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Mammal-derived respiratory lipocalin allergens do not exhibit dendritic cell-activating capacity

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Abstract

Most mammal-derived respiratory allergens belong to the lipocalin family of proteins. Determinants of their allergenic capacity are still unknown. Innate immune cells, in particular dendritic cells, have been shown to be involved in the allergenicity of some proteins. As recognition by dendritic cells is one of

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the few plausible mechanisms for the allergenicity of proteins, we wanted to investigate their role in the allergenicity of lipocalin allergens. **Therefore**, we first incubated human monocyte-derived dendritic cells with immunologically functional recombinant allergens mouse Mus m 1, dog Can f 1 and 2, cow Bos d 2, horse Equ c 1 and natural Bos d 2. Then, the surface marker expression and cytokine production of dendritic cells and their capacity to promote T cell proliferation and Th2 immune deviation in naïve CD4⁺ T cells was examined *in vitro*. We found that **near to** endotoxin-free lipocalin allergens had no effect on the activation, allostimulatory capacity or cytokine production of dendritic cells. The dendritic cells could not induce immune deviation in naïve CD4⁺ T cells. In contrast, lipopolysaccharide activated the dendritic cells efficiently. However, lipocalin allergens were not able to modify the lipopolysaccharide-induced responses. We conclude that an important group of mammal-derived respiratory allergens, lipocalins, appear not to be able to activate dendritic cells, a major component involved in the allergenicity of some proteins. It is conceivable that this incapacity of lipocalin allergens to arouse innate immunity **may be** associated with their poor capacity to induce a strong T cell response, verified in several studies.

Introduction

One of the fundamental questions in allergological research is why a relatively small number of basically harmless environmental proteins are widely allergenic, that is, **capable of inducing** T-helper type 2 (Th2) immunity in atopic individuals with diverse human leukocyte antigen (HLA) backgrounds. Although it would be advantageous to know the basis of this property, for example, for the rational development of specific allergen immunotherapy, it has remained undiscovered for most allergens. **This includes** the important animal-derived respiratory allergens of the lipocalin family [1, 2].

The lipocalin protein family contains about 20 allergens, most of them of mammalian origin [1, 2]. Several of these allergens have been reported **to**

sensitize a large proportion of genetically susceptible individuals exposed to the allergen source. For example, almost 80 % of horse dust-sensitized subjects had IgE to natural[3] or recombinant[4]Equ c 1, 80-90 % of cow dust-sensitized subjects had IgE to natural [5]or recombinant[4]Bos d 2, and near to 70 % of mouse dust-sensitized subjects had IgE to recombinant Mus m 1 [4].Despite this, *in vitro* T cell responses to these allergens have consistently been observed to be weak even in highly sensitized individuals[6-13].

Recent research has highlighted the importance of innate immunity in the allergenicity of some proteins. For example, serine and cysteine proteases are reported to activate multiple cell types of the innate immunity through protease-activated receptors [14]. Basophil leukocytes, able to produce Th2-type cytokines, may be activated directly, without IgE crosslinking, by protease allergens [15]. Importantly, dendritic cells (DCs), the pivotal antigen-presenting cells, have been observed to be involved in many allergenic processes.For example, peanut Ara h 1,**containing a nonmammalian glycan, was**recognizedby dendritic cell-specific ICAM-grabbing nonintegrin(DC-SIGN) onDCs.**Even though the capacity of the allergen to stimulate DCs was inconsistent, the recognition**activated human monocyte-derived DCs from normal donors to prime naïve allogeneic T cells for the Th2-biased phenotype, [16]. A study in a mouse model on house dust mite-driven allergic airway inflammation showed that toll-like receptor (TLR) 4-expressing lung structural cells (probable epithelial cells) together with DCs favored the Th2 differentiation [17]. Weak DC-derived production of interleukin (IL)-12, an important Th1-deviating cytokine, upon exposure to low levels of lipopolysaccharide (LPS), has also been found to promote Th2-type immunity [18]. Other adjuvant-like substances, such as E1-phytosteranes and possibly other Th2-deviating lipid mediators, contained in pollen grains, directly inhibited the IL-12 production by monocyte-derived DCs from nonatopic subjects[19, 20] and augmented Th2 polarization of naïve allogeneic CD4⁺ T cells [19].

