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Identification and Characterization of Pollen Associated Lipid Mediators (PALMs) as Immunomodulators of Human Monocyte Derived Dendritic Cells

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1 Introduction

1.1. History of allergy and disease

In the year 1819, John Bostock in a speech at the Royal Medical Society of London described his own periodically arising affection of the eyes and the chest -, which he called Catarrhus aestivus and later also hay cold (1). A half century later Charles Blackley furnished by a self attempt the proof that pollen played a role in the pathogenesis of the hay cold. However, the neologism “allergy” was formed only in 1906 by the Viennese paediatrician Clemens of Pirquet from the Greek words "allos" (different) and "ergon" (work) (2).

In the year 1923, the American allergists Coca and Cooke wanted to describe a strange, abnormal type of hypersensitivity against environmental substances which was observed only in humans and tended to occur within families without obvious prior sensitizations. They wanted to differentiate this type of hypersensitivity from other forms such as anaphylaxis and asked the philologist Perry from Columbia University for help. Perry came up with the term “atopy”, meaning “not in the right place” or “strange”. Since that time more than 80 years have passed. Yet the term “atopy” is still controversial. Nonetheless, the clinical conditions described by this name are old and have been well known for thousands of years. This is clear from classical medical literature where we find descriptions of asthma, eczema and rhinitis. The first documented atopic individual was most likely Emperor Octavianus Augustus, who suffered from extremely itchy skin, seasonal rhinitis and tightness of the chest (Suetonius: Vita Caesarum). His grandson, Emperor Claudios, suffered from symptoms of rhinoconjunctivitis. Including Augustus’s great grandnephew, Britannicus, who supposedly suffered from horse dander allergy, one can safely state that the first family hystory of atopy is documented in the Julian-Claudian family of emperors.

From the beginning of the modern history of atopy, the major difficulty in defining the conditions has been that many authors have tried to describe the clinical

symptomatology and an etiopathophysiological mechanism at the same time. However, atopy is primarily a condition of hypersensitivity to environmental substances, which can lead to a disease (namely, an atopic disease such as eczema, asthma or rhinoconjunctivitis) and in many cases to a syndrome of different diseases (including respiratory, gastrointestinal and skin symptoms) (3). The fact that allergies could be carried out by serum factors was shown in 1921 by Prausnitz and Kuestner. In 1967, the serum factor IgE was identified at the same time by the married couple Ishizaka and Johannson and Bennich (4, 5). From this time on the allergology became part of the clinical Immunology, particularly since the same period during which the Langerhans cells were recognized as antigen-presenting cells (6).

1.2. Atopic diseases

Atopic diseases mostly affect the skin, the conjunctiva and the respiratory mucosa, which define boundaries between the host and the environment.

Atopic diseases are characterized by a peculiar hyperreactivity of the target tissues toward a variety of inflammatory stimuli and by IgE hyperresponsiveness to environmental allergens. The former aspect is always present whereas the latter is not constant. The preferential development of T-helper 2 (Th2) immune responses in atopic patients has been extensively studied, whereas the cellular and molecular bases of the tissue hyperreactivity have been only marginally investigated.

Epithelial cells are the outermost components of skin and mucous membranes, and they can be activated by diverse factors to produce mediators involved in the initiation and amplification of inflammatory responses. In particular, a dysregulated production of cytokines and chemokines by epithelial cells can be primarily involved in the pathophysiology of atopic diseases. Keratinocytes and mucosal epithelial cells produce granulocyte/macrophage colony-stimulating factor (GM-CSF), a cytokine essential for the

development of dendritic cells (DCs) from precursors, but also involved in the attraction and survival of DCs. DCs are present in high number in the skin and respiratory tract of patients with atopic diseases and play a crucial role in the initiation of allergen-specific immune responses (7). Allergic diseases represent a major health problem in most modern societies (8, 9). In the past ten years we have witnessed exciting progress in understanding different facets of the complex biological phenomena that participate in an allergic reaction. However, most of the very basic questions that were formulated 20 or more years earlier are still valid, since no answer has been given to them. Characteristically, in 1978, in an article entitled, “What makes an allergen an allergen”, Aas (10) wrote “Why do some substances and some molecules act as allergens while others apparently do not?” This is a question of outmost importance since its elucidation will have an important impact on patient treatment and eventually on prophylaxis. It is possible to hypothesize that allergens themselves possess some particular molecular characteristics that are responsible for the deviation of the immune response. Alternatively, since in nature allergens enter the organism in association with many other substances, some of them, might help driving the immune response to type 2 (11).

1.3. Natural Mission of Pollen Grains

Pollen grains primarily bear a natural mission - that is the unitary adaptive function to reach a receptive stigma and to deliver two haploid nuclei to the recipient ovary in order to transmit genetic information from the male parent to the offspring (12, 13). Fertilisation of the ovules of flowering plants occurs when dehydrated pollen grains- on the receptive surface of the female (the stigma) - hydrate, germinate, and produce a pollen tube. These events occur in the lipid-rich environment formed by the pollen coat of the stigma exsudate (14, 15). Pollen tubes navigate the route from the stigma to the ovule with great accuracy.

Therefore there is evidence that unsaturated lipids in the pollen coat are involved in the cell-cell recognition required for hydration (16, 17) and for directional pollen tube growth.

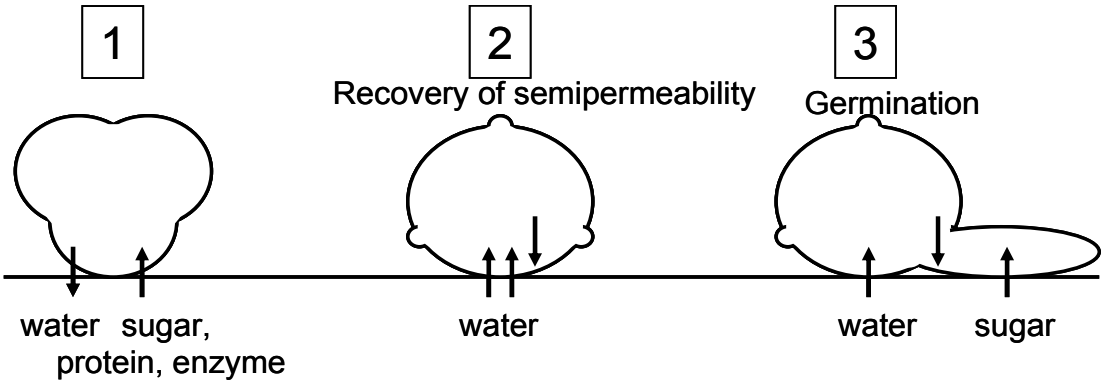

Figure 1: Hydration and transfer of organic molecules through cell membrane for pollen germination.

1.4. Pollen Grains in the Elicitation of Allergic Reactions

It is commonly believed that allergic sensitization starts with the contact between allergen and the surface of the antigen-presenting cell in the mucosa of the skin. Most studies dealing with this aspect at a pathophysiological level use allergen extracts as stimulus. Under natural exposure conditions, however, the bioavailability of allergen depends on allergen liberation from internal binding sites within the allergen carrier, e.g., the pollen grain (18-20). Proteins and glycoproteins from pollen can function as allergens, environmental molecules interacting with the human immune system to elicit an allergic response in susceptible individuals. Little is known about the physiological function of the allergenic proteins within the pollen grain even though some have been identified as defence proteins (21). Thus, the release of these proteins -harmful for humans- depends, in part, on stress situations for the plant. The majority of studies conducted to date concerning the elicitation and expression of allergic symptoms have concentrated primarily on individual allergens. However, the immune system of most individuals is not necessarily exposed to allergens in pure manner, but rather in particulate form (22, 23), either as pollen grains, their starch granules (24), or they may become associated with exogenous

particulate material such as diesel exhaust particles. The importance of grass pollen in the form of “free granular matter” as potent inducers of allergic reactions was established almost 130 years ago when Blackley (1) performed provocation tests and documented the relationship between pollen exposure and allergic symptoms. The biochemical nature of group 1 grass pollen allergens was analysed approximately 90 years later (25). Today, it is generally accepted that specific aeroallergens released from pollen cause hypersensitivity and lead to allergic diseases (e.g. bronchial asthma, allergic rhinitis, and allergic conjunctivitis), systemic effector-cell priming and mediator release. These mechanisms are thought to be initialized by a specific protein- the allergen (26). Moreover, urban air pollution, especially caused by pollutants due to traffic emission, exhibits adjuvant activities in allergen-specific IgE production and skews cytokine production to a Th2-type pattern (27).

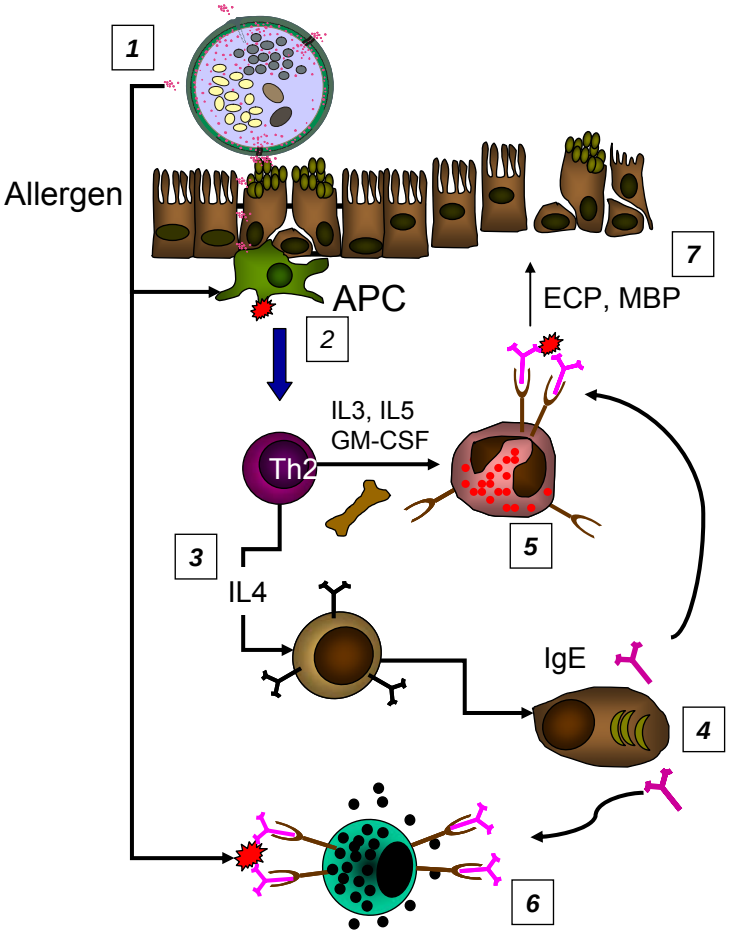

Figure 2: *Pathomechanisms of the allergic reactions***.** 1: the primary site of exposure to pollen grains is the epithelium of the upper respiratory tract, which is densely populated by immature dendritic cells (2). Pollen grains are deposited on mucosal membranes and release the allergen in the aqueous phase. Since pollen allergens are water soluble and readily available they can be taken up by antigen presenting cells such as dendritic cells which migrate into the regional lymphnodes to present the antigen to naïve T-cells with an ensuing Th2 response in the case of type I allergens. The production of IL-4 by Th2 cells, mastcells and further not identified cells lead to the isotype switching of B-cells with the resulting allergen-specific IgE production- in susceptible individuals (4). Durong the second and every upcoming encounter with the allergen the cross linking of membrane bound (e.g. eosinophils, mastcells and basophils) leads to the release of proinflammatory substances resulting in the immediate allergic reactions (5,6). Repeated allergen challenge leads to airway hyperreactivity (7).

1.5. Pollen-Associated Lipid Mediators (PALMs)

In studying the interaction of pollen grains with inflammatory cells, it has been observed that- apart from allergens- pollen grains secrete proinflammatory substances of eicosanoid-like character under humid conditions called PALMs (pollen-associated lipid mediators) (28). These PALMs are formed in the plants either via lypoxygenase activity or via autooxidative mechanisms. Pollen-associated lipid mediators exhibit strong cross-reactivity with leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2), as measured by ELISA (29). The release of the "LTB_4-like" and "PGE_2-like" substances takes place in an aqueous environment without contact with the organism or its inflammatory cells. The release is temperature, pH and time dependent. Interestingly, the lipid mediators- measured by cross-reactivity with LTB_4- reach the maximum release much faster than, for example, Bet v1. Prostaglandins and leukotriens are metabolities of arachidonic acid that are not present in birch pollen (30). Other unsaturated fatty acids, like linoleic and linolenic acid, are major components of membrane fatty acids in plants. By HPLC and GC analysis, high amounts of linoleic (LeA) and linolenic acid (LA) and its octadecanoid metabolities (9-, 12-, 13-, 16- HOTE, 9-, 13- HODE) were identified in lipid extracts (hexan-isopropanol extracts from whole pollen) from birch and grass pollen. Notably, LeA and LA as well as their auto-

oxidative products were found in aqueous pollen extracts, whereas lipoxygenase mediated enzymatic products of LeA and LA were minor in birch and even absent in grass. Interestingly, a distinctive and reproducible pattern of monohydroxylated products was observed for both grass and birch pollen extracts. Whether definable lipids function in the variable and complex reproductive process at stigmatic surfaces remains to be determined. However, their very rapid release makes them ideal candidates in these pollen-stigma negotiations. In fact trienoic fatty acids are invariably abundant in membranes of photosynthetic eukaryotes. Conservation of the high trienoic content of eukaryotes membranes throughout evolution is evidence of their importance. McConn et al. (31) described the critical requirement of trienoic fatty acids for pollen development. Using *Arabidopsis* mutant lines containing <0.1 % trienoic acid, they observed the development of tricellular but unviable, male sterile pollen. Another essential requirement for unsaturated acids such as linolenic acid in the plant life cycle appears to be a substrate of the octadecanoid pathway, which produces signaling molecules such as jasmonic acid (31). In *Arabidopsis* mutants that are defective in long-chain lipids, the pollen grains fail to hydrate on the stigma (31). This failure is interpreted as evidence that lipids in the pollen coat are involved in the cell-cell recognition required for hydration (12, 13). Furthermore Wolters-Arts et al. (32) could show that unsaturated lipids are required for directional pollen tube growth during the fertilization of the ovules of flowering plants (9, 11).

1.6. Phytoprostanes

Free radical- catalyzed oxidation of arachidonate has been shown to lead to a complex isomeric mixture of prostaglandin-like compounds termed isoprostanes. Isoprostanes have attracted considerable attention since it has been shown that isoprostanes are formed in mammals at concentrations that exceed the levels of enzymatically formed prostaglandins by at least an order of magnitude (33). Furthermore, isoprostanes are dramatically induced

by oxidative stress (34) and have been shown to be biologically active in the low nanomolar range. Therefore, it has been postulated that isoprostanes represent mediators of oxidative cell injury (35). Since isoprostanes F_2 represent chemically stable end products of lipid peroxidation, they have found to be extremely reliable and sensitive markers of oxidative stress in vivo (36). Higher plants generally do not synthesize the precursors arachidonate required for isoprostane formation, but rather utilize α-linolenic acid (the predominant polyunsaturated fatty acid in plants) for the formation of isoprostanoids and prostaglandin-like mediators of the jasmonate-type (30). Linolenic acid (C18:3) can undergo free radical-catalyzed oxidation reactions similar to arachidonate (C20:4) (37, 38) to yield C18 isoprostanoids. Because the isoprostanoids derived from linolenate are two methylene groups shorter than those from C20 acids they have been previously termed dinor isoprostanes (30, 39). However, C18 compounds derived from β-oxidation of isoprostanes (C20) in animals are also termed dinor isoprostanes, but differ from the plant dinor isoprostanes by the length of the two side chains (40). To avoid confusion of terminology, the plant prostanoids have been referred to as phytoprostanes. The phytoprostane pathway theoretically yields several classes of phytoprostanes denoted with letters that specify the prostaglandin ring system according to the prostaglandin/isoprostane nomenclature (30, 41). Each class represents a complex isomeric mixture that consists of two regioisomers (type I and type II). In addition, each regioisomer is theoretically comprised of 16 stereomers. Recent results suggest that phytoprostanes might have an evolutionary ancient function in plant host defense (42, 43). Whereas the physiological role of phytoprostanes in the life cycle of plants is just beginning to emerge, virtually nothing is known about their effects on the human immune response in health and disease. The identification of phytoprostanes in pollen grain and their effect on the innate and adaptive immune system is part of this scientific work.

