

The Impact on Allergy-Related Cells of a Birch Pollen Allergoid, with and without Monophosphoryl Lipid A, in Comparison with the Native Equivalent

Margitta Worm Dennis Ernst Markus Kraller Magda Babina

Department of Dermatology and Allergy, Charité – Universitätsmedizin Berlin, Berlin, Germany

Keywords

Allergoid · Immunomodulation · Toll-like receptor 4 · B cell · Cytokines · Basophil activation test

Abstract

Background: The clinical efficacy and safety of allergoid immunotherapy have been demonstrated in clinical trials. However, simultaneous monitoring of the immunological changes by allergoids versus allergens in the cells of the same individual has not been extensively performed, and the impact of concurrent Toll-like receptor 4 (TLR4) ligation has not been specified. **Methods:** Three types of birch allergen were utilized: glutaraldehyde-treated allergoid (extract A), the same allergoid plus monophosphoryl lipid A (MPL), i.e., TLR4 ligand (extract A*), and native allergen (extract B). Antigen-specific responses after the in vitro stimulation of blood cells with the extracts were assessed by studying co-stimulatory receptors on the B cell surface by flow cytometry, cytokine responses by ELISA, and CD63 and CD203c up-regulation (basophil activation test) in allergic versus nonallergic subjects. **Results:** HLA-DR selectively increased upon allergen or allergoid treatment in the allergic group only. The extract types elicited similar cytokine responses, with IL-6 and IL-10 production detected only in certain atopic

subjects. The allergoids revealed a strong reduction (100- to <10,000-fold) in basophil activation versus native allergen. Reactivity was undetectable in the basophils from nonallergic subjects. **Conclusion:** The allergenicity of the allergoid employed was sharply reduced when compared to the native allergen, while its immunogenicity was largely retained, especially in the presence of MPL. We also provide further evidence that allergic and nonallergic individuals show pre-existing differences in their immune repertoires.

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Introduction

Allergoids, defined as modified allergen proteins, have been elaborated as vaccines for specific immunotherapy in order to improve safety. The modification aims at sustaining the linear epitopes recognized by the major histocompatibility complex (MHC) to keep T cell activation largely intact (and thereby preserve immunoreactivity), while reducing the interaction with IgE, thereby establishing hyporesponsiveness at the level of the effector cells. Glutaraldehyde is commonly used to produce allergoids via the polymerization of native allergens such as extracts from birch or grass [1].

Clinically relevant type I allergy is mediated by a Th2-skewed immune system [2]. To reverse this imbalance, the rationale of specific immunotherapy (SIT) is to modulate the immune response away from Th2 dominance by inducing Th1 and Treg responses [2, 3]. The general SIT protocol may be supplemented by agents capable of fostering Th1-type immunity, such as Toll-like receptor 4 (TLR4) ligands. Such ligands, of which lipopolysaccharide (LPS) is the prototype, are known to possess strong immunostimulatory properties, and have been extensively evaluated as adjuvants for promoting immunity to minimally immunogenic antigens [4]. It is hypothesized that because TLR4-ligation acts as a strong Th1-inducing adjuvant, it strengthens SIT outcomes by favoring Th1 over Th2, thereby curbing any preexisting Th2 antiallergen responses. LPS itself is associated with significant toxicity, but monophosphoryl lipid A (MPL), a modified LPS of *Salmonella minnesota*, can induce similar cytokine profiles to LPS, but is at least 100-fold less toxic [4].

Various clinical studies have proved the efficacy and safety of allergoids as alternatives for allergen-specific SIT protocols [5–8]. Lesser side effects than with native allergens are accompanied by a reduced capacity to induce anaphylactic reactions in animal models [9].

Here, we investigated the impact of a birch-specific allergoid on the sensitization and effector phases of the deregulated immune response encountered in atopic subjects in comparison to their nonallergic counterparts. We report that allergoid and allergen are equipotent at the level of B cell modulation, especially if combined with MPL. Conversely, ex vivo basophil activation test (BAT) assays indicated the profound hyporesponsiveness of the allergoid versus the native allergen, corroborating safety of the preparations examined.

Materials and Methods

Extracts

The allergen derivatives used for the study were as follows: glutaraldehyde-modified birch pollen extract adsorbed to L-tyrosine (A; Pollinex Quattro, Bencard, Vienna, Austria), the same allergoid combined with MPL (A*; Pollinex Quattro), and native birch pollen extract (B; ALK-Abelló, Hamburg, Germany). Extracts were used at equivalent concentrations (50 SU/mL of allergoid is equivalent to 100,000 SQ/mL of native extract).

