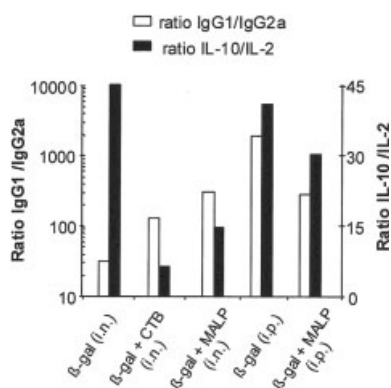


Fig. 4. Th-profile stimulated in vaccinated mice. Serum β -gal-specific IgG isotypes and cytokines from supernatants of *in vitro* β -gal stimulated spleen cells were determined in immunized mice by ELISA. Results are expressed as IgG1/IgG2a and IL-10/IL-2 concentration ratios.

$p < 0.005$) or in animals immunized using CTB as mucosal adjuvant (0.6 ng/ml, $p < 0.05$). Despite the fact that lower absolute cytokine values were observed in supernatants from cells obtained from regional lymph nodes (IL-10, 0.11 ng/ml for mice immunized i.n. with β -gal plus MALP-2 and 0.12 ng/ml for mice immunized with CTB), the general pattern was similar to that in spleen cell cultures (data not shown). The observed Th2-type dominant cytokine response pattern was in agreement with the detection of β -gal-specific IgG1 in the same animal groups (Table 1, Fig. 4). In fact, the strong stimulation of IL-10 secretion is congruent with the role played by this factor in the inhibition of cytokine synthesis by Th1 cells, the enhancement of B cells proliferation and the stimulation of IgA production [15–18].

Interestingly, although secretion of IL-10 and IL-4 was also stimulated in cells from mice vaccinated with β -gal alone by the i.p. or i.n. route, the Th1-cytokines IL-2 and IFN- γ were below the detection limit or the values did not differ from those in the control group (Fig. 5). In contrast, IL-2 was also detected in mice that received i.n. antigen mixed with either CTB (80 pg/ml vs. 7 pg/ml, $p < 0.05$) or MALP-2 (110 pg/ml vs. 7 pg/ml, $p < 0.05$). These results are in agreement with the detected IgG isotype patterns (Table 1), confirming that, although Th2 type responses are prevalent, MALP-2 also facilitates the stimulation of Th1 cells.

2.5 Intranasal administration of MALP-2 affects the expression of surface markers by NALT cells

To understand the underlying events leading to the adjuvanticity, a flow cytometric analysis of NALT cells obtained 16 h after i.n. administration of MALP-2 was performed. Approximately 6×10^6 cells were obtained from a pool of 20 animals. The analysis was focused on APC (B cells, monocytes and dendritic cells) and their

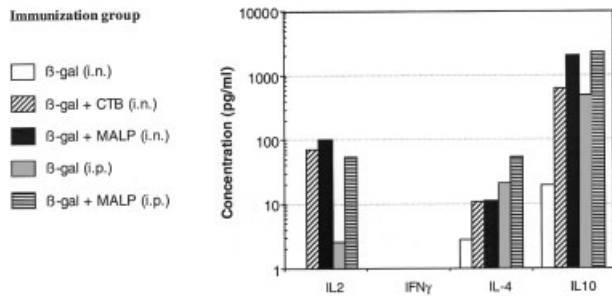


Fig. 5. Cytokines secreted by *in vitro* re-stimulated spleen cells from immunized mice. Cytokine production was measured by ELISA in the supernatants of cells cultured for 48 h (IL-2, IFN- γ) or 96 h (IL-4 and IL-10) in the presence of β -gal (20 μ g/ml). Results are expressed in pg/ml after subtraction of background values obtained with cells from non-immunized mice.

activation markers. Single staining showed a higher number of cells positive for the B cell marker B220 (70.1% vs. 56.3%), as expected due to the prevalence of B cells in NALT [19, 20]. An increment in the number of positive cells for both the myeloid marker CD11b, 15.7% vs. 3.1%, and for the leukocyte antigen CD18, 12.8% vs. 1.6%, was also observed in MALP-2 treated animals. As

shown in Fig. 6, 74.3% of the CD18⁺ cells also expressed CD11b, suggesting the surface expression of the heterodimer CD11b/CD18 (MAC-1). CD11c⁺ cells were not detected, suggesting either that dendritic cells are not involved at this stage or, alternatively, that the sensitivity is limited due to the low number of recovered cells. These results suggest that MALP-2 induces a local recruitment of B cells and monocytes/macrophages.