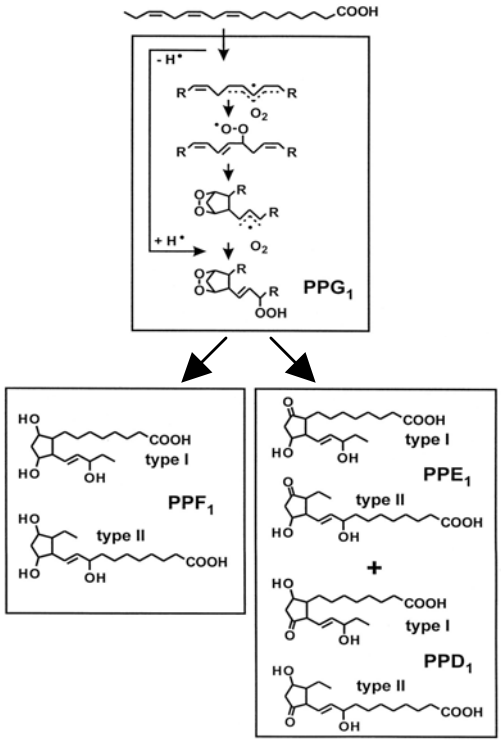

Figure 3: *Synthesis of Phytoprostanes*. Phytoprostanes are formed via autooxidation, which is initiated by free radical attack of α-linolenic acid yielding a linolenate radical that readily oxidies and cyclizes to two regioisomeric, prostaglandin G-like compounds. In vivo, PPG_1 may be either reduced to phytoprostanes F_1 (PPF_1) or converted to PPE_1 and to PPD_1. PPE_1 itself may be dehydrated and isomerised to PPB_1.

1.7. T cell biology

Efficient responses to different types of pathogens require different mechanisms of immunity. Similarly, for optimal effectiveness and minimal damage to local tissues, the character of the response must be modified to suit particular body compartments (44). The selection of effector immune functions is controlled by antigen (Ag)- specific T helper (Th) cells, which secrete Th1- and Th2- type cytokines at different ratios. Th1-type cytokines, e.g. interferon-γ (IFN-γ) promote cellular immunity by activating cytotoxic and phagocytic functions in effector cells, such as cytotoxic T lymphocytes (CTLs), natural killer (NK) cells and macrophages. In fact, Th1 cytokines induce delayed-type hypersensitivity (DTH)

reactions and IFN-γ is commonly expressed at sites of DTH reactions (45, 46). Interleukin-4 (IL-4), a Th2-type cytokine, supports B- cell production of Ag-specific antibodies, the isotype of which is determined by the cytokine cocktail produced by Ag-specific Th cells (47). Th2 cytokines encourage particularly IgE responses (48), and also enhance eosinophil proliferation and function. Accordingly, Th2 cytokines are commonly found in strong antibody and allergic responses. The characteristic cytokine products of Th1 and Th2 cells are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype. Thus, IFN-γ selectively inhibits proliferation of Th2 cells, and IL-10 inhibits cytokine synthesis by Th1 cells (49).

This cross-regulation may partly explain the strong biases towards Th1 or Th2 responses during many infections in mice and humans. In several cases, alteration of these patterns by cytokine or anti-cytokine reagents reverses host resistance or susceptibility to infection. Thus, there is ample evidence that these cytokine patterns are important in mediating resistance to several infectious agents (50).

The factors responsible for the polarization of the specific immune response into a predominant Th1 or Th2 profile have been extensively investigated in mice and humans. Strong evidence suggests that Th1 and Th2 cells do not derive from distinct lineage but rather develop from the same Th-cell precursor under the influence of environmental and genetic factors acting at the level of antigen presentation (51).

Among the environmental factors, a role has been demonstrated for the route of antigen entry, the physical form of antigen, the type of adjuvant and the dose of antigen (51). The genetic mechanisms that occur in controlling the type of Th-cell differentiation remain elusive. The environmental and genetic factors influence Th1/Th2 differentiation mainly by determining the predominance of a given cytokine in the microenvironment of the responding Th cell. The early presence of IL-4 is the most potent stimulus for Th2 differentiation, whereas IL-12 and IFNs favor Th1 development (52).

The past decade has seen the discovery of several T cell populations that appear to have major regulatory effects on T cells responding to both self-antigens and those derived from infectious agents. As is frequently the case, these regulatory T cell populations were discovered first in experimental animal models and subsequently identified in humans (53). They can broadly be divided into T cells that appear to require antigen-specific, MHC-restricted stimulation, with subsequent secretion of cytokines that down-regulate immune responses- the prototypic Tr1 and Th3 cells, which secrete IL-10 and TGF-β respectively (54)- or more innate regulatory cells that do not appear to require an in vivo "adaptive" immunization to observe their function- the CD1-restricted NKT cells with invariant TCRs, "nonclassical" NK T cells with variant TCR (55), and the $CD4^+$ $CD25^+$ regulatory T cells- T reg- (56). The current view is that $CD25^+$ T reg originate from the thymus as a distinct T cell lineage involved in the maintenance of tolerance to self Ags (57). $CD25^+$ T reg constitute 5-10% of peripheral blood CD4+ T cells, express high levels of CTLA-4, and are naturally anergic *in vitro* to mitogens (58). Upon TCR triggering, $CD25^+$ T reg inhibit $CD4^+$ and $CD8^+$ T cell proliferation and IL-2 release in an Ag- and cytokine-independent, but cell-to-cell contact-dependent fashion (59). $CD25^+$ T reg may originate also in peripheral lymphoid organs and regulate immune responses to environmental Ags (69). Moreover, $CD25^+$ T reg can regulate immune responses to microorganisms and transplantation Ags (61). Interestingly, a conspicuous number of circulating $CD25^+$ T cells co-expresses the skin-homing receptor cutaneous lymphocyte associated Ag (CLA), thus indicating their possible engagement in skin immunity (62).

1.8. Dendritic cells

Paul Langerhans first described dendritic cells (DCs) in human skin but thought these were cutaneous nerve cells (63). Steinman and Cohn (64) discovered these cells almost a century later in mouse spleen and applied the term "dendritic cells" based on their unique

morphology. For almost two decades, however, proving the specialized properties of these cells for initiating immunity required depletion of other cells like monocytes, macrophages and B cells that also presented Ag. Potent immunostimulatory activity was enriched in the remaining DCs. The paradigm emerged that Ag capture segregated to immature, peripherally distributed DCs, with Ag presentation and lymphocyte stimulation being an aquired property of mature DCs in secondary lymphoid organs. The essential and pivotal role of DCs in the onset of immunity was thus established.

Progress in the study of DC biology exploded in the 1990s. Investigators developed cytokine-driven methods for expanding and differentiating cytokine-driven DCs *ex vivo* in both mouse and human system (65-67), and further refinements continue to emerge (68-70). For the first time, sufficient numbers of DCs became accessible for large-scale study and applications. Dendritic cells are the single most central player in all immune responses, both innate and adaptive. DCs are exceptionally potent immunogens under inflammatory conditions, yet are also critical to the induction and maintenance of self-tolerance in the steady state (71). The heterogeneity of DCs and their activation states afford investigators more opportunities to define and manipulate the immune response using these specialized leucocytes. Human DCs are all bone marrow-derived leukocytes (72). They comprise at least four types defined under cytokine-driven conditions in vitro. These include conventional or “myeloid” DCs (66, 67, 69): 1) CD14+ blood monocytes-derived DCs (mo-DCs); 2) dermal DCs or interstitial DCs (DDC-IDCs); 3) Langerhans cells (LCs); and 4) plasmacytoid DCs (73). A trace population of DCs also circulates in human blood. Those DCs are lineage negative, CD11c+, CD86+ and HLA-DR bright and express CD83 after activation by brief overnight culture (74). Although not identical, they share many phenotypic and immunostimulatory features with cytokine-generated conventional DCs *in vitro*. Schakel et al. identified a unique subpopulation of human DC circulating in blood that account for 0.5-1% of blood leukocytes only; their most specific characteristic is

the expression of a cell surface protein recognized by a novel monoclonal antibody (M-DC8) which enables their isolation by a one-step immunomagnetic procedure. The isolated cells (>97% pure) present morphologically as typical dendritic cells. They express the Fc (gamma) RIII (CD16), so far not found on DC and avidly phagocytose latex beads as well as opsonized erythrocytes. These cells not ony present antigens efficiently to naive T cells but also induce purified CD8+ T cells to become alloantigen-specific cytotoxic cells (75). DCs are also a major component of lymphoid tissues, particularly the T cell areas. In the case of lymph nodes, DCs access these areas via afferent lymphatics. Investigators now know that most DCs in lymphoid organs are immature or semimature in the steady state and efficiently process self-Ags to induce and maintain tolerance (76). Under inflammatory conditions, however, DCs undergo a complex maturation process. The specifics of maturation vary with the stimulus, but the result is stimulation of innate and adaptive immunity. DCs in lymphoid tissues include populations termed “conventional” or myeloid, as well as “plasmacytoid”, the latter named because of their morphological resemblance to plasma cells (73).

Our work focused on human DCs generated in vitro with cytokines, because these have yielded ample numbers and purity for experimental and clinical evaluations. The precursors of DC progeny generated *in vitro* with cytokines should approximate resident populations of DCs that exist in vivo under steady-state conditions. The cytokine-driven DC progeny are induced populations and hence would not be found *in vivo* except under inflammatory conditions.

1.9. Allergen Uptake and Processing

At the interface of environment and organism, resident dendritic cells are in a functional immature state that is specialized to capture and process antigen.

DCs use phagocytosis, endocytosis, pinocytosis, and receptor- mediated endocytosis involving clathrin- coated pits to capture microbial pathogens, dead or dying cells, immune complexes, and other Ags for immune presentation (77). Immature dendritic cells display a large panel of cell receptors for patterns associated with foreign antigens, such as the C-lectin carbohydrate receptors (eg, mannose, Langerin, DEC205, DC-SIGN) (78). These pattern recognition receptors facilitate antigen capture and uptake and lead to an increased effectiveness in antigen presentation. In addition, dendritic cells express complement and Fc receptors that mediate capture of opsonized or antibody-bound antigens during primary and secondary antigen exposure. Ingested antigen is cleaved into peptides by proteolitic enzymes within the endocytic compartment and loaded onto newly synthesized major histocompatibility complex class II (MHC II) molecules within the acidic MHC II compartment or onto preformed MHC II molecules that have been internalized from the cell surface into less acidic endosomal vesicles. Recent reports indicate that, at least in vitro, some of the antigen processing by dendritic cells may also occur extracellularly through secretory proteases. Our current understanding is mostly based on studies using model antigens, while there is still very little data available on the mechanisms involved in uptake and processing of allergens. Recent in vitro data indicate that recombinant allergens, such as rPhl p1 or rBet v1, are primarily ingested via macropinocytosis, a mechanism that may be of particular relevance during primary sensitization. In already sensitized individuals, uptake is most likely mediated by receptor-mediated endocytosis, eg, internalization of IgE- bound allergen via the high- affinity IgE receptor that targets the allergen to the MHC II compartment.

1.10. Activation and Maturation of Dendritic Cells

DCs require some form of terminal maturation to become fully immunogenic. We now know that there are many different environmental stimuli that can mature DCs in

different ways. All of the various lymphocyte responses require activation by mature DCs, so that DC maturation is a pivotal event in the control of innate and adaptive immunity. Maturation also ensures that DCs do not revert to a less mature and less immunogenic form. A key event in the induction of primary immune responses is the migration of allergen-loaded dendritic cells from the periphery to the regional lymph nodes where they initiate systemic immune responses activating those allergen-specific T lymphocytes. The inflammatory mediators that induce this migration to areas where naïve T cells are located also convert DCs from an antigen-capturing mode into a T cell-sensitizing mode. In fact, DCs display a unique capacity to activate naïve T cells and induce the polarization of the ensuing immune response toward a T helper 1 (Th1) or a Th2 phenotype.

Local activation of immature dendritic cells is best understood in the context of pathogen-induced responses. Dendritic cells are equipped with pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs), that discriminate and are triggered by different pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), lipoteichoic acid, flagellin or bacterial DNA. While other receptors primarily mediate pathogen uptake (eg, lectins), ligation of TLRs leads to signaling that results in the activation and maturation of dendritic cells.

Activated DCs transiently increase the intensity of their Ag uptake involving increased surface expression of peptide/MHC complexes and terminate this process after several hours. This confers a relative selectivity of Ag uptake, restricting it to the site and the moment of pathogen entry. Simultaneously, activated DCs move towards draining lymph nodes, up-regulating the expression of co-stimulatory molecules such as CD40, CD80 and CD86, which allows them to prime naïve Th cells (77, 78). Upon arrival in the lymph nodes, mature DCs translate the tissue-derived information into the language of Th cells, providing them with an Ag-specific “signal 1” and a “signal 2”. Signal 1, the triggering of the T-cell receptor (TCR) by pathogen-derived antigenic peptides bound to major

histocompatibility complex (MHC) molecules, indicates the molecular identity of the pathogen. The co-stimulatory signal 2 comprises contact-dependent and humoral signals, and transmits the information about the DC-activating property of the pathogen, reflecting its pathogenic potential. The combination of signal 1 and signal 2 results in Ag-specific activation of naïve Th cells and their development into effector/memory cells. Accumulating data suggest that migrating DCs not only carry antigenic and costimulatory signals, but also are well equipped to transmit an additional signal 3 from the periphery to the lymph nodes consisting in the production of cytokines, such as IL-12, IL-18, and IL-10, thereby contributing to the initial bias of naïve Th cells towards Th1 or Th2. Since Th2 effector lymphocytes play a critical role in orchestrating allergic inflammation, allergists have become increasingly interested in the mechanisms that regulate dendritic cell biology and the factors that control the dendritic cells-mediated induction of Th2- dominated immune responses.

1.11. APC-related factors affecting the Th-cell polarization

IL-4 originating from CD4+ T cells and possibly also other sources such as mast cells, is the best known example of an APC-independent factor with a potent impact on the cytokine profile of Th cells (80). An impressive large proportion of the known Th1/Th2-polarizing mechanisms are related to APCs. The extent of TCR ligation by antigenic peptide-MHC complexes on APCs, as well as the signals provided by the interactions of CD80 and/or CD86, intracellular adhesion molecule 1 (ICAM-1) and or ICAM-2, and OX40 ligand (OX40L) with their respective T-cell counter-receptors, CD28, LFA-1 and OX40, represent the principal contact-dependent factors (81). Unfortunately, the mechanisms of their action are still far from clear and the available reports are sometimes contrasting. An apparently better defined role in Th-cell differentiation is played by soluble factors. The best explored factor within this group is IL-12, which currently appears to be the most crucial APC product, during the development of naïve precursors into Th1 cells (82-83). IL-12 is a heterodimer formed by a 35-kDa light chain (known as p35 or IL-12α) and a 40-kDA heavy chain (known as p40 or IL-12β). The two genes are unrelated and located on separate chromosomes both in humans and mice (84). Its potent and non-redundant role in the development of functional Th1 responses was demonstrated by studies in IL-12 or signal transducer and activator of transcription 4 (STAT4)- deficient animals (85) and in humans lacking a functional IL-12 receptor (85). A similar Th1-deficient phenotype can be found in mice lacking IL-18. However, IL-18 appears to act mainly as a factor supporting ongoing Th1 responses, rather than inducing Th1 differentiation in the naïve Th-cell population (80). Interestingly, a part of the modulatory activities of several other well-recognized Th1 and Th2-driving factors, such as IFN-γ, IL-4, IL-10, prostaglandin E_2 (PGE_2) and IFN-α is mediated by regulating either the production of, or the responsiveness to, IL-12 (80, 85). In accordance with the potent polarizing activity of IL-12, Ag- presentation by APCs with different abilities to produce IL-12, for instance, B cells

compared with monocytes, macrophages or DCs, results in different patterns of cytokine production in responding T cells (79).

An interesting possibility is that the type of Th-cell responses can also be differentially influenced by DCs of different lineages, such as lymphoid and myeloid DCs. Whereas lymphoid DCs are mainly considered to perform a tolerogenic function (86) due to their ability to induce apoptosis in responsive Th cells (87), myeloid DCs are often regarded as a Th1-driving APC type, as judged by their ability to produce IL-12.

The amounts of IL-12 produced can be regulated either by exogenous factors such as pathogens or endogenously produced factors.

