

REVIEW

Mammalian lipocalin allergens – insights into their enigmatic allergenicity

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Summary

Most of the important mammal-derived respiratory allergens, as well as a milk allergen and a few insect allergens, belong to the lipocalin protein family. As mammalian lipocalin allergens are found in dander, saliva and urine, they disperse effectively and are widely present in the indoor environments. Initially, lipocalins were characterized as transport proteins for small, principally hydrophobic molecules, but now they are known to be involved in many other biological functions. Although the amino acid identity between lipocalins is generally at the level of 20–30%, it can be considerably higher. Lipocalin allergens do not exhibit any known physicochemical, functional or structural property that would account for their allergenicity, that is, the capacity to induce T-helper type 2 immunity against them. A distinctive feature of mammalian lipocalin allergens is their poor capacity to stimulate the cellular arm of the human or murine immune system. Nevertheless, they induce IgE production in a large proportion of atopic individuals exposed to the allergen source. The poor capacity of mammalian lipocalin allergens to stimulate the cellular immune system does not appear to result from the function of regulatory T cells. Instead, the T cell epitopes of mammalian lipocalin allergens are few and those examined have proved to be suboptimal. Moreover, the frequency of mammalian lipocalin allergen-specific CD4⁺ T cells is very low in the peripheral blood. Importantly, recent research suggests that the lipocalin allergen-specific T cell repertoires differ considerably between allergic and healthy subjects. These observations are compatible with our hypothesis that the way CD4⁺ T-helper cells recognize the epitopes of mammalian lipocalin allergens may be implicated in their allergenicity. Indeed, as several lipocalins exhibit homologies of 40–60% over species, mammalian lipocalin allergens may be immunologically at the borderline of self and non-self, which would not allow a strong anti-allergic immune response against them.

Keywords allergen, allergenicity, animal allergen, lipocalins, mammal

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Allergy and allergenic proteins

Allergy is an immunological disorder in which the erroneous function of the immune system is directed to allergens, those ubiquitous, basically harmless environmental substances. It is still largely unanswered why the immune system of atopic individuals with diverse major histocompatibility complex (MHC) backgrounds recognizes only a limited number of them as allergens, but ignores all the numerous other substances in the environment [1–3]. Yet, this erroneous recognition results in allergic sensitization, that is, the development of T-helper type 2 (Th2) immunity and IgE production [4].

Lipocalins and lipocalin allergens

Lipocalin allergens

From the allergological point of view, lipocalins represent a significant protein group as almost all important mammalian respiratory allergens belong to this family (Table 1). Several of them are potent allergens sensitizing a large proportion of subjects sensitized to the allergen source (Table 1). In addition to these allergens, a milk allergen Bos d 5 (β-lactoglobulin) [16], and the arthropodan lipocalins, a cockroach allergen, Bla g 4 [17], a 'kissing bug' (*Triatoma protracta*) allergen, Tria p 1 [18], and a pigeon tick

Table 1. Characteristics of important mammalian respiratory allergens of lipocalin origin

Allergen	Animal	Principal source	Sensitized*, %	MM [†] , kDa	Amino acids	Isoelectric point	Glycosylation	Oligomeric state [‡]	UniProtKB accession #§	Key reference
Bos d 2	Cow	Dander	80–90	20	156	4.2	No	M	Q28133	[5]
Can f 1	Dog	Saliva, dander	50–75	22–25	156	5.2	Yes	D	O18873	[6]
Can f 2	Dog	Saliva, dander	25–40	22–27	162	4.9	Putative	M/D	O18874	[6]
Can f 4	Dog	Saliva, dander	35–60	18	158	6.5	No		D7PBH4	[7]
Cav p 1 [¶]	Guinea-pig	Dander, urine	70–87	20		4.3	No	M/D	P83507	[8]
Cav p 2	Guinea-pig	Dander, tears	55–65	17–19	154	4.3–4.5	No		F0UZ11	[9]
Cav p 3	Guinea-pig	Saliva	54	18–19	155	5.2	No		F0UZ12	[9]
Equ c 1	Horse	Dander, saliva, urine	76	22	172	3.9	Yes	D	Q95182	[10]
Equ c 2 [¶]	Horse	Dander	50	16		3.4–3.5	No		P81216 P81217	[11]
Fel d 4	Cat	Saliva	63	20	171	4.6	Putative	M	Q5VFH6	[12]
Fel d 7	Cat	Saliva	38	18	154	4.9		D	E5D2Z5	[13]
Mus m 1	Mouse	Urine, dander	66	18–21	162	4.6–5.3	No	M/D	P02762	[14]
Ory c 1 [¶]	Rabbit	Saliva, dander		17–18			Yes			[15]
Ory c 2 ^{¶, **}	Rabbit	Saliva, dander		21						[15]
Rat n 1	Rat	Urine	66	17–21	162	4.2–5.5	Yes	M/T	P02761	[14]

