Johansen P, Senti G, Martinez Gomez JM, et al. Toll-like receptor ligands as adjuvants in allergen-specific immunotherapy

ARTICLE *in* CLINICAL & EXPERIMENTAL ALLERGY · JANUARY 2006

Impact Factor: 4.77 · DOI: 10.1111/j.1365-2222.2005.02384.x · Source: PubMed

Clin Exp Allergy 2005; **35**:1591–1598 doi:10.1111/j.1365-2222.2005.02384.x

Toll-like receptor ligands as adjuvants in allergen-specific immunotherapy

P. Johansen*, G. Senti*, J. M. Martinez Gomez*, T. Storni*, B. R. von Beust*, B. Wüthrich*, A. Bot† and T. M. Kündig*

*Unit for Experimental Immunotherapy, University Hospital of Zurich, Zurich, Switzerland and †MannKind Corporation, Valencia, CA, USA

Summary

Background Allergen-specific immunotherapy (SIT) leads to long-term amelioration of T-helper type 2 (Th2)-mediated allergic symptoms and is therefore recommended as a first line therapy for allergies. The major disadvantage of SIT is its low efficiency, requiring treatment over years.

Objective In this study, we evaluated the potential of Toll-like receptor (TLR) ligands to facilitate Th1-type immune responses.

Methods The immunogenicity and therapeutic potential of the major bee venom allergen phospholipase A2 (PLA2) combined with various TLR ligands were tested in mice and compared with immune responses induced by conventional aluminium-based preparations.

Results Regarding total IgG against PLA2, TLR2/4-binding lipopolysaccharide and TLR3-binding polyriboinosinic polyribocytidylic (PolyI:C) were the superior adjuvants for prophylactic vaccination. However, TLR9-binding phosphorothioate-modified cytosine–guanosine-rich oligonucleotide (CpG), TLR-3-binding PolyI:C, and TLR2/6-binding peptidoglycan skewed the immune responses more towards IgG2a isotype and Th1 cytokines. Furthermore, in a therapeutic approach, CpG, PolyI:C and TLR7/8-binding 3M003 had immune modulating properties as they suppressed established IgE titres.

Conclusion The potential of TLR ligands to adjuvate the immunogenicity of bee venom PLA2 and to skew the Th1–Th2 balance proved very heterogeneous. With respect to SIT, CpG, PolyI:C, and 3M003 were very promising. Hence, TLR ligands should be considered as adjuvants or immune modulators in SIT in human as to improve its efficiency regarding the Th1–Th2 balance of the immune response with a likely effect on therapy duration.

Keywords adjuvants, allergy, animal models, antibodies, bee venom, Hymenoptera, vaccination
Submitted 28 July 2004; revised 5 August 2005; accepted 20 September 2005

Introduction

During allergen-specific immunotherapy (SIT), gradually increasing quantities of the allergen is administered in order to ameliorate symptoms associated with subsequent exposure. The major disadvantages of the current therapy are its long duration of multiple injections [1] and the associated high costs. SIT is also associated with a significant risk of severe allergic reactions. It is therefore of capital interest to improve SIT by reducing the required number of allergen injections, thereby shortening treatment duration, or to reduce the side-effects by optimizing the allergen type, dose and administration.

A way to improve immunotherapy or vaccines is by using adjuvants. However, the adjuvants used in SIT are salts of aluminium and calcium. These have a good safety record but produce poor T cell responses, and the antibodies are mostly of T-helper type 2 (Th2)-type, including IgE. This is disadvantageous in immunotherapies aiming at inducing cellular-mediated immunity or protection against IgE-mediated allergies. Therefore, research to improve SIT now focuses on the use of alternative adjuvants driving the immune response towards Th1.

Lately, a new class of adjuvants or so-called immune response modifiers (IRMs) has been described. Professional antigen-presenting cells carry so-called Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns on micro-organisms [2, 3]. Ligation of the TLRs activates nuclear factor-κB and leads to the expression of several cytokines and co-stimulatory molecules. It is the presence of these molecules along with the presentation of microbial antigens that activates the CD4 T cells required to initiate most adaptive immune responses. So far, a dozen mammalian TLRs have been described together with a multitude of natural and synthetic ligands [4].

This study compares different TLR ligands as adjuvants or IRMs for use in allergy vaccines. In particular, we compare the efficiency of these adjuvants in triggering Th1 immune responses and in inducing murine IgG2a against the allergen, as both parameters are considered important for successful

Correspondence: Pål Johansen, Unit for Experimental Immunotherapy, University Hospital of Zurich, Gloriastrasse 31, 8091 Zurich, Switzerland. E-mail: pal.johansen@usz.ch

SIT [5]. A major allergen of bee venom, i.e., phospholipase A2 (PLA2; Api m 1) served as a model allergen, and in order to eliminate potential differences in distribution and pharmacokinetics of the adjuvants, i.e., to assure that the adjuvant reaches the lymph node, both the allergen and the adjuvant were administered directly into the inguinal lymph nodes of mice, a procedure that has been described recently [6, 7].

TLR ligands differed in their adjuvancy, their Th1-polarization properties and the kinetics in which specific antibodies were produced. Polyriboinosinic polyribocytidylic (PolyI:C) and lipopolysaccharide (LPS) were far the most potent adjuvants with a balanced Th1/Th2-type immune response. In contrast, phosphorothioate-modified cytosine–guanosine-rich oligonucleotide (CpG) and peptidoglycan were weaker adjuvants but resulted in a stronger Th1-polarized antibody response. Finally, CpG and PolyI:C, but also the TLR-7/8-binding compound 3M003 had therapeutic potentials, as they suppressed IgE and prevented anaphylaxis in allergic mice as compared with conventional adjuvants (aluminium salts).