Many intracellular pathogens directly induce IL-12 production (84), whereas some Th2-inducing pathogens are a source of IL-12-inhibiting factors, such as prostanoids produced by some helmints (88) or IL-10 induced by *Schistosoma mansoni* eggs (89). Van der Kleij et al. stimulated dendritic cells with lipid classes derived from *Schistosoma mansoni* eggs and adult worms and found that the fraction containing phosphatidylserine (PS) polarized the maturation of dendritic cells, resulting in Th2 skewing and the development of T regulatory cells (90). Whelan et al. used a phosphorylcholine-containing glycoprotein, ES-62, secreted by the filarial nematode, *Acanthocheilonema viteae* that was found to induce the maturation of DC with the capacity to induce Th2 responses. This pathogen product was chosen because filarial nematodes are parasites that have a propensity to generate a Th2 response *in vivo* and such Th2 responses are considered to be associated with parasite longevity (91). In these cases, a direct modulation might help to select the appropriate effector mechanisms at the site of infection. Other pathogens, such as *Brucella abortus* or CpG-containing bacterial DNA, induce the early production of IL-12 by DCs (84).

Recent *in vivo* and *in vitro* data suggest that the IL-12-producing capacity of DCs can also be modulated indirectly by microenvironmental tissue factors. These factors are either

produced constitutively or mediate innate, Ag-nonspecific tissue responses to pathogens. Among the possible mechanisms of tissue- dependent modulation of the Th1- and Th2-promoting capacity of myeloid DCs, an interesting option is the modulation of their IL-12-producing capacity by inflammatory mediators at different stages of DC development. An insight into the mechanism can be obtained in an *in vitro* model of monocyte-derived DCs (67).

PGE_2 is a common inflammatory product of stromal fibroblasts and epithelia (92) and might serve as an example of one type of modulatory factor.

PGE_2 affects early development of tissue-type immature DCs, inducing IL-12 deficient cells that show the same morphology and stimulatory potential as control immature DCs, but have a distinct Th2- promoting function (93). IL-12- producing capacity can also be modulated during the final maturation of DCs. At this stage, the presence of PGE_2 results in type-2 polarized effector DCs, which produce reduced amounts of IL-12 upon interaction with Th cells and induce a bias towards Th2 cytokine production in naïve Th cells (94). The above effects of PGE_2 are dose dependent at the physiological concentrations of 10^{-9}-10^{-6} M, a wide range found in inflamed and healthy tissues in different body compartments (95). The same pattern of DC modulatory activity is likely to be shared by other cyclic AMP (c-AMP) elevating agents, such as histamine and β-adrenergic agonists (96). IL-10 represents a modulatory factor of another type. Similar to PGE_2, it inhibits IL-12 production in immature DCs but, in addition, it prevents DC development when present at the early stages (97). In contrast to PGE_2, which enhances the stimulatory potential of DCs by promoting their final maturation (98), IL-10 inhibits both the ability of DCs to produce IL-12 (94) and their stimulatory capacity (94, 98). Despite being mainly suppressive, IL-10-exposed DCs have a residual Th2-driving function (99). Their Th2- driving effect may be particularly relevant at later stages of Th-cell responses, when the stimulatory requirements are less strict. A similar, IL-10- like modulatory activity is shared by

glucocorticoids (100). In contrast to IL-10 and PGE_2, the presence of IFN-γ during DC maturation results in the development of type-1 polarized effector DCs with an elevated ability to produce IL-12 and a strong Th1-inducing capacity. Such a type 1 polarization could be induced in tissue- type DCs by local IFN-γ sources, such as NK cells, which are rapidly recruited to the site of viral infection. Although several other factors, including lipopolysaccharide (LPS), poly I:C, or CD40 ligation can induce IL-12 production or elevate its intensity, the ability to induce stable effector DCs that produce enhanced levels of IL-12 after subsequent stimulation appears to be a unique property of IFN-γ.

1.12. Chemokine and Chemokine receptors expression

Chemokines are generally low molecular weight, around 8 kDa, cytokines whose major collective biological activity appears to be that of chemotaxis of leucocytes (101-103). They play a critical role in the directed movement of leucocytes from the bloodstream into tissue as well as localization of cells within tissues. These molecules are almost exclusively secreted and act as extracellular messengers for the immune system. However, emerging data also show that various members of the chemokine gene superfamily exert a range of biological effects beyond chemotaxis, including angiogenesis and haematopoiesis (104). Gene products are assigned to the chemokine gene superfamily according to the organization of a characteristic cysteine signature in their predicted primary amino acid structure. Based on this cysteine motif, the various members can be subdivided into four families (two major and two minor), known interchangeably as either the CXC, CC, C and CX_3C, or the α, β, γ and δ subfamilies, respectively. An alternative system that relates expression to function has recently been developed (105-106). According to this system, the chemokines can be classified based on their functional expression as homeostatic/constitutive or inflammatory/inducible. Homeostatic chemokines are expressed constituvely within lymphoid tissues and appear to be

responsible for movement of thymocytes through the thymus during selection (106, 107) and for the physiological trafficking of cells including lymphocytes and DC into secondary lymphoid organs, under normal conditions or during immune response. Inflammatory chemokines are up-regulated at sites of inflammation, and play a key role in the recruitment of effector leucocytes to peripheral tissues in response to immunological challenge (108). There is also evidence that the maintenance of resident immune surveillance cells in peripheral tissues is regulated by low- level basal expression of some homeostatic and some inducible chemokines.

Chemokines exert their biological effects on target cells via ligation of cell surface receptors (109). All chemokine receptors identified to date are G protein-coupled seven transmembrane domain receptors (GPCR). The remarkable feature of the chemokine receptor superfamily, however, is their promiscuity as far as ligand binding is concerned. Relatively few receptors that bind only one ligand have been identified. Having a wide range of chemokine ligands and receptors probably allows our immune system to overcome microbial evasion strategies. Chemokine receptors are also defined as either homeostatic or inflammatory, and this classification is essentially based on function rather than function and expression, as is the case with the chemokine ligands. Migration of dendritic cells from the site of antigen capture to secondary lymphoid organs is a crucial event in the initiation and amplification of immune responses. Trafficking of DCs through tissues is regulated by the pattern of chemokine receptors expressed on DCs and the local availability of chemokines. Chemokines regulate leukocytes trafficking by inducing firm integrin-dependent adhesion of blood leukocytes to endothelial cells and by inducing directional migration (chemotaxis). Immature DCs and monocytes express receptors for inflammatory chemokines (CXC chemokine receptor [CXCR] 1, CC chemokine receptor [CCR] 1, CCR2 and CCR5), which account for the capacity of these cells to migrate to inflamed tissues where cognate ligands are produced. Maturation of DCs is associated

with the coordinated down-regulation of receptors for inflammatory chemokines and the induction of lymphoid chemokine receptors such as CXCR4, CCR4 and CCR7 (110). Thus, maturing DCs become responsive to lymphoid chemokines such as CXC ligand (CXCL) 12 (stromal-derived factor [SDF] 1), CC ligand (CCL) 2 (macrophage-derived chemokine [MDC] and CCL19 (macrophage inflammatory protein [MIP] 3 β) and they co-localize with naïve T cells in secondary lymphoid organs. DCs are also a relevant source of chemokines. Immature DCs constituvely release CCL22 and CCL17 (thymus- and activation- regulated chemokine [TARC]) (111). At early stages of maturation, DCs produce high levels of inflammatory chemokines, such as CCL2 (monocyte chemoattractant protein 1 [MCP-1]), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (regulated upon activation, normal T-cell expressed and secreted [RANTES] chemokine), CXCL-8 (interleukin-8 [IL-8]) and CXCL-10 (interferon-inducible protein 10 [IP-10]), that sustain the recruitment of circulating immature DCs, DC precursors and T cells to inflamed tissues (111). Lymphoid chemokines, including CCL-19, CCL17 and CCL22 are produced or upregulated later during DC maturation (109), providing chemotactic signals for mature DCs and for T cells in secondary lymphoid organs. Recently there has been significant progress in characterization of chemokine receptors expressed by T cells at various developmental stages and functional status: naïve T lymphocytes express a number of chemokine receptors, including CCR7 and CXCR4 which are subsequently lost after initial activation of the lymphocyte (112, 113). These receptors seem to be required for entry into secondary lymph nodes through the high endothelial venule, which express the specific ligands CCL21 and CXCL12 (114). Subsequently, lymphocytes begin to alter their chemokine receptor expression on the basis of the immune environment that develops Th1 or Th2. Lymphocytes that are skewed toward Th1-type responses (IL-12 and IFN-γ) differentially express CXCR3 and CCR5, whereas those skewed toward Th2-type cytokine production (IL-4, IL-5 and IL-13) differentially express CCR3, CCR4, CXCR4 and CCR8

(115). The chemokine receptor profile and migratory properties of regulatory T cells has also been investigated; normally resting Th cells coexpress functional Th1 and Th2 chemokine receptors and migrate in response to a variety of chemokines. Upon activation, Reg T cells are attracted exclusively by CCL1 (I-309), CCL2 (MCP-1), CCL17 (TARC) and CXCL12 (SDF-1) (115). However, these assumptions, taken from in *vitro* analyses, should be used only as a guide, as it is unclear how stable these phenotypes are in vivo.

2 Aim of the study

The majority of studies on the elicitation and manifestation of allergic disease have focused primarily on the composition and function of specific protein allergens. However, individuals are rarely exposed to purified allergens, but rather to allergens in particulate form, either as pollen grains or as pollen derived starch granules. In the context of allergy pollen grains have simply been regarded as allergen carriers, and until recently little attention has been devoted to non-protein compounds of pollen. It has been recently demonstrated that pollen, apart from the allergen, also release factors with potential immunomodulatory capacity namely eicosanoid-like substances.

Prostaglandins and leukotrienes are metabolities of arachidonic acid that is not present in birch and grass pollen. However, it is well known that unsaturated fatty acids, like linolenic and linoleic acid are major components of membrane fatty acids in plants and they can undergo an oxidation process, resulting in a series of isomeric dinor isoprostanes termed phytoprostanes. Recent data showed that linolenic/linoleic acid and their monohydroxylated derivatives act as bioactive lipids on cells of the innate immune system inducing activation and directed migration of human neutrophil and eosinophil granulocytes. These results prompted us to investigate whether water soluble substances in pollen also affected the function of human dendritic cells.

At first, we studied the impact of pollen-derived factors on the phenotypical and functional maturation of monocyte derived DC. Then, concerning the initiation phase of the immune response, we wanted to assess whether pollen derived factors had an impact on the IL-12 production and, consecutively, on the differentiation of type 2 Th cells that are known to be involved in the developing of allergies.

In an attempt to identify potential candidates responsible for the observed effects of pollen-derived factors, we quantified levels of phytoprostanes present in *Bet*.-APE. The structural similarity of α-linolenic acid derived phytoprostanes and prostaglandins

prompted us to analyze whether phytoprostanes identified in *Bet*.-APE showed similar effects on human DC.

In order to investigate the effects of water soluble pollen-derived factors on the amplification of the polarized immune response, we further analyzed whether *Bet*.-APE modulated the chemokine receptor expression and chemokine production in DC.

In fact, we wanted to assess whether *Bet*.-APE exposed DC preferentially induced type 2 Th cell differentiation and attraction, thereby providing a mechanism for the amplification of Th2 immune responses.

3 Materials and Methods

3.1. Reagents and Abs

Human rIL-4 was obtained from Promocell, human rGM-CSF from Essex, soluble CD40L (sCD40L) from Alexis. Purified LPS (*Escherichia coli* K235-derived LPS; <0.008% protein) was provided by Dr. Stephanie Vogel (University of Maryland, College Park, MD). FITC- or PE-conjugated anti–HLA-DR, anti-CD1a, anti-CD86, anti-CD80, anti-CD83, anti-CD1a, anti–IL-4, and anti–IFN-γ mAb were purchased from Becton Dickinson, anti-CD4 and anti-CD45RA microbeads from Miltenyi Biotech. The mAbs PE-conjugated anti-CCR1 anti-CCR5, anti-CXCR4, and anti- CRTh2 were purchased from Becton Dickinson (San Jose, CA). Control mouse or rat IgG were purchased from Becton Dickinson or PharMingen. Human recombinant CCL3 and CCL22 for migration assay were provided by R&D Systems (Minneapolis, MN).

3.2. Preparation of *Bet.*-APE

Birch pollen grains (*Betula alba* L.) were obtained from Allergon. *Bet.*-APE were generated by incubation of pollen grains in RPMI 1640 (30 mg/ml) for 30 min at 37°C followed by centrifugation (20 min at 3,345 *g*) and sterile filtration (0.2 μm; 24). LPS was measured by LAL assay (Cambrex Bio Science). To deplete LPS, *Bet.*-APE were eluted over polymyxin B columns (Pierce Chemical Co.) leading to LPS concentrations below the detection limit of the assay (<0.05 EU/ml). LPS-depleted *Bet.*-APE was used for subsequent experiments.

3.3. Donors

Healthy, non-atopic blood donors were characterized by screening for total and specific IgE for common allergens. Healthy control subject had no history of allergic diseases. In

addition, RAST and skin prick test results for 15 common allergens, including birch and grass pollen, were negative, and total IgE was less than 20 IU/mL. All volunteers were without medication for at least 15 days before blood sampling. The ethical committee of the Technical University of Munich approved the study, and volunteers were enrolled in the study after providing written informed consent. The investigation was conducted according to the Declaration of Helsinki.

3.4. Monocyte-derived DCs

Healthy, nonatopic blood donors were characterized by screening for total and specific IgE for common allergens as described recently. Monocyte-derived DCs were prepared from peripheral blood of healthy individuals, as described recently. In brief, adherent PBMC (>90% pure $CD14^+$ cells) were cultured at 10^6 cells/ml in RPMI 1640 supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Life Technologies) supplemented with 10% FBS, 500 U/ml human rGM-CSF (Essex Pharma) and 500 U/ml human rIL-4 (Promocell; complete DC medium) at 37°C under 5% CO_2. At day 5 cells (>95% $CD1a^+$, $CD14^-$) were harvested and recultured in complete DC medium for 24 h at 37°C with or without indicated stimuli in the presence or absence of LPS (100 ng/ml) or soluble CD40L (1 µg/ml; Alexis) followed by addition of a cross-linker (1 µg/ml). Aliquots of DC culture supernatants were assayed for IL-12 p70, IL-6, IL-10, and TNF-α by two site ELISAs using antibodies from BD Biosciences as described previously.

3.5. Flow cytometry of DCs

DCs that were either untreated or stimulated for 48 hours with LPS in the presence or absence of pollen extracts were washed and then incubated in round-bottom 96-well plates in cold phosphate-buffered saline (2% FCS and 0.01% sodium azide) with FITC-

CD83 mAb and PE- CCR5, CCR1, CXCR4 mAbs for 40 minutes at 4^{o}. Matched isotype control mAb were used in control samples. Stained cells were analyzed using a FACS Calibur flow cytometer equipped with CellQuest software (Becton Dickinson).

3.6. DC cytokine release

Aliquots of DC culture supernatants were assayed for IL-12 p70 and IL-6 by two site ELISAs using antibodies from BD PharMingen. In brief, 96-well polyvinyl chloride microtiter plates (Dynatech Laboratories) were coated with the appropriate purified capture mAb, 20C2 (anti–human IL-12 p70; BD Pharmingen), or C8.3 (anti–human IL-12 p40; BD Pharmingen). Cytokine binding was then detected with the appropriate biotinylated detection mAb, C8.6 (anti–human IL-12 p40; BD Pharmingen). The readout was then obtained by using streptavidin–horseradish peroxidase conjugate (Amersham Pharmacia Biotech) and a substrate solution containing 548 μg/ml 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS;Sigma-Aldrich) and 0.001% hydrogen peroxide (Ajax Chemicals) in 0.1 M citric acid, pH 4.2, followed by scanning the optical density at 405–490 nm. Samples for each condition were assayed in triplicate.

