

Original article

Monophosphoryl lipid A (MPL®)* promotes allergen-induced immune deviation in favour of Th1 responses

Background: Monophosphoryl lipid A (MPL®) is a nontoxic derivative of the lipopolysaccharide (LPS) of *Salmonella minnesota* R595. MPL has been used as an adjuvant in grass and tree pollen vaccines for the treatment of seasonal allergic rhinitis. Little is known about the influence of MPL on cellular responses to allergens in man. We therefore studied the effects of MPL *in vitro* on peripheral blood mononuclear cells (PBMC) obtained from patients with grass pollen hay fever.

Methods: The PBMCs from 13 subjects were cultured with grass pollen *Phleum pratense* extract (0, 2 and 20 µg/ml) and MPL (0 and 10 µg/ml; defined as an optimal concentration in preliminary studies) and after 6 days proliferative responses were measured by thymidine incorporation and cytokine production by enzyme-linked immunosorbent assay (ELISA).

Results: Proliferative responses were unaffected by the presence of MPL whereas MPL induced a significant increase in allergen-induced interferon (IFN)-γ production [allergen alone, 645 ± 466 pg/ml (mean ± SE) vs allergen + MPL, 3232 ± 818 pg/ml; $P < 0.001$]. In addition, there was a significant decrease in interleukin (IL)-5 production (4307 ± 1030 pg/ml vs 2997 ± 826 pg/ml; $P < 0.01$). Although MPL alone could induce modest increases in IL-10 production, MPL did not influence the production of this cytokine in allergen-stimulated cultures. Addition of neutralizing antibody against IL-12 resulted in 95% inhibition of MPL-induced IFN-γ production. Depletion of monocytes from the culture system abrogated the effects of MPL on elevated cytokine production.

Conclusions: In summary, use of MPL with grass pollen extract results in immune deviation of allergen-induced peripheral Th2-cell responses in favour of 'protective' Th1 responses in an IL-12 and monocyte-dependent fashion.

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Key words: adjuvants; allergy; human; T-lymphocytes; Th1/2 cytokines.

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Accepted for publication 8 September 2004

Allergen-specific immunotherapy is highly effective in the treatment of immunoglobulin E (IgE)-mediated allergy and numerous studies have demonstrated its clinical efficacy (1–3). Immunological changes that are associated with immunotherapy include decreased blood and tissue eosinophilia, a reduction of allergen-induced CD4+ T-cell infiltration and impaired basophil and mast cell recruitment (4–6). The mechanisms underlying effective immunotherapy are thought to involve induction of protective IgG antibodies (7), skewing of the disease-

associated Th2 cytokine profile towards a Th1 phenotype (8) and the generation of regulatory T cells (9, 10).

Currently aluminium hydroxide is widely used as an adjuvant for use in allergen injection immunotherapy for IgE-mediated allergic conditions such as severe allergic rhinitis (11). Whilst aluminium salts are generally safe and effective there is evidence that alum selectively promotes Th2 responses in mice (12) and may augment IgE antibody responses (13). Several new adjuvants have been tested which may allow a rational approach to vaccine development. Monophosphoryl lipid A (MPL®), a 3-deacylated MPL, is derived from the lipopolysaccharide (LPS) of *Salmonella minnesota* R595. Whilst MPL retains the immunostimulatory properties of the parent molecule it does not have its inherent toxicity (14).

MPL has been tested clinically and shown to be an effective and well-tolerated adjuvant in a number of vaccines for infectious diseases (15, 16). Immunization

Abbreviations: RAST, radioallergosorbent test; MPL, monophosphoryl lipid A; HLA, human leucocyte antigen; ICAM, intracellular adhesion molecule-1; APC, antigen-presenting cell.

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strategies utilizing MPL alone or in combination with other adjuvants have been successfully employed to enhance responses to a variety of antigens (17). Co-administration of recombinant human immunodeficiency virus (HIV) gp120 with MPL and a derivative of *Quillaja* saponin (QS21) to mice results in enhanced humoral and cellular immune responses and polarization of the T-cell response to a Th1 subtype (18). Immunization of mice with the cancer-associated antigen MUC1 mucin peptide encapsulated within microspheres, in conjunction with MPL, promotes enhanced interferon (IFN)- γ production (19). MPL is also an effective adjuvant formulated with hepatitis B surface antigen, tetanus toxoid and influenza antigens (20). In humans, MPL has been used as an adjuvant in a well-tolerated hepatitis B surface antigen vaccine (21) and in malaria vaccine preparations (22, 23).