*Percentages are based on determinations with different methods and may not be comparable.

[†]Molecular mass.

[‡]M, monomer; D, dimer; T, tetramer.

[§]UniProt Knowledgebase, <http://www.uniprot.org/>.

[¶]Only N-terminus known.

**Tentatively named.

(*Argas reflexus*) allergen, Arg r 1 [19], belong to the lipocalin family.

Previous studies with allergen extracts pointed to the possibility that there could be IgE cross-reactivity between mammalian allergens. Recently, it has been found with recombinant preparations of lipocalins that this is true at least between dog Can f 1 and Can f 2, between Can f 1 and its human homologue tear lipocalin, and between horse Equ c 1 and mouse Mus m 1 [20]. Moreover, IgE cross-reactivity has been observed between cat Fel d 4 and allergen extracts from cow, horse and dog [12]. It is of interest that IgE cross-reactivity has also been demonstrated between Fel d 4 and Can f 2, although their sequential identity is less than 22% [21]. Finally, a dog allergen, Can f 4, has been found to exhibit strong IgE cross-reactivity with a 23 kDa protein of cow [7]. In all, IgE cross-reactivity between mammalian lipocalin allergens from different sources appears not to be extensive, which is probably due to their low overall amino acid identity (see below).

Although IgE binding by definition is characteristic to allergens and certain properties of allergens, such as dimerization, can promote it [22], IgE binding does not explain allergenicity, that is, the capacity of proteins to elicit allergic sensitization (induction of Th2 cellular immunity [4]). It is conceivable, however, that IgE cross-reactivity could be implicated in regulating it, a subject outside the scope of this review.

Mammalian lipocalin allergens in the environment

Several mammals, for example, mice, guinea-pigs, dogs and cats, are kept as pets. Horseback riding is a popular hobby. As animal allergens stick to clothing, they spread everywhere, even in homes without animals. Moreover, they can give rise to occupational allergy, for example, in the agricultural environment. Laboratory animal allergy is a significant health problem world-wide.

One obvious property for a protein to be an allergen is that it is efficiently dispersed in the environment so that it can get into contact with the human immune system. As lipocalins are present in animal dander and excretions, this requirement is fulfilled (Table 1). Several other factors, such as aerodynamic properties of allergen particles, further influence the dispersion of allergens and human exposure.

It appears that the allergenicity of a protein is not simply related to its amount in the allergen source or the extent of human exposure to it. Our analyses of a dog allergen extract support this notion, as two dog allergens, Can f 1 and Can f 4, with a reported similar capacity to sensitize (Table 1) are found in the extract in different amounts: about 10% of the total protein content of a dog dander extract was composed of Can f 1, while the figure for Can f 4 was 0.5% (M. Rytkönen-Nissinen, unpublished results). Accordingly, the analysis of horse dander allergens by IgE immunoblotting indicated

that IgE-binding activity did not correlate with the relative amounts of proteins in the preparation [11]. In this context, it is of interest that some allergens, for example, from arthropods, are known to be effective sensitizers in very low ambient concentrations, whereas mammalian allergens are needed in high concentrations to sensitize [3]. Taken together, antigenicity and/or immunogenicity, although necessary for IgE production, seems not to be directly linked with allergenicity [1].

Characteristics of lipocalin proteins

Lipocalins are a large group comprising proteins from vertebrate and invertebrate animals, plants, and bacteria (<http://prosite.expasy.org/PS00213>). The family is part of a larger superfamily, calycins [23]. Typically, lipocalins are small, extracellular proteins with a capacity to bind small, principally hydrophobic molecules, to attach to specific cell-surface receptors, and to form covalent and non-covalent complexes with soluble macromolecules [23].