3.7. Purification of naïve T cells

Human $CD4^{+}$, $CD45RA^{+}$ T cells were purified from non-adherent PBMC from healthy non atopic donors using MACS (Magnetic Cell Sorting) column separators with anti-CD4 and anti-CD45RA microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Non adherent cells were washed carefully, supernatant discarded and cell pellet resuspended in 80 μL per 10^{7} total cells of MACS buffer (PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA, pH 7.2). Additionally cells were labeled by adding 20μl CD4 Microbeads then mixed well and incubated for 15 minutes in the refrigerator at 6°-12° C. Then labeled cells were washed and resuspended in 1mL of MACS buffer

according to the positive selection column type. Once the column was placed in the MACS separator and prepared by washing with appropriate amount of MACS buffer, cell suspension was applied to the column and the negative cells started to pass through it. At the end of the positive selection, the column was washed with the appropriate amount of MACS buffer, removed from separator and placed on a suitable collection tube. Then 5 mL of MACS buffer were pipetted to the column in order to flush out the positive fraction using the plunger supplied with the column. Afterwords $CD4^+$ T cells were washed, counted and a second magnetic labeling with anti-$CD45RA^+$ Microbeads was carried out in order to obtain the fraction of $CD4^+$ cells which were also positive for $CD45RA^+$ using the same procedure described above. A sample was removed in order to analyse the separation by flow cytometry and more than 98% pure $CD4^+CD45RA^+$ T cells were obtained. They were resuspended in RPMI supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Life Technologies, Chagrin Falls, OH;) and 0.05 mM 2-ME (Merck, Darmstadt, Germany; complete RPMI) complemented with 5% human serum and 100µL of cell suspension at $1x10^6$ cell/mL were plated in 96-well flat bottom plates for the mixed leucocyte reaction as described below.

3.8. Mixed leukocyte reaction - MLR

Human $CD4^+$, $CD45RA^+$ T cells were purified from nonadherent PBMC from healthy nonatopic donors using magnetic cell sorting column separators with anti-CD4 and anti-CD45RA microbeads (Miltenyi Biotec). Differently stimulated DCs (24 h) were washed and cocultured with magnetic cell sorting–purified allogeneic naive $CD4^+$, $CD45RA^+$ T cells (10^5 cells/well) in complete RPMI with 5% human serum. Cell proliferation was quantified using a BrdU cell proliferation ELISA (Amersham Biosciences). To analyze T cell polarization, DC/T cell cocultures were incubated in a 96-well plate at a DC/T ratio of 1:4 and T cells

were subsequently expanded in 24-well plates in medium supplemented with IL-2 (20 U/ml; Chiron Corp.). LPS-activated DCs (24 h) were harvested, washed twice, and used for priming to generate Th1-polarized T cells ("Th1 control"). Th2-polarized T cells ("Th2 control") were generated by using DCs that were activated (24 h) with LPS in the presence of PGE_2 (10^{-6} M; Qbiogene). In addition, neutralizing anti-IL-12 mAb (10 μg/ml; BD Biosciences) was added at the beginning of the DC/T cell coculture in order to generate a maximal Th2 polarization.

3.9. Establishment of T cell lines

In order to analyze T-cell polarization, T-cell line were generated by coculturing the differently stimulated DCs along with freshly isolated naive T cells in a flat bottom 96-well plate at a DC:T ratio of 1:4 in complete RPMI 1640 complemented with 5% human serum. In order to get a positive control for type 1 cell polarization 10μg/mL anti IL-4 mAb were added at the beginning of the coculture to the naive T cells primed with LPS plus IFNγ stimulated-DCs. The positive control for type 2 cell polarization was achieved adding 10μg/mL anti IL-12 mAb to the T cells primed with LPS plus PGE_2 exposed-DCs. Starting from day 5 of the coculture, rhu IL-2 20 U/mL was added to the line every second day and T cells were subsequently expanded in 24-well plates in medium supplemented with IL-2 (10-20U/ml, Chiron, Munich**,** Germany).

3.10. Intracellular cytokine staining

After 12 d of culture, T cells were restimulated with PMA (20 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) for 6 h and examined for intracellular IFN-γ and IL-4 accumulation. To prevent cytokine secretion, Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the final 2 h. T cells were fixed (2% paraformaldehyde), permeabilized (0.5%

saponin), and stained with FITC-conjugated mouse anti–IFN-γ and PE-conjugated rat anti–IL-4 mAb or isotype-matched control mAb and analyzed by flow cytometry as described previously.

3.11. Release of chemokines from DCs

Cell-free supernatants from DCs were tested for chemokines content by enzyme-linked immunosorbent assay (ELISA). CCL5 (RANTES) and CCL17 (TARC) were measured by using ELISA kit Duo Set from R&D Systems. A 96- well microplate was coated with 100μL per well of diluted corresponding Capture Antibody and incubated overnight at room temperature. Next, the excess of antibody was removed, and the plate was washed and incubated with 300μL of blocking reagent for 1 hour at room temperature. Then 100μL per well of cell- free supernatants and standards appropriately diluted were added to the plate for two hours at room temperature followed by a second 2 hours incubation at room temperature with the detection antibody appropriately diluted. The readout was then obtained by using streptavidin–horseradish peroxidase conjugate and the optical density was determined subtracting the wavelenght readings at 570 nm from the readings at 450 nm. CCL22 (MDC) was assayed by using Quantikine Immunoassay from R&D Systems as descibed below. In a pre-coated 96- well microplate, 100 μL of assay diluent included in the assay kit were added to each well. Next, the plate was incubated with 100μL of standards and samples for two hours at 2° -8° C. After extensive washing of the plate, a second incubation with 200μL of Conjugate antibody was performed for 2 hours at 2° -8° C. After the plate was washed, substrate solution was added and the cells were incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 1 *M* sulphuric acid, and the optical density of each sample was measured at 450 nm.

CXCL10 (IP-10) was measured using an OptEIA ELISA kit from BD Pharmingen. A 96- well microplate was coated with 100μL per well of diluted Capture Antibody and

incubated overnight at 4° C. After several washes, the microplate was incubated with 200μL of blocking buffer for 1 hour at room temperature. Further washing steps were followed from the adding of 100μL of standards and supernatants to each microwell for two hours at room temperature. The colorimetric reaction was developed by incubating the plate with a working detector solution (detection antibody plus streptavidin HRP reagent) for 1 hour at room temperature. After the plate was washed, the substrate solution was added to each microwell for 30 minutes at room temperature. The reaction was stopped by the addition of 1 *M* sulphuric acid, and the optical density of each sample was measured at 450 nm. The plates were analyzed in an ELISA reader (Thermo, Labsystems). Samples for each condition were assayed in triplicate.

3.12. T cell clones

Polarized Th1 and Th2 $CD4^+$ T cell clones were generated respectively from $CD4^+$ purified and polarized Th1 and Th2 T cell lines by limiting dilution (0.7 cells/mL) in the presence of 3.5 x 10^5 cells/well, PBMC previously treated with 10μg/mL mitomycin (Sigma), 30 U/mL IL-2 and 1μg/mL phytoemagglutinin (PHA; Sigma) in complete RPMI 1640 plus 10% FBS, 5% human serum. Clones were grown in the presence of IL-2 and periodically stimulated with 1% PHA and feeder cells or plate-coated anti-CD3 and soluble anti-CD28 (both at 1μg/mL; BD Pharmingen). Phenotype was assessed by flow cytometry.

3.13. Flow cytometry analysis of T lymphocytes

Phenotype and chemokine receptor expression of freshly isolated $CD4^+$ $CD45RA^+$ T cells was assessed by flow cytometry. Chemokine receptor expression was analyzed again after 10 days of coincubation with differently stimulated DCs. $CD4^+$ T cell clones phenotype and chemokine receptor expression was also assessed on both resting and

activated T cell clones. T cells were washed and then incubated in round-bottom 96-well plates in cold phosphate-buffered saline (2% FCS and 0.01% sodium azide) with CD4-FITC mAb and CCR5-, CXCR3-, CCR4-, CRTh2-PE mAbs for 40 minutes at 4°C. Isotype-matched control IgG was used in control samples. Stained cells were analyzed using multicolor flow cytometry and CellQuest software (FACS Calibur, Becton Dickinson, Mountain View, CA) equipped with CellQuest software (Becton Dickinson).

3.14. Migration assay

Chemotaxis of T cells was evaluated by measuring their migration through 5-μm-pore polycarbonate filters in 24-well transwell chambers (Corning Costar, Cambridge, MA). The chemotactic property of DCs supernatants was evaluated by adding 10^5 T cells suspended in complete RPMI with 0.5 % bovine serum albumin (BSA, Sigma) to the top chamber and various dilutions of the supernatants (0.6 mL) to the bottom chamber of the transwell. After 1 hour of incubation at 37° C with 5% carbon dioxide, cells that transmigrated into the lower chamber were recovered and acquired with a FACS Calibur device for 60 seconds at a flow rate of 60 μL/min. Data acquisition and analysis were restricted to events with the forward and side scatter properties of cells and not cell debris. Results are shown as the migration index, which represents the ratio between cells migrated to the lower chamber in the presence of the chemotactic substance and cells migrated in response to the medium alone.

3.15. Quantitative mRNA Analysis

Total RNA was extracted from purified DC after 6h incubation with the indicated stimuli using peqGOLD RNAPure buffer (Peqlab, Erlangen, Germany). Cells were

resuspended in 1mL of lysis buffer and 0.2mL of chloroform was further added. Next the cell lysate was centrifuged for 20' at 14.000 rpm, at 4°C. Following centrifugation, RNA remained exclusively in the aqueous phase, and 0.5mL of isopropil alcohol was added and further centrifugation was performed at 14.000 for 15' at 4°C. After centrifugation, the RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. Once removed the supernate, RNA was washed with ethanol 75% and then dried and dissolved in Rnase-free water. RNA was reverse transcribed using specific primers (Applied Biosystems). PCR-reactions (Assay on demand, Applied Biosystems, Darmstadt) were run on an ABI PRISM® 7700 Sequence Detection System device (Applied Biosystems Division of Perkin Elmer, Foster City, CA) using the following program: 10 min at 94 °C followed by 40 cycles of 15 s at 95 °C, and 60 s at 55 °C. 18s RNA served as house keeping gene.

3.16. Analysis of PPE_1, PPA_1/PPB_1 and PPF_1 by NCI GC-MS

Aqueous extracts (20 ml) of birch pollen (200 mg) were treated with 10 ml of a saturated NaCl solution in water containing 0.05% butylated hydroxytoluene (wt/vol), 20 mg of triphenylphosphine, 0.2 ml of 1 M citric acid, and isotopically labeled phytoprostane standards. Phytoprostanes were extracted with diethyl ether, purified, derivatized, and analyzed by NCI GC-MS as described previously.

3.17. Preparation of PPE_1, PPA_1/PPB_1 and PPF_1

Racemic E_1- and F_1-phytoprostanes were prepared by autoxidation of α-linolenic acid and purified as described previously. B_1-Phytoprostanes were obtained by base-catalyzed isomerization of E_1-phytoprostanes.

3.18. Statistic

Student's paired *t* test was used to compare groups and ratios of IL-4– or IFN-γ–producing T cells induced by differently stimulated DCs. $P < 0.05$ was considered significant.

4 Results

4.1. Induction of phenotypical and functional DC maturation by *Bet*.-APE

To investigate the impact of soluble factors released from pollen on the function of human DCs, immature monocyte-derived DCs were exposed to *Bet.*-APE and phenotypical and functional DC maturation was analyzed. Analysis of *Bet.*-APE by *Limulus* amebocyte lysate (LAL) test revealed substantial quantities of LPS (ranging from 10 to 500 EU/ml). Elution over polymyxin B columns allowed efficient removal of LPS (<0.05 EU/ml). LPS-depleted *Bet.*-APE was used for all subsequent experiments. Functional maturation of DCs was investigated analyzing the allostimulatory capacity of DCs in MLR. Exposure of immature DCs to *Bet*.-APE alone resulted in an enhanced proliferative response of allogeneic naive T cells (Fig.4). Furthermore, simultaneous DC stimulation with *Bet*.-APE and LPS appeared to cause additive effects (Fig.4). Exposure of immature DCs to *Bet.*-APE alone induced an up-regulation of HLA-DR surface expression, whereas the remaining maturation markers (CD40, CD80, CD83, and CD86) remained unchanged (Fig.5). When DCs were stimulated simultaneously with LPS plus *Bet*.-APE, the presence of *Bet.*-APE resulted in an additional up-regulation effect of CD80, CD86, and HLA-DR surface expression (Fig.5).

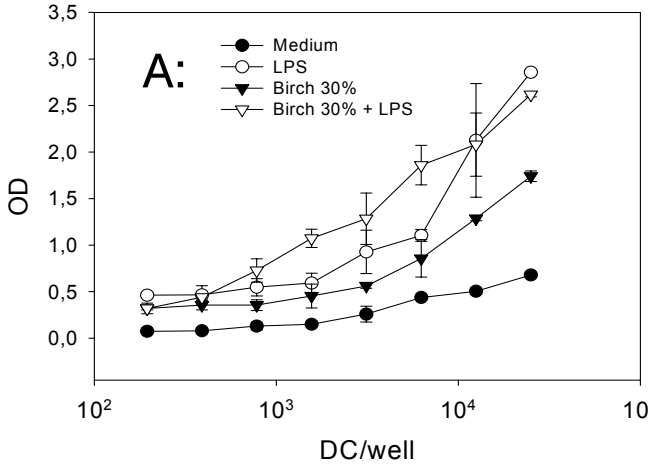

Figure 4: *Bet.-APE induce DCs allostimulatory activity***.** (A) Immature DCs were left untreated (medium) or stimulated with LPS (100ng/ml), Bet.-APE (3mg/ml) alone, or together with LPS (100ng/ml). After 24h, DCs were analysed for their capacity to induce T cell proliferation in naïve allogeneic $CD4^+$ $CD45RA^+$ T lymphocytes (10^5/well). T cell proliferation was measured by BrdU incorporation after 3 d of coculture. Results are given as mean OD±SD of triplicate cultures and are representative of five independent experiments from different nonatopic donors.

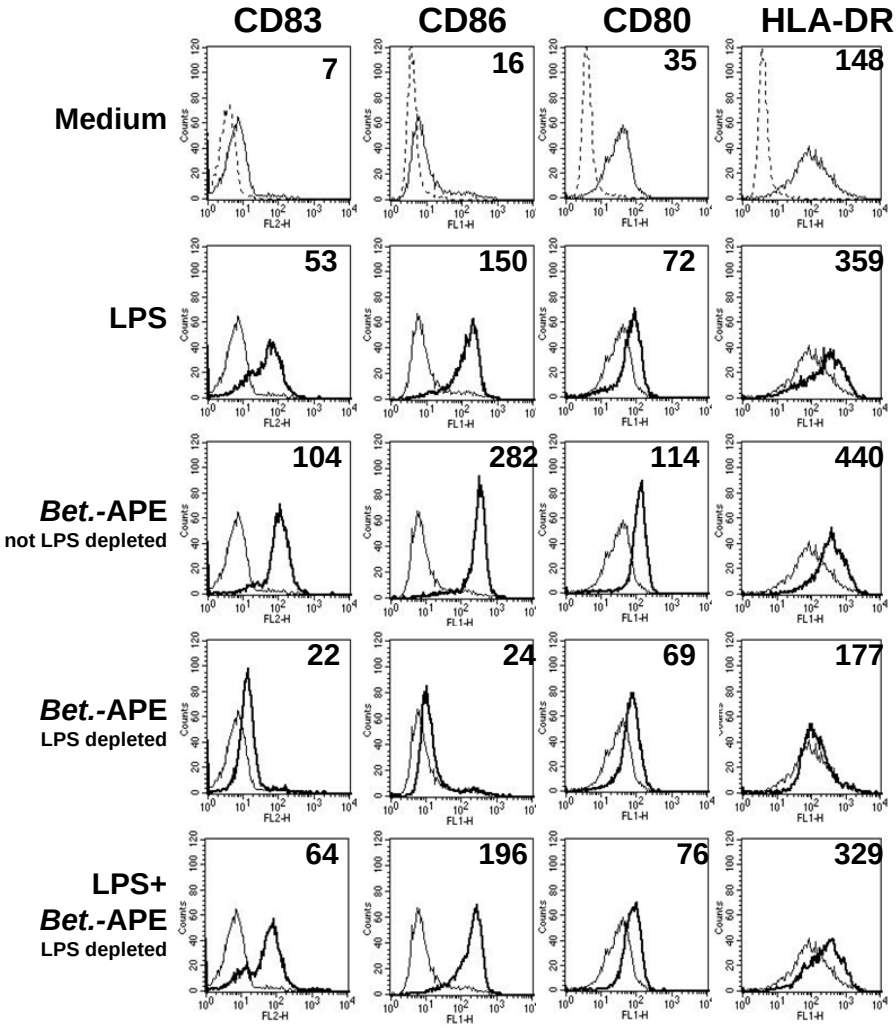

Figure 5: *Bet.-APE effects on DC maturation*. Immature DCs were left untreated (medium), stimulated with LPS (100ng/ml) or LPS-depleted *Bet*.-APE (1mg/ml), or simultaneously with both stimuli. Surface marker expression was analysed after 24 h using flow cytometry. Medium control DCs (thin line) served as reference for all other culture condition (bold line). Numbers indicate the net mean fluorescence intensity. Representative experiment of n=5 using DCs from different nonatopic donors.