MPL has also been tested as an adjuvant in allergen immunotherapy (24–26) and data shows that the combination of MPL with grass pollen extract was effective at reducing symptoms and promoting increases in serum allergen-specific IgG. There is little information on the effects of MPL on human allergen-specific T-cell responses *in vitro*. The aim of this study was therefore to examine the effects of MPL on allergen-stimulated peripheral blood mononuclear cells (PBMC) derived from grass pollen allergic individuals. We show that MPL can promote Th1 responses, which are mediated by the induction of interleukin (IL)-12. Furthermore, we show that MPL acts via monocytes to skew resulting T-helper responses and to increase accessory molecule expression.

Materials and methods

Subjects

Thirteen subjects [age median: 31, interquartile range (IQR): 23–39 years, seven males] were recruited from the allergy clinic of the Royal Brompton Hospital, London, UK or by advertisement in a local newspaper. All subjects had a history of severe summer hay fever, positive skin test reaction (median wheal diameter 8 mm, IQR: 5–9) to *Phleum pratense* (Soluprick; ALK Abelló, Hørsholm, Denmark) and positive radioallergosorbent test (RAST) (UniCap; Pharmacia, Uppsala, Sweden) test to the allergen (median value 60.8 IU/ml, IQR: 21.2–85.95). Total IgE serum levels were also measured (median value 153 IU/ml, IQR: 99.5–286.5). None of the subjects had previously received grass pollen immunotherapy. The subjects had not taken any parenteral antihistamines or topical corticosteroids for at least 2 weeks before venesection. The study was approved by the Ethics Committee of the Royal Brompton and Harefield Hospitals NHS Trust and was performed with patients' written informed consent.

Preparation of PBMC

The PBMC were isolated from heparinized blood samples by density-gradient centrifugation over Histopaque (Sigma, Poole, UK), washed twice with RPMI-1640 (Invitrogen, Paisley, UK) and resuspended in RPMI-1640 supplemented with 5% human AB serum

(Sigma), 100 U/ml penicillin/streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen).

PBMC cultures

The PBMC were resuspended at 1×10^6 cells/ml and incubated in the presence of 0, 2 and 20 $\mu\text{g/ml}$ of *P. pratense* (whole allergen extract, kindly provided by Allergy Therapeutics, Worthing, UK) at 37°C and 5% CO_2 for 6 days. Initial experiments examined the optimal dose of MPL (Allergy Therapeutics) to induce changes in cytokine production by PBMC. A dose range from 1 to 30 $\mu\text{g/ml}$ of MPL was tested and maximal changes were observed at 10 $\mu\text{g/ml}$ of MPL (data not shown). For all subsequent experiments, PBMC were cultured in the presence or absence of 10 $\mu\text{g/ml}$ of MPL. All cultures were performed in 48-well tissue culture plates (Nunc, Roskilde, Denmark) and no supplementation or change in media occurred over the 6 days of culture. Cellular proliferation was assessed by the addition of 0.5 μCi of tritiated methylthymidine (Amersham, Aylesbury, UK) to 100 μl of mixed cell suspension in a round bottomed 96-well plate in triplicate for the final 16 h of culture. IFN- γ , IL-4, IL-5, IL-10 and IL-12 concentrations in supernatants were measured using culture matched antibody pairs (PharMingen, San Diego, CA, USA) with a sensitivity <10 pg/ml. For IL-12 neutralization studies, anti-IL-12 (R&D Systems, Abingdon, UK) was added at 2 $\mu\text{g/ml}$ and control wells contained a similar concentration of an isotype control (IgG2a).