Initially, lipocalins were characterized as transport proteins for such ligands such as retinol, odorants, steroids, and pheromones. Now it has turned out that they are also involved in a wide range of other biological functions. These include, for example, the immunomodulatory activity of glycodelin (placental protein 14) [24], enzyme activity (exhibited by a few lipocalins) [25] and participation in the mediation of cell homeostasis [26].

The sequential length of lipocalins is 160–230 amino acids with an average predicted molecular mass of about 20 000 Da (without post-translational modifications) [23]. Lipocalins can be N- and/or O-glycosylated. Mammalian lipocalin allergens show an acidic isoelectric point (Table 1).

The overall amino acid identity between lipocalins is 20–30%, but it can be considerably higher. For example, human epididymal-specific lipocalin-9 exhibits identities above 50% with its rodent homologues, and identities of about 40% are found with Mus m 1, Rat n 1, Equ c 1 and Fel d 4. The amino acid identity of dog Can f 1 with human tear lipocalin (von Ebner's gland protein) is about 60% (the SIB BLAST network service, May 3, 2011 [27]). Lipocalins contain 1–3 structurally conserved regions (SCR) in defined positions of the molecule [23]. More than 90% of lipocalins have SCR1, which contains the motif glycine-x-tryptophan and SCR3, which contains arginine/lysine. SCR2 which contains the motif threonine-aspartic acid-tyrosine-x-tyrosine is found in more than 60% of lipocalins [23].

Although the sequential identity among lipocalins is low in general, they share a common three-dimensional structure [23, 28]. The central β -barrel of lipocalins, which is composed of eight anti-parallel β -strands

encloses an internal ligand-binding site. At the N-terminus, there is a 3_{10} helix, whereas at the C-terminus, there is an α -helix of variable length. Most lipocalins contain one or more intramolecular disulphide bonds. The arrangement of lipocalin molecules in a multi-subunit complex (oligomerization) is variable [29]. In all, the physicochemical and structural features of the characterized lipocalin allergens are not known to account for their allergenic capacity or to distinguish them from other lipocalin proteins [30].

Allergenicity of lipocalins

Lipocalin allergens and adjuvant-like substances

Several studies in recent years indicate that the capacity of a protein to promote sensitization (Th2 immunity) can rather be a property of a substance associated with the protein than a property of the protein itself. Such adjuvant-like substances include diesel exhaust particles [31], pollen NADPH oxidase [32] and pollen-derived adenosine [33].

As lipocalins are known to carry small hydrophobic ligands in their internal ligand-binding site, recent observations that pollen extracts from birch and several other plants contain E_1 -phytoprostanes and possibly other Th2-deviating lipid mediators are of interest [34, 35]. It has even been suggested that lipid binding can be a key characteristic for many allergens because lipids can directly activate innate immunity [36]. Although there are no data supporting the idea that lipocalin allergens would carry immunomodulatory substances favouring allergy, the hypothesis is no doubt worth further examination.

Another substance, endotoxin [or lipopolysaccharide (LPS)], a cell-wall product from Gram-negative bacteria, is probably more directly related to mammalian lipocalin allergens because bigger pets and contact with farm animals increase home LPS levels [37, 38]. Importantly, although LPS has been found to be protective against atopic sensitization [39], low concentrations of LPS are needed for optimal Th2 responses [40, 41]. Therefore, it is conceivable that in a clean urban setting, LPS may promote the allergenicity of lipocalin and other allergens. There is no indication, however, that any adjuvant-like immune-deviating substance would specifically favour sensitization to lipocalin allergens.

Direct activity against immune cells

Enzyme activity. As the allergenicity of some allergens has been found to be implicated with their enzyme activity, through multiple mechanisms [42–45], it is of interest to note that β -lactoglobulin (Bos d 5), a milk allergen, and tear lipocalin, a human homologue of dog

Can f 1, have been reported to have non-specific endonuclease activity [46]. The glutamic acid at position 128 in tear lipocalin, important for the enzyme activity, is conserved in several lipocalin allergens, such as Bos d 2, Mus m 1, Rat n 1, Equ c 1 and Can f 1. Interestingly, as the evolutionarily conserved amino acid is also found in some human lipocalins [46] and at or adjacent to the cores of the immunodominant T cell epitopes in Bos d 2 [47] and Can f 1 [48], it might be implicated in modifying human T cell repertoire in the thymus and thus T cell responsiveness to the immunodominant epitopes of the allergens (see Hypothesis, below).