4.2. *Bet*.-APE inhibits DC IL-12 production

Under control conditions (medium) DCs spontaneously released low levels of IL-12 (43.4 ± 18.4 pg/ml, *n* = 6), IL-6 (393 ± 280 pg/ml), TNF-α (<78 pg/ml, *n* = 6), and IL-10 (11.8 ± 5.3

pg/ml, *n* = 5). LPS stimulation induced an up-regulation of these cytokines 82-, 33-, 40-, and 29- fold, respectively. Interestingly, *Bet.*-APE when added simultaneously with LPS dose-dependently inhibited LPS-induced IL-12 p70 release, although it had no significant effect on basal IL-12 production. In contrast, LPS-induced IL-6, IL-10, and TNF-α release was not affected (Fig.6A). The inhibition of IL-12 p70 was not due to cytotoxic effects as determined by propidium iodide staining. *Bet.*-APE similarly inhibited the IL-12 p70 production when DCs were activated by CD40 ligation. IL-12 is a heterodimeric cytokine, consisting of covalently bound p40 and p35 subunits. Distinct genes encode each subunit and each gene is independently regulated. To investigate the effects of *Bet*.-APE on IL-12 p40 and p35 mRNA expression DCs were stimulated with LPS in the presence or absence of increasing concentrations of *Bet*.-APE. LPS stimulation resulted in a strong induction of IL-12 p40 mRNA ($[2^{\{-CT\}}]$: 1,149 ± 518, *n* = 3) whereas IL-12 p35 mRNA was induced to a lesser degree ($[2^{\{-CT\}}]$: 364 ± 164, *n* = 3). Simultaneous addition of increasing concentration of *Bet*.-APE lead to a dose-dependent inhibition of IL-12 p40 mRNA expression (Fig.6B). In contrast, *Bet*.-APE stimulation seemed to enhance the LPS-induced IL-12 p35 mRNA (Fig.6B). These results suggest that *Bet.*-APE–dependent inhibition of IL-12 p70 release is likely to be regulated at the level of IL-12 p40 mRNA expression. To rule out IL-10 as an autocrine inhibitor of IL-12 production. DCs were stimulated with LPS and *Bet*.-APE in the presence or absence of neutralizing anti–IL-10 mAb (10 µg/ml, R&D Systems). Although IL-10 neutralizing mAb restored the inhibition of IL-12 release induced by exogenous IL-10 (10 ng/m, R&D Systems) back to normal, it did not restore IL-12 production inhibited by *Bet*.-APE (Fig.6C). To exclude effects of endogenous prostaglandins such as PGE_2, DCs were stimulated in the presence of the cyclooxygenase inhibitor indomethacin (25 µg/ml). Inhibition of DC cyclooxygenase did not reverse the inhibitory effects of *Bet*.-APE, suggesting that the observed inhibition was independent of endogenous prostaglandin production (Fig.6D).

Aqueous extracts of pollen grains from a variety of different plants such as alder, hazel, lilac, mapel, and mugwort displayed similar inhibitory activity on LPS-induced DC IL-12 production (Fig.6E), although IL-6 release was not significantly affected, suggesting that the observed immunomodulatory activity is not restricted to birch pollen but rather a more general phenomenon shared by pollen grains from different species.

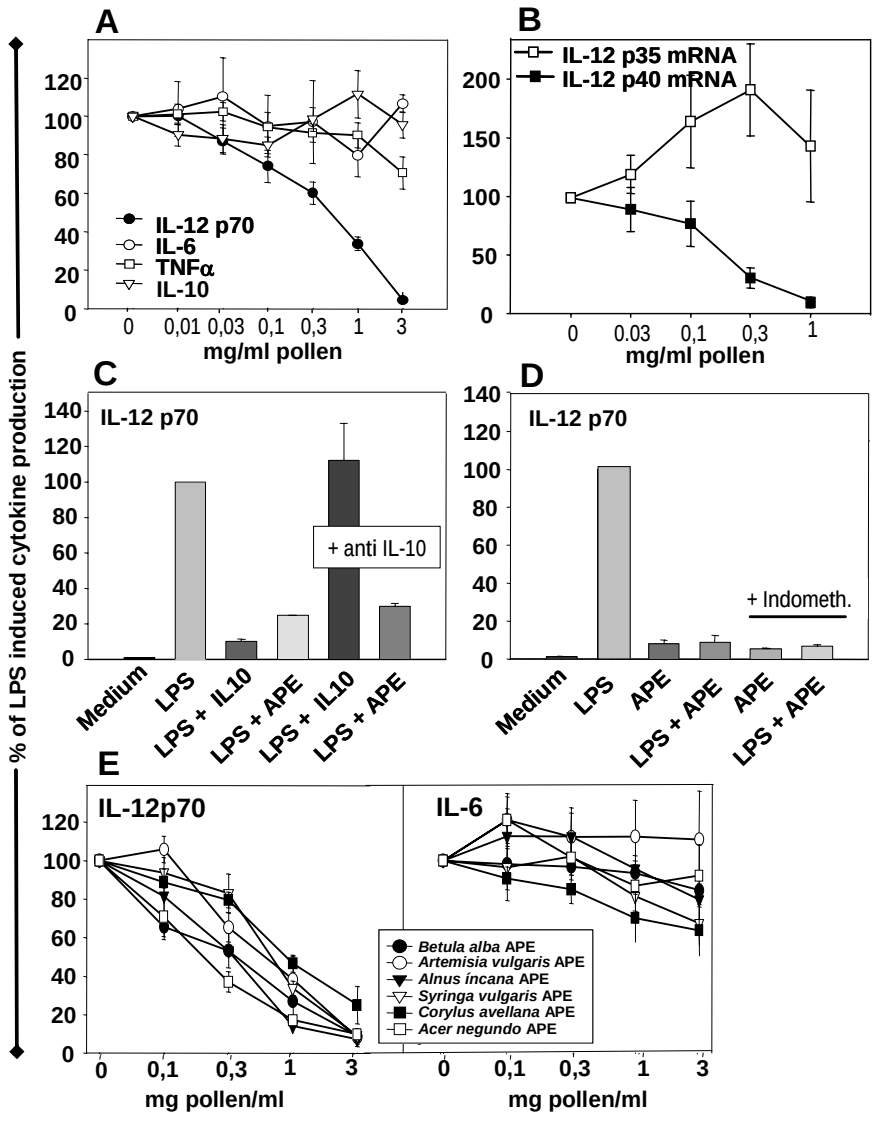

Figure 6: *Bet.-APE inhibit DC IL-12 but not IL-6 production*. (A) DCs were stimulated with LPS (100ng/ml) in the presence of increasing concentrations of *Bet*.-APE. After 24h IL-12 p70, IL-6, TNF-α and IL-10 concentrations were determined in culture supernatants. Data are presented as percentage of LPS-induced cytokine production in order to equalize donor specific variabilities (IL-12 p70 $\bar{x}$= 3,599 ±1,446 pg/ml, *n* = 6; IL-6 $\bar{x}$=13,069 ± 7,358 pg/ml, *n* = 6; TNFα $\bar{x}$= 3,068; ± 440 pg/ml, *n* = 5; IL-10: $\bar{x}$= 343 ± 149 pg/ml, *n* = 5). *Bet*.-APE reduces LPS-induced IL-12 p40 but not IL-12 p35 mRNA levels. (B) Immature DCs were stimulated for 6h with LPS in presence of increasing concentrations of *Bet*.-APE. Quantitative IL-12 p40 and p35 mRNA analysis was

performed. Data is shown as percentage of LPS-induced IL-12 p40 mRNA ([$2^{\{-\Delta\Delta CT\}}$]: 1,149 ± 518, mean ± SEM, *n* = 3) or IL-12 p35 mRNA ([$2^{\{-\Delta\Delta CT\}}$]: 364 ± 164, mean ± SEM, *n* = 3) expression. *Bet*.-APE-induced inhibition of DC IL-12 release is independent of autocrine IL-10 or endogenous cyclooxygenase products. (C and D) DC stimulations were performed in the presence of IL-10-neutralizing antibodies (10μg/ml; C) or a cyclooxygenase inhibitor (25 μM indomethacine;D). Data is presented as percentage (mean ± SEM) of IL-12 p70 production induced by LPS (C: IL-12 p70: $\bar{x}$= 2,481 ± 416 pg/ml; *n* = 4; D: IL-12 p70 $\bar{x}$= 4,126 ± 326 pg/ml; *n* = 3). APE from different species display similar effect on DC IL-12 and IL-6 production. (E) DCs were stimulated with LPS (100 ng/ml) in the presence of increasing concentrations of APE generated from different pollen species. After 24 h, IL-12 p70 and IL-6 levels were determined in culture supernantants (IL-12 p70 $\bar{x}$= 1,477 ± 426 pg/ml, *n* = 3; IL-6 $\bar{x}$= 10,776 ± 1,463 pg/ml, *n* = 3).

4.3. *Bet*.-APE exposure shifts DC polarization capacity from Th1 to Th2

The inhibitory effects of *Bet*.-APE on DC IL-12 production prompted us to analyze the phenotype of primary T cell responses induced by DCs matured in the presence of *Bet*.-APE. Naive allogeneic T cells primed by LPS-matured DCs differentiated into Th1 lymphocytes with characteristic production of large amounts of IFN-γ and low levels of IL-4 (Fig.7). In contrast, DCs activated by LPS in the presence of *Bet*.-APE displayed a dramatically reduced capacity to induce IFN-γ producing Th1 cells and a markedly enhanced capacity to induce IL-4–producing Th2 cells (Fig.7 and Table I). The *Bet*.-APE–induced shift of a primarily Th1-dominated response to a primarily Th2-dominated response was comparable to that obtained under maximal Th2-polarizing condition, i.e., when DCs where stimulated with LPS in the presence of PGE_2 and neutralizing anti–IL-12 mAb was added at the beginning of the MLR (Fig.7A and Table I). The *Bet*.-APE–induced shift from a Th1- to a Th2-dominated immune response could partially be restored, when exogenous IL-12 was added at the beginning of the DC–T cell coculture (Fig.7A), indicating that indeed inhibition of the DC IL-12 production by *Bet*.-APE plays a crucial role in the observed deviation of the immune response. However, addition of exogenous IL-12 (at concentration exceeding those measured in DC cultures) was not able to restore the response completely, suggesting that besides IL-12 other Th1-driving mediators may be inhibited by exposure of DCs to *Bet*.-APE. In contrast, when IL-4–neutralizing antibodies

were added at the beginning of the T cell–DC coculture (Fig.7B) induction of Th2 cells was almost completely abrogated, demonstrating that the Th2-polarizing effect of Bet-APE–treated DCs was clearly IL-4 dependent.

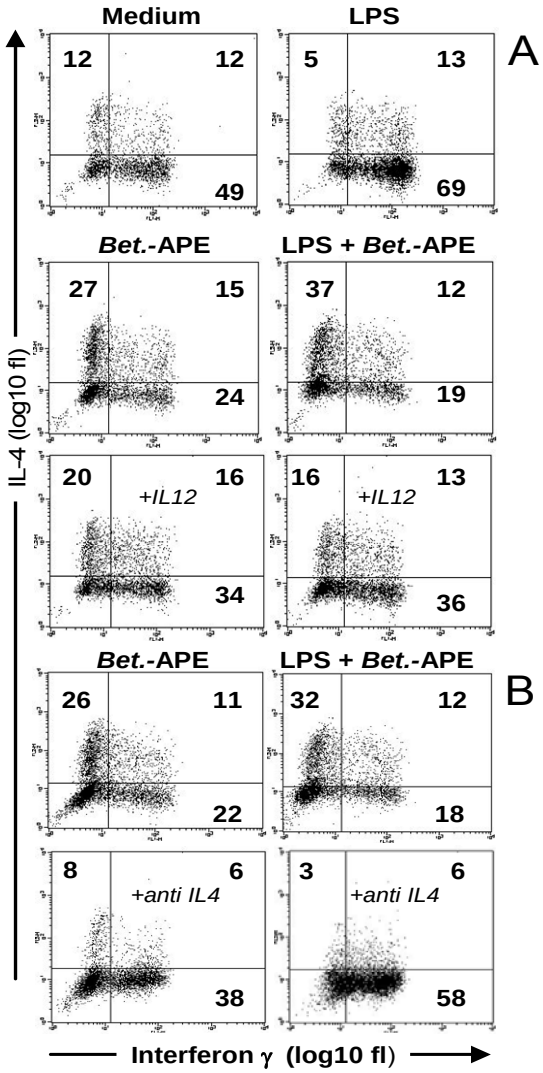

Figure 7: *DCs matured in the presence of Bet.-APE display reduced Th1- and increased Th2-polarizing capacity.* (A) DCs were left untreated or stimulated with *Bet*.-APE (3mg/ml) in the presence or absence of LPS (100ng/ml). After 24h DCs were washed and cocultured with $CD4^+$ $CD45RA^+$ allogeneic T cells (DC/T cell ratio 1:4) that were expanded for 12 d in the presence of IL-2. T cell polarization was determined by analysing intracellular IFN-γ and IL-4 accumulation via flow cytometry. *Bet*.-APE exposed DCs favoured Th2 polarization induction. (B) To address, if the *Bet*.-APE-dependent Th2 polarization could be reverted by exogenous IL-12, hrIL-12 (10ng/ml) was added at the beginning of the coculture of *Bet*.-APE/LPS-treated DCs and T cells. Representative experiment of n=3-6.

Table I. Phytoprostane E1 turn the LPS-induced Th1 in a Th2 response**.**

DC Stimulation	IL-4+	IFN-γ+	IL4+ IFN-γ+	IL-4/IFN-γ Ratio	n
Medium	13.8±6.4	37.0±3.3$^{\#}$	8.8±0.4	0.36±0.05	6
LPS	12.3±2.4	59.2±3.7*	8.3±0.8	0.22±0.05*	6
LPS + PGE_2	38,8±6,5	15,5±4,2	9.7±2.1	2,5±0,18	6
LPS + *Bet.*-APE	24.3±3.8*$^{\#}$	25.8±4.1*$^{\#}$	8.0±2.0	1.06±0.18*$^{\#}$	6
APE	22.3±2.4	36.9±2.01	9.7±0.5	0.6±0.09	6
LPS	15.7±3.1	53.7±4.3	10.5±2.1	0.30±0.17	3
LPS + PPE_1	33.3±0.3 *$^{\#}$	36.7±2.6$^{\#}$	14.3±3.3	0.92±0.06*$^{\#}$	3
LPS + PPF_1	15.0±0.5	58.7±6.1	10.7±0.3	0.27±0.04	3
LPS + PPB_1-I	18.7±3.5	55.3±6.3	11.3±1.2	0.38±0.12	3
LPS + PPB_1-II	19.0±2.6	58.0±6.0	15.7±2.8	0.35±0.08	3

4.4. *Bet*.-APE contain substancial amounts of phytoprostanes

Previously it has been shown that various classes of prostaglandin-like compounds, the phytoprostanes, apparently occur ubiquitously in plants. Notably, exceptionally high levels of F_1-phytoprostanes (PPF_1) have been observed in organic extracts of birch pollen. In an attempt to identify potential candidates responsible for the observed effects of *Bet*.-APE, we quantified levels of phytoprostanes present in *Bet.*-APE by NCI GC-MS (Fig.8; A-C and Table II). PPF_1 levels in pollen released in aqueous buffer (*Bet.*-APE) were 2.25 µg/g pollen. In addition, A_1/B_1- and E_1-phytoprostanes were detected in *Bet.*-APE. Interestingly, PPE_1 levels were found to be eightfold more abundant, whereas concentrations of PPA_1/B_1 were found to be threefold less abundant as compared with PPF_1 (Table II).