Study Subjects

Birch pollen-allergic patients were identified based on their history and a positive skin prick test (SPT). Nonallergic subjects (a negative history and negative SPT) served as controls. Patients were not on medication and did not receive SIT while donating blood to the study.

The study was approved by the University ethics committee (No. EA1/155/06), and experiments were conducted according to the Declaration of Helsinki.

Detailed descriptions of the BAT, the stimulation of peripheral blood mononuclear cells (PBMCs), flow cytometry, and ELISA can be found in the online supplement (for all online suppl. material, see www.karger.com/doi/10.1159/000455047).

Results

Enhanced HLA-DR Expression upon Antigen Stimulation

We previously observed phenotypic modulations in the B cells of patients during SIT with tree pollen allergoid in conjunction with MPL versus placebo [10].

Here, we quantified the changes of costimulatory molecules and HLA-DR on B cells upon allergoid treatment of PBMCs ex vivo. The allergoid was used with and without MPL in direct comparison and also contrasted against the native allergen extract. LPS served as a control. As depicted in Figure 1, none of the treatments produced noticeable changes in CD80, CD86, or CD54 on the B cell surface. The lack of effect was independent of whether allergic or nonallergic subjects were examined. Likewise, receptor expression remained unaffected by LPS, with the exception of a slight increase in CD86 (Fig. 1, top right). Conversely, the combination of PMA + ionomycin triggered CD54 expression independently of the allergic state of the individual (not shown).

In contrast to the results for the costimulatory receptors, HLA-DR was upregulated by the extracts in allergic but not (or much less so) in nonallergic individuals (Fig. 1, bottom right). Conversely, LPS led to augmented HLA-DR in both groups. Interestingly, the allergoid-mediated increase in HLA-DR expression was more pronounced in the presence of MPL, suggesting a synergy between allergenic stimulation and TLR4 ligation.

Together, the data imply that the allergoid employed in this study does not compromise the antigen-presenting capacity of the B cells when compared to the native allergen, especially in combination with MPL.

Cytokine Responses

We determined the allergen-driven release of cytokines in PBMCs from allergic and healthy subjects stimulated by the antigen preparations, A, A*, and B, each at several concentrations. Levels of IL-4, IL-6, and IL-10 were measured in supernatants after 24, 48, and 72 h, respectively. IL-4 was below detection throughout (not depicted). In contrast, IL-6 and IL-10 were measurable in