As scientific literature suggests that the modulationof DC function through the receptors of innate immunity determines the allergenicity of some proteins, we

sought to address the impact of mammalian lipocalin allergens (mouse Mus m 1, dog Can f 1 and Can f 2, cow Bos d 2, and horse Equ c 1[1, 2]) on the function and phenotype of human DCs. To that end, we monitored the capacity of pure lipocalin allergens to activate monocyte-derived DCs and the capacity of these DCs to stimulate and polarize naïve allogeneic CD4⁺ T cells *in vitro*. Our results suggest that lipocalin allergens are inert with respect to DCs.

Materials and Methods

Subjects and antigens

Peripheral-blood mononuclear cells (PBMCs) from four healthy nonallergic volunteers were separated by Ficoll-Paque Plus density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). The antigens used in this study were recombinant (r)mouse Mus m 1, dog Can f 1 and Can f 2, cow Bos d 2 and horse Equ c 1. They were produced in *Pichia pastoris*, as we have previously reported [4]. Natural (n) Bos d 2 was purified from bovine dander, as we have described before [21]. **Residual LPS contamination was removed from the allergen preparations by Detoxi-Gel Endotoxin Removing Columns (Thermo Fisher Scientific, Rockford, IL). All the allergen preparations were determined to be near to endotoxin-free (<0.001 EU/μg) by the Limulus assay (Lonza, Walkersville, MD).** Lipopolysaccharide (LPS) was provided by Sigma-Aldrich. The study was approved by the Ethics Committee of the Kuopio University Hospital.

Generation and activation of monocyte-derived dendritic cells

Monocytes were separated from PBMCs using CD14-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. Immature dendritic cells (iDCs) were obtained by culturing the freshly isolated monocytes for 6 days in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine, 20 μM 2-mercaptoethanol, sodium pyruvate, non-essential amino acids, 100

IU/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES (BioWhittaker) and 10% heat inactivated fetal bovine serum (BioWhittaker) together with granulocyte-macrophage colony-stimulating factor (GM-CSF) at 500 IU/ml and interleukin (IL)-4 at 400 IU/ml (MilttenyiBiotec) in 6-well plates (Corning Inc, Corning, NY). On day 3, fresh medium and cytokines were added to the plates. On day 6, cells were detached and harvested, **and assessed by FACS to be >90 % pure iDCs (CD14⁺HLA-DR⁺CD40^{low}CD80^{low}CD86^{low}CD83⁻; Fig. 1A)**. For maturing iDC, 5 x 10⁵ cells were stimulated with the allergens (100 µg/ml) in the presence or absence of LPS (100 ng/ml), or LPS alone, for 24 h in the culture medium in 48-well plates (Corning). On day 7, cell culture supernatants (0.5 ml/well) were collected and the production of IL-1β, IL-6, IL-10, IL-12p70 and tumor necrosis factor (TNF)-α was measured by the BioPlex system (Bio-Rad Laboratories, Hercules, California, USA), according to the manufacturer's protocol. On day 7, cells were also harvested and aliquots of the cells were stained with anti-CD14-PE, anti-CD86-FITC, anti-CD83-APC, anti-CD80-PE, anti-HLA-DR-FITC and anti-CD40-APC (BD Biosciences, San Jose, CA) and then analyzed by flow cytometry (BD FACSCanto II).