Monocyte depletion

Depletion of monocytes from PBMC was achieved by plastic adherence for 2 h at 37°C. Between 81.4 and 91.6% of monocytes were depleted from cultures as determined by flow cytometry. Monocyte depleted cultures and whole PBMC were incubated in the presence or absence of MPL (10 $\mu\text{g/ml}$) for 6 days.

Flow cytometry

The PBMC were cultured with *P. pratense* in the presence or absence of 10 $\mu\text{g/ml}$ of MPL for 72 h in complete media. Cells were washed twice with staining buffer [phosphate-buffered saline (PBS) + 0.1% bovine serum albumin (BSA) + 0.09% azide] and incubated with either anti-human leucocyte antigen (HLA)-DR/DP/DQ, CD40, CD54, CD58, CD86 or a control antibody (IgG1) for 30 min (all antibodies from DakoCytomation, Ely, UK except CD40; Diaclone from Immunodiagnostic Systems, Bordon, UK). Cells were additionally co-stained for CD14-PE-Cy5 (monocytes; Chemicon, Harrow, UK) and CD19-PE (B-cells; DakoCytomation). For CD80 staining, samples were stained with CD80-PE (Becton Dickinson, Mountainview, CA, USA), CD19-FITC (Becton Dickinson) and CD14-PE-Cy5. Samples were then washed twice in staining buffer and analysed using a FACScan flow cytometer with appropriate compensation settings (Becton-Dickinson) and further analysed with WinMDI (J. Trotter, The Scripps Research Institute, La Jolla, CA, USA).

Statistical analysis

Groups of data were analysed by the Wilcoxon matched-pairs signed rank test, where $P < 0.05$ was considered significant. In unstimulated cultures (medium only) 14 ± 7 pg/ml of IL-5, 10 ± 2 pg/ml of IL-10, 10 ± 3 pg/ml of IFN- γ was detected and proliferative responses were 985 ± 403 cpm.

Results

MPL in combination with grass pollen allergen induces immune deviation in favour of Th1 responses

The effects of MPL were investigated on allergen-stimulated PBMC cultures. Unstimulated cultures produced little (< 15 pg/ml) or no cytokines above detection limits of the assay whereas addition of allergen (20 μ g/ml) resulted in a significant increase in proliferative responses and increased production of IL-5 (Fig. 1). In contrast to IL-5, significant IFN- γ production was detected in only two of the 13 subjects tested and little IL-10 was produced in response to stimulation with allergen. This cytokine profile is characteristic of a Th2-biased allergic response. Addition of MPL to allergen-stimulated cultures resulted in a significant increase in IFN- γ production ($P < 0.001$) by all subjects tested and a concomitant decrease in IL-5 production ($P = 0.01$). IL-10 production and proliferative responses were not affected by the presence of MPL. We also tested the influence of MPL with a lower concentration of *P. pratense* (2 μ g/ml) and again we observed a significant increase in IFN- γ production (344.5 ± 323 pg/ml vs 1967 ± 656 pg/ml; $P < 0.01$) and a trend for a reduction in IL-5 production were observed (3485 ± 1114 pg/ml vs 2425 ± 712 pg/ml). Again MPL, did not influence allergen-stimulated IL-10 production (50.8 ± 16 pg/ml vs 78 ± 23 pg/ml) or proliferative responses ($13\,767 \pm 4497$ cpm vs $23\,388 \pm 6202$ cpm) to grass pollen allergen. Little (< 10 pg/ml) or no IL-4 was detected in any of the culture supernatants.