For the time being, the enzyme activity of lipocalin allergens other than Bos d 5 is unknown. It remains also unclear what could be the role of this activity for the allergenicity of Bos d 5, especially because it is very modest compared with that of DNase I [46]. Physiologically, it is thought that endonucleases in body fluids are involved in the antimicrobial defence by degrading foreign DNA [46, 49].

One of the mammalian lipocalin allergens mentioned above, Can f 1, has been thought to be a cysteine proteinase inhibitor as well because of its sequential homology with von Ebner's gland protein [50, 51]. The motifs crucial for the function are only partially conserved in Can f 1. Even if Can f 1 were a cysteine proteinase inhibitor, the significance of the property for the allergenicity of Can f 1 is not known.

Binding to receptors. As lipocalins characteristically have a capacity to attach to specific cell-surface receptors [23], it is noteworthy that a number of allergens have been shown to be actively recognized by the receptors of innate immunity. These include, for example, the peanut allergen Ara h 1, a glycoprotein, which is able to enhance its own uptake through binding to the dendritic cell (DC)-specific ICAM-grabbing non-integrin (DC-SIGN) on human monocyte-derived DCs [52]. The important observation was that the binding activated DCs to prime for a Th2 response, mostly by increasing interleukin (IL)-4 and IL-13 and decreasing interferon (IFN)- γ production by T cells [52]. Some other allergens, including the dog lipocalin allergen Can f 1, which is glycosylated, were found to be bound by the mannose receptor [53]. The results further suggested that the mannose receptor is involved in the Th2 polarization through the down-regulation of indoleamine 2,3-dioxygenase (IDO) activity in DCs.

In this setting, the observations of Rachmilewitz et al. with a human endogenous lipocalin, glycodelin or placental protein (PP)14, are intriguing. PP14 is an anti-inflammatory lectin-like glycoprotein abundant in the amniotic fluid during pregnancy and thought to be involved in counteracting maternal immune rejection of the fetal semiallograft. Instead of affecting DCs, it

exerts its function by attenuating T cell receptor (TCR) signalling, presumably through binding to CD45 [24, 54, 55]. This favours the development of Th2-type immunity [56]. In addition, PP14 was recently reported to promote the development of CD4⁺ T cells with a regulatory phenotype upon long-term *in vitro* culture [57].

Other receptors for lipocalins are megalin [58], the lipocalin-interacting membrane receptor (LIMR) [58], 24p3R [59] and STRA6 [60]. To our knowledge, their role in taking up lipocalin allergens has not been examined widely. However, the milk allergen β -lactoglobulin (Bos d 5) has recently been shown to be taken up by LIMR [61]. This receptor is found on a number of immune cells (B. Redl, personal communication) and it has been observed to mediate the uptake of another lipocalin allergen, Equ c 1 (B. Redl, personal communication). As to other receptors, our collaborative studies indicated that Bos d 2, Can f 1, Can f 2, Equ c 1 and Mus m 1 are not bound by megalin (S. Moestrup, personal communication).

Whether Bos d 5 and Equ c 1 are able to exhibit similar biological effects upon binding to LIMR as PP14 upon binding to its receptor is not known. It is clear, however, that this possibility needs to be explored. Regardless of the potential immunomodulatory effect, active uptake of an allergen may facilitate its access into the immune system, as speculated for Bos d 5 [61].

Nevertheless, as mammalian lipocalin allergens exist in both glycosylated and non-glycosylated forms (Table 1), glycosylation cannot be a denominator for their allergenic capacity. Moreover, mammalian lipocalin allergens appear not to be able to induce or inhibit the maturation of human monocyte-derived DCs, or to promote the proliferation or Th2 deviation of naïve CD4⁺ T cells (S. Parviainen, unpublished observations). Therefore, one characteristic of mammalian lipocalin allergens may rather be their unresponsiveness with the immune system, as reviewed in the following section.