Isolation of naïve CD45RA⁺ cells

Naïve CD45RA⁺ CD4⁺ T cells were isolated from PBMCs with the "no touch" naïve CD4⁺ T cell isolation kit (MilttenyiBiotec). The mean purity of the cells was 93.7% (range 91.0–97.6%), as assessed by staining with anti-CD4-FITC, anti-CD45RA-APC and anti-CD45RO-PE-Cy7 (BD Biosciences).

Proliferation assays

For the measurement of allostimulatory capacity of DCs on naïve CD4⁺ T cells, iDCs (0.156-10 x 10³ cells per well) were first subjected to a prior treatment with the allergens (100 µg/ml) in the presence or absence of LPS (100 ng/ml) or with LPS alone for 24 h. Next, the cells were cultured with 5 x 10⁴ CD4⁺CD45RA⁺ allogeneic T cells in triplicate in 96-well round-bottomed plates (Corning). After 3 days, ³H-thymidine was added (1 µCi per well; GE Healthcare, Little Chalfont, UK), and after additional 16 h, the cells were harvested onto glass fiber filters (Wallac, Turku, Finland). Radioactivity was

measured by scintillation counting (Wallac Micro Beta 1450) and the results expressed as counts per minute (cpm).

DC-T cell coculture experiments for Th cell differentiation

The Th1/Th2 skewing capacity of DCs was assessed in coculture experiments with naïve allogeneic CD4⁺ T cells. After a prior 24 h treatment of iDCs with the allergens (100 µg/ml), allergens plus LPS (100 ng/ml) or medium only, 5 x 10⁴ DCs were cocultured with 2 x 10⁵ allogeneic CD4⁺CD45RA⁺ T cells in 96-well plates. As a Th2 skewing control, LPS-matured DCs treated with IL-4 (100 IU/ml) and anti-IL-12 (10 µg/ml; BD Pharmingen) were used. After five days and every 2 to 3 days thereafter, fresh medium and IL-2 (10 IU/ml) were added to the cultures and they were expanded if necessary. After 14 days, the cells were collected for the analysis of intracellular IL-4 and IFN-γ. First, the T cells were stimulated with PMA (50 ng/ml) plus ionomycin (1 µg/ml; Sigma-Aldrich) for 6 h. Brefeldin A (Golgiplug, BD Biosciences) was added for the final 2 h. Then, the cells were fixed and permeabilized (Cytofix/Cytoperm Kit, BD Biosciences), according to the manufacturer's protocol. After staining with anti-human IL-4 PE and IFN-γ APC (BD Pharmingen), accumulation of IL-4 and IFN-γ was measured by flow cytometry (BD FACSCanto II).

Results

First, we cultivated immature monocyte-derived DCs from healthy individuals in medium containing **near to** endotoxin-free recombinant lipocalin allergens, mouse Mus m 1, dog Can f 1 and 2, cow Bos d 2 or horse Equ c 1 or natural lipocalin allergen Bos d 2, in the presence or absence of LPS, or LPS alone, for 24 h, adopting an approach found functional by other investigators [16, 19, 20]. We discovered that the surface markers CD40, CD80, CD83, CD86 and HLA-DR on DCs were not upregulated in the cultures without extra LPS (Fig. **1A and B**, and data not shown). However, when LPS was included in the cultures, DCs exhibited strong upregulation of the surface markers (Fig. **1A and C**, and data not shown). Importantly, the level of expression did not differ

from that induced by LPS alone(**Fig. 1C**), and **varying the LPS concentration (1-100 ng/ml) did not change the outcome (Fig. 1D).**

The capacity of lipocalin allergens to modulate the activity of **monocyte-derived**DCs was further examined by allostimulatorycoculture experiments. Purified naïve CD4⁺ T cells were cultured with increasing numbers (0.156-10 x 10³) of DCs which were subjected to a prior treatment with the lipocalin allergens or medium alone. Proliferation of the naïve T cells was dose-dependent on the number of DCs and it did not differ from the proliferation observed upon stimulation with medium-treated DCs (Fig. 2A). Moreover, T cells cultured in the presence of DCs with a prior treatment with lipocalin allergens plus LPS proliferated as well as the cells cultured in the presence of DCs matured with LPS only (Fig. 2B).