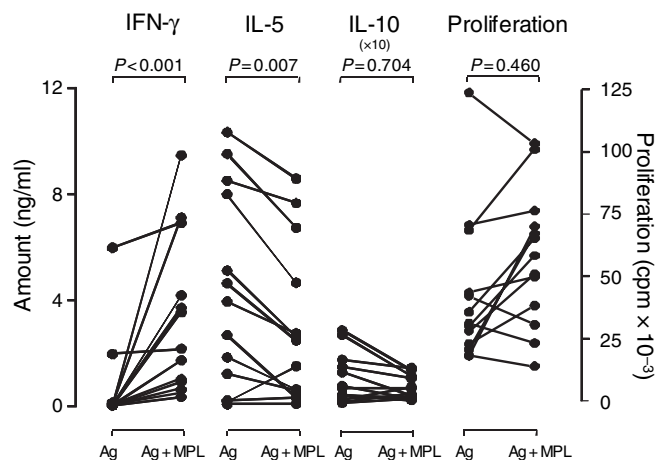


Figure 1. Effect of monophosphoryl lipid A (MPL) on allergen-stimulated cytokine production and proliferative responses. Peripheral blood mononuclear cells (PBMC) from grass pollen-sensitive donors ($n = 13$) were stimulated with 20 μ g/ml of *Phleum pratense* in the presence or absence of 10 μ g/ml MPL. After 6 days proliferative responses and cytokine production was measured. Data are shown as mean $\pm$ SE and statistical analysis was carried out using a Wilcoxon matched pairs test.

MPL increases cytokine production and proliferative responses by PBMC

In order to investigate the effect of MPL *per se* on cellular responses *in vitro*, PBMC from 13 grass pollen-allergic donors were cultured in the presence or absence of MPL (10 μ g/ml). Spontaneous T-cell proliferation and cytokine production were extremely low, whereas incubation of cells with MPL induced a significant increase in the production of IFN- γ , IL-5 and IL-10 ($P < 0.01$) (Fig. 2). In addition, proliferative responses were elevated in the presence of MPL compared with media alone ($P < 0.01$).

MPL increases IL-12 production by PBMCs from grass pollen allergic donors

In view of the observation of enhanced IFN- γ production in the presence of MPL we investigated whether MPL also could promote synthesis of the Th1-inducing cytokine IL-12. The MPL induced significant increases in IL-12 production ($P < 0.001$) at all allergen concentrations tested (Fig. 3). It is of note that, in this context, the presence of allergen in the culture system had no effect on MPL-induced IL-12 production.

Neutralization of IL-12 inhibits the effects of MPL on cytokine production

In order to determine whether the effects of MPL were IL-12-dependent we cultured PBMC with allergen and MPL in the presence of an IL-12 neutralizing antibody (Fig. 4). MPL induced a significant increase in IFN- γ by allergen-stimulated cells and IFN- γ production was significantly inhibited by the inclusion of neutralizing

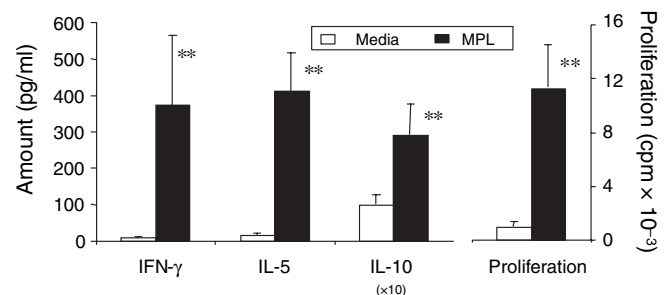


Figure 2. Effect of monophosphoryl lipid A (MPL) on cytokine production and proliferation by peripheral blood mononuclear cells (PBMC). PBMC were incubated in the presence (filled bars) or absence (open bars) of 10 μ g/ml MPL for 6 days. Interleukin (IL)-5, IL-10 and interferon (IFN)- γ production was measured by enzyme-linked immunosorbent assay (ELISA) and proliferation was measured by thymidine incorporation. Data (mean $\pm$ SE) are from 13 atopic donors and statistical analysis was carried out using a Wilcoxon matched pairs test where $**P < 0.01$.

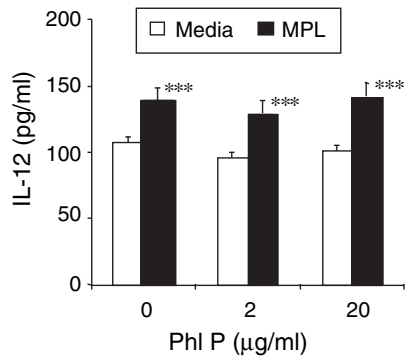


Figure 3. Effect of monophosphoryl lipid A (MPL) on allergen-stimulated interleukin (IL)-12 production. Cells from grass pollen-sensitive donors ($n = 13$) were stimulated with 0, 2 and 20 µg/ml of *Phleum pratense* in the presence or absence of 10 µg/ml MPL. After 6 days, IL-12 production was measured. Data are shown as mean $\pm$ SE and statistical analysis was carried out using a Wilcoxon matched pairs test where *** $P < 0.001$.