4.5. Effects of phytoprostanes on LPS-induced IL-12 production and on T cell polarization

E_1-Phytoprostanes inhibit LPS-induced DC IL-12 p70 production and augment DC's capacity to induce Th2 responses. Various prostaglandins have been reported to modulate human DC function and cytokine profile. The structural similarity of α-linolenic acid–derived phytoprostanes and prostaglandins prompted us to analyze whether phytoprostanes

identified in *Bet.*-APE (PPE_1, PPF_1, and PPA_1/PPB_1) showed similar effects on human DCs. For this purpose DCs were activated by LPS in the presence or absence of phytoprostanes or PGE_2 over a wide range of concentrations (Fig.9). As reported previously, PGE_2 dose dependently inhibited the LPS-induced IL-12 p70 release, whereas LPS-induced IL-6 production remained unchanged. PPF_1 and PPB_1 did neither affect the LPS-induced IL-12 p70 production nor the LPS-induced IL-6 production. However, PPE_1 markedly inhibited the LPS-induced IL-12 p70 production, without affecting the IL-6 production (Fig.9). The inhibitory effect of PPE_1 on DC IL-12 production was only observed when DCs were activated (e.g., by LPS) whereas in the absences of activation signals PPE_1 alone did neither modulate the basal IL-12 production, nor the outcome of the DC induced Th cell response. The inhibition of IL-12 release by PPE_1 was not due to cytotoxic effects, as determined by propidium iodide exclusion. In contrast to the effect on LPS-induced IL-12 production none of the phytoprostanes tested had any significant effect on LPS-induced DC maturation. In addition to phytoprostanes, we recently demonstrated that *Bet.*-APE contain substantial quantities of monohydroxylated derivatives of α-linolenic and linoleic acid, such as 9- and 13-hydroxyoctadecatrienoate as well as 9- and 13-hydroxyoctadecadienoate. Since some of these lipids have been suggested to inhibit the IL-12 production in human macrophages we analyzed their effect on human DCs. Interestingly, none of these mediators (10^{-11}–10^{-5} M) lead to an inhibition of the LPS-induced IL-12 p70 release of human DCs (unpublished data). The PPE_1-dependent inhibition of DC IL-12 production prompted us to analyze the effects of various phytoprostanes on Th1–Th2 polarizing capacity of DCs. Presence of PPE_1 but not PPF_1 or PPB_1 during LPS-induced DC activation lead to the generation of DCs that displayed an increased capacity to induce Th2 polarization in naive T cells, as determined by intracellular cytokine staining (Table I).

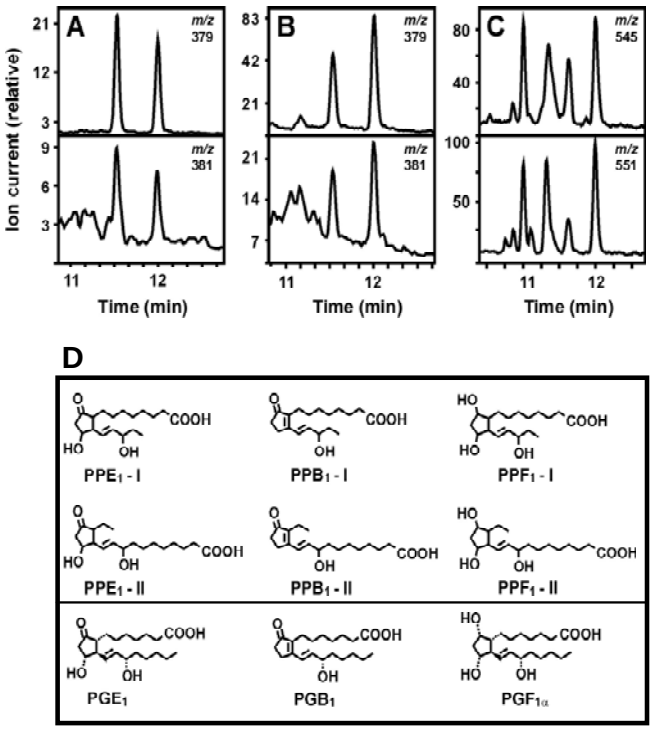

Figure 8: *Analysis of phytoprostanes in Bet.-APE.* Representative selected ion monitoring GC–NCI–MS traces of phytoprostanes from birch pollen extracts are shown. (A-C) PPE_1, PPA_1, and PPB_1 were extracted, purified, and analyzed as their corresponding pentafluorobenzyl ester, trimethylsilyl ester PPB_1 derivativesEndogenous PPF_1 derivatives are identified in the *m/z* 545 ion current chromatogram and quantitated against the $[^{18}O]_3PPF_1$ internal standard in the *m/z* 551 ion current chromatogram. (D) Prostaglandin and phytoprostane structures.

Table II. Concentration of phytoprostanes in *Bet*.-APE

	MW	Conc. in *Bet.*-APE[a] (10 mg/ml)	Conc. [a] (µg/g pollen)	n
PPE_1	326	543.6 ± 41.1 nM	17.72 ± 1.34 µg/g	3
PPF_1	328	68.6 ± 1.5 nM	2.25 ± 0.05 µg/g	3
PPA_1/B_1	308	23.8 ± 3.5 nM	0.74 ± 0.11 µg/g	3

[a] Phytoprostanes were quantified in aqueous birch pollen extracts (*Bet.*-APE 10 mg/ml) by GC-NCI-MS (see materials and methods).

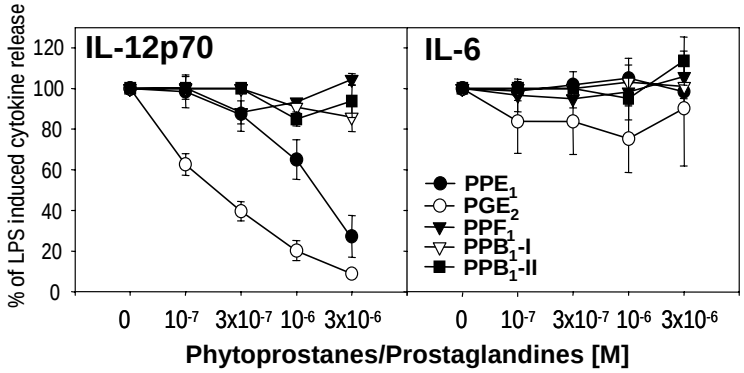

Figure. 9: *Effects of Phytoprostane E_1 on the LPS-induced IL-12 production.* DCs were stimulated with LPS (100ng/ml) in the presence of increasing concentrations of different phytoprostanes. After 24h IL-12 p70, IL-6 concentrations were determined in culture supernatants. Data are presented as percentage of LPS-induced cytokine production in order to equalize donor specific variabilities. Phytoprostane E_1, but not the other phytoprostanes, inhibit LPS-induced IL-12 production.

4.6. Dendritic cells matured by LPS together with *Bet*.-APE induce in naïve T cells Th2 chemokine receptor expression

FACS analysis was performed to study the pattern of expression of chemokine receptors in Th1 and Th2 polarized T cell line (Fig.10). Freshly isolated naïve CD4+ T cells expressed low levels of both type 1 and type 2 chemokine receptors. Naïve T cells that were primed with allogeneic LPS- matured DCs preferentially expressed Th1 type chemokine receptors CCR5 and CXCR3. Instead, the activation of allogeneic naïve T cells with DCs matured in the presence of *Bet*.-APE, resulted in an induction of Th2 type chemokine receptors CCR4 and CRTh2.

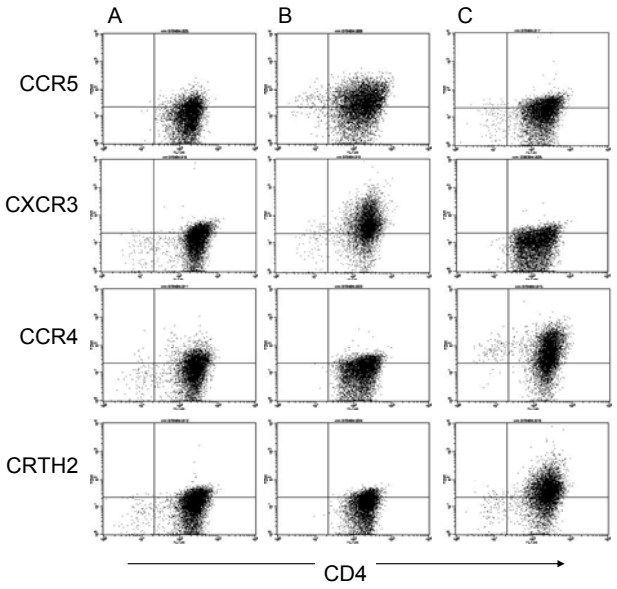

Figure 10: *Bet.-APE exposed DCs induce the differentiation of CD4$^+$ Th2 type T lymphocytes***.** Dendritic cells were stimulated with medium (A), LPS (B 100ng/mL) or *Bet*. –APE (C aqueous pollen extracts from birch) with LPS and subsequently coincubated with allogeneic naïve T-cells. The ensuing T-cell line was expanded and analyzed on day 10 for the expression of the Th1-chemokine receptor CCR5, CXCR3 and the Th2 chemokine receptors CCR4 and CRTh2 (PE) and CD4 (FITC).

4.7. *Bet*.-APE induce CXCR4 and downregulate CCR1 and CCR5 expression

Expression of chemokine receptors on the membranes of immature or mature DCs stimulated with APE was assessed by flow cytometry (Fig.11A). In LPS- matured DCs the expression of CXCR4 was up-regulated in comparison to immature DCs. Instead, DCs treated with APE up-regulated membrane expression of CXCR4 though CD83 was not detected. On the contrary, CCR1 and CCR5 were expressed by immature DCs but completely disappeared on DCs treated with APE, LPS, or both. Real time- PCR was also done in order to investigate the mRNA expression of chemokine receptors in DCs stimulated with APE (Fig.11B). LPS matured DC expressed consistent levels of CCR1, CCR5 and lower levels of CXCR4 messenger RNA (mRNA). Consistently with protein

data, incubation with 3mg/mL of APE strongly down-regulated CCR1 and CCR5 mRNA. In addition, APE further increased CXCR4 mRNA levels induced by LPS.

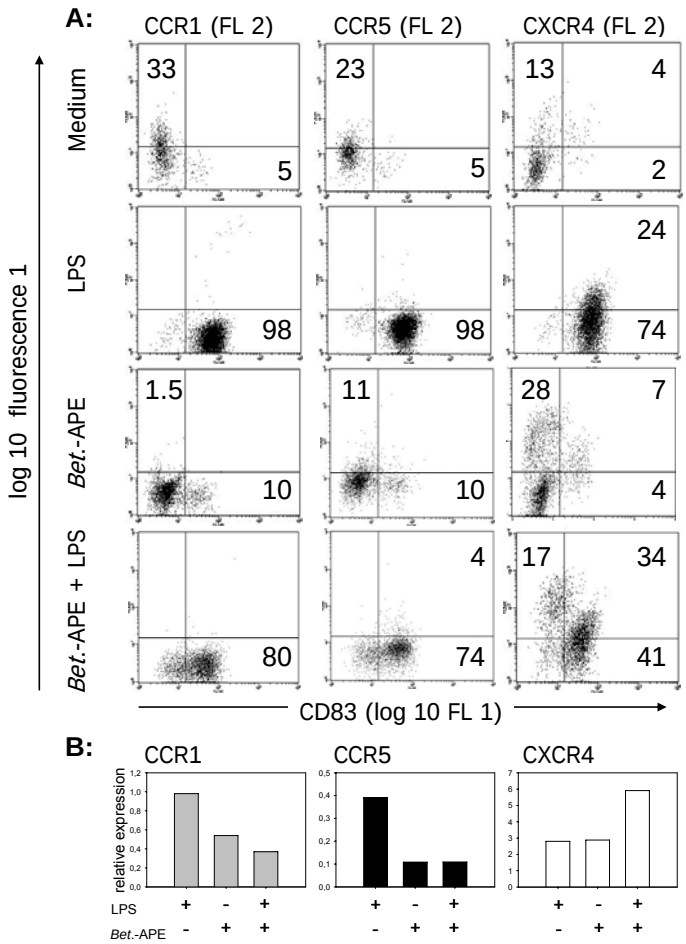

Figure 11: *Bet.-APE up-regulates CXCR4 and reduces CCR1 and CCR5 on DC membranes*. (A) Immature DCs were untreated, stimulated with 3 mg/mL APE, or induced to mature with 100 ng/mL LPS in the absence or presence of APE. After 48 hours, DCs were stained with mAbs against the indicated surface molecules or with isotype-matched control Ig. Numbers indicate the percentage of positive cells. Results are representative of 5 independent experiments. (B) Immature DCs were not treated, stimulated with 3mg/mL APE or induced to mature with 100ng/mL LPS in the absence or presence of APE. After 6 hours, total RNA was extracted and real time-PCR done to study the expression of chemokine receptor mRNA.

4.8. DCs matured with LPS in the presence of *Bet.*-APE showed chemotaxis to CCL19 but not to CCL4 and CCL16

The functional activity of chemokine receptors was examined by measuring cell migration (Fig.12) in response to agonistic chemokines. Since the presence of PGE_2 has been

described to be important for the response to the CCR7 and CXCR4 ligands, DCs were stimulated with PGE_2 ($1x10^{-5}$) plus LPS or PGE_2 alone as positive control. Immature DCs showed a base line, non-directional migration to all stimuli used that was comparable to medium control. After LPS stimulation, base line migration to medium and migration towards the CCR1 and CCR5 ligands (CCL16 and CCL4, respectively) was absent, while high migratory activity was observed to the CCR7 ligands CCL21 and CCL19. The CXCR4 ligand CXCL12 induced only a weak migratory response in LPS-stimulated DCs. When PGE_2 was added to the LPS stimulated culture, the migration towards CCL19 and CCL21 was enhanced 2.2 and 1.2 fold, respectively. Notably, PGE_2 enhanced the migration of LPS-stimulated DCs towards CXCL12 55 fold. Similary, when DCs were co-stimulated with LPS and *Bet*.-APE the migration towards CXCR4 ligand CXCL12 was enhanced 37 fold. In contrast, the migration to the CCR7 ligands CCL21 and CCL19 did not change significantly when *Bet*.-APE was added. The stimulation with PGE_2 or *Bet*.-APE in the absence of LPS led only to a marginal variation in the random migration but not in the directed migration towards on of the tested ligands.

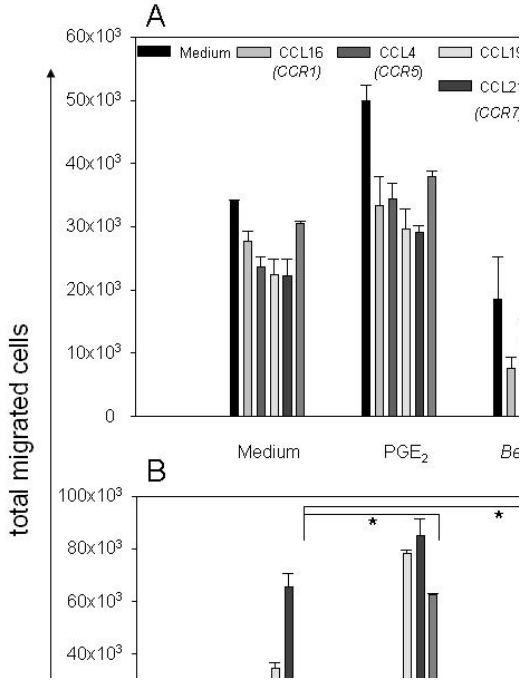

Figure 12: *Bet.-APE treated DCs migrate in response to CCL19 but not to CCL4 and CCL16***.** Monocyte derived DCs were incubated for 24 hours with (A) medium, *Bet*.-APE (3 mg/ml), PGE_2 ($1x10^{-6}$) or (B) LPS, LPS plus PGE_2, LPS plus *Bet*.-APE. Stimulated DCs were processed for migration assays in 24 well transwell chambers. As migratory stimuli the following chemokines were chosen: 1 µg/ml CCL16 (ligand for CCR1), 100 ng/ml CCL4 (ligand for CCR5), 30ng/ml CCL21, 100ng/ml CCL19 (both ligands for CCR7) and 10ng/ml CXCL12 (ligand for CXCR4). Data are expressed as total migrated cells +/- SD. Shown is one representative experiment of 5 performed in triplicate. Asterisk indicates significant changes of DC maturation by PGE_2 or *Bet*.-APE compared to LPS-stimulation alone ($p \leq 0.05$).