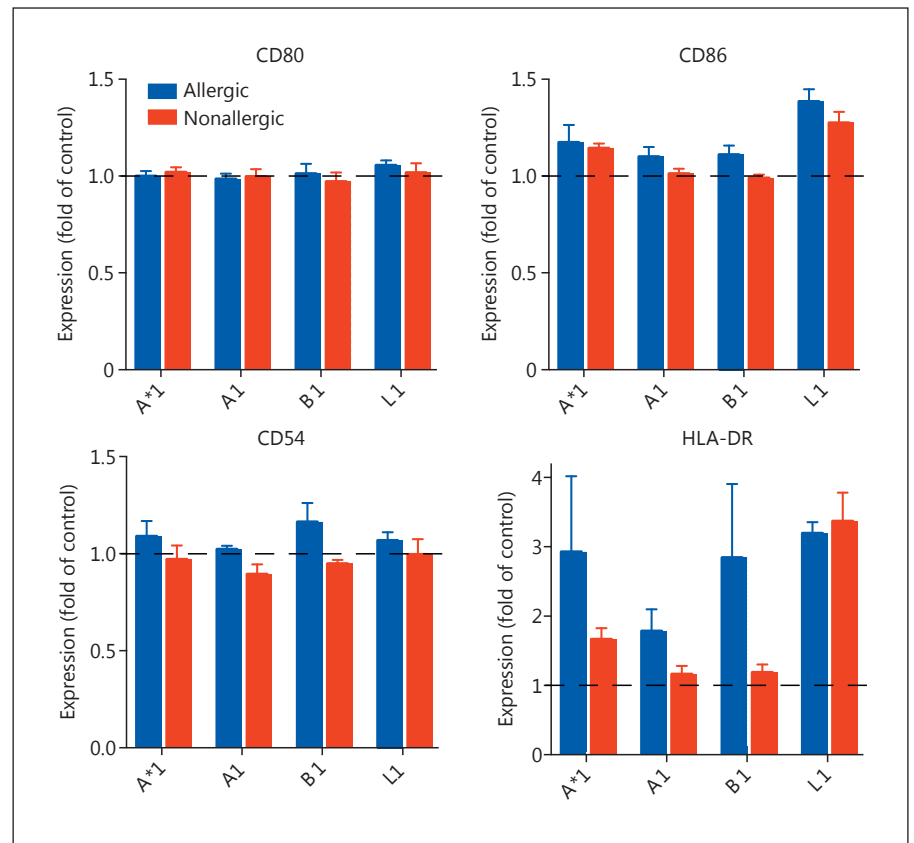


Fig. 1. B cell surface receptors. PBMCs from allergic and nonallergic subjects were treated with A, A*, or B for 48 h, or with LPS as a control, and cell surface receptor expression was determined by flow cytometry. Fold change in mean fluorescence intensity (versus at baseline) was separately calculated for each donor and summarized for the allergic ($n = 4$) and nonallergic groups ($n = 6$), respectively.

distinct settings and in certain donors. The data are summarized in Figure 2.

Responses varied across donors, but in the case of IL-6, PBMCs from allergic individuals displayed more vigorous responses at earlier time points whereas (selected) nonallergic subjects developed a delayed response after stimulation with A or B alone, i.e., without TLR4 ligation. IL-6 responses were potentiated by MPL (A*), and this was comparable between groups. IL-6 elicited by A* therefore possibly resulted from TLR4 ligation by MPL in the first place, as LPS triggered a similar pattern, with no difference between groups. There were different responses for IL-10 and IL-6. In fact, IL-10 production in response to A, B, and even A*, was confined to the allergic group, where 3 of 4 individuals included showed detectable IL-10 under certain conditions. As expected, LPS triggered IL-10 expression in all donors. This appeared largely independent of the allergic state, although there was a slight tendency to increased IL-10 in the nonallergic group. We concluded that while only selected subjects respond with IL-6 or IL-10 expression after stimulation by allergen or allergoid, such responses are generally stronger in allergic subjects.

Basophil Activation

To estimate the degree of (hypo)allergenicity of the allergoids, we used the BAT on 4 subjects with a proven allergy to birch (and a control group of nonallergic individuals). Serial dilutions of the extracts A, A*, and B were employed. Medium only and anti-IgE served as the negative and positive control, respectively. Induction was recorded for both CD63 and CD203c (Fig. 3). In all donors tested, birch pollen allergoids revealed a strong (100- to >10,000-fold) reduction of basophil-activating capacity. There were interesting differences across subjects, however, and donors are therefore depicted individually in Figure 3. Donor 2 showed basically no reactivity at all, while donors 3 and 4 were responsive only to the highest concentration of the allergoid ($\geq 200 \mu\text{g/mL}$). In contrast, donor 1 displayed a more vigorous response. All donors potentially reacted to the native allergen. CD203c tended to give somewhat higher responses with the allergoid, but data for CD63 and CD203c were largely in agreement. Interestingly, a trend to further reduction in the presence of MPL could be observed in those patients who reacted to the allergoid (particularly evident in donors 1 and 3).