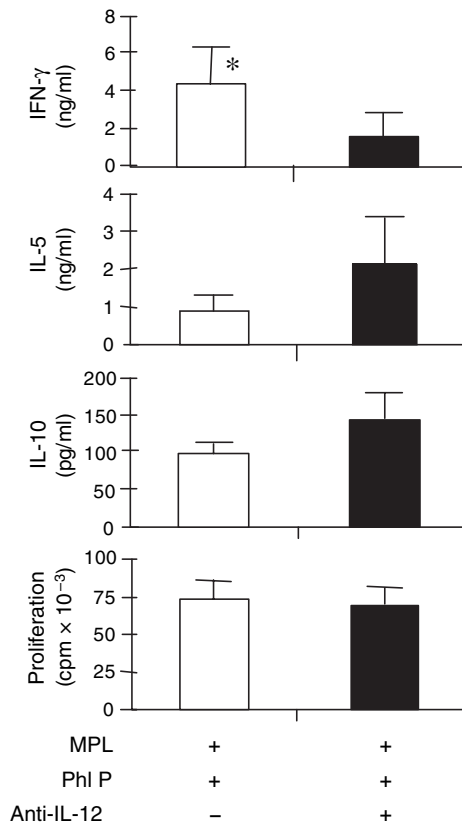


Figure 4. Effect of neutralization of interleukin (IL)-12 on allergen and monophosphoryl lipid A (MPL)-induced cytokine profiles and proliferative responses. Cells from allergic donors ($n = 6$) were stimulated with allergen (20 µg/ml) and MPL (10 µg/ml) for 6 days in the presence of a neutralizing antibody to IL-12 (2 µg/ml) or an isotype control antibody. Proliferative responses and IL-5, IL-10 and interferon (IFN)-γ production was measured. Data (mean $\pm$ SE) was statistically analysed using a Wilcoxon matched pairs test where * $P < 0.05$.

IL-12 ($P = 0.03$). Also, decreases in IL-5 production were no longer apparent although this did not reach statistical significance. The IL-10 production and proliferative responses were unaffected by the neutralization of IL-12.

MPL acts via monocytes to promote cytokine production

MPL has been reported to bind to toll-like receptors (TLR) and CD14 on monocytes. We therefore studied the influence of MPL on PBMC populations depleted of monocytes. These further experiments reconfirmed that co-culture of PBMC with MPL resulted in the increased production of IL-12, IFN-γ and IL-5 (Fig. 5). However, in cultures depleted of monocytes, MPL did not influence cytokine production compared with controls.

Influence of MPL on accessory molecule expression on monocytes

The effects of MPL on the expression of accessory molecules involved in antigen presentation were examined. The PBMC were cultured with allergen in the presence or absence of MPL for 72 h and expression of accessory molecules were measured by flow cytometry. MPL had no effect on the expression of HLA, CD40, CD58, CD80 or CD86 (Table 1). In contrast, MPL induced significantly higher expression of CD54 on allergen-stimulated monocytes ($P < 0.01$). Addition of neutralizing IL-12 reduced the expression of MPL-induced CD54 expression (control: 858 ± 108 , MPL: 920 ± 89 ; MPL + α IL-12: 873 ± 82 , $n = 3$).

