81%, respectively) than in all other groups (χ^2, $P<0.05$). As expected and also shown in Figure 1c, IgG4 anti-Dsg1 was detected in a large number of FS, PF, and PV patients (90, 77, and 33% respectively), which is a known feature of these diseases. These findings suggest a persistent antigenic stimulation of the immune system of individuals living in the endemic areas of FS.

In summary, our findings further support the notion that the anti-Dsg1 response in FS patients may be initiated by sensitization to an environmental allergen(s). The crossreactive IgE, IgM, and pathogenic IgG4 anti-Dsg1 response may be the serological markers of these immunological responses.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Evidence for Non-Allergic Mast Cell Activation in Pollen-Associated Inflammation

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TO THE EDITOR

The occurrence of allergies is rapidly increasing in the Western world, with allergic rhinoconjunctivitis to airborne allergens being the most common condition. The diagnosis of allergic rhinoconjunctivitis is based on a history of clinical symptoms, typically during the pollen season, and skin testing with the respective allergen. A positive skin prick test (SPT) is taken as an indication for IgE-mediated mast cell (MC) degranulation. As it has recently been shown that pollen release not only allergens but also bioactive mediators that can activate and modulate immune cells (Traidl-Hoffmann *et al.*, 2009), we hypothesized that non-allergic pollen-derived factors can induce inflammation both in patients and in non-allergic mice. We, therefore, assessed the levels of specific IgE against birch or ambrosia in 184 consecutive patients seen at the outpatient clinics of the Allergie-Centrum-Charité in Berlin, Germany, with a positive history for allergic symptoms to birch (146 patients) or ambrosia (38 patients), and positive SPT to the respective allergens. In accordance with previously reported findings (Bodtger *et al.*, 2003), we found that 13% of our patients with positive SPT to birch had

Abbreviations: APE, aqueous pollen extract; MC, mast cell; SPT, skin prick test

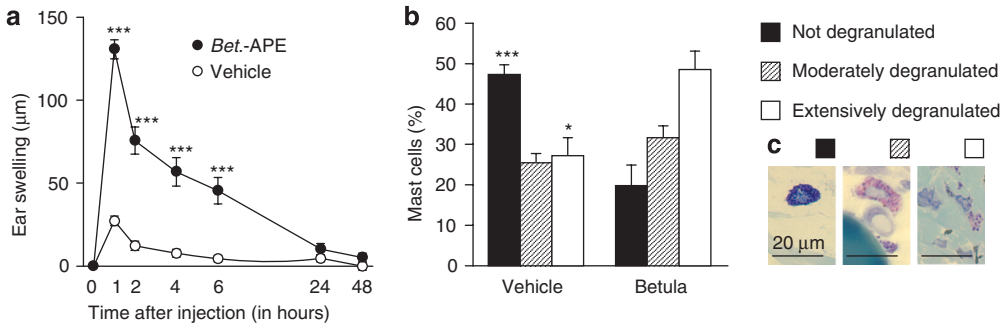

Figure 1. IgE-independent skin inflammation by aqueous pollen extracts from birch (*Bet.*-APEs) is associated with increased mast cell (MC) degranulation. *Bet.*-APE or vehicle (100 μg each) was injected intradermally (in 20 μl Dulbecco's phosphate-buffered saline) into the ears of C57BL/6 mice. MC degranulation was assessed by quantitative histomorphometry in alkaline Giemsa-stained sections. Data are derived from three independent experiments, (**a**) $n \geqslant 11$ per group, (**b**) $n = 4$; ***$P < 0.005$, *$P < 0.01$. Images in **c** are representative of not degranulated, moderately degranulated, and extensively degranulated MCs; original magnification ×100, bar = 20 μm.

very low serum levels of specific IgE against birch (CAP class 0 or 1, i.e., specific IgE $<0.7\,\mathrm{kU\,l^{-1}}$; Supplementary Table S1 online and Supplementary Figure S1 online). More strikingly, we observed very low specific IgE against ambrosia in almost 30% of our patients, with a positive history of allergic reactions to ragweed and a positive SPT to ambrosia (Supplementary Table S1 online and Supplementary Figure S2 online). These data are very similar to recently published results showing 8 of 33 patients (24%) with very low specific IgE radioallergosorbent test (RAST) value for ragweed but positive history and SPT (Stokes *et al.*, 2005).