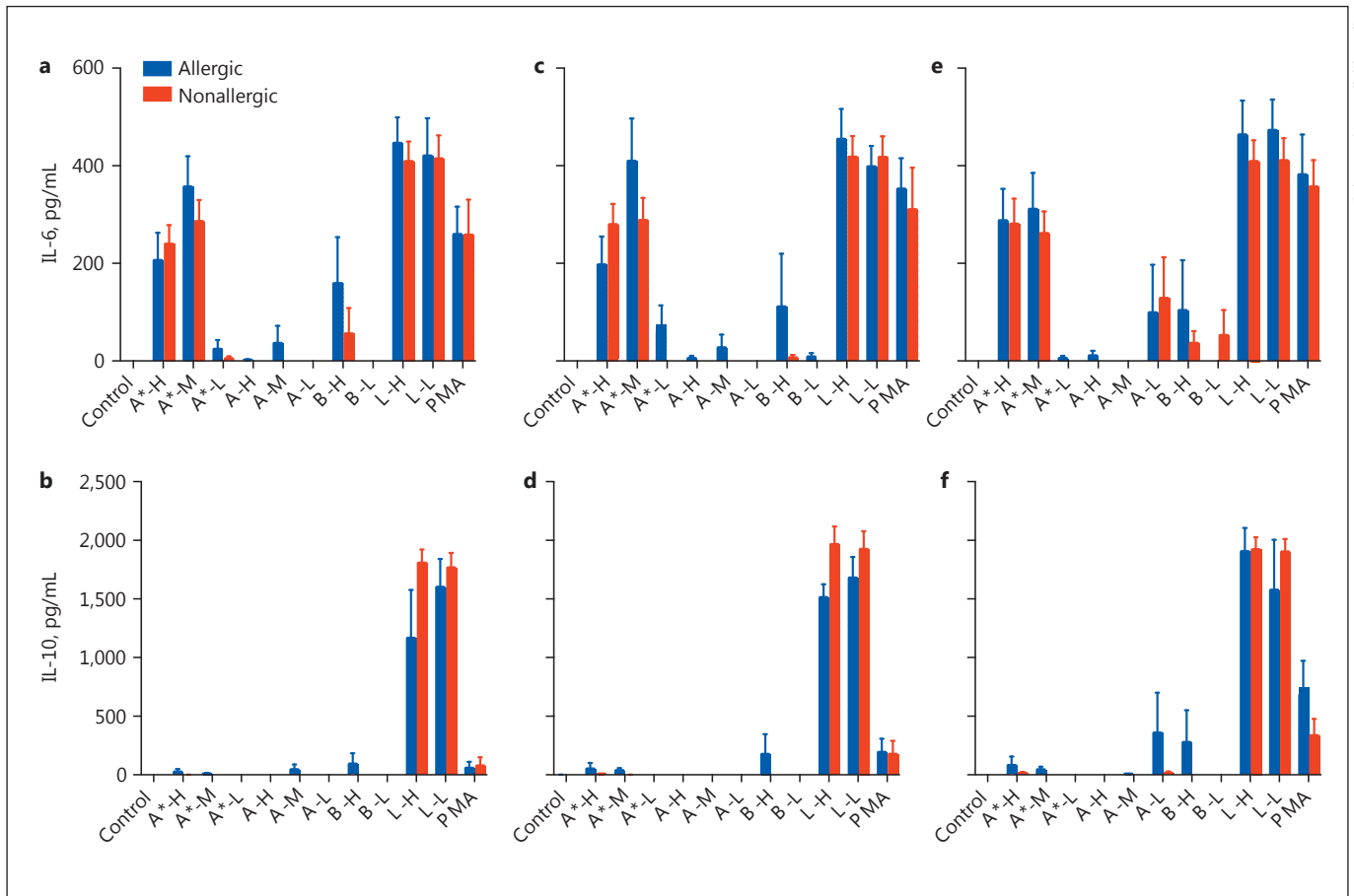


Fig. 2. Cytokine responses induced by allergoid versus allergen in PBMCs from allergic and nonallergic subjects. PBMCs were treated with A*, A, or B (see Methods) at a low (L), medium (M), and high (H) concentration. PMA (20 ng/mL) was used. LPS was used at 1 and 0.1 $\mu\text{g/mL}$. L = 0.5 SU/mL, M = 5 SU/mL, and H = 50 SU/

mL for the allergoid with/without MPL, i.e., A/A*. L = 10,000 SQ/mL and H = 100,000 SQ/mL for the native birch pollen extract, B. Cell-free supernatants were collected after 24 h (a, b), 48 h (c, d), and 72 h (e, f). IL-6 and IL-10 levels were assessed by ELISA. $n = 4$ (allergic) and $n = 6$ (nonallergic).

In the nonallergic group, none of the subjects showed nonspecific activation with any extract, though all subjects reacted with anti-IgE (online suppl. Fig. S1).

Collectively, our results demonstrate a clear-cut reduction in basophil activation by the allergoid when compared to the allergen, underscoring its hypoallergenic character.

Discussion

This study was undertaken to dissect key allergy-related cellular responses to 1 allergoid preparation from birch pollen in direct comparison to its native counterpart. The potential benefit from a Th1 adjuvant was like-

wise addressed by analyzing the allergoid in the presence or absence of MPL. The aim was to uncover the early immune modulation *ex vivo* in patients not undergoing SIT. This strategy allowed for different parallel measurements on cells from the same subjects. Three comparative settings were considered: (i) allergoid versus allergen with respect to allergen-specific immune modulation and effector cell reactivity, (ii) the impact of MPL on allergoid-mediated responses, and (iii) patterns of reactivity in allergic versus nonallergic individuals.