Next, we wanted to define the profiles of cytokines secreted by the **monocyte-derived**DCs by measuring IL-1 β , IL-6, IL-10, IL-12p70 and TNF- α in cell culture supernatants. Immature DCs cultured for 24 h with the allergens or with medium only released either none or low levels of the cytokines (Fig. 3 and data not shown). In contrast, stimulation with LPS induced a strong production of all the cytokines but, importantly, allergens had no modulatory effect on the LPS-induced cytokine synthesis (Fig. 3 and data not shown).

Finally, we examined whether lipocalin allergens can modulate **monocyte-derived**DCs to prime naïve T cells for the Th2 phenotype. We found that naïve allogeneic CD4⁺ T cells primed by LPS-matured DCs exhibited a tendency for the Th1-skewed phenotype (Fig. 4A and 4B). DCs activated by LPS in the presence of the lipocalin allergens exhibited the similar phenotype (Fig. 4B). A clear Th2 bias was seen with T cells that were primed with LPS-matured DCs in the presence of IL-4 and neutralizing anti-IL-12 mAb (Fig. 4A and 4B). Importantly, T cells primed by DCs exposed to the allergens alone did not show a bias for a polarized phenotype (Fig. 4B).

Discussion

The allergenic capacity of proteins has remained elusive up to recent times. This probably results from the fact that the process of sensitization was incompletely understood until the Th1/Th2 hypothesis was presented and later established. Therefore, it is now clear that the rational basis for searching the allergenic determinants of proteins is to discover factors which promote their Th2-inducing capacity.

Several cell populations, including CD4⁺ T cells and APCs, such as DCs, are involved in shaping a favorable cytokine microenvironment for sensitization. For the Th2 immunity, IL-4, produced by several cell types, including Th cells, is crucial [22]. Its effects are, however, counterregulated by several other cytokines, including IL-12 which is a potent Th1-deviating cytokine produced by DCs [18, 22]. Therefore, factors which attenuate the activation of DCs and thus the production of IL-12 are likely to promote the Th2 immunity.

As found functional in several studies [16, 19, 20], we adopted an approach involving monocyte-derived DCs and naïve allogeneic CD4⁺ T cells from nonatopic individuals to examine the DC-activating and Th2-deviating capacity of allergenic lipocalin proteins *in vitro*. Although we have previously shown that our natural and recombinant lipocalin allergens are stimulatory to the CD4⁺ T cells of subjects sensitized to them [6, 13, 23, 24], these allergens had no effect on the surface marker expression or cytokine production of immature monocyte-derived DCs, the capacity of the DCs to induce T cell proliferation or to promote Th2 deviation in naïve CD4⁺ T cells. They were neither able to modify the response induced by LPS. Therefore, one important component of the innate immunity, DCs, **reported** to be involved in the allergenicity of some proteins, for example peanut Ara h 1 or mite Der p 1, **did not play an apparent role here with lipocalin allergens. Interestingly, the DC-activating capacity of pure Ara h 1 [16] or Der p 1 [25] were also reported to be inconsistent or nonexistent, which may point to other mechanisms**

involved, such as Erk 1/2 phosphorylation [16]. In all, a common property to all these allergens may be their weak capacity to activate monocyte-derived DCs *in vitro*. Our results do not tell, however, whether other types of DCs, including plasmacytoid DCs [26], could have produced a different outcome in corresponding experiments.