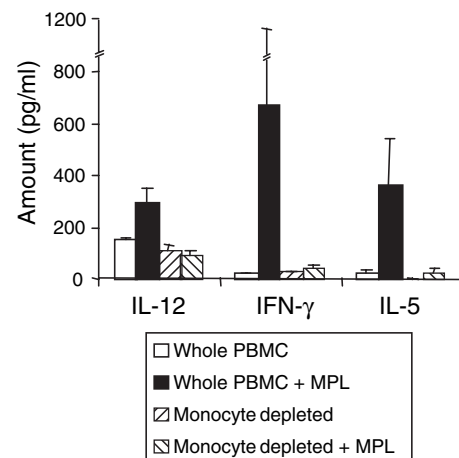


Figure 5. Effect of monophosphoryl lipid A (MPL) on peripheral blood mononuclear cell (PBMC) cultures depleted of monocytes. Monocytes were depleted from PBMC cultures by plastic adherence and the remaining cell population was cultured with MPL at 10 µg/ml. Whole PBMC populations were also stimulated with MPL under identical conditions. After 6 days production of IL-5, IL-12 and interferon (IFN)-γ was measured by enzyme-linked immunosorbent assay (ELISA).

Table 1. Effect of MPL on monocyte accessory molecule expression

	Medium	MPL	P-value
HLA	2118 ± 539	1807 ± 414	0.201
CD40	166 ± 8	171 ± 7	0.21
CD54	681 ± 80	843 ± 110	0.002
CD58	317 ± 16	315 ± 21	0.84
CD80	138 ± 19	150 ± 20	0.36
CD86	290 ± 15	297 ± 23	0.36

Cells were cultured with 20 µg/ml of allergen in the presence or absence of MPL (10 µg/ml). After 72 h the expression of accessory molecules was assessed on CD14⁺ monocytes by flow cytometry. Data represents the mean fluorescence intensity ± SE. P-values are calculated using a Wilcoxon matched pairs test and significant values are shown in bold.

MPL, monophosphoryl lipid A; HLA, human leucocyte antigen.

Discussion

Stimulation of PBMC derived from grass pollen allergic subjects with allergen induces the production of predominantly Th2-associated cytokines including IL-4, IL-5 and IL-13 (27, 28). In this study, we show that incubation of PBMC with grass pollen allergen and the bacterial LPS-associated molecule MPL could induce preferential allergen-dependent increases in IFN- γ , and decreases in IL-5 consistent with immune deviation in favour of a Th1-phenotype. In contrast, MPL had no effect on the allergen-induced production of the regulatory cytokine IL-10. Using a neutralizing antibody, we show this shift in cytokine production is dependent upon IL-12. In addition, this effect of MPL is related to monocytes since depletion of this cell type from our culture system inhibited the effects of the adjuvant. Moreover, MPL selectively promotes enhanced expression of accessory molecules on monocytes. Thus, the Th1-inducing potential of the adjuvant MPL is both IL-12- and monocyte-dependent.

Immunization strategies utilizing MPL alone or in combination with other adjuvants have been successfully employed to enhance responses to a variety of antigens (17–23). Recently, MPL has been used as an adjuvant in a successful vaccination preparation used for grass and tree-pollen immunotherapy (24–26, 29, 30). MPL, in conjugation with modified whole grass pollen (allergoid) adsorbed to L-tyrosine, reduce allergic symptoms in patients suffering from hay fever (30). Clinical improvement was accompanied by an increase in serum allergen-specific IgG concentrations, which inhibited allergen-induced immediate basophil histamine release (26). Increases in allergen-specific IgG was also associated with blunting of seasonal increases in allergen-specific IgE.

MPL has been shown to be effective at promoting humoral responses in animal models (31). Injection of antigen with MPL, in conjunction with L-tyrosine, can induce IgG2a antibodies, which are indicative of a Th1-response in mice. Moreover, injection of MPL

co-administered with keyhole limpet haemocyanin to presensitized rats could prevent antigen-specific IgE responses induced by injection of the antigen alone.

In the murine system, MPL can promote activation of antigen-presenting cells (APC) *in vitro*. Incubation of dendritic cells, B cells or macrophages with MPL resulted in increased ability to prime naïve T cells *in vitro* (32). Moreover, MPL-treated APC had the ability to mount both Th1- and Th2-associated antibody responses as characterized by the production of IgG2a and IgG1 antibodies respectively. Pretreatment of APC with MPL increased their immunostimulatory properties and resulted in the increased production of IFN- γ , IL-4 and IL-5 by B-cells and macrophages. The MPL also has the ability to up-regulate expression of co-stimulatory molecules on dendritic cells (33). High doses of MPL increased surface expression of HLA-DR, CD80, CD86 and CD40. Other studies in both murine and humans systems have shown that MPL may up-regulate CD80 and/or CD86 on APC (18, 32–34). In our study, we did not observe changes in expression of these molecules on monocytes and this may have been due to the relatively low concentration of MPL used compared with other reports. However, we did observe a highly significant increase in the expression of CD54 (intracellular adhesion molecule-1, ICAM-1). The regulation of ICAM-1 has been linked to IL-12 and IL-18 (35) and we show that neutralization of IL-12 can indeed counteract the MPL-induced up-regulation of this receptor.