Cellular immune response to mammalian lipocalin allergens

Cellular immune response to mammalian lipocalin allergens is weak. Although mammalian lipocalin allergens efficiently induce the production of specific IgE, peripheral blood mononuclear cells (PBMCs) from clinically allergic subjects sensitized to cow Bos d 2, dog Can f 1 or horse Equ c 1 unexpectedly proliferate very weakly upon stimulation with the allergens [47, 62, 63]. Accordingly, the PBMC response to rat Rat n 1 has also been reported to be weak [64]. Consistent with these observations, Bos d 2 was found to be a weak immunogen for several inbred mouse strains with different MHC haplotypes, and the control antigen hen egg lysozyme (HEL) elicited a stronger spleen cell response by

the BALB/c mouse than Bos d 2 [65]. Further flow-cytometric carboxyfluorescein succinimidyl ester (CFSE) dilution and cytokine ELISPOT analyses showed that the number of spleen cells responding to Bos d 2 was smaller than that to HEL [66]. Moreover, an analysis of the proliferation of peripheral blood CD4⁺ T cells from 15 Can f 1-allergic subjects by the CFSE dilution assay suggested that the precursor frequency of the lipocalin allergen-reactive memory CD4⁺ T cells is significantly lower than that for the microbial antigen streptokinase and tubercle bacillus-derived preparation, purified protein derivative [67] (and S. Parviainen, unpublished results).

Regulatory control of responsiveness to mammalian lipocalin allergens. Although regulatory T cells, such as naturally occurring CD4⁺CD25⁺FoxP3⁺ and inducible type 1 regulatory (Tr1) T cells, are evidently involved in establishing tolerance in specific immunotherapy [68], their role in maintaining tolerance to common respiratory allergens in healthy subjects without allergy is less clear [69–72]. When we explored the unresponsiveness to Bos d 2 in a murine model, we found that the weak cellular immune response to Bos d 2 was not mediated by cytokines, such as IL-4, IL-10, or transforming growth factor (TGF)- β . Neither was increased cell death involved in the phenomenon [66]. Our recent results further support the idea that the weak cellular reactivity to mammalian lipocalin allergens is not governed by Tregs. When CD4⁺CD25⁺FoxP3⁺ Tregs were depleted from PBMCs with anti-CD25-magnetic beads *in vitro*, specific proliferative responses of the Can f 1-allergic subjects or healthy dog owners upon stimulation with the allergen were not increased [67]. Accordingly, only low and similar levels of IL-10 were found in the cultures of Can f 1-allergic and healthy dog owners. Moreover, neutralization of IL-10 did not enhance the responses of either of the subject groups to Can f 1 [67].

Few T cell epitopes on mammalian lipocalin allergens. Whereas plant allergens often contain multiple T cell epitopes several of which are recognized by each individual [73, 74], the situation appears to be different with mammalian lipocalin allergens. On average, an individual recognizes three epitopes in Bos d 2, Can f 1 or Equ c 1 [47, 48, 63]. The total number of epitopes detected in Bos d 2, for example, was seven and the maximal number one individual's T cells could recognize was five [47].

The T cell epitopes in the lipocalin allergens examined (Bos d 2, Can f 1, Equ c 1 and Rat n 1) are not randomly distributed along the molecules, but are clustered in a few regions [47, 48, 63, 64]. In Bos d 2 and Equ c 1, one of the epitope regions in the carboxy-termi-

nal end of the molecules proved to be immunodominant, whereas in Can f 1, the T cell reactivity is less focused. As several of the epitope regions colocalize when the sequences are aligned [48, 63, 64], it is conceivable that the T cell epitopes between lipocalins colocalize in general. In this respect, it is noteworthy that the first colocalizing epitope region contains the signature motif G-X-W, which is also present in human endogenous lipocalins.