Phenotypic alterations at the B cell and cytokine responses of PBMCs were considered. In the B cells, the costimulatory molecules CD54, CD80, and CD86 were not affected, and unresponsiveness was equivalent for the 2 groups, and was likewise independent of the allergen

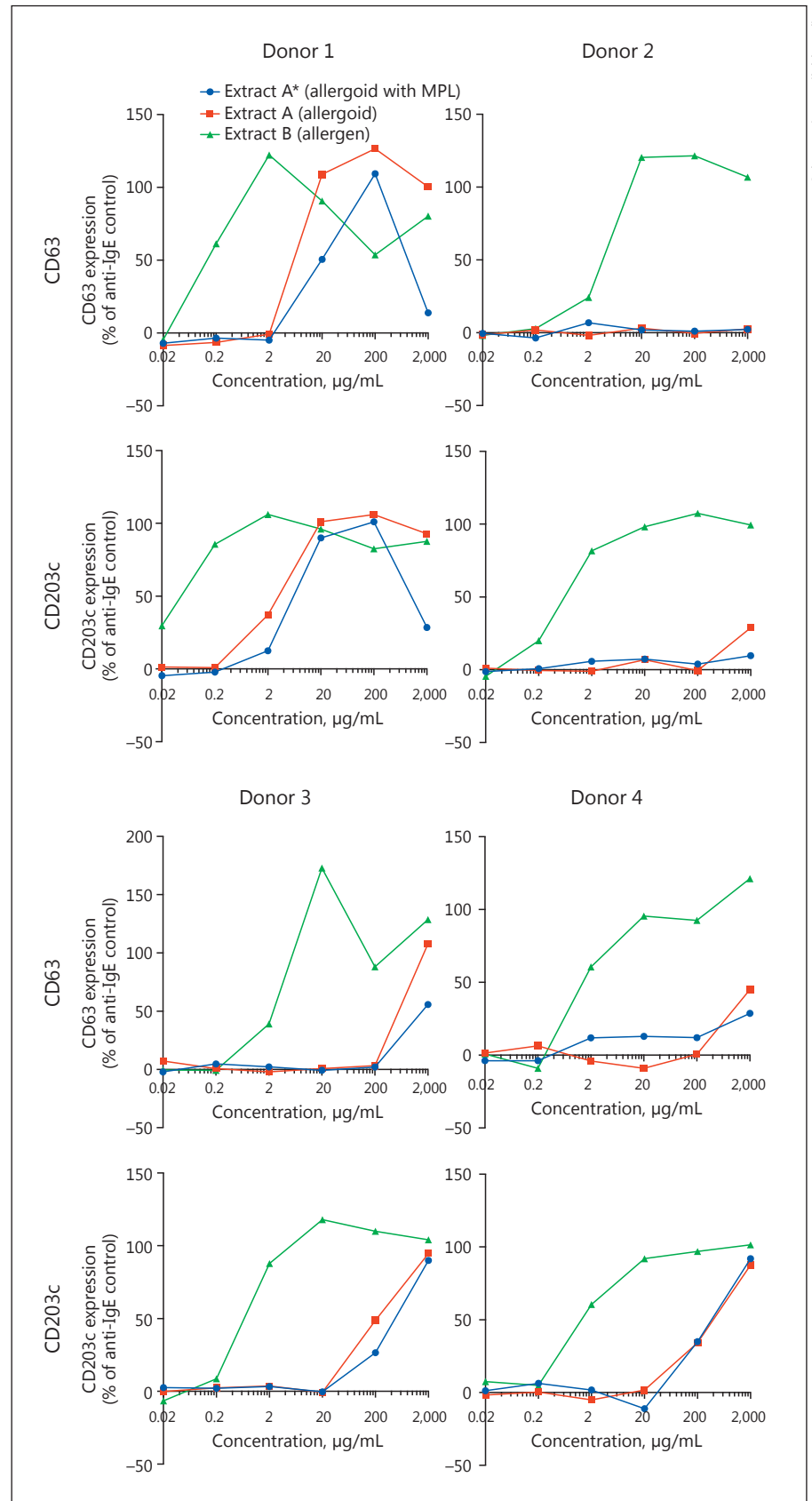


Fig. 3. BAT reveals the hypoallergenicity of the allergoid preparation. The test was performed on heparinized whole blood (details in Methods) section using serial dilutions of allergoid (A or A*) and allergen (B). To only account for the increase in staining resulting from IgER aggregation (either by anti-IgE or allergen extracts), the value obtained for the vehicle control was subtracted. The values were normalized to the response to anti-IgE of the same donor to account for differences in stimulability. Changes in both CD63 and CD203c were considered. Results for 4 allergic subjects are depicted individually. Note that responsiveness to allergoid allergen was reduced 100- to >10,000-fold depending on the donor.

preparation. However, we observed a specific increase in HLA-DR expression, and a difference between allergic and healthy subjects, with only the former developing reactivity. HLA-DR induction on B cells by allergen/allergoid may correct the tendency of atopic individuals to hypoexpress the receptor [11, 12].