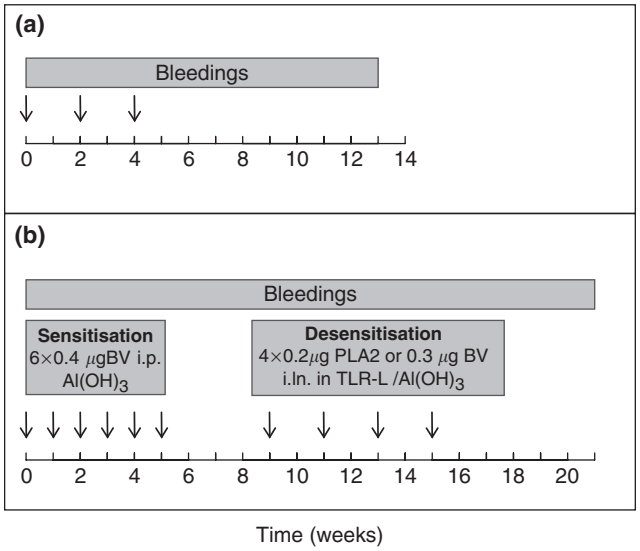

Fig. 1. Schematic illustration of immunization protocols. Porphylactic (a) and therapeutic (b) protocols. Arrows indicate injections.

Material and methods

Allergens and adjuvants

Purified bee venom PLA2 was purchased from Sigma-Aldrich (Buchs, Switzerland), and bee venom extract from ALK-Abello was purchased through Trimedal (Bruttisellen, Switzerland). *Staphylococcus aureus* peptidoglycan (PGN), *Eschericia coli* LPS and PolyI:C were purchased from Invivogen (San Diego, CA, USA), whereas lipoteichoic acid (LTA), and flagellin were kindly provided to us by Martin F. Bachmann. The experimental TLR-7/8-binding compound 3M003 [8] was a gift from 3M (St. Paul, MN, USA) and is a more recent analogue of imiquimod and resiquimod. Aluminium hydroxide (Alu-Gel-S® Suspension) was from Serva (Heidelberg, Germany), and phosphorothioate-modified CpG oligodeoxynucleotide 1668 (5′-TCC-ATG-ACG-TTC-CCT-GAC-GTT-3′) was synthesized by Microsynth (Balgach, Switzerland).

Table 1. Description of adjuvants used for intralymphatic immunizations

Adjuvant	Dose*	TLR-L number	Description and source
LTA	1.25 μg	2	Lipoteichoic acid from *Staphylococcus aureus*
PGN	2 μg	2 and 6	Peptidoglycan from *S. aureus*
LPS	2 μg	2 and 4	Lipopolysaccharide from *Eschericia coli*
PolyI:C	2 μg	3	Synthetic analogue of dsRNA
Flagellin	3 μg	5	*S. thyphimurium*
3M003	2 μg	7 and 8	Small synthetic antiviral imidazoqionoline
CpG	1 nmol	9	Synthetic dsDNA with repeating C and G motives
$Al(OH)_3$	60 μg†	–	Aluminium hydroxide

*The chosen doses were based on predictions where the ligands has previously been shown the be effective as well as on our own dose–response testing (data not shown).
†Injected amount with respect to Al^{3+}.
LTA, lipoteichoic acid; PGN, peptidoglycan; LPS, lipopolysaccharide; PolyI:C, polyriboinosinic polyribocytidylic; CpG, phosphorothioate-modified cytosine–guanosine-rich oligonucleotide.

Immunization

Young female CBA/J mice from Harlan (Horst, the Netherlands) were anaesthetized with ketamin and xylazin by intraperitoneal injection and immunized thrice on a semi-weekly interval by intralymphatic injection of 0.1 μg purified PLA2, adjuvant and saline contained in a total of 10 μL (Table 1) into the inguinal lymph node (Fig. 1a). In a therapeutic approach, mice were sensitized by six weekly injections of 50 μL bee venom extract (0.1 μg) in aluminium hydroxide (300 μg Al^{3+}) prior to the therapeutic vaccination by intralymphatic injections of PLA2 (Fig. 1b). Serum was prepared from clotted blood taken at different time-points. The sera were frozen and kept at −20 °C until analysed by ELISA. For induction of anaphylactic responses, immunized mice were injected intraperitoneally with 15 μg of PLA2 in saline. Rectal temperature was monitored with a calibrated digital thermometer after 30 min. All animal experiments were performed according to guidelines formulated by the Veterinary authorities of the Kanton Zurich.

Antibody determination by enzyme linked immunosorbent assay

For detection of PLA2 antibodies, microtitre 96-well plates (Nunc Maxisorb) were coated at 4 °C overnight with 100 μL of 5 μg/mL PLA2 in buffered carbonate (pH 9.4). Plates were washed with PBS–0.05% Tween 20 (PBST) and saturated with 150 μL of 2.5% skimmed dry milk (PBSTM) at room temperature (RT) for 1 h. After washing, serial dilutions of individual sera in 100 μL PBSTM were added to the plates, which were incubated at RT for 2 h. Plates were washed and incubated with 1 μg/mL goat anti-mouse IgG1 or IgG2a conjugated to biotin (BD Biosciences Pharmingen, San Diego, CA, USA) in 100 μL PBSTM at RT and for 2 h. After further washing and incubation with 100 μL of a

1 : 1000 dilution of strepatavidin-conjugated HRP (BD Biosciences Pharmingen) at RT for 1 h, the plates were washed and added 100 μL of the enzyme substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (Sigma-Aldrich) in 1 M sodium dihydrogen phosphate. The absorption was read at 405 nm after 20 min incubation. End-point titres were defined as the highest serum dilution that resulted in an absorbance value three standard deviations higher than that of negative control sera from non-immunized mice.

Cytokine-secretion assay

To evaluate cytokine secretion from lymphocytes in immunized mice, spleens were isolated from mice 2 weeks after the last injection of PLA2. After homogenization of spleens and lysis of red blood cells, 5×10^5 splenocytes were cultured with 20 μg/mL PLA2 protein in 200 μL IMDM (Gibco, Invitrogen Basel, Switzerland) supplemented with L-glutamine (4 mM), fetal calf serum (FCS) (10%), mercaptoethanol (75 μM), as well as streptomycin and ampicillin. Supernatants were collected after 96 h incubation and IL-4, IL-10, and IFN-γ concentrations determined by ELISA from R&D Systems (Abingdon, UK).