These findings imply that allergic and/or non-allergic components of pollen can enhance or even induce allergy-like symptoms even in the absence of specific IgE, e.g., by directly activating MCs in the skin or mucosa (Mattila *et al.*, 2010). Indeed, extracts of birch pollen have previously been shown to directly activate neutrophils, eosinophils, and dendritic cells (Traidl-Hoffmann *et al.*, 2002, 2005; Plotz *et al.*, 2004), and ragweed pollen extracts can reportedly induce IgE-independent histamine release from rat basophilic leukemia cells (Chodaczek *et al.*, 2009). To assess the potential of pollen-derived substances to induce IgE-independent allergy-like inflammation, we prepared aqueous pollen extracts (APEs) from birch pollen (from *Betula alba* sp.), as previously described (Gilles *et al.*, 2009), and injected the APE of birch pollen (*Bet.*-APE) or vehicle intradermally into the ears of naïve C57BL/6 mice. After injection, ear swelling was measured as a parameter of skin inflammation at various timepoints. Injection of *Bet.*-APE resulted in a rapid and pronounced inflammatory skin reaction (Figure 1a), closely mimicking an IgE and antigen-mediated allergic skin inflammation (Metz *et al.*, 2006). Furthermore, quantitative histomorphometry (Metz *et al.*, 2009) in alkaline Giemsa-stained plastic-embedded 1-μm sections of the ears 6 hours after injection revealed that *Bet.*-APE ear swelling is associated with an increased percentage of extensively degranulated MCs (Figure 1b and c).

To further characterize the mechanisms of *Bet.*-APE-induced skin inflammation, we injected various concentrations of *Bet.*-APE into the ears of MC-deficient Kit^{W}/Kit^{W-v} mice and their respective wild-type $Kit+/+$ littermates. Whereas *Bet.*-APE injection resulted in strong and dose-dependent ear swelling in $Kit+/+$ mice, virtually no signs of inflammation were observed in the ears of MC-deficient mice (Figure 2a and b). Similar results were obtained for APE derived from *Ambrosia artemisiifolia* pollen (Supplementary Figure S3 online). In addition to their profound deficiency in MCs, Kit^{W}/Kit^{W-v} mice also lack melanocytes and interstitial cells of Cajal, and display some other abnormalities such as slight anemia (Metz *et al.*, 2007). Therefore, we selectively repaired the MC deficiency of the ear skin of some Kit^{W}/Kit^{W-v} mice by engraftment with bone marrow-derived cultured MCs, as described previously (Metz *et al.*, 2006), and injected *Bet.*-APE into the ears of MC-engrafted Kit^{W}/Kit^{W-v} mice (Kit^{W}/Kit^{W-v} + bone marrow-derived cultured MCs), normal MC-deficient mice (Kit^{W}/Kit^{W-v}), and wild-type controls ($Kit+/+$). As shown in Figure 2c, the inflammatory skin reaction in response to injection of APE was fully restored in Kit^{W}/Kit^{W-v} mice after the engraftment with MC (Figure 2c), formally proving that MCs are indeed responsible for Bet-APE-induced skin inflammation.

To characterize the nature of the MC-activating agent within the extracts of birch and ambrosia pollen, we prepared different fractions of the APEs of birch and ambrosia pollen. To obtain allergen-free APE fractions, the total extracts were ultra-filtered using 3 kDa cutoff filters (Amicon ultra YM3, Millipore, Schwalbach, Germany). Interestingly, depending on the type of pollen, different substances seem to be responsible for the inflammatory response: in the case of *Bet.*-APE, the effect was mediated by a fraction enriched for compounds <3 kDa (Gilles *et al.*, 2010) (Figure 2d). In contrast, APE from ambrosia exhibited inflammatory potential mainly in the fraction with molecules larger than 3 kDa (Supplementary Figure S4 online). Future investigations will have to identify the exact nature of the MC-activating substances within the respective pollen extracts.