The rapid impact of allergoids on *in vitro* cytokine responses outside of ongoing SIT have remained ill-defined, as the common approach is to assess such responses in PBMCs before/after SIT, a strategy that precludes the comparison of different antigen preparations on the same subject. In studies investigating SIT, increases in IL-10 and IFN γ [13, 14] and decreases in IL-4 were described [14]. In our study, the cytokine responses showed substantial variability among the donors, and not all allergic individuals were responsive to either allergen or allergoid in this setting. If viewed in aggregate, however, the allergoid tended to increase IL-6 and IL-10 in a way obviously dependent on the allergy state of the respective individual, especially in the case of IL-10. The induction of IL-10 is notable, because the potency to induce T cell IL-10 responses in the course of SIT is viewed as predictive of outcome [2, 3]. In addition, low IL-10 production early in life has been associated with the development of atopic dermatitis [15]. Our findings of rapid effects at the level of PBMCs are in agreement with previous reports on immunological responses to allergoid-SIT already after a few injections [16], and are likewise compatible with allergen-specific IL-10 responses in B cells [17].

Because B lymphocyte responses to allergens are under the control of T lymphocytes, both phenotypic changes at the B cell surface and cytokine production likely depend on memory T cell responses. This is supported by the reported correlation between allergen-specific IgE responses and T cell proliferation induced by the same allergen, while recall responses to tetanus toxoid display no such correlation [18]. In addition, direct effects on allergen-specific B cells may also contribute to the overall response [17, 19].

As detailed earlier, a major aim of the allergoid concept is to reduce IgE-dependent hypersensitivity. By comparing the potential of the 3 regimens to induce basophil responses in allergic patients directly *ex vivo* without prior SIT, our study firmly established the hypoallergenic nature of the allergoid utilized by comparing 2 independent strategies for assessing basophil activation. In fact, different activation markers can serve to detect allergen-mediated basophil stimulation, with CD63 and CD203c being most common [20, 21]. Employing both strategies in parallel, we documented largely similar outcomes, and

both markers were clearly able to distinguish between allergic and nonallergic subjects. The usefulness of CD203c for the estimation of the hypoallergenic features of allergen preparations has been reported previously [22].

Notably, though hypoallergenicity was consistent, the 4 allergic donors included presented different patterns, ranging from no reactivity at all to a well-developed response (in donor 1). Allergen extracts contain a wide variety of allergenic determinants and the sensitization of allergic subjects can be extremely heterogeneous. The distinct patterns observed probably reflect the differences in IgE entities, recognizing variable epitopes on the allergens and/or binding with different avidity. The hypoallergenicity unearthed here matches early reports contrasting allergen with allergoid in SPT and BAT responses [22, 23]. However, reduced allergenicity (the key rationale behind the allergoid concept) is not always fulfilled. In fact, Lund et al. [24] compared 4 different allergoid preparations with a native allergen, revealing that 1 even displayed greater allergenicity than the unmodified allergen. The potential to induce immediate allergic responses therefore needs to be tested for each allergoid on an individual basis to prove reduced allergenicity in comparison to the native extract.

Taken together, within the limitations of the study, we were able to pinpoint selected immunomodulatory effects for the allergoid, which appeared to be similarly immunogenic to its native counterpart at inducing anti-allergen responses, but also displaying profound hypoallergenicity. The combination with MPL tended to further modify the responses, which warrants further investigation into the mechanism of simultaneous TLR4 ligation.

Acknowledgement

This work was supported by Bencard.

Disclosure Statement

M.W. received a consulting fee from Bencard and has acted as principal investigator in several clinical trials from Bencard.

Author Contributions

M.W. designed the study, D.E. and M.K. performed the experiments and analyzed the data, and M.B. interpreted the data and wrote the paper. M.W. revised the paper. All authors approved the final version of the manuscript.