Characteristics of T cell epitopes of mammalian lipocalin allergens. Two T cell epitopes from two distinct lipocalin allergens, Bos d 2 and Can f 1, have been characterized in detail [75, 76]. The epitope of Bos d 2, situated in peptide (p)127–142, is immunodominant, whereas the epitope of Can f 1, situated in p105–120, is one of a few of comparable importance. The pivotal finding with both epitopes was that they are suboptimal; optimal (or heteroclitic, that is, more potent than the natural one) peptide analogues (altered peptide ligands, APLs) of the natural epitopes were able to stimulate human T cell clones at 10–100-fold lower concentrations than the natural ligands [75, 76]. The heteroclitic activity of these peptides was attributed to their more efficient recognition by TCR as they bound to the restricting human leucocyte antigen (HLA; human MHC) alleles with affinities similar to those of the natural ligands. Staining the Bos d 2 p127–142-specific T cell clones with HLA class II tetramers confirmed that the TCR avidity of the clones for the heteroclitic ligands (superagonists) was higher than that for the natural one [77].

In addition to their enhanced capacity to induce proliferation in T cell clones, the heteroclitic peptide analogues were found to have a qualitative impact on the T cell response *in vitro*. For example, upon stimulation with the heteroclitic analogues of p127–142 of Bos d 2, specific T cell clones exhibited greater Th1/Th2 cytokine ratios than upon stimulation with p127–142 at equimolar concentrations [77]. Importantly, when human CD4⁺ T cell lines (TCLs) were induced with p127–142 and its heteroclitic analogue pN135D, the lines induced with the natural ligand were predominantly of the Th2/Th0 phenotype, whereas the lines induced with the heteroclitic analogue were of the Th1/Th0 phenotype [78]. A similar qualitative change in the cytokine phenotype was not seen, however, in the TCLs induced with the heteroclitic analogues of p105–120 of Can f 1, although they mostly tended to be more stimulatory than the natural peptide for PBMCs [76]. In murine experiments, nevertheless, a homologue of p127–142 of Bos d 2 gave rise to a qualitative shift: when the spleen cells from the BALB/c mice primed with p127–142 (or the corresponding peptide from horse Equ c 1) were stimulated with p127–142 (or the peptide from Equ c

1), the response was Th2-biased, whereas when the cells from the mice primed with a homologous bacterial peptide from *Spiroplasma citri* were stimulated with the bacterial peptide, the response was Th0-biased [79]. In all, these experiments suggest that T cell recognition may be implicated in modifying the quality of immune response, as discussed in more detail later.

Another feature associated with the T cell epitopes of mammalian lipocalin allergens may be degeneracy in their recognition. We found that one of the human T cell clones specific to the immunodominant epitope of Bos d 2 (p127–142), for which a search motif for homologous ligands was constructed, recognized five of the nine homologous peptides tested [75]. Four of these search peptides were of human origin and one of bacterial origin. Another clone also recognized one of the search peptides of human origin, although the search motif was not designed for it. The clones did not recognize peptides corresponding to p127–142 from other lipocalins. In experiments with the BALB/c mouse, T cell cross-reactivity was also demonstrated between p127–142 and a few non-lipocalin peptides as well as a peptide from horse Equ c 1 [79]. Interestingly, two Can f 1-specific TCLs out of four from a Can f 1-allergic subject recognized a corresponding peptide of tear lipocalin, a human protein homologous to Can f 1 [80]. Whether the degenerate TCR recognition of endogenous proteins is implicated in the activation of mammalian lipocalin allergen-reactive T cells, as is proposed for the activation of autoreactive T cells [81, 82], is not known.

T cell repertoires specific to mammalian lipocalin allergens. Recent studies suggest that allergen-specific CD4⁺ T cell repertoires can markedly differ between allergic and non-allergic subjects [69, 70, 83]. This also appears to be the case with mammalian lipocalin allergen-specific T cells in that the frequency of Can f 1 and Bos d 2-specific memory Th cells in allergic subjects is higher than in non-allergic ones [67, 84]. When we stained Bos d 2_{127–142}-specific CD4⁺ T cells with DR0401/p127–142 HLA class II tetramers in the cultures derived from peripheral blood CD4⁺ memory T cells after a short *ex vivo* expansion, the analyses revealed that the cultures of four of five subjects with allergy, but only two of five cultures of subjects without allergy, contained tetramer-positive cells [84]. The mean frequency ($\pm$ SEM) of the cells in the cultures was $0.38\% \pm 0.19\%$ for allergic subjects and about half of that for non-allergic subjects. As tetramer-positive responses could not be detected in the cultures of three subjects without allergy, the mean frequency of specific memory T cells in their peripheral blood appears to be even lower. It was calculated that the frequency of Bos d 2_{127–142}-specific memory T cells in the peripheral blood of subjects with allergy is in the range of 10^{-5} and 10^{-6} circulat-

ing CD4⁺ T cells [84]. When the frequency was assessed with a complementary method, the split-well assay, it was in the same range. Here, we also corroborated the frequency difference of memory Th cells between subjects with and without allergy, as it was possible to induce about three times more specific long-term TCLs from allergic subjects than from non-allergic subjects [84]. Results from our study on Can f 1 allergy are consistent with those of studies reviewed above [67].