Flow cytometry

Intracellular cytokine production was assessed after 4 h stimulation at 37 °C of single-cell suspensions of lymphocytes with phorbol dibutyrate and ionomycin (both at 500 ng/mL) in the presence of brefeldin A (10 μg/mL). The cells were then incubated with anti-CD16/CD32 for Fc-recptor blocking and stained on ice and in PBS/FCS 2% with fluorescent-labelled antibodies (Abs) against CD4, CD8 and CD44 (all Abs from BD Biosciences, Franklin Lakes, NJ, USA). Cells were subsequently fixed with 1% paraformaldehyde and permeabilized with 0.1% Nonidet P-40 before staining with Ab against IFN-γ. Four-colour cytometry were performed on a FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Results

To evaluate the effect of TLR ligands on the antibody response, mice were immunized trice semi-weekly with PLA2 and a TLR ligand (Fig. 1a); the compounds were mixed within an hour before injection into the inguinal lymph node. Figure 2 illustrates PLA2-specific IgG2a (Fig. 2a) and IgG1 (Fig. 2b) antibodies in sera as well as the ratio of IgG2a to IgG1 (Fig. 2C) titres at different time-points; the ratio of IgG2a to IgG1 antibodies in murine serum is typically used as a qualitative measure for the relative strength of Th1 (IgG2a)- and Th2 (IgG1)-type immune responses. Seven weeks after the first and 3 weeks after last injection, notable seroconversion was observed in mice receiving LPS, PolyI:C or CpG. The responses were completely different in nature, with CpG inducing only IgG2a, LPS predominantly IgG1, and

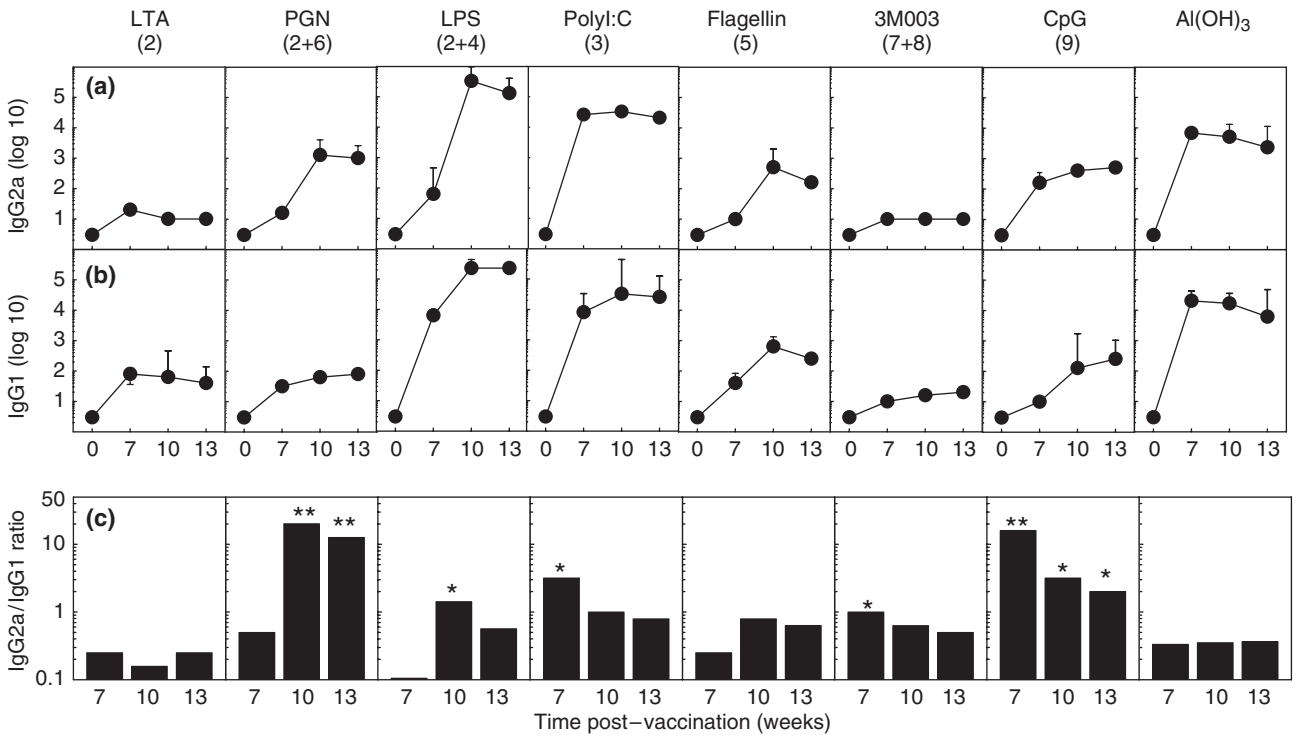

Fig. 2. Isotype profile of anti-phospholipase A2 (PLA2) serum antibodies. Isotype profile of anti-PLA2 serum antibodies in CBA/J mice vaccinated on days 1, 15, and 29 with 0.1 μg PLA2 and either of the indicated Toll-like receptor ligands or the reference adjuvant $Al(OH)_3$. The sera were obtained over 13 weeks and analysed by ELISA for anti-PLA2 IgG2a (a) and IgG1 (b). The ratio of IgG2a to IgG1 titres was calculated as a measure for T-helper type 1 (Th1)/Th2 immune response balance (c). IgE antibodies were not detected upon intralymphatic immunization (not shown). The serological titres were defined as the inverse of the highest dilution yielding an absorbance higher than that of a negative serum plus three standard deviations and expressed as geometric means ± standard error ($n = 3$–4). Pre-immunization levels of anti-PLA2 antibodies were measured on sera from five mice taken the day before first injection. The results show one representative out of two immunization experiments. Significant differences are indicated (** $P < 0.01$; * $P < 0.05$).

with PolyI:C inducing high antibodies titres of both IgG1 and IgG2a isotypes. This is also highlighted in the resulting isotype ratios. For the other TLR ligands tested, only low levels of IgG1 and IgG2a antibodies in these early sera were detected when compared with background levels. When PLA2 was administered together with the conventional adjuvant aluminium hydroxide, a balanced profile of IgG2a and IgG1 antibodies was produced, but the titres were lower than those produced by PolyI:C- or LPS-containing vaccines. Also, the ratio of IgG2a to IgG1 was shifted towards Th2 when compared with PolyI:C, CpG, and peptidoglycan. None of the vaccines induced detectable IgE antibodies against PLA2 upon intralymphatic injection (data not shown).