Our results do not **either** rule out the possibility that other arms of the innate immunity could be involved in the allergenicity of lipocalin allergens. At least epithelial cells, basophils and a new subset of non-B non-T cells are also known to participate in generating a Th2-skewing microenvironment [22]. However, apart from a few cysteine protease allergens [14, 15] and mite Der p 2 (an analog of a protein required for LPS to bind to TLR4) [27], no allergen molecule has been reported to directly promote Th2 immunity by affecting other cells of the innate immunity than DCs [22]. Instead, their function may be modified by adjuvant-like substances associated with allergens, as mentioned in Introduction. Therefore, one area worth investigating can be the small hydrophobic ligands the lipocalins are known to carry [1], although our results here with natural Bos d 2 do not support the idea. As to glycan moieties, they are unlikely to be implicated in the allergenicity of lipocalins because lipocalin allergens exist both glycosylated and nonglycosylated [1, 2].

Taken together, our results highlight the notion that diverse mechanisms are likely to be involved in the allergenicity of proteins. For **some allergens, such as mammalian** lipocalin allergens, their incapacity to arouse innate immunity by activating DCs may be a factor contributing to their allergenicity, as in the absence of cytokines from DCs promoting Th1 or Th17 development other players in the immune response, such as T cell recognition, may be decisive for the outcome [26]. Thus, weak activation of the innate immunity, shown here, associated with the weak T cell recognition of mammalian lipocalin allergens, verified in several studies [6-13], can be a combination promoting **specific** Th2 immunity [2]; it is well known that weak T cell receptor (TCR) signal strength favors the differentiation of naïve T cells to the Th2 phenotype [22]. **In genetically susceptible individuals, however,**

several other factors, such as favorable cytokine environment at epithelial barriers, is most probably also involved in generating Th2 immunity to allergenic proteins [22].

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Figure legends

Figure 1. **Lipocalin allergens do not activate DCs.** Immature monocyte-derived DCs were incubated for 24 h together with the allergens (100 µg/ml) without or with LPS. **The expression of CD14, CD83, CD40, CD80, CD86 and HLA-DR on LPS-stimulated DCs (solid gray) and unstimulated DCs (bold line) compared to an isotype control (dashed line) (A).** The change

in median fluorescence intensity (MFI) of CD40, CD83 and CD86 relative to unstimulated DCs upon stimulation with the allergens (B), allergens together with 100 ng/ml LPS (C) and LPS (1-100 ng/ml) with and without nBos d 2 (D). Results represent the mean $\pm$ SEM of four independent experiments with DCs from four separate donors.

Figure 2. Lipocalin allergens do not affect the allostimulatory capacity of DCs. Immature monocyte-derived DCs were incubated together with allergens (100 μ g/ml) without (A) or with LPS (B). After 24 h, DCs were analyzed for their capacity to induce T cell proliferation on naïve allogeneic CD4⁺ T cells. Results are representative of four **independent experiments with DCs from four separate donors.**

Figure 3. Lipocalin allergens do not affect the cytokine production of DCs. Immature monocyte-derived DCs were incubated for 24 h together with the allergens (100 μ g/ml) $\pm$ LPS. The concentrations of secreted IL-1 β (A), IL-10 (B) and IL-12p70 (C) were measured in the supernatants. Results represent the mean $\pm$ SEM of **four independent experiments with DCs from four separate donors.**

Figure 4. Lipocalin allergens do not affect the Th1/Th2-polarizing capacity of DCs. Immature monocyte-derived DCs were incubated together with the allergens (100 μ g/ml) $\pm$ LPS. After 24 h, DCs were washed and cocultured with naïve allogeneic CD4⁺ T cells and IL-2 for 14 days. T cell polarization was determined by analyzing intracellular IFN- γ and IL-4 production by flow cytometry after restimulation with PMA and ionomycin in the presence of brefeldin A. A representative staining of T cells cocultured with immature DCs, LPS-stimulated DCs or LPS-stimulated DCs together with IL-4 and anti-IL-12 (Th2-polarizing control) is shown in (A). Results are expressed as a ratio of IFN- γ /IL-4 relative to unstimulated DCs (B), and represent the mean $\pm$ SEM of four **independent experiments with DCs from four separate donors.**