We have also shown that the effects of MPL on allergen-induced responses is dependent on monocytes as MPL had no effects on cytokine production or proliferation of PBMC cultures depleted of this cell type. A recent study has identified the receptors on monocytes responsible for MPL-binding (36). These data show that, like the parent molecule LPS, MPL may bind to the TLR-2 and TLR-4 to promote proliferative responses and cytokine production. Interestingly, MPL-induced production of TNF- α , IL-10 and IL-12 are differentially effected by the addition of neutralizing antibodies against either TLR-2 or TLR-4. In addition, intracellular signalling molecules utilized by the receptors differ as both receptors induced phosphorylation of extracellular signal-related kinase whereas only TLR-4 was responsible for the phosphorylation of p38.

One proposed mechanism of allergen immunotherapy is the induction of cells producing regulatory cytokines such as IL-10 and transforming growth factor (TGF)- β . Therefore, promotion of IL-10 may be a desirable feature of an adjuvant used in allergen immunotherapy. Indeed, other studies have demonstrated that MPL alone may increase IL-10 production by human peripheral blood monocytes and whole PBMC cultures from normal subjects (36). In contrast, our data showed that the addition of MPL to allergen-stimulated cultures revealed no such increase and overall IL-10 production was low although our study utilized PBMC from grass pollen

allergic subjects. Using similar culture media and methodology to those of Martin et al. (36) we were also able to detect large amounts of IL-10 at early time-points although these levels were unaffected by the addition of allergen or MPL (data not shown).

An alternative mechanism associated with allergen immunotherapy is the deviation of allergen-specific Th2 profile in favour of a Th1 response (37). It is well documented that MPL can induce Th1 cytokines (18, 33, 36) and we aimed to test this adjuvant on Th2-dominated allergic response *in vitro*. IL-4 is the main factor responsible for promoting Th2 responses (27); however, IL-4 levels were at or below detection limits in our assay. We therefore measured IL-5 production as a marker for both allergic (38) and Th2 responses. Results show that MPL deviated cytokine production in favour of Th1 responses in allergen-stimulated cultures although MPL alone could induce both Th1 and Th2 cytokines. Others have shown that MPL can induce IL-10 production (36) but, to our knowledge, this is the first report to show the influence of MPL on human IL-5 production. As T cells have recently been shown to express TLRs (39) it is possible that MPL acts directly on these cells to induce cytokine production. In atopics who are biased towards Th2 responses the direct stimulation of T cells by MPL may inevitably result in the production of both Th1 as well as Th2 responses.

Here, we show that MPL can induce IFN- γ production both alone and when used in combination with allergen, in a dose-dependent manner. In contrast, whilst MPL can promote IL-12 production (36), addition of allergen to cultures has no effect on this cytokine. Also, neutralization of IL-12 can abrogate increases in spontaneous IFN- γ production. Therefore, it seems that one mechanism by which MPL acts is to enhance IL-12 production, albeit at low levels, by monocytes which, in turn, promotes allergen-stimulated T cells to preferentially produce IFN- γ .

In conclusion, we show that the addition of MPL to allergen-stimulated human PBMC cultures *in vitro* resulted in immune deviation in favour of Th1 responses in an IL-12 and monocyte-dependent fashion. These data are consistent with initial studies reporting the clinical efficacy and enhanced IgG production (25, 29) when allergen-MPL conjugate is employed *in vivo* in the treatment of seasonal pollinosis.

Acknowledgments

Funding for this work was from the Clinical Research Committee of the Royal Brompton and Harefield Hospitals NHS trust.

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