The kinetics of the antibody production was also affected by the adjuvant. PolyI:C produced its maximum levels of antibodies within 3 weeks of the final boost injection. LPS caused a delay of 3 weeks for both the IgG1 and the IgG2a isotypes, and the IgG2a titre increased relatively more than the IgG1. In 7-week sera from CpG-injected mice, IgG1 was hardly detectable but increased at 10 and 13 weeks indicating a delay in the IgG1 antibody production. However, the serum concentration of IgG1 was geometrically only 30–50% of the titres induced by LPS or PolyI:C. At the later time-points, also peptidoglycan and flagellin moderately improved PLA2 immunogenicity. Peptidoglycan induced predominantly IgG2a, whereas flagellin induced both IgG2a and IgG1. 3M003 and lipoteichoic acid did not improve the immunogenicity of PLA2 (Fig. 2), despite being tested at a wide range of doses (results not shown).

After the last bleeding, mice from selected groups (aluminium hydroxide, PolyI:C, LPS, CpG, and 3M003) received another boost injection of PLA2 and adjuvant. Two weeks later, spleen cells were isolated, and analysed directly for intracellular IFN-γ (Fig. 3) or analysed for IFN-γ, IL-4 and IL-10 secretion after 4 days cultivation with PLA2 (Fig. 4). It was an overall higher percentage of IFN-γ-producing cells

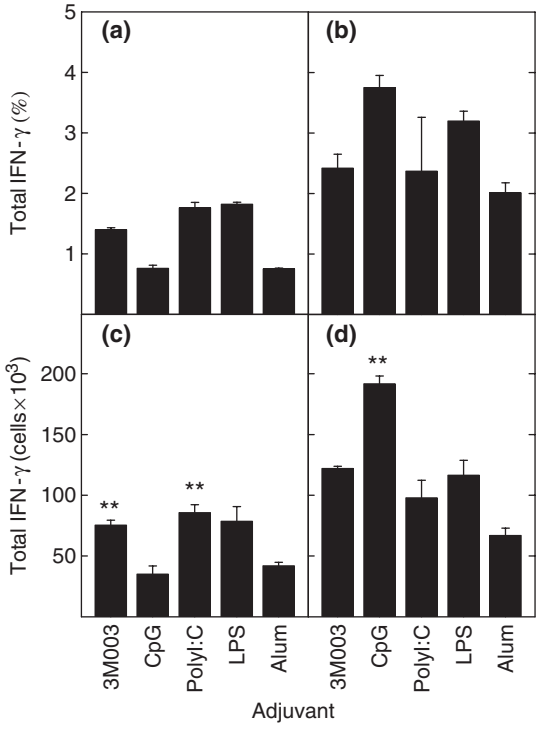

Fig. 3. Intracellular IFN-γ in CD4 and CD8 T cells. The mice described in Fig. 2 were given another boost injection of phospholipase A2 and various Toll-like receptor ligands (3M003, phosphorothioate-modified cytosine–guanosine-rich oligonucleotide, polyriboinosinic polyribocytidylic, and lipopolysaccharide, 10 weeks after the third injection. Splenocytes were isolated 2 weeks thereafter, and intracellular IFN-γ was measured by flow cytometry after 4 h stimulation of cells with PdBu and ionomycin in the presence of brefeldin A. Cells were gated on CD4 (a+c) or CD8 (b+d) positive lymphocytes and the results show the means (± SD) for three mice as percent of T cell subset (a+b) or as absolute numbers (c+d) in the spleen. Statistical differences are indicated when significant difference to the $Al(OH)_3$ control ($P<0.01$). The results show one representative out of two immunization experiments.

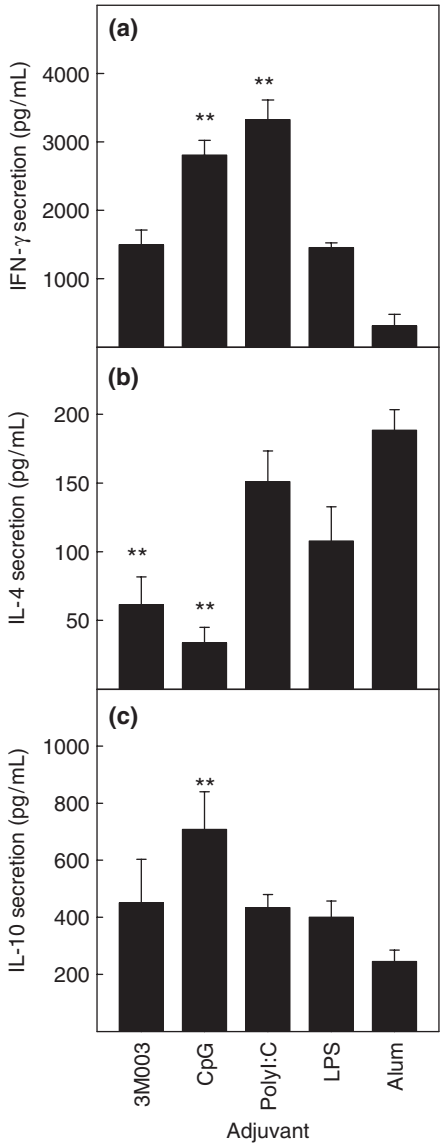

Fig. 4. Secretion of cytokines *in vitro*. Splenocytes from mice described in Fig. 2 were analysed for their secretion of IFN-γ (a), IL-4 (b) and IL-10 (c) after *in vitro* stimulation with 10 μg/m phospholipase A2 for 4 days. The results show cytokine concentrations in supernatants as determined by ELISA after subtraction of spontaneous cytokine secretion, which was determined upon *in vitro* stimulation with an irrelevant antigen (ovalbumin). Statistical differences are indicated when significant difference to the $Al(OH)_3$ control ($P<0.01$). The results show one representative out of two immunization experiments.