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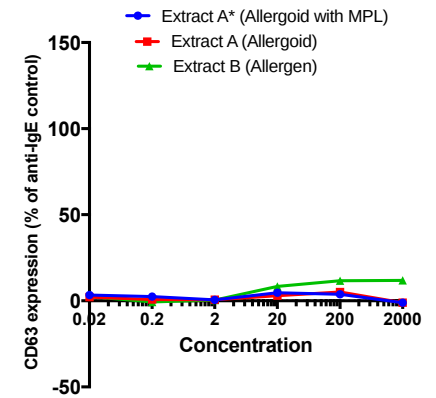
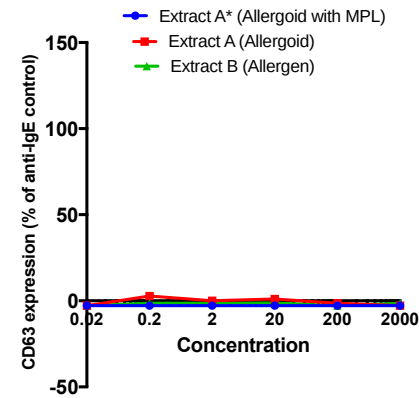
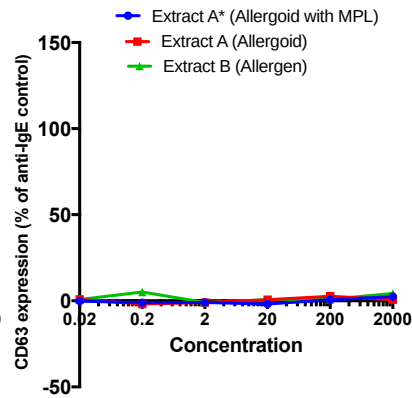
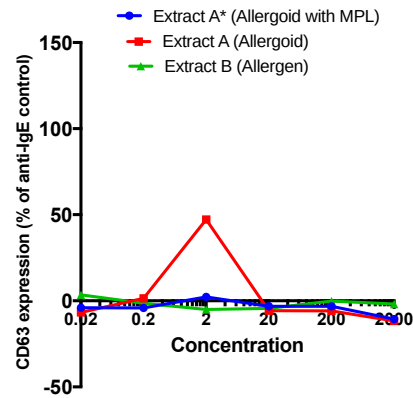
Non-allergic 1

Non-allergic 2

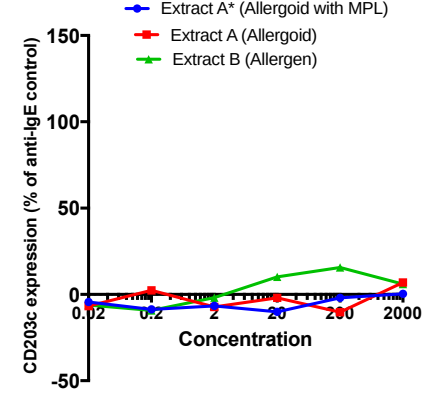
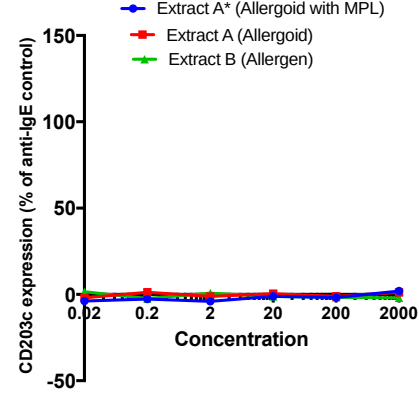
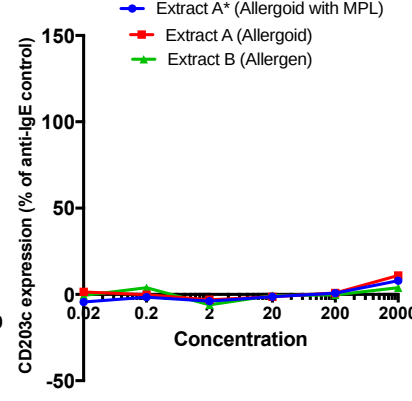
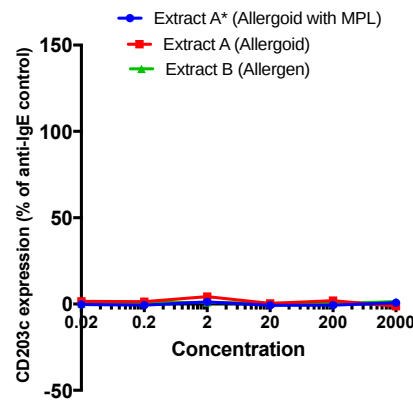
Non-allergic 3