Taken together, our data show that water-soluble mediators from birch and ambrosia pollen can induce MC-dependent but IgE-independent allergy-like inflammation in murine skin. Using this

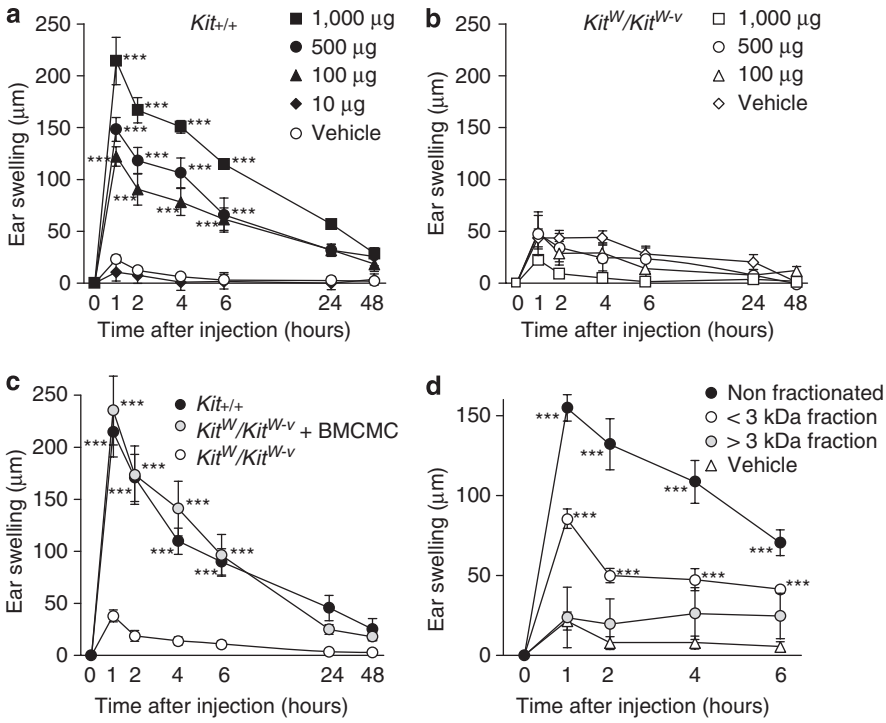

Figure 2. Aqueous pollen extracts from birch (*Bet.*-APEs) induce mast cell (MC)-dependent skin inflammation in mice. Ears of naïve wild-type *Kit*+/+ mice (**a**, **c**), MC-deficient $Kit^{W}/Kit^{W\text{-}v}$ mice (**b**, **c**), $Kit^{W}/Kit^{W\text{-}v}$ mice that have been locally and selectively engrafted with MCs ($Kit^{W}/Kit^{W\text{-}v}$ + bone marrow-derived cultured mast cell (BMCMC), (**c**)) or C57BL/6 mice were injected intradermally with 100 µg *Bet.*-APE or as indicated (in 20 µl Dulbecco's phosphate-buffered saline). (**a**, **b**) $n \geq 6$ mice/group, (**c**) $n \geq 12$, (**d**) $n = 4$; $^{***}P < 0.005$.

model, we provide a proof of concept for pollen-associated but allergen-independent inflammation. It has to be noted, however, that one has to be careful in extrapolating from the murine model system to the patients. For example, in ''real life'', pollen components will rarely be injected intradermally. Nevertheless, in some tissues such as the upper airway epithelium, pollen-derived substances could penetrate and get in direct contact with MCs. In the skin of patients with inflamed skin, e.g., patients with atopic dermatitis, barrier defects provide greater access for toxins, microbes, and allergens (Hanifin, 2009). This could also facilitate direct contact of pollen components with MCs. Furthermore, although human MCs also express a huge amount of potent inflammatory mediators and share many of the same mechanisms of activation, there are certain functional differences between murine and human MCs (Bischoff, 2007; Metz *et al.*, 2008).