deriving from CD8- than from CD4-positive T cells (Fig. 3a and b), with CpG showing the strongest tendency. The same was evident with regard to the absolute number of cytokine-producing cells (Fig. 3c, d). The capacity to produce IFN-γ correlated with the amount of cytokine secreted as measured by ELISA. Highest amounts of IFN-γ were produced by cells from mice immunized using PolyI:C and CpG (Fig. 4a), the concentrations measured being significantly higher than those obtained from mice immunized with LPS or 3M003 ($P<0.01$). Interestingly, whereas marginal anti-PLA2 antibodies could be detected after immunizing mice with PLA2 and 3M003, this vaccine regime apparently generated IFN-γ-producing cells as observed both in FACS (Fig. 3) and in ELISA (Fig. 4). CpG caused significantly reduced IL-4 secretion (Fig. 3b) when compared with 3M003 and PolyI:C ($P<0.01$) or LPS ($P<0.05$), despite triggering the strongest IL-10 production (Fig. 4c).

The combination of TLR ligands and aluminium hydroxide had synergistic effects (Fig. 5). PolyI:C or 3M003 was unable to induce PLA2-specific antibodies after three intralymphatic injections of bee venom extract; note that PolyI:C was a strong adjuvant for the purified PLA2 (Fig. 2). The aluminium-based vaccine-induced strong IgG1 (Fig. 5a) and detectable IgG2a (Fig. 5b) responses. When combining aluminium hydroxide with PolyI:C or 3M003, the IgG1 response increased to the same level as did aluminium hydroxide alone, and more importantly, IgG2a titres increased to levels significantly higher than for the control vaccine. Moreover, the ratio of IgG2a to IgG1 showed that both TLR ligands had immunomodulatory properties by skewing the antibody responses towards a Th1 phenotype (Fig. 5c). Similar results were obtained for combination of PolyI:C or 3M003 with alum and with purified PLA2 (results not shown).

Finally, the immunotherapeutic potential of TLR ligands tested in mice were first sensitized by six weekly intraperitoneal injections of 0.4 μg bee venom extract (Fig. 1b). Four weeks after the last sensitization, the mice were treated with 4 semi-weekly intralymphatic injections of 0.2 μg PLA2. As illustrated, sensitized mice had established titres of IgG1 and IgE prior to the PLA2 treatment (time-point zero in Fig. 6).

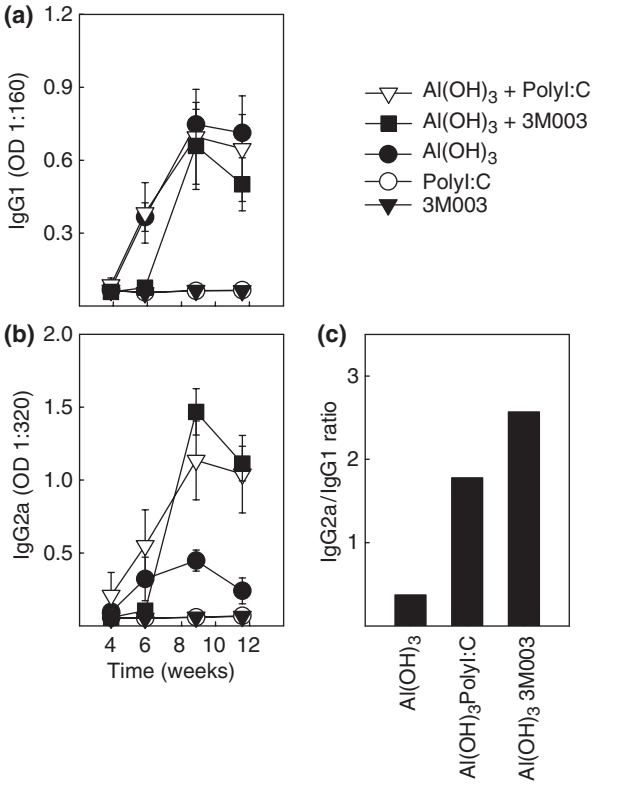

Fig. 5. Toll-like receptor ligands in combination with aluminium hydroxide. Isotype profile of anti-phospholipase A2 (PLA2) serum antibodies in CBA/J mice vaccinated by 3 semi-weekly intralymphatic injections of 0.3 μg bee venom extract in $Al(OH)_3$ (•, polyriboinosinic polyribocytidylic (PolyI:C) (○), 3M003 (▼), as well as in the combinations $Al(OH)_3$ and PolyI:C (▽), or $Al(OH)_3$ and 3M003 (■). Anti-PLA2 IgG1 were analysed by ELISA of 1:160 dilution of sera (a) and IgG2a at the dilution of 1:320 (b) and expressed as means ± standard error ($n=3$). The IgG2a/IgG1 ratios were calculated on sera prepared 12 weeks after initiating the desensitization (c).