Taking into account the results on CD4⁺ memory T cells, it is intriguing that the frequency difference between Bos d 2-allergic and healthy subjects appears to be minimal in naïve T cells [84]. When we stained Bos d 2_{127–142} tetramer-positive cells in the naïve T cell-derived cultures from subjects with or without allergy, they were detected in the cultures of all subjects. Moreover, the mean frequencies were largely the same in both groups, $0.81\% \pm 0.38\%$ and $0.61\% \pm 0.35\%$ respectively. The frequency of naïve cells in the peripheral blood was estimated to be within the same range as that of memory T cells, between 10^{-5} and 10^{-6} circulating CD4⁺ T cells. When the alternative approach, the split-well assay, was employed, Bos d 2_{127–142}-specific long-term TCLs of naïve origin could be generated from all but one non-allergic subject. Here, we found, however, that they could be induced from allergic subjects (8.5%) about twice as often than from healthy subjects [84].

These findings suggest that although present *ex vivo*, the naïve allergen-specific Th cells of healthy individuals had not expanded efficiently in the long-term *in vitro* culture. As TCR avidity is of fundamental importance for recruiting fit T cell clones from a large repertoire of T cells [85], we analysed it with HLA class II tetramers as a factor accounting for the phenomenon; it is known that the capacity of T cells to bind HLA class II tetramers directly correlates with the structural TCR avidity [77, 86, 87]. We found out, indeed, that the intensity of tetramer staining of the *ex vivo*-expanded Bos d 2_{127–142}-specific CD4⁺ T cells was higher in the naïve T cell-derived cultures from allergic subjects than in those from non-allergic ones [84]. Therefore, the finding points to the higher structural TCR avidity in the cells of allergic subjects than in those of non-allergic ones. Results from a complementary analysis with long-term TCLs generated by the split-well method were parallel, as 14 of the 17 TCLs of memory or naïve origin (82%) from allergic subjects stained with the specific tetramers, whereas only one of the five TCLs (20%) from non-allergic subjects showed a positive staining ($P = 0.02$) [84]. Results from the study of Can f 1-allergic and healthy dog owners support these notions. We found that the functional TCR avidity of Can f 1-specific memory T cells, estimated by the effective concentration 50 (EC50) values (the concentration of Can f 1

needed to induce a half-maximal proliferative response), was higher ($P = 0.02$) in the Can f 1-specific TCLs from allergic subjects than in those from healthy subjects [67]. Importantly, 33% of the TCLs from allergic subjects, but none of those from healthy subjects, could be classified as being of higher functional TCR avidity ($EC_{50} \leq 2$ mg/mL; $P = 0.01$) [67].

The divergent characteristics of T cell repertoires of allergic and healthy subjects were further reflected in the cytokine production: both Bos d 2 and Can f 1-specific memory cell-derived TCLs from allergic subjects produced predominantly IL-4 and were of a Th2 phenotype, whereas those from healthy subjects were of a Th0 phenotype [67, 84]. One interesting finding was that the memory cells from allergic subjects, defined as being of higher functional TCR avidity, produced significantly more IL-4 and IL-5 than the low-avidity T cells [67]. Moreover, a subtle Th2 bias was seen in the TCLs of naïve origin from subjects with allergy compared with those without allergy [84].