Non-allergic 4

CD63



CD203c



Suppl. Fig. 1

SHORT COMMUNICATION

Online Supplement

Impact of a birch pollen allergoid, with and without monophosphoryl-lipid A, on allergy related cells in comparison with the native equivalent

Running Title: Immunomodulation by allergoid with and without MPL

Margitta Worm, Dennis Ernst, Markus Kraller Magda Babina

Department of Dermatology and Allergy, University Medicine Berlin, Charité Campus Mitte, Germany

Key words: allergoid, immunomodulation, TLR4, B cell, cytokines, basophil activation test

Correspondence: Prof. Dr. Margitta Worm, Department of Dermatology and Allergy, Charité Campus Mitte, Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany

Phone No. 49-30-450518105, Fax No. 49-30-450518931, email: margitta.worm@charite.de

SUPPLEMENTARY METHODS

Basophil activation test

Heparinized whole blood samples from birch pollen allergic patients and non-allergic control subjects were stimulated with the three classes of extracts defined above at tenfold serial dilutions (2000; 200; 20; 2; 0,2; 0,02 µg/ml) for 15 minutes in RPMI 1640 medium (#FG1215) supplemented with Penicillin and Streptomycin (#A2213) and 10% FCS (#SO113), all from BioChrom, Berlin, Germany. Medium alone served as negative and anti-human IgE (mix of HP6029 #9250-01 and HP6061 #9240-01, both from SouthernBiotech/Biozol, Eching, Germany) instead of allergen as positive control. After stimulation erythrocytes were lysed by adding FACS Lysing Solution (#349202, BD Biosciences, Heidelberg, Germany).

Following fixation, cells were stained with specific antibodies and analyzed on a MacsQuant Flow-cytometer (Miltenyi Biotec, Bergisch Gladbach). Basophils were identified as CD3-negative (#130-094-363 Miltenyi Biotec) and CCR3-positive (#FAB155A, R&D Systems, Wiesbaden, Germany) cells. Basophil activation was determined by measuring expression of CD203c (#IM3575 from Beckman-Coulter, Krefeld, Germany) and CD63 (#557288 from BD Biosciences). Non-specific binding of antibodies was blocked by prior addition of Beriglobin (#17940321T, CSL Behring, Marburg, Germany). Net stimulation was determined by subtracting baseline positivity, and the values obtained for extract-triggered stimulation were normalized to the net positive control (mix of two anti-IgE Abs, see above) to account for differences in basophil stimulability across individual subjects.

PBMC stimulation

PBMCs were isolated by Bicolll (#BCR.L6115, BioChrom) and stimulated for 24, 48 or 72 hours in RPMI 1640 medium/10% FCS with different concentrations (50, 5 or 0.5 µg/ml) of A, A* or the comparator B (at 100000 and 10000 SQ/ml). LPS (at 1 µg/ml and 0,1µg/ml, #L2880, Sigma Aldrich, USA) and PMA (at 20 ng/ml, #P8139, Sigma Aldrich) alone or in combination with Ionomycin calcium salt (at 2×10^{-7} mol/L, #I0634, Sigma Aldrich) served as control.

Flow-cytometry

Cell surface receptors were quantified 48 h post stimulation of PBMC by flow-cytometry. B cells were identified as CD19-positive cells (#130-098-064, Milteny Biotec). Antibodies against (co-)stimulatory receptors were as follows: anti-CD54 (#555511), anti-CD80 (#340294), anti-HLA-DR (#347402), all from BD Biosciences, and anti-CD86 (#130-094-876, Miltenyi Biotec). Non-specific binding of antibodies was blocked by prior addition of Beriglobin, as above.

ELISA

Cytokine levels were measured in PBMC supernatants by ELISA kits according to the manufacturer's recommendations (IL-6 #DY206, IL-4 #Dy204, IL-10#DY217B, all from R&D Systems).

SUPPLEMENTARY FIGURE LEGEND

Suppl. Figure 1

No responsiveness of basophils from non-allergic subject to either allergen or allergoid

The basophil activation test was performed as described in Fig. 3 with the only difference that whole blood from non-allergic subjects was used.