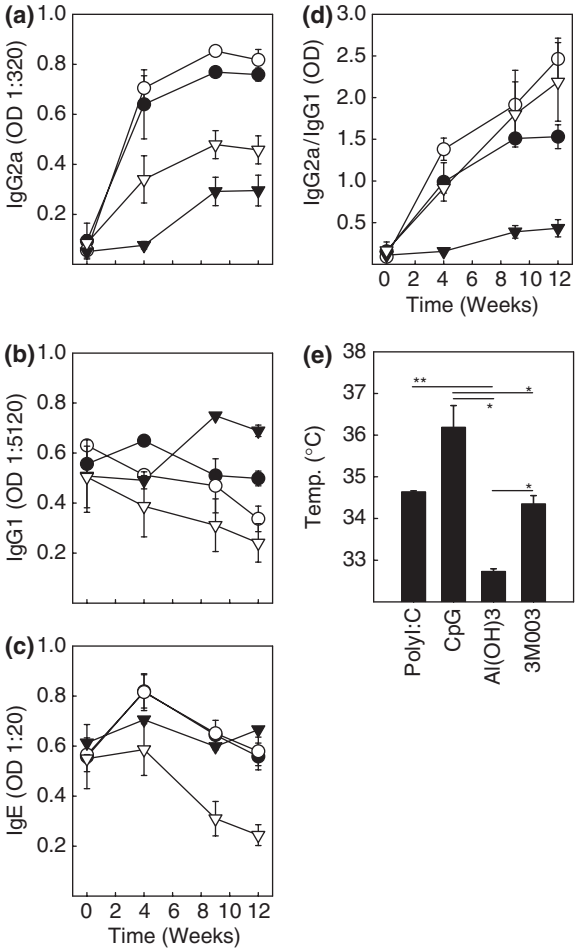

Fig. 6. Toll-like receptor ligands as adjuvants in allergen-specific immunotherapy. Isotype profile of anti-phospholipase A2 (PLA2) serum antibodies in CBA/J mice sensitized six times weekly with 0.4 μg bee venom extract in $Al(OH)_3$ by intraperitoneal injections. Four weeks later (time-point zero in Fig. 6), the mice were therapeutically vaccinated with 0.2 μg PLA2 in $Al(OH)_3$ (▼), polyriboinosinic polyribocytidylic (PolyI:C) (•), phosphorothioate-modified cytosine–guanosine-rich oligonucleotide (CpG) (○), or 3M003 (▽) by intralymphatic injections as described above ($n=4$). This was repeated four times on semi-weekly intervals. The sera were obtained over 12 weeks and analysed by ELISA for anti-PLA2 IgG1 (a), IgG2a (b) and IgE (c). The ratio of IgG2a to IgG1 titres (OD) was calculated as a measure for T-helper type 1 (Th1)/Th2 immune response balance (d). Twelve weeks after the first de-sensitization, mice were challenged with 30 μg PLA2 intraperitoneally and anaphylaxis was measured as temperature drop after 30 min (e). Significant differences are indicated (** $P<0.01$; * $P<0.05$).

After the PLA2 de-sensitization, titres of IgG2a (Fig. 6a) increased extensively when PolyI:C, CpG, and 3M003 were used as adjuvants. Four to twelve weeks after initiating desensitization, the anti-PLA2 IgG2a titres were significantly higher than those raised with aluminium hydroxide as adjuvant ($P<0.05$). Aluminium hydroxide produced a slower onset of IgG2a production than did the TLR ligands, which probably reflects its depot effect, and the titre did not reach those of the TLR ligands. Moreover, de-sensitization with CpG and 3M003 ligands reduced anti-PLA2 IgG1 antibody titres, whereas aluminium hydroxide maintained or even increased this isotype (Fig. 6b). 3M003 also produced a decrease in IgE titres (Fig. 6c). Nine ($P<0.05$) and 12 ($P<0.01$) weeks after the start of the SIT, the titres were significantly reduced. The other adjuvants tested had only little effect on long-term IgE titres. Nonetheless, the overall immune modifying properties of all three are illustrated by the prominent increase in the IgG2a to IgG1 antibody ratio (Fig. 6d). The significance of this Th1 polarization is also underlined by the correlation of this ratio with the protection against allergic anaphylaxis as measured by the drop in temperature after intraperitoneal injection of a high but non-lethal dose of PLA2 (Fig. 6e). While four intralymphatic injections with the aluminium-based vaccine were not sufficient to de-sensitized mice, with regard to the temperature drop upon injection, CpG-treated mice were practically cured after four injections. PolyI:C- as well as 3M003-treated mice showed an improved allergic condition.

Discussion

Current treatment of type-I hypersensitivity is symptomatic with antihistamines, β_2-antagonists and adrenalines. As causative treatment, SIT is effective but a laborious procedure with dozens of injections yearly until the patient tolerates for the given allergen. However, SIT carries the risk of allergic reactions including anaphylaxis, for which safer strategies have been proposed, e.g., the use of recombinant allergens with reduced IgE binding [9–12], as well as pre-treatment with antihistamines [13, 14].

It is though that compounds that can control the over-expression of Th2 cytokine-secreting cells or skew the Th1–Th2 balance towards Th1 would be of clinical advantage [15]. This is why the discovery of TLRs may open new avenues in SIT [16, 17] as well as in the treatment of cancer and infectious diseases [18–20]. We evaluated the capacity of TLR ligands to enhance protection and allergen-specific Th1 immune responses as opposed to the Th2-triggering properties of disease as well as of conventional adjuvants, i.e., salts of aluminium. Our results revealed that especially PolyI:C and CpG, but also 3M003, LPS, and peptidoglycan may have important adjuvant properties with potential application in SIT.

During prophylactic immunization (Figs 2–4) PolyI:C, LPS, CpG, and peptidoglycan adjuvanted strong immune responses against PLA2, comparable or higher than the titres produced by the control vaccine consisting of PLA2 and aluminium hydroxide. The kinetics, magnitude and isotype profile of the Ig response depended on the ligand. LPS was the strongest adjuvant, but CpG, PolyI:C, and peptidoglycan skewed the immune response more strongly towards Th1. Furthermore, CpG and PolyI:C, and to a less extent 3M003, strongly increased IgG2a in bee venom-sensitized mice, and, most interestingly, they had immunomodulatory effect on existing IgE and IgG1 titres in such mice. The functional consequence of this was that CpG-, PolyI:C-, 3M003-treated mice, significantly more than aluminium-treated mice, withstood anaphylaxis provoked by a high-dose of PLA2 (Fig. 6e). Similar properties have been observed for other protein vaccines [21] and been ascribed to increased expression of T-bet [22], which inhibits IgG1 and IgE switching in B cells. The outcome of CpG- and LPS-mediated Ig switching in B cells is different, with CpG and LPS preferentially stimulating IgG2a and IgG1 production, respectively (Fig. 2c). The difference is likely a result of the cytokine milieu produced by the two TLR ligands [23]. CpG produced predominantly IFN-γ, whereas LPS caused less IFN-γ and more IL-4 production (Fig. 3). Indeed, LPS can promote both Th1- and Th2-related Ig production, depending on the cytokine milieu, whereby IFN-γ stimulates IgG2a production and IL-4 stimulates IgG1 and IgE production [24, 25]. By the same token, the poor protection (Fig. 6e) and the IgG1- and IgE-favoured antibody responses produced by aluminium-adjuvanted PLA2 (Fig. 6a–d) may be explained by this adjuvants preferential Th2-cytokine profile with little IFN-γ (Fig. 4a) and much IL-4 (Fig. 4b) production.