The inflammatory effects of pollen components observed in mouse skin may account for the seasonal allergy-like clinical symptoms experienced by some patients. In the patients reported here, we considered specific IgE levels $<0.7\,\mathrm{kU\,l^{-1}}$ (CAP class 0 and 1) to be of limited clinical relevance. However, especially in those patients with low levels of specific IgE, non-allergic substances from pollen may enhance the reactivity of MCs and thus promote aggravation of allergic diseases. No detectable levels of specific IgE ($<0.1\,\mathrm{kU\,l^{-1}}$) against birch or ambrosia were found in 3.6 and 5.3% of the symptomatic patients with positive SPT, respectively (Supplementary Figures S1 and S2 online). In these patients, a direct effect of non-allergic pollen mediators on MCs similar to the observed effects in mice seems possible. Susceptibility for this non-allergic pollen-associated inflammation may depend on the status of the epithelial barrier or differences in the sensitivity of MCs between individuals (i.e., because of genetic differences, differences in the microenvironment, or simultaneous minimal activation by specific IgE and the respective allergen in those patients with low levels of specific IgE).

Another interesting speculation is that these mediators could also serve as adjuvants in sensitization to allergens. McLachlan *et al.* (2008) recently demonstrated that antigen-specific serum IgG responses to a vaccine antigen can be largely increased by co-administration of a MC activator. Our data presented here indicate that pollen can provide both MC activation and allergen challenge at the same time, and it will be of great interest to investigate whether this may also lead to enhanced production of IgE and thus increased sensitization to the respective allergen.

All animal care and experimentation were conducted at Charité-Universitätsmedizin Berlin in accordance with current federal, state, and institutional guidelines.

In all investigations involving patients, the patients' written, informed consent was obtained and the Declaration of Helsinki protocols were followed.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Calcipotriol Induces Autophagy in HeLa Cells and Keratinocytes

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TO THE EDITOR

Calcipotriol is used as a first-line topical agent in the treatment of psoriasis. Inhibition of keratinocyte proliferation, repression of growth signals, and modulation of T-cell signaling have been proposed to contribute to the clinical effects of calcipotriol (Scott *et al.*, 2001). However, the precise mechanism of action of calcipotriol and related vitamin D analogs remains poorly understood. Recent studies have implicated vitamin D in autophagy induction in a variety of cell types including MCF-7 breast carcinoma cells (Hoyer-Hansen *et al.*, 2007), human myeloid leukemia cells (Wang *et al.*, 2008), and human monocytes (Yuk *et al.*, 2009). During autophagy, intracellular contents are enveloped in double-layered membrane vesicles that fuse with lysosomes for degradation (Mizushima *et al.*, 2010). This ''recycling'' process is highly regulated and may be activated by a variety of stimuli including infections, starvation, misfolded proteins, and mitochondrial stress. In this study we find that calcipotriol, a commonly prescribed topical analog of vitamin D, also induces autophagy in both HeLa cells and keratinocytes.

Abbreviations: NHK, normal human keratinocyte; VDR, vitamin D receptor

The induction of autophagy is characterized by specific histological and biochemical changes (Mizushima *et al.*, 2010). Under basal conditions, microtubule-associated protein 1 light chain 3 beta (LC3) is a diffuse cytosolic protein. After autophagy induction, LC3 is proteolytically cleaved, lipidated, and localizes to autophagosomal membranes, forming punctate subcellular structures. Using a HeLa cell line that expresses a green fluorescent protein-LC3 (GFP-LC3) fusion protein (Orvedahl *et al.*, 2010), we assessed whether calcipotriol alters the subcellular distribution of LC3. Indeed, calcipotriol treatment (40 nM for 24 hours) caused a striking induction of GFP-LC3