To further characterize the recruited populations, the expression of makers relevant for T cell stimulation was analyzed in B220⁺ and CD18⁺ cells (Fig. 6). The expression of the co-stimulatory molecule CD80 as well as that from the MHC class I molecules was up-regulated, whereas that of CD54 (ICAM-1) was unaffected in B220⁺ cells of MALP-2-treated mice. Similar to the observation in B220⁺ cells, a consistent increment in the expression of MHC class I and CD80 molecules was observed in CD18⁺ cells from MALP-2-treated mice (Fig. 6). In addition, the expression of the adhesion molecule CD54 (ICAM-1) was also up-regulated in CD18⁺ cells. The expression of both MHC class-II molecules and CD86 was marginally modified in both CD18⁺ and B220⁺ cells (data not shown).

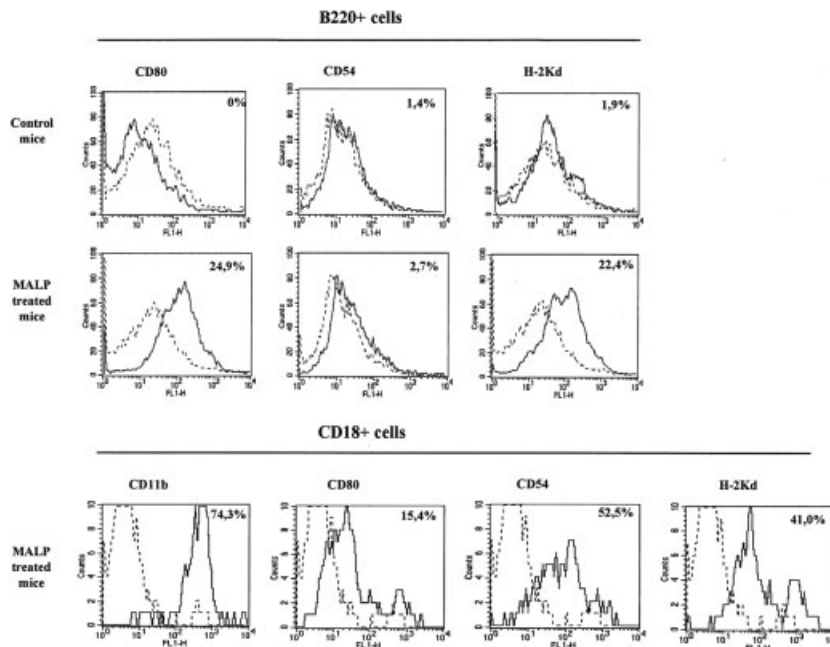


Fig. 6. Flow cytometric analysis of B220⁺ and CD18⁺ cells isolated from NALT of mice 16 h after i.n. administration of MALP-2 (0.5 μ g). The dotted and solid lines represent the histograms obtained using isotype controls and marker-specific antibodies, respectively. The percentages were calculated after subtraction of the values obtained from the isotype controls. The analysis is representative of two independent experiments performed with pooled cells from 20 mice each.

3 Discussion

Most infectious agents that are pathogenic for humans or animals infect the susceptible hosts through the mucosa. Then, disease develops upon local replication and colonization until a critical mass is reached, or, alternatively, by penetration and systemic dissemination to target organs. This has led to the concept that for a successful vaccination not only an efficient systemic immune response, but also an efficient local response at the mucosal portal of entry is highly desirable. This, in fact, may allow the infection process to be blocked at its very early stage, also reducing the risk of microbial transmission to other susceptible hosts. Unfortunately, soluble antigens administered by the mucosal route are usually poorly immunogenic. Therefore, different strategies have been developed to increase their immunogenicity. One of them is the co-delivery of antigens with mucosal adjuvants.