It is known that different TLRs have distinct patterns of cellular expression [26], as well as effect on dendritic cells (DCs) and lymphocytes [27]. The TLR4 is expressed on myeloid DCs and monocytes, and is essential for the recognition of LPS from Gram-negative bacteria. In contrast, TLR9 seems to be expressed in the endosomal compartment of plasmacytoid DCs and B cells [26], and may be essential for the recognition intracellular viral and bacterial DNA [28]. Considering the fact that a TLR7 is expressed on both DC subsets [29, 30], and been shown to priming allergen-specific CD4 T cell responses [31], it was surprising that 3M003 did not more strongly stimulate antibody responses against PLA2 and that it only did so in a therapeutic model (Fig. 5) and not in a prophylactic model (Fig. 2) unless combined with aluminium hydroxide (Fig. 6). However, for the induction of neutralizing antibody responses, it is likely that monocytes and B cells are more important than DCs, which are especially potent inducers of cytotoxic responses through CD8 T cells [32]. This is reflected in the results showing that 3M003 induced poor antibody responses in the prophylactic immunization model (Fig. 2), relatively stronger cellular responses in terms cytokine secretion from T cells (Figs 3–4), strong antibody response in the therapeutic model (Fig. 6), and that other TLR7/8 ligands have proven promising for the induction of T cell-mediated immune responses [29, 33, 34]. However, in line with a recent report on the immunomodulatory effect of another TLR 7/8 ligand R-848 [31], 3M003 was capable of ameliorating allergy in sensitized animals (Fig. 5), suggesting that the compound has immunomodulatory properties rather than priming properties—at least with regard to antibody production. This could be associated with the relative expression of TLR7 and 8 on naïve and memory B cells. While TLRs were not or only barely expressed on naïve human B cells from peripheral blood, TLR6, 7, and 9 were highly expressed on memory B cells [35]. Also in murine splenocytes mRNA for TLR7 was up-regulated in memory B cells (paper in preparation). This

might explain why in sensitized mice, the TLR7 ligand 3M003 had a strong immunomodulatory effect without having good priming properties.

For SIT, it would perhaps be of advantage to combine the depot properties of aluminium hydroxide with the Th1-triggering properties of some TLR ligands. Indeed, an additive or synergic effects were observed with the combination of aluminium hydroxide and 3M003 after three intralymphatic immunizations. While 3M003 and PolyI:C together with bee venom extract failed to induce antibody responses, the combination of either TLR ligand with aluminium produced much stronger immune responses than did aluminium hydroxide and the allergen extract alone (Fig. 5). This furthermore suggests an immunomodulatory role of these TLR ligands in vaccination. If the immune response is triggered by another compound, e.g., aluminium salts, the TLR ligand can act complementary in adjusting both the quantity and the quality of the immune response. This was also observed by Vasilakos et al. [36] for the TLR7 ligand R-848 and for CpG, in which the immune response to ovalbumin was strengthened and shifted towards Th1 by combining either TLR ligand with an aluminium salt.

Another reason for the superiority of PolyI:C, CpG, and LPS in our study may be a consequence of their chemical charge. Plurivalent ions, which can neutralize oppositely charged proteins make these proteins more hydrophobic and Th1 inducing [37]. Bee venom PLA2 has an isoelectric point of approximate 8.5 (our observation on an IEF gel), hence, is positively charged at physiological pH. Negatively charged adjuvants such as PolyI:C, CpG, and LPS will therefore form a complex with PLA2, which is likely to trigger Th1-type responses. Such a complex can also be expected to delay the clearance of the antigen and thereby to increase its persistence and immunogenicity [38–40]. Other adjuvants such as the TLR 7/8 ligand 3M003 may not form the required complex or depot, making them less appropriate for induction of strong antibody responses.

Taken together, based on their primary effect on the innate and adaptive immunity, TLR ligands are likely to meet requirements for new vaccines regarding the induction of immunity, or to modify existing vaccines as to tune the type or strength of the induced immunity. Regarding the strength of TLR ligands in improving anti-PLA2 antibody responses, our data suggest that PolyI:C, LPS, and CpG are the most powerful priming adjuvants. On the other hand, if the goal of immunization were to specifically trigger the Th1 arm of the immunity, we show that CpG, PolyI:C, and 3M003 as well as peptidoglycan might be adjuvants of choice for improvement of SIT.

Acknowledgements

We thank Susi Pena-Rodriguez for technical assistance, Martin F. Bachmann for providing flagellin and lipoteichoic acid and 3M Pharmaceuticals for providing 3M003.

References

1 Durham SR, Walker SM, Varga EM et al. Long-term clinical efficacy of grass-pollen immunotherapy. N Engl J Med 1999; 341:468–75.

2 Thoma-Uszynski S, Stenger S, Takeuchi O et al. Induction of direct antimicrobial activity through mammalian Toll-like receptors. Science 2001; 291:1544–7.

3 Akira S. Mammalian Toll-like receptors. Curr Opin Immunol 2003; 15:5–11.

4 O'Neill LA. TLRs: professor Mechnikov, sit on your hat. Trends Immunol 2004; 25:687–93.

5 Bousquet J, Lockey R, Malling HJ et al. Allergen immunotherapy: therapeutic vaccines for allergic diseases. World Health Organization. American Academy of Allergy, World Health Organization. Ann Allergy Asthma Immunol 1998; 81:401–5.