4.9. *Bet*.-APE significantly reduced LPS-induced release of CXCL10 and CCL5, increased CCL22 and did not significantly change production of CCL17

In the next series of experiments, we investigated the effect of APE on chemokine production by DCs. In these cells, APE increased the production of CCL22 and strongly inhibited secretion of CXCL10 and CCL5 induced by LPS. CCL17 production was just

slightly increased in the presence of 3 mg/mL APE, the highest concentration of APE used in the experiments. Suppression of CXCL10 was already evident at the dose of 0.1mg/mL of APE, whereas the minimal APE concentration inhibiting CCL5 secretion was 0.3mg/mL. The enhancement of CCL22 was evident at an APE dose of 1 mg/mL (Fig.13). The protein data were confirmed also at mRNA level (Fig.14). In fact, LPS induced expression of CXCL10 and CCL5 mRNA was significantly down-regulated in the presence of *Bet*.- APE whereas CCL22 mRNA production was strongly induced. Also an increase in the CCL17 mRNA levels was detected in the presence of APE. Furthermore, it was observed that 3 mg/mL of Bet.-APE alone induced the production of low levels of CCL17 and CCL22 mRNA.

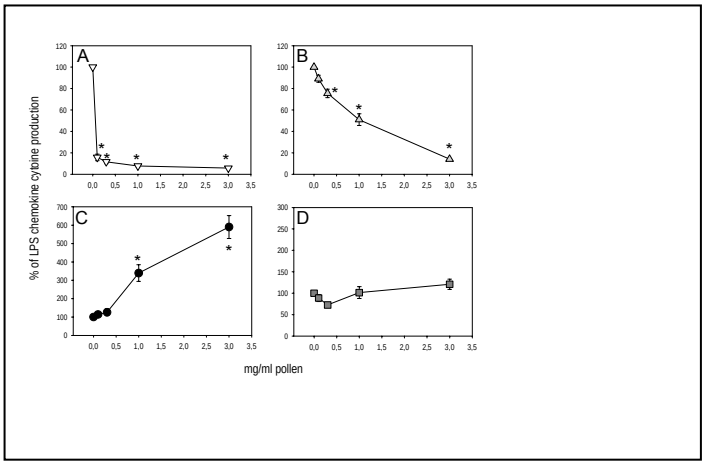

Figure 13. *Bet.-APE inhibits CXCL10 and CCL5 release and augments CCL22 secretion in a dose-dependent fashion whereas CCL17 production is not affected.* Immature DCs were stimulated with 100 ng/mL LPS and a graded concentration *of Bet*.-APE. After 24 hours of incubation, CXCL10 (A), CCL5 (B), CCL22 (C), and CCL17 (D) were measured in culture supernatants by ELISA. Results are given as the percentage of LPS induced chemokine production.

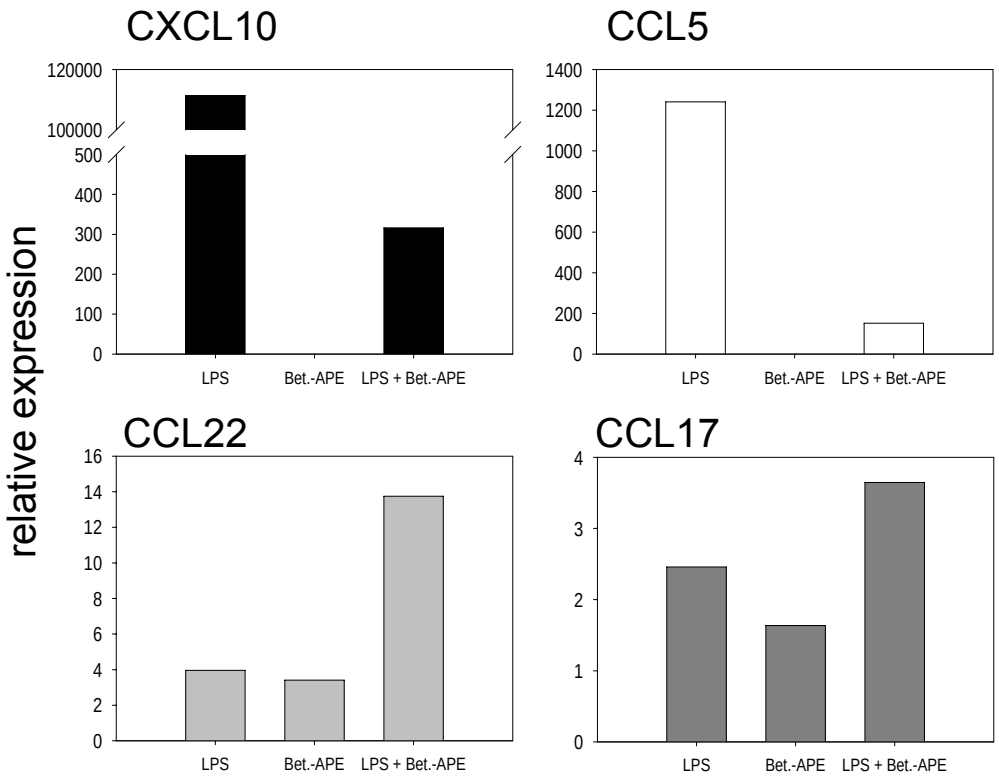

Figure 14: *Bet.-APE inhibits the LPS induced expression of CXCL10 and CCL5 mRNA and increases the levels of CCL22 and CCL17 mRNA.* DCs were stimulated for 6 hours with LPS together with APE. Total RNA was extracted and analyzed for Th1 and Th2 chemokine mRNA expression.

4.10. *Bet*.-APE enhance the capacity of DCs to attract type 2 T lymphocytes

As previously shown, *Bet*.-APE significantly changed DCs chemokine production, thus it was investigated whether the capacity of DCs to attract different polarized T lymphocytes was modified (Fig.15). Differently polarized T cell lines were established and characterized for chemokine receptor expression (Fig.15A). Next, the migratory behaviour was analyzed. Type 1 and type 2 T cells showed a higher level of migration to supernatants from LPS-stimulated DCs compared with supernatants from immature DCs with type 1 cells migrating more efficiently than type 2 cells to LPS according to the higher production of Th1 chemokines. Consistent with the production of CCL22 and CCL17 by immature DCs,

type 2 cells migrated more efficiently than type 1 cells to supernatants from immature DCs stimulated with APE. However, supernatants from DCs matured in the presence of APE showed a strongly reduced capacity to attract type 1 lymphocytes, whereas attraction of type 2 cells was enhanced (Fig. 15B).

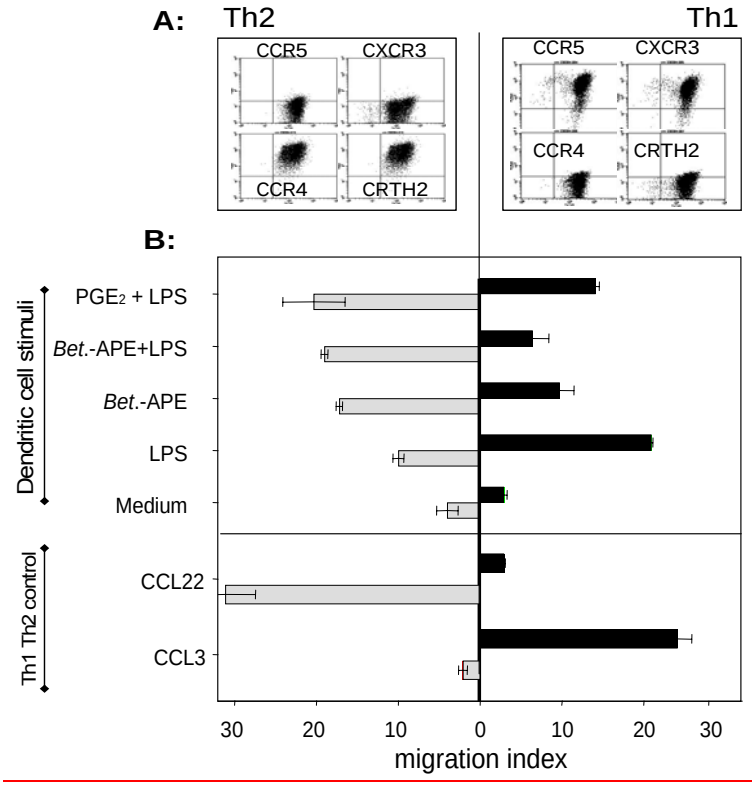

Figure 15: *Bet.-APE- stimulates human monocyte derived dendritic cells to attract preferentially Th2 cells.* (A) Chemokine receptor expression on polarized T cell line. (B) DCs were stimulated for 24 hours with LPS together with APE or APE alone. Cell free supernatants diluted in complete medium and 0.5% BSA were used for cell migration studies of polarized Th1 and Th2 T- cell lines. Shown are results with supernatants from immature DCs that were either untreated or stimulated with 3 mg/mL APE and results with supernatants from DCs treated with 100 ng/mL LPS alone and LPS plus APE**.**

5 Discussion

Pollen grains are one of the most common inducers of allergic symptoms. Upon contact with mucosal surfaces of the upper respiratory tract, pollen grains rapidly release proteins/allergens into the aqueous phase. On the basis of a genetic susceptibility atopic individuals develop allergen-specific Th2 biased immune responses that ultimately lead to clinical manifestations of IgE mediated hypersensitivity (19). While the biology of Th2 cells in the effector phase of allergy is well understood, little is known about the mechanisms that control the initial Th2 polarization in response to exogenous allergens. In this study, we found that stimulation of immature DCs with aqueous birch pollen extracts (*Bet*.-APE) increased membrane expression of CD83, CD86, CD80 and HLA-DR and this effect was mostly due to the presence of LPS in *Bet*.-APE. However, LPS-free *Bet*.-APE was able to enhance DCs capacity to promote proliferation of naive allogeneic T cells. On the other hand, *Bet*.-APE dose-dependently inhibited LPS-induced production of IL-12 whereas *Bet*.-APE showed an augmented capacity to initiate Th2 responses in vitro. In addition, we demonstrated that *Bet*.-APE contain substantial quantities of phytoprostanes that structurally resemble prostaglandins. In fact, E_1 phytoprostane markedly inhibited LPS-induced IL-12 production and as a consequence, DC matured in the presence of PPE1 displayed an increased capacity to induce Th2 type T cells differentiation. The current study also show that *Bet*.-APE stimulation lead to up-regulation of the lymphoid chemokine receptor CXCR4 and the coordinated down-regulation of CCR1, CCR5 on DCs. Functionally, *Bet*.-APE -exposed DCs migrated to CCL19, whereas chemotaxis to CCL4 was reduced. Thus, *Bet*.-APE appears to set DCs for enhanced migration to lymphoid organs. *Bet*.-APE also up-regulate the production of CCL22 and inhibited the LPS-induced secretion of CXCL10 and CCL5 in DCs. As a consequence, we found that stimulation of DCs with *Bet*.-APE enhanced migration of type 2 T cells, suggesting an increased capacity to amplify type 2 immune responses.

5.1. Effects of Pollen Associated Lipid Mediators on the Adaptive Immune Response

It has been demonstrated that pollen in addition to liberating protein allergens rapidly release various bioactive lipids into the aqueous phase (29). These pollen-associated lipid mediators (PALMs) were shown to stimulate and attract cells of the innate immune system, such as neutrophil and eosinophil granulocytes (122, 123). In fact, although it is generally accepted that chemoattractants are generated by activated cells of the local microenvironment, little attention has been paid so far to exogenous sources of these mediators. Recent reports about the effects of parasite-derived prostaglandin E_2 in schistosomiasis infection (124) and the existence of dermatophyte- derived lipid mediators inducing granulocyte migration (125) suggest that exogenous lipid mediators may also modulate local immune responses in the host. Here, we describe an interaction between pollen derived lipid structures with the adaptive immune sytem. We demonstrated that pollen release soluble factors that synergise with LPS and CD40L in the induction of phenotypical and functional final maturation of human monocyte-derived dendritic cells (MDDC). Birch aqueous pollen extract (*Bet*.-APE) promoted maturation resulted in $CD1a^+$, $CD83^+$, $CD86^+$ DCs which produced only low amounts of IL-12, augmented their capacity to promote proliferation of naïve allogenic T cells and bias the development of naïve Th cells toward the production of Th2-type cytokines. Further analysis revealed that *Bet*.-APE contain considerable amounts of LPS which is also present under natural exposure conditions. When MDDC were costimulated with *Bet*.-APE and LPS or CD40L a dose dependent blockage of the LPS (CD40L) induced IL12-p70 production was observed while other cytokines such as IL-6, IL-10, IL1β and TNF-α were not impaired. The same was true when *Bet*.-APE were purified for LPS by means of polymyxin B columns that ruled out a sole LPS effect. Thus, soluble factors from pollen are capable to antagonize the LPS induced IL-12 release. The declined IL-12 production was verified on RNA level showing

that simultaneous addition of increasing concentration of *Bet*.-APE lead to a dose-dependent inhibition of IL-12 p40 mRNA expression. In contrast *Bet*.-APE stimulation seemed to enhance the LPS induced IL-12 p35 mRNA. These results suggest that *Bet*.-APE dependent inhibition of IL-12 p70 release is likely to be regulated at the level of IL-12 p40 mRNA expression. As a consequence of blocked IL-12 production, DCs matured in the presence of *Bet*.- APE showed an impaired capacity to initiate Th1 responses in vitro. In fact, naïve T cells stimulated with mature DCs exposed to *Bet*.-APE produced lower levels of IFN-γ, but higher amounts of IL-4 in comparison to naïve T cells stimulated with LPS- matured DCs. Addition of exogenous IL-12 at the time of T cell priming partially restored a Th1 polarization, suggesting that the suppression of IL-12 production in maturing DCs was not the sole mechanism responsible for the immune deviation induced by *Bet*.-APE. However, when IL-4-neutralizing antibodies were added at the beginning of the coculture, DCs matured in the presence of *Bet*.-APE were not able to induce a Th2 type T cell differentiation any longer. These results demonstrated that the Th2-polarizing effect of *Bet*.-APE-treated DCs was clearly IL-4 dependent.

DC maturation is an important control point in the development of immune responses, as it converts Ag-capturing DCs into potent immunostimulatory APCs and can also determine whether peripheral tolerance or immunity is induced (126).

Maturation of DCs is stimulated by factors signaling tissue danger such as microorganisms, dying cells, or proinflammatory cytokines. Different pathogen- associated molecular patterns (PAMPs) also constitute a physiologic activation stimulus via Toll- like receptors (TLRs), pattern recognition receptors expressed by both plasmacytoid and conventional DCs (127). Many PAMPs still remain to be characterized and it is not clear whether allergens or allergens carriers, such as pollen grains, contain similar molecular patterns that can trigger TLRs. It is well conceivable that at the site of allergen exposure, such as allergen- or allergen carrier- associated molecular patterns (AAMPs) could directly

induce the activation and maturation of dendritic cells. Alternatively, some allergens may have direct dendritic cells- activating potential because of their intrinsic enzymatic activity, as it was recently reported for one of the major house dust mite allergens- the cystein protease Der p 1 (128).

For many aeroallergens, the bioavailability depends on the allergen liberation from internal binding sites within the allergen carrier, such as pollen grains. In addition, it has been recently demonstrated that bioactive mediators get rapidly release upon pollen contact with the acqueous phase (29). Among others, eicosanoid- like lipid mediators are released within minutes, clearly preceding the liberation of protein allergens. Even though in vivo effects of these mediators are still somewhat uncertain, it seems very likely that they may act as adjuvants, leading to the activation and modulation of dendritic cell function at the site of allergen exposure (129).

Recently, a variety of factors has emerged that can limit DC maturation. For example intracellular cAMP-elevating agents, such as cholera toxin and β_2-adrenoreceptor agonists inhibit IL-12 and TNF-α and enhance IL-10 expression by LPS-stimulated DCs (44). Besides IL-10, glucocorticoids, and vitamin D3 interfere with DC maturation as a whole by blocking the up-regulation of presenting and co-stimulatory molecules (97), the release of IL-12 and TNF-α and maintain DCs in an Ag-capturing state (130).