The majority of the mucosal adjuvants described up to now are microbial-derived products, such as the toxin of *Vibrio cholerae* and the heat-labile enterotoxin of *Escherichia coli* [1, 4]. Since their intrinsic toxicity limits their use in humans, non-toxic derivatives have been generated to overcome this problem [4, 21–23]. However, it seems that i.n. administration of toxins, toxoids or even B subunits alone can promote a GM1-dependent retrograde transport of antigen and adjuvant into neuronal tissues [24]. Therefore, there is an urgent need for novel and non-toxic mucosal adjuvants. In addition, the availability of a broader palette of mucosal adjuvants will facilitate the modulation of the obtained immune responses, using different adjuvants according to the specific needs (e.g. activation of Th1 or Th2 cells, induction of CTL). This would also allow overcoming problems such as a reduced efficacy resulting from a pre-existing immunity against one particular adjuvant, in that different adjuvants can be selected for vaccine preparations against different pathogens.

Due to its intrinsic characteristics (e.g. macrophage stimulatory activity and biochemical properties), we decided to evaluate the usefulness of the synthetic *Mycoplasma*-derived lipopeptide MALP-2 as a mucosal adjuvant. The dosage for MALP-2 was established according to preliminary studies in which MALP-2 was administered to mice by the s.c., i.d., i.p., oral or i.n. routes. In all protocols, co-administration of MALP-2 resulted in a significant increase in the production of β -gal-specific antibodies (data not shown). In the present study, we analyzed in detail the immune response stimulated against a model antigen in the presence of MALP-2 after vaccination by the i.n. or the i.p. route, and compared it with that obtained using CTB as adjuvant.

The use of MALP-2 resulted in a significant enhancement of both humoral and cellular β -gal-specific responses, using a dose of 0.5 μ g per administration. A threefold molar excess of CTB (10 μ g) was necessary to stimulate systemic or mucosal humoral responses, which were comparable to those obtained using MALP-2 as mucosal adjuvant. Regarding the cellular responses, spleen cells from mice immunized with MALP-2 showed a significantly ($p < 0.05$) higher proliferation than those from animals vaccinated with CTB.

Interestingly, humoral and cellular responses were more efficient after i.n. vaccination than i.p. vaccination with β -gal and MALP-2. This result suggests a differential local activity of MALP-2, which might be related to either its bioavailability or to the regional distribution of specific receptors on target cells. Furthermore, the presence of high concentrations of β -gal-specific IgA in lung and vaginal lavages demonstrated that MALP-2 was not only able to prime the local mucosal immune system, but was also able to direct an efficient cellular homing to distant mucosal sites. A comparison of the contribution of β -gal-specific antibodies to the total IgA in lavages versus that in serum showed that local IgA production rather than transudation of serum IgA had occurred.

Previous work has demonstrated that immunization with β -gal results in the induction of immune responses characterized by a dominant Th2-like response profile [25, 26]. In this report, antibody isotypes and cytokine patterns were used as indicators of the T helper bias induced by different vaccination protocols. The administration of β -gal alone per i.n. or i.p. route induced, as expected, a clear Th2-type response. However, the activation of Th1 cells was also observed, when β -gal was co-administered with MALP-2. Nevertheless, the dominant response pattern remained Th2, since the Th1 component did not exceed 10% of the total response. These results can be explained by the strong general MALP-2-mediated activation of the innate immune system, which may lead to the amplification of all signals to a detectable level. Alternatively, co-administration of MALP-2 might shift the pure Th2 response to a mixed Th1/Th2 response pattern. Interestingly, similar response patterns were obtained following co-administration of MALP-2 and CTB. Therefore, it can be hypothesized that both CTB and MALP-2 can exploit, at least in part, a common pathway to exert their activity as adjuvants.

To characterize the potential mechanism of action of MALP-2, a cytofluorimetric analysis of NALT cells was performed 16 h after administration. The results showed that there is a local recruitment and activation of B220⁺ and MAC-1⁺ cells, likely B cells and monocytes/macrophages. This is in agreement with our previous report on

macrophage influx in response to chemoattractants after i.p. injection of MALP-2 [11]. The adhesion receptor MAC-1 (CD11b/CD18) has an important role in the trans-endothelial migration of leukocytes, as well as in the activation of T cells. Interestingly, the key adhesion molecule ICAM-1 (CD54, [27]) was highly expressed by MAC-1⁺ cells. Double staining studies also allowed to establish that there is an up-regulation in the expression of CD80 and MHC-class I molecule both in B220⁺ and MAC-1⁺ cells from MALP-2-treated mice. Previous studies have demonstrated that CD80 provides an important co-stimulatory signal for naïve T cell priming through CD28 [28]. This result is consistent with the role of B cell help and the extraordinary anti- β -gal antibody production obtained after immunization. Thus, the recruitment and activation of APC in NALT may explain, at least in part, the *in vivo* activity of MALP-2 in the induction of humoral and cellular immune responses.