6 Maloy KJ, Erdmann I, Basch V et al. Intralymphatic immunization enhances DNA vaccination. Proc Natl Acad Sci USA 2001; 98:3299–303.

7 Johansen P, Haffner AC, Koch F et al. Direct intralymphatic injection of peptide vaccines enhances immunogenicity. Eur J Immunol 2005; 35:568–74.

8 Gorden KB, Gorski KS, Gibson SJ et al. Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. J Immunol 2005; 174:1259–68.

9 Valenta R, Kraft D. From allergen structure to new forms of allergen-specific immunotherapy. Curr Opin Immunol 2002; 14:718–27.

10 Schmid-Grendelmeier P, Holzmann D, Himly M et al. Native Art v 1 and recombinant Art v 1 are able to induce humoral and T cell-mediated in vitro and in vivo responses in mugwort allergy. J Allergy Clin Immunol 2003; 111:1328–36.

11 Tresch S, Holzmann D, Baumann S et al. In vitro and in vivo allergenicity of recombinant Bet v 1 compared to the reactivity of natural birch pollen extract. Clin Exp Allergy 2003; 33: 1153–8.

12 Ferreira F, Ebner C, Kramer B et al. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. FASEB J 1998; 12: 231–42.

13 Halmerbauer G, Hauk P, Forster J, Urbanek R, Kaufmehl K, Koller DY. In vivo histamine release during the first minutes after deliberate sting challenges correlates with the severity of allergic symptoms. Pediatr Allergy Immunol 1999; 10:53–7.

14 Muller U, Hari Y, Berchtold E. Premedication with antihistamines may enhance efficacy of specific-allergen immunotherapy. J Allergy Clin Immunol 2001; 107:81–6.

15 Chisholm D, Libet L, Hayashi T, Horner AA. Airway peptidoglycan and immunostimulatory DNA exposures have divergent effects on the development of airway allergen hypersensitivities. J Allergy Clin Immunol 2004; 113:448–54.

16 Akdis CA, Kussebi F, Pulendran B et al. Inhibition of T helper 2-type responses, IgE production and eosinophilia by synthetic lipopeptides. Eur J Immunol 2003; 33:2717–26.

17 Rhodes J. Discovery of immunopotentiatory drugs: current and future strategies. Clin Exp Immunol 2002; 130:363–9.

18 Weigel BJ, Rodeberg DA, Krieg AM, Blazar BR. CpG oligodeoxynucleotides potentiate the antitumor effects of chemotherapy or tumor resection in an orthotopic murine model of rhabdomyosarcoma. Clin Cancer Res 2003; 9:3105–14.

19 Jain VV, Kitagaki K, Kline JN. CpG DNA and immunotherapy of allergic airway diseases. Clin Exp Allergy 2003; 33:1330–5.

20 Klinman DM. Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat Rev Immunol 2004; 4:249–58.

21 Chu RS, McCool T, Greenspan NS, Schreiber JR, Harding CV. CpG oligodeoxynucleotides act as adjuvants for pneumococcal polysaccharide-protein conjugate vaccines and enhance antipolysaccharide immunoglobulin G2a (IgG2a) and IgG3 antibodies. Infect Immunol 2000; 68:1450–6.

22 Liu N, Ohnishi N, Ni L, Akira S, Bacon KB. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. Nat Immunol 2003; 4:687–93.

23 Gould MP, Greene JA, Bhoj V, DeVecchio JL, Heinzel FP. Distinct modulatory effects of LPS and CpG on IL-18-dependent IFN-gamma synthesis. J Immunol 2004; 172:1754–62.
24 Snapper CM, Pecanha LM, Levine AD, Mond JJ. IgE class switching is critically dependent upon the nature of the B cell activator, in addition to the presence of IL-4. J Immunol 1991; 147:1163–70.
25 Rothman P, Lutzker S, Cook W, Coffman R, Alt FW. Mitogen plus interleukin 4 induction of C epsilon transcripts in B lymphoid cells. J Exp Med 1988; 168:2385–9.
26 Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, Wagner H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. Eur J Immunol 2002; 32:1958–68.
27 Lore K, Betts MR, Brenchley JM et al. Toll-like receptor ligands modulate dendritic cells to augment cytomegalovirus- and HIV-1-specific T cell responses. J Immunol 2003; 171:4320–8.
28 Hemmi H, Takeuchi O, Kawai T et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408:740–5.
29 Jurk M, Heil F, Vollmer J et al. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol 2002; 3:499.
30 Kadowaki N, Ho S, Antonenko S et al. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. J Exp Med 2001; 194:863–9.
31 Brugnolo F, Sampognaro S, Liotta F et al. The novel synthetic immune response modifier R-848 (Resiquimod) shifts human allergen-specific CD4+ TH2 lymphocytes into IFN-gamma-producing cells. J Allergy Clin Immunol 2003; 111:380–8.
32 Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392:245–52.
33 Hemmi H, Kaisho T, Takeuchi O et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol 2002; 3:196–200.
34 Dummer R, Urosevic M, Kempf W, Kazakov D, Burg G. Imiquimod induces complete clearance of a PUVA-resistant plaque in mycosis fungoides. Dermatology 2003; 207:116–8.
35 Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood 2003; 101:4500–4.
36 Vasilakos JP, Smith RM, Gibson SJ et al. Adjuvant activities of immune response modifier R-848: comparison with CpG ODN. Cell Immunol 2000; 204:64–74.
37 Gupta RK. Aluminum compounds as vaccine adjuvants. Adv Drug Deliv Rev 1998; 32:155–72.
38 Kundig TM, Bachmann MF, Ohashi PS, Pircher H, Hengartner H, Zinkernagel RM. On T cell memory: arguments for antigen dependence. Immunol Rev 1996; 150:63–90.
39 Maruyama M, Lam KP, Rajewsky K. Memory B-cell persistence is independent of persisting immunizing antigen. Nature 2000; 407:636–42.
40 Gray D, Skarvall H. B-cell memory is short-lived in the absence of antigen. Nature 1988; 336:70–3.