Bet.-APE acted independently of the above-cited mechanism since its activity was not affected by neutralization of endogenous IL-10. Also PGE_2, like the other cAMP-elevating agents, has been reported to inhibit TNF-α and IL-12, but not IL-10 production by monocytes and DCs (131). However, experiments performed blocking PG synthesis by indomethacin showed that the production of TNF-α and IL-12 was not restored, indicating that *Bet*.-APE acted independently of endogenously produced PGs. Modulation of the surface markers by PALMs was also not responsible for the Th2 switch- as it was

described for example for Der p 1 to cleave proteolytically CD40 (44)- since the surface markers of *Bet*.-APE-stimulated DCs were similar to those stimulated with LPS.

5.2. Identification of polarizing lipids in Aqueous Pollen Extract

It has been shown that various classes of prostaglandin-like compounds, the phytoprostanes, apparently occur ubiquitously in plants (43).

Phytoprostanes are formed via autooxidation, which is initiated by free radical attack of α-linolenic acid yielding α linolenate radical that readily oxidizes and cyclizes to two regioisomeric, prostaglandin G-like compounds (132). In vivo, PPG_1 may be either reduced to PPF_1 or converted to PPE_1, which itself may be dehydrated and isomerized to PPB_1 (30).

In this study, we demonstrated for the first time that nonenzymatically formed phytoprostanes such as PPE_1, PPF_1 and PPB_1 are present in aqueous pollen extract in nanomolar concentrations as identified and quantified by a negative ion chemical ionization gas chromatography-mass spectrometry method. Levels of PPF_1 in organic extracts of birch pollen appear to be approximately 15 times more abundant in organic as compared with aqueous extracts (39). These differences might reflect different extraction efficiencies as well as varying concentrations in pollen from different sources. Phytoprostane levels in organic extracts reflect lipid peroxidation in pollen, and may only be of limited relevance for estimates of natural exposure levels on mucus membranes.

In conrast, analysis of phytoprostanes levels spontaneously released into the aqueous phase of the buffer used in this study, more closely mimics physiological exposure conditions. PPF_1 levels in pollen released in aqueous buffer (*Bet*.-APE) were 2.25 μg/g pollen. In addition, A_1/B_1- and E_1-phytoprostanes were detected in *Bet*.-APE. Interestingly, PPE_1 levels were found to be threefold less abundant, whereas

concentration of PPA_1/B_1 were found to be threefold less abundant as compared with PPF_1.

PPE_1, PPF_1 and PPB_1 were tested in their capacity to antagonize the LPS induced IL-12 production but only PPE_1 resulted to hamper the LPS or CD40-induced IL-12 release. While the crude aqueous pollen extracts induced a functional and phenotypical maturation going alone with a low production of IL-12, the effect of phytoprostane E_1 on the IL-12 production was dependent on the presence of a maturation signal such as LPS. Furthermore, T lymphocytes primed with DCs matured in the presence of PPE_1 displayed a reduced production of IFN-γ and a higher release of IL-4 compared to T cells activated with LPS-matured DCs. It is well known that migrating DCs, provide lymph-node-based naïve Th cells with three signals: “signal 1”, (Ag), giving information about the identity of the invader; “signal 2”, (costimulation), giving information about the Ag-related pathogenic potential; and “signal 3”, mediating the DC-dependent component of the early polarization of primary Th-cell responses (44). This third signal, resulting from the character of a pathogen-induced, non-specific tissue response, reflects both the nature of the pathogen and the type of infected tissue, and allows a swift selection of appropriate effector mechanisms. Although the mechanism of transmission of “signal 3” appears to involve a particular level of IL-12 production by polarized DCs, by analogy to “signal 2”, it is likely to depend on several different mechanisms. These might involve additional soluble DC products as well as contact-dependent mechanisms, including the density of antigenic peptide-MHC complexes or particular levels of distinct co-stimulatory molecules on the DC surface (79, 80). In this respect, ICAM-1 and OX40L, the surface molecules that are differentially regulated by IFN-γ and PGE_2 are of special interest. Such a DC-dependent primary polarization can be influenced by additional factors that might be present in the lymph nodes which, like IL-4, TGF-β or IFN-α, can modulate the responsiveness of naïve Th cells to IL-12, or act in an IL-12- independent manner (81). Our study proposes that

signal 3 can be modulated not only by endogenous mediators but also by exogenous- in this case, pollen derived lipid mediators (PALMs). Regarding the *in vivo* situation we might expect that the observed *in vit*ro effects of the pollen-derived soluble factors would partly depend on their concentration in the nasal or bronchial mucosa. However, since the concentration of linolenic and linoleic acid is very high in pollen (123, 133), the chronic exposure to pollen grains could possibly lead to high concentration of the products of these fatty acids in the mucosa. These fatty acids derivatives might represent the exogenous factors that influence the DC dependent primary polarization inhibiting the LPS- induced IL12p70 production. We could hypothesize that such a perturbation of "signal 3" might be one of the factors responsible for the induction of the Th2 type immune response against allergic diseases. In conclusion, we suggest that DCs that have been conditioned by pollen derived lipid mediators will provide the initial driving force for Th2 cell development.

5.3. Influence of *Bet.-APE* on chemokine receptor expression in DCs and in T cells

Heterogeneity in leukocyte trafficking behavior is determined by expression patterns of chemokine receptors and adhesion molecules (134). Chemokines regulate leukocyte trafficking by inducing firm integrin-dependent adhesion of blood leukocytes to endothelial cells and by inducing directional migration (chemotaxis). Since pollen-derived lipid factors modified the capacity of mature DCs to produce IL-12 and consequently the outcome of the ensuing Th cell polarization, we wanted to assess whether they also might induce effects on the ability of DCs to attract different polarized Th cells.

We investigated the effects of *Bet*.-APE on the expression of chemokine receptors on DCs. It is well known that DC maturation results in a switch in chemokine receptor expression with down-regulation of receptors for inflammatory chemokines CCR1 and CCR5 and induction of the lymphoid chemokine receptor CXCR4. The current study

shows that *Bet*.-APE modifies this pattern of chemokine-receptor expression leading to the up-regulation of CXCR4 and the coordinated down-regulation of CCR5 and CCR1 both in immature and LPS-matured DCs. These results were confirmed both at protein and mRNA level. Interestingly, pre-DC2 cells have also been shown to express CXCR4 and migrating to CXCL12 (SDF-1β), indicating a role for this system in DC precursor entry into tissues (135). Since CXCL12 is constituvely expressed in lymphoid tissue (136), this could be critical for maintenance of DC levels in such tissues.

It has been previously reported that cholera toxin induced DC maturation associated with membrane CXCR4 expression (137). Thus, cholera toxin might lead to CXCR4 expression on DC membranes by elevating the intracellular levels of cyclic adenosine monophosphate (c-AMP). Consistent with this hypothesis, Cole et al. (138) reported that c-AMP up-regulates membrane CXCR4 expression on lymphocytes by decreasing receptor internalization without affecting the level of gene expression. Since PGs are well- known c-AMP elevating factors, it would be tempting to speculate that eicosanoid- like mediators released from *Bet*.-APE could induce CXCR4 up-regulation through this mechanism.

As already observed, *Bet*.-APE turn DCs into Th2 inducing antigen presenting cells and these results were confirmed also at the level of T cell chemokine receptor expression. In fact naive T cells primed with DCs matured in the presence of *Bet*.-APE preferentially expressed CCR4 and CRTh2, well known Th2- type chemokine receptors, whereas the Th1- type chemokine receptors CCR1 and CCR5 were prominent on naive T cells activated with LPS- matured DCs (106).

5.4. Effects of *Bet. APE* on chemokine release in DCs

Bet.-APE also affected the pattern of chemokine release from DCs by up-regulating the constitutive production of CCL22 (macrophage-derived chemoattractant [MDC]) and inhibiting the LPS-induced secretion of CXCL10 (interferon-inducible protein 10 [IP-10]) and CCL5 (regulated upon activation, normal T- cell expressed and secreted [RANTES]) whereas the production of CCL17 (Thymus- and activation- regulated chemokine [TARC]) did not significantly changed. These results were confirmed also at mRNA level.

CXCL10 preferentially attract Th1 lymphocytes thanks to the high CXCR3 and CCR5 expression. Also CCL5 is considered a Th1 type chemokine since it is the ligand for both CCR1 and CCR5. CCL17 and CCL22 are known as specific Th2 chemoattractants (139). Several reports have shown that the preferential chemoattractant properties of these chemokines are found not only in vitro but also in vivo using model mice with several Th2- related diseases. In the mouse model of airway inflammation, macrophages in lungs (resident and infiltrating macrophages) produced CCL22 and the neutralization of CCL22 by the administration of specific Ab in vivo reduced Th2 recruitment to the inflammatory sites and inhibited airway hyperreactivity. Many reports have indicated that Th2 cytokines, IL-4 and IL-13, are strong inducers of CCL22 and CCL17 from APCs (140). However, several data indicated that PGE_2 up-regulated CCL22 production by LPS- stimulated DCs (141). There are several reports indicating that PGE_2 also suppresses chemokine mRNA expression and chemokine production in various types of cells. Those effects might depend on the accumulation of intracellular cAMP. For example, PGE_2 or intracellular accumulation of cAMP suppresses CCL5 production by murine mesangial cells (142) and CXCL-10 mRNA expression by cultured keratinocytes (143).

The pattern of chemokine release from DCs matured in the presence of *Bet*.-APE suggested an altered capacity of DCs to attract T-cell subsets, since *Bet*.-APE up-regulate the production of theTh2-related chemokine CCL22 and inhibit the release of the LPS-

induced Th1 chemokines CXCL10 and CCL5. As a result, in order to assess whether APE might enhance Th2 recruitment, we tested the capacity of supernatants from DC cultures to induce migration of type 1 or type 2 polarized T cells.

We found that DCs matured in the presence of APE attracted type 1 polarized T-cell lines and Th1 clones less efficiently compared to LPS- activated DCs, whereas migration of type 2 T cells was enhanced. These results confirmed the hypothesis that DCs exposed to *Bet*.-APE have a diminished capacity to amplify type 1 immune responses and at the same time, favour the attraction of type 2 Th cells to the site of infection.

In conclusion, it was established that *Bet*.-APE change the chemokine-receptor aspect of DCs, conferring on them an enhanced capacity to localize to lymph nodes.

We also observed an additional mechanism by which *Bet*.-APE can promote immune deviation toward a type 2 response, that is, by preventing DC recruitment of type 1 and enhancing the migration of type 2 T lymphocytes to inflamed tissues.

These findings support the hypothesis that pollen grains, upon contact with the aqueous phase of the mucosa of the upper respiratory tract release soluble factors that regulate DCs function and might ultimately lead to the development of antigen presenting cells with an increased ability to amplify Th2 immune responses.

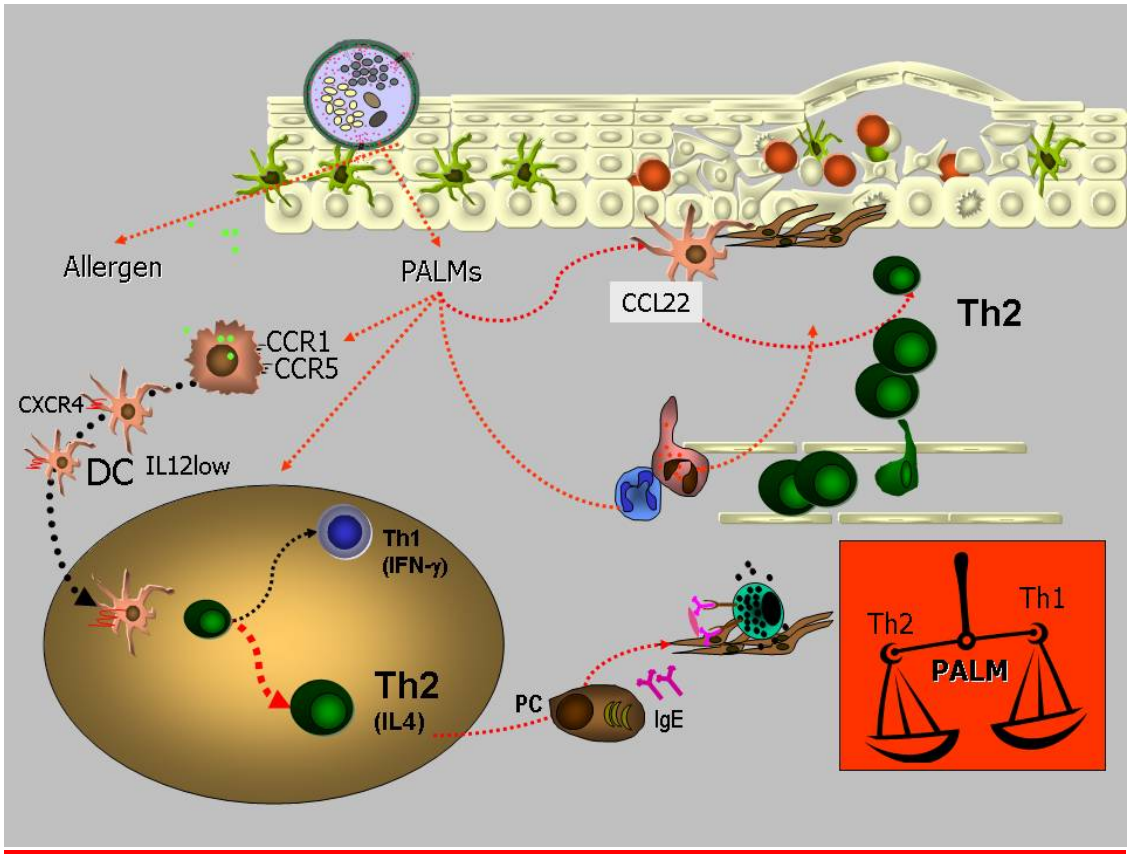

Figure 16: In conclusion we hypothesized that upon contact with aqueous mucosa, pollen granules release soluble mediators that modulate dendritic cell phenotypical and functional maturation. In fact, those soluble factors might inhibit IL-12 production and might account for DCs IL-12 low homing to lymh nodes where they could favour the differentiation of Th2 type T cells. Those pollen associated lipid mediators may also induce not only elicitation but also the amplification of Th2 immune responses by upregulating the production of Th2-type chemokines in DCs.

6 Summary

Pollen grains, as one of the most common inducers of type I allergy, are thought to induce allergy in susceptible individuals by the release of a specific protein. However, the epithelium of the upper respiratory tract- being the primary site of exposure to pollen grains - is not only exposed to the protein but to the whole pollen grain. It has been recently demonstrated that pollen grains not only function as allergen carriers but are also a rich source of bioactive lipid mediators stimulating and attracting cells of the innate immune system. In this study we show that soluble factors from pollen also stimulate the adaptive immune system in terms of phenotypical and functional maturation of monocyte derived dendritic cells. Chemokine production in immature and mature dendritic cells was also analyzed in the presence or absence of aqueous pollen extracts (*Bet*.-APE) from birch. Exposure to *Bet*.-APE induced upregulation of MHC class II, CD83, CD86 and increased allostimulatory activity. However, *Bet*.-APE -promoted DC maturation led to a low IL-12 production in comparison to CD40L or LPS. Furthermore, costimulation of *Bet*.-APE and LPS showed that *Bet*.-APE blocked LPS induced IL12 production while IL-6, IL-10 and TNF-α release remained unchanged. Consequently, the ensuing T-cell response of naïve T-cells stimulated by *Bet*.-APE -matured DC resulted in a Th2 subtype. As one causative substance in *Bet*.-APE for this effect, phytoprostane E_1 was identified to antagonize the LPS- induced IL-12 production. We also examined the effects of *Bet*.-APE on chemokine-receptor expression and chemokine production by DCs. *Bet*.-APE strongly induced expression of CXC chemokine receptor 4 on both immature and lipopolysaccharide (LPS)-stimulated DCs. In contrast, *Bet*.-APE reduced CCR5 and CCR1 expression on immature DCs. These effects were confirmed at both messenger RNA and protein levels. Furthermore, *Bet*.-APE significantly reduced LPS-induced production of interferon-inducible protein 10 (CXCL10) and regulated upon activation, normal T-cell expressed and secreted chemokine (CCL5); increased secretion of macrophage-derived chemokine

(CCL22) and did not change production of thymus and activation-regulated chemokine (CCL17). Consistent with these findings, supernatants from *Bet*.-APE- treated mature DCs attracted Th1 type T cells less efficiently whereas migration of Th2 type T cells was enhanced. Our data suggest that *Bet*.-APE provide a signal for enhanced lymph node localization of DCs, but that it may, at the same time, diminish the capacity of DCs to amplify type 1 immune response increasing the outcome and amplification of a Th2 type immune response.

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