In conclusion, this study has demonstrated that MALP-2 is a novel and potent mucosal adjuvant without the need to conjugate the target antigen with the active lipopeptide. No adverse effects or signs of acute or chronic toxicity were observed in vaccinated animals during the entire course of these experiments. In contrast to protein adjuvants, the intrinsic poor immunogenicity of lipids and the short peptide moiety minimize the risk of appearance of MALP-2-specific immune responses, thereby allowing its inclusion in vaccines against different pathogens. In fact, after i.n. immunization, no MALP-2-specific antibodies were detectable (data not shown). The well-defined structure and chemical properties of synthetic MALP-2, its well-established synthesis, as well as its high potency, stability during storage and batch-to-batch consistency will facilitate its use in vaccine formulations. Thus, MALP-2 constitutes a promising adjuvant for the design of new vaccination strategies aimed at the delivery of antigens by the mucosal route.

4 Materials and methods

4.1 Mice and cell cultures

Six- to eight-week-old female BALB/c (H-2d) mice were purchased from Harlan Winkelmann GmbH (Borchen, Germany) and treated in accordance with local and European Community guidelines. Cells were grown in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 50 μ g/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol and 1 mM L-glutamine (GIBCO BRL, Karlsruhe, Germany) and maintained at 37°C in a humidified 5% CO₂ atmosphere.

4.2 Immunization protocols

Groups of five mice each were immunized on days 1, 14 and 21 with 50 μ g β -gal (Boehringer, Mannheim, Germany), alone or with 0.5 μ g synthetic MALP-2 [10] or 10 μ g CTB purified from native CT (ICN Biomedicals Inc., OH). MALP-2 was synthesized at the GBF as previously described [10]. For i.n. immunization, 10 μ l were applied to each naris, whereas, for the i.p. injection, β -gal with or without MALP-2 was resuspended in 250 μ l PBS.

4.3 Sample collection

Serum samples were collected at different time points (days 0, 13, 20 and 30) after immunization and stored at -20°C prior to determination of β -gal-specific antibodies. At day 31, mice were killed and the final sampling was performed. Vaginal and lung lavages were obtained by flushing the organs with several aliquots of PBS supplemented with 50 mM EDTA, 0.1% BSA, and 10 mM PMSF with a final volume of 1 ml. Lavages were then centrifuged to remove debris (10 min at 3,000 \times g), and supernatant fluids were stored at -20°C. Submandibular lymph nodes and spleens were removed and pooled for analysis of cellular immune responses.

4.4 Detection of β -gal-specific IgG in serum

Antibody titers were determined by ELISA as previously described [29]. Briefly, 96-well Nunc-Immuno MaxiSorp assay plates (Nunc, Roskilde, Denmark) were coated with 100 μ l β -gal (Boehringer, Mannheim, Germany) at 5 μ g/ml in 0.05 M carbonate buffer (pH 8.2) per well. Serial twofold dilutions of sera in PBS with 1% BSA and 0.05% Tween 20 were added (100 μ l/well), and plates were incubated for 2 h at 37°C. After washing, biotinylated γ -chain-specific goat anti-mouse IgG (Sigma Chemie, Deisenhofen, Germany) was added as secondary antibody. Plates were incubated for an additional 1 h at 37°C. After four washes, 100 μ l of peroxidase-conjugated streptavidin (Sigma Chemie) was added to the wells and plates were incubated at 37°C for 30 min. After four washes, reactions were developed with ABTS in 0.1 M citrate-phosphate buffer (pH 4.35) containing 0.01% H₂O₂. Endpoint titers were expressed as the reciprocal log₂ of the last dilution, which gave an optical density at 405 nm of 0.1 units above the values of the negative controls after 30 min of incubation.