Hypothesis for the Th2-deviated immune response to mammalian lipocalin allergens

Information on the physicochemical and immune characteristics of lipocalin allergens draws one's attention to the role of their recognition by the adaptive immune system in that the distinctive feature of human and murine cellular immune reactivity to mammalian lipocalin allergens is weakness, as reviewed above. This observation is in accordance with the hypothesis we have previously proposed: the allergenicity of mammalian lipocalin allergens may be associated with the adaptation of the immune system to the presence of endogenous lipocalins [88]. In other words, evolutionary relatedness of human endogenous lipocalins with exogenous lipocalin allergens (sequential similarity) may have resulted in the absence of high-avidity lipocalin allergen-reactive T cells due to thymic deletion, and this would result in a weak cellular reactivity that would favour Th2 immunity against them. It is well-documented that suboptimal T cell recognition of an antigen favours the development of Th2-type immune responsiveness [4, 89–97]. In this setting, it is noteworthy that several of the epitope regions of distinct mammalian lipocalin allergens have been found to colocalize [48, 63], as this may indicate that these regions are also antigenic for T cells in human endogenous lipocalins.

The significance of adaptive immunity for shaping the magnitude and quality of a specific immune response is further emphasized by recent investigations. It has been discovered that the repertoire of T cells with naïve phenotype contains distinct peptide-MHC-specific populations the size of which differs between individu-

als [98]. Importantly, naïve T cell repertoires appear to play a central role in determining which peptides induce a T cell response [99, 100] or which epitopes are immunodominant [101]. Therefore, it can be speculated that the composition of the naïve T cell repertoire can be a factor, which ultimately 'selects' some proteins as allergens as well as can shift the fine balance of immunity in favour of the Th2 predominance. In accordance with this view, it was recently reported that if the T cell repertoire does not contain cells with high-affinity TCRs, stimulation through TCR favours the development of Th2-type immunity [102].

In this context, it is of interest to pay attention to the observation indicating that the highest level of sensitization to cat Fel d 1 is found in children exposed to intermediate levels of the allergen, whereas tolerance tends to develop at higher levels of the exposure [103]. Thus, the dose–response relationship between exposure to cat and sensitization appears to be bell-shaped. The protective effect is thought to result from a modified Th2 response involving the synthesis of specific IgG4. Although there is some controversy between the studies on the protective effect of high allergen exposure, the effect has also been observed, for example, upon exposure to dust from dog [104], rat [105] and house dust mite [106]. In an experimental human study of nasal neoantigen exposure, specific IgE was found to exhibit a bell-shaped dose–response, while the specific IgG response went up with the increasing exposure [107]. Thus, these observations are concordant with the data pointing to the anti-allergenic T cell response upon strong TCR signal strength.

Two other studies, based on sequential homology comparisons, further suggest that evolutionary relatedness may play a role in the allergenicity of proteins. In one study, it was shown that 25 of 30 randomly selected allergen sequences from UniProt Knowledgebase had no bacterial homologues, whereas non-allergen control sequences from the same species as the allergens had a large number of such homologues [108]. Another study suggests that animal food allergens have an upper limit, about 60%, for homology with human proteins above which they are rarely allergenic [109]. When we analysed the reviewed lipocalin allergens with a known complete amino acid sequence for the sequential similarity with bacterial proteins, we found that the amino acid identities were mostly situated in short stretches of the sequences. The maximal level of identity with a bacterial protein, 42%, was shown for Bla g 4, whereas all other allergens exhibited lower identities in such stretches (the SIB BLAST network service, July 28, 2011 [27]). As several mammalian lipocalins are known to exhibit homologies of 40–60% over species, it can be hypothesized that mammalian lipocalin allergens may reside immunologically in

the borderline of self and non-self, which may make them prone to induce Th2-type immunity [110, 111].

Conclusions

Even though lipocalin allergens compose a significant group of allergens, which are widely found indoors, the basis for their capacity to sensitize has remained enigmatic. For the time being, they are not known to exhibit any physicochemical, functional or structural property that would account for their allergenicity. As information on the role of the T cell repertoire for the quantitative and qualitative outcome of an immune response has accumulated, it is conceivable that T cell recognition can be an important factor in the sensitization process. It can be speculated that specific CD4⁺ T

cells must exhibit a certain avidity window for the epitopes of a mammalian lipocalin allergen to permit sensitization to take place: if the protein is recognized as truly foreign, a non-allergenic Th1-type response is likely; if it resembles the immunological self, the protein is probably tolerated; if it is able to cross the threshold for TCR activation, without being too stimulatory (the borderline of self and non-self), the Th2-type immune response is possible.

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