4.5 Measurement of β -gal-specific IgG isotypes

The amount of β -gal specific IgG subclasses present in serum samples were determined using an isotype-specific ELISA as previously described [29]. To determine the sample concentration, standard curves were obtained by coating the wells with an IgG-isotype-specific goat anti-mouse anti-

body (Sigma Chemie). Plates were further incubated with serial dilutions of purified mouse IgG1, IgG2a, IgG2b or IgG3 antibodies (Dianova, Hamburg, Germany). After washing, the following secondary antibodies were added: biotin-conjugated rat anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Birmingham, GB). The plates were then developed as described above (see Sect. 4.4). The reported values represent the mean \pm SEM of five mice per group.

4.6 Determination of total and β -gal-specific IgA

The amount of total and β -gal-specific IgA present in serum, lung washes and vaginal lavages was determined by ELISA as previously described [29]. To establish the IgA standard curve, plates coated with goat anti-mouse IgA (Sigma Chemie) as capture antibody were further incubated with serial dilutions of purified mouse IgA (Dianova, Hamburg, Germany). As secondary antibody, biotinylated goat anti-mouse IgA (Sigma Chemie) was used, plates were developed as described above (see Sect. 4.4). To compensate for variations in the efficiency of recovery of secretory antibodies between animals, the results were normalized and expressed as percentage of β -gal-specific IgA with respect to the total amount of IgA present in the sample.

4.7 T cell proliferation assays

Lymph node and spleen cell suspensions were adjusted to 5×10^6 cells/ml in complete medium, cells were seeded at 100 μ l/well in a flat-bottom 96-well microtiter plate (Nunc) and plates were incubated for 4 days in the presence of different concentrations of β -gal. Each concentration was tested in triplicate. During the final 18 h of culture, 1 μ Ci [3 H]thymidine (Amersham International, Freiburg, Germany) was added to each well. Cells were then harvested on paper filters (Filtermat A; Wallac, Freiburg, Germany) by using a cell harvester (Inotech, Wohlen, Switzerland), and the amount of incorporated [3 H]thymidine into the DNA of proliferating cells was determined by a γ -scintillation counter (Wallac 1450, Micro-Trilux). The results are expressed as the arithmetic mean of [3 H]thymidine uptake in cpm.

4.8 Cytokines determination

Culture supernatants from proliferating cells were collected on days 2 and 4, and stored at -70°C . Determinations of IFN- γ , IL-2, IL-4, and IL-10 were performed by ELISA using commercial antibodies from BD-PharMingen, according to the manufacturer's instructions. Briefly, 96-well microtiter plates were coated overnight at 4°C with purified rat anti-mouse IFN- γ , anti-IL-2, anti-IL-4 or anti-IL-10 mAb (BD-PharMingen). After three washes, plates were then blocked and supernatants added to the wells. A standard curve was generated for each cytokine by using the corresponding

recombinant murine cytokine (BD-PharMingen). Plates were incubated at room temperature for additional 4 h. After washing, biotinylated rat anti-mouse IFN- γ , IL-2, IL-4, or IL-10 mAb (PharMingen) were added to the wells and incubated for 1 h at room temperature. After six washes, streptavidin-peroxidase conjugate was added and incubated for 30 min at room temperature. The plates were then developed with ABTS in 0.1 M citrate-phosphate buffer (pH 4.35) containing 0.01% H_2O_2 .

4.9 Preparation of NALT cells and flow cytometry

Two groups of 20 mice were used for the isolation of cells from NALT. Sixteen hours before sampling, one group received 0.5 μ g of MALP-2 by i.n. route. NALT cells were prepared as previously described [19, 20]. For the flow cytometric experiment, approximately 10^5 cells for each staining were first incubated with mouse Fc Block (BD PharMingen) for 1 h at 4°C . Then, cells were stained for 1 h at 4°C with FITC-conjugated antibodies against CD11b, CD14, CD40, CD86, CD80, CD54, CD11c, H-2K d , and I-A d , and/or PE-conjugated antibodies against CD18, CD45R/B220, and CD11b (BD PharMingen). Irrelevant labeled antibodies of the same isotype were used as negative control in all experiments. After washing, the stained cells were fixed in 1% paraformaldehyde in PBS. NALT cells were gated according to the physical characteristics of forward and side scatter in comparison to splenic cells and peritoneal macrophages. The analysis of 5,000 gated events was performed using a FACS-Sort and the CellQuest software (Becton Dickinson, Mountain View, CA).

4.10 Statistical analysis

Comparisons between experimental groups were made by using the Student's *t*-test; $p < 0.05$ was considered as significant.

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